Inflammation and Parkinson's Disease: The Effect of Poly(I:C) in a Toxin-based model of Parkinson's Disease

by

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Abstract

Parkinson's disease (PD) has been linked to exposure to a variety of environmental and immune agents in which inflammatory and oxidative processes appear to be involved. Evidence suggests that the damaging effects of immune agents stem from their regulatory actions upon microglial cells. The pesticide paraquat also affects microglia. In light of this, the aim of the current study was to determine whether a viral challenge prior to toxin exposure would exacerbate PD-like pathology, and whether these effects were associated with inflammation. Mice received a supra-nigral infusion of poly(I:C), followed by a regimen of paraquat injections. Indeed, poly(I:C) priming followed by paraquat exposure resulted in an enhancement of dopamine neuron loss in the substantia nigra pars compacta. The combination of immune and chemical challenge was associated with marked microglial activation, as well as oxidative stress. These findings suggest that viral-induced neuroinflammation is sufficient to render midbrain dopamine neurons vulnerable to further toxin exposure.

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List of Abbreviations

PD	Parkinson's disease
SNc	Substantia nigra pars compacta
DAT	dopamine transporter
CYP2D64	cytochrome P450 debrisoquine 4-hydroxylase
GSTT1	glutathione S-transferase T1
GSTP1	glutathione S-transferase P1
6-OHDA	6-hydroxydopamine
BBB	blood brain barrier
ROS	reactive oxygen species
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahyropyridine
MAO-B	monoamine oxidase B
MPP+	1-methyl-4-phenylpyridinium
iNOS	inducible nitric oxide synthase
NADPH	nicotinamide adenine dinucleotide phosphate
BDNF	brain-derived neurotrophic factor
LPS	lipopolysaccharide
TNF	tumor necrosis factor
NGF	nerve growth factor
NT	neurotrophin
ICAM	inter-cellular adhesion molecule
MHC	major histocompatibility complex
IL	Interleukin
IFN	Interferon
Bcl-2	B-cell lymphoma 2
COX-2	cyclooxygenase 2
PET	positron emission tomography
NSAIDs	nonsteroidal anti-inflammatory drugs
TLR	toll-like receptor
PAMP	pathogen-associated molecular pattern
TIR	Toll/IL-1 receptor
MyD88	myeloid differentiation factor-88
NF-κB	nuclear factor κB
PRR	pattern recognition receptor
MAPK	mitogen-activated protein kinase
JNK	c-Jun N-terminal kinase
poly(I:C)	polyinosinic:polycytidylic acid poly
CXCL	chemokine (C-X-C motif) ligand
TH	tyrosine hydroxylase
DCX	doublecortin
DAB	diaminobenzidine
SVZ	subventricular zone
dsRNA	double-stranded RNA
CSF	cerebrospinal fluid
GABA	gamma-Aminobutyric acid

SGZ	subgranular zone
BrdU	bromodeoxyuridine
GDNF	glial cell line-derived neurotrophic factor
FGF	fibroblast growth factor
TIRAP	TIR homology domain-containing adaptor protein
TRIF	TIR domain-containing adaptor inducing IFN-β
IRF	IFN regulatory factor
MAL	MyD88 adaptor-like

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General Introduction

Background

Parkinson's Disease (PD) is a progressive neurodegenerative disorder affecting approximately 3% of the population over the age of 65, making it the second most common neurodegenerative disorder worldwide (Whitton PS, 2007; Orr et al., 2002). The degeneration of dopamine producing neurons of the substantia nigra pars compacta (SNc) results in a marked reduction of dopamine release from the downstream terminals located in the dorsolateral striatum. The loss of these dopamine neurons leads to a dysregulation of basal ganglia signalling that ultimately produces increased inhibition of the thalamus and decreased excitation of the motor cortex, culminating in the characteristic motor deficits observed in PD (DeLong and Wichmann, 2007).

The typical motor deficits seen in PD include tremor, rigidity, bradykinesia and postural instability (Tufekci et al., 2011). These symptoms typically do not present themselves until there has been a 50% loss of dopaminergic neurons in the SNc, which results in about an 80% reduction of dopamine release from terminals in the striatum (Whitton PS, 2007). While disease diagnosis and treatments are primarily based on motor symptoms, idiopathic PD patients exhibit non-motor symptoms as well, including impaired olfaction, disordered sleep, constipation, anxiety and depression (Shulman et al., 2011; DeLong and Wichmann, 2007). The non-motor symptoms seen in PD are likely the result of extra-nigral neuronal degeneration, particularly in the brainstem, thalamus and the cerebral cortex (DeLong and Wichmann, 2007). Indeed, the locus coeruleus (source of much of the brains norepinephrine) degenerates in PD to a degree even greater

than that of the SNc (McMillan et al., 2011; Zarow et al., 2003). Likewise, pathological protein aggregates and abnormal neuronal morphology have been reported to occur in hippocampal, as well as brainstem nuclei (e.g. dorsal vagal complex) of PD patients (Shi et al., 2010; Grinberg et al., 2010; Braak et al., 2003). Importantly, the non-motor symptoms (particularly depression) often precede motor symptoms by as much as 20 years (Shulman et al., 2011), suggesting that such co-morbid symptoms are not simply secondary to the primary motor pathology that dominates the disease.

Motor symptoms respond well to dopamine replacement therapy in the early stages of the disease. PD is treated almost exclusively with L-DOPA, the precursor to dopamine, combined with carbidopa, which prevents peripheral breakdown of L-DOPA. Unfortunately, this therapy becomes ineffective as the disease progresses, underscoring the need for treatments that not only alleviate symptoms, but halt or even reverse progression. Indeed, as is the case for virtually all neurological conditions, there are presently no clinical treatments that actually inhibit the neurodegenerative process in PD. Moreover, the fact that non-motor symptoms are generally not affected by dopamine treatments further reinforces the idea that PD is a multi-spectrum disease with wide ranging pathology affecting numerous neural systems (Shulman et al., 2011).

Besides the neuronal loss observed, a defining histological hallmark of PD is the widespread accumulation of pathological Lewy bodies (Tufekci et al., 2011). These are abnormal intracytoplasmic aggregates, composed primarily of excessive amount of the synaptic protein, α-synuclein, present in neurons and axons (Orr et al., 2002; Dunnett and Bjorklund, 1999). Lewy bodies are most often present in the SNc, but can also be found in various other regions, including the hypothalamus and locus coeruleus (Braak et al.,

2003; Halliday et al., 2011). While genetic mutations in α-synuclein are most often affiliated with autosomal dominant forms of PD, accumulation of Lewy bodies are also present in the brains of idiopathic PD patients (McGeer and McGeer, 2008). Although the exact role of α-synuclein in PD has not been fully established, recent studies have indicated that the mutated form of the protein could contribute to DA neuronal degeneration through its pro-inflammatory and pro-oxidative effects (McGeer and McGeer, 2008).

There are a number of factors that have been proposed to be mechanistically involved in the neurodegenerative process that occurs in PD. In particular, the presence of increased lipid peroxidation and elevated iron levels in post-mortem PD brains has highlighted the role of oxidative stress in PD pathology (Olanow and Tatton, 1999). Moreover, defects in complex I of the mitochondrial electron transport chain and the ubiquitin protein degradation system have also been observed in the SNc dopamine neurons of PD patients (McCoy and Cookson, 2011; McNaught et al., 2001). There have even been a few recent reports indicating that reduced trophic support, coupled with deficits in the anti-apoptotic buffering capacity of SNc dopamine neurons could contribute to the especially prominent vulnerability of these neurons (Nagatsu et al., 2000; Lev et al., 2003). Although the exact relationship between all these biological events remains unresolved, it has been hypothesized that these changes may all be a part of secondary, deleterious events set in motion by a single, or multiple causative factors (Liu, 2006; Hunot and Hirsch, 2003). One potential common mechanistic thread that likely contributes to virtually all pro-death pathways in PD is the consistent finding of enhanced activation of the neuroinflammatory system in PD brains, as well as in animal

models of the disease (Tufecki et al., 2011; McGeer and McGeer, 2008; Nagatsu and Sawada, 2005). The present thesis will focus primarily upon the contribution of neuroinflammatory processes involving the brain's immunocompetent cells, the microglia, in a toxin based model of PD.

We and many other researchers are in support of a multi-hit hypothesis of PD, speculating that disease onset involves multiple "hits" with various environmental toxins possibly combined with genetic vulnerabilities. Given the multi-factorial nature of idiopathic PD, treatments should aim to either simultaneously modulate several pro-death pathways or seek to influence very early processes that could "feed into" these disparate deleterious pathways. In this regard, we propose that manipulation of the inflammatory response holds great promise as a common treatment strategy for many subtypes of PD. Indeed, inflammatory factors are known to influence apoptotic, oxidative stress and mitochondrial processes (Orr et al., 2002).

Animal models of PD

Several genetic mutations have been associated with early-onset PD cases, specifically proteins responsible for encoding parkin, α-synuclein, PTEN-induced kinase 1, DJ-1, ubiquitin carboxyl-terminal esterase L1 and leucine-rich repeat kinase 2 (Liu, 2006). However, these familial forms of PD are rare and upwards of 95% of the cases of PD are not clearly linked to genetics (Liu, 2006; Orr et al., 2002). Hence, substantial effort has been devoted to uncovering potential environmental causes of the disease (Cichetti et al., 2009). Gene-environment interactions may be particularly important for PD onset. Indeed, the penetrance of monogenic forms of PD is variable, insinuating that

environmental input may modulate the development of PD in patients with a pathogenic mutation (Horowitz and Greenamyre, 2010). In fact, certain genetic mutations have been reported to enhance susceptibly to environmental insults and hence, might contribute to the more common late-onset idiopathic cases of the disease. In this regard, a recent study revealed that individuals who possessed a combination of mutations of the dopamine transporter (DAT), and who had substantial life-long pesticide exposure were at greater risk for developing PD than did individuals with either the genetic factor or pesticide exposure alone (Kelada et al., 2006). Moreover, the recent findings that polymorphism within certain environment responsive genes encoding effector proteins critical for cellular detoxification and xenobiotic metabolism, including cytochrome P450 debrisoquine 4-hydroxylase (CYP2D64) and glutathione S-transferase T1 and P1 (GSTT1 and GSTP1, respectively), modified the risk of developing PD, suggests that environmental toxicants might contribute to PD in genetically vulnerable individuals (Singh et al., 2008; Wilk et al., 2006).

The first experimental evidence clearly showing that administration of a specific toxin could provoke a PD-like syndrome was documented in studies showing that central infusion of the dopamine analogue, 6-hydroxydopamine (6-OHDA), had marked neurodegenerative effects upon SNc neurons (Ungerstedt and Arbuthnott, 1970). In fact, the reliability and low-cost of the toxin has kept it one of the most commonly used models of PD to this day (Blandini et al., 2008). 6-OHDA does not cross the blood-brain barrier (BBB), and therefore is infused directly into the SNc or medial forebrain bundle. Animals subject to this toxin are often infused unilaterally, resulting in hemi-parkinsonian behavioural symptoms, characterized by excessive turning and impaired

gait; with the contralateral side often used as a control (Blandini et al., 2008). This toxin produces a massive lesion of nigral dopaminergic neurons almost immediately. Striatal infusions have also been used, in which striatal terminals suffer immediate damage, with progressive death of SNc neurons through what is termed a 'dying back' mechanism (Blandini et al., 2008).

In parallel with its neurodegenerative and behavioural consequences, 6-OHDA infusion produces marked microglial activation and production of inflammatory mediators (including cytokines and prostaglandins) (Miller et al., 2009). Yet, perhaps the most significant pathological aspect of 6-OHDA administration involves its oxidative stress effects. In this regard, 6-OHDA is taken up via the DAT, where it accumulates and simultaneously undergoes auto-oxidation, resulting in the production of reactive oxygen species (ROS) (Blandini et al., 2008). At the same time, 6-OHDA has also been demonstrated to cause neuronal damage by interfering with complex I on the electron transport chain (Blandini et al., 2008). Hence, neuronal demise in this model is believed to be ultimately caused by the overwhelming oxidative stress and impaired energy production resulting from the toxin. However, as will become apparent as a major thread of this thesis, pro-inflammatory processes could be a common driving force behind the oxidative and other pro-death mechanisms ultimately engaged by toxin exposure.

A more recent animal model emerged in the 1980s, largely as a result of the unexpected finding that several illicit drug users who mistakenly injected the meperidine analogue, 1-methyl-4-phenyl-1,2,3,6-tetrahyropyridine (MPTP), rapidly developed irreversible parkinsonism (Langston et al., 1983). This provided the first concrete and causal example of toxin exposure inducing parkinsonism in humans. MPTP can be

administered systemically or centrally, as it is highly lipophilic and easily crosses the BBB. Once in the brain, MPTP undergoes a series of glial-dependent chemical changes, partially mediated by monoamine oxidase B (MAO B), and ultimately is oxidized to 1-methyl-4-phenylpyridinium (MPP+), the active neurotoxin. MPP+ is subsequently taken up by striatal dopamine neurons via DAT, where it accumulates within the inner mitochondrial membrane. At this site, MPP+ inhibits complex I by interrupting electron transport, resulting in a depletion of ATP energy stores (Nagatsu and Sawada, 2006). Much like 6-OHDA, substantial data also indicates that MPTP induces marked neuroinflammation, characterized by robust microglial activation, coupled with excessive oxidative stress load (e.g. increased inducible nitric oxide synthase (iNOS) and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity) (Wu et al., 2002). Two glaring caveats of both the MPTP and 6-OHDA models are: 1. their inability to induce the formation of the hallmark Lewy bodies, and 2. the fact that these toxins are not naturally present in the environment and therefore lack external validity as potential causative agents.

Environmental Toxins and PD

The growing list of environmental toxins associated with PD includes various heavy metals (e.g. iron, manganese), pesticides, bacteria and viruses (Dick et al., 2007; Weisskopf et al., 2010; Mattock et al., 1998). Pesticides in particular have received considerable attention as potential triggers of PD, although definitive evidence for a causal role has yet to be established. Epidemiological studies have shown that a progressively greater odds ratio for developing PD was associated with pesticide

exposure (Dick et al., 2007), and several other epidemiological studies have implicated specific pesticides, including rotenone (an organic insecticide) and paraquat (a chemical herbicide still widely used throughout the world), in neurological pathology (Priyadarshi et al., 2001; Firestone et al., 2005). Indeed, a sharp increase of PD incidence was seen in agricultural areas that use these pesticides (Tsui et al., 1991; Semchuk et al., 1992; Petrovitch et al., 2002; Baldi et al., 2003). In particular, the non-selective herbicide, paraquat (N,N'-dimethyl-4,4'-bipyridylium ion), has been shown to dose-dependently increase the risk of developing PD as a function of cumulative pesticide exposure (McCormack et al., 2002; Liou et al., 1997).

Paraquat is among the most widely used herbicides worldwide, although its use is currently restricted in the United States and was banned in Europe in 2007 (Cichetti et al., 2009). Paraquat is thought to enter the brain through neutral amino acid transporters, and is taken up into cells in a sodium-dependent fashion (Cichetti et al., 2009). Once paraquat has entered parenchymal cells, it acts as a redox cycling agent and indirectly induces mitochondrial toxicity (Cichetti et al., 2009). Additionally, paraquat has been reported to downregulate trophic factors, such as brain-derived neurotrophic factor (BDNF), while at the same time inducing marked pro-inflammatory effects involving excessive microglial activation (Mangano et al., 2011, Litteljohn et al., 2010, Purisai et al., 2007). As will be discussed in ensuing sections, one mechanism through which microglia can contribute to the neurodegenerative cascade is through their well documented release of the superoxide radical following toxin exposure (Halliday and Stevens, 2011; Liu, 2006; McGeer and McGeer, 2004).

Rotenone, a pesticide, like paraquat, has been epidemiologically linked to PD and has been used to recapitulate PD-like features in rodents (Tanner et al., 2011; Cichetti et al., 2009). Indeed, rotenone was demonstrated to promote a loss of SNc dopamine neurons, the production of abnormal Lewy bodies and motor abnormalities (Liu, 2006; Betarbet et al., 2000). Rotenone is a member of the isoflavone family and, in a manner analogous to MPTP, is known to inhibit complex I of the mitochondrial chain (Betarbet et al., 2000). Since rotenone is highly lipophilic it easily passes the BBB into the brain parenchyma, where it promotes dysfunction of mitochondrial complex I, ultimately leading to the production of oxidative radicals (Cichetti et al., 2009).

In addition to having greater ecological validity than the MPTP and 6-OHDA models of PD, paraquat and rotenone have been shown to provoke histopathological changes that more closely resemble the disease, particularly the aberrant fibrillization and aggregation of ubiquinated α-synuclein containing Lewy body inclusions (as occurs in PD patients) (Uversky et al., 2001; Manning-Bog et al., 2002; Yang et al., 2007; Fernagut et al., 2007). Yet, one potential caveat to the paraquat model is the fact that some authors have reported mild to nonexistent effects of the pesticide upon the striatal dopamine terminals (McCormack et al., 2002; Thiruchelvam et al., 2000; Perry et al., 1986). At this juncture, it is clear that paraquat can have deleterious effects upon SNc dopamine neuronal soma (this has been repeatedly demonstrated in numerous laboratories), but the impact of the pesticide on downstream dopaminergic processes is less well defined.

PD has long been considered a 'threshold' disease, meaning that clinical symptoms of PD will only appear after dopaminergic neuron numbers have decreased past a certain threshold (Barlow et al., 2007). The threshold for PD is believed to be

around 80% for the loss of nigrostriatal dopaminergic neurons (Calne and Langston, 1983; Barlow et al., 2007). The 'multi-hit hypothesis' of PD purports that damage to the dopaminergic system through repeated exposure to multiple environmental insults eventually leads to the decline of dopaminergic neurons below such a threshold, and therefore to the onset of PD symptoms (Weidong et al., 2009; Thiruchelvam et al., 2000a). Furthermore, following one 'hit', the nigrostriatal system is likely to be vulnerable to various secondary insults (Weidong et al., 2009). The accumulating evidence does seem to suggest that it is highly likely that PD is triggered by multiple exposures to low doses of environmental toxins, that act either additively or synergistically (Ling et al., 2004). The fact that toxin exposure levels could be minimal and sporadic, however, makes epidemiological detection exceedingly difficult. Ultimately, it is highly plausible that multiple 'hits' culminating throughout life and consistently targeting a specific region (in this case the nigrostriatal dopmainergic neurons) would eventually act together to push nigrostriatal damage past the threshold, thereby enabling the presentation of clinical symptoms (Barlow et al., 2007).

A number of studies support the multi-hit hypothesis and some have even suggested that the initial 'hit', which initiates the neurodegenerative process, could occur prenatally. In fact, Mattock et al. (1988) proposed that intra-uterine influenza could lead to later PD development, based on the observation of a significant correlation between idiopathic PD risk and prenatal influenza exposure. Numerous animal studies also support a link between PD and prenatal toxin exposure. For example, prenatal administration of the endotoxin, lipopolysaccharide (LPS), resulted in the birth of offspring with fewer SNc dopamine neurons (Ling et al., 2002). This can be extrapolated

to cases of bacterial vaginosis that commonly occur in pregnant mothers (Cicchetti et al., 2009; Dammann and Leviton, 1998). Also, animals prenatally exposed to LPS exhibited long-term elevated brain levels of tumor necrosis factor (TNF)-α, implying increased inflammation in these animals (Ling et al., 2002). Furthermore, Ling et al. (2004) later demonstrated a subsequent vulnerability to pesticide exposure at adulthood in animals prenatally exposed to LPS. Specifically, the combination of prenatal LPS and postnatal rotenone synergistically decreased the number of SNc dopamine neurons and substantially increased levels of inflammatory microglial cells compared to treatment groups treated with either toxin alone (Ling et al., 2004).

Underscoring the importance of pesticide exposure and the multi-hit hypothesis, some evidence suggests that exposure to certain combinations of heavy metals and pesticides may synergistically provoke conformational changes in α-synuclein, favouring the development of PD pathology (Uversky et al., 2002). In fact, a recent study revealed that exposure to a combination of iron and paraquat synergistically increased α-synuclein aggregation and fibrillization and augmented the extent of oxidative stress-induced neurodegeneration (Peng et al., 2007). Similarly, although the dithiocarbamate pesticide, maneb, had no effect on SNc dopamine neurons alone, when co-administered with paraquat, it synergistically enhanced nigrostriatal damage and associated glial reactivity (Thiruchelvam et al., 2000b). Our own work demonstrated an augmented loss of dopamine neurons and protracted microglial inflammatory reaction in mice primed with a single dose of LPS followed by later treatment with paraquat (Mangano and Hayley, 2009). These data support the notion of inflammation being a key event in rendering nigrostriatal neurons vulnerable to later toxin exposure.

Inflammation and PD

Until relatively recently, the brain was seen as an immune-privileged organ that, by virtue of tight junctions formed between cerebral microvascular endothelial cells (i.e., the BBB), was impenetrable to noxious, blood-borne molecules and circulating immune cells in the periphery (Orr et al., 2002). However, it has become apparent that the microenvironment of the brain is, in fact, capable of both mounting and sustaining an inflammatory immune response (Aloisi, 2001). Yet, inflammation in the brain is unique, in that it occurs without antibodies and without significant infiltration of T cells into the parenchyma (McGeer and McGeer, 2004) and instead centers around microglial activation and production of cytokines, prostaglandins and other inflammatory and oxidative messengers (McGeer and McGeer, 2004). In this regard, a short-lived inflammatory microglial responses to transient CNS insult might very well be expected to restrict damage to and promote healing of viable brain tissue (e.g., removal of cellular debris, release of trophic factors) in a manner analogous to a short-lived immune response in the periphery (Wyss-Coray and Mucke, 2002; Teismann et al., 2004). Indeed, projections from microglia are continuously sampling their microenvironment, scavenging and clearing cellular debris (McGeer and McGeer, 2008; Nimmerjahn et al., 2005; Orr et al., 2002). However, unchecked CNS inflammation of a chronic (i.e., genetic mutation or prolonged exposure to environmental toxin) or sustained/self-perpetuating nature is, indeed, capable of mediating profound neuropathologic effects (Tansey et al., 2008; Rogers et al., 2007).

Microglial cells are central to all immune/inflammatory events that occur within the CNS. Essentially, together with astrocytes, microglia work to maintain a homeostatic microenvironment within the brain, responding to changes in the CNS such as neurotransmitter imbalances as well as infiltrating peripheral immune cells (Bessis et al., 2007). Importantly, microglia display certain characteristics reminiscent of peripheral antigen-presenting cells (e.g. macrophages or dendritic cells), and are thought to interact with T cells to facilitate removal of any CNS pathogens (Liu, 2006). Microglia rapidly respond to imbalances in ion homeostasis, invading pathogens, the presence of foreign substances, and events which precede pathological changes (Liu, 2006; Kreutzberg, 1996). In response to such insults, microglia proliferate and display dramatic functional and morphological changes indicative of an activated state (Kreutzberg, 1996; Halliday and Stevens, 2011). These changes are comprised of adaptation of an amoeboid morphology, the up-regulation of a variety of receptors and the secretion of multiple molecules involved in inflammation and phagocytosis, such as cytokines, oxygen and nitrogen free radicals, chemokines, fatty acid metabolites and trophic factors (Liu, 2006; Aloisi, 2001; McGeer and McGeer, 2004; Kreutzberg, 1996).

Microglia are often referred to as a double-edged sword, in that often times they are protective in the short term, but in chronic conditions they eventually overwhelm local anti-oxidant and anti-inflammatory controls and result in cytotoxic effects upon neighbouring neurons (Kreutzberg, 1996). The reactivity state of microglia varies along a spectrum ranging from resting to hyperactive and is under the strict control of several regulatory proteins. Whether microglia take on a more M1 (state normally associated with the respiratory burst and can often have neurotoxic consequences) or M2 (basal

state, often release neuroprotective factors) phenotype depends on the signals they receive (Michelucci et al., 2009). For the most part, microglia normally take on an M2 phenotype, acting as sentinels slowly proliferating and removing any debris by phagocytosis in the CNS (Davalos et al., 2005; Kumar and Jack, 2006; Nimmerjahn et al., 2005). Compelling evidence suggests that microglial cells in an M2 state perform neuroprotective functions in PD, at least in the short term, by secreting trophic factors such as nerve growth factor (NGF), neurotrophin (NT)-3 and BDNF (Baquet et al., 2005; Peterson and Nutt, 2008). However, as the disease progresses, cytotoxic substances promoted by dopamine cell death (e.g. enhanced extracellular debris, oxidative stress and pro-inflammatory cytokine release) cause microglia to undergo significant elevations in both activation markers and cell adhesion molecules, resulting in a more hyper-reactive state, characteristic of the M1 phenotype (Gao and Hong, 2008).

Despite the fact that microglia will undergo functional changes with "normal" aging (heightened activation and reduced proliferative ability) the degree of activation in age-dependent neurodegenerative diseases appear to be different (Conde and Streit, 2006a; Conde and Streit, 2006b). Indeed, in the case of PD, microglial cells undergo extreme morphological changes and adopt an M1 phenotype, wherein their processes retract inward and the cell adopts an amoeboid-like appearance, eventually becoming phagocytic, akin to the perivascular macrophage. Paralleling these morphological changes, these hyper-active microglia will enhance the expression of inter-cellular adhesion molecule (ICAM), major histocompatibility complex (MHC) class II, complement receptors while at the same time releasing interleukin (IL)-1β, IL-6,

interferons (IFNs) and TNF-α in an attempt to combat infections (Glezer et al., 2007; Hirsch et al., 2005; Przedborski et al., 1996).

Sporadic and familial cases of PD all display substantial microglial activation, particularly in the SNc and striatum (McGeer and McGeer, 2004). Animal studies have likewise demonstrated that dopaminergic cells are particularly vulnerable to inflammatory attack. For example, direct infusion of LPS into the SNc dose-dependently affects dopaminergic cell survival, but direct infusions into the hippocampus and cortex produced no effects on neurons (McGeer and McGeer, 2008). Moreover, humans who accidentally self-administered MPTP demonstrated high levels of activated microglia in the SNc, prevalent up to 20 years after exposure (Langston et al., 1999). Interestingly, these people also demonstrated progressive parkinsonian symptoms, suggesting that inflammation and activated microglia could have a role in progression of PD. This observation also supports the notion that an inflammatory reaction in the SNc can become self-sustaining, even years after the initiating agent has been removed (McGeer and McGeer, 2008). Reactive microglia are a common occurrence in all established animal models of PD (Erazi et al., 2011; Tufecki et al., 2011; Cichetti et al., 2009; Blandini et al., 2008; Nagatsu and Sawada, 2005; McGeer and McGeer, 2004) and even α-synuclein can act as an inflammatory stimulant for these cells (McGeer and McGeer, 2008).

In addition to microglial changes, proinflammatory cytokines and neurotrophins have been reported to be altered in the brains of PD patients (Nagatsu and Sawada, 2006). Particularly, a multitude of cytokines have been found to be upregulated in the basal ganglia and cerebrospinal fluid (CSF) of PD brains (McGeer and McGeer, 2004; Mogi et

al., 1996). Mogi et al. (1996) have shown increases in a number of cytokines in these regions, including TNF-α, IL-1β, IL-6, IL-2 and IL-4. These investigators have also reported decreased neurotrophin levels in nigrostriatal regions of human post-mortem PD brains, such as BDNF and NGF, as well as increases in apoptosis-related molecules, such as B-cell lymphoma 2 (Bcl-2) and Fas (Nagatsu and Sawada, 2006; Mogi et al., 1999). Animal models of PD further confirm that the increase in inflammatory cytokines and decrease in neurotrophic factors are likely detrimental factors in PD (Hirsch and Hunot, 2009). In fact, MPTP models of PD have provided immunohistochemical, biochemical and gene array data demonstrating increased expression of proteins and/or mRNA for IL-1β, IL-6, TNF-α, iNOS, cyclooxygenase 2 (COX-2), as well as subunits of the NADPH oxidase complex (Liu, 2006). Furthermore, LPS administration or 6-OHDA promotes increased expression of IL-1β, TNF-α and iNOS and NADPH oxidase subunits (Liu, 2006).

The presence of reactive microglia and cytokines in post-mortem PD brains was initially deemed a secondary effect of massive neuronal loss in the terminal stages of the disease (Liu, 2006). However, more recent empirical evidence has indicated that reactive microglia are not only present throughout PD disease progression, but that they have an active role in the onset and advancement of the disease (Liu, 2006). Indeed, the growing consensus is that neuroinflammation is part of the pathologic process and precedes dopaminergic cell death (Hunot and Hirsch, 2003). For instance, Ouchi et al. (2005) conducted a positron emission tomography (PET) analysis in early stage PD patients which demonstrated a correlation between activated microglia and dopaminergic terminal loss, once again suggesting a primary detrimental contribution of microglia to the

progression of the disease. Moreover, cultured microglia have been shown to directly contribute to neurotoxic effects on co-cultured midbrain dopaminergic neurons (McGeer and McGeer, 2004).

Anti-inflammatory agents that inhibit microglial activation, such as minocycline, have been shown to decrease dopaminergic cell death (Radad et al., 2010). Indeed, several large epidemiological studies have reported that chronic use of nonsteroidal anti-inflammatory drugs (NSAIDs) significantly reduces the risk of PD by about 45% (Liu, 2006; McGeer and McGeer, 2004; Chen et al., 2003). One such study monitored middle to old-aged male and female participants for upwards of 10 years. A protective effect associated with NSAID use was observed in both genders (Chen et al., 2003). A follow-up study by this same group demonstrated that regular use of ibuprofen, but not other NSAIDs, was associated with a similar decreased incidence of PD (Chen et al., 2005). A meta-analysis of several large epidemiological studies revealed that moderate use of NSAIDs was associated with a 15% reduction in PD risk and this reduction increased to 29% with further regular long-term use (Gagne and Power, 2010). Evidence from various animal models of PD corroborates these observations. For example, aspirin was found to protect against MPTP-induced dopamine depletion in mice through inhibition of COX-2 (an enzyme crucial in the production of pro-inflammatory lipid mediators, such as prostaglandins) ((Nagatsu and Sawada, 2005; Liu, 2006). Furthermore, pre-treatment with NSAIDs in MPTP and 6-OHDA animal models of PD protected against nigrostriatal degeneration (for review, see Esposito et al., 2007). In summary, the protective effects mediated by acetylsalicylic acid (aspirin), salicylic acid, ibuprofen and COX-2 selective

inhibitors are convincing, although controversy behind their exact mechanisms of action still exists (Esposito et al., 2007).

Toll like receptors (TLRs) and PD

In addition to acting as antigen presenting cells, microglial cells respond to microbes by recognizing specific structural components that are essential for the survival of those invading organisms. These unique molecules are referred to as pathogen-associated molecular patterns (PAMPs) (Olson and Miller, 2004) and are recognized by mammalian toll like receptors (TLRs). (Okun et al., 2009). TLRs are ubiquitously expressed in immune-related cells, including cells located in the brain parenchyma. Indeed, evidence has indicated TLR presence on microglia, astrocytes, oligodendrocytes and, more recently, neurons (Okun et al., 2009). While TLRs are primarily recognized for their role in innate immunity, through recognition and subsequent response to various PAMPs, they have also been shown to play a role in bone metabolism, neurogenesis and brain development (Okun et al., 2009).

TLRs are a single membrane spanning protein, comprised of a leucine-rich extracellular domain responsible for PAMP recognition, as well as a cytoplasmic Toll/IL-1 Receptor (TIR) domain which initiates downstream signalling (Kawai and Akira, 2007). Each TLR, alone or in combination with other TLRs, recognize distinct PAMPs that include lipids, lipoproteins, nucleic acids and proteins (Okun et al., 2009). Presently, 13 different types of TLRs have been identified and each is responsible for recognizing a different structural component (Lee et al., 2007). Agonist recognition specificity is achieved by TLR homo- or hetero-dimerization (Trudler et al., 2010). Most often, TLR

signalling is dependent on expression of the adaptor protein, Myeloid differentiation factor 88 (MyD88). TLR3 and TLR4 are the sole TLRs that have been shown to elicit responses through a MyD88-independent signalling pathway (Okun et al., 2009). Upon activation by PAMPs, TLRs initiate downstream signalling which ultimately lead to activation of the transcription factor nuclear factor κB (NF-κB), which in turn induces the release of pro-inflammatory cytokines in an attempt for host defence (Trudler et al., 2010; Okun et al., 2009). TLRs work in conjunction with other pattern recognition receptors (PRRs) such as CD14 and scavenger receptors to remove pathogens, toxic cellular debris (amyloid fibrils, aggregated synuclein, prions) and apoptotic cells accumulating within the brain parenchyma and the cerebrospinal fluid. An innate immune response often results in collateral damage to nearby tissue as a result of TLR-induced microglial response or recruitment of leukocytes to the injured area (Kawai and Akira, 2007).

TLR regulation is essential for maintaining homeostasis, as over-activation of TLRs has been associated with infectious and inflammatory diseases (Trudler et al., 2010). The damaging effects elicited by TLR activation are likely instigated by the TLRs ability to increase pro-inflammatory cytokine release, as well as increase activation of members of the mitogen-activated protein kinase (MAPK) family, including p38 and c-Jun N-terminal kinase (JNK), which are involved in transcription of genes and regulation of mRNA stability (Trudler et al., 2010).

The involvement of TLRs in neurodegenerative disorders such as Alzheimer's disease, multiple sclerosis and ischemic stroke have recently been established (Landreth and Reed-Geaghan, 2009; Racke and Drew, 2009; Hamanaka and Hara, 2011), yet little

is known about TLR functioning in Parkinson's disease. The current data (albeit very sparse) on TLRs in PD has predominantly implicated TLR4 and TLR3 agonists in the disease (Dutta et al., 2008; Deleidi et al., 2010). The membrane spanning TLR4 receptor specifically recognizes LPS, which is present on gram-negative bacteria (Okun et al., 2009). In contrast, TLR3 is localized intracellularly, and is recognized by nucleic acids present on viruses (Okun et al., 2009). Indeed, the TLR3 signalling pathway is activated in response to double stranded RNA of viral origin, including polyinosinic:polycytidylic Acid (poly(I:C)) (Okun et al., 2009).

Agonists for various TLRs will trigger the secretion of various types' pro-inflammatory mediators. Specifically, LPS for TLR4 and poly(I:C) for TLR3 will augment the secretion of multiple cytokines including IFN-α, IFN-β, IL-1β, IL-6, IL-10, IL-12, IL-18, TNF-α coupled with nitric oxide and chemokines such as macrophage-inflammatory protein-1α, monocyte chemotactic protein-1, and chemokine ligand 5 (also referred to as RANTES) (Olson and Miller, 2004). Stimulation of a TLR will result in a customized immune response; for instance, TLR4-mediated microglial activation and TLR3 signalling were observed to induce the strongest pro-inflammatory response compared with other TLRs, characterized by secretion of IL-12, TNF-α, IL-6, chemokine (C-X-C motif) ligand (CXCL)-10, IL-10, and IFN-β (Jack et al., 2005). Furthermore, Castano et al. (1998) demonstrated that intranigral injection of the TLR4 agonist LPS caused a rapid and sustained microglial activation, as well as decreased dopamine levels in the SNc and striatum and fewer nigrostriatal tyrosine hydroxylase (TH) immunoreactive neurons. Moreover, this LPS-induced degenerative effect was attenuated when animals are co-treated with naloxone, an opioid receptor antagonist (Liu et al.,

2000). Indeed, protection elicited by naloxone appeared to be mediated through an anti-inflammatory effect, as seen through decreased microglial activation in naloxone-treated animals (Liu et al., 2000).

TLR3 has also been speculated to having a role in PD pathogenesis. There have been various reports of neurological symptoms in patients exposed to viruses, including those with the highly pathogenic H5N1 virus, as well as the 'Spanish' influenza outbreak in 1918 (Ravenholt and Foege, 1982; Jang et al., 2009). In these reports, patients have presented with mild encephalitis, coma, and motor disturbances (Jang et al., 2009). Moreover, intense inflammatory reactions as well as cytokine dysregulation has been reported in H5N1 cases (Gambotto et al., 2007). Jang et al. (2009) conducted a study to determine whether the H5N1 influenza virus could indeed cause neurological pathology. Mice intra-nasally exposed to the H5N1 virus displayed motor disturbances, including ataxia, tremor and bradykinesia. Moreover, immunohistochemical analysis confirmed entry of the H5N1 virus into the CNS, and its presence was verified in the SNc, neurons and microglia, among other regions. Finally, increased α-synuclein phosphorylation and aggregation was observed, as well as an increase in apoptotic cells, prolonged reactive microgliosis, as well as a significant loss of dopaminergic neurons in the SNc (Jang et al., 2009). Along these lines, direct injection of the TLR3 agonist poly(I:C) into the SNc of adult rats was very recently reported to induce long-term glial activation in both the SNc and the dorsolateral striatum (Deleidi et al., 2010).

Research Objectives

The present thesis is focused upon determining whether exposure to a viral related challenge (poly(I:C)) would enhance the neurodegenerative effects of later exposure to

the pesticide, paraquat. Our hypothesis is essentially that poly(I:C) priming will augment the SNc dopamine neuronal loss triggered by paraquat exposure and that such an effect will be associated with an augmented microglial inflammatory/oxidative response. This proposition is in line with the so called multi-hit hypothesis of PD, wherein the disease is believed to stem from time-dependent changes that occur over time following multiple toxin exposures.

While previous work from our laboratory revealed that the TLR4 agonist, LPS, was able to augment the damaging effects of later paraquat exposure, it is unknown whether such a sensitization effect would generalize across other inflammatory stimuli. In this regard, it was presently of interest to assess whether TLR3 activation, using poly(I:C), would have effects comparable to LPS and if common neuroinflammatory pathways underlie enhanced vulnerability of SNc dopamine neurons to environmental toxins.

Materials and methods

Animals

Male C57BL/6 mice were obtained (Charles River, Laprarie, Quebec, Canada) at 10-12 weeks of age and given one week to acclimatize. Animals were single-housed in standard polypropylene cages, and were maintained on a 12 hour light/dark cycle. Mice were provided with a*d libitum* food and water, and rooms were temperature and humidity controlled. All experimental tests were approved by the Carleton University Committee for Animal Care and were conducted in adherence to the guidelines stipulated by the Canadian Council for the Use and Care of Animals in Research.

Experimental Paradigm

Animals underwent stereotaxic cannula implantation (described below), with cannulae situated directly above the substantia nigra pars compacta (SNc), and were given one week convalescence prior to experimentation. Mice were then infused with the toll-like receptor 3 agonist, polyinosinic:polycytidylic acid (poly(I:C)) (5μg/2μl) (InvivoGen), or saline. Following infusions, mice (n=8) received intraperitoneal injections with paraquat (1,1'-dimethyl-4,4'-bipyridinium dichloride; Sigma Aldrich, 10 mg/kg), or an equivalent volume of saline (Sigma), 3 times a week for 3 weeks, for a total of 9 injections, as previously described (Brooks et al., 1999; Mangano et al., 2011b). Mice began receiving the paraquat or saline injections at 2, 7 or 14 days following intra-SNc poly(I:C) or saline infusion in order to investigate whether varying exposure times

differentially affect paraquat toxicity. Animals were then euthanized at 7 days following the final injection. Treatment groups are summarized below (Table 1).

Mice were overdosed by sodium pentobarbital injection and thereafter perfused with ice cold saline followed by 4% paraformaldehyde. Whole brains were dissected out and stored in 20% sucrose solution (comprised of 20% sucrose, 0.02% sodium azide, dissolved in 0.1M PBS) at 4°C until further analysis.

Surgery

Animals underwent cannula implantation surgery as previously described (Mangano et al., 2011b). Briefly, mice were anesthetized with oxygen-enriched isoflurane, and placed in a stereotaxic apparatus. All mice were then implanted with indwelling cannulae directly above the SNc (bregma: anterior-posterior -3.16mm, ± lateral 1.2 mm, ventral -4.0mm). Animals were given one week rest post-surgery before experimentation commenced. Infusions were delivered through polyethylene tubing connected to a Hamilton microliter syringe, using a Harvard Apparatus Pico Plus syringe pump. The drug was dissolved into 2 µl of saline and delivered over a 5 minute period, followed by 2 minutes rest to ensure proper delivery. Infusions were conducted between 0830h and 1400h in order to avoid variability attributed to diurnal variations. Furthermore, animals were unrestrained during the infusion process to minimize stress.

Behavioural Analysis

Home-cage locomotor activity was monitored during a complete 12-h light/dark cycle using a Micromax infrared beam-break apparatus (Accuscan Instruments,

Columbus, OH, USA). Locomotor activity was measured during the 3rd and 4th weeks of experimentation (15 and 22 days after the infusion), as well as 24h prior to sacrifice. Animals were given 1h to acclimatize to the room prior to assessment. Total locomotor activity was assessed by recording the number of infrared beam-breaks over a 24h-period, as described previously (Crocker et al., 2003).

Motor coordination was examined by way of a pole test, as previously described (Litteljohn et al., 2008; Sedelis et al., 2000). This test consists of a 50 cm pole (diameter, 0.8 cm), with a plastic ball at the top. The pole was covered with non-toxic adhesive tape to increase traction. Home-cage bedding was placed at the bottom of the pole to encourage the animals to descend. Animals were placed head upward directly below the plastic top. Motor coordination is necessary for the mouse to rotate downward and climb to the ground. Latency to rotate 180° and latency to climb down the pole was recorded, with maximum test duration set at 90s. Testing was conducted under dim lighting conditions between 0900h and 1400h on the days following the 1st and 2nd micromax recordings (16 and 23 days following the infusion). The animals were recorded for 3 trials, divided by a 1 minute rest period between tests.

Immunohistochemical Analysis

Fixed brains were cryostat sectioned and 20 μm coronal sections of the striatum and SNc were mounted directly onto gelatin-coated slides. Both SNc and striatal sections were analyzed for tyrosine hydroxylase (TH) staining as a representative measure of nigrostriatal dopamine neurons. SNc sections were also stained for the microglial and oxidative stress markers, CD11b and $gp91^{PHOX}$, respectively. In order to provide an index

of potential neuroplastic changes that could influence behavioural outputs, striatal sections were stained for doublecortin (DCX).

Sections were incubated overnight at 4°C in mouse anti-TH (1:1000, ImmunoStar), rat anti-CD11b (1:1000, AbDSerotec), goat anti-gp91^{PHOX} (1:1000, Santa Cruz) or goat anti-DCX (1:1000, Santa Cruz). Sections were further incubated with their respective biotinylated antibodies (biotin anti-mouse (1:500, Jackson ImmunoResearch), biotin anti-rat (1:500, Jackson ImmunoResearch), biotin anti-goat (1:1000, Jackson ImmunoResearch)) for 2 hours at room temperature. Primary and secondary antibodies were diluted in 0.01M PBS, pH 7.3, containing 2% BSA with 0.3% Triton X-100 and 0.01 sodium azide. Sections were further incubated in horseradish peroxidase-conjugated streptavidin tertiary (1:1000 for gp91^{PHOX} and DCX, 1:500 for TH and CD11b, Jackson ImmunoResearch) at room temperature for 2h and dissolved in 0.01M PBS, pH 7.3, containing 2% BSA and 0.3% Triton X-100. Antibodies were visualized by incubation with diaminobenzidine (DAB; Sigma-Aldrich) for 10 minutes on a shaker table. TH-stained SNc sections were further counterstained with cresyl violet (Sigma-Aldrich), and all sections were dehydrated with serial alcohol washes and cover-slipped with clearene (Surgipath).

The microglial state of activation on Cd11b-stained sections was rated using a scale previously described by our lab (Mangano et al., 2011b). In brief, cells were rated on a 0-3 scale. A score of 0 reflects the lowest state of activation, where microglia are in their resting state, identified by highly ramified, thin processes. A score of 1 indicates an intermediate state of activation, in which less than 10 cells within the SNc were moderately activated. A score of 2 was given when more than half of the visible cells

were in an intermediary or active state, characterized by rounded soma and no or few processes. A score of 3 was attributed to those sections which showed the highest level of activation, in which the majority of cells exhibited a highly active state.

Similarly, $gp91^{PHOX}$ reactivity was rated on a scale comparable to that described above. A score of 0 reflected little to no $gp91^{PHOX}$ reactivity. A score of 1 indicated low to moderate $gp91^{PHOX}$ immunostaining, in which $gp91^{PHOX}$+ microglial cells were present in relatively low numbers. A score of 2 signified elevated $gp91^{PHOX}$ activity, where numerous activated $gp91^{PHOX}$+ microglial cells were observed. Finally, a score of 3 denoted high $gp91^{PHOX}$+ staining, in the presence of numerous highly reactive microglia.

All ratings (both Cd11b and $gp91^{PHOX}$) were conducted twice by the same rater, who was kept blind to all treatments. The ratings yielded an >90% inter-rating overlap, and all scores per section were averaged for an overall representation of total nigral glial cell and oxidative activity (Cd11b and $gp91^{PHOX}$, respectively).

Quantification of TH+ Neurons

Quantification of SNc dopamine producing neurons was achieved by way of serial section analysis of the number of SNc TH+ cells, at bregma levels from -3.08 through to -3.40. Using a double blind procedure, the total number of TH+ cells was counted across multiple bregma levels in 8 animals per treatment group. Midbrain TH+ counts from each bregma level and animal were compared across treatment groups. The same midbrain sections were also used to evaluate the total number of TH- cells, which represents the number of surviving neurons. Cresyl violet stained neurons from each bregma level and animal were quantified and compared across treatment groups.

Striatal quantification photomicrographs were obtained for each animal using the same exposure time. Image J software was used to determine the background threshold for each striatal section and the total number of white (background) and black (TH+) pixels. All images were converted into an 8-bit format, where the grayscale varied from 0 to 255. The area of interest was selected and the upper and lower threshold values were used across all images to separate the features of interest from the background. The upper and lower thresholds were determined using an automatic thresholding option, a modified version of the IsoData method. This algorithm divides the image into the object of interest and background by taking an initial threshold (histogram-derived), followed by the averages of the pixels at or below the threshold and pixels above are computed. The averages of those two values are computed, the threshold is incremented and the process is repeated until the threshold is larger than the composite average. The data were then presented as a histogram with the number of black (object of interest) and white (background) pixels present.

Statistical Analysis

All data were analyzed by ANOVA, followed by Fisher's planned comparisons ($P<0.05$) where appropriate. Data were evaluated using a StatView (version 5.0) statistical software package from the SAS Institute, Inc.

Treatment Group	Infusion	Delay	Injection	Sac time
1	Sal	2 days	Sal	7 days
2	Sal	2 days	PQ	7 days
3	Poly I:C	2 days	Sal	7 days
4	Poly I:C	2 days	PQ	7 days
5	Poly I:C	7 days	PQ	7 days
6	Poly I:C	14 days	PQ	7 days

Table 1. *Breakdown of treatment groups*

Results

Behavioural Analysis

Total home-cage locomotor activity (during an entire 24h cycle) was assessed during the 3rd and 4th week of the paraquat injection regimen (at 15 and 22 days following the initial poly(I:C) or saline infusion), as well as 24h prior to sacrifice. The ANOVAs revealed no significant differences between groups in the first ($F_{5,52}$=.736, $p>0.05$), and final ($F_{5,49}$=.599, $p>0.05$) home-cage assessments. However, at the second time point (i.e. 22 days after the initial infusion), a significant difference in activity was evident between the treatment groups ($F_{5,47}$=2.858, $p<0.05$). The posthoc analyses revealed that administration of either paraquat or poly (I:C) alone, or in combination significantly reduced total locomotor activity compared with saline treated animals (Figure 1).

Insert Figure 1 about here

Analysis of pole test performance was used to investigate any PD-like hind-limb and forelimb coordination deficits that might have been induced by paraquat with or without poly(I:C) priming. The pole test was conducted once, during the 2nd week following saline/poly(I:C) infusion. Latency to turn, as well as latency to descend the pole were both recorded for 3 trials. No behavioural deficits were found in any treatment groups ($F_{5,42}$=.166, $p>0.05$; Table 2).

Insert Table 2 about here

Quantification of TH+ Neurons

Nigral dopaminergic cell bodies were counted at bregma levels -3.08 through -3.40 for all treatment groups. The ANOVAs revealed that the number of nigral tyrosine hydroxylase (TH)+ neurons varied as a function of treatment, particularly at bregma levels 3.08 and 3.28 ($F_{5,29}$= 8.669, $p<0.05$; $F_{5,30}$=4.654, $p<0.05$, respectively). As shown in Figure 2, groups treated with paraquat or poly(I:C) alone demonstrated a modest, non-significant decrease in substantia nigra pars compacta (SNc) dopamine neurons (approx. 10-15%). However, poly(I:C) priming followed by subsequent paraquat administration led to a significant decrease of SNc dopamine neurons (25-50% relative to saline-treated controls; $p < 0.05$). The magnitude of the decrease in TH+ neurons varied depending on the timing between poly(I:C) infusion and the commencement of paraquat injections. In this regard, the most pronounced TH+ reduction was observed with intervals of 2 or 7 days (with somewhat less after 14 days) interspersed between the poly(I:C) and later paraquat treatments.

Insert Figure 2 about here

In order to ascertain whether the loss of TH+ staining reflected a genuine neurodegenerative effect and whether the impact of paraquat was limited to dopamine neurons, all slides were counterstained with cresyl violet. In this regard, quantification of SNc cresyl-violet stained TH- neurons revealed no significant differences among the treatment groups ($F_{5,28}$=.804, $p>0.05$, $F_{5,29}$=1.791, $p>0.05$; bregma levels -3.08 and -3.28, respectively; Figure 3). Hence, it appears that the effects of paraquat were more selective

for TH+ neurons and that this was a genuine neurodegenerative effect. Indeed, one would expect increased cresyl violet TH- neuronal numbers if paraquat was simply inducing a suppression of TH expression in the absence of neuronal loss. However, if anything, paraquat provoked a trend towards reduced TH- neuronal counts that was reminiscent of that observed for the TH+ dopamine neurons. Furthermore, combined exposure to poly(I:C) and paraquat caused a more marked effect. Specifically, post hoc comparison revealed that mice that received paraquat 2 days following poly(I:C) infusion did display reduced TH- levels (at bregma level 3.28), relative to their saline treated counterparts ($p < 0.05$). Thus, although the effect were much more pronounced for TH+ SNc neurons, poly(I:C) infusion + paraquat treatment did appear to impact non-dopaminergic neurons.

Insert Figure 3 about here

Microglial assessment

Morphological changes in microglia were determined as a means of indicating differing degrees of glial activation, as a representation of the inflammatory state of the SNc. In order to assess morphological changes of microglia, SNc sections were immunostained using anti-CD11b (complement receptor marker) and rated for appearance. The state of microglial activation was determined by rating (with the rater completely blind to the treatments) using a 0-3 point rating scale with 0 representing the lowest state of activation (with thin highly ramified branches) and 3 signifying the highest (with amoeboid-like appearance). The ANOVA revealed that microglial ratings of morphological appearance approached significance as a function of treatment

($F_{5,19}$=2.596, p=.0595). As shown in Figure 4, Fisher's posthoc comparisons revealed that although paraquat and poly(I:C) alone did not affect microglial ratings, enhanced ratings of glial morphology were evident in mice that were pre-treated with the viral analogue and later exposed to the pesticide ($p < 0.05$). However, the time interval interspersed between the poly(I:C) and paraquat treatments did not influence this outcome. In general, the poly(I:C) – paraquat combination provoked changes in morphology that were essentially characteristic of an intermediate microglial activation; with moderately compacted soma with shortened and thickened projections.

Insert Figure 4 about here

In order to assess the oxidative potential of activated microglia, SNc sections were stained with anti- $gp91^{PHOX}$. Indeed, $gp91^{PHOX}$ is the inducible catalytic subunit of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase enzyme found on microglia. This membrane bound enzyme is responsible for the generation of the oxidative radical, superoxide. Presence of $gp91^{PHOX}$ immunoreactivity was rated in a blind fashion using a scale similar to that described above for CD11b. In brief, a scale of 0-3 was used, with 0 representing little to no $gp91^{PHOX}$ immunoreactivity, and 3 indicating very high activity. Indeed, $gp91^{PHOX}$ activity varied significantly between treatment groups ($F_{5,23}$=3.166, $p<0.05$). As shown in Figure 5, administration of poly(I:C) or paraquat alone induced a modest increase in $gp91^{PHOX}$ activity. However, combined exposure of poly(I:C) and paraquat induced a marked very obvious elevation of $gp91^{PHOX}$

immunoreactivity, particularly in mice that received poly(I:C) 2 days prior to the paraquat injections ($p < 0.05$), relative to saline treatment.

Insert Figure 5 about here

Striatal assessment

Densitometry measures were employed to quantify TH+ terminals in the striatum. Surprisingly, there were no differences found between groups with regards to striatal dopaminergic terminal coverage ($F < 1$; data not shown).

Given the lack of effect of paraquat upon striatal terminals, it was of interest of investigate the possibility that compensatory processes might have been up-regulated over time in response to the pesticide exposure. In this regard, previous studies found increased striatal neuronal branching and enhanced neurogenesis within the adjacent subventricular zone (SVZ) occurred in response to neuronal injury (Wei et al., 2011; Kreuzberg et al., 2010; Parent et al., 2002; Peng et al., 2008; Finkelstein et al., 2000). To this end, striatal sections were immunostained for doublecortin (DCX; a measure of immature neurons). However, the total number of DCX+ cells present in the SVZ ipsilateral to the poly(I:C) infusion did not significantly vary between the treatment groups ($F < 1$; data not shown). Similarly, there did not appear to be any DCX+ new neurons induced to migrate from the SVZ into the striatum ($F < 1$; data not shown).

Figure 1. *Home-cage locomotor activity varies between treatment groups.*
Total home-cage locomotor activity was monitored for 24h at 3 separate time points; 15 and 22 days following infusion as well as 24h prior to euthanization. No significant differences between treatments groups were found for the first and final micromax test ($F_{5,52}$=.736, p<.05; $F_{5,49}$=.599, p<.05, respectively). However, the middle time point (22 days after infusion), a significant treatment effect was seen for all treatments. * $p < .05$, relative to saline-treated controls. Data are expressed as a mean ± SEM; n=6-15

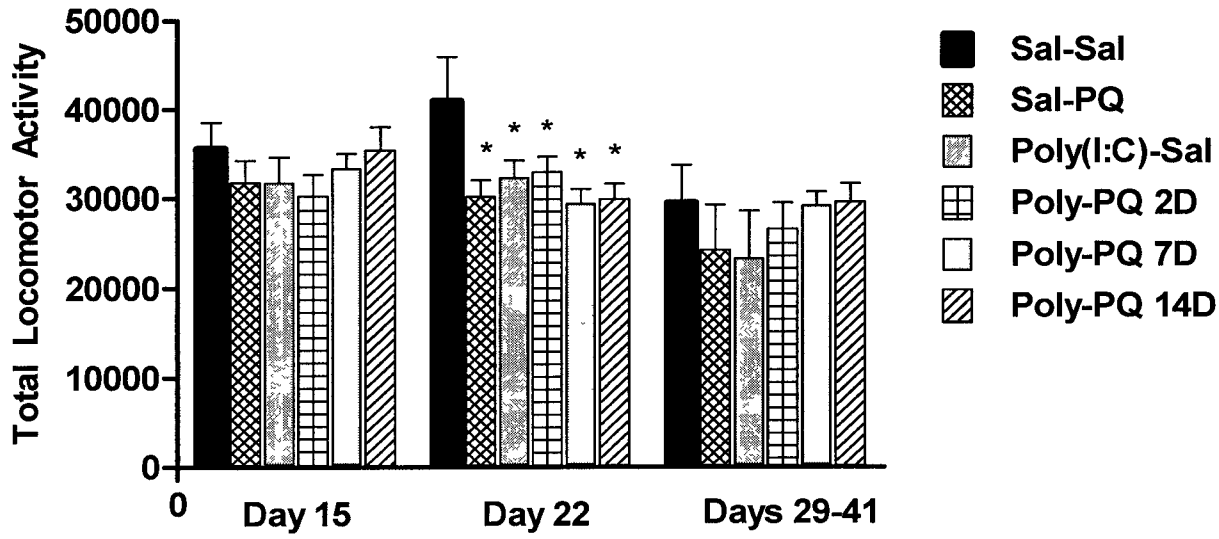
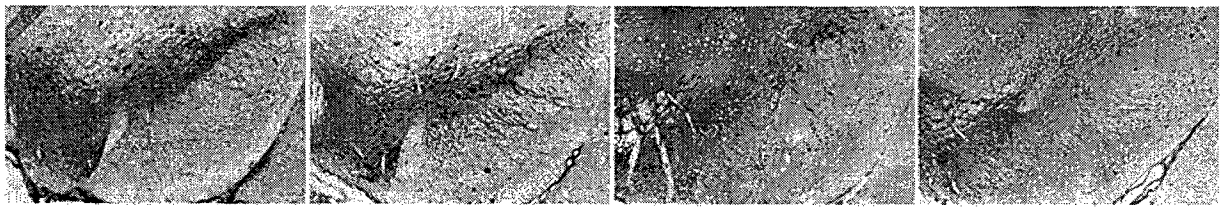

Total Locomotor Activity
50000
40000
30000
20000
10000
0
0
Day 15
Day 22
Days 29-41
Sal-Sal
Sal-PQ
Poly(I:C)-Sal
Poly-PQ 2D
Poly-PQ 7D
Poly-PQ 14D

Figure 2. *Priming of the SNc with poly(I:C) prior to paraquat exposure exacerbates dopaminergic neuron loss.*
SNc TH+ neurons were quantified from bregma level -3.08 to -3.40 for all treatment groups. Paraquat alone (i.p.; 10 mg/kg 3 times a week for 3 weeks) caused a modest, statistically insignificant effect relative to saline-treated controls. Likewise, a single supra-nigral infusion of poly(I:C) (5μg/2μl) provoked non-significant decrease in TH+ neurons. Combined exposure to poly(I:C) followed by paraquat induced a statistically significant reduction in TH+ neuronal counts, most notably at bregma levels -3.08 and -3.28. This degeneration was most prominent when poly(I:C) was given 2 or 7 days prior to the commencement of paraquat injections; by 14 days the neuronal loss became less pronounced. a) Representative photomicrographs demonstrating the degree of nigral TH+ neuron loss in paraquat, poly(I:C), and the combination treatment groups compared with saline-treated controls. b) Quantification of TH+ neuronal counts in the SNc for bregma levels -3.08 and -3.28 depicted in bar graphs. * $p < .05$, relative to saline-treated controls. All data expressed as mean ± SEM, n = 5-7

a) Sal-Sal Sal-PQ Poly (I:C)-Sal Poly (I:C)-PQ



b)

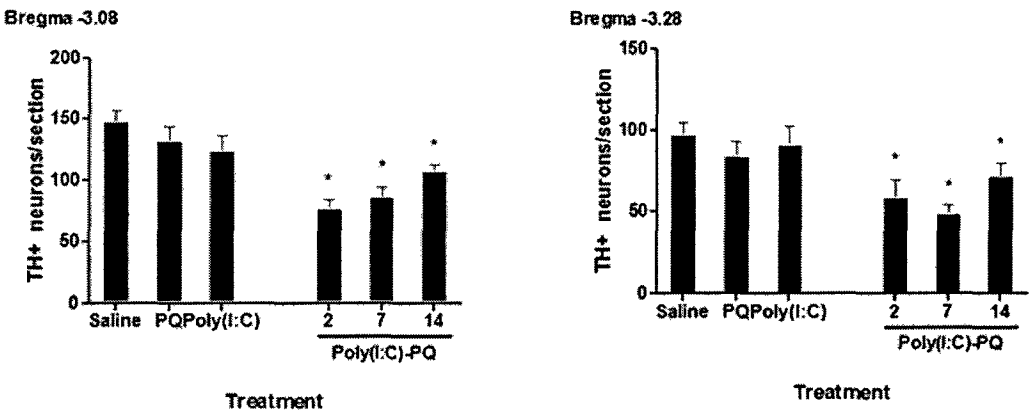
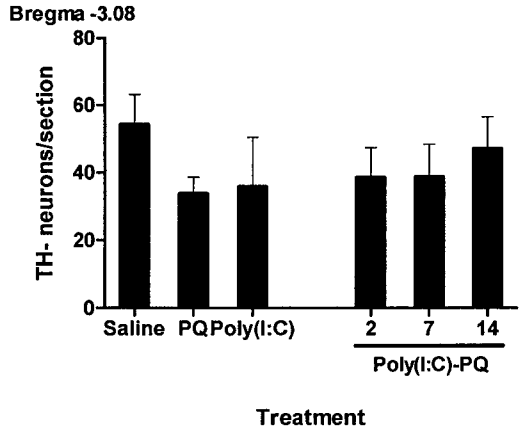


Figure 3. *Priming of the SNc with poly(I:C) prior to paraquat exposure results in variable modest nigral non-dopaminergic neuron loss.*
Cresyl-violet stained TH- neurons of the SNc from bregma level -3.08 to -3.40 were quantified as a measure of non-dopaminergic neurons. A trend in TH- neuronal counts was seen that approached statistical significance. Treament with paraquat alone (i.p., 10 mg/kg 3 times a week for 3 weeks) or nigral infusion of poly(I:C) (5μg/2μL) alone caused a modest non-significant decline in TH- counts across bregma levels. However, combined exposure to poly(I:C) followed by paraquat treatment resulted in a signifcant decrease in TH- counts that was evident at bregma -3.28 when poly(I:C) was administered 2 days prior to paraquat. * $p < .05$, relative to saline-treated controls. Data expressed as mean ± SEM, n =4-7.


Bregma -3.08
TH- neurons/section
80
60
40
20
0
Saline
PQ
Poly(I:C)
2
7
14
Poly(I:C)-PQ
Treatment

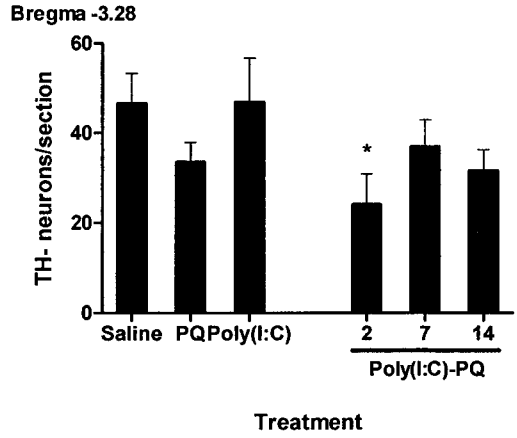
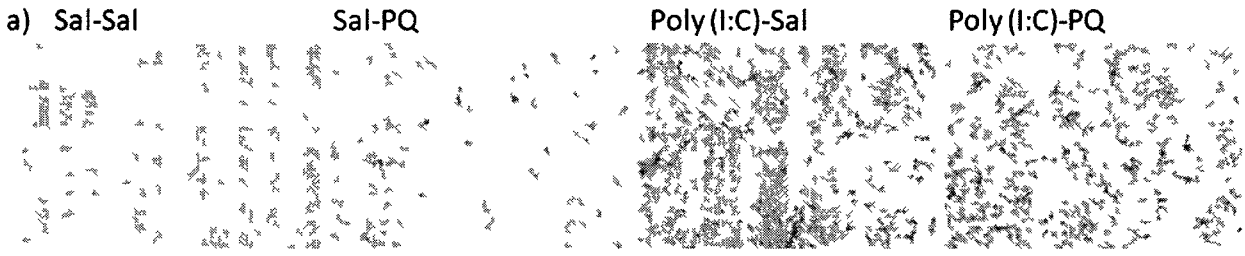

Bregma -3.28
TH- neurons/section
60
40
20
0
*
Saline
PQ
Poly(I:C)
2
7
14
Poly(I:C)-PQ
Treatment

Figure 4. *Intra-SNC administration of poly(I:C) followed by paraquat exposure resulted in microglial activation.*
SNc sections were stained with anti-Cd11b as a microglial marker. Sections were blindly rated using a scale of 0-3, a score of 0 representative of the lowest state of activation and 3 indicating a highly activated state. All sections were rated twice with >90% inter-rating overlap, and all scores per section were averaged as a representation of total nigral microglial activation. Paraquat alone (i.p., 10 mg/kg 3 times a week for 3 weeks) resulted in a modest elevation in microglial activation relative to saline-treated controls. However, a single intra-SNc infusion of poly(I:C) (5μg/2μL) produced marked microglial activation (although not statistically significant). The combination of poly(I:C) followed by paraquat induced significant microglial activation, regardless of the time interval between poly(I:C) infusion and the commencement of paraquat injections. a) Representative photomicrographs demonstrating the degree of nigral microglial activity in paraquat, poly(I:C), and the combination treatment groups compared with saline-treated controls. b) Quantification of arbitrary rating units of microglial activation for bregma levels -3.08 and -3.28 depicted in bar graphs. * $p < .05$, relative to saline-treated controls. Data expressed as mean ± SEM, n =4-5


a) Sal-Sal
Sal-PQ
Poly (I:C)-Sal
Poly (I:C)-PQ

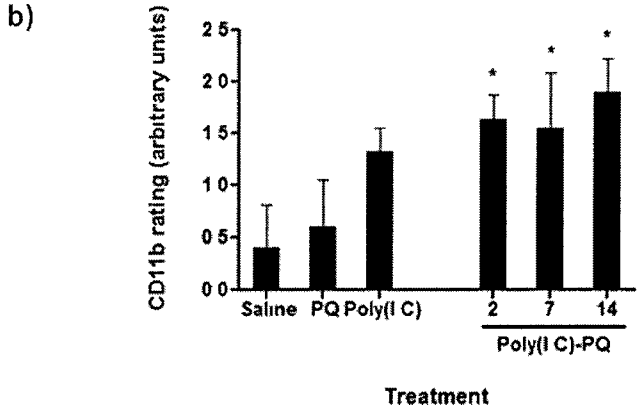
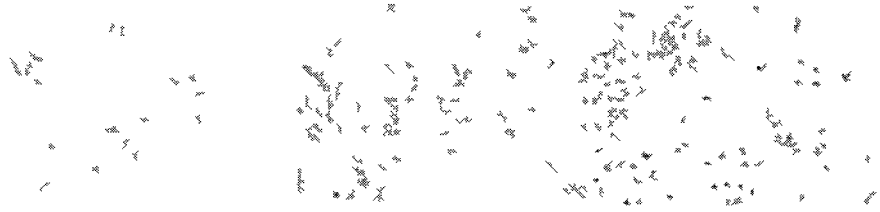
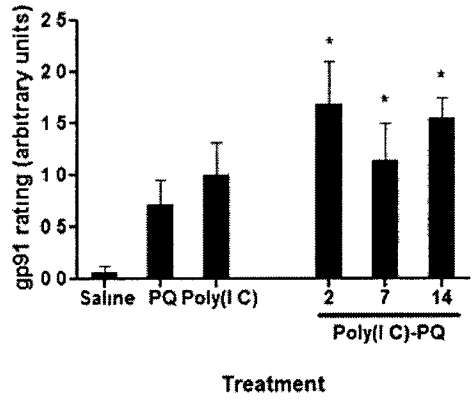

b)
CD11b rating (arbitrary units)
25
20
15
10
05
00
Saline
PQ
Poly(I C)
2
7
14
Poly(I C)-PQ
Treatment

Figure 5. *Intra-SNC administration of poly(I:C) followed by paraquat exposure significantly increased oxidative stress.*
SNc sections were stained with anti-gp91^{PHOX} as a marker of oxidative stress. Sections were blindly rated using a scale of 0-3, a score of 0 representative of the lowest induction of gp91^{PHOX} and a rating of 3 indicative of high oxidative stress. All sections were rated twice with >90% inter-rating overlap, and all scores per section were averaged as a representation of total nigral oxidative stress. Paraquat (i.p., 10mg/kg 3 times a week for 3 weeks) or a single intra-SNc infusion of poly(I:C) (5µg/2µL) led to a modest, non-significant increase in gp91^{PHOX}. However, the combination of poly(I:C) followed by paraquat resulted in significantly elevated expression of gp91^{PHOX} immunostaining relative to saline-treated controls. These effects were not affected by differing time points between poly(I:C) infusion and the first paraquat injection. a) Representative photomicrographs demonstrating the degree of gp91^{PHOX} immunoreactivity in paraquat, poly(I:C), and the combination treatment groups compared with saline-treated controls. b) Quantification of arbitrary rating scale units representating of gp91^{PHOX} immunoreactivity in the SNc for bregma levels -3.08 and -3.28 depicted in bar graphs. * $p < .05$, relative to saline-treated controls. Data expressed as mean ± SEM, n =4-6

a) Sal-Sal Sal-PQ Poly (I.C)-Sal Poly (I.C)-PQ



b)



Pole Test Data

	Trial 1		Trial 2		Trial 3	
	Latency to Turn	Latency to Descend	Latency to Turn	Latency to Descend	Latency to Turn	Latency to Descend
Saline	4.057±1.357	7.252±1.278	3.032±1.050	5.212±1.012	4.792±1.808	4.122±.681
PQ	4.89±1.329	7.100±.753	2.303±.589	4.944±.749	6.867±.939	4.576±.481
Poly (I:C)	5.568±1.655	6.543±1.340	4.184±.866	6.033±1.282	4.992±1.102	6.110±1.411
Poly (I:C)-PQ 2 Day	4.722±.837	5.392±.502	5.347±.928	4.072±.339	6.269±1.347	11.188±6.591
Poly (I:C)-PQ 7 Day	3.147±.634	6.663±.457	2.909±.720	5.727±1.068	4.906±2.641	11.455±6.558
Poly (I:C)-PQ 14 Day	2.745±.236	7.133±.514	5.243±1.380	7.887±2.832	7.180±1.809	6.392±1.613

Table 2. *Pole Test Data.*
Latency to turn and latency to descend for 3 pole test trials. Data expressed as mean ± SEM, n=8

Discussion

Several lines of evidence suggest that environmental events may be involved in the origins of Parkinson's disease (PD). Although genetic vulnerability may obviously play some role, accumulating evidence does suggest that exposure to certain environmental toxins, particularly pesticides, may be triggers for the production of PD (Betarbet et al., 2000; Giasson and Lee, 2000; Landrigan et al., 2005; Hatcher et al., 2008). Indeed, a much greater likelihood for developing PD occurs with pesticide exposure (Dick et al., 2007), and several other epidemiological studies have implicated specific pesticides, including rotenone (an organic insecticide) and paraquat (a chemical herbicide still widely used throughout the world) (Priyadarshi et al., 2001; Firestore et al., 2005; Fong et al., 2007; Dhillon et al., 2008; Costello et al., 2009). A sharp increase in PD incidence has been observed in agricultural areas using these pesticides (Tsui et al., 1991; Semchuk et al., 1992; Petrovitch et al., 2002; Baldi et al., 2003). Of course it should also be mentioned that high ambient air levels of bacterial products, mostly lipopolysaccharides, associated with livestock, insects and composted organic material are also present in such agricultural centres (Rylander, 2002). Similarly, viral infections have been epidemiologically linked to the development of PD and very recently certain human viruses have been shown to directly kill substantia nigra pars compacta neurons (SNc) in rodents (Jang et al., 2009). Indeed, infectious and non-infectious immunological agents alone can alter permeability of the blood brain barrier (BBB), affect neuron signalling through well circumscribed pathogen detection pathways and in sufficient

concentrations can damage midbrain dopamine neurons (Mangano and Hayley, 2009; Gao et al., 2002).

Interestingly, "immune toxins" activate many of the same CNS mechanisms as chemical insults, and hence, both categories of toxins may reinforce each other's actions on the brain. In this regard, we have shown, as have others, that administration of a bacterial insult greatly enhanced the neurotoxic effects of a subsequently applied chemical toxin (Carvey et al., 2003; Mangano & Hayley, 2009). In addition to a reduction in the overall number of neurons, early life or prenatal exposure to a bacterial endotoxin provoked a dramatic long-term sensitization of the inflammatory immune response, such that the neuroinflammatory and neurodegenerative actions of pesticides applied during adulthood were greatly enhanced (Ling et al., 2004; Barlow et al., 2007). Moreover, the risk for inflammatory-associated neurodegenerative disease is further increased in individuals with a history of traumatic head injury (or other cerebral trauma) with such insults further perpetuating neuroinflammatory cascades and the accumulation of toxic beta-amyloid and α-synuclein (Dick et al., 2007; Crawford et al., 2007; Uryu et al., 2003).

The basic hypothesis of the present investigation was that manipulation of the neuroinflammatory response using an agent that mimics viral infection (polyinosinic:polycytidylic acid (poly(I:C)) would augment the degree of dopamine neuronal death produced by the herbicide, paraquat, and that this effect would be connected to an exaggerated neuroinflammatory response within the SNc. As will be elaborated upon shortly, our findings did clearly support our hypothesis and are

consistent with a prominent role for inflammatory processes in the evolution of neuronal death following viral and environmental toxin exposure.

Viral and bacterial involvement in PD

Besides chemical agents, pathogenic microorganisms have been implicated in PD. Cases of parkinsonian-like syndromes have been associated with infections including, poliovirus, arbovirus, herpes simplex virus and encephalitis (Takahashi and Yamada, 1999; Tsui et al., 1991; Casals et al., 1998). A viral hypothesis proposed for PD has suggested the possibility that infection prenatally or early in life with some (yet to be discovered) latent virus(es) may instigate the disease (Takahashi and Yamada, 1999). The long incubation period and slow evolution of damage provoked by the virus could certainly be envisaged to correspond with the insidious time-course for PD onset. For instance, cases of Parkinsonism associated with von Economo encephalitis have been reported to occur years after infection (Dickman, 2001). Likewise, post-encephalitic cases of Parkinsonism that were associated with the influenza epidemic of 1918 (Casals et al., 1998) have been attributed to cytotoxic effects of the virus on the developing SNc within the intra-uterine environment (Mattock et al., 1988).

Cases of Parkinsonism have been reported in HIV infected individuals and it was suggested that accompanying infections, such as toxoplasmosis, may exacerbate the impact of the virus upon basal ganglia functioning (Koutsilieri et al., 2002). Another virus implicated in PD, the Japanese encephalitis virus, reduced the number of dopamine neurons and provoked marked gliosis within the SNc of infected rats (Ogata et al., 1997). The PD-like behavioral symptoms provoked by the Japanese encephalitis virus, most

notably bradykinesia, were significantly improved by L-DOPA treatment (Ogata et al., 1997).

In addition to viruses, prenatal or early life exposure to a pathogen of bacterial origin may also play a role in PD. In fact, recent animal studies have demonstrated that rats receiving prenatal administration of the bacterial endotoxin, lipopolysaccharide (LPS), displayed substantial degeneration of dopaminergic neurons (Carvey et al., 2003). It was also noted that rodents exposed to low concentrations of pesticides early in life were much more susceptible to the neurotoxic consequences of dopaminergic toxins later in life (Cory-Slechta et al., 2005). It may be that early exposure to immunogenic events (viral, bacterial or chemical) provokes mild neuroinflammation (e.g. microglial activation, cytokine release) that over time may cause neurodegeneration or render dopamine neurons vulnerable to degeneration in response to normally low grade insults (Carvey et al., 2003).

In the present investigation, we were interested in determining whether the viral analogue, poly(I:C) pre-treatment would time-dependently influence the neurodegenerative impact of later exposure to paraquat. Firstly, the rationale for using poly(I:C), which is essentially double stranded RNA (dsRNA; as occurs during intermediate steps of viral replication), is based upon numerous previous reports demonstrating that this agent induces immune and brain changes that closely mimic (albeit slightly more modest) those evident with a "true" live virus (Alexopoulou et al., 2001). A second more practical reason for working with poly(I:C) is out of concerns for safety, given that live viral work would require highly specialized containment facilities

and might not only pose a hazard for experimenters, but also animals themselves could develop systemic toxicity that could compromise the experimental findings.

Neuroinflammatory and neurodegenerative effects of poly(I:C)

Just as inflammation of the peripheral nervous system is characterized by an immediate neutrophilic infiltration, coupled with later (24-48 hours) mobilization of monocytic cells (Gabay 2006), CNS pathology also is typically associated with a marked neuroinflammatory response. In this regard, the most consistent finding across human post-mortem and animal toxin models of PD has been the basic finding of highly activated inflammatory microglia (the brain's own specialized macrophage-like immunocompetent cells) being associated with dopaminergic neurodegeneration (McGeer and McGeer, 2004; Langston et al., 1999; Erazi et al., 2011; Tufecki et al., 2011; Cichetti et al., 2009; Blandini et al., 2008). Similarly, emerging data are beginning to show that pro-inflammatory cytokines, particularly interferon (IFN)-γ and tumor necrosis factor (TNF)-α, likely regulate the deleterious effects of microglia in response to a host of environmental toxins that are relevant for PD (Mangano et al., 2011a; Mount et al., 2007; Barcia et al., 2011).

Recent work has specifically implicated the pro-inflammatory cytokine, IFN-γ, in dopamine neuronal loss and nigrostriatal dysfunction provoked by 1-methyl-4-phenyl-1,2,3,6-tetrahyropyridine (MPTP) administration in mice (Mount et al., 2007). The importance of this cytokine in PD likely stems from its ability to promote microglial oxidative responses; in fact, IFN-γ is considered a most potent mediator of microglial activation (Delgado, 2003). This is particularly relevant given that elevated levels of

IFN-γ, as well as other pro-inflammatory cytokines, including interleukin (IL)-1β and TNF-α have been reported in the blood, cerebrospinal fluid (CSF), and SNc of PD patients (Gribova et al., 2003; Mogi et al., 2007; Mount et al., 2007; Widner et al., 2002). Moreover, postmortem analyses of brain tissue revealed numerous signs of inflammation, including microglial activation and increased levels of several pro-inflammatory cytokines (IL-1, IL-2, IL-6 and TNF-α), as well as expression of elements of the complement cascade important in mediating antibody dependent cytotoxicity (Czlonkowska et al, 2002, 2005; Sawada et al., 2006; McGeer et al., 2002,2003).

Certain brain regions appear to be especially vulnerable to immune insults, as indicated by reports that infusion of the gram-negative bacterial endotoxin, LPS, directly into the motor regulatory SNc (but not other regions e.g., thalamus, cortex) provoked substantial degeneration of the local dopamine producing neurons (McGeer and McGeer, 2008). The particularly high concentration of microglia within the SNc, coupled with the finding that *in vitro* LPS exposure was only toxic to midbrain dopamine neurons in the presence of co-cultured microglia (Gao et al., 2003a, 2003b), emphasizes the importance of these inflammatory glial cells in the central effects of the endotoxin.

Our current data clearly revealed that poly(I:C) alone, as well as in conjunction with paraquat exposure did affect SNc dopamine neurons and microglia, as well as certain behavioural responses. Although poly(I:C) infusion alone (just above the SNc) caused a very modest non-significant reduction in the number of surviving SNc tyrosine hydroxylase (TH)+ dopamine neurons (at bregma level -3.08), it did promote robust microglial responses, in terms of both morphological status (using CD11b staining) and oxidative potential (assessed by elevated gp91 staining). This finding is in agreement

with previous reports reporting that a single poly(I:C) infusion (albeit of higher concentration) also led to a decrease of TH+ neurons in the SNc (Deleidi et al., 2010). However, the most dramatic effects were observed within the context paraquat exposure, wherein poly(I:C) pre-treatment greatly increased the degree of dopamine neuronal loss and microglial activation in response to later paraquat exposure.

As mentioned earlier in the thesis, this study was based upon our previous work demonstrating that LPS exposure greatly augmented the neuronal loss engendered by subsequent paraquat exposure (2 days after LPS) (Mangano & Hayley, 2009). In contrast to our previous findings using LPS, the present results indicated that timing between the poly(I:C) and paraquat administration did not appear to be of paramount importance. However, the 2 and 7 day delays following priming with the viral analogue were associated with a slightly (but not significantly) greater neurodegenerative effect being evident than when 14 days had elapsed before pesticide exposure. These findings are totally consistent with our previous findings demonstrating that LPS priming similarly sensitized the neurodegenerative effects of paraquat when exposure to the pesticide occurred at a time of maximal microglial activation (i.e. 2 days) (Mangano and Hayley, 2009). We currently found that poly(I:C) induced a more protracted activation of microglia within the SNc that was still evident after 14 days, whereas the effects of LPS upon CD11b+ microglia were more transient (evident after 2 days but returning towards baseline by 7 days) in the previous study. Although the time-course of microglial activation differed between LPS and poly(I:C), both studies showed a strong relationship between the SNc microglial response and extent of dopamine neuron loss within the SNc.

The fact that our previous study revealed that LPS actually had a neuroprotective effect at the level of the striatum when pre-treatment occurred 7 days prior to paraquat exposure underscores the importance of timing in shaping the neuronal impact of inflammatory insults. Indeed, mice that received LPS-paraquat (7 day) treatment had greater dopamine terminal coverage within the striatum than did mice that received paraquat alone (Mangano & Hayley, 2009). Correspondingly, the state of CD11b+ microglia had returned to resembling control mice by this 7 day time. However, the current poly(I:C) treatment induced a more prolonged microglial response that, once again, corresponded with the neurodegenerative effects. It is possible that employing a longer delay between poly(I:C) priming and paraquat exposure would result in a diminished microglial response and neuroprotective consequences as observed with LPS. Indeed, our working hypothesis is that the activation state of microglia at the time of initiation of the paraquat regimen is the critical factor influencing the degree of neuronal pathology induced. Specifically, and consistent with a multi-hit model of PD (Weidong et al., 2009; Thiruchelvam et al., 2000a; Ling et al., 2004), when organisms are exposed to multiple overlapping environmental insults, the state of the microenvironment shapes the impact of the insults. Indeed, when microglia are in an activated state they would likely be far more prone to release substantial deleterious oxidative and inflammatory factors when faced with subsequent insults while still in such a state. Conversely, it is possible that should microglia have passed from a highly activated state into their more quiescent "resting" state just prior to exposure to a second inducing stimulus, then they could conceivably become refractory to the second challenge or even up-regulate protective factors in an attempt to cope with the repeated perturbations.

Although paraquats most potent effects were restricted to TH+ SNc neurons, it should be mentioned that some TH- nigral neurons were also affected. While the loss of TH- neurons was not significant, a clear trend was apparent, most notably in animals treated with paraquat alone or the poly(I:C) and paraquat combination. These TH- cells likely represent a GABAergic neuronal population, given that GABAergic neurons occupy surrounding regions (McCormack et al., 2002). This observation suggests that paraquat-induced neurodegeneration may not be solely specific to dopamine neurons. While it is widely accepted that dopamine neurons are particularly vulnerable to increased oxidative stress and inflammation (Olanow and Tatton, 1999; Loeffler et al., 1994; Pei et al., 2007), it is possible that, given the marked pro-inflammatory and pro-oxidative state observed in the SNc, non-dopaminergic neurons can succumb to such a volatile microenvironment as well. Indeed, LPS infusion alone was sufficient to significantly decrease TH-negative neurons in the rat SNc (Liu et al., 2000), re-inforcing the idea that highly reactive microglia are sufficient to induce non-dopaminergic neuron death. However, the mechanisms behind this observation were unexpected and require further research.

Oxidative stress and PD

We report here that in response to paraquat or poly(I:C) treatment, alone or in combination, $gp91^{PHOX}$ immunoreactivity was increased. $gp91^{PHOX}$ is the inducible catalytic subunit of the microglial nicotinamide adenine dinucleotide phosphate (NADPH) oxidase enzyme, which in turn is responsible for the generation of superoxide (Babior, 1999). Therefore, the increased presence of $gp91^{PHOX}$ immunoreactivity

indicates an augmented ability of microglia to release the superoxide radical. Superoxide is dangerous because it is a reactive oxygen species (ROS) intermediate, acting as the starting material for generating a variety of highly toxic oxidants including hydrogen peroxide, hydroxyl radicals, singlet oxygen and peroxynitrite (Anrather et al., 2006). Indeed, stimulation of NADPH activity is the predominant mechanism through which microglia produce neurotoxic ROS (Block et al., 2007, Babior, 1999).

Oxidative stress has received much attention in PD research. Indeed, dopamine neurons in particular are vulnerable to oxidative stress, as dopamine metabolism itself yields hydrogen peroxide and other ROS (Olanow and Tatton, 1999). Futhermore, dopamine neurons have lower levels of glutathione (needed to clear hydrogen peroxide) compared with other cell types (Loeffler et al., 1994, Pei et al., 2007). Indeed, high oxidative damage has been seen in post-mortem PD brains (Jenner and Olanow, 1996). Alterations in brain iron content, impaired mitochondrial function, alterations in antioxidant protective systems (superoxide dismutase and reduced glutathione) and evidence of oxidative damage to lipids, proteins and DNA have been seen in PD brains (Jenner and Olanow, 1996). Post-mortem analysis of PD brains also revealed an up-regulation of gp91 within the SNc, suggesting that NADPH oxidase may be chronically activated in these patients (Gao et al., 2003a; Wu et al., 2003).

It is widely accepted that oxidative radicals play a critical role in paraquat-induced cell death. Paraquat is a redox cycling agent capable of generating large amounts of superoxide by electron transfer reactions with microglial NADPH-oxidase and nitric oxide synthase (NOS) (Day et al., 1999, Ramachandiran et al., 2007, Bonneh-Barkay et al., 2005). The degenerative effects of most dopamine neurotoxins, including paraquat,

can be completely prevented using mice lacking a functional NADPH oxidase or by administering pharmacological inhibitors for iNOS and NADPH prior to the administration of a toxin (Gao et al., 2003a, Gupta et al., 2010, Le et al., 2001, Liberatore et al., 1999). Indeed, murine microglia treated with paraquat demonstrated a time and dose-dependent release of superoxide that was entirely dependent on NADPH oxidase and NOS activity (Bonneh-Barkay et al., 2005). Furthermore, mice pre-treated with a superoxide dismutase mimetic prior to receiving a paraquat infusion into the SNc were completed protected from paraquat induced behavioural and neuropathological effects (Mollace et al., 2003).

Besides chemical toxins, it is well known that inflammatory immune challenges potently induce the production of oxidative stress factors. NADPH oxidase is an integral part of the antimicrobial host defense system and is designed to kill microbes, and therefore is expressed upon exposure to an immune challenge (Anrather et al., 2006). Indeed, NADPH oxidase-mediated generation of superoxide by activated microglia is a major contributor to LPS-induced neurotoxicity (Gao et al., 2002, Anrather et al., 2006). In fact, microglial-derived superoxide contributes to about 50% of LPS-induced dopamine neurotoxicity (Qin et al., 2004, Pei et al., 2007).

Taken together, the induction of $gp91^{PHOX}$ activity in response to poly(I:C), paraquat, or the combination that is reported in the current study is likely attributable to increased microglial activation in response to immune and/or toxin challenge.

Behavior and compensatory processes

Systemic exposure to paraquat has been shown to induce dose-dependent loss of dopamine neurons in the SNc of mice (McCormack et al 2002). However, there have been discrepant reports regarding the degree of degeneration in striatal terminals of mice exposed to paraquat, with some reporting no differences in nigrostriatal terminals when compared with control animals (Cichetti et al., 2005; McCormack et al 2002). In the present investigation, we found that regardless of treatment, paraquat, poly(I:C) or a combination failed to induce a loss of dopamine terminals in the striatum. In agreement with this observation was the general lack of behavioural effects observed. Indeed, there was only a transient reduction of home-cage activity evident and no significant changes in co-ordination produced by the treatments. The reduction of home-cage activity was seen during the second micromax test (22 days post-infusion) of the poly(I:C)-paraquat treatment regimen but was absent by the end of the experiment during the 24 hours period prior to sacrifice. Furthermore, a pole test, which is designed to tap into forelimb and hind-limb deficits, failed to reveal any signs of co-ordination impairment. In light of these observations, we sought to determine whether potential compensatory mechanisms might have been induced following the treatments.

It is conceivable that compensatory downstream processes provoked by soma loss (e.g. changes in dendritic branching patterns, up-regulation of neurotrophins, or alterations of brain monoamine systems) could account for the discrepant behavioural and striatal findings. This possibility has been suggested before (McCormack et al 2002, Cichetti et al 2005), however evidence of such compensatory mechanisms is sparse.

In order to investigate the possibility of new neurons in response to injury, immunohistochemical analysis for doublecortin (DCX) in striatal sections was employed.

Extensive evidence supports the existence of adult neurogenesis that is active throughout life (Im et al., 2010, Goldman, 1998). Specifically, the existence of neural stem cells and progenitors in the subventricular zone (SVZ) and the subgranular zone (SGZ) of the dentate gyrus of the adult mammalian brain has been reported (Goldman, 1998). Normally, newborn neurons migrate from the SVZ toward the olfactory bulb along the rostral migratory stream, where they continue to migrate radially until they reach their final destination and differentiate into GABAergic periglomerular and granule neurons (Im et al., 2010; Kreuzberg et al., 2010). However, SVZ neural stem cells have been shown to be recruited into the cortex or striatum in response to ischemic brain injury (Parent et al., 2002; Kreuzberg et al., 2010; Im et al., 2010). Moreover, stroke was reported to enhance SVZ neurogenesis (Kreuzberg et al., 2010). Indeed, focal stroke in adult rats showed markedly increased SVZ neurogenesis and chains of neuroblasts extending from the SVZ to the peri-infarct striatum (Parent et al 2002). Finally, neuroblasts in the injured striatum appear to differentiate into a region-appropriate phenotype (Parent et al., 2002). Increased neurogenesis in the SVZ and the SGZ has been reported in other neurodegenerative conditions as well, most notably in Alzheimers disease (Jin et al., 2004), and Huntington's disease (Curtis et al., 2003). Less evidence exists supporting the possibility of increased neurogenesis in a PD model, however, it has been reported that MPTP-treated mice exhibited increased neurogenesis (in the form of increased bromodeoxyuridine (BrdU) and DCX immuno-positive cells) in the SGZ, SVZ and striatum (Peng et al., 2008).

In light of these reports, the possibility of new neuronal recruitment to the striatum from the SVZ in response to striatal injury was of interest. DCX-positive cells

were counted in the in the SVZ, as well as the striatum, both ipsilateral to the infusion. No significant differences between treatment groups were found. The fact that MPTP is taken up in the striatal terminals of dopamine neurons (Nagatsu and Sawada, 2006), whereas paraquat is thought to act predominantly on SNc dopamine cell bodies, could account for the lack of paraquat-induced effects on striatal neurogenesis.

Based on these observations, it appears that the recruitment of new neurons is likely not accountable for the lack of striatal and behavioural deficits seen in the current study. However, other potential compensatory mechanisms should be investigated. For instance, microglia might enter a refractory condition or switch into a phenotypically distinct state once the effects of the insult subside. Microglia are often dichotomized into two unique phenotypic states, referred to as M1 and M2. M1 is the classic "activated" phenotype, wherein microglia are responding to some pathogenic or foreign threat and correspondingly up-regulate pro-inflammatory and pro-oxidative defence mechanisms (Michelucci et al., 2009). It is in this state that these glial cells are thought to exert their deleterious consequences on neighbouring neurons (Michelucci et al., 2009). In contrast, microglia are more often in the so called M2 state, in which they perform important housekeeping functions (e.g. scavenging cellular debris), provide nutritive support, buffer extra-cellular composition and express neurotrophic factors, such as brain derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF) (Davalos et al., 2005; Kumar and Jack, 2006; Nimmerjahn et al., 2005). Indeed, fibroblast growth factor (FGF)-2 levels were elevated within the striatum in parallel with the protection of striatal terminals observed with LPS pre-treatment 7 days before paraquat (Mangano and Hayley, 2009). It remains to be determined whether similar neurotrophic

or other protective mechanisms might be up-regulated with time following poly(I:C) administration.

The possibility of enhanced dendritic branching has been posited by others as well. Indeed, compensatory axonal sprouting of surviving striatal dopamine terminals following 6-OHDA lesioning has been reported (Finkelstein et al., 2000). Moreover, dopamine transporter (DAT) immunoreactive varicosities in the caudate putamen did not differ between controls and treated animals until SNc dopamine neuron loss surpassed 70% (Finkelstein et al., 2000). These findings suggest that substantial compensation in the form of sprouting and new dopaminergic synapse formation occurs following lesions of the SNc. Given the fact that cell loss in the present study did not exceed 70%, it remains plausible that compensation in the form of dendritic branching could account for the lack of striatal or behavioural deficits found.

Furthermore, toxin-induced injury could result in an increase in TH activity, which catalyzes the rate-limiting reaction in dopamine synthesis. Measurements of striatal TH activity have supported this idea, in which a significant paraquat-induced increase in TH activity was seen, as high as 70% (McCormack et al., 2002; Thiruchelvam et al., 2003). Moreover, 6-OHDA induced partial lesion of the nigrostriatal system was reported to provoke a time-dependent increase in both the synthesis and release of dopamine in surviving dopaminergic terminals (Blanchard et al., 1995). In fact, striatal TH protein content was significantly lower at 1 month post-lesion compared with 6 months post-lesioning, signifying a gradual increase in TH levels in response to nigrostriatal injury (Blanchard et al., 1995). Given these observations, it is possible that

enhanced dopamine synthesis and release may offset the effects of striatal terminal damage and aid in restoring healthy dopamine levels.

Overall, sufficient evidence exists to support the idea of multiple compensatory pathways in response to nigrostriatal injury in PD-like animal models. While we cannot say with certainty which, if any, compensatory changes could account for the protection of striatal dopamine terminals, as well as the lack of behavioural deficits seen in the current study, it is highly plausible that any of the factors described above may have offset any degenerative effects at the level of the striatum. Future studies investigating the possibility of new dendritic branching, as well as TH levels and dopamine turnover rates, would be of interest in a paraquat model of PD.

TLR-signaling and neurodegeneration

The role of the toll-like receptor (TLR)-4 agonist LPS in PD pathology has been studied extensively. However, despite the fact that epidemiological and experimental evidence suggests that viral exposure may contribute to PD onset and progression (Ravenholt and Foege, 1982; Jang et al., 2009), limited research has been done with respect to TLR3 activation. Therefore, the premise of this study aimed at further understanding the role of the viral-analogue, poly(I:C), in a toxin-based model of PD.

Studies have shown that immune infection may interact with environmental insults (i.e. pesticides) to produce exaggerated neuroinflammatory, neurodegenerative and behavioural changes in neurological patients (Liu et al., 2003). Indeed, co-administration of LPS with the pesticide, rotenone, synergistically augmented dopamine neuron degeneration in midbrain-microglial co-cultures (Gao et al., 2003b). Inflammatory

insults may also sensitize dopamine neurons to subsequent environmental toxin exposure. For example, animals pre-natally exposed to LPS were especially vulnerable to pesticide exposure (rotenone) later in life (Ling et al., 2004). Futhermore, infusion of LPS into the SNc sensitized dopamine neurons, such that the neurodegenerative effects of the pesticide paraquat were greatly increased when administered 2 days following the endotoxin (Mangano and Hayley, 2009). We report here that poly(I:C) also sensitized nigral dopamine neurons to subsequent paraquat exposure. This sensitization was most apparent when poly(I:C) was administered 2 or 7 days prior to paraquat exposure, although effects at 14 days were still significant.

Interestingly, poly(I:C) and LPS differ in their ability to sensitize dopamine neurons to further toxin exposure. As previously discussed, both TLR agonists produced a similar degree of nigral dopamine neuron loss at the 2 day priming time point (approximately 50%), whereas at the 7 day priming interval, they exhibited opposing effects (Mangano and Hayley, 2009). The transient vulnerability of dopamine neurons exposed to LPS could have to do with the relatively small dose used (0.1μg), whereas we used a much higher dose of poly(I:C) (5μg). However, differences between TLR3 and TLR4 activity in response to agonist binding have been reported (Doyle et al., 2003, Jack et al., 2005). Indeed, murine bone-marrow derived macrophages treated with TLR3 or TLR4 agonists demonstrated that TLR3 is capable of producing enhanced and sustained antiviral activity (i.e. induction of type-1 IFN gene expression) compared with TLR4 (Doyle et al., 2003). Unlike TLR4, TLR3 is able to enhance its own expression (via an IFN-β-mediated positive feedback loop) thereby promoting an even stronger antiviral response (Doyle et al., 2003). Moreover, cultured human microglia were found to

produce higher levels of IFN-β when treated with poly(I:C) compared with LPS (Jack et al., 2005).

Differences in adaptor protein binding and downstream signalling between TLR3 and TLR4 could account for differences in their immune activity. TLRs recognize pathogen associated molecular patterns (PAMPs) through activation of signalling cascades via Toll/IL-1 receptor (TIR) domain-containing adaptors, such as myeloid differentiation factor-88 (MyD88), TIR homology domain-containing adaptor protein (TIRAP) and TIR domain-containing adaptor inducing IFN-β (TRIF) (Yamamoto et al., 2003). These signalling cascades can lead to activation of phosphoinositide 3-kinase, Jun N-terminal kinase (JNK), p38, nuclear factor kappa B (NF-κB) and IFN regulatory factor (IRF)-3, ultimately leading to the induction of target genes involved in inflammation and antimicrobial activity (Doyle et al 2003, Takeuchi and Akira, 2001). The adaptor protein MyD88 is common to all TLRs, whereas TIRAP is specifically involved in TLR2- and TLR4-mediated signalling (Yamamoto et al., 2003). Indeed, inhibition of the TIRAP/MyD88 adaptor-like (MAL) signalling pathway has been shown to block TLR4 but not TLR3 signalling, supporting the notion that differing signalling pathways likely account for the different antiviral activity elicited by either TLR3 or TLR4 (Doyle et al., 2003). TRIF is a third adaptor protein that has recently been shown to activate IFN-β expression via TLR3 and TLR4 (Yamamoto et al., 2003). Given the fact that MyD88-dependent signalling has been shown to be dispensable for induction of IFN-β by TLR3, TLR3 likely mediates its effects through TRIF (Alexopoulou et al., 2001; Doyle et al., 2003). TLR3 and TLR4 have been shown to enhance IFN-β expression, as well as several IFN-inducible genes (RANTES, IP-10 and MCP-1) through TRIF (Yamamoto et al.,

2003). However, studies with cell cultures demonstrated an inability of TLR3 to activate NF-κB or JNK in the absence of TRIF, whereas TLR4 suffered only a minor decrease in their activation (Yamamoto et al., 2003). Overall, while downstream TLR signalling pathways are beyond the scope of the current study, differential adaptor protein binding and signalling activity likely play a key role in mediating the distinct effects of TLR3 versus TLR4 activity.

Another factor worth discussing is the role of other cell types in response to TLR agonist activation. TLRs are predominantly expressed on microglia, although recent evidence has shown that TLRs are also present on astrocytes, oligodendrocytes, and neurons (Okun et al., 2009). Indeed, our interests were primarily in the contribution of microglial-induced inflammation and oxidative factors in response to poly(I:C) priming, yet it remains possible that poly(I:C) acted through other cell types, or even directly on dopamine neurons. Indeed, high levels of TLR3 have been shown to be expressed on nigral dopamine neurons, and were up-regulated following intra-nigral poly(I:C) exposure (Deleidi et al., 2010). Interestingly, different TLR expression on various cell types has been documented. Specifically, human astrocytes have been shown to express high levels of TLR3, as well as selective TLR3 upregulation in response to pro-inflammatory cytokines or TLR agonists (Okun et al., 2009, Bsibsi et al., 2006). Furthermore, human astrocytes were found to respond solely to poly(I:C) treatment by secretion of IL-6, chemokine (C-X-C motif) ligand (CXCL)-10 and IFN-β, and exhibited no response to LPS (Jack et al., 2005). Indeed, a study conducted by Jack et al. (2005) suggests a pro-inflammatory and possibly detrimental contribution of astrocytes in response to TLR3 activation. Furthermore, TLR3 expressed on astrocytes demonstrated

an inflammatory response upon viral infection (Park et al., 2006). Indeed, rats intra-nigrally infused with poly(I:C) displayed highly activated astrocytes (Deleidi et al 2010). However, others have reported increased astrocytic production of neuroprotective mediators in response to poly(I:C) (Bsibsi et al., 2006). Taken together, these findings suggest that the difference between poly(I:C) and LPS priming may lie in their ability to act on various cell types, however much more work needs to be done in this area.

Conclusions

The present thesis underscores the importance of inflammatory and oxidative factors in the onset and progression of PD-like neurodegeneration. Furthermore, the current thesis reinforces the idea that PD likely originates from combined exposures to multiple environmental insults (possibly in combination with genetic vulnerabilities), which accumulate throughout life. Indeed, we have shown that a viral challenge rendered nigral dopamine neurons vulnerable to further toxin exposure. This enhanced toxin sensitivity likely stems from a viral-induced neuroinflammatory state, rendering midbrain dopamine neurons more susceptible to further inflammatory attack. Furthermore, we have shown that the severity of dopamine neuron loss was associated with highly activated microglia, as well as enhanced oxidative stress.

The results presented in the current study reinforce the idea that modulation of neuroinflammation is likely a key element in the treatment or prevention of PD. Future therapies for PD might be considered by targeting critical inflammatory mechanisms that are often triggered by environmental exposures to various immune or chemical toxins.

References

Alexopoulou L, Holt AC, Medzhitov R, Flavell RA. Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. Nature. 2001;413(6857):732-8.

Aloisi, F. Immune Function of Microglia. Glia 2001;36:165-179

Anrather J, Racchumi G, Iadecola C. NF-kappaB regulates phagocytic NADPH oxidase by inducing the expression of gp91phox. J Biol Chem. 2006;281(9):5657-67

Babior BM. NADPH oxidase: an update. Blood. 1999;93(5):1464-76

Baldi I, Cantagrel A, Lebailly P, Tison F, Dubroca B, Chrysostome V, Dartiques JF, Brochard P. Association between Parkinson's disease and exposure to pesticides in southwestern France. Neuroepidemiology 2003;22(5):305-310

Barcia C, Ros CM, Annese V, Gomez A, Ros-Bernal F, Aguado-Yera D, Martinez-Pagan ME, de Pablos V, Fernandez-Villalba E, Herrero MT. (2011) IFN-γ signalling, with the synergistic contribution of TNF-α, mediates cell specific microglial and astroglial activation in experimental models of Parkinson's disease. Cell Death Dis. 2:e142

Barlow BK, Cory-Slechta DA, Richfield EK, Thiruchelvam M. The gestational environment and Parkinson's disease: Evidence for neurodevelopmental origins of a neurodegenerative disorder. Reprod Toxicol 2007;23(3):457-70

Baquet ZC, Bickford PC, Jones KR. Brain-derived neurotrophic factor is required for the establishment of the proper number of dopaminergic neurons in the substantia nigra pars compacta. J Neurosci 2005;25(26):6251-9

Bessis A, Bechade C, Bernard D, Roumier A. Microglial control of neuronal death and synaptic properties. Glia 2007;55(3):233-8

Betarbet R, Sherer TB, MacKenzie G, Garcia-Osuna M, Panov AV, Greenamyre JT. Chronic systemic pesticide exposure reproduces features of Parkinson's disease. Nat Neurosci 2000;3(12):1301-6

Blanchard V, Chritin M, Vyas C, Savasta M, Feuerstein C, Agid Y, Janoy-Agid F, Raisman-Vozari R. Long-Term Induction of Tyrosine Hydroxylase Expression: Compensatory Response to Partial Degeneration of the Dopaminergic Nigrostriatal System in the Rat Brain. J Neurochem. 1995;64(4):1669-79

Blandini F, Armentero MT, Martignoni E. The 6-hydroxydopamine model: news from the past. Parkinsonism Relat Disord 2008;14(Suppl2):S124-9

Block ML, Zecca L, Hong JS. Microglia-mediated neurotoxicity: uncovering the molecular mechanisms. Nat Rev Neurosci. 2007;8(1):57-69

Bonneh-Barkay D, Reaney SH, Langston WJ, Di Monte DA. Redox cycling of the herbicide paraquat in microglial cultures. Brain Res Mol Brain Res. 2005;134(1):52-6

Braak H, Del Tredici K, Rub U, de Vos RA, Jansen Steur EN, Braak E. Staging of brain pathology related to sporadic Parkinson's disease. Neurobiol Aging 2003;24(2):197-211

Brooks AI, Chadwick CA, Gelbard HA, Cory-Slechta DA, Federoff HJ. Paraquat elicited neurobehavioural syndrome caused by dopaminergic neuron loss. Brain Res 1999;823(1-2):1-10

Bsibsi M, Persoon-Deen C, Verwer RW, Meeuwsen S, Ravid R, Van Noort JM. Toll-like receptor 3 on adult human astrocytes triggers production of neuroprotective mediators. Glia. 2006;53(7):688-95

Calne DB, Langston JW. Aetiology of Parkinson's disease. Lancet 1983;2(8365-66):1457-9

Carvey PM, Chang Q, Lipton JW, Ling Z. Prenatal exposure to the bacterotoxin lipopolysaccarhide leads to long-term losses of dopamine neurons in offspring: a potential, new model of Parkinson's disease. Front Biosci. 2003;8:s826-37

Casals J, Elizan TS, Yahr MD. Postencephalitic parkinsonism-a review. J Neural Transm. 1998;105(6-7):645-76

Castano A, Herrera AJ, Cano J, Machado A. Lipopolysaccharide intranigral injection induces inflammatory reaction and damage in nigrostriatal dopaminergic system. J Neurochem 1998;70(4):1584-92

Chen H, Jacobs E, Schwarzschild MA, McCullough ML, Calle EE, Thun MJ, Ascherio A. Nonsteroidal anti-inflammatory drug use and the risk for Parkinson's disease. Ann Neurol 2005;58(6):963-7

Chen H, Zhang SM, Herman MA, Schwarzschild MA, Willet WC, Colditz GA, Speizer FE, Acherio A. Nonsteroidal anti-inflammatory drugs and the risk of Parkinson disease. Arch Neurol 2003;60:1059-64

Cicchetti F, Drouin-Ouellet J, Gross RE. Environmental toxins and Parkinson's disease: what have we learned from pesticide-induced animal models? Trends Pharmacol Sci 2009;30(9):475-83

Cicchetti F, Lapointe N, Roberge-Tremblay A, Saint-Pierre M, Jimenez L, Ficke BW, Gross RE. Systemic exposure to paraquat and maneb models early Parkinson's disease in young adult rats. Neurobiol Dis. 2005;20(2):360-71

Conde JR, Streit WJ. Microglia in the aging brain. J Neuropathol Exp Neurol 2006a;65(3):199-203

Conde JR, Streit WJ. Effect of aging on the microglial response to peripheral nerve injury. Neurobiol Aging 2006b;27(10):1451-61

Cory-Slechta DA, Thiruchelvam M, Barlow BK, Richfield EK. Developmental pesticide models of the Parkinson disease phenotype. Environ Health Perspect. 2005;113(9):1263-70

Costello S, Cockburn M, Bronstein J, Zhang X, Ritz B. Parkinson's disease and residential exposure to maneb and paraquat from agricultural applications in the central valley of California. Am J Epidemiol 2009;169(8):919-26

Crawford FC, Wood M, Ferguson S, Mathura VS, Faza B, Wilson S, Fan T, O'Steen B, Ait-Ghezala G, Hayes R, Mullan MJ. Genomic analysis of response to traumatic brain inury in a mouse model of Alzheimer's disease (APPsw). Brain Res. 2007;1185:45-58

Crocker SJ, Smith PD, Jackson-Lewis V, Lamba WR, Hayley SP, Grimm E, Callaghan SM, Slack RS, Melloni E, Przedborksi S, Robertson GS, Anisman H, Merali Z, Park DS. Inhibition of calpains prevents neuronal and behavioural deficits in an MPTP mouse model of Parkinson's disease. J Neurosci 2003;23(10):4081-91

Curtis MA, Penney EB, Pearson AG, van Roon-Mom WM, Butterworth NJ, Dragunow M, Connor B, Faull RL. Increased cell proliferation and neurogenesis in the adult human Huntington's disease brain. Proc Natl Acad Sci U S A. 2003;100(15):9023-7

Czlonkowska A, Ciesielska A, Gromadzka G, Kurkowska-Jastrzebska I. Estrogen and cytokines production-the possible cause of gender differences in neurological diseases. Curr Pharm Des. 2005;11(8):1017-30

Czlonkowska A, Kurkowska-Jastrrzebska I, Czlonkowski A, Peter D, Stefano GB. Immune processes in the pathogenesis of Parkinson's disease-a potential role for microglia and nitric oxide. Med Sci Monit. 2002;8(8):RA165-77

Dammann O, Leviton A. Infection remote from the brain, neonatal white matter damage, and cerebral palsy in the preterm infant. Semin Pediatr Neurol 1998;5(3):190-201

Davalos D, Grutzendler J, Yang G, Kim JV, Zuo Y, Hung S, Littman DR, Dustin ML, Gan WB. ATP mediates rapid microglial response to local brain injury in vivo. Nat Neurosci 2005;8(6):752-8

Day BJ, Patel M, Calavetta L, Chang LY, Stamler JS. A mechanism of paraquat toxicity involving nitric oxide synthase. Proc Natl Acad Sci U S A. 1999;96(22):12760-5

Deleidi M, Hallett PJ, Koprich JB, Chung CY, Isacson O. The Toll-Like Receptor-3 Agonist Polyinosinic:Polycytidylic Acid Triggers Nigrostriatal Dopaminergic Degeneration. J Neurosci 2010;30(48):16091-101

Delgado M. Inhibition of intereferon (IFN) gamma-induced Jak-STAT1 activation in microglia by vasoactive intestinal peptide: inhibitory effect on CD40, IFN-induced protein-10, and inducible nitric-oxide synthase expression. J Biol Chem. 2003;278(30):27620-9

DeLong MR and Wichmann T. Circuits and Circuit Disorders of the Basal Ganglia Arch Neurol 2007;64(1): 20-4

Dhillon AS, Tarbutton GL, Levin JL, Plotkin GM, Lowry LK, Nalbone JT, Shepherd S. Pesticide/environmental exposures and Parkinson's disease in East Texas. J Agromedicine 2008;13(1):37-48

Dick FD, De Palma G, Ahmadi A, Scott NW, Prescott GJ, Bennett J, Semple S, Dick S, Counsell C, Mozzoni P, Haites N, Wettinger SB, Mutti A, Otelea M, Seaton A, Soderkvist P, Felice A. Environmental risk factors for Parkinson's disease and parkinsonism: the Geoparkinson study. Occup Environ Med 2007;64(10): 666-72

Dickman MS. von Economo encephalitis. Arch Neurol. 2001;58(10):1696-8

Doyle SE, O'Connell R, Vaidya SA, Chow EK, Yee K, Cheng G. Toll-like receptor 3 mediates a more potent antiviral response than Toll-like receptor 4. J Immunol. 2003;170(7):3565-71

Dunnett SB and Bjorklund A. Prospects for new restorative and neuroprotective treatments in Parkinson's disease. Nature 1999;399:A32-9

Dutta G, Zhang P, Liu B. The lipopolysaccharide Parkinson's disease animal model: mechanistic studies and drug discovery. Fundam Clin Pharmacol 2008;22(5):453-64

Erazi H, Ahboucha S, Gamrani H. Chronic exposure to aluminum reduces tyrosine hydroxylase expression in the substantia nigra and locomotor performance in rats. Neurosci Lett 2011;487(1):8-11

Esposito E, Di Matteo V, Benigno A, Pierucci M, Crescimanno G, Di Giovanni G. Non-steroidal anti-inflammatory drugs in Parkinson's disease. Exp Neurol 2007;205(2):295-312

Fernagut PO, Hutson CB, Fleming SM, Tetreaut NA, Salcedo J, Masliah E, Chesselet MF. Behavioral and histopathological consequences of paraquat

intoxication in mice: effects of alpha-synuclein over-expression. Synapse 2007;61(12):991-1001

Finkelstein DI, Stanic D, Parish CL, Tomas D, Dickson K, Horne MK. Axonal sprouting following lesions of the rat substantia nigra. Neuroscience. 2000;97(1):99-112

Firestone JA, Smith-Weller T, Franklin G, Swanson P, Longstreth WT Jr, Checkoway H. Pesticides and risk of Parkinson disease: a population-based case-control study. Arch Neurol 2005;62(1):91-95

Fong CS, Wu RM, Shieh JC, Chao YT, Fu YP, Kuao CL, Cheng CW. Pesticide exposure on southwestern Taiwanese with MnSOD and NQO1 polymorphisms is associated with increased risk of Parkinson's disease. Clin Chim Acta 2007;378(1-2):136-141

Gabay C. Interleukin-6 and chronic inflammation. Arthritis Res Ther. 2006;Suppl2:S3

Gagne JJ, Power MC. Anti-inflammatory drugs and risk of Parkinson disease: a meta-analysis. Neurology 2010;74(12):995-1002

Gambotto A, Baratt-Boyes SM, de Jong MD, Neumann G, Kawaoka Y. Human infection with highly pathogenic H5N1 influenza virus. Lancet 2008;371(9622):1464-75

Gao HM, Jiang J, Wilson B, Zhang W, Hong JS, Liu B. Microglial activation-mediated delayed and progressive degeneration of rat nigral dopaminergic neurons: relevance to Parkinson's disease. J Neurochem. 2002;81(6):1285-97

Gao HM, Hong JS. Why neurodegenerative diseases are progressive: uncontrolled inflammation drives disease progression. Trends Immunol 2008;29(8):357-65

Gao HM, Liu B, Hong JS. Critical role for microglial NADPH oxidase in rotenone-induced degeneration of dopaminergic neurons. J Neurosci. 2003a;23(15):6181-7

Gao HM, Hong JS, Zhang W, Liu B. Synergistic dopaminergic neurotoxicity of the pesticide rotenone and inflammogen lipopolysaccharide: relevance to the etiology of Parkinson's disease. J Neurosci. 2003b;23(4):1228-36

Giasson BI, Lee VM. A new link between pesticides and Parkinson's disease. Nat Neurosci 2000;3(12):1227-1228

Glezer I, Simard AR, Rivest S. Neuroprotective role of the innate immune system by microglia. Neuroscience. 2007;147(4):867-83.

Goldman SA. Adult neurogenesis: from canaries to the clinic. J Neurobiol. 1998;36(2):267-86

Gribova IE, Gnedenko BB, Poleshchuk W, Morozov SG. Level of interferon-gamma, tumor necrosis factor alpha, and antibodies to them in blood serum from Parkinson disease patients. Biomed Khim. 2003;49(2):208-12

Grinberg LT, Rueb U, Alho AT, Heinsen H. (2010) Brainstem pathology and non-motor symptoms in PD. J Neurol Sci 2010;(1-2):81-8

Gupta SP, Patel S, Yadav S, Singh AK, Singh S, Singh MP. Involvement of nitric oxide in maneb- and paraquat-induced Parkinson's disease phenotype in mouse: is there any link with lipid peroxidation? Neurochem Res. 2010;35(8):1206-13

Halliday G, Lees A, Stern M. Milestones in Parkinson's disease- Clinical and pathological features. Mov Disord 2011;26(6):1015-21

Halliday GM and Stevens CH. Glia: Initiators and Progressors of Pathology in Parkinson's Disease. Mov Disord 2011;26(10):6-17

Hamanaka J, Hara H. Involvement of Toll-Like Receptors in Ischemia-Induced Neuronal Damage. Cent Nerv Syst Agents Med Chem 2011;Epub ahead of print

Hatcher JM, Pennell KD, Miller GW. Parkinson's disease and pesticides: a toxicological perspective. Trends Pharmacol Sci 2008;29(6):322-329

Hirsch EC, Hunot S. Neuroinflammation in Parkinson's disease: a target for neuroprotection? Lancet Neurol 2009;8(4):382-97

Hirsch EC, Hunot S, Hartmann A. Neuroinflammatory processes in Parkinson's disease. Parkinsonism Relat Disord 2005;Suppl 1:S9-S15

Horowitz MP and Greenamyre JT. Gene-Environment Interactions in Parkinson's Disease: The Importance of Animal Modeling. Clin Pharmacol Ther 2010;88(4):467-74

Hunot S, Hirsch EC. Neuroinflammatory processes in Parkinson's disease. Ann Neurol 2003;53 (suppl 3):S49–S60

Im SH, Yu JH, Park ES, Lee JE, Kim HO, Park KI, Kim GW, Park CI, Cho SR. Induction of striatal neurogenesis enhances functional recovery in an adult animal model of neonatal hypoxic-ischemic brain injury. Neuroscience. 2010;169(1):259-68

Jack CS, Arbour N, Manusow J, Montgrain V, Blain M, McCrea E, Shapiro A, Antel JP. TLR signaling tailors innate immune responses in human microglia and astrocytes. J Immunol 2005;175(7):4320-30

Jang H, Boltz D, Sturm-Ramirez K, Shepherd KR, Jiao Y, Webster R, Smeyne RJ. Highly pathogenic H5N1 influenza virus can enter the central nervous system and induce neuroinflammation and neurodegeneration. Proc Natl Acad Sci U S A 2009;106(33):14063-8

Jenner P, Olanow CW. Oxidative stress and the pathogenesis of Parkinson's disease. Neurology. 1996;47(6 Suppl 3):S161-70

Jin K, Galvan V, Xie L, Mao XO, Gorostiza OF, Bredesen DE, Greenberg DA. Enhanced neurogenesis in Alzheimer's disease transgenic (PDGF-APPSw,Ind) mice. Proc Natl Acad Sci U S A. 2004;101(36):13363-7

Kawai T, Akira S. TLR signalling. Semin Immunol 2007;19:24-32

Kelada SN, Checkoway H, Kardia SL, Carlson CS, Costa-Mallen P, Eaton DL, Firestone J, Powers KM, Swanson PD, Franklin GM, Longstreth WT Jr, Weller TS, Afsharinejad Z, Costa LG. 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 2006;15(20):3055-3062

Koutsilieri E, Sopper S, Scheller C, ter Meulen V, Riederer P. Parkinsonism in HIV dementia. J Neural Transm. 2002;109(5-6):767-75

Kreutzberg GW. Microglia: a sensor for pathological events in the CNS. Trends Neurosci 1996;19: 312-318

Kreuzberg M, Kanov E, Timofeev O, Schwaninger M, Monyer H, Khodosevich K. Increased subventricular zone-derived cortical neurogenesis after ischemic lesion. 2010;226(1):90-9

Kumar S, Jack R. Origin of monocytes and their differentiation to macrophages and dendritic cells. J Endotoxin Res 2006;12(5):278-84

Landreth GE, Reed-Geaghan EG. Toll-like receptors in Alzheimer's disease. Curr Top Microbiol Immunol 2009;336:137-53

Landrigan PJ, Sonawane B, Butler RN, Trasande L, Callan R, Droller D. Early environmental origins of neurodegenerative disease in later life. Environ Health Perspect 2005;113(9):1230-1233

Langston JW, Ballard P, Tetrud JW, Irwin I. Chronic Parkinsonism in humans due to a product of meperidine-analog synthesis. Science 1983;219(4587):979-80

Langston JW, Forno LS, Tetrud J, Reeves AG, Kaplan JA, Karluk D. Evidence of active nerve cell degeneration in the substantia nigra of humans years after 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine exposure. Ann Neurol. 1999;46: 598–605

Le W, Rowe D, Xie W, Ortiz I, He Y, Appel SH. Microglial activation and dopaminergic cell injury: an in vitro model relevant to Parkinson's disease. J Neurosci. 2001;12(21):8447-55

Lee MS, Kim YJ. Signaling pathways downstream of pattern-recognition receptors and their cross talk. Annu Rev Biochem 2007;76:447-80

Lev N, Melamed E, Offen D. Apoptosis and Parkinson's disease. Prog Neuropsychopharmacol Biol Psychiatry 2003;27(2):245-50

Liberatore GT, Jackson-Lewis V, Vukosavic S, Mandir AS, Vila M, McAuliffe WG, Dawson VL, Dawson TM, Przedborski S. Inducible nitric oxide synthase stimulates dopaminergic neurodegeneration in the MPTP model of Parkinson disease. Nat Med. 1999;5(12):1403-9

Ling Z, Chang QA, Tong CW, Leurgans SE, Lipton JW, Carvey PM. Rotenone potentiates dopamine neuron loss in animals exposed to lipopolysaccharide prenatally. Exp Neurol 2004;190(2):373-83

Ling Z, Gayle DA, Ma SY, Lipton JW, Tong CW, Hong JS, Carvey PM. In Utero Bacterial Endotoxin Exposure Causes Loss of Tyrosine Hydroxylase Neurons in Postnatal Rat Midbrain. Mov Disord 2002;17(1):116-24

Liou HH, Tsai MC, Chen CJ, Jeng JS, Chang YC, Chen SY, Chen RC. Environmental risk factors and Parkinson's disease: a case-control study in Taiwan. Neurology 1997;48(6):1583-1588

Litteljohn D, Mangano E, Hayley S. Cyclooxygenase-2 deficiency modifies the neurochemical effects, motor impairment and co-morbid anxiety provoked by paraquat administration in mice. Eur J Neurosci 2008;28(4):707-16

Litteljohn D, Mangano E, Clarke M, Bobyn J, Moloney K, Hayley S. Inflammatory mechanisms of neurodegeneration in toxin-based models of Parkinson's disease. Parkinsons Dis 2010;713517

Liu B. Modulation of Microglial Pro-inflammatory and Neurotoxic Activity for the Treatment of Parkinson's Disease. AAPS J 2006;8(3):E606-21

Liu B, Gao HM, Hong JS. Parkinson's disease and exposure to infectious agents and pesticides and the occurrence of brain injuries: role of neuroinflammation. Environ Health Perspect. 2003;111(8):1065-73

Liu B, Jiang JW, Wilson BC, Du L, Yang SN, Wang JY, Wu GC, Cao XD, Hong JS. Systemic infusion of naloxone reduces degeneration of rat substantia nigral dopaminergic

neurons induced by intranigral injection of lipopolysaccharide. J Pharmacol Exp Ther 2000;295(1):125-32

Loeffler DA, DeMaggio AJ, Juneau PL, Havaich MK, LeWitt PA. Effects of enhanced striatal dopamine turnover in vivo on glutathione oxidation. Clin Neuropharmacol. 1994;17(4):370-9

Mangano E, Hayley S. Inflammatory priming of the substantia nigra influences the impact of later paraquat exposure: Neuroimmune sensitization of neurodegeneration. Neurobiol Aging 2009;l30(9):1361-78

Mangano EN, Litteljohn D, So R, Nelson E, Peters S, Bethune C, Bobyn J, Hayley S. Interferon-γ plays a role in paraquat-induced neurodegeneration involving oxidative and proinflammatory pathways. Neurobiol Aging 2011a;Epub ahead of print

Mangano EN, Peters S, Litteljohn D, So R, Bethune C, Bobyn J, Clarke M, Hayley S. Granulocyte macrophage-colony stimulating factor protects against substantia nigra dopaminergic cell loss in an environmental toxin model of Parkinson's disease. Neurobiol Dis 2011b;43(1):99-112

Manning-Bog AB, McCormack AL, Li J, Uversky VN, Fink AL, Di Monte DA. The herbicide paraquat causes up-regulation and aggregation of alpha-synuclein in mice: paraquat and alpha-synuclein. J Biol Chem 2002;277(3):1641-1644

Mattock C, Marmot M, Stern G. Could Parkinson's disease follow intra-uterine influenza?: a speculative hypothesis. J Neurol Neurosurg Psychiatry 1988;51(6):753-6

McCormack AL, Thiruchelvam M, Manning-Bog AB, Thiffault C, Langston JW, Cory-Slechta DA, Di Monte DA. Environmental risk factors and Parkinson's disease: selective degeneration of nigral dopaminergic neurons caused by the herbicide paraquat. Neurobiol Dis 2002;10(2):119-27

McCoy MK, Cookson MR. Mitochondrial quality control and dynamics in Parkinson's disease. Antioxid Redox Signal 2011;Epub ahead of print

McGeer PL and McGeer EG. Inflammation and neurodegeneration in Parkinson's disease. Parkinsonism Relat Disord 2004;1:S3-7

McGeer PL, McGeer EG. Innate immunity, local inflammation, and degenerative disease. Sci Aging Knowledge Environ. 2002;29:re3

McGeer PL and McGeer EG. Glial Reactions in Parkinson's Disease. Mov Disord 2008;23(4): 474-83

McGeer PL , Yasojima K , McGeer EG . Association of interleukin- 1 beta polymorphisms with idiopathic Parkinson's disease. Neurosci Lett 2002;326 : 67 - 69 .

McMillan PJ, White SS, Franklin A, Greenup JL, Leverenz JB, Raskind MA, Szot P. Differential response of the central noadrenergic nervous system to the loss of locus coeruleus neurons in Parkinson's disease and Alzheimer's disease. Brain Res 2011;1373:240-52

McNaught KS, Olanow CW, Halliwell B, Isacson O, Jenner P. Failure of the ubiquitin-proteosome system in Parkinson's disease. Nat Rev Neurosci 2001;2(8):589-94

Michelucci A, Heurtaux T, Grandbarbe L, Morga E, Heuschling P. Characterization of the microglial phenotype under specific pro-inflammatory and anti-inflammatory conditions: Effects of oligomeric and fibrillar amyloid-beta. J Neuroimmunol 2009;210(1-2):3-12

Miller RL, James-Kracke M, Sun GY, Sun AY. Oxidative and inflammatory pathways in Parkinson's disease. Neurochem Res 2009;34(1):55-65

Mogi M, Harada M, Narabayashi H, Inagaki H, Minami M, Nagatsu T. Interleukin (IL)-1 beta, IL-2, IL-4, IL-6 and transforming growth factor-alpha levels are elevated in ventricular cerebrospinal fluid in juvenile parkinsonism and Parkinson's disease. Neurosci Lett 1996;211(1):13-6

Mogi M, Kondo T, Mizuno Y, Nagatsu T. P53 protein, interferon-gamma, and NF-kappaB levels are elevated in the parkinsonian brain. Neurosci Lett. 414(1):94-7

Mogi M, Togari A, Kondo T, Mizuno Y, Komure O, Kuno S, Ichinose H, Nagatsu T. Brain-derived growth factor and nerve growth factor concentrations are decreased in the substantia nigra in Parkinson's disease. Neurosci Lett 1999;270(1):45-8

Mollace V, Iannone M, Muscoli C, Palma E, Granato T, Rispoli V, Nistico R, Rotiroti D, Salvemini D. The role of oxidative stress in paraquat-induced neurotoxicity in rats: protection by non peptidyl superoxide dismutase mimetic. Neurosci Lett. 2003;335(3):163-6

Mount MP, Lira A, Grimes D, Smith PD, Faucher S, Slack R, Anisman H, Hayley S, Park DS. Involvement of interferon-gamma in microglial-mediated loss of dopaminergic neurons. J Neurosci. 2007;27(12):3328-37

Nagatsu T, Mogi M, Ichinose H, Togari A. Changes in cytokines and neurotrophins in Parkinson's disease. J Neural Transm Suppl 2000;60:277-90

Nagatsu T and Sawada M. Inflammatory Process in Parkinson's Disease: Role for Cytokines. Curr Pharm Des 2005;11(8):999-1016

Nagatsu T, Sawada M. Cellular and molecular mechanisms of Parkinson's disease: neurotoxins, causative genes, and inflammatory cytokines. Cell Mol Neurobiol 2006;26(4-6):781-802

Nimmerjahn A, Kirchhoff F, Helmchen F. Resting Microglial Cells Are Highly Dynamic Surveillants of Brain Parenchyma in Vivo. Science 2005;308(5726):1314-8

Ogata A, Tashiro K, Nukuzuma S, Nagashima K, Hall WW. A rat model of Parkinson's disease induced by Japanese encephalitis virus. J Neurovirol. 1997;3(2):141-7

Okun E, Griffioen KJ, Lathia JD, Tang SC, Mattson MP, Arumugam TV. Toll-Like Receptors in Neurodegeneration. Brain Res Rev 2009;59(2): 278-292

Olanow CW, Tatton WG. Etiology and Pathogenesis of Parkinson's Disease. Annu Rev Neurosci 1999;22:123-44

Olson JK, Miller SD. Microglia initiate central nervous system innate and adaptive immune responses through multiple TLRs. J Immunol 2004;173(6):3916-24

Orr CF, Rowe DB, Halliday GM. An inflammatory review of Parkinson's disease. Prog Neurobiol 2002;68(5):325-340

Ouchi Y, Yoshikawa E, Sekine Y, Futatsubashi M, Kanno T, Ogusu T, Torizuka T. Microglial activation and dopamine terminal loss in early Parkinson's disease. Ann Neurol 2005;57:168-175

Parent JM, Vexler ZS, Gong C, Derugin N, Ferriero DM. Rat forebrain neurogenesis and striatal neuron replacement after focal stroke. 2002;52(6):802-13

Park C, Lee S, Cho IH, Lee HK, Kim D, Choi SY, Oh SB, Park K, Kim JS, Lee SJ. TLR3-mediated signal induces proinflammatory cytokine and chemokine gene expression in astrocytes: differential signalling mechanisms of TLR3-induced IP-10 and IL-8 gene expression. Glia. 2006;53(3):248-56

Pei Z, Pang H, Qian L, Yang S, Wang T, Zhang W, Wu X, Dallas S, Wilson B, Reece JM, Miller DS, Hong JS, Block ML. MAC1 mediates LPS-induced production of superoxide by microglia: the role of pattern recognition receptors in dopaminergic neurotoxicity. Glia. 2007;55(13):1362-73

Peng J, Peng L, Stevenson FF, Doctrow SR, Andersen JK. Iron and paraquat as synergistic environmental risk factors in sporadic Parkinson's disease accelerate age-related neurodegeneration. J Neurosci 2007;27(26):6914-6922

Peng J, Xie L, Jin K, Greenberg DA, Andersen JK. Fibroblast growth factor 2 enhances striatal and nigral neurogenesis in the acute 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine model of Parkinson's disease. 2008;153(3):664-70

Perry TL, Yong VW, Wall RA, Jones K. Paraquat and two endogenous analogues of the neurotoxic substance N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine do not damage dopaminergic nigrostriatal neurons in the mouse. Neurosci Lett 1986;69(3):285-9

Peterson AL, Nutt JG. Treatment of Parkinson's disease with trophic factors. Neurotherapeutics 2008;5(2):270-80

Petrovitch H, Ross GW, Abbott RD, Sanderson WT, Sharp DS, Tanner CM, Masaki KH, Blanchette PL, Popper JS, Foley D, Launer L, White LR. Plantation work and risk of Parkinson disease in a population-based longitudinal study. Arch Neurol 2002;59(11):1787-1792

Priyadarshi A, Khuder SA, Schaub EA, Priyadarshi SS. Environmental risk factors and Parkinson's disease: a metaanalysis. Environ Res 2001;86(2):122-127

Przedborski S, Dhawan V, Donaldson DM, Murphy PL, McKenna-Yasek D, Mandel FS, Brown RH Jr, Eidelberg D. Nigrostriatal dopaminergic function in familial amyotrophic lateral sclerosis patients with and without copper/zinc superoxide dismutase mutations. Neurology 1996;47(6): 1546-51

Purisai MG, McCormack AL, Cumine S, Li J, Isla MZ, Di Monte DA. Microglial activation as a priming event leading to paraquat-induced dopaminergic cell degeneration. Neurobiol Dis 2007;25(2):392-400

Qin L, Liu Y, Wang T, Wei SJ, Block ML, Wilson B, Liu B, Hong JS. NADPH oxidase mediates lipopolysaccharide-induced neurotoxicity and proinflammatory gene expression in activated microglia. J Biol Chem. 2004;279(2):1415-21

Racke MK, Drew PD. Toll-like receptors in multiple sclerosis. Curr Top Microbiol Immunol 2009;336:155-68

Radad K, Moldzio R, Rausch WD. Minocycline Protects Dopaminergic Neurons Against Long-Term Rotenone. Toxicity Can. J. Neurol. Sci 2010;37: 81-85

Ramachandiran S, Hansen JM, Jones DP, Richardson JR, Miller GW. Divergent mechanisms of paraquat, MPP+, and rotenone toxicity: oxidation of thioredoxin and caspase-3 activation. Toxicol Sci. 2007;95(1):163-71

Ravenholt RT, Foege WH. 1918 influenza, encephalitis lethargic, parkinsonism. Lancet 1982;2(8303):860-4

Rogers J, Mastroeni D, Leonard B, Joyce J, Grover A. Neuroinflammation in Alzheimer's disease and Parkinson's disease: are microglia pathogenic in either disorder? Int Rev Neurobiol 2007;82:235-246

Rylander R. Endotoxin in the environment- exposure and effects. J Endotoxin Res. 2002;8(4):241-52

Sawada M, Imamura K, Nagatsu T. Role of cytokines in inflammatory process in Parkinson's disease. J Neural Transm Suppl. 2006;70:373-81

Sedelis M, Hofele K, Auburger GW, Morgan S, Huston JP, Schwarting RK. MPTP susceptibility in the mouse: behavioural, neurochemical and histological analysis of gender and strain differences. Behav Genet 2000;30(3):171-82

Semchuk KM, Love EJ, Lee RG. Parkinson's disease and exposure to agricultural work and pesticide chemicals. Neurology 1992;42(7):1328-1335

Singh M, Khan AJ, Shah PP, Shukla R, Khanna VK, Parmar D. Polymorphism in environment responsive genes and association with Parkinson disease. Mol Cell Biochem 2008;312(1-2):131-138

Shi M, Huber BR, Zhang J. Biomarkers for cognitive impairment in Parkinson disease. Brain Pathol 2010;20(3):660-71

Shulman JM, De Jager PL, Feany MB. Parkinson's Disease: Genetics and Pathogenesis. Annu. Rev. Pathol. Mech. Dis 2011;6:193-222

Takahashi M, Yamada T. Viral etiology for Parkinson's disease-a possible role of influenza A virus infection. Jpn J Infect Dis. 1999;52(3):89-98

Takeuchi, O, Akira S. Toll-like receptors: their physiological role and signal transduction system. Int. Immunopharmacol.2001;1:625.

Tanner CM, Kamel F, Ross GW, Hoppin JA, Goldman SM, Korell M, Marras C, Bhudhikanok GS, Kasten M, Chade AR, Comyns K, Richards MB, Meng C, Priestley B, Fernandez HH, Cambi F, Umback DM, Blair A, Sandler DP, Langston JW. Rotenone, paraquat, and Parkinson's disease. Environ Health Perspect 2011;119(6):866-72

Tansey MG, McCoy MK, Frank-Cannon TC. Neuroinflammatory mechanisms in Parkinson's disease: potential environmental triggers, pathways, and targets for early therapeutic intervention. Exp Neurol 2008;208(1):1-25.

Teismann P, Schulz JB. Cellular pathology of Parkinson's disease: astrocytes, microglia and inflammation. Cell Tissue Res 2004;318(1):149-161

Thiruchelvam M, Brockel BJ, Richfield EK, Baggs RB, Cory-Slechta DA. Potentiated and preferential effects of combined paraquat and maneb on nigrostriatal dopamine systems: environmental risk factors for Parkinson's disease? Brain Res 2000a;873(2):225-234

Thiruchelvam M, Richfield EK, Baggs RB, Tank AW, Cory-Slechta DA. The nigrostriatal dopaminergic system as a preferential target of repeated exposures to combined paraquat and maneb: implications for Parkinson's disease. J Neurosci 2000b;20(24):9207-9214

Thirulchelvam M, McCormack A, Richfield EK, Baggs RB, Tank AW, Di Monte DA, Cory-Slechta DA. (2003) Age-related irreversible progressive nigrostriatal dopaminergic neurotoxicity in the paraquat and maneb model of the Parkinson's disease phenotype. Eur J Neurosci. 18(3):589-600

Trudler D, Farfara D, Frenkel D. Toll-Like Receptors Expression and Signaling in Glia Cells in Neuro-Amyloidogenic Disease: Towards Future Therapeutic Application. Mediators Inflamm 2010;ID 497987

Tsui JK, Calne DB, Wang Y, Schulzer M, Marion SA. Occupational risk factors in Parkinson's disease. Can J Public Health 1991;90:334-337

Tufekci KU, Genc S and Genc K. The Endotoxin-Induced Neuroinflammation Model of Parkinson's Disease. Parkinsons Dis 2011;Jan 18; 2011:487450

Ungerstedt U, Arbuthnott GW. Quantitative recording of rotational behavior in rats after 6-hydroxy-dopamine lesions of the nigrostriatal dopamine system. Brain Res 1970;24(3):485-93

Uryu K, Giasson BI, Longhi L, Martinez D, Murray I, Conte V, Nakamura M, Saatman K, Talbot K, Horiquchi T, McIntosh T, Lee VM, Trojanowski JQ. Age-dependent synuclein pathology following traumatic brain injury in mice. Exp Neurol. 2003;184(1):214-24

Uversky VN, Li J, Fink AL. Pesticides directly accelerate the rate of alpha-synuclein fibril formation: a possible factor in Parkinson's disease. FEBS Lett 2001;500(3):105-108

Uversky VN, Li J, Bower K, Fink AL. Synergistic effects of pesticides and metals on the fibrillation of alpha-synuclein: implications for Parkinson's disease. Neurotoxicology 2002;23(4-5):527-536

Wei B, Nie Y, Li X, Wnag C, Ma T, Huang Z, Tian M, Sun C, Cai Y, You Y, Liu F, Yang Z. Emx1-expressing neural stem cells in the subventricular zone give rise to new interneurons in the ischemic injured striatum. Eur J Neurosci. 2011:33(5):819-30

Weidong L, Chen S, Jankovic J. Etiopathogenesis of Parkinson's Disease: A New Beginning? Neuroscientist 2009;15(1):28-35

Weisskopf MG, Weuve J, Nie H, Saint-Hilaire MH, Sudarsky L, Simon DK, Hersh B, Schwartz J, Write RO, Hu H. Association of cumulative lead exposure with Parkinson's disease. Environ Health Perspect 2010;118(11):1609-13

Whitton PS (2007) Inflammation as a causative factor in the aetiology of Parkinson's disease. Br J Pharmacol 2007;150(8):963-76

Widner B, Leblhuber F, Fuchs D. Increased neopterin production and tryptophan degradation in advanced Parkinson's disease. J Neural Transm. 2002;109(2):181-9

Wilk JB, Tobin JE, Suchowersky O, Shill HA, Klein C, Wooten GF, Lew MF, Mark MH, Guttman M, Watts RL, Singer C, Growdon JH, Latourelle JC, Saint-Hilaire MH, DeStefano AL, Prakash R, Williamson S, Berg CJ, Sun M, Goldwurm S, Pezzoli G, Racette BA, Perlmutter JS, Parsian A, Baker KB, Giroux ML, Litvan I, Pramstaller PP, Nicholson G, Burn DJ, Chinnery PF, Vieregge P, Slevin JT, Cambi F, MacDonald ME, Gusella JF, Myers RH, Golbe LI. Herbicide exposure modifies GSTP1 haplotype association to Parkinson onset age: the GenePD Study. Neurology 2006;67(12):2206-2210

Wu DC, Jackson-Lewis V, Vila M, Tieu K, Teismann P, Vadseth C, Choi DK, Ischiroulos H, Przedborksi S. Blockade of microglial activation is neuroprotective in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine mouse model of Parkinson disease. J Neurosci 2002;22(5):1763-71

Wu DC, Teismann P, Tieu K, Vila M, Jackson-Lewis V, Ischiropoulos H, Przedborksi S. 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. 2003;100(10):6145-50

Wyss-Coray T, Mucke L. Inflammation in neurodegenerative disease--a double-edged sword. Neuron 2002;35(3):419-432

Yamamoto M, Sato S, Hemmi H, Hoshino K ,Kaisho T, Sanjo H, Takeuchi O, Sugiyama M, Okabe M, Takeda K, Akira S. Role of Adaptor TRIF in the MyD88-Independent Toll-Like Receptor Signaling Pathway. Science 2003;301: 640.

Yang W, Tiffany-Castiglioni E. The bipyridyl herbicide paraquat induces proteasome dysfunction in human neuroblastoma SH-SY5Y cells. J Toxicol Environ Health A 2007;70(21):1849-1857

Zarow C, Lyness SA, Mortimer JA, Chui HC. Neuronal loss is greater in the locus coeruleus than nucleus basalis and substantia nigra in Alzheimer and Parkinson diseases. Arch Neurol 2003;60(3):337-41