Neuroinflammation potentates the degenerative effects of environmental toxins upon the substantia nigra pars compacta: a role for pro-inflammatory cytokines

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Abstract

Most cases of Parkinson's disease (PD) are idiopathic, with several environmental factors potentially contributing to disease onset and progression, including pesticides, heavy metals and viral or bacterial infections. For instance, the pesticide, paraquat induced a loss of dopamine (DA) neurons within the substantia nigra pars compacta (SNc) in exposed rodents. Since dopaminergic toxins and immune challenges implicated in PD may stimulate similar neuroinflammatory pathways, it is of interest to assess the interactive effects between these challenges. To this end, experiments were conducted to determine the individual and interactive effects of paraquat and the bacterial endotoxin, lipopolysaccharide (LPS) upon survival of DA neurons, as well as glial activation and cytokine variations. Accordingly, mice received either saline or paraquat (5 mg/kg or 10 mg/kg, 3 times/week for 3 weeks) or infusion of LPS (0.1μg or 3.0μg) into the SNc and were sacrificed at several different times. In a separate experiment, animals were infused with either saline or LPS into the SNc and then 2 days later received the saline or paraquat treatments. It was found that paraquat caused dose-dependent loss of DA neurons within the SNc coupled with signs of motoric disturbances consistent with a PD-syndrome, including impaired muscle tone, gait and posture. At the same time, paraquat elevated levels of several cytokines within the SNc, striatum and plasma including the pro-inflammatory cytokines, TNF–α, IL-1β, IL-6, as well as the anti-inflammatory cytokine, IL-10. Interestingly, pre-treatment with LPS attenuated these paraquat induced cytokine alterations and concomitantly prevented the loss of DA terminals within the striatum associated with the pesticide, suggesting a neuroprotective de-sensitization effect was engendered by the endotoxin. Taken together, these data suggest that multiple inflammatory and environmental insults may interact to shape the evolution of PD and that pro-inflammatory cytokines may be important mediators of this disease.

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Figure 15. Intra-SNc infusion of LPS provoked alterations of IL-1β and IL-2 levels within plasma upon later exposure to paraquat. Intra-SNc infusion of LPS followed by chronic paraquat exposure resulted in a de-sensitization of IL-1β only with the lower (5 mg/kg) of paraquat. Paraquat alone seemed to cause a non-significant increase in the levels of circulating IL-1β; however pre-treatment with LPS significantly prevent this rise in IL-1β. A similar pattern was observed with IL-2, where paraquat alone caused a significant increase in the levels of IL-2 with exposure to the lower (5 mg/kg) and higher (10 mg/kg) dose of paraquat, which was significantly attenuated with pre-treatment with LPS, $*p < 0.05$ relative to saline treated mice.

Figure 16. Intra-SNc infusion of LPS provoked alterations of TNF-α, IL-6 and -10 levels within plasma upon later exposure to paraquat. Pre-treatment with an intra-SNc infusion of LPS attenuated the elevation of circulating TNF- α caused by chronic paraquat exposure, resulting in a de-sensitization of TNF-α only with the higher (10 mg/kg) of paraquat. A similar pattern was observed with IL-10, where paraquat alone caused a non-significant increase in the levels of IL-10, which was significantly attenuated with pre-treatment with LPS. The levels of IL-6 remained unchanged with exposure to paraquat or with pre-treatment of LPS. It seems that LPS alone did not provoke alterations of any of the cytokines, $*p < 0.05$ relative to saline treated mice.

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1. Introduction

1.1 Background on Parkinson's disease

After Alzheimer's disease, Parkinson's disease (PD) is the most common age-related neurodegenerative disorder (Nagatsu and Sawada, 2005). According to a study conducted by the National Institute of Neurological Disorders and Stroke in 2004, more then 500 000 Americans are afflicted with PD and internationally, approximately 17 out of 100 000 people per year are diagnosed with the disease (Landrigan et al., 2005). Less than 10% of all PD cases appear to be familial, with the majority of cases being of unknown origin or idiopathic. A large scale twin study was conducted in order to determine the genetic and the environmental risks involved in both types of PD. Not surprisingly, this study found that environmental risk factors do not play an important role in familial PD, whereas the incidence of idiopathic PD was correlated with exposure to environmental toxins found in certain areas, such as agricultural centers and industrial manufacturing plants (Landrigan et al., 2005; Longroscino et al., 2005; Nagatsu and Sawada, 2005).

Pathologically, both familial and idiopathic PD are characterized by selective loss of dopaminergic neurons of the substantia nigra pars compacta (SNc) resulting in reduced striatal dopamine (DA) and the cardinal clinical features of PD (Betarbet et al., 2000; Beutler et al., 1999; Bonnet and Houeto, 1999; Brebner et al., 2000; Hirsch et al., 1998; Langdrigan et al., 2005). PD is a disorder of the motor system characterized by dysregulation of basal ganglia functioning. In this respect, dis-inhibition of striatal interneurons provides faulty input to the globus pallidus and thalamus, ultimately

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culminating in reduced activation of motor regulatory cortical regions (Blandini et al., 2000). Interestingly, the debilitating motor deficits such as tremor, slow and decreased movements (bradykinesia), muscle stiffness, paucity of voluntary movements, and postural instability only become evident after approximately 60-80% loss of dopaminergic neurons (Blandini et al., 2000; Bonnet and Houeto, 1999; Di Monte, 2003; Hirsch et al., 1998; Hunot and Hirsch, 2003; Langston et al., 1983).

PD is also characterized by the presence of intracellular eosinophilic inclusion bodies referred to as Lewy bodies (Manning-Bog et al., 2003). These inclusions are primarily composed of fibrillar α-synuclein (α-SN), a ubiquitous protein. Studies using transgenic mice expressing mutated forms of human α-SN, have reported degeneration of DA neurons and motor deficits similar to those seen in PD (Przedborski et al., 1996). However, others have reported no effect of α-SN or that the protein may actually have neuroprotective properties (Cicchetti et al., 2005; Manning-Bog et al., 2003; Striet, 2004).

1.2 Neurotoxin animal models of PD: MPTP and 6-OHDA

The two most commonly used and widely validated animal models of PD are those involving 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and 6-hydroxydopamine (6-OHDA). Essentially, MPTP is a thermal breakdown product of a meperidine-like form of synthetic heroin that was accidentally discovered to induce Parkinsonism in a group of drug users in the early 1980s (Landrigan et al., 2005; Salach et al., 1984). Systemic exposure to MPTP has been demonstrated in numerous studies

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over the past two and a half decades to provoke SNc dopaminergic degeneration coupled with depletion of striatal dopamine in mice and primates (Landrigan et al., 2005; Przedborski et al., 1996; Rowe et al., 1998). The behavioral symptoms associated with MPTP namely, akinesia, tremor and impaired gait, are similar to those seen with PD patients. MPTP is highly lipophilic; it readily crosses the blood brain barrier (BBB) and is quickly metabolized by monoamine oxidase. It is converted into its active metabolite MPP+ and taken up by the dopamine transporter (DAT), where it has neurotoxic effects on the dopamine cell (Landrigan et al., 2005; Salach et al., 1984). Early studies with MPTP determined that MPP+ inhibits complex I of the mitochondrial electron transport chain.

In contrast to MPTP, 6-OHDA is not able cross the BBB. Typically it is directly infused into the either the SNc or striatum where it exerts deleterious effects on local neurons and terminals (Hirsch et al., 2003). 6-OHDA is a hydroxylated analogue of dopamine that may be generated by auto-oxidation of endogenous dopamine (Blum et al., 2001). It seems plausible that the highly reactive metabolic nature of dopamine itself may contribute to PD neurodegeneration.

1.3 Environmental Risk factors associated with PD: Pesticides

Epidemiological studies have concluded that there is a higher risk of developing PD with exposure to certain environmental toxins. In particular, well water, farming, air pollutants, heavy metals (e.g. iron, manganese) and rural living have all been linked with PD (Caals et al., 1998; Cicchetti et al., 2005; Di Monte, 2003; Peng et al., 2004; Rochet

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et al., 2004; Seonghan et al., 2004; Takahashi et al., 1989; Thiruchelvam et al., 2000; Wu et al., 2003). Moreover, etiological links have been documented between PD and pesticide use. Undeniably, accumulating evidence has linked exposure to commonly used pesticides and PD (Andersen, 2003; Cicchetti et al., 2005; Thiruchelvam et al., 2000; Wu et al., 2003). Along these lines, animal studies have found that continuous infusion of rotenone, using osmotic minipumps, elicited degeneration of SNc dopaminergic neurons and destruction of non-dopaminergic neurons within the basal ganglia and brainstem (Betarbet et al., 2000). More recent studies have also correlated high clusters of PD in rural areas with high usage of the herbicide paraquat and / or fungicide maneb (Cicchetti et al., 2005; Thiruchelvam et al., 2000). These toxins are commonly used in overlying areas and it has been suggested that they may interact synergistically to increase the risk of PD-onset (Andersen, 2003; Cicchetti et al., 2005; Thiruchelvam et al., 2000; Wu et al., 2003).

1.4 Paraquat as a novel pesticide model of PD

The present series of experiments involve manipulations of the herbicide paraquat, as a model of idiopathic PD. This pesticide may provide a model of this disease superior to that of existing models (e.g. MPTP) since it provokes histopathological changes closely aligned to PD and has greater ecological validity. In fact, paraquat is the third most commonly used pesticide in the world. Presently, thirteen countries have recognized the dangerous side-effects of both direct and indirect exposure to paraquat and have banned the use of this herbicide. Curiously, Canada and the United States are not included in this

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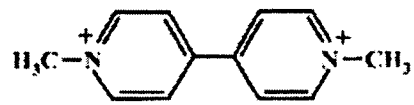
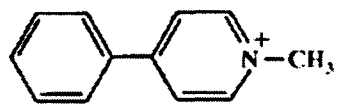
list (Cicchetti et al., 2005). Paraquat is chemically similar to the active metabolite of MPTP, MPP+ (Figure 1). While both paraquat and MPP+ provoke depletion of the nigrostriatal dopaminergic neurons, post-mortem and in vivo studies have shown that only paraquat provokes Lewy body formation (Blandini et al., 2000; Lawerence et al., 1998; Manning-Bog et al., 2002; McCormack et al., 2002; Thiruchelvam et al., 2000; Tsui et al., 1991). Paralleling the epidemiological findings, several studies have found that repeated systemic administration of the pesticide provoked selective destruction of SNc dopamine neurons. Surprisingly, striatal dopamine (DA) levels were not altered by paraquat, suggesting that compensatory mechanisms may have been provoked by the surviving neurons (McCormack et al., 2002). It is presently unclear how paraquat impacts neuronal survival. However, accumulating evidence suggests that activation of neuroinflammatory cascades plays an important role in the degeneration of dopamine (DA) neurons caused by paraquat (Casals et al., 1998; Di Monte, 2003; Takahashi et al., 1989 and 1999; Thiruchelvam et al., 2000, Tsui et al., 1991).



Paraquat



MPP^+

FIG. 1. Chemical structures of paraquat and MPP^+.

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1.5 Mechanisms of neurodegeneration: PD toxin models

Three interrelated mechanisms may underlie the deleterious neurodegenerative effects caused by MPTP, 6-OHDA and certain pesticides: (1) inhibition of complex I of the mitochondrial respiratory chain, (2) direct oxidative stress factors and (3) provocation of neuroinflammatory cascades. Alterations in the mitochondrial respiratory chain are possible sources of reactive oxygen species (ROS); free radicals are subsequently generated and require ionization by antioxidants (Calabrese et al., 2000; Hunot et al., 1996; Jenner et al., 2001; Przedborski et al., 1996). If free radical accumulation exceeds that of antioxidants, the cell undergoes a state of oxidative stress, resulting in extensive damage to DNA, proteins, and lipids (Calabrese et al., 2000; Hunot et al., 1996; Jenner, 2001). Interestingly, all of the toxins mentioned above are inhibitors of complex I of the mitochondrial respiratory chain.

Rotenone and MPTP are particularly potent complex I inhibitors, hindering oxidation of nicotinamide adenine dinucleotide (NAD+) substrates and α-ketoglutarate dehydrogenase, culminating in various detrimental alterations, such as decreased ATP levels, loss of mitochondrial membrane potential, faulty intracellular calcium buffering and free radical generation (Blum et al., 2001). Paraquat can be reduced intracellularly by NADPH-cytochrome P-450 reductase to the cation radical form of paraquat, which in turn, causes an electron transfer to molecular oxygen, ultimately creating the superoxide anion (Figure 2). This reaction has the ability to generate substantial amounts of ROS (DeGray et al., 1991; McCormack et al., 2005). Accumulation of ROS and chronic oxidative stress may be particularly relevant to PD, given that diminished activity of complex 1 in the mitochondrial respiratory chain coupled with elevated levels of

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oxidative stress factors have been reported in the SNc of Parkinsonian patients (DeGray et al., 1991; McCormack et al., 2005).

$$PQ^{2+} + e^- \rightarrow PQ^{\cdot+}$$

$$PQ^{\cdot+} + O_2 \rightarrow PQ^{2+} + O_2^{\cdot-}$$

Figure 2. *The role superoxide plays in paraquat-mediated cytotoxicity.* One electron is transferred from paraquat to molecular oxygen creating superoxide, which is then free to interact with other free radicals and elicit cellular damage.

Although mitochondrial dysfunction itself can elicit oxidative neuronal damage, 6-OHDA and MPTP can provoke oxidative stress independent of the mitochondria. Postmortem analysis of PD brains showed compelling evidence of oxidative stress by displaying diminished levels of reduced glutathione and oxidative modifications to DNA, lipids, and proteins (Sherer et al., 2003). 6-OHDA can generate cytotoxic metabolites, such as quinones, superoxide radicals, hydrogen peroxide and the hydroxyl radical in a manner similar to endogenous dopaminergic auto-oxidation and interactions with monoamine oxidase (Blum et al., 2001). 6-OHDA is taken up into the DA neuron via high affinity reuptake mechanisms; it can directly damage neurons by means of amplifying the amount of free radicals due to its cytotoxic metabolites, ultimately causing oxidative stress.

Likewise, accumulation of free radicals directly attributed to rotenone-induced dopaminergic damage was attenuated with pre-treatment of an anti-oxidant (e.g. alpha-tocopherol) (Sherer et al., 2003). Systemic MPTP and paraquat can induce superoxide as well as nitric oxide (NO) formation, which together can create the highly reactive and destructive peroxynitrate radical (Beal et al., 1998). As well, MPTP may also impair the

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functioning of endogenous protective free radical scavengers, such as glutathione, metallothionein and manganese superoxide dismutase (Beal et al., 1998).

Finally, neuroinflammatory processes also may contribute to oxidative damage provoked by environmental toxins (Czlonkowska et al., 2002; Kurkowska-Jastrzebska et al., 1999). In this respect, it is of interest to mention that each of the environmental agents used to induce experimental Parkinsonism also elicited profound immune activation and neuroinflammation. This is not surprising given that a primary role of immunological functioning is to rid the body of such environmental antigens. Accordingly, excessive activation of central and peripheral immune factors (such as cytokines) stimulated by these challenges may contribute to neuronal tissue damage evident in PD.

1.6 Neuroinflammation and PD: Microglia and Astrocytes

The brain is considered to be an immune-privileged site since it is protected by the BBB. However, immune responses do, in fact, normally occur within the brain and involve a small number of infiltrating leukocytes as well as resident glial cells. Neuroinflammation typically refers to activation of brain processes that resemble innate mediated immune responses of the periphery (Thiruchelvam et al., 2000; et al., Tsui et al., 1991). The brain's glial cells (astrocytes and microglia) are the resident immune cells of the central nervous system (CNS). Astrocytes provide glia-neuron contact, maintain ionic homeostasis and buffer excess neurotransmitters and neurotrophic factors. Conversely, microglia act as immuno-surveillance cells and serve as the host defense mechanism against invading microorganisms and foreign substances. Both types of glial cells release

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pro-inflammatory messengers, called cytokines, such as interleukins (ILs), interferons (IFNs) and tumor necrosis factor-α, upon detection of injury or infection. These pro-inflammatory messengers are capable of amplifying and sustaining an immune response locally (at glial cells) or through orchestrating the activation and possibly central infiltration of circulating leukocytes (McCormack et al., 2002; Seongham et al., 2004).

Under normal physiological conditions, resting microglia has a morphological appearance characterized by thin, highly ramified processes. In response to injury or infection, microglial cells become activated and undergo morphological changes and adopt an amoeboid-like appearance with enhanced expression of surface receptors, including the major histocompatability complex and complement receptors (Hirsch et al., 2003; Nagatsu and Sawada, 2005; Przedborski et al., 1996; Yang et al., 2005). Microglia act in a fashion similar to peripheral macrophages, serving to eliminate foreign pathogens through phagocytosis, up-regulation of antigen presentation complexes (MHC II), as well as the production of prostaglandins and oxidative factors, such as nitric oxide (NO) and superoxide. Highly activated microglia are considered the hallmark of neuroinflammation, and these cells serve as the only fully immunocompetent cells within the brain.

It has become clear that chronic activation of microglia for extended periods of time has neurotoxic consequences. Both *in vivo* and *in vitro* procedures have demonstrated that 6-OHDA, MPTP (or its metabolite MPP+) and rotenone can induce substantial activation of microglia (Czlonkowska et al., 2002; Gao et al., 2002; Kurowska-Jastrzebska et al., 1999; Le et al., 2001). Profound microgliosis was detected in the SNc of monkeys exposed to systemic MPTP treatment 5 to 14 years earlier, suggesting that progressive

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long-term; neuroinflammatory processes may be associated with relatively brief toxin exposure (McGeer et al., 1995). As well, microglia were significantly amplified in mice treated acutely with systemic MPTP (four 10 mg/kg doses spaced 1 hr apart) with morphological changes indicative of activation (e.g. cellular thickening) that was evident for up to 4 and 14 days within the striatum and SNc, respectively (Kurowska-Jastrzebska et al., 1999; Sugama et al., 2003). It is of interest to note that this microglial response occurred long before dopaminergic neuronal death was evident (14-21 days). This imparts more support for the neuroinflammatory model of PD. It suggests that the inflammatory response seen in both postmortem analysis of PD brains and animal studies is associated with deleterious effects, rather than serving only as neurotrophic support. Additionally, advancing age, which is a clear risk factor for PD, has also been associated with profound microglia activation, whereby older mice (9-12 months) displayed greatly enhanced microglial reactivity following MPTP relative to younger animals (3 months) (Sugama et al., 2003).

In addition to MPTP, 6-OHDA and certain pesticides, the bacterial endotoxin, lipopolysaccharide (LPS) has been also demonstrated to induce the selective loss of dopaminergic SNc neurons through activation of microglia (Carvey et al., 2003; Kim et al., 2000; Landrigan et al., 2005). In fact, infusion of LPS into the hippocampus, thalamus and cortex of rats did not induce substantial neuronal loss, suggesting that SNc dopaminergic neurons are especially vulnerable to immunogenic insults. The particularly high concentration of microglia within the SNc may contribute to the enhanced vulnerability of these dopamine neurons (Kim et al., 2000; Landrigan et al., 2005). In vitro experiments revealed that the neurodegenerative consequences of LPS on

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mesencephalic neurons were only evident in the presence of co-cultures including microglia (Landrigan et al., 2005). Since LPS potently induces circulating cytokine production and may also stimulate central cytokine expression, this may be one mechanism through which the endotoxin causes dopaminergic degeneration (to be discussed shortly).

To determine the mechanistic role of activated microglia in dopaminergic loss, several co-culture systems have been established using a combination of embryonic ventral mesencephalic neurons and post-natally obtained microglia. Using this approach, microglia (but not astrocytes) co-cultured with mesencephalic neurons were found to contribute to MPTP provoked neuronal injury (Gao et al., 2002). Their deleterious effects were linked to NADPH oxidase, the main ROS-producing enzyme during inflammation (Wu et al., 2003). In this regard, neurons obtained from mice genetically lacking NADPH or treated with the pharmacological inhibitor, apocynin, were largely resistant to MPTP toxicity (Gao et al., 2003). Likewise, knockout mice lacking molecular subunits (gp91pnox) required for functioning of NADPH oxidase, were resistant to rotenone induced dopaminergic loss (Gao et al., 2003).

Corresponding to the *in vitro* data, NADPH oxidase is increased within the SNc of human PD patients and MPTP treated rodents (Wu et al., 1999). Similarly, NADPH oxidase deficient mice displayed substantially less dopaminergic neuron loss in response to systemically delivered MPTP as compared to wild type treated animals (Wu et al., 1999). Furthermore, systemic MPTP treatment promotes microglial inducible nitric oxide synthase (iNOS) expression, ultimately increasing the production of cellular NO. This neuronal messenger is suspected of being one of the many sources of toxic free

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radicals in the brain, since it reacts with superoxide forming peroxynitrite, which evidently is an extremely potent oxidizing agent (Calabrese et al., 2000; Hunot et al., 1996). Studies using mice lacking iNOS display substantially reduced dopaminergic loss (Rowe et al., 1998). Treatment with minocycline, a tetracycline derivative that inhibits microglial activation, prevented MPTP induced nigrostriatal degeneration and reduced levels of NADPH oxidase, iNOS and nitrotyrosine (marker of NO activity) expression (Wu et al., 2003). Accordingly, inflammatory microglia reactivity likely contributes to ongoing degeneration through the release of highly reactive oxidative species.

1.7 Neuroinflammation and PD: adaptive immune mechanisms

In contrast to the role of glial mediated inflammatory processes in PD, much less attention has focused upon the impact of adaptive immune responses in the disease. Indeed, cytokines may orchestrate lymphocyte activity and influence adaptive responses (e.g. antibody mediated complement deposition, cell mediated cytotoxicity) that may damage CNS tissue. T-lymphocytes are mainly peripheral immune agents that possess receptors to govern cell mediated immune responses (McGeer and McGeer, 1995). Definitive evidence supporting the presence of T lymphocytes within PD brain is lacking. There is some degree of alteration in peripheral lymphocyte immunity, including reduced levels of T cells within the bloodstream and impaired proliferative responses to mitogens in PD patients (Czlonkowska et al., 2002).

Post-mortem analyses have indicated that major histocompatability complex (MHC) class II-positive reactive microglial cell levels are elevated within the SNc and MHC class 1

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within the striatum of PD patients (Baba et al., 2005; Hunot and Hirsch et al., 2003; Lekiw and Bazan, 2000). MHC-II acts as a marker for microglial activation and is specific for CD4 helper T cells, which are designed to help initiate immune responses to antigen presentation. On the other hand, MHC class I initiates immune responses to intracellular viral or bacterial infections by mobilizing CD8 cytotoxic T cells (Castano et al., 2002; Hirsch et al., 2003; Hunot and Hirsch, 2003). Specifically, this finding provides evidence for CD4-CD8 T cell infiltration across the BBB, thus suggesting the possibility of cross-talk between glial cells and T cells within the brain parenchyma (Hunot and Hirsch, 2003). A minute amount of CD8-positive T lymphocytes have been discovered in the vicinity of degenerating DA neurons in the SNc of PD patients. Likewise, systemic MPTP induced expression of the pro-inflammatory cytokine, IFN-γ, which among other things is a potent regulator of MHC-II functioning and antigen presentation pathways (Baba et al., 2005; Hunot and Hirsch, 2003). Additionally, it has been estimated that 30% of PD patients have auto-antibodies reactive against basal ganglia neurons, raising the possibility of humoral antibody involvement in the disease (Abramsky and Litvin, 1978).

A fascinating emerging neuroimmune theory of PD suggests that formation of neoepitopes (new antigens) within the basal ganglia recruits a specific destructive immune reaction (Lee et al., 2000; Rothwell, 1999). Specifically, accumulation of toxic dopamine auto-oxidation metabolites, particularly quinone, may cause tissue alterations favoring neoepitope creation (Lee et al., 2000). Consistent with this proposition, antibodies from a subset of PD patients but not subjects with other neurological diseases, recognized epitopes from dopamine quinone modified proteins (Rothwell, 1999). Thus,

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PD patients displayed antibodies against neoepitopes produced by altered dopamine metabolism, which could contribute to or amplify ongoing inflammatory and degenerative response (Rothwell, 1999). Animal studies revealed that direct intra-SNc infusion of purified antibodies from PD patients but not age matched disease controls, induced complement activation and dopaminergic neuronal death in several rodent species (He et al., 2002). Importantly, these effects were prevented in mice lacking the Fc receptors, which are critical for antibody mediated activation of microglial cells (He et al., 2002). Indeed, through binding and clustering of the Fc membrane antibody receptors, specific antibodies can provoke the release of oxidative species, such as superoxide radicals, from microglia. Together, these findings raise the possibility that intricate disturbances of humoral or cellular branches of immunity may influence progression of PD. However, at this point it remains to be determined if these immunological alterations play a primary role in genesis of the condition, or rather are recruited as secondary messengers stemming from ongoing neuronal death/survival processes.

1.8 Cytokine cascades and Parkinson's disease: Mechanisms of neurodegeneration

Cytokines have been implicated in acute and chronic cell death (Barone et al., 1998; Rochet et al., 2004). Clinical studies revealed increased levels of pro-inflammatory cytokines in post-mortem brains as well as in blood of patients with PD, stroke, head injury, multiple sclerosis, and Alzheimer's (Barone et al., 1998; Bruce et al., 1996; Rochet et al., 2004; Sheng et al., 1998; Yang et al., 2005). Although these findings have been reiterated in animal models it is still uncertain whether these cytokines a

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neuroprotective or neurodestructive role. It may be that relatively low endogenous cytokine levels act in a protective capacity to buffer against damage related to death processes, whereas relatively high levels of these factors may contribute to neuronal damage (Bruce et al., 1996). Indeed, low levels of cytokines can provoke the release of potentially beneficial trophic factors and free radical scavengers; nevertheless, elevated levels may activate inflammatory cascades or even induce apoptotic death (self destructive programmed death mechanism). For instances mice genetically lacking the pro-inflammatory cytokine TNF-α receptors (thereby removing the influence of endogenous TNF-α) have been shown to be susceptible to ischemic injury. However, administration of exogenous TNF-α at the time of ischemia exacerbated neuronal death (Bruce et al., 1996). Likewise, administration of the endogenous IL-1 antagonist, IL-1ra, reduces infarct size in response to middle cerebral artery occlusion and prevented the accumulation of inflammatory infiltrates within the area of damage, suggesting a prominent destructive role for IL-1 in acute cerebrovascular insults (Barone et al., 1998). Consequently, many variables such as concentration influence how immunotransmitters affect the CNS, whether it is protective or deleterious. Indeed, the timing of exposure and the interactive effects of cytokines profoundly influence the state of microglia activation and likely the role they play in pathological conditions.

Although there is scant evidence concerning the role of cytokines in PD, postmortem brain tissue from PD patients often contains increased expression of cytokines, such as IL-1β, IL-6, IFN-γ, TNF-α, as well as the TNF-α receptor superfamily member, Fas. Likewise, cDNA microarray studies indicated that MPTP treated mice displayed similar alterations of pro-inflammatory cytokine genes within basal ganglia brain regions

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(Mandel et al., 2000; Nagatsu et al., 2000; Park et al., 2003). In terms of mechanistic studies, two laboratories have recently reported altered basal ganglia responses to MPTP in TNF-α deficient knockout mice (Sriram et al., 2002). Although one report indicated that TNF-α deletion protected striatal terminals and normalized dopamine levels in MPTP treated mice, the other found increased dopamine metabolism in the absence of any evidence of neuroprotection in the MPTP null mice (Striet, 2004). In concordance with the latter finding, new finding found that mice lacking the TNF-α receptor superfamily receptor, Fas, displayed attenuated dopaminergic neurodegeneration and associated microgliosis (Gao et al., 2003). Interestingly, IL-6 knockout mice displayed increased SNc dopaminergic soma and striatal terminal degeneration following MPTP, suggesting enhanced sensitivity to the toxin in the absence of the cytokine (Bolin et al., 2002). Thus, in keeping with the trophic actions reported for IL-6, endogenous levels of the cytokine may actually protect neurons against insults.

There are several mechanisms through which cytokines may influence the survival or death of dopaminergic neurons. Although this section will evaluate some of these pro-death mechanisms, it should be underscored that many cytokines (at least within the immune system) act as growth factors promoting cellular differentiation and proliferation. It has been well established that pro-inflammatory cytokines, such as TNF-α, Fas and IFN-γ as well as several chemokines (subcategory of chemo-attractant cytokines), can promote cellular death through apoptotic, excitotoxic or oxidative processes (Lawrence et al., 1998). For instance, the recently reported low levels of the intracellular Fas death domain (FADD) in PD patients prompted the assertion that FADD expressing neurons may selectively die through apoptosis in PD (Hartmann et al., 2001).

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Other evidence for classical apoptotic pathways implicated in PD includes reports of increased levels of caspase-3 and –8 which act as downstream effectors of FADD, Bax and related death pathways (Hartmann et al., 2001). However, evidence also exists for inflammatory mechanisms of action for Fas. For instance, Fas knockout mice were reported to display diminished microglial activation within the SNc and striatum in response to MPTP compared to wild type mice (Gao et al., 2003). Fas also promoted central expression of the chemokine IL-8 and have numerous inflammatory effects in common with its superfamily member, TNF-α (Park et al., 2003).

Cytokines may also influence neurodegenerative processes through excitotoxic pathways. Indeed, IL-1β exacerbated the degree of excitotoxic cell death promoted by glutamate through N-methyl-d-aspartate (NMDA) and AMPA receptors, whereas infusion of the endogenous IL-1β antagonist, IL-1Ra, prevented striatal excitotoxicity (Lawrence et al., 1998; Nagatsu and Swada, 2005; Patel et al., 2003). Interestingly, TNF-α was recently demonstrated to alter communication between microglia and astrocytes, thereby facilitating the development of excitotoxicity (Bezzi et al., 2001). Thus, cytokines may promote neuronal death directly or indirectly though their impact upon glial cells. In fact, by way of their potent autocrine stimulatory effects, cytokines may also amplify the release of any oxidative or other death factors released from the microglia in which they originate.

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1.9 Neuroimmune-neurotoxin interactions in PD: synergistic effects

The fact that PD often occurs in distinct clusters suggests that environmental factors (e.g., toxins) related to certain geographical areas may confer vulnerability to disease. Environmental distribution of many chemical agents often overlap and it is certainly conceivable that multiple toxins may synergistically influence neuronal processes. Likewise, one can surmise that the combination of various toxins (e.g. pesticides) with immunological events (e.g. viral, bacterial pathogens) may interact in a similar fashion. Consistent with this proposition, co-administration of LPS with rotenone synergistically augmented dopaminergic degeneration in mesencephalon-microglia co-cultures, through the release of ROS (Gao et al., 2002; Gao et al., 2003). Early-life exposures to a combination of the fungicide, maneb and paraquat have produced deleterious effects on the nigrostriatal DA system far beyond their individual effects (Cicchetti et al., 2005).

Interestingly, many of the behavioral deficits produced by MPTP are only evident in the presence of other chemical agents or stressors, suggesting possible synergistic interactions among these treatments (Sedelis et al., 2001). For instance, it has been reported that MPTP only induced akinesia and cataplepsy deficits when co-administered with the pesticides, diethyldithiocarbamate (DDC) or maneb (Miller et al., 1991; Takahashi et al., 1989). It was also reported that the imposition of a stressor (transportation stress) was necessary to realize the behavioral effects of MPTP (Sedelis et al., 2001). Taken together, these studies indicate that consideration of the interactive effects of multiple factors is warranted when considering the environmental triggers implicated in PD.

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1.10 Cytokine-provoked neuronal sensitization: Implications for PD

Various environmental events (e.g. stressors, infections) have been shown to exert long term effects upon biological processes, such that these systems may be hyper-responsive to subsequent challenges. Indeed, LPS, cytokines (IL-1β, TNF-α) and even psychologically stressful events were reported to sensitize neurochemical pathways, such that exaggerated monoamine and neuroendocrine responses were evident upon later exposure (1-28 days) to these very same or similar insults (Tilders et al., 1993). Thus, cytokines administered at concentrations low enough not to produce discernable behavioural effects, may elicit central neuronal sensitization upon re-exposure. Although these sensitization effects were previously argued to have relevance with respect to psychiatric conditions such as depression, their relevance to neurological states has not been explored. Moreover, there is a lack of data regarding the possibility that these immunogenic or stressful insults may sensitize neural pathways or substrates that are affected in neurodegenerative conditions, such as PD (Tilders et al., 1993). In this respect, one aim of the present investigation is to determine if a potent immunogenic agent, LPS, enhances the impact of the PD relevant toxin paraquat, upon neuroinflammatory and degenerative processes. Indeed, it is important to evaluate whether a cross-sensitization of inflammatory / behavioural / neurodegenerative substrates is apparent between these insults.

Although the existing literature assessing the protracted effects of immune insults upon DA functioning is limited, there are a few studies demonstrating that such challenges applied early in life confer increased vulnerability to the development of PD-like pathology. For instance, prenatal exposure to LPS at embryonic days 10-11 resulted in a

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substantial reduction of the number of SNc dopaminergic neurons evident in adult rats (Blandini et al., 2000). In addition, prenatal LPS exposure engendered a long-term increase of striatal TNF-α that was even evident in mice 120 days of age (Ling et al., 2004). As the overall number of neurons within the SNc, as revealed by microtubule-associated protein-2 (MAP-2) immunoreactivity, was not reduced by LPS, it appears that at the dose used the endotoxin selectively impacted dopamine neurons. It was suggested that dopamine containing neurons may be particularly vulnerable to the effects of early TNF-α exposure, possibly through its inhibitory effects on important growth factors, such as nurr-1 or sonic hedgehog (Blandini et al., 2000).

Early exposure to pro-inflammatory environmental toxins may also act to sensitize neurons to the deleterious actions of subsequent nigrostriatal insults. For instance, combined treatment with the pesticides paraquat + maneb from postnatal days 5-19 sensitized rodents to the damaging effects of these challenges months later (Thiruchelvam et al., 2000). Indeed, re-exposure to the combination of these pesticides in adulthood provoked a significantly greater SNc neuronal loss and striatal dopamine depletion coupled with pronounced motor impairment relative to animals not exposed to the toxins early in life (Thiruchelvam et al., 2000). Although not measured in this study, it will be recalled that these pesticides readily instigate neuroinflammatory activation, particularly as indicated by microgliosis.

The time course for neurodegeneration following inflammatory challenges is consistent with the slow progressive nature of PD. For instance, continuous intra-SNc infusion of a low dose of LPS for several weeks (closely mimicking a typical neuroinflammatory state)

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elicited a maximal microglia response after 2 weeks but SNc degeneration was not evident until 4-6 weeks later (Di Monte, 2003). Likewise, exposure to MPTP and head injury has been associated with protracted elevations (often for years) of microglia and cytokines (Liu et al., 2003). The latter may explain cases of PD linked to repeated blows to the head, as observed in boxers. In any case, it is conceivable that such ongoing neuroinflammation would sensitize individuals to the degenerative consequences of subsequent environmental insults. We believe that microglia activation may lead to cytokine release that contributes to pathology through two primary mechanisms: (1) the promotion of glial derived reactive oxygen species and (2) activation of intracellular neuronal apoptotic or excitotoxic death processes, possibly through stimulation of various mitogen activated protein kinase pathways. Alternatively, the possibility should not be dismissed that low-grade dopaminergic injury may recruit peripheral immune responses (e.g. antibody dependent cytotoxicity) that may further amplify any ongoing degeneration.

1.11 Does LPS influence the neurodegenerative actions of paraquat: neuroinflammatory cross-sensitization?

Given the overwhelming evidence revealing that different classes of environmental agents (immune vs. non-immune; chemical vs. organic) may instigate PD-like pathology, it is of interest to establish whether such diverse toxins have common signaling mechanisms. In this regard, we propose that inflammatory cytokines may act as general messengers mediating the pathology elicited by these varied insults. Moreover, it may be that exposure to one insult confers increased susceptibility to the deleterious consequences of alternate insults through activation of common cytokine pathways. This

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is an important point, since individuals may be exposed to various combinations of environmental insults at intermittent times throughout one's life. It may be that the timing of exposure to these insults shapes their CNS consequences. Indeed, several studies found that psychological and immunological stressors induce time-dependent sensitization effects (Tilders et al., 1993), such that even a single exposure enhanced the neurochemical and behavioral effects of subsequent challenges.

To this end, it is of interest to determine if similar immunological insults enhance the neurodegenerative consequences of later environmental toxins implicated in PD. Thus, we plan to undertake experiments involving administration of LPS and subsequent exposure to paraquat. These studies will assess whether endotoxin exposure enhances the degree of microglial activation and expression of cytokines provoked by later paraquat treatment. Moreover, we will evaluate the impact of these treatments upon nigrostriatal DA neuronal survival in an attempt to relate cytokine variations to the neurodegenerative effects of the toxins applied. Importantly, these experiments will simultaneously assess multiple cytokines after several time intervals following the LPS and paraquat treatments in the hope of finding novel combinations of these immunotransmitters over time. These studies should aid in the identification of future inflammatory targets and their temporal pattern of expression in relation to PD like pathology.

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2. Materials and Methods

All experiments conducted complied with the guidelines of the Canadian Council on Animal Care and protocols approved by the Carleton University Animal Care Committee. C57BL/6 male mice (8-10 weeks of age for Experiment 1 and 10-12 weeks of age for Experiments 2-4) were single housed one week prior to initiation of experiments. Animals were subject to a 12:12 light-dark cycle and room temperature was maintained at 21°C. Food and water were provided *ad libitium*.

Four major experiments were conducted in this thesis. The first two experiments deal with the inflammatory and neurodegenerative consequences of the herbicide paraquat, with a particular emphasis upon time-dependent cytokine variations and their relation to loss of DA neurons. In the third and fourth experiments, we seek to establish an inflammatory model of PD using the bacterial endotoxin, LPS. Specifically, we assess the time-course of nigrostriatal cytokine changes provoked by LPS and evaluate whether LPS pre-treatment influences the central effects of later paraquat exposure. It is our contention that such different classes of immune and non-immune insults may have overlapping central effects. We will initially describe the procedures unique to each of these and then sections will follow that describe in depth the methodologies common to these experiments.

2.1 Experiment 1:

Injection procedure: Importantly, paraquat may provide an excellent model of PD since it induces the formation of alpha-synuclein containing Lewy bodies (as observed in PD)

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and it is actually still used agriculturally throughout much of the world. Thus, an initial experiment was performed to assess whether paraquat induces a dose-dependent loss of nigrostriatal DA neurons. Accordingly, mice were administered either saline or paraquat (5 mg/kg or 10 mg/kg; i.p.) three times a week for three consecutive weeks; consequently, all animals received a total of nine injections. Animals were sacrificed 5 days following their final paraquat or saline injection either by rapid decapitation or transcardial perfusion. In decapitated mice, striatal and SNc nuclei were removed by micropunch and later subjected to cytokine analyses. Following fixation, perfused tissue was used for immunohistochemistry. A clinical rating scale was devised to measure the hallmark symptoms of PD (e.g. gait, posture, and muscle tone) just prior to sacrifice. Additionally, home-cage activity levels were assessed in a subset of mice 24 hrs prior to sacrifice. N=8/group.

2.2 Experiment 2

Injection procedure: To determine the time-dependent effects of paraquat upon behavioural and cytokine responses, a second experiment was performed in which mice were sacrificed at 2 and 24 hours following exposure to paraquat. Specifically, C57BL/6 mice received either saline or paraquat (10 mg/kg; i.p.) three times a week for either one, two, or three consecutive weeks, receiving a total of three, six, or nine injections. Mice were decapitated, trunk blood collected and brains removed. SNc, striatum and hippocampal nuclei were removed by micropunch. Cytokine levels were then assessed in plasma isolated from blood and brain nuclei using a beadlyte mouse multi-cytokine detection system in conjunction with a Luminex bead assay machine. Additionally,

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home-cage activity levels were assessed immediately following injection, with data being collected until the time of sacrifice. N=6/group.

2.3 Experiment 3

Surgery and LPS injection: LPS can act as an immune toxin and at low doses the endotoxin can be used to stimulate activation of glial cells with minimal neurodegenerative effects. Therefore, all C57BL/6 mice received an infusion of either saline or LPS (0.1μg or 3.0μg at doses with negligible degenerative consequences). Using a Harvard Apparatus Pump (Pico Plus Syringe), 2 μl of fluid was delivered, over a 5 minute period, into the SNc by a 10μl Hamilton syringe. Six days prior to the infusion, animals underwent stereotaxic surgery, whereby a custom designed indwelling cannula was implanted, just above the SNc (bregma: anteroposterior, -3.16mm; lateral -/+ 1.2mm; depth, -4.0mm). Again to assess time-dependent cytokine changes, all animals were sacrificed at three different time points: 90 min, 2 days or 7 days following infusion and plasma and brains were collected for cytokine and glial variations. Additionally, home-cage activity levels were assessed 90 minutes prior to sacrifice. N=11/group

2.4 Experiment 4

Surgery and injections: Immune challenges may influence the impact of environmental toxins, such as pesticides. Indeed, combinations of various pesticides and bacterial infections may be associated with the onset of PD. Thus, we sought to determine whether administration of a bacterial endotoxin sensitized neuroinflammatory processes within

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the SNc, such that animals display an exaggerated vulnerability to subsequently encountered environmental toxins. Accordingly, C57BL/6 male (n= 8/group) mice received a stereotaxic surgery wherein indwelling cannulae were implanted into the SNc (bregma: anteroposterior, -3.16mm; lateral -/+ 1.2mm; depth, -4.0mm) and animals were given a 6 day convalescence period before any treatments were administered. Thereafter, an infusion of either saline or LPS (0.1μg; a dose expected to activate microglia but to have minimal degenerative effects in and of themselves) was given in 2 μl of fluid delivered into the SNc over a 5 minute period. Two days following the LPS/saline infusion, mice were administered either saline or paraquat (5 or 10 mg/kg; i.p.) three times a week for a three week period. Finally, mice were sacrificed 5 days following their last paraquat or saline injection and half of the animals were perfused for immunohistochemical quantification of DA neurons, while plasma and micropunched brain nuclei (SNc, striatum, hippocampus) were removed from the remaining animals for cytokine analyses. In terms of behavioural analyses, a rating scale was devised to measure the hallmark symptoms of PD (e.g. gait, posture, and muscle tone) one hour following the final paraquat injection. Additionally, home-cage activity levels were assessed by a beam-break apparatus in a subset of mice 24 hrs prior to sacrifice.

2.5 General methods:

Behavioral measures: Behavioural testing was conducted between 0800 and 1100 hr to minimize influences of circadian variations.

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Ratings of PD-like symptoms: A qualitative index of overall motor functioning was devised by our laboratory in order to verify motor deficits in mice that received paraquat. Mice were evaluated on Day 20, 1 hr post final i.p. injection. The scale consisted of 3 criteria that captured the hallmark behavioral deficits observed in Parkinson's patients; mice were assessed in a double-blind procedure in order to increase high inter-reliability. A high-speed digital video camera was used to film the animals for 60 seconds in a 90 cm^3 arena and in the vertical position while being examined from all angles; front, lateral, ventral and rear views.

Posture: Mice were examined to assess gross curvature or arching of the spine when placed in a novel 90 cm^3 arena for 60 sec. Essentially, our early observations revealed that paraquat animals often displayed such a hunched posture. Animals received a rating of 0 for normal posture and a 1 if an arched back appearance became apparent at some point during the time in the open field. Mice that immediately displayed the arched appearance and remained so for the entire duration in the arena got a rating of 2.

Gait: Animals were evaluated on the style of step-by-step motion utilized upon being placed in a 90 cm^3 arena. If the paw placement appeared normal with a well coordinated gait a rating of 0 was assigned. Gait disturbances involved the presence of one of two abnormalities: 1. dragging the hind-paws in an uncoordinated manner or 2. shuffling of the hind limbs producing a "start-stop" pattern of motion. If the mouse displayed either one of the abnormal behaviours a rating of 1 was given and if both abnormal behaviours were present a rating of 2 was given.

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Muscle Tone: Mice were briefly (10 sec) suspended in the vertical position and hind-legs were scrutinized for limb placement and struggling behavior. Uncharacteristic muscle tone was evident when animals presented splayed hind-paws or spread apart in a clumsily fashion. The splayed hind limb placement was further assessed when mice were placed in a 90 cm^3 arena. Animals showing mildly splayed hind-paws with an absence of normal struggling in the vertical position were assigned a rating of 1. Those that failed to show any sign of struggle in the vertical position and had severe splayed hind-paws in both the vertical and horizontal positions were given a rating of 2. Mice with a robust struggle reflex in the vertical position and no signs of splayed limbs were given a normal rating of 0.

Home-cage activity: Undisturbed locomotor activity was evaluated in the home cage using a Micromax beam-beam apparatus (AccuScan, CA) equipped with 16 photocells interfaced into a digital analyzer. Essentially, mice are placed, while remaining in their home cages, into a series of metal boxes (12 x 18 cm), in which horizontal and vertical movements are detected by photocells and then analyzed by a proprietary software package (Microlyze).

Immunohistochemistry: Following transcardial perfusion [ice cold 0.9% saline followed by 4 % paraformaldehyde (PFA)], cryostat-cut coronal sections (14-um thick) were prepared for the striatum and the SNc. Every 10^{th} section for the striatum and every 5^{th} section for the SNc were processed. Survival of DA neurons was assessed by counting the number of TH+ (rate limiting DA enzyme, 1:1000, ImmunoStar) neurons present

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throughout the rostral-caudal axis of the SNc. Estimates of total stained neurons in the SNc will be calculated using Abercrombie's correction (Hunot et al., 2003; Sheng et al., 1998). Density of the TH+ terminals within the striatum were determined as we previously reported (Hunot et al., 2003; Sheng et al., 1998), using a commercially available densitometry software program.

Multiplex Luminex procedure: The Luminex 100 is a suspension based bead array system that uses xMAP technology. This system can detect up to 100 different analytes in a single 50 µl sample. Sets of microspheres (5.6 µm beads) are internally dyed with different ratios of fluorophores, each conjugated to a different capture probe (cytokine specific antibody). Following incubation, a classification laser identifies the particular cytokine bound and a second reporter laser quantifies the signal present. Numerous mouse and human multiplex cytokine kits (with ~ 10-20 cytokines on each panel) are commercially available. In the present study, we used a custom-designed kit encompassing both pro-inflammatory (IFN-γ, TNF-α, IL-1β, IL-2, IL-5, IL-6, IL-12) and anti-inflammatory (IL-4, IL-10) cytokine detection (Upstate, Cell Signaling Solutions).

Tissue Preparation: Prior to cytokine detection within plasma samples, a mouse serum diluents kit (Cat # 43-007) was used to dilute plasma supernatants. Brain nuclei were prepared using a specialized Bio-Plex cell lysis kit, (Cat # 171-304012). Briefly, the lysis buffer (extraction buffer) was prepared by adding 5 ml of solution buffer (Bio-Rad), with 20µl of factor 1, 10µl of factor 2 and 20µl of PMSF to make a 500 Mm concentration solution. Thereafter, frozen brain tissue (- 80°C) was washed with a wash buffer, 60µl of

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lysis buffer added and samples were immediately homogenized. The samples were then stored at -80°C for 20 min. The samples were homogenized for a second time and centrifuged at (6000 rpm) for 10 min at 4°C. Levels of overall protein were then determined using a commercially available BioRad detection system (Cat #500-0006).

Cytokine detection/quantification: A Beadlyte Mouse Multi-Cytokine Detection System 2 kit (Upstate, Cell Signaling, Cat # 48-004) was used in conjunction with the Luminex 100 system. First, a 5000pg Multi-Cytokine 2 standard was suspended in 1ml of Standard Serum Diluent (SSD), and vortexed on medium speed for 15 seconds. A series of serial dilutions were then performed to cover a range of standards (from 5000-6.9 and 0 pg/ml). Subsequently, 25µl of Beadlyte Cytokine Assay Buffer was placed in each well and a gentle vacuum applied to filter excessive liquid. Plates were blotted dry and 25µl of plasma diluents and 25µl of plasma added to each well. The filter plate was then incubated on a shaker for 20 minutes. Following incubation, samples were vortexed at medium speed for 15 seconds and then sonicated for an additional 15 seconds. To finalize the initial reaction, 25µl of bead solution was added to each well and the plate was covered and vortexed on low speed.

Plates were placed on a plate shaker and incubated overnight at 4°C in a dark room. Following removal of excessive liquid, brain tissue samples were re-suspended in 50µl of Beadlyte Cytokine Assay Buffer. For plasma samples, this wash step was conducted twice using 75µl of Beadlyte Cytokine Assay Buffer. Next, following a short vortex and vacuum step, 25µl of Biotin conjugated Beadlyte Anti-Mouse Cytokine was added to

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each well. After 1.5 hours of incubation with the biotin antibodies in a dark room, 25µl of diluted beadlyte streptavidin-PE was added to each well, covered, and mixed by vortex at a low speed. The antibody was incubated for 30 minutes in the dark at room temperature on a plate shaker. Finally, 25µl of Beadlyte stop solution was added to each well to halt the reaction and samples were re-suspended in 125µl of sheath fluid and read in the Luminex 100 instrument. Once the filter plate was placed in the Luminex 100 instrument, 100 different bead sets were distinguished based on its internal dye ratios. The antibodies bound to the bead surface act as targets for the substrates. Reporters are also tagged with a fluorescent label and also bind to the target. The substrate being measured is unique to the particular bead set.

Statistical Analysis: All data was analyzed using appropriate ANOVAs, followed by Newman Keul's post hoc comparisons (unless otherwise stated).

3. Results

3.1 Experiment 1: Dose-dependent effects of Paraquat

Paraquat dose-dependently induced a loss of TH+ neurons within the nigrostriatal system. As shown in **Figure 3** and consistent with previous reports (Ciccetti et al., 2005; McCormack et al., 2003; Shimizu et al., 2003), paraquat dose-dependently reduced TH+ staining within the SNc and this effect was dependent upon the rostro-caudal level of the SNc. Indeed, the repeated measures ANOVA revealed a significant interaction between paraquat treatment and SNc bregma level $F(1, 13) = 4.409$, $p = 0.0345$. The planned comparisons revealed a significant reduction of TH+ staining following only the higher

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dose (10 mg/kg) of the herbicide relative to control ($p < 0.05$), and this effect was present at bregma levels -3.16, -3.28 and -3.40 (**Figure 3**). Overall, it appeared that paraquat provoked a loss of approximately 30% of the DA neurons within the SNc, however, the possibility exists that these neurons did not degenerate but merely ceased to express TH. Yet, our preliminary data using cresyl violet staining (which non-selectively labels all neurons within the SNc) argue against such phenotypic changes. Indeed, paralleling the reduction of TH staining, a loss of SNc cresyl violet stained neurons was provoked by paraquat, suggesting that a genuine neurodegenerative effect was elicited.

Insert Figure 3 about here

Paraquat elicited marked time-dependent behavioural alterations: Ratings of gait, posture and muscle tone. With regard to these measures, ratings by independent observers revealed that paraquat-treated mice exhibited hunched posture, splayed hind-limbs and impaired gait relative to vehicle-treated animals. When briefly (10 sec) vertically suspended by the tail, vehicle treated control mice displayed a normal degree of struggling and attempted to right themselves. In contrast, mice that were chronically administered paraquat, at least at the lower 5 mg/kg dose, failed to show a normal righting response. Furthermore, these same mice displayed a marked lack of hind-limb muscle tone when placed in the open field. Indeed, the overall ANOVA revealed significant differences in hind-limb muscle tone between different treatment groups, $F(2,11) = 4.638$, $p < 0.05$. As depicted in **Figure 4**, the follow-up planned comparisons revealed that only the lower dose (5mg/kg) of paraquat was associated with a severe

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Figure 3: Paraquat promotes dopaminergic degeneration within the SNc. Depicted on the left are representative photomicrographs indicating reduced tyrosine hydroxylase (TH) staining within the substantia nigra (SNc) in response to chronic systemic (i.p.) injection of saline (Sal) or the herbicide paraquat (5 or 10 mg/kg, 3 times/week for 3 weeks). The right bar graph depicts quantification of TH+ neuronal loss across multiple levels of the SNc. Paraquat elicited a dose-dependent decrease in TH immunoreactivity across several levels of the SNc, $*p < 0.05$ relative to saline treated mice.

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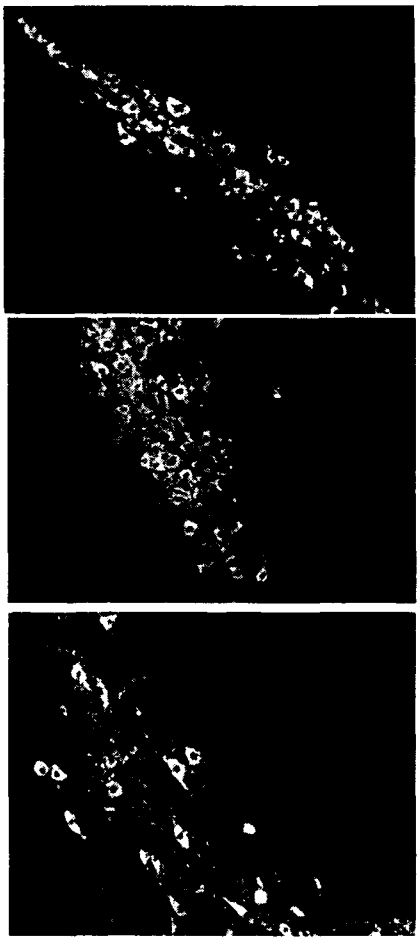

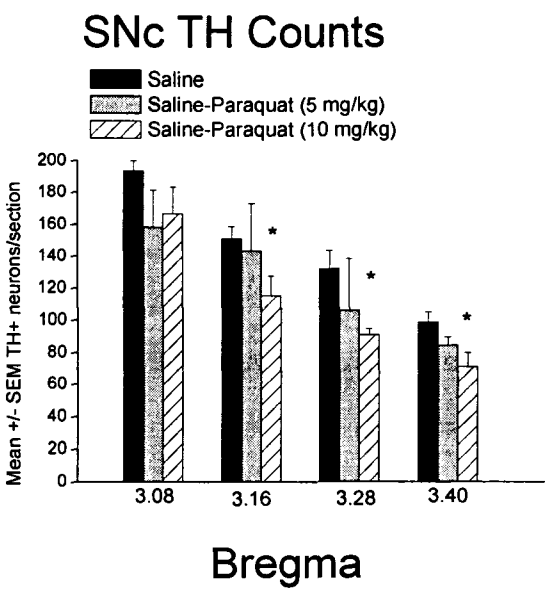

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splay of the hind-limbs with little sign of struggling when placed in the vertical position ($p < 0.05$). In effect, paraquat treated mice appeared less active overall in both undisturbed and challenged conditions. Surprisingly, the higher dose (10 mg/kg) of paraquat did not seem to affect the muscle tone of mice to any measurable extent.

Consistent with the proposition that paraquat induced a PD-like syndrome, both the high and low doses of the herbicide severely impaired posture and gait (**Figure 4**). Indeed, the overall ANOVAs revealed significant differences between the treatments groups for both posture and gait, Fs (2, 11) = 5.549 and 5.175, p = 0.01 and 0.02, respectively. The follow-up planned comparisons revealed that both the low and high doses of paraquat did in fact induce higher ratings of impairment for these measures, relative to saline treated animals ($p < 0.05$). Mice treated with paraquat also showed severe postural impairments immediately after being placed in an open field. These were characterized by extreme curvature of the spine throughout the duration (2 min) of exploration in the apparatus. In addition to postural disturbances, paraquat-treated mice had marked gait abnormalities relative to vehicle treated controls, as evidenced by poor paw placement and a start-stop shuffling pattern of movement within the open field. It is unlikely that these motoric disturbances stemmed from non-specific stressor effects associated with the combined paraquat treatment and testing procedures given that none of the tested animals displayed excessive micturition or defecation upon open-field testing nor did they display typical signs of distress or sickness (e.g. piloerection, ptosis).

Insert Figure 4 about here

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Figure 4. Chronic systemic exposure to paraquat (3 times a week for 3 weeks) provoked behavioural disturbances. Mice were assessed 20 days following initiation of the experiment at a time when the neurodegenerative effects of paraquat were believed to be stable. Three different behavioral measures were used to assess PD-like symptoms. Increased rating is indicative of impairment associated with the treatments. Panel A depicts ratings of muscle tone (e.g. hind-limbs splayed apart in a clumsily fashion). Panel B depicts posture (e.g. spine was bent or arched upwards forming a hump). Panel C depicts ratings of gait (shuffling, start-stop movement, and dragging paws). Paraquat impaired muscle tone at the lower dose, whereas the herbicide provoked disturbances of gait and posture at both doses relative to saline treated animals, $*p < 0.05$ relative to saline treated mice.

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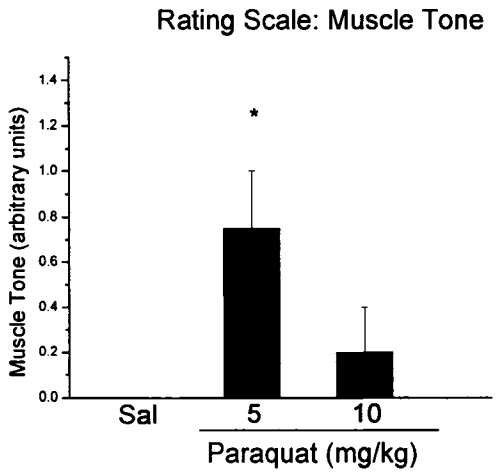

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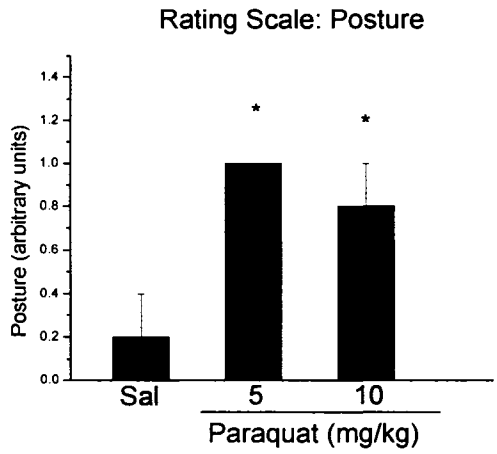

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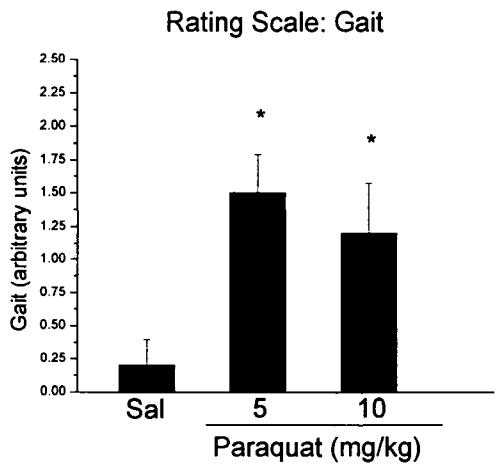

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Paraquat causes peripheral variations of a panel of pro- and anti-inflammatory cytokines. Systemic injections of paraquat resulted in changes of numerous plasma cytokines; however, these effects were, for the most part, discernible only via *a priori* post-hoc comparisons and not by way of omnibus F-tests (**Table 1**).

Insert Table 1 about here

IL-6, IL-10 and TNF-α. No significant differences between treatment groups for the pro-inflammatory cytokines TNF-α and IL-6 or for the anti-inflammatory cytokine IL-10 were observed with the overall ANOVA, *F*s (2, 11) = 2.461, .929 and 2.494, p = .131, .424 and .128, respectively. However, it was specifically predicted that paraquat would cause a rise in IL-6 and TNF-α since previous studies have demonstrated elevations of these cytokines in PD patients (Mogi et al., 1998). Accordingly, the planned comparisons revealed that the higher dose of paraquat did in fact cause long-term changes to TNF-α ($p < 0.05$), as seen five days following the final paraquat injection (**Figure 5**). There is also reason to suppose that the anti-inflammatory cytokine, IL-10, may have been elevated since this cytokine typically acts to counter the normally elevated TNF-α levels, thereby preventing the development of autoimmunity. Along these lines, the planned comparisons indicated that although changes of IL-0 followed a similar trend to that of TNF-α, this trend failed to reach significance (**Figure 5**). Similarly, no significant changes to the levels of circulating IL-6 were evident.

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Table 1. Paraquat induced cytokine changes in plasma 5 days following final exposure.

	Plasma Cytokine Concentrations (pg/ml)						
	TNF-α	**IL-6**	**IL-10**	**IFN-γ**	**IL-12**	**IL-1 ß**	**IL-2**
F*	2.461	.929	2.495	1.724	2.722	1.877	4.23
***p*-value**	.1309	.424	.1278	.2231	.1095	.1989	.043

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Insert Figure 5 about here

IL-2 and IL-1β. The ANOVA revealed significant differences between the treatments groups for IL-2, F (2, 11) = 4.230, p = 0.043. The planned comparisons revealed that the low dose of paraquat elevated levels of the pro-inflammatory cytokine IL-2 as compared to controls, whereas the high dose of paraquat had a modest non-significant effect (**Figure 6**). While IL-1β followed a very similar pattern of expression to IL-2, the overall F-test for the main effect of paraquat was not significant F (2, 11) = 1.877, p = 0.199. Yet, the planned comparison revealed a significant elevation of circulating plasma IL-1β levels ($p < 0.05$). However, again only the lower paraquat dose was effective in this respect (**Figure 6**). Once again, the justification for conducting these follows up comparisons stems from our initial proposition that IL-1β plays an important role in PD from other studies demonstrating changes in this cytokine in PD patients as well as rodents infused with the DA toxin, 6-OHDA (Ling et al., 2004).

Insert Figure 6 about here

The ANOVAs for the remaining five cytokines, IL-4, IL-5, IL-12, GM-CSF and IFN-γ failed to reach significance. Out of these immunotransmitters, *a priori* predictions were made for IFN-γ, based upon preliminary data from our laboratory demonstrating that mice lacking the IFN-γ receptor were resistant to the neurodegenerative effects of MPTP (Mount, Lira, Smith, Hayley & Park, 2006, submitted). However, the post-hoc

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Figure 5: Paraquat induced cytokine changes in circulating levels of TNF-α, IL-6 and -10. Using a multiplex laser-based bead assay, circulating cytokine levels were assessed in mice that received chronic systemic injections of paraquat (5 or 10 mg/kg 3 times/week for 3 weeks) or saline. Paraquat was found to provoke a dose-dependent elevation of plasma TNF-α and a trend towards increased IL-10 concentrations (panels A and C). However, the herbicide did not affect plasma IL-6 levels (panel B), *$p < 0.05$ relative to saline treated mice.

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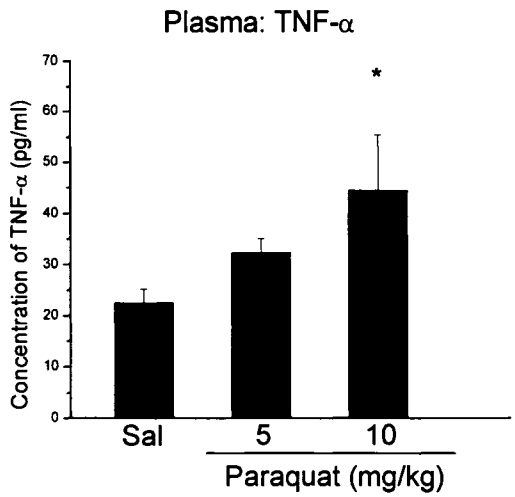

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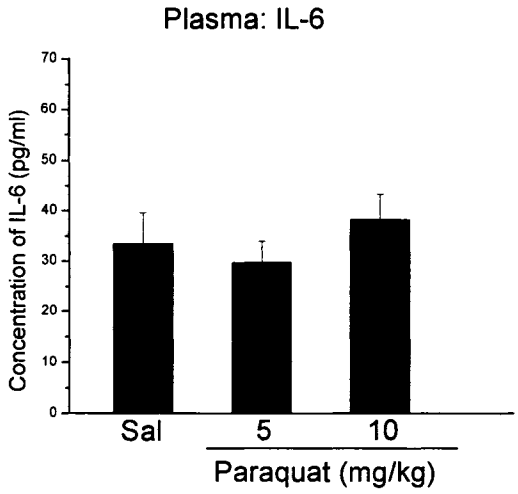

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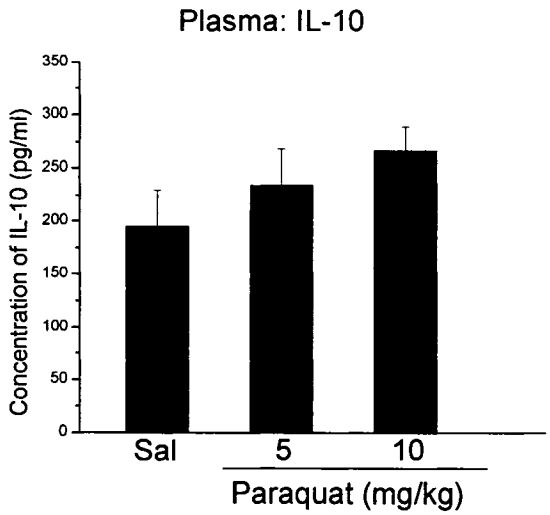

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Figure 6: Paraquat induced alterations in circulating levels of IL-1β and IL-2. Chronic systemic injection of paraquat (5 or 10 mg/kg 3 times/week for 3 weeks) provoked alterations of plasma levels of the pro-inflammatory cytokines interleukin-1β (IL-1β) and IL-2. In this respect, the lower paraquat dose induced a significant rise of levels of both these cytokines (top and bottom panels). *$p < 0.05$ relative to saline treated mice.

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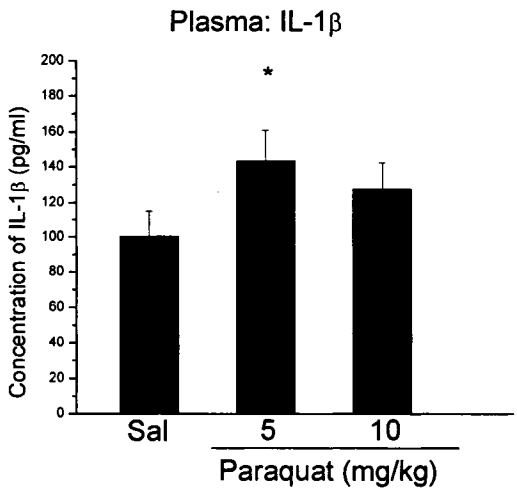

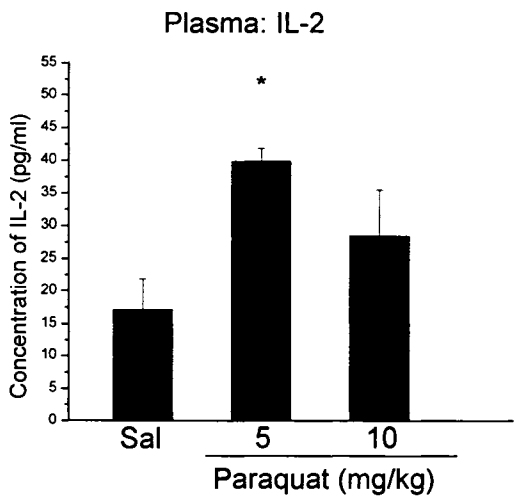

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comparisons failed to reveal any significant effect of paraquat upon circulating levels of IFN-γ. At this juncture, it is unclear whether changes in these cytokines would be apparent at different intervals following paraquat. Indeed, cytokine responses are often rapid and short lasting, owing to their relatively short half lives (~15-30 min), their pulsatile pattern of release and the fact that efficient endogenous mechanisms exist for their removal. Accordingly, any cytokine changes observed at this later time (5 days after the last paraquat injection) may reflect protracted changes of expression of these factors associated with the ongoing neurodegenerative processes provoked by paraquat. Yet, it is of interest to determine if early, more transient cytokine fluxes were elicited by the herbicide; such variations may indicate whether these cytokines play a major role in the initiation of the degenerative process or may be activated secondarily by this of associated processes. To this end, an extensive time-course was performed to evaluate the temporal effects of paraquat.

3.2 Experiment 2: paraquat timecourse

As shown in **Table 2**, paraquat alone had little effect on circulating cytokine levels with the exception of IL-6, at various times during the injection procedures. It should be underscored that these times were prior to that evaluated in Experiment 1. Namely, plasma cytokine changes were evaluated 2 and 24 hrs after the 3rd, 6th and 9th injections of paraquat. In contrast, Experiment 1 involved cytokine assessment 5 days following the final (9th) paraquat injection. Thus, cytokine differences between these initial two studies may be related to timing of sampling relative to any ongoing neurodegenerative processes. As noted, IL-6 was significantly influenced by treatment with paraquat.

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This cytokine was modestly elevated 2 hrs following the 3rd paraquat injection and then dramatically increased after the 6th exposure to the herbicide. Interestingly, levels returned to baseline by the 9th exposure to paraquat, suggesting that IL-6 may be involved in the early to intermediate effects of the herbicide.

Insert Table 2 about here

3.3 Experiment 3: LPS timecourse

Intra-SNc LPS alters home-cage activity. The level of home-cage activity of animals was substantially influenced by intra-SNc infusion of LPS. Indeed, the overall ANOVA revealed significant differences in photocell beam breaks among the treatment groups in the 30 minutes prior to sacrifice, $F(6, 60) = 7.960$ $p < 0.001$. As shown in **Figure 7**, the follow-up comparisons revealed that both the high and low doses of LPS reduced home-cage activity ($p < 0.05$) when the endotoxin was administered 90 min prior to sacrifice. In addition to this relatively acute effect, LPS also suppressed behavioral activity when mice were tested 2 days following the intra-SNc infusion; however, surprisingly this effect only reached significance for the lower dose of LPS ($p < 0.05$).

Insert Figure 7 about here

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Table 2. Paraquat effects on circulating cytokines.

	Plasma Cytokine Concentrations (pg/ml)						
	TNF-α	**IL-6**	**IL-10**	**IFN-γ**	**IL-12**	**IL-1 ß**	**IL-2**
F*	.284	2.107	.767	1.724	.553	.311	.933
***p*-value**	.9674	.04	.6338	.2231	.8092	.9577	.5005

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Figure 7. Evaluation of home-cage activity. The home cage activity was significantly influenced by intra-SNc infusion of LPS. Reduced home-cage activity was evident in mice exposed to the low (0.1 μg) and high (3.0 μg) dose of LPS when the toxin was administered 90 minutes prior to sacrifice (i.e. 60 minutes before testing). LPS also suppressed behavioural activity when mice were tested 2 days following the intra-SNc infusion; surprisingly this effect only reached significance for the lower dose of LPS. Seven days following infusion of LPS, mice displayed activity levels that were not significantly different from saline treated controls for wither of the doses. However, the lower dose of LPS did provoke a modest increase of activity at the 7 day interval (~23 %). Therefore, it seems that LPS had a rather dramatic immediate effect upon SNc dependent behavioural processes that was still evident several days following the single endotoxin exposure, $*p < 0.05$ relative to saline treated mice.

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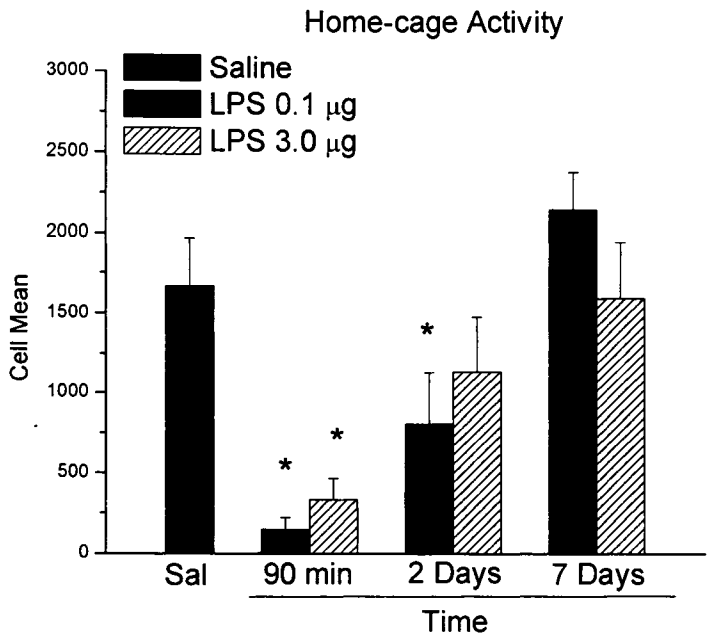

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Intra-SNc LPS causes peripheral and central variations of a panel of pro- and anti-inflammatory cytokines. Direct infusion of LPS into the SNc resulted in a multitude of cytokine changes within the SNc and striatum, as well as in circulation (**Table 3**).

Insert Table 3 about here

TNF-α, IL-6 and IL-10. The overall ANOVAs revealed significant differences between the treatment groups in circulating plasma levels of both TNF-α and IL-6, as well as the anti-inflammatory cytokine IL-10, *F*s (6, 70) = 12.857, 11.938 and 5.04, $p < .0001$, < .0001, .0002, respectively (**Figure 8**). The follow-up comparisons revealed that LPS at the low (0.1 μg) and high dose (3.0 μg) caused a short-lived acute elevation of plasma levels of all three of these cytokines at 90min, which was not seen at either 2 or 7 days following the endotoxin. As depicted in **Figure 8**, this pattern was also evident in the SNc for TNF-α and IL-6, *F*s (6, 69) = 7.617 and 3.472, $p < .0001$ and .0047, respectively; however, the results obtained for IL-10 were highly variable F (6, 69) = 1.191, $p = .3216$ (**Figure 8**). Of particular note, is that both of the macrophage derived cytokines, TNF-α and IL-6, exhibited similar patterns of expression in plasma and SNc, whereas no such trend was evident for IL-10, which is typically released by activated T cells.

Insert Figure 8 and 9 about here

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Table 3. Intra-SNc LPS administration induced variations in plasma as well as the SNc and Striatum.

	TNF- α	IL-6	IL-10	IFN-γ	IL-12	IL-1ß	IL-2
	Plasma Cytokine Concentrations (pg/ml)						
F*	12.857	11.938	5.04	n.s	n.s.	4.228	n.s
***p*-value**	<.0001	<.0001	.0002	n.s	n.s.	.0011	n.s.
	SNc Cytokine Concentrations (pg/ml)						
F*	7.617	3.472	n.s	n.s	n.s.	2.721	n.s
***p*-value**	<.0001	.0047	n.s	n.s	n.s	.0197	n.s
	Striatum Cytokine Concentrations (pg/ml)						
F*	n.s	5.64	n.s	n.s	n.s.	3.82	n.s.
***p*-value**	n.s	.0006	n.s	n.s	n.s.	.0069	n.s.

n.s = not significant ($p > .05$)

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Figure 8. Intra-SNc infusion of LPS provoked alterations of TNF-α, IL-6 and -10 levels within plasma and the SNc. Intra-substantia nigra (SNc) infusion of lipopolysaccharide (LPS) provoked early elevations of TNF- α and IL-6 within both the plasma and the SNC at 90 min following either dose of the endotoxin (panels A, B, D & E, respectively). Similarly, plasma concentrations of the anti-inflammatory cytokine, IL-10, were increased at 90 minutes (panel C), however, this cytokine remained unchanged within the SNc at all time points (panel F), $*p < 0.05$ relative to saline treated mice.

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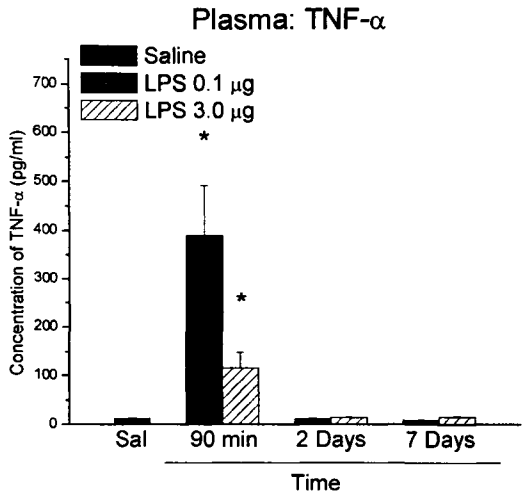

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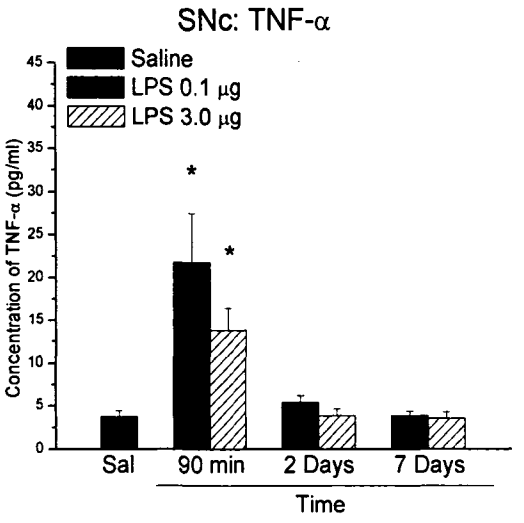

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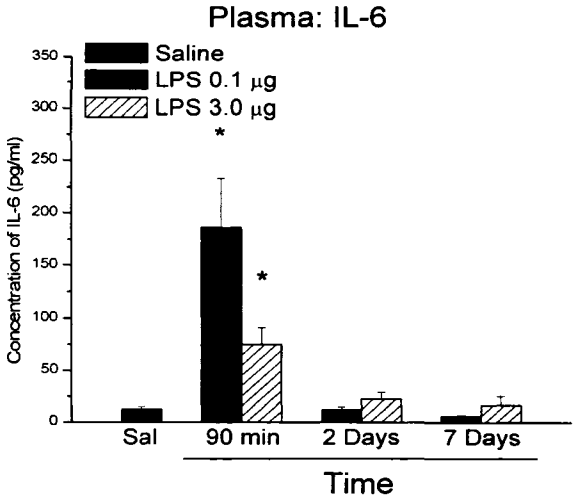

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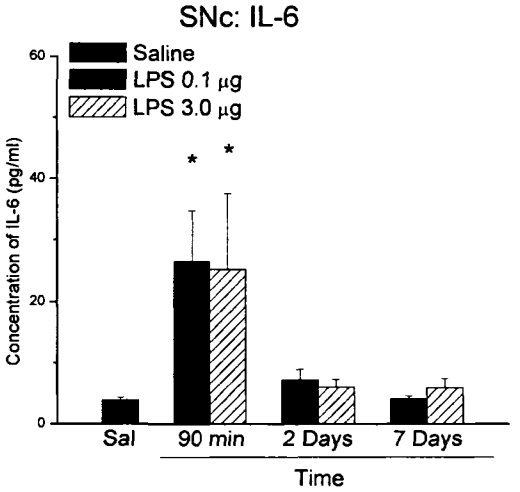

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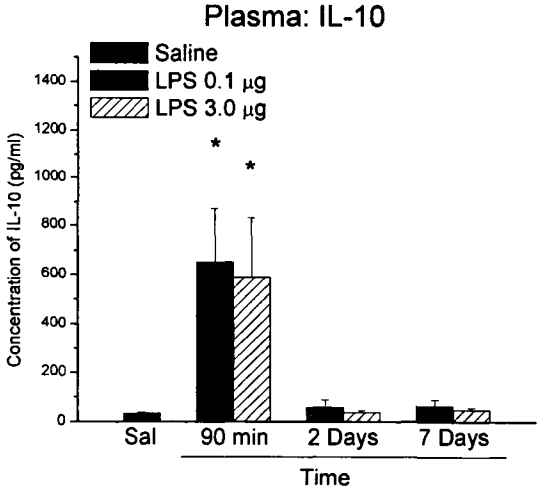

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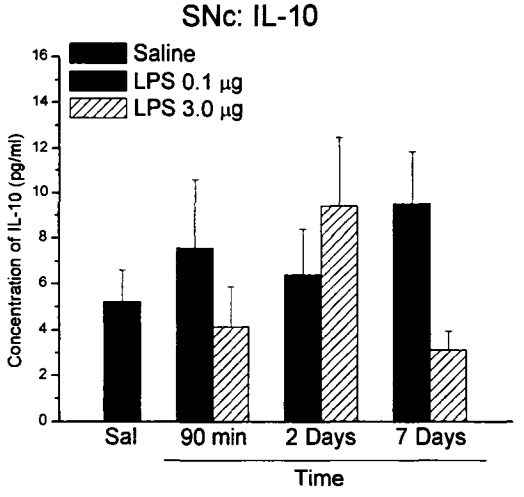

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Figure 9. The pattern of LPS induced alterations of TNF-α, IL-6 and -10 within the striatum were clearly different from that within the SNc. Within the Striatum, concentrations of TNF-α , and IL-10 were not significantly affected by the treatments (panel A & C). However, striatal IL-6 was increased by both doses of LPS, peaking at 90 minutes and remaining elevated for the entire 7 days following exposure to the higher endotoxin dose (panel B). Although IL-6 was still increased at the 2 day sampling interval following the lower LPS dose, concentrations of the cytokine returned to basal levels at 7 days after this treatment. *$p < 0.05$ relative to saline treated mice.

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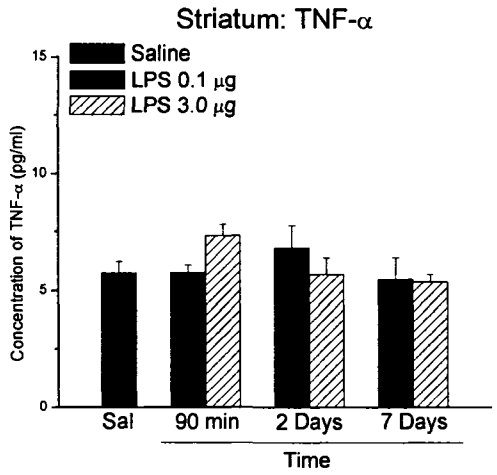

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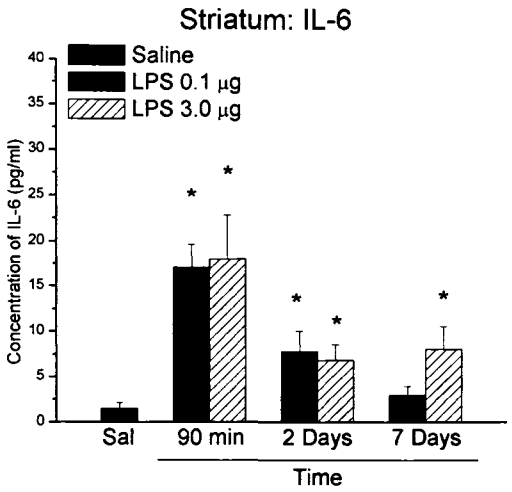

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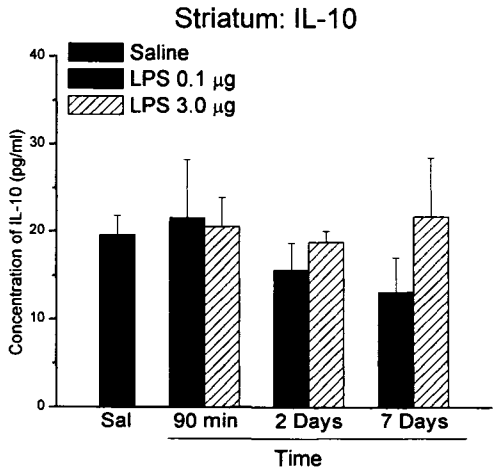

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Although IL-6 and TNF-α followed a similar pattern of expression in both plasma and SNc, these cytokines exhibited markedly distinct patterns of expression within the striatum. The ANOVA revealed that the levels of IL-6 varied as a function of the treatment groups F (6, 29) = 5.64, p = .0006 (**Figure 9**). Follow up comparisons confirmed that both doses of LPS were equally effective in provoking a marked rise of IL-6 within the striatum at 90 min and was still modestly elevated 2 days following endotoxin infusion with LPS. Conversely, the ANOVAs failed to detect the presence of any significant differences between treatment groups for striatal levels of either TNF-α or IL-10 (**Figure 9**).

IL-1β. The ANOVA revealed significant differences between the treatments groups for the pro-inflammatory cytokine IL-1β within plasma and both the SNc and striatal brain regions; *Fs* (6, 69) = 4.228, 2.721 and 3.82, p = .0011, .0197 and .0069, respectively. As shown in **Figure 10**, the follow-up comparisons revealed that an intra-SNc infusion of LPS caused a dose-dependent increase in plasma of IL-1β, which was only significant at the higher dose of LPS (3.0 μg). As well, a biphasic response was evident for plasma levels of IL-1β, entailing a short-lived acute elevation at 90min that returned to basal levels at 2 days post-infusion followed by a second peak at 7 days post-infusion ($p <$ 0.05). As seen in **Figure 10**, the lower dose of LPS (0.1 μg) provoked an acute significant elevation of IL-1β in the SNc at 90min, which returned to basal levels by 2 days and remained down at 7 days following the infusion; as such, nigral expression of IL-1β did not follow a biphasic pattern. With respect to IL-1β expression within the striatum, the higher dose of LPS, but not the lower dose, resulted in an acute elevation of

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IL-1β levels at 90min that returned to basal levels at 2 days post-infusion and remained down at 7 days post-infusion. Curiously, the lower dose of LPS elicited a relatively delayed increase in striatal IL-1β levels that was only evident at 2 days and returned to basal levels at day 7. It may be that multiple mechanisms account for these divergent patterns of expression between brain regions.

Insert Figure 10 about here

IFN-γ, IL-2 & IL-12. The overall *F*-tests for the remaining cytokines, including IFN-γ, IL-2 and IL-12, failed to reveal any significant findings for either circulating plasma levels or protein levels within the SNc or striatum (**Table 3**). However, for both doses of LPS, an apparent biphasic trend was apparent for plasma levels of IFN-γ, as demarcated by dual peaks at 90min and 7 days post-infusion. Also, there is an apparent delayed elevation in plasma IL-2 levels at 7 days post-infusion, although this effect did not reach significance.

3.4 Experiment 4: Influence of LPS pre-treatment upon later paraquat exposure

Paraquat dose-dependently induced a loss of TH+ neurons within the SNc irrespective of prior exposure to LPS. Paralleling the effects of paraquat found in Experiment 1, the herbicide treatments again were associated with dose-dependent reductions of TH+ staining within the SNc at bregma levels -3.16 and -3.28. Indeed, the ANOVAs for the main effect of paraquat at these SNc levels were Fs (1, 25) = 6.12 and 5.32, $p < 0.01$, respectively. The planned comparisons revealed that the number of TH+ neurons counted

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Figure 10. Intra-SNc LPS administration induced IL-1β variations in plasma as well as the SNc and Striatum. In the case of IL-1β, levels of the cytokine varied as a function of the intra-SNc infusion. There was a clear dose response change evident for IL-1β plasma concentrations, wherein only the higher dose of LPS influenced circulating levels of the cytokine. Interestingly, there was a biphasic pattern of IL-1β changes with increased levels at the earliest time point, 90 minutes and again at the later 7 day time point. Within the SNc, the lower LPS dose provoked significant rise of SNc IL-1β by 90 minutes which returned to baseline by 2 days after the endotoxin (panel B). At the level of the striatum, intra-SNc LPS increased IL-1β concentrations at 90 minutes but not at the two later intervals. In this instance, the effect was only provoked by the higher of the two endotoxin doses (panel C) *$p < 0.05$ relative to saline treated mice.

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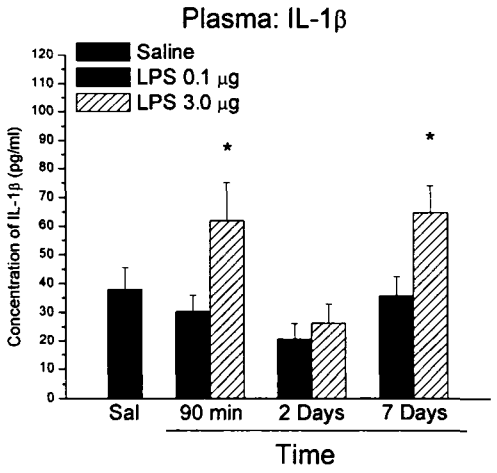

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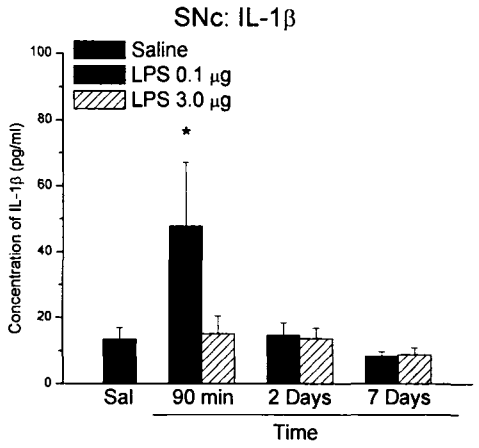

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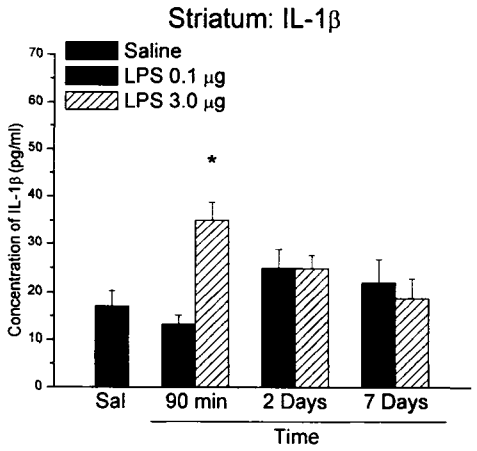

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in mice that received the 10 mg/kg dose of paraquat (at both bregma levels) were significantly lower that that of saline treated controls ($p < 0.05$). However, none of the paraquat injection x LPS infusion interaction ANOVAs reached significance, indicating that the effect of paraquat was not influenced by the endotoxin pretreatment. Likewise, the main effect for LPS infusion did not reach statistical significance, indicating that the endotoxin did not provoke a loss of TH+ neurons. Thus, contrary to our initial hypothesis, LPS pre-treatment did appear to sensitize processes that influenced the actions of paraquat upon SNc levels of TH+ neurons (**Figure 11**).

In contrast to the SNc, LPS pre-treatment did influence the impact of paraquat upon TH+ immunoreactivity within the striatum. Specifically, as shown in **Figure 12**, paraquat treatment itself dose-dependently reduced the density of TH+ terminals within the striatum; however, this effect was greatly diminished in mice that had also been pre-treated with LPS. Yet, LPS treatment alone did not alter striatal TH+ immunoreactivity, relative to saline treated controls. Thus, it appeared that pre-treatment with the endotoxin had a de-sensitizing effect, such that it attenuated the impact of later paraquat upon striatal TH+ expression.

Insert Figure 11 and 12 about here

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Figure 11. LPS and paraquat promote dopaminergic degeneration within the SNc. Tyrosine hydroxylase (TH) staining within the substantia nigra (SNc) was reduced in response to central SNc infusion of either saline (Sal) or lipopolysaccharide (LPS) in addition to subsequent systemic (i.p.) injection of saline (Sal) or the herbicide paraquat (5 or 10 mg/kg, 3 times/week for 3 weeks). LPS pre-treatment did not influence the actions of paraquat upon SNc levels of TH+ neurons Paraquat elicited a dose-dependent decrease in TH+ neurons across multiple levels of the SNc (panel A and B), *$p < 0.05$ relative to saline treated mice.

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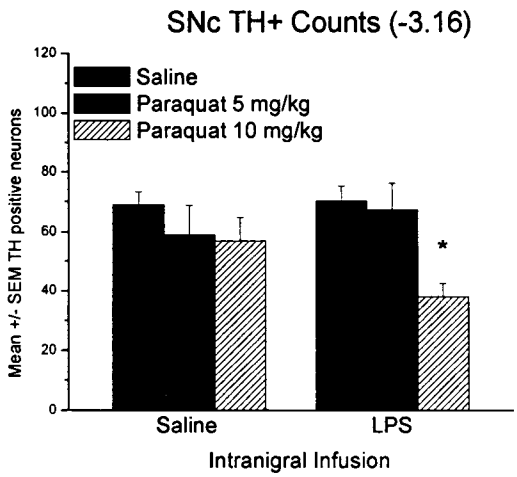

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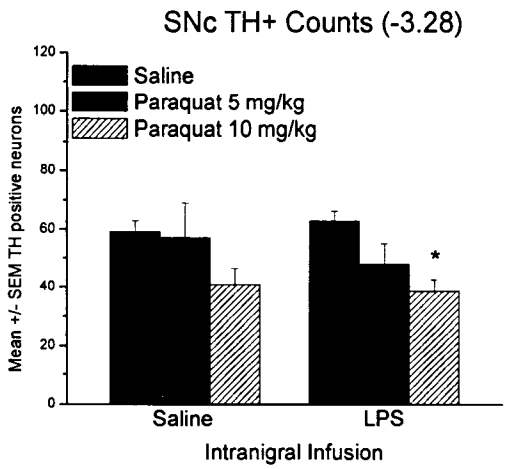

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Figure 12. LPS pre-treatment de-sensitized dopamine terminals in the striatum to later exposure to paraquat at two doses (5 and 10 mg/kg). The eight representative photomicrographs depict that contrast to the SNc, LPS pre-treatment did influence the impact of paraquat upon TH+ immunoreactivity within the striatum. Paraquat treatment itself dose-dependently reduced the density of TH+ terminals within the striatum; however, this effect was greatly diminished in mice that had also been pre-treated with LPS. Yet, LPS treatment alone did not alter striatal TH+ immunoreactivity, relative to saline treated controls. Pre-treatment with the endotoxin had a de-sensitizing effect that attenuated the impact of later paraquat upon striatal TH+ expression, $*p < 0.05$ relative to saline treated mice.

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LPS pre-treatment did not augment the behavioural alterations of paraquat: Ratings of muscle tone, posture and gait. With regard to these measures, ratings by independent observers revealed that paraquat-treated mice displayed hunched posture, splayed muscle tone and impaired gait relative to vehicle-treated animals, similar to those results delineated in Experiment 1.

Indeed, the overall ANOVA revealed significant differences in hind-leg muscle tone between different treatment groups, F (5, 54) = 3.112, $p < .0153$. As depicted in **Figure 13**, the follow-up planned comparisons revealed that both the lower (5mg/kg) and the higher (10 mg/kg) doses of paraquat caused severe splayedness of the hind-limbs with signs of struggling when placed in the vertical position ($p < 0.05$). Although the higher dose of paraquat alone did not affect the muscle tone, when these animals were pre-treated with LPS they displayed severe impaired muscle tone, suggesting an interactive effect of these insults. Surprisingly, LPS exacerbated the effects of paraquat only at the higher paraquat dose, indicating the possibility of an inverse U shaped sensitization. Importantly, LPS alone did not affect muscle tone indicating that any inflammation associated with this challenge did not cause progressive changes in muscle tone, rather that paraquat was required to trigger such effects. As well, it should be noted that we assessed behavioral ratings at several other time intervals, including after the first and second weeks of paraquat treatment (data not shown), but for the sake of clarity these data are not shown. Briefly, there were not apparent behavioral changes at the one week time but after two weeks the PD-like behavioral disturbances clearly resembled those evident after the third week, as reported in this thesis.

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In contrast to the effects LPS had on hind-leg muscle tone, surprisingly the endotoxin did not influence the effects of paraquat on overall posture and gait (**Figure 13**). The overall ANOVAs revealed significant differences between the treatments groups for posture and gait, omnibus *F*s (5, 54) = 4.491 and 5.088, p = 0.0017 and 0.0007, respectively. Specifically, follow-up planned comparisons revealed that the low and high doses of paraquat did in fact increase ratings of postural and gait abnormalities ($p < 0.05$). Mice treated with paraquat, regardless of whether they were pre-treated with LPS, showed severe postural impairments immediately upon being placed in an open field. In particular, they displayed extreme curvature of the spine prior to and throughout the duration of exploration **(Figure 13)**. Also, paraquat-treated mice displayed marked gait abnormalities, as evidenced by awkward paw placement and a start-stop, shuffling pattern of gait.

Insert Figure 13 about here

LPS pre-treatment attenuated the impact of paraquat upon plasma cytokines: A de-sensitization effect. The LPS and paraquat treatments altered circulating levels of several cytokines including, IFN-γ, IL-1β, IL-2, IL-10, IL-12 and TNF-α, but had no affect upon levels of IL-4, IL-5, IL-6 and GM-CSF. In the case of IFN-γ, a significant main effect was evident for the LPS infusion F (1, 22) = 6.01, $p < 0.05$, in the absence of any significant main effects for paraquat or the interaction between the two treatments.

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Figure 13. Intra-nigral infusion of LPS or Saline followed by chronic systemic paraquat injections 2 days later (3 times a week for 3 weeks). The mice were assessed on Day 20 following initiation of the experiment. Three different behavioral measures assessed PD-like symptoms. Increased rating is indicative of impairment associated with the treatments. Panel A depicts ratings of muscle tone (e.g. hind-legs were splayed apart in a clumsily fashion). Panel B depicts ratings of gait (e.g. Shuffling, start-stop movement, and dragging paws). Panel C depicts posture (e.g. the spine seemed to be bent or arched upwards forming a hump). Paraquat alone impaired muscle tone at the lower dose, whereas the herbicide provoked disturbances of gait and poster at both doses, $p \leq 0.05$ relative to saline. It was evident that LPS pre-treated mice later exposed to paraquat had markedly impaired muscle tone, gait and posture relative to those who received a saline or those who received a LPS alone, $*p < 0.05$ relative to saline treated mice.

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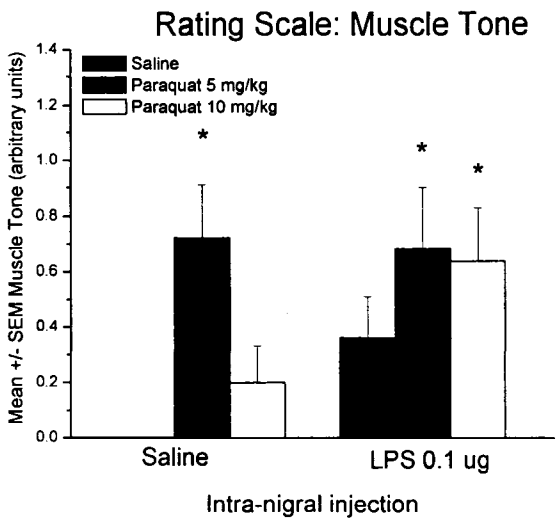

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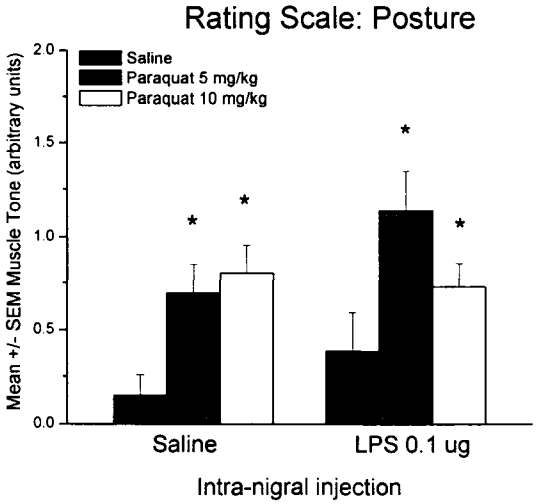

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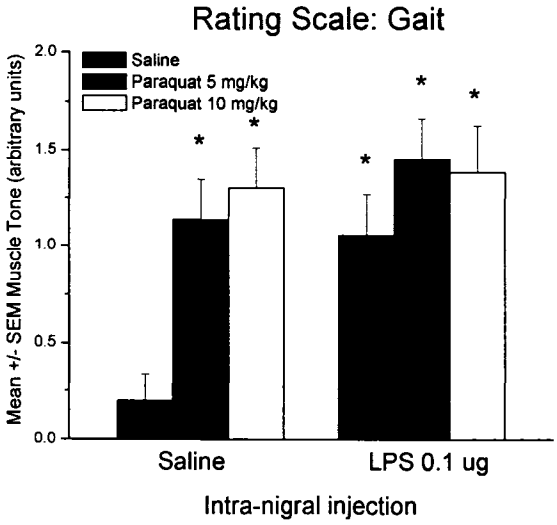

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Interestingly, the follow up comparisons revealed a reduction of plasma IFN-γ levels in mice infused with the endotoxin 26 days earlier, relative to those that received saline infusion at this time (**Figure 14**). It will be recalled that sampling at earlier time points following a single intra-SNc infusion of LPS (90 min, 2 and 7 days), conducted in Experiment 3, did not reveal any IFN-γ variations.

A robust LPS x paraquat interaction was evident for IL-12, F (1, 22) = 5.37, $p < 0.01$. The comparisons revealed that both doses of paraquat increased circulating IL-12 concentrations in saline pre-treated animals, however, both doses were also equally effective in suppressing levels of this cytokine in animals previously exposed to LPS ($p < 0.05$). In fact, as shown in **Figure 14**, the animals that received both the LPS and paraquat treatments had IL-12 levels even lower than that of saline only treated controls.

Insert Figure 14 about here

Evaluation of plasma IL-1β revealed that the impact of paraquat varied as a function of LPS pre-treatment, with the overall interaction F (2, 19) = 5.84, $p < 0.01$. Evaluation of the simple effects indicated that among the saline pre-treated mice, later administration of 5 mg/kg of paraquat induced a 40 % rise of IL-1β above that of animals receiving saline on this occasion (**Figure 15**). In contrast, LPS pre-treated animals that were later administered paraquat actually displayed a 36 % reduction of IL-1β levels, relative to mice that were injected with saline at this time. Thus, it appeared that LPS pre-treatment altered the cytokine stimulatory actions of later paraquat exposure.

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Figure 14. Intra-SNc infusion of LPS provoked alterations of IFN-γ and IL-12 levels within plasma upon later exposure to paraquat. The lower dose of paraquat alone caused a non-significant increase in the levels of circulating IFN-γ; however, pre-treatment with LPS significantly prevent this rise in IFN-γ. A similar pattern was observed with circulating levels of IL-12, where paraquat alone caused a significant increase in the levels of IL-12 with exposure to the higher dose of paraquat, which was significantly attenuated with pre-treatment with LPS (panel B). It seems that LPS alone did not provoke alterations of any of the cytokines (panel A, B). Intra-SNc infusion of LPS followed by chronic paraquat exposure resulted in a de-sensitization of both IFN-γ and IL-12 with exposure to the low (5 mg/kg) and high (10 mg/kg) doses of paraquat (panel A and B), $*p < 0.05$ relative to saline treated mice.

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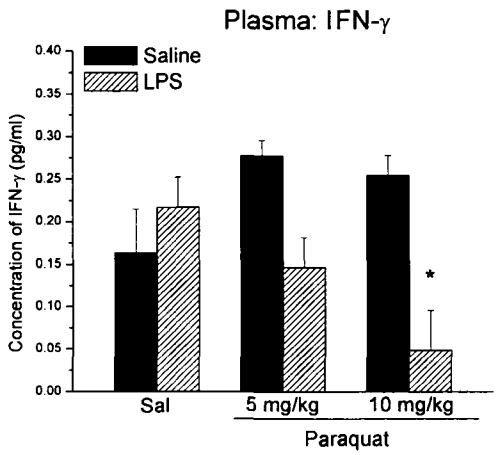

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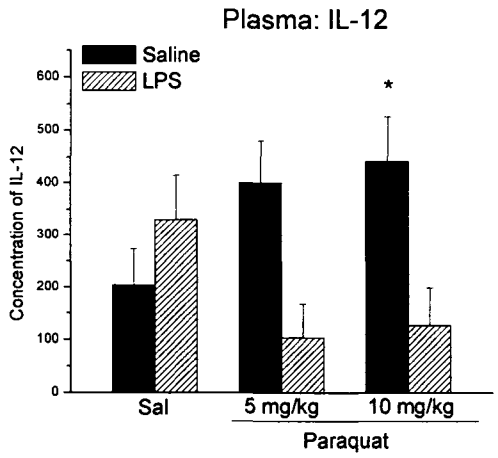

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Plasma levels of IL-2 did not significantly vary as a function of interaction between the LPS and paraquat treatments, nor was the main effect for paraquat significant. However, the ANOVA revealed a significant effect for LPS pre-treatment, $F(1, 22) = 11.28$, $p < 0.01$, wherein IL-2 levels were diminished by the endotoxin. However, inspection of **Figure 15**, clearly shows that mice that received saline on the day of sacrifice did not significantly differ from one another irrespective of whether or not they had been initially pre-treated with the endotoxin, indicating that LPS infusion alone did not affect levels of the cytokine. Interestingly, paraquat did elevate IL-2 levels in saline pre-treated animals but had no such effect among those previously exposed to LPS, again suggesting that the endotoxin blunted the impact of later paraquat exposure.

The LPS x paraquat interaction for the anti-inflammatory cytokine, IL-10, approached significance, $F(1, 20) = 3.18$, $p = 0.06$. The comparisons showed that while paraquat dose-dependently elevated IL-10 concentrations in saline pre-treated animals, the herbicide actually reduced levels of the anti-inflammatory cytokine in mice previously exposed to the endotoxin ($p < 0.05$; **Figure 14**). Once again, LPS infusion alone did not affect levels of this cytokine after this relatively long interval (26 days), compared to the robust elevations for IL-10 described in Experiment 3 at earlier times.

Insert Figure 15 about here

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Figure 15. Intra-SNc infusion of LPS provoked alterations of IL-1β and IL-2 levels within plasma upon later exposure to paraquat. Intra-SNc infusion of LPS followed by chronic paraquat exposure resulted in a de-sensitization of IL-1β only with the lower (5 mg/kg) of paraquat (panel A). Paraquat alone seemed to cause a non-significant increase in the levels of circulating IL-1β; however pre-treatment with LPS significantly prevent this rise in IL-1β. A similar pattern was observed with IL-2, where paraquat alone caused a significant increase in the levels of IL-2 with exposure to the lower (5 mg/kg) and higher (10 mg/kg) dose of paraquat, which was significantly attenuated with pre-treatment with LPS (panel B). *$p < 0.05$ relative to saline treated mice.

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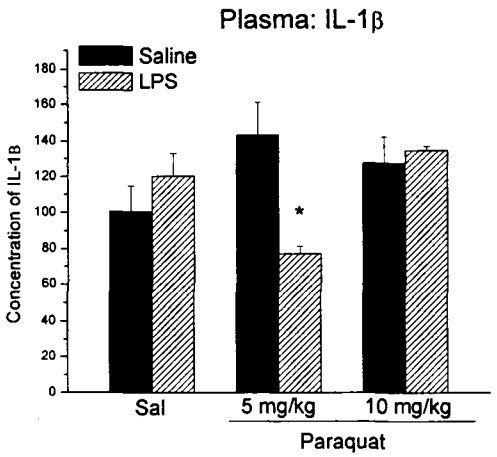

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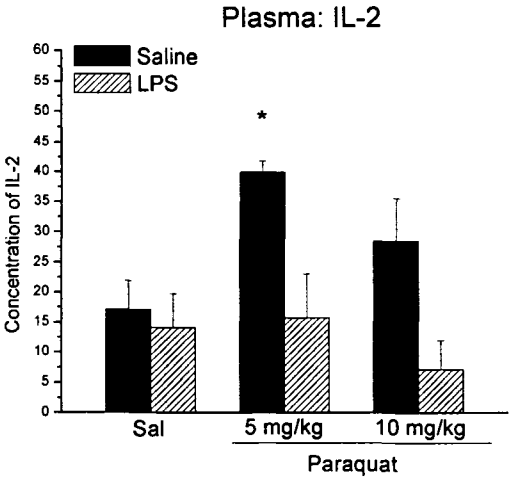

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Although the LPS x paraquat interaction and the main effect for paraquat both failed to reach significance, a significant main effect for LPS upon plasma TNF-α levels was apparent, $F(1, 22) = 10.40$, $p < 0.01$. As shown in **Figure 16**, there was a definite trend towards elevated TNF-α concentrations in saline pre-treated animals, while the herbicide clearly had absolutely no effect upon the cytokine in LPS pre-treated mice. Thus, although the interaction missed significance there was a definite indication of a de-sensitization effect. Indeed, it appears that intra-SNc LPS infusion blunted the impact of later paraquat exposure, at least at this 2 day interval, upon TNF-α as well as IL-1β, Il-2, IL-10 and IL-12.

Insert Figure 16 about here

Discussion

Postmortem studies and rodent toxin models of PD indicate that activated inflammatory immune mechanisms transpire through the course of the neurodegenerative disease. However, whether such effects play a primary role as a neurodegenerative culprit or rather a secondary reaction to ongoing tissue damage remains to be determined. Nonetheless, numerous studies have implicated inflammatory processes as one of many mechanisms that at least in part influence the progression of PD. The present study further demonstrated that endogenous inflammatory factors within the basal ganglia are implicated in PD following exposure to inflammatory and environmental insults. Consistent with the notion that cytokines may play a role in PD, our present findings

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Figure 16. Intra-SNc infusion of LPS provoked alterations of TNF-α, IL-6 and -10 levels within plasma upon later exposure to paraquat. Pre-treatment with an intra-SNc infusion of LPS attenuated the elevation of circulating TNF- α caused by chronic paraquat exposure, resulting in a de-sensitization of TNF-α only with the higher (10 mg/kg) of paraquat (panel A). A similar pattern was observed with IL-10, where paraquat alone caused a non-significant increase in the levels of IL-10, which was significantly attenuated with pre-treatment with LPS (panel C). The levels of IL-6 remained unchanged with exposure to paraquat or with pre-treatment of LPS (panel B). It seems that LPS alone did not provoke alterations of any of the cytokines (panel A, B, C), $*p < 0.05$ relative to saline treated mice.

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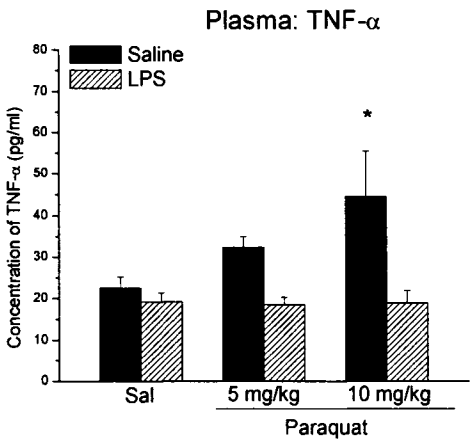

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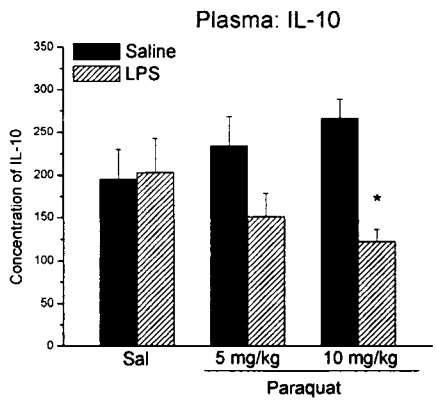

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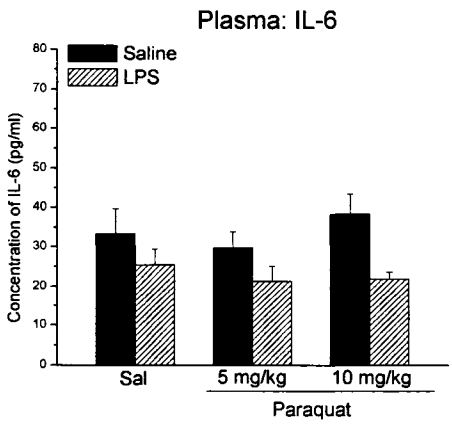

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revealed variations of several pro-inflammatory cytokines, particularly IL-6 and TNF-α, following treatments that elicited a PD-like syndrome.

The present investigation revealed that the pesticide paraquat, which is agriculturally applied in more than 130 countries (Deer, 2004), elicited DA neuronal degeneration and several behavioural deficits that reflect the cardinal motoric disturbances evident in PD. As well, a second series of experiments conducted as part of this thesis involved central infusion of LPS in order to assess the direct effects of a bacterial endotoxin on SNc functioning. In this regard, we demonstrated time-dependent activation of SNc microglial cells and behavioral disturbances following intra-SNc LPS infusion. Finally, it was of interest to determine if a cross-sensitization occurs between these two separate classes of toxins. Specifically, does LPS pre-treatment enhance the central impact of later paraquat exposure? This is an important issue given that exposure to multiple toxins likely occurs under natural environmental conditions. Moreover, it is our contention that these disparate toxins may elicit or activate common inflammatory signaling pathways within the CNS. Interestingly, our findings actually suggested that LPS pre-treatment diminished some of the neurodegenerative actions of paraquat and concomitantly reduced the ability of the herbicide to provoke cytokine variations. Characterizing the nature of such inflammatory-induced CNS effects has important implications for understanding interactions between multiple environmental toxins and the onset and progression of neurological disturbances. The role of pro-inflammatory cytokines in neurodegeneration may help uncover clues as to possible inflammatory substrates that should be targeted in treatments for idiopathic PD.

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4.1 Paraquat Induced Neurodegeneration and behavior alterations

The most widely used animal model of PD involves administration of the meperidine analog, MPTP; which induces nigrostriatal degeneration comparable to that observed in PD patients. Indeed, selective loss of approximately 50-60% of rodent SNc neurons occurs following sub-chronic MPTP administration (Przedborski et al., 1996; Langston et al., 1983). These effects stem largely from the ability of MPTP to induce mitochondrial dysfunction, elevate levels of oxidative stress, as well as release of pro-inflammatory cytokines and gliosis (Cleren et al., 2005; Shimohama et al., 2003; McCormack et al 2003). However, MPTP is not a perfect model of PD because it does not produce all of the typical histological features of PD, nor is this toxin found in the environment. In contrast, several pesticides that are found in many different geographic areas produce histological features more closely aligned with PD. Indeed, rotenone and paraquat elicit marked accumulation of abnormal deposits of α-SN and Lewy bodies, coupled with signs of inflammation (Betarbet et al., 2000; Gao et al., 2003; DiMonte 2003). Furthermore, the herbicide, paraquat, may be a particularly ethologically relevant, given that it binds strongly to soil particles and has a very long half life (~ 3 yrs). In contrast, other pesticides, such as rotenone break down much more rapidly and do not remain in soil for longer than 1-3 days (Deer, 1993). Accordingly, individuals are more likely to encounter chronically high levels of paraquat over time. However, paraquat, unlike MPTP, is somewhat less potent in its neurodestructive actions, typically producing a loss of 25-30% of nigrostriatal DA neurons (Brooks et al., 1999; Cicchetti et al., 2005; McCormack et al., 2002; McCormack et al., 2005; Thiruchelvam et al., 2000). Yet, behavioral alterations are clearly evident following exposure to these compounds. Although

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traditionally it has been thought that the clinical signs of PD are only apparent after approximately 80% loss of DA neurons, recent findings have challenged this position. In fact, a number of reports suggest that at least subtle cognitive and motoric symptoms may be evident in PD patients, long before clinical diagnosis is made (Brooks et al., 1999).

In the present investigation, there were obvious signs of motor dysfunction in mice coupled with an approximate 30% loss of SNc DA neurons following chronic paraquat exposure. However, it may be argued that a subset of these symptoms (e.g. impaired gait, reduced locomotion, poor posture), stem from the systemic toxic actions of paraquat (Ciccetti et al., 2005). Indeed, the toxic effects of paraquat upon several organs, especially the lungs, liver and kidneys have been elucidated (Deer, 2004). If in fact, paraquat was having such systemic toxic actions then it would be expected that mice would display signs of general illness and respiratory dysfunction (Deer, 2004). To assess this possibility, all animals were rated for signs of sickness (e.g. ptosis, piloerection) prior to motor testing/rating. To this end, no obvious signs of sickness were evident in the present investigation nor were there any indications of pulmonary disturbances upon visual inspection of mice. Thus, paraquat may produce a clinical syndrome with fairly high face validity for PD.

When considering the mechanisms of PD, it is important to recognize that the genetic background of humans likely contributes to the impact any environmental toxins may have. Indeed, to this date, several genetic mutations have been linked to PD, including DJ-1, α-SN, Parkin and UCHLI (Hashimoto et al., 2003; Michel et al., 2002). Each of

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these mutations has been demonstrated to enhance the development of PD by either reducing endogenous neuroprotective mechanisms (DJ-1), creating abnormal protein aggregations (α-SN) or even modifying normal cellular housekeeping functioning (UCHLI) (Chen et al., 2005; Choi et al., 2004). Essentially, any and all of these mutations may also render the organism vulnerable to alternate insults. For instance, mutations of UCHLI impair protein ubiqunation that is required for normal proteasome functioning for protein folding and removal of degraded protein fragments (Hashimoto et al., 2003; Klein et al., 2006).

4.2 Paraquat and the Blood Brain Barrier: possible transport mechanisms

Although measurable levels of pesticides have been found within the SNc of postmortem PD patients, the mechanism used to transport these across the BBB are not fully understood and appears to be rather controversial (Hald and Lotharius, 2005; Kortekaas et al., 2005). This is an important point since compelling evidence indicates that impaired BBB occurs in PD and influences the progression of the disease (Bahat-Stroomaza et al., 2005; de Boer and Gaillard; Kortekaas et al., 2005; Tomas-Camardiel et al 2004). The movement of paraquat across the BBB cannot be explained by simple diffusion; however, paraquat is a hydrophilic compound that may be transported by a carrier molecule or may penetrate in areas where the BBB is more permeable to compounds, such as the circumventricular organs, including the area postrema, (Hayley et al., 2004; Shimizu et al., 2001).

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In vitro studies have shown that paraquat is not a substrate for the dopamine transporter, DAT, unlike the chemically similar compound MPTP (Richardson, et al., 2005). Interestingly, pre-treatment with L-valine (a protein with a high affinity for neutral and basic amino transport mediators), which acts as a substrate for the BBB associated transporter LAT-1, prevented paraquat neurotoxicity (McCormack et al., 2003). Yet to date, there is no information available as to whether paraquat interacts with this transporter. Along these lines, mice pretreated with L-valine followed by systemic paraquat showed significantly lower levels of paraquat accumulation in the brain. Thus, it has been proposed that paraquat may be taken up by a neutral amino acid transport system and then transported into the brains parenchyma (Shimizu, 2001). As well, P-glycoprotein, another carrier-mediated transport molecule located at the BBB, may also be important in transporting toxins into the brain during BBB dysfunction (de Boer and Gaillard 2005; Drodzik et al., 2003; Kortekaas et al., 2005).

P-glycoprotein is an ATP-dependent transmembrane efflux pump coded by the Multidrug resistance 1 gene (MDR1) intended to transport numerous groups of lipophilic compounds across the BBB. According to Schinkel *et al* (1994), MDR1a knockout mice exposed to the pesticide, ivermectin, were 100-fold more sensitive to its neurotoxic effects in addition to the excessive accumulation of the pesticide in the brain of these mice. Pesticides are substrates for P-glycoprotein, which ultimately prevents pesticides from entering the central nervous system (Drodzik et al., 2003). A recent postmortem study involving evaluation of 107 PD patients (30 early onset and 77 late onset patients; 59 exposed to pesticides and 48 non-exposed) and 103 controls found that individuals

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with a mutated form of 3435T allele of the MDR1 gene, which encodes P-glycoprotein were five times more likely to develop PD in the exposed subgroup when compared to the non-exposed group (Drodzik). This mutation presumably predisposes individuals to the damaging effects of pesticides due to the reduction of pesticides bound to the P-glycoprotein receptor, indicating an enhanced likelihood of entry of putative toxins into the CNS.

The factors that may be responsible for triggering intra-cellular death cascades in DA neurons, such as free radical nitrogen and oxygen species, as well as pro-inflammatory cytokines are capable of increasing the permeability of the BBB (Bahat-Stroomaza et al., 2005; de Boer and Gaillard 2005; Drodzik et al., 2003). Interestingly, vascular inflammation can compromise the function of the BBB (Carvey et al., 2005) and provoke changes in blood vessel branching, such as the microangiogenesis that has been detected in PD patients (Barcia et al 2004). In addition to these findings, multiple studies have suggested that there is a correlation between abnormalities of BBB function in PD patients and loss of DA neurons (Kortekass, 2005 and Barcia 2004 and 2005, and Faucheux, 1999). A follow-up study exposing rats to a unilateral injection of 6-OHDA to either the striatum or medial forebrain bundle resulted in a patchy leakage in the BBB associated with SN and striatum and this effect was even observed 34 days following the lesion (Carvey et al., 2005). Moreover, Lynch *et al* (2004) showed that microglia was activated prior to breakdown of BBB and that TNF-α has a prominent role in contributing to such barrier disruption (Mark & Miller, 1999; Lynch *et al.*, 2004). Thus, multiple factors ranging from pesticides to stressors may enhance BBB permeability in PD,

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thereby augmenting the progression of disease by allowing the infiltration of potentially harmful immune cells or soluble messengers.

4.3 Neuroinflammation and apoptotic cell death following paraquat administration

Several studies have looked at the neurotoxic effects of the inflammatory mediators released from microglia on local dopaminergic neurons. Although there is a lack of data concerning paraquat, rotenone was reported to provoke degenerative effects that were reversed by NADPH inhibitors, suggesting the importance of NADPH dependent microglial release of superoxide and NO dopaminergic neuronal loss (Cicchetti et al., 2005; Gao et al., 2003; Hald and Lotharius 2005; Minghetti et al., 2005; Ringheim and Conant 2004). As well, chronic administration of paraquat over several weeks elicited microglial activation, coupled with an accumulation of oxidative species within the SNc (Cicchetti et al., 2005). In contrast to these animal studies, nothing is known about the long term histopathological consequences of paraquat exposure in humans. However, postmortem investigations of humans exposed to MPTP did reveal increased levels of activated microglia in the vicinity of DA neurons exhibiting signs of degeneration for up to 16 years after the last exposure to the drug (Hald and Lotharius 2005). Similarly, activated microglia were evident within the SNc of primates years after a single administration of MPTP and these glial variations were correlated with loss of local DA neurons (McGeer et al., 2003).

Microglial NADPH-oxidase appears to be a key player in the production of multiple oxidative radicals involved in PD, including superoxide and NO, and may even influence

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cytokine production (Choi et al., 2005; Gao, 2003; Wu et al., 2003). NADPH-oxidase is a multimeric enzyme, which remains inactive in resting microglia, but once activated, the entire cytosolic complex translocates to the microglial membrane to form a functional oxidative radical producing enzyme (Choi et al., 2005, Gao, 2003 and Wu et al., 2003). In addition to promoting oxidative radicals, NADPH activation has been linked to stimulation of the c-Jun-JNK (c-Jun N terminal kinase) signaling pathway (Choi et al., 2005; Hayley et al., 2004; Gao, 2003). Indeed, the toxins MPTP and rotenone were reported to stimulate NADPH-dependent superoxide production which subsequently triggered c-Jun NH2-terminal kinase (JNK) activation and phophorylation of c-Jun (Wu et al., 2003).

Several studies have postulated that the c-Jun-JNK pathway may be involved in PD suggesting that inhibiting JNK may be an effective method in averting dopaminergic loss of the SNc (Peng et al., 2004). Essentially, this pathway involves sequential phosphorylation of multiple small proteins by a series of protein kinases culminating in activation of immediate early genes, c-Jun and c-Fos, which then regulate widespread gene transcription (Peng et al., 2004; Waetzig et al., 2005). In terms of evidence for JNK in PD, one recent study reported that exposure to the JNK inhibitor SP600125 attenuated paraquat induced cell death in vivo, supporting a role for JNK in paraquat neurotoxicity (Peng et al., 2004). Along these lines, rodents exposed to chronic paraquat displayed elevated levels of JNK and phosphorylated c-Jun within the SNc and striatum (Waetzig et al., 2005; Peng et al., 2004). Moreover, there are several mechanisms through which JNK may influence neuronal survival; however, the majority of these are dependent upon

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phosphorylation/activation of c-Jun. As already alluded to, phosphorylated c-Jun modulates cellular processes, including cellular proliferation, differentiation and apoptosis, through its transcriptional effects upon numerous pro-death and inflammatory processes (Waetzig et al., 2005). In particular, phosphorylation of c-Jun was reported to be an essential step in the engagement of an apoptotic signal cascade in DA neurons provoked by MPTP and paraquat (Choi et al., 2005; Hayley et al., 2004; McCormack et al., 2005; Peng et al., 2004; Saibara et al., 2003). As well, inhibition of c-Jun by dominant negative adenoviral vectors or pharmacological blockade prevented both the neurodegenerative and neuroinflammatory actions of MPTP (Crocker et al., 2001; Hayley et al., 2004).

Among the downstream gene products activated by c-Jun that may be important in PD, the TNF-α superfamily member and pro-apoptotic factor Fas appears to be of great importance. Expression of Fas is enhanced by c-Jun phosphorylation through actions of the immediate early gene that influence the promoter region of the Fas gene (Ivanov et al., 2002). A variety of insults, including stroke, seizure and toxin exposure, increase Fas in a c-Jun dependent manner (Brecht et al., 2003). In the case of PD, Fas-deficient mice demonstrated attenuated activation of SNc microglia and loss of DA neurons in response to MPTP treatment (Hayley et al., 2004). Although the pro-apoptotic actions of Fas are likely important in toxin induced neurodegeneration, the contribution of pro-inflammatory processes also warrant attention. In this respect, Fas and several associated intra-cellular TNF-α receptor adapter proteins have been implicated in the release of pro-inflammatory cytokines, such as IL-1β, -6, -8 and TNF-α from astrocytes, monocytes and

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macrophages (Choi et al., 2005; Hayley et al., 2004). In light of this, Fas may contribute to degeneration by exacerbating/amplifying ongoing inflammatory cytokine cascades. Taken together with the current study, these findings suggest that microglial activation within the SNc plays a pivotal role in toxin exposure and dopaminergic cell loss.

4.4 Pro-inflammatory cytokine induced DA neurodegeneration: possible mechanism for paraquat neurotoxicity

Neuroinflammation is regulated by many signaling factors, such as cytokines; these pleiotrophic proteins play a pivotal role in neuroimmunopathological processes following injury and neurodegeneration (Ciesielska et al., 2003, Rousselet et al., 2002). Undeniably, it appears that pro-inflammatory cytokines participate in events associated with dopaminergic degeneration of the nigrostriatal pathway. It will be recalled that postmortem analysis found elevated levels of TNF-α, IL-1β, -4, -6 and transforming growth factor in the cerebrospinal fluid and the striatum (Mogi et al., 1998; Rousselet et al., 2002; Shen et al., 2005). As well, altered levels of apoptotic-related modifiers bcl-2, and Fas were also found in the brains of PD patients.

In the present investigation, paraquat had few effects upon plasma cytokine levels during the course of the injection procedures, the notable exception being a dramatic rise of IL-6 between the end of the first and second weeks of the three week treatment regimen used for Experiment 2. Interestingly, however, as revealed in Experiment 4, paraquat was found to elicit dose-dependent elevations of TNF-α , IL-1β, IL-2, IL-12 and IFN-γ, when plasma was obtained five days following the final injection of the herbicide. Thus, it appears that IL-6 may be important early on in the physiological impact of paraquat,

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whereas other inflammatory cytokines only change with the passage of time following insult with the toxin. These later occurring cytokine variations may be more important for progressive neurodegenerative processes that are believed to be manifested only after several weeks.

Analyses of cytokine protein levels of chronically treated MPTP mice revealed markedly elevated levels of IL-1β within the striatum coupled with significantly reduced concentrations of the neuronal growth factor, NGF (Gayle et al., 2002). It was suggested that IL-1β may act as a neurotoxic mediator through its oxidative effects, while reduced NGF levels would engender a vulnerability of DA neurons (Gayle et al., 2002; Hald and Lotharius, 2005). In addition to IL-1β, TNF-α has also been implicated in several neurological diseases, including PD (Hirsch et al., 2005; Jenner, 2001; Mogi et al., 1998; Sriram et al., 2002). In fact, TNF-α is generally considered a potent neurotoxin and inflammatory mediator, yet its function in neurodegenerative processes is highly controversial and widely debated. Several studies suggest that relatively low endogenous levels of TNF-α may actually have important neuroprotective functions, whereas higher exogenously applied concentrations of the cytokine appear to have deleterious central consequences (Bruce et al., 1996; Rothwell, 1999). It seems that this cytokine is pleiotrophic by nature with its function depending upon which of its two receptors is bound. Ligation of receptor 1 (R1) is important for the induction of apoptosis, cytotoxicity, fibroblast proliferation, and NF-*k*b activation, while receptor 2 (R2) seems to be important for cellular proliferation (Ciesielska et al., 2003; Mogi et al., 1998; Rousselet et al., 2002). Interestingly, double knockout mice lacking both TNF-α

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receptors were completely protected against the neurodegenerative and behavioral effects of MPTP (Sriram et al., 2002). Specifically, the double knockout mice were protected against striatal DA nerve terminal loss, and associated reactive gliosis. Therefore, TNF-α was suggested to be involved with early stages of damage that occur in the MPTP model (Sriram et al., 2002).

Increased levels of TNF-α following paraquat supports the notion that this cytokine plays a critical role at several stages of the degenerative processes. Specifically, we speculate that TNF-α is acting as an early initiator of microglial activation and is acting to augment the release of radical species and inflammatory cascades over the course of paraquat administration. This proposition is consistent with the well established role for TNF-α in oxidative, excitotoxic and apoptotic signaling under situations of acute (stroke, seizure) and chronic (viral meningitis, multiple sclerosis) central injury. Collectively, our data supports the notion that TNF-α is an essential component of the deleterious effects on the dopaminergic neurons within the SNc.

4.5 Intra-LPS infusion as a model for PD

Notably, the vast majority of PD-like animal models (i.e. MPTP, 6-OHDA) reproduce aspects of the neuroinflammatory response seen in post-mortem PD brains; however they are unable to reproduce a state of progressive DA loss within the SNc. In contrast, as will be discussed shortly, LPS exposure has been reported to recruit relatively long term inflammatory processes within the nigrostriatal system and, with sufficiently high doses, elicit progressive neurodegeneration (Kim et al., 2000; Ling et al., 2004). Thus, LPS

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exposure may more closely mimic the endogenous mechanisms which may be activated in PD patients. In fact, it may be that a chronic state of inflammation or a critical threshold is reached in PD which causes homeostatic mechanisms to be overwhelmed resulting in the progressive loss of DA neurons.

The bacterial endotoxin LPS has potent stimulatory effects upon the innate immune system (Castano et al., 2002). For instance, *in vitro* studies have shown that exposure to LPS elevated levels of a wide range of markers indicative of an inflammatory state including, NF_kB, cyclo-oxygenase-2 (COX-2), NO, scavenger receptor, several pro- and anti-inflammatory cytokines, as well as mitogen activated protein kinases (Herber et al., 2006). Similarly, *in vivo* administration of LPS also stimulates expression and/or release of these inflammatory factors from microglia within the brain parenchyma, such effects were observed following either central or peripheral routes of administration (Herber et al., 2006). In light of such potent immune activating effects, LPS-induced inflammation is believed to serve as a good animal model for examining the role of inflammation in PD (Castano et al., 2002; Herber et al., 2006). Indeed, LPS infusion into the ventricles or brain parenchyma instigates an inflammatory response causing heightened expression of complement receptor 3, MHC-II, CD45, F4/80 and the pro-inflammatory cytokines, IL-1β, IL-6 and TNF-α (Herber et al., 2006).

Although LPS is a strong inducer of macrophage/microglia cells within the SNc and striatum, its impact on nigrostriatal neurons remains unclear. Indirectly, the endotoxin may cause degeneration of the nigrostriatal dopaminergic neurons (Castano et al., 2002;

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Shibata et al, 2003), through its effects upon glial-dependent inflammatory processes. Substantial amounts of evidence suggest that the neurodegenerative effects of LPS are time-dependent and regionally specific (Kim et al., 2000; Herber et al., 2006; Iravani et al., 2005; Shibata et al, 2003). More specifically, acute infusion of LPS into the SNc was reported to provoke neurodegeneration of DA neurons, whereas infusion into the cortex, thalamus or the hippocampus had no such effects upon local neurons (Herber et al., 2006). In addition to acute administration, one existing report demonstrated that chronic infusion of LPS into the SNc by osmotic mini-pump induced maximal activation of microglia after 2 weeks and loss of DA neurons that was only evident after a one month interval (Iravani et al., 2005). The enhanced sensitivity of the neurons within the SNc has been attributed to the abundance of microglia in this region. Along these lines, mesencephalic neuron-glia co-cultures exposed to LPS displayed dose-dependent activation of microglia and release of NO and TNF-α, which in turn had damaging effects upon dopaminergic and other mesencephalic neurons (Gao et al., 2003).

Our preliminary findings, not included in the present thesis, indicated that even a relatively low (0.1 μg) dose of LPS had potent activating effects on SNc microglia that appeared to peak at 2 days and remained evident at 7 days following the infusion. Interestingly, this time course differed from that of several of the cytokines measured within the SNc in the present investigation. For instance, IL-6 and TNF-α were acutely elevated within the SNc at 90 min following LPS of both doses. Thus, these cytokines may be rapidly produced by cells other than local microglia. Indeed, it was recently found that intra-SNc infusion of LPS in rats provoked similar discrepancies in microglial

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activation and cytokine levels (Ling et al., 2006). It may be that these cytokines stem from infiltration of peripheral immune cells. In any case, this topic will be dealt with further in the ensuing section devoted to cytokine variations. Consistent with the present SNc findings, intra-hippocampal injection of LPS was recently reported to maximally increase cd11b after 3 days following the endotoxin and remained in such an activated state after 28 days. Thus, LPS may be able to activate microglia at several distinct brain regions, but the neurodegenerative consequences appear to be most apparent at the SNc.

The relationship between toxin-induced neurodegeneration and microglial activation is complex but likely involves activation of innate recognition elements that provide the first line of defense against pathogens. Of these, the Toll-like receptors (TLR) are classified as type-1 orphan receptors that posses an extracellular portion with leucine-rich repeats in addition to cytoplasmic similarities to the intracellular portion of IL-1R. Stimulation of TLRs initiates the formation of the toll-interleukin-1 receptor (TIR) signaling module, whereby several molecules act concomitantly as a single functional unit. TIRs are composed of numerous adaptor molecules such as Myd88 and kinases. Toll-like receptor-4 (TLR4) acts as the signal transducing receptor for LPS on monocytes and macrophages (Lehnardt et al., 2003; Pawate et al., 2004). Notably, TLR4 receptors are expressed on microglial cells and not astrocytes, and have been suggested to play a critical role in the central consequences of LPS challenge. In the absence of TLR4 receptors, several neuronal populations were protected from injury following *in vitro* or *in vivo* LPS application (Lehnardt et al., 2003) Additionally, interaction of LPS with TLR4 stimulates downstream MAP kinase and NF_kB signaling cascades (Aderem and

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Smith, 2004; Barry et al., 2005). As mentioned previously, LPS stimulation of iNOS and TNF-α expression in glial cells required intracellular signaling via ERK and p38 MAP kinases. As well, inhibitors of the alternate MAP kinase, JNK, by SP600125 prevent iNOS, TNF-α, IL-6 and COX-2 expression (Chen et al., 2005; Pall, 2002; Pawate et al., 2004; Peng et al., 2004). It is important to mention that LPS is merely one potential ligand for TLR4, meaning other endogenous/exogenous ligands may potentially bind to this receptor, ultimately contributing to neurotoxic and neurodegenerative effects of these receptors.

4.6 Cytokine variations elicited by LPS: a possible mechanism for PD

Although all of the factors through which microglia may influence neuronal survival are not completely understood, release of glial derived oxidative species clearly have toxic effects on the nigrostriatal system. As already alluded to, paraquat elicited the release of inflammatory cytokines that may regulate or at least enhance the production of such oxidative factors. For instance, TNF-α and IL-1β have been reported to mediate the neurotoxic effects of LPS on dopaminergic neurons in culture through the expression of iNOS and NADPH (Hald and Lotharius, 2005; Mander and Brown, 2005; Shibata et al., 2003). In concordance with past studies, the results for this thesis demonstrated that intra-nigral injection of LPS caused dramatic elevation in the cytokines TNF-α and IL-6 in the blood as well as in the SNc, 90min following the endotoxin injection. Interestingly these cytokines were only transiently elevated, returning to baseline levels by 2 days following the infusion. Alterations of TNF-α may be of particular interest given the recent accumulation of evidence to implicate the pro-death actions of this cytokine in PD.

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Indeed, prenatal LPS administration induced protracted nigrostriatal elevations of TNF-α and administration of LPS or similar inflammatory agents (e.g. thrombin) induced microglial expression of TNF-α (Carvey et al., 2003; Lee et al., 2005). Anti-inflammatory agents, such as minocycline and 3-hydroxymorphinan, have also emerged as having neuroprotective properties at dopamine neurons through their ability to inhibit TNF-α activity (Zhang et al., 2005; Prezborski). As well, two recent reports indicated that TNF-α deletion protected striatal terminals and either normalized dopamine levels in MPTP treated mice or increased DA metabolism, without necessarily affecting neuronal survival (Sriram et al., 2002).

Paralleling the TNF-α and IL-6 changes, the anti-inflammatory cytokine IL-10 displayed a similar pattern of expression within circulation but was not changed within the SNc. Interestingly, IL-1β plasma levels were up-regulated by LPS in a biphasic fashion, such that an early 90 min peak was followed by a return to baseline by 2 days and a second significant rise at the later 7 day interval following the endotoxin treatment. This complex pattern of expression may reflect alternate, temporally sensitive mechanisms that control distinct phases of the inflammatory cascades. In contrast, SNc levels of this cytokine were only elevated at the early 90 min interval, much like that of IL-6 and TNF-α.

It is unclear as to how intra-SNc LPS infusion elevated peripheral cytokine levels. This outcome may have arisen from several different mechanisms, including the activation of SNc projections to peripheral immune regulatory centres or activation of local

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immunocompetent microglia, which subsequently signal circulating immune elements. Alternatively, the possibility exists that a small concentration of LPS may have leaked into circulation or stimulated lymphocytes residing within the SNc or adjacent brain regions. Both of these latter explanations are not likely given the small volume infused and low concentration of such lymphocytes likely present within the SNc. In fact, our preliminary studies involving i.p. injection of 0.1 or 3.0 μg of LPS produced plasma cytokine variations that were actually of lower magnitude than those observed following the intra-SNc route of administration (Hayley et al., unpublished observations). In support of a role for the SNc in modulating peripheral immunity, SNc lesions were reported to reduce mitogen induced T lymphocyte proliferation 2 weeks after lesioning, but to enhance this response by 2-6 weeks after the insult (Deleplanque et al., 1992). Similarly, MPTP treatment that provoked a loss of SNc neurons also diminished the proliferation of splenocytes in response to treatment with LPS or the T cell mitogen, concanavalin A (Bieganowska et al., 1993).

It may be that LPS increased circulating cytokines by impairing the ability of SNc soma to release dopamine, which is know to restrain cytokine levels. Indeed, dopamine itself has been suggested to act in a similar manner to anti-inflammatory cytokines, in that the monoamine inhibited the release of Th1 and other pro-inflammatory cytokines, including IL-2, IFN-γ and TNF-α. Also, dopamine agonists have even been used as anti-inflammatory treatments in cases of shock and systemic lupus erythematosus (Oberbeck et al., 2006; Sookhai et al., 2000). It is likely that dopamine utilization was affected by LPS in the present study given the marked alterations of home-cage activity that were

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evident. Expanding upon these early studies, the present investigation revealed that doses of LPS that elicited marked neuroinflammation in the absence of overt SNc neurodegeneration influenced peripheral immune functioning.

Less marked cytokine changes were evident within the striatum when compared to the changes within the SNc or plasma. The changes that did occur tended to appear at later times or were more protracted in duration, as was the case for IL-6, which was elevated at 90 min and remained so for the entire 7 day testing interval. Interestingly, the only other cytokine out of the 10 that were assayed within the striatum that was influenced by LPS was IL-1β, which was increased at the 90 min interval. This is particularly significant since both IL-1β and IL-6, unlike TNF-α, have been reported to have a neuroprotective role in PD (Bolin et al., 2002). For instance, infusion of IL-1β protected DA neurons from 6-OHDA and MPTP toxicity and induced dendritic branching from residual neurons following SNc lesion (Saura et al., 2003). Moreover, IL-6 knockout mice displayed enhanced degeneration of SNc neurons and striatal terminals following MPTP, suggesting enhanced sensitivity in the absence of endogenous levels of the cytokine (Bolin et al., 2002). The LPS induced cytokine changes at the level of the striatum may be viewed as compensatory responses aimed at strengthening this region against any future damage. It is interesting to speculate that intra-SNc LPS infusion may have activated nigrostriatal DA fibre projections resulting in these cytokine variations within the striatum.

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It is not clear how these cytokine variations may influence neuronal survival, however, it has been previously shown that neutralizing antibodies for either TNF-α or IL-1β diminished neuronal toxicity induced by LPS and IFN-γ in neuron-microglia cultures by approximately 50% (Hald and Lotharius, 2005). It may be that the early rise of several of these cytokines serves to activate the initial inflammatory cascade, such that microglia reactivity only becomes apparent after several days. During such early stages of activation the cytokines may be acting upon microglia, placing them in a primed state while at the same time directly binding to DA neurons. Indeed, several cytokine receptors, at least that of TNF-α, exist on SNc DA neurons (Hald and Lotharius, 2005). By way of either direct cytokine interactions at DA receptors or via microglia inflammatory species, DA neurons may then be vulnerable to the impact of subsequent challenges. In this regard, the final section of this thesis deals with the possibility that, following LPS, these cytokine and glial alterations may sensitize neurons to the effects of later exposure to paraquat.

4.7 LPS pre-treatment enhances the central impact of paraquat: Cross-sensitization

As already alluded to, many factors likely influence the onset and the progression of PD. Interestingly, although several epidemiological studies have implicated a number of environmental agents, in the majority of cases no one particular toxin or incident may be identified that actually caused the disease. In actuality, it may be that repeated exposure to low doses of multiple toxins act synergistically to provoke or at least worsen the disease, meaning that PD may be a result of "multiple hits" involving one or many different toxins (Ling et al., 2004). This interesting hypothesis first suggested by Di

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Monte (2003) implies that synergistic interactions among multiple toxins may be responsible for PD. In concordance with this hypothesis, combined MPTP and LPS synergistically induced selective and progressive degeneration of DA neurons *in vitro* and ROS released from microglia were essential for this synergistic neurotoxicity (Gao et al., 2003). More specifically, in the absence of microglia or the presence of inhibitors of ROS, LPS combined with either MPP+ or with MPTP failed to produce dopaminergic degeneration (Barry et al., 2005). As well, a recent study which looked at combined neurotoxicity of maneb and paraquat revealed that the dual combination of these pesticides synergistically influenced DA neuron losses in aged mice (Thiruchelvam et al., 200). It is reasonable to suggest that multiple acute toxin exposures could produce progressive dopaminergic cell loss by achieving a critical threshold of inflammation. Such inflammation may then initiate a self-sustaining cascade of events leading to enhanced DA loss which may ultimately lead to neurodegeneration.

In addition to the synergistic actions observed with concomitant administration of LPS and toxins, these insults may interact over time such that they sensitize or reinforce each others consequences. For instance, a series of *in vivo* experiments conducted by Carvey and associates (2004, 2004 and 2006) demonstrated that prenatal LPS induced a relatively permanent state of neuroinflammation, which modified the impact of later exposure to several different toxins. Indeed, rodents re-exposed to LPS as adults following prenatal endotoxin administration exhibited a profound and progressive loss of DA neurons and augmented expression of TNF-α within the striatum (Ling et al., 2006). As well, prenatal LPS exposure, by injecting gravid females with the endotoxin, greatly

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enhanced the impact of rotenone administration upon striatal DA levels, after a protracted period of 17 months (Ling et al., 2004). These findings suggest that early exposure to an inflammatory event sensitized neuronal functioning, such that adult animals displayed a relatively permanent increased in vulnerability to DA toxins. Thus, in addition to the aforementioned synergistic effects of LPS with such toxins, the endotoxin may also act over time to sensitize the impact of these challenges. As well, it should be considered that LPS may be acting to produce neuro-developmental deficits in the DA circuits. Indeed, rats that simply received *in utero* LPS in the absence of a later challenge, had a lower number of TH+ neurons as adults (Ling et al., 2004).

According to a multi-hit hypothesis, accumulative intermittent toxin exposure at any point in an individual's life may contribute to the evolution of PD. Expanding upon this hypothesis, one premise behind the current thesis is that multiple insults may provoke a PD-like syndrome through activation of inflammatory processes. Specifically, LPS exposure is hypothesized to stimulate microglia and pro-inflammatory cytokine release, which subsequently enhances the vulnerability of DA neurons to the impact of paraquat. However, contrary to our initial hypothesis, LPS pre-treatment did not alter the impact of later paraquat exposure upon the loss of DA neurons within the SNc. Indeed, comparable losses of TH+ cells were found within this region following paraquat with or without pre-treatment with the endotoxin. Surprisingly, however, intra-SNc LPS actually reduced the loss of TH+ terminals within the striatum following paraquat exposure. Thus, it appeared that LPS pre-treatment has a neuroprotective effect against subsequent paraquat exposure. This finding is of considerable importance and it raises the possibility that

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certain temporal combinations of insults may actually have favorable outcomes. This finding may be interpreted in a manner similar to previous studies demonstrating that LPS pre-treatment protected against the damage elicited by a later ischemic challenge (Barone et al., 1998). Indeed, endotoxin exposure 1-2 days prior to occlusion of the middle cerebral arteries induced a state of ischemic tolerance, wherein the infarct volume was dramatically reduced by the LPS pre-treatment (Barone et al., 1998). This effect was suggested to stem from enhanced expression of growth factors, heat shock proteins, and molecular chaperones, provoked by LPS (Barone et al., 1998). However, little is known regarding the potential role of cytokines in such a de-sensitized response.

Our finding that LPS pre-treatment has a de-sensitizing effect upon the impact of paraquat at the level of the striatum but not the SNc is particularly intriguing in light of the fact that it is well know that toxins, such as MPTP, often differentially influence SNc and striatal functioning (Landrigan et al., 2005; Przedborski et al., 1996; Salach et al., 1984). Indeed, the timing and degree of degeneration of these two interconnected regions often varies dramatically. Additionally, well established dopaminergic neuroprotective agents often selectively protect the SNc soma without influencing the degeneration of downstream striatal terminals (Przedborski et al., 1996; Hayley et al., 2005). The neuroprotective effect of LPS in the current thesis may stem from several potential mechanisms including, the endotoxin; 1. provoking a de-sensitization of neurodestructive pathways normally activated by paraquat, or 2. activating alternate, normally protective factors that counteract those stimulated by the pesticide. In either case, changes in cytokines may play a role, just as they have been speculated to modulate some of the

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delayed protective consequences of ischemic tolerance induced by LPS (Barone et al., 1998).

Paralleling the neuroprotective effect of LPS at the striatum, endotoxin pre-treated mice that were later exposed to paraquat displayed greatly reduced circulating cytokine levels, relative to those that received the pesticide in the absence of LPS pre-treatment. Thus, it appears that LPS may have de-sensitized the cytokine response, such that the inflammatory immune response was less responsive to the toxin challenge. This finding is contrary to our initial expectations but is consistent with previous reports of immunological studies demonstrating the phenomenon of endotoxin tolerance. Indeed, it is well known, especially using *in vitro* systems, which repeated LPS administration results in a dampening of the release of pro-inflammatory cytokines and acute phase proteins (Chen et al., 2005). This effect was dependent upon the timing, with a 16 hr delay between two systemic LPS injections resulting in a profound de-sensitization in the ability of the second injection to provoke TNF-α and IL-6 changes without affecting the actions of the endotoxin upon IL-1β levels (Chen et al., 2005). Interestingly, the present results indicate that such a phenomenon might also occur between the two heterogeneous insults, LPS and paraquat. Accordingly, this would suggest the possibility that paraquat may be acting upon immune pathways similar to those activated by LPS. This is a novel finding, since little is known about the general immunological effects of paraquat outside a few toxicological reports indicating that the pesticide provoked inflammation within the lungs and possibly other peripheral organs (Day et al., 1999).

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The present findings highlight the importance of cytokines not only as regulators of immune processes, but also as messengers that may be involved in central signaling during nigrostriatal neurodegenerative processes. It is our contention that cytokines may help transform peripheral inflammatory signals provoked by environmental toxins into brain changes that have important functional consequences. Indeed, paraquat induced disturbances of several ratings of PD-like behaviors were found to correlate with elevations of circulating pro-inflammatory cytokines. Moreover, direct inflammation within the SNc, as provoked by LPS, caused profound time-dependent changes of nigrostriatal cytokines that are likely involved in concomitant processes necessary for neuronal repair. Yet, chronically persisting elevations of these cytokines may actually damage neurons through actions upon free radical or apoptotic pathways. Given that PD likely originates from the interactive effects of multiple environmental insults upon a possibly compromised genetic background, it is necessary to evaluate the joint effects of more than one causative agent. In this regard, the present data support the notion that a pre-existing neuroinflammatory state (induced by LPS) may inhibit some of the deleterious consequences of later toxin exposure and such an effect may arise from alterations of the ratios of different cytokines. Finally, the assessment of multiple cytokines simultaneously, as conducted in the present thesis, may provide important clues as to how these pleiotrophic immunotransmitters may interact over time to ultimately shape the evolution of disease states such as PD.

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