Age and IFN-γ Deficiency Modulate the Impact of Repeated Paraquat Exposure in a Mouse Model of Parkinson's Disease

by

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Abstract

Parkinson's disease (PD) is a chronic and progressive age dependent disease characterized by its severe motor impairment, which accompanies a loss of dopamine producing neurons in the substantia nigra pars compacta (SNc). The herbicide paraquat, has been observed to dose-dependently provoke a loss of dopaminergic neurons in the SNc. Additionally a substantial body of research has provided evidence for a role of chronic inflammation in PD. In the present thesis, we assessed the influence of age of exposure to paraquat and whether re-exposure at an advanced age (following a four month delay) would augment the neurodegenerative effect of the herbicide. We also endeavored to determine whether deficiency in the pro-inflammatory cytokine interferon-γ (IFN-γ) would alter the long-term impact of paraquat, as we previously found this cytokine to be integral to the neurodegenerative consequences of a more restricted paraquat regimen in young adult mice. Our results revealed that paraquat provoked a significant loss of dopamine neurons in the SNc, and evoked behavioural alterations late in adulthood of wild types, these effects were totally absent in IFN-γ knockout. A sensitized response was observed within the locus coeruleus (LC), such that the re-exposure regimen was associated with a virtual complete ablation of "healthy" tyrosine hydroxylase expressing neurons. Taken together, these data support our previous findings that IFN-γ is a key regulatory of the neurodegenerative response to environmental toxin exposure and that age influences the magnitude of such effects.

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1. Introduction

1.1 Background on Parkinson's Disease

Parkinson's disease (PD) is a chronic and progressive disease characterized by muscle rigidity, resting tremor, bradykinesia, and akinesia, all of which worsen as the disease progresses (Caline, et al., 1983; Greenamyre, et al., 2004; Israel, et al., 2008; Blandini, et al., 2007). In North America, approximately 0.3% of the general population is currently afflicted. However, the incident rate of PD increases to 1 – 2% by the age of sixty five and doubles further still for individuals over 85 years of age (Blandini, et al., 2000; Weintraub, et al. 2008).

The motor symptoms associated with PD result from a dysfunction of the basal ganglia network (VanItallie, 2008). In particular, disruption of basal ganglia communication and resultant motor deficits originate from the death of dopaminergic neurons of the substantia nigra pars compacta (SNc). These dopaminergic neurons of the SNc project to the dorsal region of the striatum (ST) where dopamine is released, and acts upon local GABAergic (γ-aminobutyric acid) inhibitory neurons (Betarbet et al., 1997, Betarbet et al, 1999; Betarbet et al., 2000; Goetz et al., 2007; Tepper et al., 2007). The loss of striatal dopamine input results in a disruption in the normal functioning of GABA neurons, which subsequently acts in a feed-forward manner to disturb the delicate communication to the globus pallidus and thalamic basal ganglia regions (see appendix: *Neuroanatomy of the Murine Brain*). Ultimately, such faulty communication results in attenuated thalamic drive to the motor cortex and the manifestation of cardinal motor features of PD, including a paucity of movement (bradykinesia) resting tremor, and difficulty in the purposeful initiation and cessation of movement (Hunot and Hirsch.,

2003; Litteljohn et al., 2008; Mangano and Hayley, 2009). Current consensus suggests that these behavioural symptoms only become clinically apparent when there is a 50-70% loss of dopamine neurons in the SNc (Blandini et al., 2000; Kedar, 2003; Weintraub et al. 2008; Mangano and Hayley, 2009). Currently the etiology of PD is unknown and investigators in this field have postulated everything from viral infection, auto-immune disease, genetics, and environmental toxins as potential origins of the disease.

Neuronal loss is not isolated to the nigrostriatal pathway, in fact, post-mortem analysis of PD brains reveal a less pronounced yet substantial loss of norepinephrine producing neurons in the locus coeruleus (LC), along with loss of neocortical and hippocampal cells (Scatton et al., 1982; Betarbet et al., 2000). In addition to the nigrostriatal degeneration, a definitive PD diagnosis requires the presence of a significant number of Lewy bodies (Schulz-Schaeffer, 2010). Lewy Bodies are insoluble protein aggregates forming fibrils, composed mainly (but not exclusively) of the protein α-synuclein (Rochet et al., 2004). Hence, PD, like all central nervous system disease, is obviously a very complex condition which involves disturbances in multiple neurotransmitter systems.

1.2 Idiopathic Neurotoxins

Even though some forms of PD are highly heritable, the overwhelming majority (>90%) are believed to be idiopathic in origin (Caline and Langston, 1983; Hunot and Hirsch, 2003; Litteljohn et al., 2011). Indeed, twin studies have demonstrated very low concordance rates and in fact, have provided little evidence of a heritable component in the majority of cases of PD (McCormack et al., 2002; Shimizu et al., 2003; Litteljohn et

al., 2008). Thus, not surprisingly researchers have looked to environmental influences as a role in the development of PD. In this regard, epidemiological studies have consistently demonstrated a positive correlation between PD incidence and proximity to agricultural centers, industrial manufacturing plants, heavy metals and air pollution (Liou, 1997; McCormack, 2002; Di Monte, 2003; Wu, 2003). In some areas contaminated with these pesticides, the rate incidence of PD for males over fifty is as high as 7%, a great increase over that of 1% observed in males over 65 in the general population (McCormack, 2002; Dick, 2007).

Although there has yet to be agreement on any particular naturally occurring toxin that maybe responsible for PD, the pesticides rotenone and paraquat (see appendix: *Chemical Structure of Paraquat*), as well as a number of heavy metals, including manganese, iron and lead have been implicated in the progression of PD-pathology (Tanner and Goldman, 1996; Ritz and Yu, 2000; Priyadarshi et al., 2001; Peng et al., 2007). Indeed, case-control studies and meta-analyses have unveiled significant correlations between PD and prolonged occupational exposures to pesticides and heavy metals (Priyadarshi et al., 2001; Abbott et al., 2003; Gorell et al., 2004). The herbicide, paraquat, has emerged as a pesticide highly correlated with PD. For instance, a Taiwanese cohort study found that exposure to paraquat had a greater association with PD in comparison to other pesticides (Liou et al., 1997). As will be discussed more fully in subsequent sections, paraquat can promote pathological features resembling PD-like syndrome in rodents, however, its long-term histopathological consequences in humans is presently unknown. Of course, there is the always the possibility that some yet to be identified genetic factor will emerge over time and explain a larger proportion of PD

cases. However, it seems more likely that a combination of an intrinsic genetic vulnerability may act in a synergistic manner with environmental exposures to jointly influence the emergence of PD pathology. Dopamine neurons in the SNc appear to be far more vulnerable to damage caused by neurotoxins than are other cells in the central nervous system (CNS). This vulnerability has been posited to stem from the high concentrations of iron and neuromelanin, as well as the intrinsically high metabolic rate of SNc dopamine neurons (Blandini et al. 2000; Betarbet et al., 2002; Gonzalez-Hernadez et al., 2010). Moreover, post-mortem analyses have revealed reduced levels of anti-oxidants (e.g. glutathione) and elevated levels of lipid peroxidation (indicative of reactive oxygen species induced oxidative stress) in the SNc of PD patients (Dexter et al., 1989; Dexter et al., 1994; Alam et al., 1997). These vulnerability factors would necessarily lead to the dopamine neurons being especially susceptible to oxidative stressors and would put strain on the cells defense and repair mechanisms (Fahn and Cohen, 1992; Betarbet et al., 2000; McCormack et al., 2002; Gonzalez-Hernadez et al., 2010). Such factors become especially important when considering the impact of environmental toxins, including pesticides, given that many of these compounds act by affecting redox cycling or the production of pro-oxidant species (Betarbet et al., 2002; McCormack et al., 2002; Thiruchelvam et al., 2003; Wu et al., 2003; Mangano et al., 2011, Tanner et al., 2011).

1.3 Established Models of Parkinson's Disease

6-OHDA and MPTP are the most commonly employed neurotoxins used in rodents and primates to elicit PD-like pathology (German et al., 1996; Bergman et al., 1998;

Blum et al., 2001; Bove et al., 2005; Carrasco et al., 2005; Quintero et al., 2006). 6-OHDA is a hydroxylated analogue of dopamine, which is capable of producing severe peripheral and central lesions. Given that this dopamine toxin cannot penetrate the blood brain barrier (BBB), it must be administered either directly into the ST, SNc or ascending medial forebrain bundle. The exact mechanism by which 6-OHDA is taken up by dopamine neurons is currently unknown; however, due to its specificity it is likely that the toxin is taken-up by the dopamine transporter. It has been demonstrated that cytosolic accumulation of 6-OHDA will generate cytotoxic metabolites (e.g. quinones, superoxide radicals, hydrogen peroxide and the hydroxyl radicals) in a manner similar to endogenous dopaminergic auto-oxidation upon encountering monoamine oxidase (Blum et al., 2001; Rodriguez-Pallares et al., 2007; Gomez-Lazaro et al., 2008). In addition to 6-OHDA's ability to generate free radicals, this toxin has also been associated with an up-regulation of the pro-apoptotic factor Bax(Blum et al., 2001; Jordan et al., 2004; Gomez-Lazaro et al., 2007; Gomez-Lazaro et al., 2008).

There are clear limitations to using 6-OHDA as an animal model of PD; for one, the requirement of administration of 6-OHDA directly into the nigrostriatal pathway. Secondly and most importantly, the time course for 6-OHDA induced dopamine cell death is very rapid. This clearly is not consistent with the very slow progressive nature of pathology observed in PD. (Bove et al., 2005). Another drawback to using 6-OHDA as a model for PD lies in its inability to cause the formation of Lewy bodies, a characterizing feature of the disease.

Some of the drawbacks associated with the 6-OHDA model may be addressed by using the meperidine derivative, MPTP. This toxin is highly lipophilic and easily crosses

the BBB preferentially targeting several midbrain dopamine neuronal populations, namely the A8 (retrorubral nucleus), A9 (substantia nigra), and A10 (ventral tegmental area) regions (German et al., 1996). Systemic exposure to MPTP has been used over the past two and a half decades to provoke SNc dopaminergic degeneration coupled with depletion of striatal dopamine in mice and primates (Czlonkowska et al., 1996; Przedborski et al., 1996; Bergman et al., 1998; Boulet et al., 2008). MPTP is metabolized by monoamine oxidase B and converted into its active metabolite, MPP+, which is taken up by the dopamine transporter and into dopaminergic terminals. Early studies with MPTP determined that MPP+ is a particularly potent mitochondrial complex I inhibitor. The inhibition of mitochondrial complex I eventual culminates in decreased adenosine triphosphate (ATP) levels, loss of mitochondrial membrane potential, faulty intracellular calcium buffering and free radical generation (Blum et al., 2001).

More recent studies have demonstrated that MPTP can also induce an inflammatory response that can be detrimental to dopamine neurons. Important for the present thesis, numerous studies have demonstrated that MPTP induces robust microglial activation and stimulation of inflammatory messengers (Czlonkowska et al., 1996; Wu et al., 2002; Feng et al., 2003; Barcia et al., 2004; Hayley et al., 2004; Bolin et al., 2005). Moreover, pharmacologically blocking the microglia response can prevent MPTP induced degeneration of dopamine neurons (Wu et al., 2002). Unlike 6-OHDA, non-human primate exposure to low doses of MPTP have been reported cause a Parkinsonian-like syndrome that mimics many of the clinical attributes of PD, including bradykinesia and resting tremors (Bergman et al., 1998; Mounayar et al., 2007; Boulet et al., 2008; Israel and Bergman, 2008). Somewhat perplexingly however, long-term evaluation of MPTP

has revealed that some of the neurochemical (striatal sopamine levels) and behavioural deficits do normalize over time, probably resulting from compensatory mechanisms eventually being recruited (Mounayar et al., 2007; Boulet et al., 2008).

1.4 More Recent Models of Parkinson's Disease, Rotenone and Paraquat

In the search for a more environmentally valid model, investigators began looking into pesticides such as the insecticide rotenone and the herbicide, paraquat. These pesticides have both been implicated in epidemiological studies concerning PD (Brooks et al., 1998; Panov et al., 2005; Dick, 2007). Rotenone is a lipophilic pesticide which is able to cross the BBB and damage mitochondria leading to cell death in dopamine neurons of the SNc following chronic systemic administration (Sherer et al., 2003a; Panov et al., 2005; Kwong et al., 2006; Emborg, 2007). Rotenone also inhibits NADH and complex I of the electron chain in mitochondria. As with MPP+, this leads to mitochondrial dysfunction, an inability to produce ATP, and eventually the build up and release of cytochrome c into the cell (Sherer et al., 2003a; Betarbet et al., 2000; Littlejohn et al., 2011). Despite the fact that rotenone inhibits mitochondrial complex 1 in many brain regions, midbrain dopaminergic neurons appear to be the most sensitive, suggesting that these neurons are intrinsically vulnerable (Betarbet et al., 2002; Sherer et al., 2003). Moreover, free radical accumulation has been attributed to rotenone-induced dopaminergic damage and was attenuated with anti-oxidant (e.g. alpha-tocopherol) pre-treatment (Sherer et al., 2003b). As cell degeneration progresses, stereotypical behavioural symptoms associated with PD begin to emerge, including decreases in home-cage activity, muscle rigidity and loss of limb co-ordination (Betarbet et al., 2000; Tanner

and Goldman, 2011). Chronic rotenone administration in mice also causes the formation of PD-characteristic cytoplasmic inclusions, α-synuclein Lewy Bodies, in the SNc (Panov et al., 2005; Kwong et al., 2006; Emborg, 2007), which are believed to reflect disturbances of protein folding, among other pathological processes occurring in the PD brain. .

Rotenone has several advantages over older PD models in that it has actually been shown to be present at elevated levels in individuals in agricultural areas that come into regular contact with the pesticide (Betarbet et al., 2000; McCormack et al., 2002). However, rotenone can produce substantial variability in the severity of symptoms provoked, and in some cases, behavioural symptoms can be entirely absent despite extensive cell death (Betarbet et al., 2002). Furthermore, rotenone often produces a very high level of systemic toxicity and sickness that can confound the interpretation of the behavioral consequences of the pesticide (Tanner and Goldman, 2011; Sherer et al., 2003b).

The pesticide that has been most often implicated in epidemiological studies, and the only pesticide observed to have a dose dependent relationship between lifetime cumulative exposure and increased risk of PD is the bypyridyl herbicide, paraquat (Liou et al., 1997; Petrovitch et al., 2002; Bateman, 2008). This herbicide can provoke histopathological and behavioural changes reminiscent of PD in rodents and non-human primates (Brooks et al., 1999; Thiruchelvam et al., 2000; Thiruchelvam et al., 2003; Cicchetti et al., 2005; Ossowska et al., 2005; Fernagut et al., 2007; Litteljohn et al., 2008; Litteljohn et al., 2009; Mangano and Hayley, 2009). Paraquat is also ecologically valid as it is currently the third most commonly used pesticide in the world. Although thirteen

countries (which are largely within the European Union) have recognized the dangerous side-effects of both direct and indirect exposure to paraquat and have banned the use of this herbicide; curiously, Canada, the United States, Mexico and much of Central and South America are not included in this list (Cicchetti et al., 2005). Paraquat can accumulate in especially high levels within the brain (where it has a half life of approximately one month) through inhalation, oral ingestion or even dermal contact (Thiruchelvam et al., 2000; Brooks et al., 1999; Cory-Slechta, 2005a).

Paraquat is chemically similar to the active metabolite of MPTP, MPP^+ and can reliably provoke a progressive loss of nigrostriatal dopamine neurons (McCormack et al., 2002a; Andersen, 2003; Peng et al., 2004; Ossowska et al., 2005; Richardson et al., 2005; Peng et al., 2007; Purisai et al., 2007; Yang and Tiffany-Castiglioni, 2007; Yang et al., 2007; Somayajulu-Nitu et al., 2009). Although the degree of neuronal loss induced by paraquat is less than that induced by MPTP (~30% versus 50%); like rotenone, paraquat can provoke the formation of Lewy bodies (McCormack et al., 2002; Manning-Bog et al., 2003) . In addition to these pathological hallmarks, paraquat can trigger behavioural disturbances reminiscent of PD. In particular, mice chronically treated with paraquat experienced a loss of striatal dopamine coupled with reduced locomotor activity, which was evident by impaired vertical forelimb and hindlimb co-ordination (Li et al., 2005; Litteljohn et al., 2008; Litteljohn et al., 2009). Similarly, aged mice (five or eighteen months) concomitantly exposed to paraquat and the fungicide maneb experienced robust motor deficits, which remained stable even three months following toxin exposure (Thiruchelvam et al., 2003; Ossowska et al., 2005). Paraquat treatment can also trigger non-motoric behavioural disturbances, including diminished performance on a forced

swim test and open field (Chen et al., 2008; Litteljohn et al., 2008; Litteljohn et al., 2009), which is often taken to reflect affective disturbances. The underlying mechanism responsible for paraquat induced dopaminergic cell death remains unknown. However, our own work and that of others suggest that that activation of neuroinflammatory cascades, along with the production of oxidative radicals play an important role (Litteljohn et al., 2008; Mangano and Hayley, 2009; Litteljohn et al., 2009).

1.5 Mechanism of Action for Paraquat Induced Degeneration

Paraquat is able to create similar neurodegenerative and behavioural deficits as observed following MPTP administration, though the mechanism through which this occurs is not entirely clear. One possibility is that paraquat promotes mitochondria instability and dysfunction by increasing membrane permeability by affecting endogenous permeability transition pores (Brooks et al., 1999; Dick et al., 2007; Gomez-Lazaro et al., 2007). Indeed, paraquat was reported to shift the gating potential of these pores causing them to remain open under resting conditions (Dick et al., 2007; Gomez-Lazaro et al., 2007), leading to membrane depolarization, uncoupling, and matrix swelling. Other studies have provided evidence that paraquat interferes with oxidative phosphorylation and the electron transport chain leading to mitochondrial dysfunction (Palmeira et al., 1995). Although paraquat may alter mitochondrial functioning in this fashion, many investigators now believe that the pesticide exerts the majority of its toxic effect through redox cycling, oxidative stress, and neuroinflammation (di Monte, 2003).

Paraquat acts as a redox cycling agent, ultimately interacting with microglia and neuronal cells to both accept and donate electrons, resulting in oxidative free radical

production (McCormack et al., 2005; Richardson et al., 2005). Such effects stem from the fact that paraquat has two positively charged ions allowing it to generate large quantities of reactive oxygen species upon contact with molecular oxygen (Richardson et al., 2005). Paraquat is believed to have a particular affinity for the NADPH oxidase enzyme complex found on microglial cells (Mizuno et al., 1987; Blum et al., 2001; Peng et al., 2004; Cristovao et al., 2009; Gonzalez-Hernadez et al., 2010). In this case, paraquat induces the production of the oxidative radical, superoxide, from microglia, which can have very toxic consequences upon neighbouring dopamine neurons (Gao and Hung, 2008; Cristovao et al., 2009; Gonzalez-Hernadez et al., 2010). Additionally, paraquat can also act in an exitotoxic manner by opening NMDA receptor channels and promoting an influx Ca(2+) (Dick et al., 2007; Gomez-Lazaro et al., 2007). This can lead to further oxidative species, including nitric oxide (NO being formed). Once created, NO is able to aid in the formation of peroxynitre anions; free radicals capable of directly damaging mitochondria and inducing dysfunction of cellular organelles (Dick et al., 2007; Gomez-Lazaro et al., 2007).

In many cases the degree of cellular damage caused by paraquat when administered alone at concentrations low enough to be relevant to human levels of exposure is somewhat limited (~ 30%) (McCormack et al., 2002; Cory-Slechta et al., 2005b; Mangano and Hayley, 2009; Berry et al., 2010). However, priming the SNc with an immunological toxin (lipopolysaccharide; LPS) prior to paraquat exposure or concomitant exposure of paraquat with the fungicide maneb greatly augmented the degree of dopamine cell death in the SNc (from ~ 30 to 50% dopamine cell loss) (Mangano and Hayley, 2009; Purisai et al., 2007). Similarly, exposure to a sub-toxic

dose of iron synergistically augmented the effects of paraquat on SNc dopamine neurons and this was related to a reduction in number of available anti-oxidants and increase in the pro-apoptotic transcription factor, c-Jun N-terminal kinases (JNK) (Peng et al., 2007). Likewise, aged mice (five or eighteen months) concomitantly exposed to paraquat and the fungicide maneb experienced robust motor deficits, which remained stable even 3 months following toxin exposure (Thiruchelvam et al., 2003; Ossowska et al., 2005).

Support for a neuroinflammatory component of PD has been gaining considerable attention in recent years for a variety of reasons. Not the least of which being that numerous studies have shown that paraquat administration leads to an up-regulation of activated microglia, cytokines and other inflammatory factors (Czlonwska et al., 1996; McCormack et al., 2002; Mangano and Hayley, 2009; Litteljohn et al., 2011). As already alluded to neuroinflammatory processes are likely fundamental for the oxidative damage provoked by toxins in dopaminergic neurons (Czlonkowska et al., 1996; Kurkowska-Jastrzebska et al., 1999). In this respect, it is of interest to mention that each of the environmental agents discussed thus far have been shown to induce signs of neuroinflammation. This is not surprising given that a primary role of inflammatory immunological functioning is to rid the body of such neurotoxins. 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 The Role of Cytokines, Microglia and Other Neuro-Inflammatory Processes

Presently there is an ongoing debate as to the role microglia play in PD, whether they are primarily responsible for dopamine degeneration or a secondary reaction acting to remove the dead/dying neurons. In reality, it is likely that microglia provide some

beneficial support to neurons, as well as release toxic by-products as a consequence of respiratory bursts. During non-pathological conditions microglia detect and react to modifications of the local environment in order to restore homeostasis (Davalos et al., 2005; Nimmerjahn et al., 2005). In the case of injury or disease, communication between neurons and microglia, endothelial cells and circulating leukocytes occur through ATP gradients and the release of heat shock proteins from sick/dying cells (Matzinger et al., 2002; Popovich and Longbrake, 2008). As neurons succumb to metabolic stress, they release ATP into the extracellular space, inducing the propagation of calcium waves in nearby astrocytes which intern causes the releases of more ATP. Subsequently, microglia cells will recognize these ATP gradients and migrate along these gradients and facilitate the removal of the dead / sick cells (Cotrina et al., 2000; Davalos et al., 2005; Koizumi and Fujishita, 2007). In this regard, microglia perform an essential neuroprotective housekeeping function (Popovich and Longbrake, 2008). In the case of PD, these 'danger' signals (e.g. ATP or heat-shock proteins) released from injured and dying cells may be subtle and occur over a prolonged period of time lead to a chronic up-regulation of inflammatory status (Popovich and Longbrake, 2008).

Initially it was thought that microglia were evenly distributed in the brain; however, Lawson et al 1990 showed that there seems to be a particularly high concentration of microglia in the SN, olfactory telencephalon, basal ganglia, and hippocampus, which coincidently are regions most affected by PD and Alzheimer's Disease, respectively. Post-mortem analysis of PD brains demonstrated profound microgliosis in the SNc and to a lesser extent the ST (McGeer et al., 1988). This was supported by Langston et al, 1996 who showed that humans exposed to MPTP displayed

heightened microglial activation in the SNc 16 years following MPTP exposure (Langston et al., 1996). . In this regard, the high concentration of microglia may explain why these brain regions are preferentially targeted in the aforementioned diseases. The fact that the SNc contains a higher density of microglial cells then the cortex and other brain regions is one explanation as to why the SN may be especially vulnerable to immunological and environmental toxic insults (Kim et al., 2000).

The reactivity state of microglia varies along a spectrum ranging from resting to hyperactive and is under the strict control of several regulatory proteins. Whether microglia take on a more M1 (state normally responsible for respiratory bursts and associated with neurotoxic consequences) or M2 (basal state often release neuroprotective factors) phenotype depends on the signals they receive (Whitton, 2007; Michelucci et al., 2009). For the most part, microglia normally take on an M2 phenotype, acting as sentinels slowly proliferating and removing any debris by phagocytosis in the CNS (Davalos et al., 2005; Nimmerjahn et al., 2005; Kumar and Jack, 2006). The immunological phenotype (e.g. surface receptor expression) of this state is characterized by low expression of MHC proteins, CD45, CD14 and CD11b receptors (Kreutzberg et al., 1996; Aloisi et al., 1998).

Together with astrocytes, microglia work to maintain a homeostatic microenvironment within the brain, responding to changes in the CNS such as neurotransmitter imbalances as well as infiltrating peripheral immune cells (Kreutzberg et al., 1996; Aloisi et al., 1998). Compelling evidence suggests that microglial cells in an M2 state perform neuroprotective functions in PD, at least in the short term, by secreting trophic factors such as nerve growth factor (NGF), neurotrophin (NT)-3 and brain

derived neurotrophic factors (BDNF) (Peterson and Nutt, 2008). In the case of PD, microglia cells undergo extreme morphological changes and adopt an M1 phenotype, wherein their processes retract inward and the cell adopts an amoeboid-like appearance, eventually becoming phagocytic, akin to the perivascular macrophage. Paralleling these morphological changes, these hyper-active microglia will enhance the expression of ICAM, MHC class II, complement receptors while at the same time releasing IL-1β, IL-6, IFNs and TNF-α in an attempt to combat infections (Glezer et al., 2007; Przedborski et al., 1996)

An M1 microglial phenotype may be implicated in the progression of toxin-induced degeneration of the nigrostriatal pathway (McGeer et al., 2003; Barcia et al., 2004) and likely responsible for enhancing dopamine neuronal loss especially following an immunological insult such as LPS. Indeed, priming the SNc with LPS augmented the loss of dopamine neurons provoked by paraquat and this effect appeared to be dependent upon the manifestation of an M1 phenotypic state of microglial (Mangano and Hayley, 2009). Corroborating these findings, blocking the induction of an M1 microglial state using anti-inflammatory drugs such as minocycline, dexamethasone, 3-hydroxmorphinan and NSAIDs protected SNc dopaminergic neurons in various animal models of PD (Wu et al., 2003; Kurkowska-Jastrzebska et al., 1999; Cassarejos et al., 2006; Quintero et al., 2006). Thus, in contrast to the beneficial effects M2 microglia, hyper-reactive M1 microglia can produce excessive amounts of inflammatory mediates that can be neurotoxic and lead to neurodegeneration (McGeer et al., 2003; Block et al., 2007).

One such inflammatory factor which is upregulated by M1 microglia is the T-helper type 1 (Th1) pleiotropic pro-inflammatory cytokine, interferon-γ (IFN-γ), which

has been observed to be an important player in PD-like pathology. In fact, our own work and that of others revealed that PD patients incurred significantly higher levels of IFN-γ in serum and the nigrostriatal pathway when compared to aged matched controls (Gribova et al., 2003; Mount et al., 2007; Managano et al., 2011). Paralleling these human studies, genetic ablation of IFN-γ attenuated the neurodegenerative effects of MPTP on the nigrostriatal system in mice and blunted the neuroinflammatory response engendered by the toxin (Gribova et al., 2003; Mount et al., 2007; Litteljohn et al., 2009). Similarly, *in vitro* co-cultures consisting of midbrain neuronal + microglia were protected from rotenone's toxicity when microglial cells were taken from mice deficient for the receptor for IFN-γ, suggesting that the cytokine is likely originating from and acting through microglia (Gribova et al., 2003; Mount et al., 2007).

IFN-γ originally known as macrophage activating factor is very important in the facilitation of a chronic inflammatory response. IFN-γ is a 20 or 25 kD glycoprotein secreted by a variety of cells most notably those involved in the immune system T leukocytes and natural killer cells (Schroder et al., 2004). IFN-γ mediates numerous inflammatory process, such as BBB permeability, immune cell trafficking, pathogen recognition, antigen processing and presentation, inhibition of cellular proliferation and microbicidal actions (Schroder et al., 2004; Sakar and Fisher, 2006; Mangano and Hayley, 2009; Dutheil 2010). This cytokine has also been shown to be involved in the mediation of sickness behavior, the promotion of anorexia, increasing sleep, and reduction in long term potentiation a key process in learning and memory (Schroder et al., 2004).

Although IFN-γ is virtually undetectable in the healthy brain, central levels of the cytokine become apparent following acute traumatic injury, as well as in association with autoimmune conditions, such as MS (Mana et al., 2006). Indeed, it is well established that IFN-γ is produced in the periphery by lymphoid cells (mainly $CD4^+$ lymphocytes and natural killer cells) and these cells can infiltrate the nigrostriatal system following sufficient challenge (Mount et al., 2007). Moreover, emerging evidence indicates that IFN-γ may also be produced by resident brain microglia, albeit in low concentrations (Kawanokuchi et al., 2006; Mount et al., 2007). In fact, a recent study reported IFN-γ in the supernatants of immune challenged microglia, even in the absence of T cells (Kawanokuchi et al., 2006; Mount et al., 2007).

Neurotoxicity caused by IFN-γ may be directly attributed to its ability to promote transcription of several pro-oxidative factors such as iNOS and $gp91^{PHOX}$ [rate-limiting catalyst required for nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) oxidase activation] (Sarkar and Fisher, 2006). As well, IFN-γ if co-administered with LPS can also enhance the production of several pro-inflammatory cytokines from microglia including IL-6 and TNF-α (Lafortune et al., 1996). This is particularly important given that chronic activation of TNF-α can exaggerate a neuroinflammatory responses and enhance dopamine neuronal loss via p55 TNF-R1 mediated apoptosis (Hayley et al., 2004).

Binding of IFN-γ to its receptor (IFN-γ-R) on microglia can induce a series of events that are mediated by JAK/STAT. Essentially IFN-γ-R will remain inactive until IFN-γ catalyzes the activation of the enzymes JAK1 and JAK2, which are linked to the cytoplasmic tail of the receptor. Thereafter, two STAT molecules are recruited and bind

to the paired docking sites on the activated receptor complex located on the JAKs (O'Shea et al., 2002; Murray et al., 2007). During this activation process, the STAT molecules are phosphorylated and dissociate from the IFN-γ-R in order to form homo- or hetero-dimers.

In the present thesis, we assessed the impact of paraquat in aged wild type and IFN-γ knockout mice. This work directly follows up on our previous findings showing that IFN-γ deficiency rendered mice less susceptible to both MPTP and paraquat (Mount et al., 2007; Mangano et al., 2011). The present experiments sought to determine whether age and schedule of exposure to paraquat would play an important role in the ability of IFN-γ deficiency to modulate the impact of the pesticide. In particular, as will be described shortly we were interested in whether exposure to paraquat relatively young age (four months) would sensitize mice to the later impact of the pesticide at eight months of age and if IFN-γ would be important for any such effect. Importantly, the IFN-γ deficient mice show no overt developmental defects, but do have compromised ability to fight tumor development and infections (Dalton et al., 2003; Huang et al., 2003; Schroder et al., 2005). Humans who lack the one of the two types of IFN-γ receptors also exhibit a similar phenotype as the knockout mice (Doffinger et al, 2000; Schroder et al., 2005; Sologuren et al., 2011).

1.7 Long Term Effects of Exposure to Environmental Toxins

As already discussed, substantial evidence favors a link between multiple environmental toxin exposures and the onset of PD (Beterabet et al., 2000; Thiruchelvam et al., 2011; Greenamyre and Hastings, 2004; Cicchetti et al., 2005; Li et al., 2005;

Litteljohn et al., 2011; Tanner and Goldman, 2011). However the environmental model is not a perfect fit as even individuals with the greatest exposure to toxic insults do not always necessarily develop PD (Seidler et al., 1996; Tanner and Goldman, 1996; Berry et al., 2010). Furthermore, disease progression is not rapid, and instead takes place over the course of many years, (over 10 - 20 years) (Hunot et al., 2003; Kedar et al., 2003; Shimizu et al., 2003), suggesting complex and time-dependent interplay between the effects of multiple environmental stressors and individual sensitivity. Age is the one factor that has been unequivocally linked to the onset of PD, and has been posited to be related to a natural decline in the resiliency and number of SNc dopamine neurons (Blandini et al., 2000; de Lau et al., 2004; Sarkar and Fisher, 2006; Chung et al., 2009; Litteljohn et al., 2011).

In 1956 Harman, developed a free radical theory of aging which argued that aging is a consequence of free radical damage (Harman et al., 1956; Yu and Yang, 1996; Sarkar and Fisher, 2005; Chung et al., 2009). Building from these basic observable premises, Harman proposed that reactive oxygen species would "react to a certain extent with other cellular constituents including the nucleoproteins and nucleic acids". Yu and Yang in 1996 expanded on Harman's theory to create the oxidative stress hypothesis by incorporating a state of chronic inflammation, acting in a positive feedback loop where more reactive oxygen and nitrogen species are created due to the tissues inflamed state. These theories suggest that aging might be viewed as acting as form of priming, to increase the degree of damage that a neurotoxic insult could inflict on already sensitized dopamine neurons. Similarly, the inflammatory theory of aging could be applied to many neurological disorders, including PD as already mentioned postmortem PD tissue and

animal modles of PD show considerable pro-inflammatory pathology (Sarkar and Fisher., 2006; Whitton, 2007; Chung et al., 2009; Litteljohn et al., 2011).

Ultimately, we posited that increased activation of inflammatory and oxidative cascades that undoubtedly occur with advanced age would enhance the neurodegenerative impact of the environmental toxin, paraquat. Moreover, we hypothesized that early paraquat exposure (at four months of age) would augment the neurodegenerative effects of later paraquat exposure in aged animals (eight months). Such a hypothesis is supported by studies revealed that LPS or toxin exposure had long-term consequences on microglia, cytokines and neuronal survival (Langston et al., 1999; McGreer et al., 2003; Peng et al., 2004; Qin et al., 2007). It is also of interest to evaluate whether the pro-inflammatory cytokine, IFN-γ, mediates the long-term impact of paraquat. As outlined above, IFN-γ is a critical player in innate and adaptive immune responses, and is the most potent endogenous microglial activator. This latter point is of upmost importance given the plethora of data supporting a deleterious role for activated microglia in PD (McGreer et al., 2003; Mana et al., 2006; Mount et al., 2007; Litteljohn et al., 2009; Gonzalez-Hernandez et al., 2010; Litteljohn et al., 2011).

1.8 Research Objectives

The over-arching goal of this thesis is to investigate how IFN-γ deficiency can modulate the impact of differing schedules of paraquat exposure in aged mice. The hypotheses postulated in this thesis are as follows:

1.a) peripheral paraquat administration will cause selective dopaminergic cell death in the SNc,

1.b) as well as cause an up-regulation of inflammatory factors/microglia activation in brain 2.a) the degree of damage in aged individuals will be greater than that observed in younger mice,

2.b) the degree of inflammation will be exaggerated in aged animals.

3) paraquat administration will sensitize animals to later re-exposure to the pesticide

4.a) animals genetically deficient in IFN-γ will be protected from paraquat induced cell damage, and

4.b) the degree of immune activation observed in IFN-γ mice will be diminished

2. Materials and Methods

2.1 Animals

C57BL/6J and IFN-γ knockout mice were initially obtained from Jackson labs (Bar Harbor, ME, USA). Development of the IFN-γ knockout mouse has been described previously (Dalton, 1993). IFN-γ knockout animals were descended from breeding multiple generations of heterozygous mice at Carleton University. All animals were placed in single housing at 10 weeks of age in standard (27 x 21 x 14 cm) polypropylene cages, and maintained on a twelve hour light/twelve hour dark cycle (8am to 8pm light) in a temperature controlled room (21°C). An *ad libitium* diet of Ralston Purina Mouse chow and water was provided for all experimental animals.

All animals were treated in adherence to the guidelines provided by the Canadian Council of Use and Care of Animals in Research, with the testing paradigm approved by the Carleton University Committee for Animal Care.

2.2 Experimental Procedure

Wild type and IFN-γ knockout mice (n = 13-15 per group) received two sets of intraperitoneal injections (i.p) of paraquat (7 mg/kg; 1,1'-dimethyl-4,4'-bipyridinium dichloride; Sigma Aldrich) or an equivalent volume of physiological saline solution (Sigma Aldrich). This experiment sought to determine whether paraquat might have age-dependent sensitization effects and if so, if the pro-inflammatory cytokine, IFN-γ, is important for such effects. To this end, WT and IFN-γ knockout mice were administered our "typical" paraquat injection regimen at early adulthood and then later re-exposed to a

lesser series of paraquat injections at a more advanced age (to assess whether the initial priming injection had protracted consequences and additionally if advanced age enhances vulnerability). The first round of injections began at four months of age and consisted of three injections a week for three weeks. Animals underwent behavioural testing at the end of the first set of injections, and then again after a six week rest period with no treatment. At eight months of age, the animals began their second round of treatment consisting of one injection a week for three weeks (i.e. 12 injections in total for the study). Animals then underwent a third and final round of behavioural testing at the end of their second round of treatment. Behavioural testing consisted of home cage activity (MicroMax® apparatus), open field anxiety testing, and a pole test designed to assess forelimb and hindlimb coordination. Mice were sacrificed one week following their last injection.

2.3 Spontaneous Home-Cage Activity

Measurements of spontaneous home-cage activity measures were obtained using MicroMax® photo sensor beam break cages (MKRO model; Accuscan Instruments, Columbus, OH, USA). Activity was analyzed during the active/dark cycle of the animals' day. In order to reduce intra-specific variability only activity between 21:00 and 09:00h was assessed. To ensure that all animals had an opportunity to adjust to their new environments and to further remove any discrepancies caused by circadian rhythms, animals were placed in the MicroMax(R) cages between 012:00 and 15:00h and allowed to adjust to their new surroundings until the assessment period. Home cage locomotor activity was assessed one day prior to the 9^{th} injection (experimental day 17), again after

a six week rest period (experimental day 55) and again one day prior to the final 12th injection (experimental day 134).

2.4 Physical Coordination and Strength Assessment

Physical coordination and dexterity was assessed using the pole test as previously described (Sedelis, 2000). Briefly this test consists of a 50cm tall wooden pole (0.8 cm in diameter), wrapped in anti-slip grip tape, with a plastic ball at the top which prevents mice from being able to perch at the top of the pole. As a form of incentive for the mouse to descend the pole the bedding of each mouse was spread around the base when it was to perform the test. Animals were placed facing the top of the pole and were recorded in their latency to rotate 180^{o} and descend the pole to their bedding. At each testing period animals underwent three trials with a two minute break between trials, and had a maximum latency of 90 seconds in which to descend to the base of the pole. Latency scores were taken as indices of coordination and motor impairment.

Behavioural testing was always conducted between 11:00h and 15:00h in order to reduce influence due to circadian rhythms, and intra-specific variation. This motor coordination task was administered three times throughout the course of the study, one day following the 9th injection (experimental day 19), after a six week rest period (experimental day 57) and one day after the final 12th injection (experimental day 136).

2.5 Open Field Exploratory Task

The test for assessment of exploratory behaviour in an open consists of 1600cm^2 (40cm x 40cm) Plexiglas arena, with a white opaque covering. Animals were

individually allowed to explore the open field environment for 15 min, during which time their movements were tracked via an automated video tracking system (EthoVision, Attleboro, MA, USA). This tracking system was used to divide the apparatus into a series of square concentric zones. The latency to first enter the inner zone, the frequency of entries into the inner zone, and time spent inside (exploring) the inner zone, were assessed by this program in order to make assessments about the exploratory behaviour of the animals, and from there inference into their state of anxiety. Behavioural testing was conducted between 11:00h and 15:00h in order to reduce variation due to circadian rhythms.

2.6 Brain Dissection Technique

One week following the final injection, animals were sacrificed through a lethal dose of pentobarbital (5-Ethyl-5-(1-methylbutyl)-2,4,6(1H,3H,5H)-pyrimidinetrione). Animals then underwent perfusion using 0.9% saline followed by 4% paraformaldehyde. Solutions were maintained at 0°C in ice baths for the duration of the procedure. The perfused brains were left submerged in paraformaldehyde for 24hours before being transferred to 20% sucrose, 0.2% sodium azide, diluted in isotonic phosphate buffered saline (PBS) where it was maintained with regular solution changes. Coronal sections of the ST, SNc, and LC, as in the mouse brain atlas of Franklin and Paxinos (1997), were cut at 14-μm using a Themo Scientific (Rockford IL, USA) cryostat.

2.7 Dopaminergic Cell Survival

The surviving number of dopamine neurons was determined by counting cell bodies that were positive for TH+, the rate limiting enzyme for dopamine, present in the SNc and in the LC. After cryostat-sectioning, the sections were incubated over-night in the primary antibody TH (1:3000; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) diluted in a solution containing 1% BSA, 0.5% Triton X-100, 0.05% Tween 20 and 0.05% sodium azide diluted in 0.01M PBS. Thereafter, sections were incubated with biotinylated rabbit anti-mouse secondary (1:500; Jackson ImmunoResearch Laboratories, West Grove, PA, USA, two hour incubation) and horseradish peroxidase-conjugated streptavidin tertiary (1:500; Jackson ImmunoResearch Laboratories, West Grove, PA, USA, two hour incubation). Sections were then visualized by reaction with diaminobenzidine (DAB; Sigma-Aldrich) for fifteen minutes and dehydrated with serial alcohol washes before cover slipping with clearene (Surgipath, Winnipeg, MB, Canada).

Survival of TH+ neurons within the SNc was assessed through serial section analysis of the TH+ cells within the SNc across brema levels -2.7 to -3.88. Using a double blind procedure, every tenth section of every animal in each treatment group was analyzed. The total number of stained neurons in the SNc was then estimated using Abercrombie's correction (Mouton, 2002). In the LC, changes in the cellular morphology of neurons were qualitatively assessed by rating differing degrees of soma shape and density/intensity of stain throughout bregma levels -5.44 to -5.88. The same staining procedure was also performed on ST sections, with the analysis focused on the degree of TH+ projection fibres present at bregma level 0.74, as measured through densitometry. Estimations on the density of TH+ projections in the ST was accomplished

using ImageJ software (National Institute of Mental Health, Maryland, USA). Projection density was determined at the 20X objective, brightness level was held constant for all sections, and were then converted to black and white images. The ratio of black pixels to white pixels was then calculated, and this ratio was used as an indicator of the degree of staining in the ST and therefore the degree of dopaminergic projections present.

2.8 Microglia Activation

Microglia were visualized using CD11b, a complement receptor marker present on microglia and which has been previously observed to be sensitive to the microglia cells state of activation (Mangano & Hayley, 2009). Tissue was incubated overnight at 4°C with CD11b anti-serum (1:1000; AbD Serotec, Raleigh, NC, USA). Sections were than visualized by incubating with the appropriate AlexaFluor secondary antibody (1:200; Invitrogen, Carlsbad, CA, USA) for two hours at room temperature. Then, microglia activation was assessed using semi-qualitative rankings of the morphology of these cells in the SNc. This scale has previously been described by our lab (Mangano E. D., 2011) in brief cells are assessed on a 0-3 scale. A score of 0 is given when microglia are in their quiescent state, identified by cells possessing many highly ramified thin processes. A score of 1 was reflective of an intermediary reactive state, in which less than 10 cells within the SNc were moderately active, indicated by the thickening and reduction in number of the thin processes observed in quiescent microglia cells. A score of 2 was given when more than half of the visible cells were in an intermediary or active state, characterized by spherical soma and no or few processes. A score of 3 was

attributed to those sections which showed the highest level of activation, in which the majority of cells exhibited a highly active state.

2.9 Growth Factor Recruitment

BDNF immunostaining was evaluated given our prediction that time-dependent changes in trophic support might underlie the progressive damaging consequences of paraquat exposure. BDNF was assessed using rabbit polycolonal primary antibody (1:1000; Jackson ImmunoResearch Laboratories, West Grove) incubated overnight at 4°C. Biotinylated goat anti-rabbit secondary antiserum (1:500; Jackson ImmunoResearch Laboratories, West Grove, PA, USA, two hour incubation) and horseradish peroxidase-conjugated streptavidin tertiary (1:500; Jackson ImmunoResearch Laboratories, West Grove, PA, USA, two hour incubation) were then to sections. Finally, slides were incubated with DAB (Sigma-Aldrich) for fifteen minutes, and dehydrated with serial alcohol washes before cover slipping with clearene (Surgipath, Winnipeg, MB, Canada). Again using a double blind procedure, 3 animals from each treatment group were analyzed. Images were taken at the 0.74 bregma level for each animal, and a representative image was chosen from each one. These representative photos were then qualitatively compared for differences in the amount and pattern of staining.

2.10 Statistical Analysis

All data was analyzed by 2 (genotype; wild-type vs. IFN-γ knockout) × 2 (initial treatment; saline vs. paraquat) x 2 (re-exposure treatment; saline vs. paraquat) ANOVAs followed by Fisher's planned comparisons ($P < 0.05$) where appropriate. Due to

deterioration in the quality of some tissue samples, a few animals were not included, and as a result the degrees of freedom for the statistical analyses varied within and across some brain regions and/or histological measures. All statistical analysis was accomplished using the StatView (Version 6.00) statistical software package available from the SAS Institute.

3. Results

3.1 Paraquat Treatment Influences Dopaminergic Cell Death in the Basal Ganglia Network of Wild Type but not IFN-γ Deficient Mice

Neither the three way interaction (Paraquat Treatment at Four Months x Paraquat Treatment at Eight months x Genotype) nor did any of the two-way interactions reach significance with regards to the number of TH+ neurons within the SNc. However, our *a prior* hypothesis that IFN-γ deficient animals would be protected from the damaging effects of paraquat prompted us to conduct separate analysis of IFN-γ deficient and wild type animals.

When wild type animals were analyzed without IFN-γ knockout mice, main effects of both treatment at four months of age ($F_{1,37} = 4.29$, $P < 0.05$) and treatment at eight months of age ($F_{1,37} = 4.65$, $P < 0.05$) were revealed. These main effects were reflective of the fact that wild type animals which received paraquat treatment at either four or eight months of age exhibited a moderate but statistically significant (~30%) decrease in TH+ positive SNc neurons, relative to their saline treated counterparts ($p < 0.05$). However, there were no significant differences between animals which received paraquat at four months and those which received paraquat at eight months, nor were these different from those that received the herbicide on both occasions. Consistent with our proposed hypothesis, there were no significant treatment differences in SNc TH+ neuron counts among IFN-γ deficient mice.

In order to further investigate what consequences paraquat may have in the basal ganglia network, densitometry analysis of TH+ projections in the ST was conducted. In

contrast to the damage observed in the SNc of wild-type animals, semi-qualitative rating of the ST revealed no significant changes regardless of genotype or treatment (Fig. 2).

Figure 1: Dopaminergic Cell Survival is Impacted in the Substantia Nigra Following Paraquat Treatment

Counts of TH+ neurons in the Substania Nigra of wild-type (pictured on the left) and IFN-γ deficient mice (pictured on the right) after intra-peritoneal saline treatment or paraquat (7mg/kg). Treatment is depicted by the colour of the bars with; saline treatment at four and eight months being depicted by the white bars, saline treatment at four months and paraquat treatment at eight is depicted by the light grey bars, paraquat treatment at four months and saline treatment at eight is depicted by the dark grey bars, and paraquat treatment at both four and eight months is depicted by the black bars.

Error bars represent mean ± SEM. ANOVA, $*P < 0.05$.

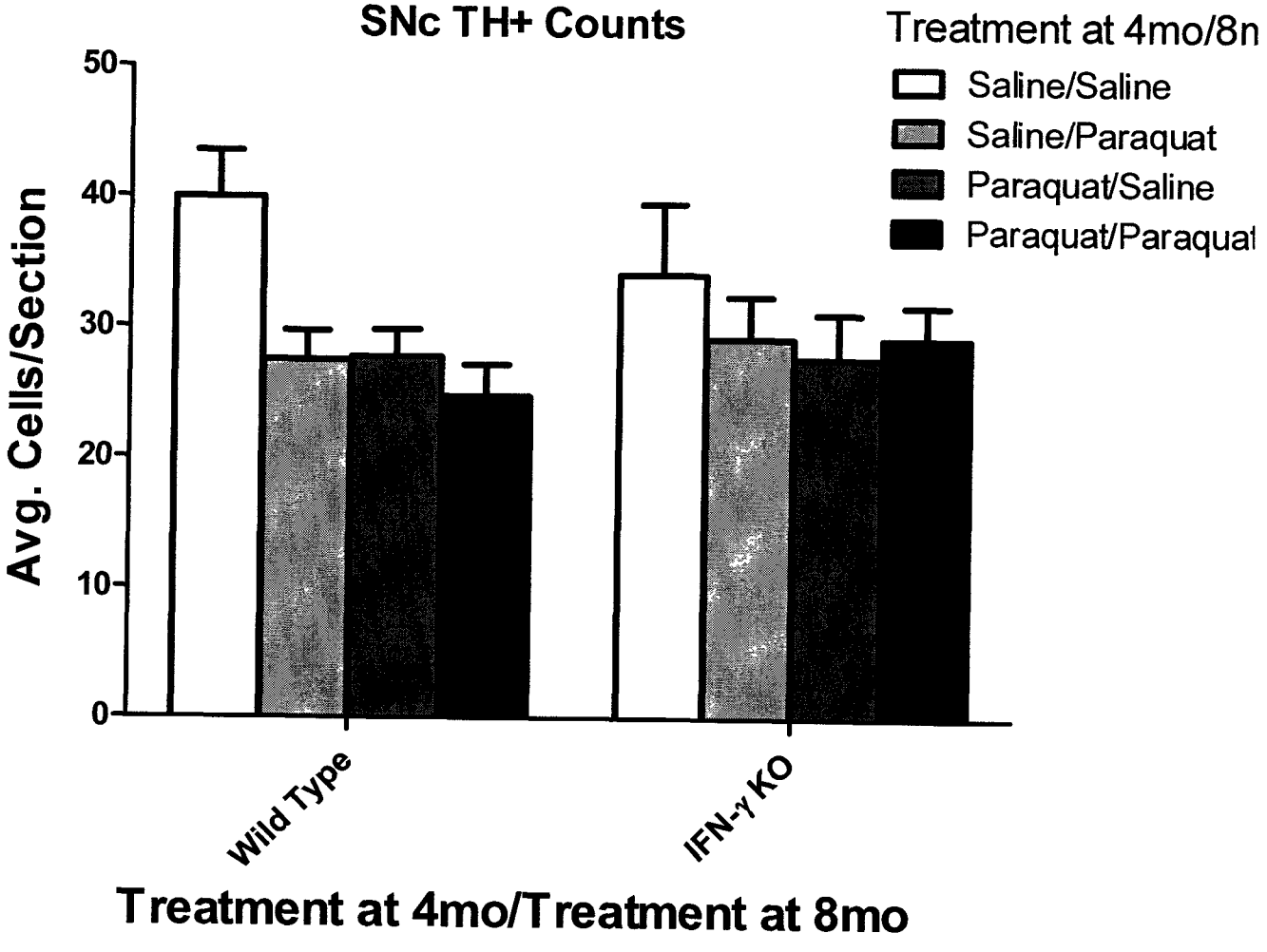
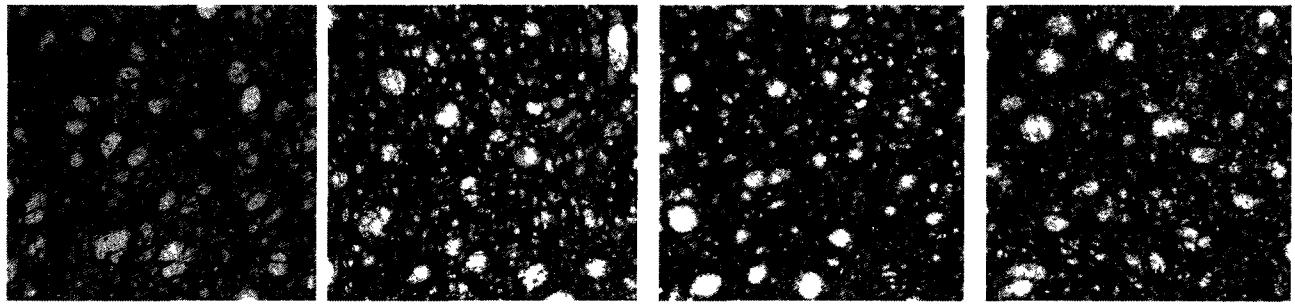

SNc TH+ Counts
Treatment at 4mo/8n
Saline/Saline
Saline/Paraquat
Paraquat/Saline
Paraquat/Paraquat
Avg. Cells/Section
50
40
30
20
10
0
Wild Type
IFN-γ KO
Treatment at 4mo/Treatment at 8mo

Figure 2: Dopaminergic Projections in the Striatum are Un-Affected Following Paraquat Treatment

Top Panel: Photomicrographs using the 20x magnification depicting typical levels of immune-staining of TH+ terminals within the ST of wild-type mice that received either saline at both time points (A), saline treatment at four month of age and paraquat at eight (B), paraquat administered at four months and treated with saline at eight months of age (C), and those which received paraquat treatment at both time points (D). IFN-γ deficient mice are not pictured, as they did not differ from wild-type animals and no treatment differences were observed.

Bottom Panel: The table at the bottom depicts ratings of the density of TH+ projections in the ST of wild type animals, measured through densometric analysis discussed in the methodology. These results suggest there is no difference in the density of dopaminergic projections in the ST.

Tyrosine Hydroxylase Positive Staining in the ST of WildType Animals



Sal/Sal Sal/PQ PQ/Sal PQ/PQ

Table 1: Mean (± SEM) Measure of TH+ Projections in the Striatum

Striatum	Wild-type (C57/Bl6)		IFN-γ knockout	
	Ratio	Error	Ratio	Error
Saline/Saline	.453	± .089	.468	± .033
Saline/Paraquat	.424	± .083	.443	± .046
Paraquat/Saline	.396	± .086	.435	± .030
Paraquat/Paraquat	.498	± .080	.418	± .045

3.2 Paraquat Exposure Impacts the Health of Catecholamine Cells in the Locus Coeruleus in IFN-γ Deficient Mice

As already mentioned, the LC shows substantial degeneration in PD, often to an extent greater than that of the SNc (Zarow et al., 2003; Delaville et al., 2011; McMillan et al., 2011; Pavese et al., 2011). This combined with the fact that brainstem noradrenergic alterations likely contribute to the co-morbid depressive pathology often observed in PD (Braak and Del Tredici, 2009; Delaville et al., 2011), prompted us to assess whether paraquat might affect the survival of noradrenergic neurons within the LC. To this end, qualitative analysis of the state of the LC revealed that paraquat induced morphological abnormalities in TH+ neurons, which correlated with the degree of extent of exposure to the herbicide. Specifically, while animals exposed to paraquat on only one occasion (at either four or eight months of age) they displayed moderately abnormal neuronal morphology. Animals that were pre-treated at four months and then re-exposed at the later eight month time clearly displayed the most profound cellular alterations (both in terms of cell size and shape). Indeed, cell morphology in these re-exposed mice was characterized by shrunken or shriveled shape, and a lack of clear dendritic projections. Unlike alterations in the SNc, differences in the LC were consistently observed in both IFN-γ deficient and wild type mice.

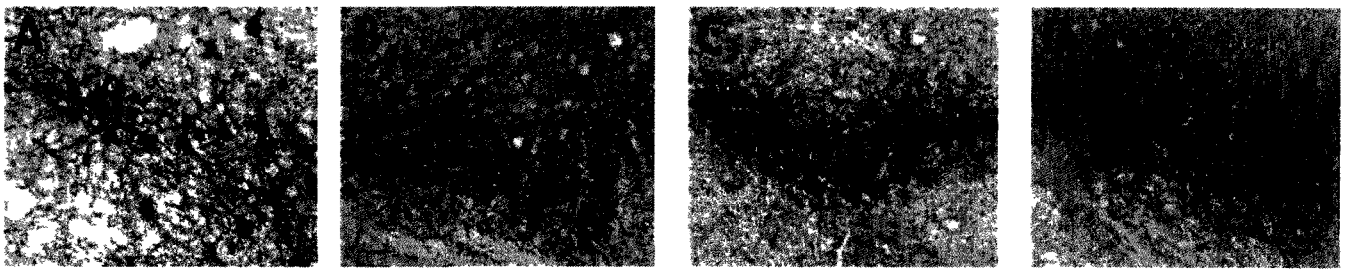
Figure 3: Morphological Appearance of Catecholaminergic Neurons in the Locus Coeruleus Following Paraquat Treatment

Top Panel: Photomicrographs depicting typical immune-stained TH+ cells within the LC of wild-type mice. Animals received either saline at both time points (A), saline treatment at four month of age and paraquat at eight (B), paraquat administered at four months and treated with saline at eight months of age (C), and those which received paraquat treatment at both time points (D).

Bottom Panel: Photomicrographs depicting typical immune-stained TH+ cells within the LC of IFN-γ deficient mice using the 20x magnifying lens. Animals received either saline at both time points (E), saline treatment at four month of age and paraquat at eight (F), paraquat administered at four months and treated with saline at eight months of age (G), and those which received paraquat treatment at both time points (H).

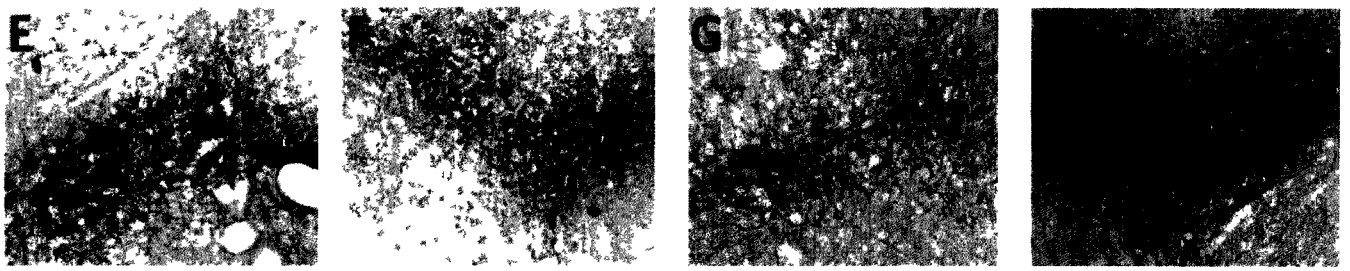
Wild type and IFN-γ deficient mice both displayed severe morphological alterations in TH+ cells, when they receive paraquat at both four and eight months of age (D and H).

TH+ Staining in the LC of Wild-type Animals



Sal/Sal Sal/PQ PQ/Sal PQ/PQ

TH+ Staining in the LC of IFN-γ Deficient Animals



Sal/Sal Sal/PQ PQ/Sal PQ/PQ

3.3 Age and Paraquat Treatment Invoked Motor Distortions in Wild Type Mice

In order to evaluate potential PD-like motor deficits provoked by paraquat and whether IFN-γ deficiency plays a role in such effects, spontaneous home cage activity and performance on a pole test (for forelimb and hindlimb coordination) were determined in IFN-γ knockout and wild-type mice. Animals were assessed during the first round of paraquat/saline injections, after a six week recovery period, and again following the second round of injections. Surprisingly, neither of these behavioural tasks revealed a typical PD-like deficit which is typically characterized by reduced locomotion and impaired coordination. Rather (as is described below) paraquat treated mice displayed heightened activity over time.

A Paraquat Treatment at Four Months x Genotype interaction reached significance for home cage locomotor activity at four months of age ($F_{1,107} = 4.37$, $P < 0.05$). Due to this significant interaction and our *a prior* hypothesis that IFN-γ deficient animals will be protected from the damaging effects of paraquat, wild type and IFN-γ knockout mice were analyzed separately for home-cage locomotor activity.

When wild type animals are analyzed without IFN-γ deficient mice, they exhibited no treatment effects at the first round of behavioural testing, but did show a significant main effect for paraquat treatment at four months of age, on both the second round of testing at approximately six months of age ($F_{1,57} = 4.63$, $p < 0.05$), as well as the third round of behavioural analysis at approximately nine months of age ($F_{1,55} = 4.27$, $P < 0.05$). Specifically, wild-type animals which received paraquat treatment at four months of age displayed significantly greater home-cage activity compared to their saline

treated counterparts ($p < 0.05$). However, no significant interaction or effect of treatment at eight months of age was observed in wild type mice.

When IFN- γ deficient mice were analyzed separately from wild type animals, it was observed that, paralleling the alterations in SNc neuronal counts, these animals did not differ in their home-cage activity across the treatment conditions (Fig. 4).

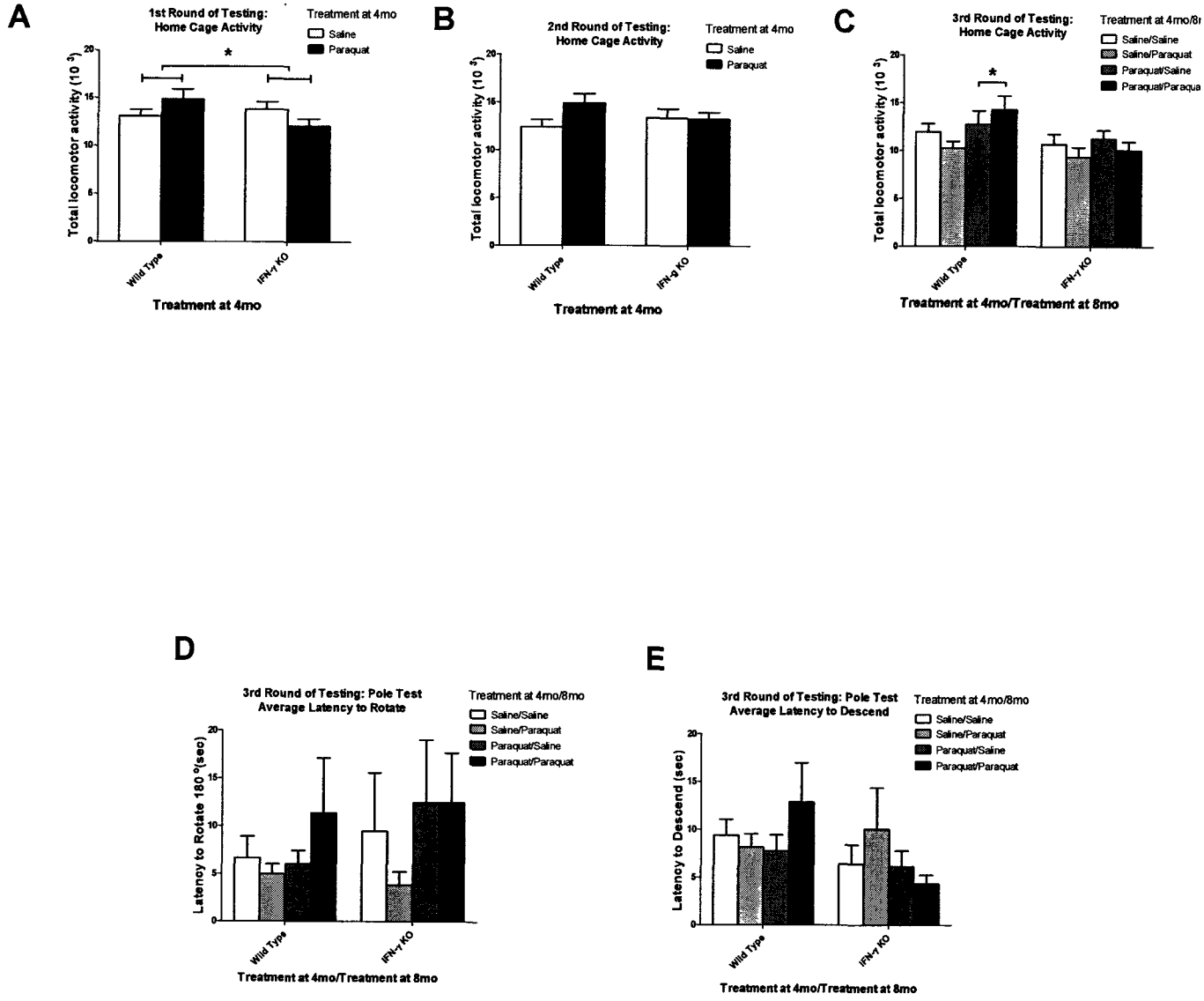
In contrast to the home-cage activity changes, neither paraquat treatment nor genotype affected performance on the pole test. This lack of impairment was apparent for both the time to turn 180^{o} when placed at the top of the pole, as well as the latency to descend to the bottom of the pole, and was identical at all the three assessment times.

Figure 4: Locomotor Activity in the Home Cage and Performance in the Pole Test

Top panel: Spontaneous locomotor activity, expressed as the total number of infrared beam breaks (1.0×10^3; mean ± SEM) between 21:00 and 05:00 of the dark cycle, among wild-type (bars to the left) and IFN-γ knockout (bars to the right) mice. Mice were assessed directly following 12 paraquat/saline injections at four months of age (A), at approximately six months of age (B) or following the 15^{th} and final injection at approximately nine months of age (C). In panels A and B, saline treatment at four months of age is depicted by white bars and paraquat treatment is depicted by black bars. In panel C, saline treatment at four and eight months being depicted by the white bars, saline treatment at four months and paraquat treatment at eight is depicted by the light grey bars, paraquat treatment at four months and saline treatment at eight is depicted by the dark grey bars, and paraquat treatment at both four and eight months is depicted by the black bars.

Bottom panel: Average latency to turn 180° (D) and average latency to descend pole (E) (mean ± SEM), among WT (bars on the left) and IFN-γ knockout (bars on the right) mice at the 3^{rd} and final round of behavioural testing either saline of paraquat administration. Treatment is depicted by the same gradient of colors in the above panel. Animals were subject to a maximum testing duration of 90 sec.

Error bars represent mean ± SEM. ANOVA, $*P < 0.05$.


A
1st Round of Testing:
Home Cage Activity
Treatment at 4mo
Saline
Paraquat
Total locomotor activity (10 3)
Wild Type
IFN-γ KO
Treatment at 4mo
B
2nd Round of Testing:
Home Cage Activity
Treatment at 4mo
Saline
Paraquat
Total locomotor activity (10 3)
Wild Type
IFN-g KO
Treatment at 4mo
C
3rd Round of Testing:
Home Cage Activity
Treatment at 4mo/8r
Saline/Saline
Saline/Paraquat
Paraquat/Saline
Paraquat/Paraqua
Total locomotor activity (10 3)
Wild Type
IFN-γ KO
Treatment at 4mo/Treatment at 8mo
D
3rd Round of Testing: Pole Test
Average Latency to Rotate
Treatment at 4mo/8mo
Saline/Saline
Saline/Paraquat
Paraquat/Saline
Paraquat/Paraquat
Latency to Rotate 180 °(sec)
Wild Type
IFN-γ KO
Treatment at 4mo/Treatment at 8mo
E
3rd Round of Testing: Pole Test
Average Latency to Descend
Treatment at 4mo/8mo
Saline/Saline
Saline/Paraquat
Paraquat/Saline
Paraquat/Paraquat
Latency to Descend (sec)
Wild Type
IFN-γ KO
Treatment at 4mo/Treatment at 8mo

3.4 Paraquat Induced Alterations of Open Field Exploratory Behaviour of Wild Type and IFN-γ Deficient Mice

Although no significant differences were observed with respect to the frequency of entries into the inner zone of the open field, the duration in and latency to enter the inner zone did vary as a function of genotype. Indeed, at the first round of behavioural testing (four months), a main effect of genotype was observed for the duration of time spent exploring the inner zone of the open field arena ($F_{1,107} = 6.08$, $p < 0.05$). Specifically, IFN-γ deficient mice spent significantly more time overall exploring in the inner zone than wild type animals. This main effect also still remained at six months of age ($F_{1,105} = 3.599$, $p < 0.05$) and also at eight months of age ($F_{1,98} = 9.67$, $p < 0.01$). Importantly, the only significant effect in the open field involving paraquat occurred in the form of a Paraquat Treatment at Eight Months x Genotype interaction for duration spent in the inner zone at the final round of behavioural analysis at nine months of age ($F_{1,99} = 5.63$, $p < 0.05$). To further explore this interaction ANOVA analysis was conducted separately for each genotype. These additional ANOVA tests revealed that paraquat treatment at eight months of age significantly reduced the duration that wild type mice spent exploring in the inner zone of the open field ($F_{1,54} = 4.73$, $p < 0.05$), whereas no such effect was observed for the IFN-γ knockouts.

At the first behavioural testing session immediately following the initial round of paraquat treatment (at four months), IFN-γ knockout mice displayed a significantly shorter latency to enter into the inner zone of the open field than wild type animals ($F_{1,103} = 5.26$, $p < 0.05$). Curiously, this effect reversed by the second testing session (at six months), such that IFN-γ deficient animals now showed a longer latency to enter the

inner zone relative to wild type mice ($F_{1,103} = 4.03$, $p < 0.05$). In contrast, no significant treatment or genotypes effects were observed at the final testing time point when the mice were approximately nine months of age.

Figure 5: Repeated Assessment of Exploratory Behaviour in an Open Field Environment

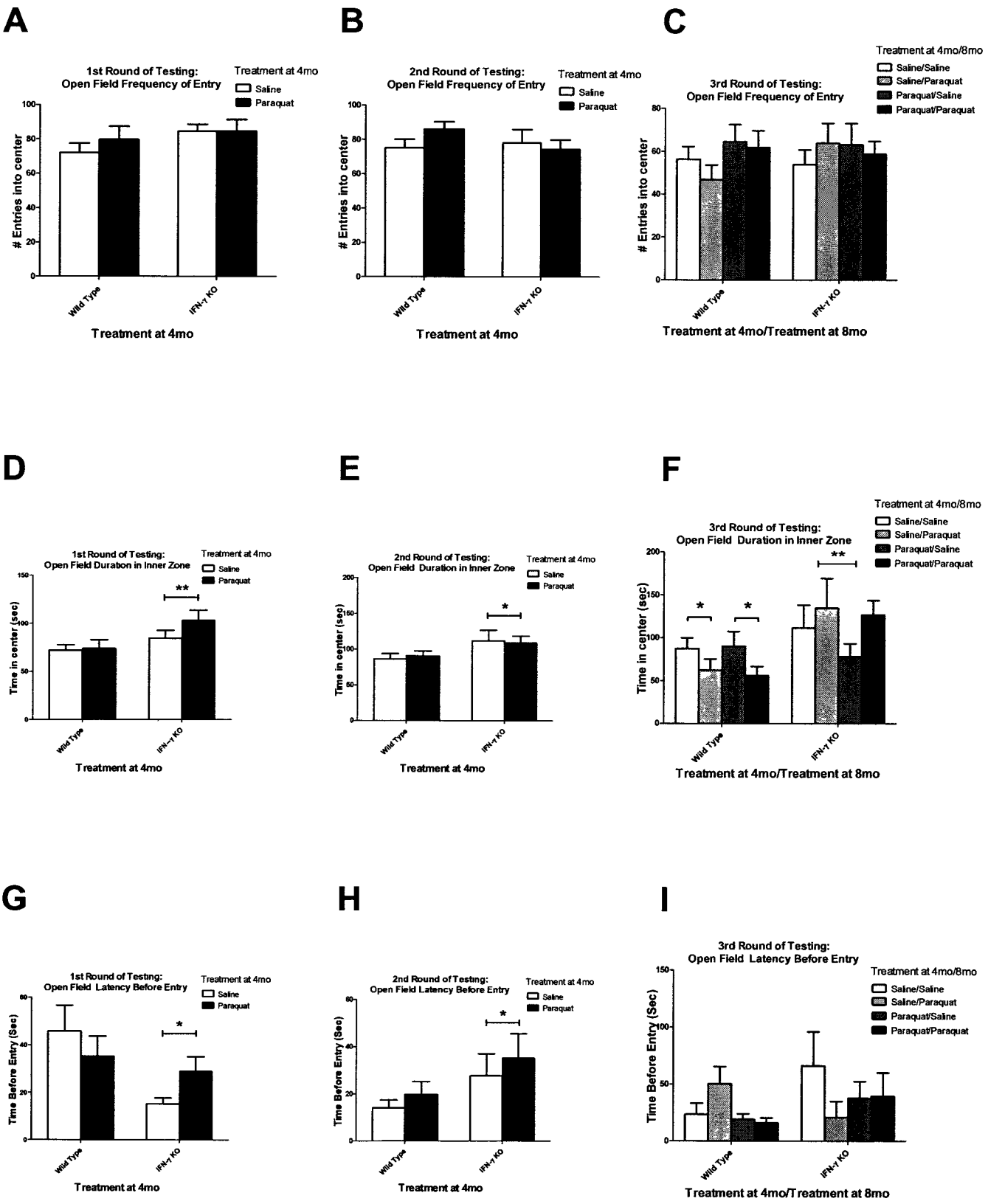
The first column (A, D, G) depicts open field measures at four months of age. The second column (B, E, H) depicts open field measures at six months of age. The third column (C, F, I) depicts open field measures at nine months of age. In the first two columns saline treatment at four months of age is depicted by white bars, and paraquat treatment is depicted by black bars. In the third column treatment is depicted by a gradient in the colour of bars with; saline treatment at four and eight months being depicted by the white bars, saline treatment at four months and paraquat treatment at eight is depicted by the light grey bars, paraquat treatment at four months and saline treatment at eight is depicted by the dark grey bars, and paraquat treatment at both four and eight months is depicted by the black bars.

Top panel: Frequency of entries mice made into the center zone of the open field arena. No significant differences were observed in this measure.

Middle panel: Duration of time animals spent in the center zone of the open field arena. Basally (at both four and six months of age) IFN-γ deficient mice are spending more time exploring the inner square than wild-type mice. At the third and final testing period when analyzed separately by genotype, wild type animals which received paraquat injections at eight months of age were spending significantly less time exploring the inner square

Bottom panel: Latency until entry mice into the center zone of the open field arena. No treatment differences were observed, however IFN-γ knockout animals do enter the inner square significantly earlier than wild type mice at the first round of behavioural testing, but are significantly delayed by the second round of behavioural testing six weeks later. No differences are observed at the third round of open field assessment in this measure.

Error bars represent mean ± SEM. ANOVA, $*P < 0.05$.


A
1st Round of Testing:
Open Field Frequency of Entry
Treatment at 4mo
Saline
Paraquat
Entries into center
Wild Type
IFN-γ KO
Treatment at 4mo
B
2nd Round of Testing:
Open Field Frequency of Entry
Treatment at 4mo
Saline
Paraquat
Entries into center
Wild Type
IFN-γ KO
Treatment at 4mo
C
3rd Round of Testing:
Open Field Frequency of Entry
Treatment at 4mo/8mo
Saline/Saline
Saline/Paraquat
Paraquat/Saline
Paraquat/Paraquat
Entries into center
Wild Type
IFN-γ KO
Treatment at 4mo/Treatment at 8mo
D
1st Round of Testing:
Open Field Duration in Inner Zone
Treatment at 4mo
Saline
Paraquat
**
Time in center (sec)
Wild Type
IFN-γ KO
Treatment at 4mo
E
2nd Round of Testing:
Open Field Duration in Inner Zone
Treatment at 4mo
Saline
Paraquat
*
Time in center (sec)
Wild Type
IFN-γ KO
Treatment at 4mo
F
3rd Round of Testing:
Open Field Duration in Inner Zone
Treatment at 4mo/8mo
Saline/Saline
Saline/Paraquat
Paraquat/Saline
Paraquat/Paraquat
**
*
*
Time in center (sec)
Wild Type
IFN-γ KO
Treatment at 4mo/Treatment at 8mo
G
1st Round of Testing:
Open Field Latency Before Entry
Treatment at 4mo
Saline
Paraquat
*
Time Before Entry (Sec)
Wild Type
IFN-γ KO
Treatment at 4mo
H
2nd Round of Testing:
Open Field Latency Before Entry
Treatment at 4mo
Saline
Paraquat
*
Time Before Entry (Sec)
Wild Type
IFN-γ KO
Treatment at 4mo
I
3rd Round of Testing:
Open Field Latency Before Entry
Treatment at 4mo/8mo
Saline/Saline
Saline/Paraquat
Paraquat/Saline
Paraquat/Paraquat
Time Before Entry (Sec)
Wild Type
IFN-γ KO
Treatment at 4mo/Treatment at 8mo

4. Discussion

Even normal aging is associated with an up-regulation of inflammatory processes, diminution of antioxidant defenses and modest neuronal loss (Stark and Pakkenberg, 2004; Sarkar and Fisher, 2005; Chung et al., 2009; Di Giovanni et al., 2009). Interestingly, the SNc appears to be especially vulnerable to the ravages of advanced age, likely owing to inherent iron and neuromelanin content, as well as the high oxidative potential of the dopamine neurons (Dexter et al., 1989; Blandini et al. 2000; Betarbet et al., 2002; Gonzalez-Hernadez et al., 2010). In the case of PD, repeated exposure to environmental toxins has been speculated to augment or speed up the normal aging processes, as well as recruit pathological inflammatory and oxidative pathways (McCormack et al., 2002; Stark and Pakkenberg, 2004; Swada et al., 2008; Gonzalez-Hernadez et al., 2010).

We endeavored to elucidate the potential inflammatory processes that might underlie the impact of age and repeated environmental toxin exposure in the provocation of PD-like neuropathology. In this regard, it was of interest to determine whether the pro-inflammatory cytokine, IFN-γ, would be involved in age-dependent paraquat effects. Indeed, we previously found this cytokine to be critical for the neurodegenerative impact of paraquat in young adult mice and IFN-γ deficiency was also found to greatly attenuate the microglial-dependent neuro-inflammatory effects of the herbicide (Litteljohn et al., 2009; Mangano et al., 2011). Moreover, the current consensus of a "multi-hit" hypothesis for PD suggests that multiple exposures to different toxins at various time points over the course of one's life ultimately shapes the evolution of the disease. Hence, it was of interest to assess whether re-exposure to paraquat at advanced age following previous

exposure months earlier would result in augmented neuronal pathology and if any such effects might be mediated by IFN-γ-microglia-dependent pro-inflammatory processes.

In the present investigation, we observed that paraquat induced the degeneration of neurons within the SNc, as well as the LC in wild type mice. Specifically, there was a significant loss of SNc TH+ dopamine neurons apparent following treatment with paraquat at either four or eight months of age. However, there was no indication of a sensitization or even an additive effect with regards to cell loss in mice that received paraquat treatment at both ages. This clearly contrasts with our previous work showing that LPS priming of the microglial response within the SNc greatly increased the degree of dopaminergic neuron loss provoked by later paraquat exposure (Mangano & Hayley, 2009). Of course, LPS is a far more robust inflammatory stimulator than paraquat and it might also be the case that the protracted delay between paraquat treatment sessions used in the current investigation (four months) was too long to produce a sensitized response. Yet, it is important to underscore that the paraquat dose employed at eight months of age was decidedly less than that used to treat the four month old animals (owing to issues of increased peripheral toxicity with advanced age), but the extent of neuronal loss was comparable. Hence, it did appear that the older mice were more vulnerable to the impact of the pesticide.

The neuronal pathology evident within the LC is particularly significant given that this brain region degenerates in PD patients (Zarow et al., 2003; Delaville et al., 2011; McMillan et al., 2011) but is often neglected in both clinical and experimental PD studies. Indeed, to our knowledge, no studies have evaluated the impact of paraquat or any other pesticides upon brainstem noradrenergic neurons. This is all the more

surprising in light of the fact that major depression is commonly observed in PD patients and is thought to be related to noradrenergic, as well as serotonergic deficits (Remy et al., 2005). While our previous findings revealed that paraquat affected noradrenergic utilization within the LC (Litteljohn et al., 2009), the present thesis is the first to demonstrate that the pesticide can provoke actual neuronal pathology in this brain region. Interestingly, unlike the loss of SNc dopamine neurons, the pathological effects evident in the LC appeared to be augmented with re-exposure to paraquat at eight months of age following treatment four months earlier. Indeed, while a small number of abnormal TH+ pyknotic cells were observed within the LC in mice which received only one round of paraquat treatment, the majority of TH+ neurons within the LC of animals that received paraquat at both four and eight months of age displayed such abnormal cellular morphology.

The neurodegenerative effects observed in response to paraquat were associated with altered home-cage activity and open field exploration. Paralleling the histological findings, age again appeared to be a factor in that the behavioural changes only became apparent at the final round of testing late in adulthood. Surprisingly, instead of the hypothesized bradykinesia (reduction in activity) that typically characterizes PD; we observed an elevation of activity levels in wild type mice treated with paraquat. The fact that paraquat actually induced increased activity suggests that hyperactivity might occur with advanced age in response to the pesticide. This could conceivably be related to changes in the dopamine producing capacity of surviving neuronal terminals, as well as the activation of alternate systems over time following toxin exposure. Along these lines, it is important to note that we did not observe a significant reduction in striatal TH+

terminal staining, suggesting that even in the presence of a loss of SNc soma the downstream terminal density was preserved. This is not unprecedented and the possibility that the behavioural outcome could be related to the recruitment of compensatory processes over time will be discussed in the ensuing sections.

Consistent with our previous findings, IFN-γ deficiency prevented the neuronal loss within the SNc that was engendered by paraquat, and this effect was apparent irrespective of age of exposure. Correspondingly, the IFN-γ null mice also did not exhibit home-cage activity changes in response to paraquat. However, IFN-γ deficient animals appeared to basally different from their wild type counterparts in that they display a less "anxious-like" profile, as indicated by greater exploration of the open field arena. Of course, this finding could also be interpreted as reflecting some changes in neural systems important for novelty seeking or exploratory behaviors independent of anxiety per se. Yet, the degree of cell pathology in the LC of knockouts was similar to that observed in wild type animals. Again, subsequent sections of this thesis will discuss the importance of IFN-γ in the neuro-inflammatory response and the onset of PD.

4.1 Multi-hit models of PD: Effects of Age

Epidemiological studies have provided a strong foundation for multi-factorial etiologies stemming from a combination of both genetic and a variety of interacting environmental components (Beterabet et al., 2000; Greenamyre and Hastings, 2004; Cicchetti et al., 2005; Li et al., 2005; Litteljohn et al., 2011; Tanner and Goldman, 2011; Thiruchelvam et al., 2011). Of the proposed factors influencing risk of PD, aging is the strongest and, in fact, the only unequivocal correlate risk factor (Blandini et al., 2000; de Lau et al., 2004; Sarkar and Fisher, 2006; Sawada et al., 2008; Chung et al., 2009;

Litteljohn et al., 2011). In fact, many of the features of PD are reminiscent of an exaggerated version of normal aging. Even with healthy aging the number of neurons in the SNc and concentration of dopamine decreases, along with impairment in reaction time and movement accompanied by stopped posture and shuffling gait, albeit to a much lesser degree than in PD. Despite these similarities it is unlikely the PD is merely the result of accelerated aging, given that levodopa, a standard drug treated used to ameliorate PD motor impairment, failed to produce any alteration in the motor abilities of elderly non-parkinsonian individuals (Calne and Langston, 1983; Jackson-Lewis et al., 1995).

In the present study, we did find that aged wild type mice appeared to be more susceptible to the neurodegenerative effects of paraquat. Specifically, exposure of eight month old mice to one third of the overall paraquat dose that four month old animal received resulted in a comparable degree of dopamine neuronal loss. This would certainly be in agreement with the numerous reports that behavioral deficits and neuronal impairment are more easily triggered in animals of advanced age in response to a variety of insults, including, cerebral stroke, head injury, toxin exposure and inflammatory insults (Tatton et al., 1992; Susman et al., 2002; Thiruchelvam et al., 2003; Rosen et al., 2005; Popa-Wagner et al., 2007; Pectu et al., 2008; Richmond et al., 2011). Yet, it should be underscored that different time periods had elapsed between insult and sacrifice in these separate treatment groups. In this regard, the extra four months that passed for the early treated mice could conceivably have influenced the degree of neurodegeneration. Evidence arguing against such a scenario would be the reports of paraquat, as well as MPTP treated mice displaying a relatively stable lesion by 2-4 weeks into dosing

regimens comparable to that of the present investigation (Czlonkowska et al., 1996; Barcia et al., 2004).

Our results support the contention that PD could result, in certain cases, from environmental toxin exposure being superimposed on an already compromised aged individual. This would be represented by a time course in which individuals exposed to environmental toxins would show progressive dopamine neuron decreases at the same rate as non-exposed aged individuals, but would possess fewer neurons to begin with from the initial insult(s). Indeed, normal aging is associated with ~7-10% reduction in the number of midbrain dopamine neurons with each passing decade (Fearnley and Lees, 1991; Ma et al., 1999; Stark and Pakkenberg, 2004: Di Giovanni et al., 2009). This would mean that individuals suffering from prior insult would drop below the critical threshold of dopaminergic cells in the SNc necessary for proper motor functioning much earlier than normally aging individuals. However, it is important to bear in mind that disease progression varies dramatically between individuals. In particular, variations in compensatory mechanisms in the aging brain could contribute to alterations in the severity and progression of disease.

4.2 Primary and co-morbid behavioural effects of paraquat

The current study provided evidence for a role of age and/or the passage of time in modulating the impact of paraquat on the development of Parkinsonian symptoms in mice. In particular, wild type mice which were administered paraquat at four months of age exhibited a hyper-active profile of home-cage activity at nine months of age, but not at either of the earlier time points. Additionally, wild type mice which received paraquat at eight months of age showed decreased time exploring the inner portion of the open

field arena following treatment. This provides strong evidence for a role of aging given that these older animals showed immediate behavioural alterations following paraquat treatment, whereas the four month old paraquat treated mice only displayed such behavioral effects months later (at nine months of age). Animals which received paraquat at both time points showed a combination of behavioural alterations, with both increased home-cage activity and decreased open field exploration, suggesting that they were, not surprisingly, the most adversely affected. Yet, it should again be mentioned that their loss of dopamine neurons was not greater than those of animal that received paraquat at only one time, however, pathology within the LC was greatly augmented in the paraquat re-exposed animals. Hence, it might be that brainstem noradrenergic impairment contributed to the observed motor activity and exploratory behaviors. Indeed, it has been reported that LC norepinephrine loss contributes to the motor deficits induced by MPTP and that in fact, no significant motor pathology was evident in the absence of a noradrenergic lesion (Rommelfanger et al., 2007). Some studies have even gone so far as to suggest that an early loss of noradrenergic neurons within the LC results in a loss of trophic support to the SNc and hence, influences the later degeneration of these nigral dopamine neurons (Srinivasan and Schmidt, 2003). Whatever the case, the contribution of the noradrenergic system, along with obvious dopamine deficits, to PD-like pathology should be considered in greater detail.

Animal models of PD often focus on degeneration in the nigral-striatal pathway and fail to consider many of the other areas in the brain which are also affected in PD. Outside of the basal ganglia network, several brain regions implicated in affective pathology are affected in PD, including the prefrontal cortex, hypothalamus,

hippocampus, ventral tegmental area, and the LC (Delaville et al., 2011). In the past 50 years, a growing body of research has provided evidence for a key role of norepinephrine neurons of the LC in the expression of both the motor and non-motor deficits observed in PD. In fact, this brain region has been observed to degenerate to an even greater degree than the SNc in PD patients (Shiba et al., 2000; Shulman et al., 2001; Zarow et al., 2003). The LC provides norepinephrine projections to the spinal cord, brain stem, cerebelleum, hypothalamus, thalamic relay nuclei, amygdale, basal telencephalon and the cerebral cortex. When norepinephrine is released, it is capable of modulating synaptic transmission, altering membrane potential, exciting neurons, and aiding in synaptic plasticity in all of these brain regions. In effect, it is highly likely that norepinephrine released from the LC is affecting not only motor and exploratory behaviors, but also the sleep/wake cycle, vigilance and a host of other behavioral processes. Indeed, the presently observed increased home-cage activity and reductions in open field exploratory behaviour promoted by paraquat treatment could be indicative of agitation or anxiety-like symptoms that are often co-morbid in PD.

The non-motor symptoms commonly associated with PD include autonomic dysfunction, deterioration in sensory abilities, sleep difficulties, depression and anxiety. In fact, co-morbid non-motor symptoms have been documented to occur in up to 88% of all PD cases (Aarsland et al., 1999; Shulman et al., 2001; Simuni and Sethi, 2008). Of the neuropsychiatric symptoms associated with PD, the most prevalent are depression, occurring in up to 50% of all cases, and anxiety, present in as many as 40% of all cases (Aarsland et al., 1999; Ravina et al., 2007; Simuni and Sethi, 2008; Selikhova et al., 2009; Weintraub and Burn, 2011). Time spent exploring the exposed inner zone of an

open field arena has been repeatedly assessed by a variety of different experiments to be a measure of anxiety-like behaviour (Litteljohn et al., 2008; Nautiyal et al., 2008; Litteljohn et al., 2009; Peruga et al., 2011; Wu et al., 2010, Zhang et al., 2011). Likewise, increased activity in the home-cage can be indicative of an anxious or agitated state (Xiangdong et al., 2002; Litteljohn et al., 2009). As neuropsychiatric symptoms are often documented to appear prior to the onset of motor symptoms in PD (Shiba et al., 2000; Ravina et al., 2007; Wolters, 2009; Weintraub and Burn, 2011), the anxiety-like effects observed in the current wild type animals could reflect the early stages of pathology which could eventually culminate in gross motor impairment.

The fact that behavioural symptoms appeared long after initial paraquat administration suggests that time-dependent processes that involve either the gradual up-regulation of pro-death processes or the down-regulation of normally protective processes could occur. It is well established that as an individual ages, anti-oxidant and other endogenous protective mechanisms become less efficient and eventually allow for the expression of pathology associated with a previously suppressed insult (Calne and Langston, 1983; McGreer 1988; Thiruchelvam et al., 2003). This could explain the hyper-active profile observed in wild type animals at nine months of age that received paraquat four months earlier. Furthermore, this is in line with the fact that equivalent levels of dopamine neuronal death were observed in the SNc of wild-type animals regardless of when the insult was administered; however, in both cases behavioural alterations only become apparent late in adulthood. From such a perspective, exposure to paraquat early in adulthood could be producing "silent" lesion(s) in the SNc, in which clinical features are not manifested until the number of already reduced dopaminergic

neurons fall below a critical threshold. In effect, normal aging processes might bring the already compromised SNc below such a critical threshold. This proposition is in agreement with previous findings indicating that early life exposure to toxins augments the impact of later pesticide exposure in adulthood (Thiruchelvam et al., 2003; Ossowska et al., 2005), and that motor difficulties are often not observed until the lesion produced by such insults results in a critical levels of dopamine depletion (Blandini et al., 2000; Kedar, 2003; Ossowska et al., 2005; Weintraub et al. 2008; Mangano and Hayley, 2009).

Although the behavioural alterations induced by paraquat in wild type animals were generally not observed in IFN-γ null mice, the knockout animals did show basal differences (i.e. with only vehicle treatment) in their anxiety-like behaviour (as assessed by exploration in the open field). This indicates the possibility that deletion of this cytokine may have affected processes in the central nervous system which are important for anxiety or motivation. Although the IFN-γ knockouts showed increased time in the centre of the open field at several different testing sessions, the latency to enter the center zone of the arena differed dramatically at different times of testing. Specifically, mice lacking the cytokine showed reductions of latency to enter the inner zone (indicative of a less "anxious-like" state) at the first testing session (just after the four month injection regimen) but displayed the opposite pattern of an increased latency (indicative of a more "anxious-like" state) by the second testing session several weeks later. These data suggest that the functional effects of IFN-γ deletion follow a highly complex temporal course. It is tempting to speculate that age was a critical factor in shaping the nature of the behavioral changes observed in the knockouts. However, the cumulated experience that these animals experienced should also be carefully considered when interpreting

behavioral outcomes. In this regard, the repeated testing, handling and injections (either vehicle or paraquat) that all mice experienced might be thought of as interacting with the genetic mutation over time in a very novel fashion. It could be considered that IFN-γ deficiency from birth resulted in changes in the development of key neural cytoarchitecture that are fundamental for dealing with repeated mild stressors of the nature that these animals experienced. Yet, this latter possibility is not obvious given the numerous studies that have failed to report any gross neuronal deficits in these IFN-γ null mice.

A final caveat that bears mention is the fact that it was possible that the behavioral measures presently used were too crude to pick up subtle early motor deficits. Indeed, although we failed to detect any signs of coordination deficits in any of the treatment groups, the pole test measure used was very crude (simply assesses time to turn and descend a vertical pole) and more sophisticated tasks could conceivably have tapped into more subtle deficits. Indeed, we currently have newer automated behavioral instruments, including 1, rotoraod beams, which will allow us to evaluate balance in a very precise manner (ability to stay on a variable moving horizontal column) and 2. a Catwalk digitized runway, which will allow for detection of any abnormalities of gait (as determined by pressure sensitive detectors that track the paw placement and movement of the animal).

4.3 Inflammation as a pro-death process in PD: modulatory effects of IFN-γ

A large body of evidence has provided support for a role for inflammation and immune system activation in the initiation and propagation of chronic degenerative diseases. Although neuro-inflammation has been repeatedly implicated in PD (Hunot and

Hirsch, 2003; Gao and Hong, 2008; Popovich and Longbrake, 2008; Qian et al., 2010), the nature of inflammatory processes has never been fully elucidated. Currently, there is still confusion over which factors and mechanisms are involved in PD. Furthermore, it is unclear whether these factors play a primary role in promoting neurodegeneration or are a secondary reaction to damage occurring through the progression of the disease (Hunot and Hirsch, 2003; Gao and Hong, 2008;Chung et al., 2009; Litteljohn et al., 2011). Neuro-inflammatory microglia cells are thought to contribute to neurodegeneration in PD largely through the oxidative free radicals they produce (Koizumi and Fujishita, 2007; Gao and Hong, 2008; Litteljohn et al., 2008; Mangano and Hayley, 2009). In this study, we assessed the influence of deletion of the pro-inflammatory cytokine, IFN-γ, given that this cytokine is the most potent activator of microglia and has been observed to be highly elevated in the blood and cerebral spinal fluid of PD patients (Gribova et al., 2003; Mount et al., 2007). Despite evidence suggesting that IFN-γ was reported to be necessary for the pathological and behavioural symptoms associated with MPTP or paraquat exposure (Mount et al., 2007; Mangano et al., 2011), the influence of the cytokine has not been evaluated in aged mice or with protracted exposure to such toxins.

As already mentioned briefly, our work here has shown that mice genetically lacking IFN-γ from birth were resistant to the effects of paraquat. IFN-γ deficient animals showed no behavioural alterations when treated with paraquat nor did they show any signs of degeneration of the nigral-striatal pathway. The degree of protection from the effects of paraquat observed in IFN-γ deficient mice is consistent with a critical role of this cytokine, and neuro-inflammatory process in PD pathology. However, IFN-γ deficient mice were not entirely protected as signs of degeneration were still observed in

the LC. The depressive, anxious and cognitive impairments observed in PD patients, discussed previously, have been theorized to be the result of deterioration in the hypothalamus, hippocampus and LC (Chanyachukul et al., 2004; Fernagut et al., 2007; Lin et al., 2008). Intriguingly, in this study, catecholaminergic cells of the LC were present but appeared as small shrunken masses. This appearance is common to cells currently undergoing pyknosis, a form of cell necrosis in which the nucleus fragments, condenses and is destroyed leaving the un-nucleated cell behind. Along with the the fact that paraquat failed to induce a loss of striatal dopamine neurons, this provides an indication that paraquat might have mechanistic effects quite distinct from more traditionally used toxins, such as MPTP or 6-OHDA. Indeed, these other toxins typically induce fairly rapid mitochondrial dysfunction, coupled with some apoptotic factors being induced (German et al., 1996; Bergman et al., 1998; Blum et al., 2001; Bove et al., 2005; Carrasco et al., 2005; Quintero et al., 2006). In contrast, the pyknotic-like neuronal pathology currently observed is not usually associated with substantial mitochondrial impairment (German et al., 1996; Blum et al., 2001; Przedborski and Vila, 2001; Yong-Kee et al., 2011). Further investigation is required to assess whether paraquat induces degeneration in other stressor-sensitive brain regions, such as the hypothalamus or hippocampus. We believe that the hippocampus might be especially vulnerable to paraquat given a recent study showing that paraquat induced oxidative stress radicals in these brain region (Chen et al., 2010), and our own recent findings demonstrating that systemically administered paraquat actually accumulated within the hippocampus at substantially higher levels than the SNc, ST or frontal cortex (Litteljohn et al., 2011, unpublished observations).

It is important to note that despite the protection observed in IFN-γ knockout mice, we did not find clear signs of increased microglial activation using CD11b immunostaining. It is possible that this was due to the timing and/or intensity of our insults, such that we missed the window of opportunity for detecting microglia during their active period. Comparably, it is possible that a low level of chronic up-regulation of neuro-inflammatory processes was initiated but that our measurements were not sensitive enough to detect these changes. Indeed, when a microglia cell sustains an active state over a very long period of time some key morphological characteristics used to visually assess the active state become more subtle (Whitton, 2007; Michelucci et al., 2009). It is unlikely that neuroinflammatory process were not initiated by paraquat administration given that our previous studies, as well as those of many other laboratories, have reliably observed microglia activation to occur in parallel with degeneration in the SNc (Czlonwska et al., 1996; McCormack et al., 2002; Gao et al., 2003; Chen et al., 2010; Mangano and Hayley, 2009; Mangano et al., 2011). Our future analyses will focus on assessment of microglial factors more proximal to the neurodegenerative processes, including the enzyme, NADPH-oxidase, which is responsible for the production of oxidative radicals.

4.4 Compensatory mechanisms in PD

It has been commonly observed that striatal dopamine levels must drop between 60-80% before clinical PD symptoms become apparent in human populations (Blandini et al., 2000; Kedar, 2003; Ossowska et al., 2005; Weintraub et al. 2008; Mangano and Hayley, 2009). However, smaller lesions have been observed to result in motor impairment in murine models (McCormack et al., 2002; Manning-Bog et al., 2003;

Fernagut et al., 2007; Litteljohn et al., 2008; Litteljohn et al., 2009; Mangano and Hayley, 2009). In the current study, despite significant degeneration of the SNc and accompanying behavioral alterations evident in paraquat treated wild type animals, there were no significant loss of striatal dopamine projections (and hence, likely very little affect on dopamine output). This lack of degeneration of the ST in the presence of an obvious SNc lesion is not unprecedented, as it has likewise previously been observed in other mouse studies involving systemic administration of paraquat (Choi-Lundberg et al., 1998; Thiruchelvam et al., 2000; McCormack et al. 2002; Cicchetti et al. 2004; Boulet et al. 2008). Whether this paradoxical finding suggests a fundamental problem with the paraquat model or some other yet to be identified compensatory mechanism and/or parallel systems being affected remains to be determined.

One proposed mechanism for this compensation could be that intra-striatal neurons are reacting to the paraquat insult by increasing their dendritic aborization. Thus, there could be a modest reduction of dopamine terminal density in the ST but that the surviving terminals increased their branching patterns resulting in a failure to detect differences in striatal coverage. Indeed, previous studies have observed up regulation in the density of these striatal projections in both MPTP animal models and in studies concerning human PD populations (Betarbet et al., 1997; Mitsumoto et al., 1998; Mao et al., 2001; Palfi et al., 2002). It is also possible that in the current study the maintenance of projections from the SNc to the ST could have been the result of the formation of new dendritic branches from local GABAergic neurons. Indeed, Zhou reported that GABA producing striatal inter-neurons up-regulate a host of transcription factor (e.g. delta Fos B, c-Jun) (Zhou et al., 1996), which could influence local dendritic arbors. Alternatively

it could be that preserved cells of the SNc are augmenting their degree of axonal collaterals in order to maintain connections in the ST. This type of compensation has been observed in observed in 6-OHDA models, but maybe too slow a process to account for the compensation (Anglade et al., 1995; Finkelstein et al., 2000; Stanic et al., 2003). It is quite possible that both forms of compensation could be occurring in our study.

In addition to changes in striatal dendritic projections, it is possible that alterations in neurogenesis and growth factors related to this processes might occur in PD. Indeed, MPTP and 6-OHDA lesions were previously reported to impair adult hippocampal neurogenesis and reduce levels of the trophic factor, BDNF (Mao et al., 2001). Similarly, reductions of a number of growth factors, including BDNF and GDNF (glia derived neurotrophic factor), were reported in postmortem PD brain tissue (Parain et al., 1999; Mogi et al., 1991; Howells et al., 2000; Mogi et al., 2001). Accordingly, central infusion of GDNF has been reported to have neuroprotective effects and/or facilitate functional recovery in virtually all animal models of PD (Bjorklund et al., 1997; Chauhan et al., 2001; Palfi et al., 2002; Jollivet et al., 2004; Collier et al., 2005). Interestingly, although the SNc is not a traditional neurogenic niche (as are the hippocampal dentate gyrus and olfactory cortex), evidence of neurogenesis was reported in MPTP treated rodents (Bedard et al., 2002). Thus, nigrostriatal circuits might have previously unrecognized capability of a certain degree of neural renewal of self repair.

Given the increasing recognition that perturbations of BDNF and neurogenesis both can have dramatic consequences for affective and cognitive functioning, it might be expected that disturbances of these fundamental neuroplastic processes could contribute to many of the neuropsychiatric co-morbid features evident in PD. Of course, alterations

of such factors at nigrostriatal regions would likewise be expected to impact upon midbrain dopamine activity and hence, motor functioning. Our own work previously demonstrated that paraquat reduced BDNF levels within the SNc and hippocampus of male mice but interestingly increased hippocampal levels in females (Litteljohn et al., 2011). We hypothesized that that the BDNF reductions evident in males (which actually occurred prior to oxidative stress factor induction and neuronal loss) served to render dopamine neurons vulnerable to the onslaught of the oxidative radicals that subsequently occurred; in contrast, the elevation in females might help explain their resiliency in general to PD-like symptoms (Litteljohn et al., 2011).

It was very surprising that no significant BDNF differences between the treatment groups were evident in the present investigation. This negative finding could stem from technical difficulties, as BDNF antibodies are notoriously unreliable between different batches even from the same vendor. It is also possible that subtle effects were missed, owing to the gross observation and quantification method used in the present investigation. In this regard, more rigorous stereological methods might have detected subtle BDNF variations. Alternatively, it would be of interest to evaluate whether changes in neurogenesis at the subventricular zone might have occurred with time and advanced age following paraquat exposure. Indeed, it was reported that following cerebral stroke, neurogenesis is increased within the subventricular zone and that these new neurons migrate into the ST to the site of lesion in order to presumably invoke some reparative or recovery functions (Popa-Wagner et al., 2007; Pectu et al., 2008). It would be of interest to determine whether similar processes could occur using toxin based PD modeling approaches. Whatever the case, a better understanding of the potential of

various neural system to reorganize following toxin exposure is of paramount importance for steps forward in PD research. The present findings represent in early step in evaluating changes in multiple systems over a protracted time scale following exposure to an environmentally relevant insult. It is also clear from the present results that the pro-inflammatory cytokine, IFN-γ, is a potential critical factor in at least some aspects of the neurodegenerative response to paraquat treatment.

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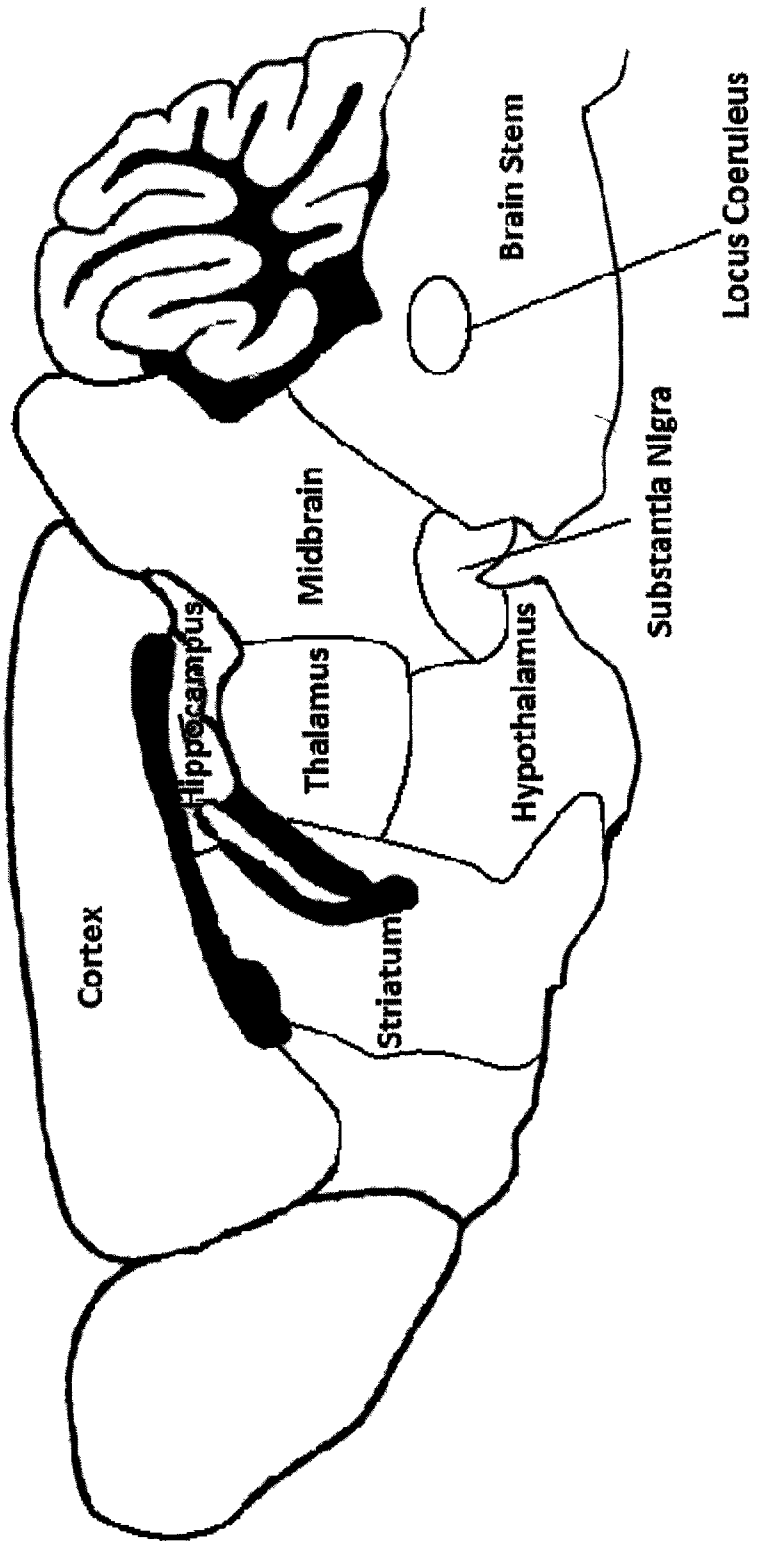
Appendix

Neuroanatomy of the Murine Brain



Chemical Structure of Paraquat