The behavioral, corticoid and hippocampal effects of chronic low-dose paraquat exposure in middle- and- advanced-aged mice: Potential implications for prodromal Parkinson's disease

by

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Abstract

The current study assessed whether exposure to the Parkinson's disease (PD) relevant toxicant, paraquat, induced non-motor behavioural symptoms implicated in PD, and whether the pesticide had stressor-like effects, as indicated by changes in corticosterone (CORT) and hippocampal glucocorticoid receptor (GR) expression. "Middle-aged" and "advanced-age" mice (aged 5 or 13 months) received a low-dose of paraquat (1mg/kg i.p. or vehicle 1x/weekX16weeks). While the present investigation failed to observe any signs of depressive-like outcomes, surprisingly aged paraquat treated mice displayed increased sucrose preference as opposed to the expected anhedonic-like deficit. Furthermore, paraquat transiently affected spatial memory, both enhancing and impairing performance. The chronic paraquat challenge elevated plasma CORT and phosphor-GR of middle aged mice only. Advanced-aged mice displayed reduced hippocampal GR expression and no increase in CORT in relation to aged matched controls. These data suggest that chronic low-dose paraquat may age-dependently influence hypothalamus-pituitary-adrenal axis functioning, with potential ramifications for prodromal PD.

Table of Contents

Abbreviations

5-HT = 5-hydroxytryptamine or serotonin
6-OHDA = 6-hydroxydopamine
AAR = alternate arm return
Ach = acetylcholine
AMPA = l-α-amino-3-hydroxy-5-methylisoxazole-4-propionate
ANOVA = Analysis of variance
ATP = adenosine triphosphate
BBB = blood brain barrier
BDNF = brain derived neurotrophic factor
CAT = catalase
CNS = central nervous system
CORT = corticosterone
COX = cyclooxygenase
DA = dopamine
DAT = dopamine transporter
EPM = elevated plus maze
EU = European Union
FST = forced swim test
GFAP = glial fibrillary acidic protein
GSH = glutathione
HPA = hypothalamic-pituitary-adrenal
IFN-γ = interferon-gamma
IL = interleukin
iNOS = inducible nitric oxide synthase
JNK3 =c-Jun N-terminal kinase 3
LB = Lewy body
LC = locus coeruleus
MAP = mitogen-activated protein
MCP-1 = monocytic chemotactic protin-1
MHC = major histocompatibility complex
MMX = micromax
MPTP = 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MPP^+ = 1-methyl-4-phenylpyridinium
NA = noradrenaline
NADPH – nicotinamide adenine dinucleotide phosphate
NE = norepinephrine
NF-κB = nuclear factor- kappa B
NMDA = N-Methyl-D-aspartic acid
NO = nitric oxide
Nrf2 = nuclear factor erythroid 2-related factor 2
NSAIDS = non-steroidal anti-inflammatory drugs
OFT = open field test
PD = Parkinson's disease
PET = positron emission tomography

PFC = prefrontal cortex
PHOX = phagocytotic oxidase
ROS = reactive oxygen species
SAR = same arm return
SAB = spontaneous alternation behavior
SN = substantia nigra
SNc = substantia nigra pars compacta
SOD = superoxide dismutase
SPT = sucrose preference test
TNF = tumor necrosis factor
TH = tyrosine hydroxylase
TrkB = tyrosine-related kinase B
UPS = ubiquitin-proteasome system
VMAT = vesicular monoamine transporter
VTA = ventral tegmental area

List of Tables

List of Figures

Introduction

Parkinson's disease (PD) is a progressive age-related neurodegenerative disorder, estimated to occur in 0.2% of humans 60 years of age and rising precipitously thereafter (de Rijk et al, 2000). One of the defining biological hallmarks in all forms of PD is the progressive degeneration of neuromelanin-containing dopamine (DA) neurons in the ventrolateral and caudal portions of the substantia nigra in a region termed the substantia nigra pars compacta (SNc) (Damier et al., 1999). Such neuronal loss, in turn, causes decreased monoamine release in the downstream caudate and putamen (striatum), the main site of innervations and termination for SNc projection neurons (Schulz & Falkenburger, 2004). This defective nigrostriatal pathway gives rise to the clinical motor control deficits that are characteristic of PD, including muscle rigidity, postural instability, and bradykinesia (slowness of movement). These cardinal motor symptoms usually become manifest after an estimated 30-50% of DAergic neurons in the SNc, and 50-80% of DA in the striatum, has already been lost (Jankovic, 2005; Wu et al., 2011). In fact, nigrostriatal damage is believed to occur up to 6 years before clinical diagnosis via onset of motor symptoms, suggesting a relatively slow and progressive onset of the illness (Marek et al., 2001).

In addition to the defective nigrostriatal pathway that results in basal ganglia dysregulation, a second feature known as Lewy Body (LB) inclusions are also a defining characteristic. LBs present themselves throughout the brain parenchyma and are found in areas other than the SNc, such as the olfactory bulbs, amygdala, and entorhinal cortex of the hippocampal formation (Wakabayashi et al., 2007). Within the neuron, LBs are found in the soma and dendrites (effectively termed Lewy neurites); and these inclusions are

comprised of accumulated misfolded protein aggregates, fibrillar α-synuclein, parkin, and ubiquitin (Schulz & Falkenburger, 2004).

Given the presence of LBs in PD, it has been hypothesized that these inclusions may play a role in the development of behavioral symptoms, as they may contribute to neuron dysfunction and degeneration (Schulz & Falkenburger, 2004). For example, it may be that LBs are disrupting otherwise adaptive intracellular processes within DA neurons, thus leading to neuronal damage and dysfunction (Schulz & Falkenburger, 2004). It is also possible that LBs themselves induce oxidative factors causing damage to host neurons via induction of cell death pathways (Schulz & Falkenburger, 2004). In addition, their presence may be indicative of other destructive processes triggered by the true underlying mechanism. That is, their presence may be a result of oxidative stress induced by inflammation, mitochondrial dysfunction and subsequent adenosine triphosphate (ATP) decline; and could also indicate and, in fact, propagate problems associated with mechanisms relating to protein degradation such as the ubiquitin-proteosome system (UPS) or the autophagy-lysosome pathway (Malkus et al., 2009; Schulz & Falkenburger, 2004). Of note, although these inclusions are a hallmark of PD-related pathology, no direct link between the presence of LBs and DA cell death has been found (Schulz & Falkenburger, 2004). Hence, it remains unclear whether LBs directly contribute to DA neuron cell death, if LBs are merely a by-product or epiphenomena of the neurodegenerative process in PD, or if the presence of LBs are simply an age-related phenomenon (Schulz & Falkenburger, 2004).

PD: More than just Motor Disturbances

While the "principal" behavioral symptoms of PD include the motor disturbances resulting from DA denervation from the striatum to the SNc, the disease is in fact not nigrostriatal- or- DA-specific. In fact, PD is a multi-system disease affecting not only the DAergic nigrostriatal pathway, but also the cholinergic, serotonergic, GABAergic, noradrenergic, and extra-nigrostriatal DAergic pathways (e.g. frontal and limbic DA systems) (Schrag, 2004). For example, the presence of LBs and neurodegeneration has been shown to occur in the locus coeruleus (where the cell bodies for noradrenaline (NA) are located; also referred to as norepinephrine; NE); the nucleus basalis of Meynert (where the cell bodies for acetylcholine (Ach) are located); the raphe (where the cell bodies for serotonin (5-HT) are located); and the ventral tegmental area (VTA) (the origin of the DAergic mesocorticolimbic system) (Ferrer, 2011). While the dysregulation and/or degeneration observed in these areas may contribute to the cardinal motor deficits that define clinical PD, it is also likely that dysfunction in these areas gives rise to other behavioral characteristics. Indeed, many non-motor behaviors such as olfactory dysfunction, cognitive alterations, sleep disturbances, and neuropsychiatric features (e.g. anxiety and depression) are co-morbid with the disease (for a review see Ferrer, 2011). It has been suggested that these co-morbid behaviors may result from defects in one or the combination of multiple transmitter pathways (including the nigrostriatal DA pathway); yet, the pathophysiology that underlies these symptoms remains largely unknown (Ferrer, 2011).

Interestingly many of these co-morbid behaviors occur in what is known as the prodromal phase of PD, before the presence of any motor symptoms, and perhaps even

before the onset of any nigrostriatal damage (Spiegel et al., 2006). It has even been suggested that identifying and combining some of these behaviors may help in pre-screening for clinical PD, perhaps in conjunction with other biomarker modalities (e.g., genetic screens) (Schlossmacher & Mollenhauer, 2010).

Olfactory Dysfunction in PD

In PD, olfactory dysfunction comes in the form of hyposmia, which is the diminished ability to fully detect or smell different odours, or anosmia, which is the complete lack of smell often seen in extreme cases (for a review see Doty, 2011). Hyposmia is perhaps the number one co-morbid symptom that occurs in individuals with idiopathic PD, present in at least 90% of patients with some evidence suggesting that it occurs in familial cases as well (Doty, 2011). Importantly, olfactory dysfunction has been posited to occur before SNc cell death, and has been defined as a prodromal symptom and possible risk factor of the disease (Doty, 2011). One hypothesis known as the Braak staging hypothesis, although not without controversy, outlines the progression of PD whereby LB inclusions present themselves in a caudo-rostral linear-like path (Braak et al., 2003). That is, LB inclusions first appear in the lower brain stem, as well as olfactory bulbs and anterior olfactory nucleus, before manifesting in other, deeper areas (Hawkes et al., 2010). The presence of these inclusions in the olfactory bulbs in the beginning stages supports the notion that disturbances in smell are one of the first symptoms observed before any nigrostriatal damage and motor behavior dysfunction occurs (Braak et al., 2003; Hawkes et al., 2010). Indeed, the level of olfactory dysfunction remains the same throughout disease progression after clinical onset, suggesting a consistency in the

biological mechanisms involved (Doty, 2011). While olfactory dysfunction is a definite co-morbid symptom in most sporadic PD cases, its etiology and biological underpinnings still remain widely unknown (Ferrer, 2011). The presence of the inclusions observed likely occurs with a myriad of complicated, complex molecular and biological mechanisms that give rise to this deficit (Ferrer, 2011).

Anxiety and Depression in PD

In addition to olfactory deficits, neuropsychiatric disturbances such as anxiety and depression are now also considered to be defining characteristic of PD (for a review see Kano et al., 2011). Interestingly, research suggests that depression is more likely to occur in individuals with PD than in any other neurological disorder (e.g. multiple sclerosis) (Kano et al., 2011). It is estimated that this symptom occurs in up to 50% of individuals with PD, although many cases likely remain undiagnosed as there is a high degree of overlap between characteristics of this disturbance with those of motor deficits (Kano et al., 2011; Tan, 2012). Indeed, some suggest that depressive symptoms may occur in up to 90% of individuals with PD indicating that this is an important feature that needs to be addressed clinically (Tan, 2012).

Anxiety is similarly estimated to occur in up to 49% of PD patients and, like depression, it is believed that anxiety episodes may occur *a priori* to the onset of frank motor impairment (Bogdanova et al., 2012; Kano et al., 2011). This has led some to suggest that neuropsychiatric problems, like the aforementioned olfactory deficits, may be considered as possible risk factors for PD (Kano et al., 2011). Similar to olfactory dysfunction, the neurobiology of depression and anxiety in PD is thought to be extremely

complex and remains largely unknown (Kano et al., 2011). While it is likely that these neuropsychiatric symptoms are a direct result of the PD-related pathology for some individuals, it is also likely that other variables that are external to PD pathology may come into play (Schrag et al., 2001). For example, environment factors (e.g. environmental toxins), personality factors, and psychosocial stress are all features that can give rise to depression and/or anxiety in PD (Schrag et al., 2001).

One possible biological explanation for depression in PD could be the nigrostriatal DAergic neuron degeneration which may give rise to depression symptoms in some patients (Kano et al., 2011). However, clinically significant symptoms of depression and anxiety may present themselves at any stage of PD including the premotor phase, and DA agonists are not always effective in patients (Kano et al., 2011). In addition, while symptoms have been shown to worsen as the disease progresses, this does not always occur in a linear-like fashion that is analogous to the progressive DA nigrostriatal degeneration, suggesting that this pathway may not be the only one involved (Gallagher & Schrag, 2012). It is also possible that disturbances in the neurotransmitter pathways involving NE, 5-HT and DA (e.g. mesocorticolimbic), are culprits either alone or in combination (Burn, 2002; Dymecki et al., 1996; Gallagher & Schrag, 2012; Tan, 2012). Furthermore, involvement of other biological mechanisms (e.g. gliosis and inflammatory cascades, decreased trophic support, receptor polymorphisms, neurotransmitters) may also contribute in this regard (Litteljohn et al., 2010b).

Located in the brainstem, the locus coeruleus and raphe nuclei are the main epinergic and 5-HTergic brain centers, respectively. Cells in these brainstem areas send projections throughout the brain and have been highly implicated in depressive,

cognitive, and/or anxiety like behaviours (Southwick et al., 2005; Morilak & Frazer, 2004). Due to their location in the brainstem, these areas are believed to be two of the first brain areas affected in PD (i.e. before the onset of any motor symptoms) (Hawkes et al., 2010). This suggests that these areas are involved in PD related depression, and might give reason as to why depression may occur before clinical diagnosis.

Cognitive Deficits in PD

In addition to the other co-morbid behaviors outlined above, cognitive deficits are also a common characteristic of PD, and range from mild to severe impairment (see Bosboom et al., 2004). While severe cognitive impairment in PD patients is generally expressed in the form of dementia (effectively termed PD with dementia (PDD)) and often occurs at later stages, other more mild disturbances involve problems in executive functioning (e.g., deficits in attention, working and spatial memory) and are generally subtle events observed early in the disease time course (Ferrer, 2009). One important consideration is that a correlation between LBs and early cognitive impairment is not observed in the cerebral cortex, one of the main areas associated with executive functioning (Ferrer, 2011). Instead, early impairment is believed to be the result of a number of other factors which include; synaptic dysfunction via α-synuclein aggregation in the synapse, oxidative stress and damage resultant from mitochondria dysfunction, and damage that results from other transmitter systems innervating the prefrontal cortex (PFC) (Ferrer, 2011). For example, degeneration of DA neurons within the VTA in PD is also observed, although to a lesser extent than is seen in the nigrostriatal area (Ferrer, 2011). Damage to the VTA affects DAergic innervation of the PFC and hippocampus

(via the mesocorticolimbic pathway), two areas which have been associated with executive functioning and visuo-spatial memory, respectively (Ferrer, 2009). Likewise, degeneration to the DAergic nigrostriatal system may also give rise to cognitive dysfunction via disrupted basal ganglia cortical signalling (Shohamy et al., 2004).

To date, animal toxin models of PD have largely (although not exclusively) focused on identifying the neurobiology that gives rise to the cardinal motor symptoms (Taylor et al., 2010). Given that PD is more widespread than just these deficits, these models should also effectively reproduce other characteristics observed in PD. This will allow for a more accurate understanding of the underlying progressive pathophysiology of this complex constellation of behaviors (Taylor et al., 2010), which will in turn foster the development of more targeted or appropriate treatment agents. Moreover, neuroprotective and restorative agents that halt or slow progression of the disease could also stem from a better understanding of PD pathogenic mechanisms. As a first step to this process, the current thesis will be largely focused on identifying and validating the role that a chronic low-dose oxidative stressor may have on the development of select non-motor behaviours commonly observed in PD.

Multiple epidemiological studies have been conducted in order to identify risk factors that might work in a synergistic or additive manner, including variation in select genes that have been linked with an increased risk of developing PD (e.g., LRRK2) (Horowitz & Greenamyre, 2010). Outside of aging (the number one risk factor of PD), some environmental risk factors that have been consistently identified through epidemiological investigation include, but are not limited to: pesticides (e.g. the herbicide paraquat or the insecticide rotenone), biphenyls, viruses, and heavy metals (e.g. iron,

manganese) (Schapira & Jenner, 2011). Indeed, the extensively studied pesticide, paraquat, and insecticide, rotenone, have been identified as potential risk factors for PD; and both agents have produced PD-like behavior and biological characteristics following systemic exposure in rodents. Furthermore, the brain and behavioural changes provoked by these pesticides are comparable to the pathology induced by the commonly used DA toxins, 6- hydroxydopamine (6-OHDA) and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (for a review see Bové & Perier, 2011).

Neuroinflammatory factors in PD pathology

One factor that has consistently been implicated as a mechanistic contributor to PD is neuroinflammation. Neuroinflammation is the central nervous system's (CNS) rapid coordinated response to perceived injury (Kraft & Harry, 2011). The brain's inflammatory processes differ from that of the peripheral immune response, because it is generally devoid of leukocytes due to the tight junction of the blood brain barrier (BBB) (Aloisi, 2001). Instead, microglia, which are the brain's resident phagocytic immune cells, and astrocytes (another type of glial cell), serve as the brains resident activators and responders to perceived insult (Neumann et al., 2009). Together with astrocytes, microglia primarily serve to: effectively maintain and facilitate neuronal survival and function, destroy and eliminate pathogens, clear toxic cellular debris and aberrant proteins, and promote repair of damaged tissue, all with the goal of maintaining CNS homeostasis (Neumann et al., 2009; Simard et al., 2006; Streit, 2002).

When at "rest" or in a quiescent state, microglia are in a constant surveying state in which they actively scan the microbe environment of the brain parenchyma

(Nimmerjahn et al., 2005; Hanisch & Kettenmann, 2007). Upon activation via peripheral influence (e.g. pathogens, cytokines, peripheral immune cells, etc.) or central recognition (e.g. via decreased CD200 expression on neurons, α-synuclein, extracellular ATP, neurotransmitter imbalance, cytokines, chemokines, etc.), microglia cells rapidly proliferate and undergo a morphological and functional transformation paralleled by an increase in metabolic activity and surface receptor expression (Block et al., 2007). For example, in response to infection or injury, chemokines (e.g. monocytic chemotactic protein-1 (MCP-1), interferon (IFN) - inducible protein 10), will attract microglia cells to the site of injury whereupon microglia are directed to release a variety of inflammatory messengers such as pro-inflammatory cytokines (e.g. interleukins (ILs), IFNs, tumor necrosis factor-α, (TNF-α)) and oxidative molecules (Aloisi, 2001; Litteljohn et al., 2010b). These messengers are not only capable of sustaining an inflammatory immune response at the site of injury, but are also capable of coordinating the infiltration of leukocytes from the periphery. Such signalling leads to further sustained expression and release of inflammatory and oxidative factors (e.g. cytokines, reactive oxygen species (ROS), reactive nitrogen species (RNS), prostaglandins, etc.) that can be toxic to neurons when not under proper control, or under increased activation and secretion (e.g. via prolonged exposure to a low dose of environmental toxins) (Giulian & Baker, 1986; Hanisch, 2002; Hanisch & Kettenmann, 2007; Nimmerjahn et al., 2005; Tansey et al., 2008; Vitkovic et al., 2000).

Evidence strongly implicates microglia and inflammation as being major contributors to neuron dysfunction and degeneration in PD (Litteljohn et al., 2010b). For example, in the human brains of post-mortem PD patients who were "accidentally"

exposed to the DA toxin, MPTP, when injecting opiates 16 years before death, the SNc degeneration observed was coupled with heightened microglia activation, suggesting that neuroinflammation plays an on-going role that contributes to the progressive pathology of PD (Langston et al., 1999). In addition, post-mortem brains and animal toxin models have also shown augmented microglia cell activation (Imamura et al., 2003), as well as several other biological markers that indicate pro-inflammatory involvement in PD (e.g. cytokines, inflammatory enzymes, inflammatory transcription factor nuclear-factor κB (NF-κB) (Mogi et al., 1996; Hirsch et al., 2012; Teismann et al., 2003; Wilms et al., 2003; Zhang et al., 2011). It has also been repeatedly demonstrated that microglia activation occurs prior to the degeneration of neurons (McGeer et al., 1988; Purisai, et al., 2007). Furthermore, post-mortem brains have also shown an up-regulation of CD4+ and CD8+ positive T cells, which would seem to argue in favour of a microglia-directed peripheral immune response (Brochard et al., 2009). Interestingly, the use of non-steroidal anti-inflammatory drugs (NSAIDS) that are specific inhibitors of the inflammatory immune enzyme, cyclooxygenase (COX)-2, has been found to be a negative risk factor for PD, decreasing risk by up to 19% (Chen et al, 2003; Chen et al., 2005). Taken together, all of this evidence suggests a definite key role of microglia cells in PD pathology. However, the specific role that neuroinflammation plays in the pathogenesis of PD is unclear. For example, it may be that microglia activation observed in PD patients as well as animal studies is secondary to initial triggers/insults. In this regard, much attention has been afforded to the possibility that microglia are recruited by secondary messengers secreted from "sick or dying" neurons and thus stem from ongoing neuronal death/survival processes (Hirsch et al., 2012). Alternatively, microglia

activation may be directly activated by an exogenous substance (e.g. pesticide / heavy metals) resulting in microglia release of several toxic factors (e.g. free radicals, pro-inflammatory cytokines), which ultimately contribute to DA cell death (Hirsch et al., 2012; Horowitz & Greenamyre, 2010).

In vivo and *in vitro* studies have offered compelling evidence supporting the hypothesis that the particularly high concentration of microglia within the SNc contributes to the enhanced vulnerability of the DA neurons in PD (Hirsch et al., 2012). For example, several studies have shown that MPTP and paraquat induced microglia activation occurs prior to DA cell death (Czlonkowska et al. 1996, McCormack et al., 2002). In fact, a single injection of paraquat was enough to cause microglia activation (Purisai et al., 2007) suggesting that microglia activation is necessary for DA cell death. Along these lines, the bacterial endotoxin, lipopolysaccharide (LPS) has also been shown to contribute to DAergic cell death in the SNc by activating microglia cells (Gayle et al., 2002). In particular, direct administration of LPS into several rat brain regions, including the SNc, hippocampus, thalamus and cortex only produced substantial neuronal loss in the SNc (Kim et al., 2000), suggesting that the high microglia concentration observed in the SNc may be the source to neuron vulnerability. Furthermore, several *in vitro* experiments also revealed that the neurodegenerative consequences of LPS on mesencephalic cells were only evident in the presence of microglia (Gayle et al., 2002); and blocking the activation of these cells with the tetracycline derivative, minocycline, prior to treatment with several DA toxins prevented DA degeneration (Silva Bastos et al., 2011).

The pro-inflammatory cytokines released from activated glial cells, including TNF-α, IFN-γ and IL-1β, have been implicated as major contributors to neuronal dysfunction observed in PD (for a review see Litteljohn et al., 2010b). Indeed, toxin models including MPTP, 6-OHDA, rotenone and paraquat have all demonstrated enhanced pro-inflammatory brain and/or blood cytokine expression (Litteljohn et al. 2010b). For example, in an MPTP model in which macaques where exposed to chronic administration of low doses of the toxin, an increase in the expression of TNF-α and IFN-γ in the blood serum was observed over a four year period (Barcia et al., 2011). It was therefore suggested that these two cytokines are responsible for the sustained and prolonged activation of microglia cells (Barcia et al., 2011). In concordance with post-mortem observations, the brains did indeed exhibit an increase in the morphological change of both microglia and astroglial cells within the SNc, as shown by the MHC-II markers and glial fibrillary acidic protein (GFAP), respectively (Barcia et al., 2011). An increase in the expression of both TNF-α and IFN-γ was also observed in the SNc, and this expression was positively correlated with degree of cell death (Barcia et al., 2011). These findings provide support for the notion that the above mentioned pro-inflammatory cytokines (and perhaps others) are active contributors to degeneration (Barcia et al., 2011). Furthermore, when looking at genetic mouse models in which either TNF-α or IFN-γ had been genetically knocked-out (KO), after acute MPTP administration no activation of glial cells or SNc cell death was observed in the SNc in either of the two KO models (the opposite was found in wildtype mice), suggesting that the two cytokines work synergistically to induce/sustain glial cell activation (Barcia et al., 2011).

Further evidence to support pro-inflammatory cytokine mediated cell death comes from other toxin models as well. For example, it appears that LPS increases levels of the pro-inflammatory cytokines, IL-1β and TNF-α, as well as inducible nitric oxide (NO) synthase (iNOS) on glial cells (Iravani et al., 2002; Iravani et al., 2012). This could lead to prolonged secretion of NO that results in destruction and activation of cell death pathways (Irvani et al., 2002; Irvani et al., 2012). Indeed, in a genetic KO model in which the pro-inflammatory cytokine IFN-γ was ablated, Mangano et al. (2011) demonstrated that these mice were protected from the neurodestructive and inflammatory/oxidative effects (e.g., microglia activation, pro-inflammatory cytokine upregulation, enhanced oxidative and inflammatory enzyme activity) following a 3-week paraquat challenge. This observation supports experimental findings that are indicative of IFN-γ inducing expression of pro-inflammatory and pro-oxidative factors, which in turn mediate DA cell death. Indeed, IFN-γ has been repeatedly shown to enhance production of microglia as well as expression of: other pro-inflammatory cytokines including TNF-α, oxidative factors such as NO and superoxide (discussed below), and has even been shown to induce direct neurotoxicity through activation of the IFN-γ receptor l-α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) GluR1 on neurons (Mizuno et al., 2008).

Other evidence supporting the role of neuroinflammation in PD pathology comes from the additive interaction of toxin models 6-OHDA, paraquat or rotenone with the inflammatory stimulant LPS (Hritcu et al., 2011). For example, Mangano and Hayley (2009) demonstrated that priming the SNc with direct LPS infusion in mice leads to highly activated microglia as expressed by CD11b staining 2 days after exposure with modest activation at 7 days post exposure. Interestingly, when paraquat was administered

at the 2 day time point when microglia activation was at its highest expression, a reduction in DA neurons was observed as indicated by the number of immune-reactive tyrosine hydroxylase (TH) neurons present in the SNc (Mangano & Hayley, 2009). This effect was additive in comparison to the reduction in DA provoked by either paraquat or LPS alone (Mangano & Hayley, 2009). In another study in which 6-OHDA-treated rats were prenatally exposed to LPS (thus activating a pro-inflammatory state in the brain), an additive effect was also shown such that there were fewer DA neurons in the SNc observed than if given each toxin alone (Ling et al., 2004).

It is important to note that pro-inflammatory cytokine expression and thus neuroinflammation observed in animal toxin models is not nigrostriatal-specific, and that inflammation may affect brain areas associated with non-motor behaviors (and the genesis of non-motor symptoms). For example, while chronic administration of MPTP in mice was shown to increase levels of the pro-inflammatory cytokines, TNF-α, IL-1β, and IFN-γ, in the striatum, the toxin also reportedly increased levels of IFN-γ in the cortex. In fact, cortical IFN-γ expression was found to correlate with both NE and DA turnover in cortical and striatal regions, as well as with SNc cell death (Luchtman et al., 2009). In addition, paraquat exposure has also been demonstrated to increase TNF-α expression in areas implicated in co-morbid neuropsychiatric disturbances, including the hippocampus and PFC (Mitra et al., 2011). In another study by Litteljohn et al. (2010), mice exposed to a chronic paraquat regimen displayed differential expression of 5-HT and NE activity in the PFC, and decreased levels of NE in the hippocampus, while this effect was not observed in IFN-γ KO mice. It seems likely, then, that inflammation plays an important role in the development of both motor and non-motor symptoms in PD.

Oxidative Stress in PD pathology

There is overwhelming evidence outlining the important role that oxidative stress plays in DAergic cell death; however, similar to glial activation the upstream mechanisms driving oxidative stress are presently not known (Patten et al., 2010). Free radicals are formed during many biochemical functions including ATP production and activation of microglia membrane bound enzyme nicotine adenine dinucleotide phosphate (NADPH) oxidase (Patten et al., 2010). The rate of free radical production is normally balanced by antioxidant consumption (Patten et al., 2010). Oxidative stress occurs when there is an imbalance between the formation and removal of the pro-oxidants (favoring free radical production) (Schulz & Falkenburger, 2004). In this regard, oxidative stress plays a critical role in the progression of neurodegeneration. In particular with PD, oxidative stress may occur through multiple upstream pathways involving pro-apoptotic signaling factors, pro-inflammatory cytokines, mitogen activated protein (MAP) kinase signaling factors, etc. (Litteljohn et al., 2010b).

It is possible that some of the intrinsic intracellular processes that occur in DA neurons, such as the synthesis, vesicular packaging, and breakdown of DA may be one of many underlying causes of oxidative stress observed in PD (Patten et al., 2010; Drechsel & Patel, 2008). DAergic neurons are unique because they are more vulnerable to free radical damage in comparison to other neurons (Hald & Lotharius, 2005). Indeed, DA neurons have very high oxidative potentials, such that DA auto-oxidation results in the production of numerous free radicals including, quinones and oxygen radicals during this degradation process (Hald & Lotharius, 2005). In addition to this, high levels of

unregulated cytosolic DA alone may be sufficient to cause neurodegeneration (Hald & Lotharius, 2005), although this remains speculative. In order to prevent excessive levels of cytosolic DA, the amine is packaged into synaptic vesicles by vesicular monoamine transporter (VMAT2) (Zheng et al., 2006). DA is then transported into the vesicle in exchange for protons (Zheng et al., 2006). If any of these mechanisms are in disarray, free-radical production results that may be toxic to DA neurons (Hald & Lotharius, 2005).

A second major source of oxidative damage is caused by metabolic stress and mitochondrial dysfunction that results in a drop in intracellular ATP levels disrupting the homeostasis of the cell causing massive release of dopamine, free radical production, activation of N-Methyl-D-aspartic acid (NMDA) and non-NMDA receptors (Drechsel & Patel, 2008; Pivovarova & Andrews, 2010; Zhou et al., 2008). Occasionally, during electron transfer down the electron transport chain towards oxygen, electrons are sometimes prematurely leaked to oxygen, resulting in the formation of the toxic free-radical superoxide (Hald & Lotharius, 2005). The extent of damage caused by superoxide radicals is dependent on how quickly antioxidants (e.g. superoxide dismutase; SOD, catalase; CAT, glutathione; GSH) can reduce or neutralize the formation of these free radicals (Hald & Lotharius, 2005). If the homeostatic environment is disturbed for a prolonged period of time, then peroxides and free radicals are generated, which are capable of damaging cellular DNA and lipids (Hald & Lotharius, 2005). Interestingly, CAT (breaks down hydrogen peroxide) and not SOD attenuated microglia proliferation induced by the pro-inflammatory cytokines, TNF-α and IL-1β (Mander et al., 2006). Therefore, NADPH oxidase generated hydrogen peroxide is important for generating

and/or sustaining an inflammatory cascade and is sufficient to stimulate vigorous microglia proliferation; this illustrates the importance of ROS in maintaining a previously initiated inflammatory response (Mander et al., 2006).

Accumulating evidence supports a role for microglia in ROS generation by NADPH oxidase in the pathogenesis of PD (Miller et al., 2009). As previously mentioned, microglia act as the brain's first line of defence against invading pathogens; free radicals generated by microglia cells assist in the removal of these toxic pathogens. However, in a state of chronic activation microglia can be linked to neurodegeneration by producing neurotoxic factors including quinolinic acid, free radicals (superoxide anions and hydrogen peroxide), NO, chemokines and pro-inflammatory cytokines (Litteljohn et al., 2010b). In particular, several co-culture studies using a combination of mesencephalic neurons and microglia cells demonstrated that neurodegeneration caused by MPTP occurred concomitantly with ROS generation through the microglia membrane bound NADPH oxidase (Anantharam et al., 2007). Likewise, neurons obtained from mice genetically lacking NADPH or treated with the pharmacological inhibitor, apocynin, were largely resistant to MPTP toxicity (Gao et al., 2003), providing more evidence supporting the notion that microglia generated ROS are necessary for MPTP provoked DA cell loss.

The mechanism by which the multi-subunit enzyme NADPH oxidase generates free radicals has been thoroughly investigated (Kim et al., 2005). In a resting state the enzyme remains inactive as the core phagocyte oxidase components ($p40^{PHOX}$, $p47^{PHOX}$ and $p67^{PHOX}$) reside in the cytosol (Kim et al., 2007). In order to activate the NADPH oxidase, $p47^{PHOX}$ is phosphorylated and the entire cytosol complex migrates to the cell

surface and binds with nucleotide-binding proteins Rac1A and Rac2, where the activated form of the enzyme catalyzes the reaction of oxygen with NADPH to form superoxide (Sumimoto, 2008). The majority of superoxide produced by microglia cannot penetrate cellular membranes and is therefore unlikely to trigger intraneuronal toxic events in a DAergic neuron (Patten et al., 2010). Superoxide can, however, react quickly with iNOS in the extracellular space forming the stable oxidant peroxynitrite (Hald & Lotharius, 2005). This compound can readily penetrate the outer membrane of DA neurons resulting in lipid peroxidation, DNA damage and nitration of tyrosine residues (Jomova et al., 2010). In fact, genetic deletion of gp91 or $p67^{PHOX}$ (essential subunit for the NADPH activation) attenuated DA loss caused by rotenone (Zhou, 2012), paraquat (Wu et al., 2005) and MPTP (Anantharam et al., 2007). Moreover, a marked up-regulation of gp91 was also observed in human PD patients (Miller et al., 2009). To this end, mounting evidence demonstrates a critical role for NADPH oxidase in DA cell death induced by several commonly used neurotoxicants. Accordingly, inflammatory microglia reactivity likely contributes to ongoing degeneration through the release of highly reactive oxidative species (Zhou, 2012).

Aging and PD

Aging is the number one risk factor in PD (Schapira & Jenner, 2011). In fact, as one ages their risk of developing this neurological disorder after 80 years of age increases approximately 5% every five years (de Rijk et al, 2000). It is well known that as one advances in age antioxidant and protective mechanisms involving brain derived neurotrophic factor BDNF (among other things) are altered, and an increase in

oxidative/inflammatory stress is seen. These changes over time render an individual more susceptible to toxic insults, and may even promote an exaggerated response to the neurodegenerative effects of toxin exposure. Indeed animal PD toxin models using MPTP, 6-OHDA, and paraquat have all shown augmented nigrostriatal damage in advanced aged rodents in relation to their younger counterparts, supporting the notion that advancing age is itself an important contributor to PD risk.

Paraquat: An Oxidative Stressor Implicated in PD

Paraquat is a fast acting herbicide that targets green plant tissue and is possibly the most common herbicide used today throughout the United States and Canada (Jiao et al., 2012; Thiruchelvam et al., 2000). In structure, paraquat resembles the active metabolite 1-methyl-4-phenylpyridinium (MPP^+) of the established DA neurotoxin, MPTP (Bové & Perier, 2011). Upon recognition of this structural similarity, paraquat exposure has since been identified as a potential environmental risk factor for PD in various epidemiological studies (Wang et al., 2011). Amidst these findings, the European Union (EU) has since banned the use of paraquat; however, countries including our own have yet to do so. In fact, developing countries such as China and India are reported to have increased their use of the pesticide (Bové & Perier, 2011). Over the past 20 years, much work has been devoted to ascertaining whether the pesticide can induce biological and behavioral characteristics that are associated with PD in order to give rise to further understanding of the pathogenic mechanisms involved in the disease, with the hopes that it could be used as a model to identify protective and restorative factors (Bové & Perier, 2011).

In this regard, studies have demonstrated that paraquat can be transported across the tight-junction BBB via a neutral amino acid transporter (Shimizu et al., 2001), and that systemic administration in rodents can, at times, induce SNc cell death and striatal monoamine depletion that differs in magnitude depending on the dose and length of exposure (McCormack et al., 2002; Litteljohn et al., 2010b). Coupled with this nigrostriatal damage, paraquat has also been shown to reproduce some of the motor deficits observed in PD, though not as reliably as MPTP and 6-OHDA (reviewed in Litteljohn et al., 2010b). Furthermore, paraquat exposure in rodents has also produced other neurotoxic biological characteristics associated with PD including microglia activation, LB like aggregates containing α-synuclein, pro-inflammatory cytokine expression, and oxidative stress via mitochondria complex inhibition or microglia activation (Litteljohn et al., 2010b). Upon entry into the brain, paraquat is distributed across areas including the PFC, hippocampus, olfactory bulbs, and the SNc (Peng et al., 2007). While many studies have reported that degeneration after paraquat exposure is only selective for neurons in the SNc, others have demonstrated that evidence of dysfunction occurs in other areas as well, albeit in a time dependent fashion (Litteljohn et al., 2010b). For example, it was reported that paraquat exposure results in modest cell death in the locus coeruleus (Fernagut et al., 2007), and VTA (Ossowska et al., 2006); mitochondrial dysfunction in the frontal cortex (Czerniczyniec et al., 2011; Mitra et al., 2011), and hippocampus (Chen et al., 2010); oxidative stress in the frontal cortex (Czerniczyniec et al., 2011; Mitra et al., 2011), and hippocampus (Chen et al., 2011); differential expression of ROS scavengers in the hippocampus (Chen et al., 2010; Mitra et al., 2011); LB like structures in the frontal cortex as well as in different regions of the

hippocampus including the dentate gyrus (Mitra et al., 2011), CA3 (Mitra et al., 2011), and CA1 (Mitra et al., 2011); decreased TH expression in frontal cortex (Mitra et al., 2011), locus coeruleus (Fernagut et al., 2007) and hippocampus (Mitra et al., 2011) ; increased hippocampal α-synuclein expression (Mitra et al., 2011); evidence of apoptosis or necrosis via the formation of pyknotic nuclei in the frontal cortex (Mitra et al., 2011), as well as in the dentate gyrus (Mitra et al., 2011), CA1 (Chen et al., 2010), and CA3 of the hippocampus (Mitra et al., 2011); evidence of the neuroinflammatory marker TNF-α in PFC, and hippocampus (Mangano et al., 2011; Mitra et al., 2011); reductions of NE in hypothalamus and cortex (Chanyachukul et al., 2004). In addition, data from our lab suggest that paraquat induces differential sex expression of the neurotrophic factor BDNF in the hippocampus, such that male mice show a decrease in the trophic factor when exposed to paraquat (Litteljohn et al., 2011; Mangano et al., 2011). Furthermore, paraquat exposure has also been shown to produce other behavioral deficits concomitant with PD-like motor impairment, including olfactory dysfunction (Czerniczyniec et al, 2011), anxiety like behavior (Czerniczyniec et al., 2011; Litteljohn et al., 2009), and memory impairment (Chen et al., 2010). Nonetheless, it has yet to be determined whether paraquat can induce depressive like behaviors in rodents.

Yet, while the majority of studies have demonstrated that paraquat can induce PD relevant brain changes, and to a lesser extent, behavioral changes, some groups have failed to find such effects (Bové & Perier, 2011). This puts into question the use of paraquat as an experimental model. It is likely though that a number of factors are involved that result in these discrepant findings. For example, strain of the rodent, age of the strain, the dosing regimen that was used coupled with chronic versus acute exposure,

time of sacrifice, compensatory mechanisms, and stereological methods all appear to be factors that may come into play (Litteljohn et al., 2010b).

Although discrepancy and inconsistency has been demonstrated, one cannot ignore the fact that paraquat has produced PD-like lesions and behavioral characteristics concomitant with the disease, nor the valuable insight it has provided into the possible neurobiological mechanisms involved in PD. Indeed, we readily admit that paraquat does not lead to PD on its own, as is evidenced by the fact that there are areas where paraquat is currently being used and no development of clinical PD characteristics has been observed (Bové & Perier, 2011). It is probable though that this pesticide works in combination with a number of other factors (e.g. other pesticides, genetic influences, aging) and that this exposure coupled with these other factors mediates or potentiates PD-like pathology (Cory-Slechta et al., 2005).

The older an individual is, the more likely various mechanisms have gone awry as a result of the aging process, thus making an individual more vulnerable to toxic effects such as those induced by paraquat exposure. Indeed, McCormack et al. (2002) demonstrated an age dependent effect of paraquat exposure such that the extent of toxin-induced SNc cell death increased as the age of the animal increased (i.e. 6 months, 5 months, 18 months). In addition, Peng et al. (2007) demonstrated that age not only induced paraquat SNc cell loss at 24 months, but that this loss was also exacerbated when aged mice were pre-exposed to iron. This was a similar finding to Thiruchelvam et al. (2003), who found mice 18 months of age exposed to paraquat and maneb were not only susceptible to paraquat exposure displaying a progressive locomotor activity reduction at various time points, but this susceptibility resulted in a progressed decrease in SNc DA

neurons and a marked decrease in TH within the striatum as shown 2 weeks and 3 months following the last treatment. Furthermore, this progression in nigral cell loss coupled with decreased TH activity in the striatum was not shown in mice exposed to paraquat at 5 months or 6 weeks of age (although a decrease in DA nigral cell count and locomotor activity was observed). Studies have also demonstrated that paraquat administration increases NADPH oxidase and microglia activity to a greater degree in older (12 month) versus younger (2 months) mice (Purisai et al., 2007; Peng et al., 2009). This evidence clearly suggests that age of exposure markedly influences the effects of paraquat exposure. However the relationship between aged animals and paraquat exposure on the progression of co-morbid behaviors has yet to be investigated.

The Current Study

PD models are generally geared towards assessment of motor deficits observed as a result of injury to the nigrostriatal system and generally ignore important non-motor behaviors associated with the disease, putting the validity of the model into question (Drechsel and Patel, 2008). In addition, toxin based models normally use very high doses that induce neural damage quite rapidly; which of course, contrasts the actual clinical scenario, wherein sporadic PD emerges very slowly and likely results from long term exposure to a combination of risk factors (Drechsel & Patel, 2008). Furthermore, the validity of these models is further compromised as the use of younger animals is common, ignoring aging as a factor (Schapira & Jenner, 2011). Hence, we were interested in assessing the non-motor symptoms provoked by long-term exposure to a relatively low dose of paraquat and whether such effects vary as a function of age in

order to identify whether paraquat can be used as a pre-clinical model, and to add to research looking to address the validity of this toxin to be used in PD research. We hypothesized that because the aging process makes one more susceptible to toxic insult, chronic low dose paraquat exposure would induce cognitive, depressive and anxiety-like behavioural disturbances in an age-dependent manner. Specifically we hypothesized that the effects of low doses of this oxidative stressor would increase with age, such that mice chronically exposed to paraquat at a later age will develop more pronounced depressive-like, olfactory, cognitive and anxiety like symptoms. In addition, we sought to determine whether this chronic low dose paraquat regimen influenced plasma corticosterone (CORT), glucocorticoid receptor (GR) expression in the hippocampus, as well as protein BDNF levels in an age-dependent manner in order to help clarify possible mechanisms underlying some of the behavioral changes.

Methods

Animals

Forty-two male C57/BL6J mice (Charles River, Laprarie, Quebec, Canada) aged 5 (middle aged) and 13 (advanced aged) months, were single housed in standard (27cm X 21cm X 14cm^3) fully transparent polypropylene cages. Mice were maintained on a 12 hour light/dark cycle in a temperature controlled (21 degrees) environment with ad libitum food and water. All mice were acclimated to the vivarium prior to commencement of the experiment. Test protocols were approved by the Carleton University Committee for Animal Care and in very strict accordance to the guidelines outlined by the Canadian Council for the Use and Care of Animals in Research.

Treatment administration and general procedures

Animals received intraperitoneal (i.p) treatment of paraquat (1,1'-dimethyl-4,4'-bipyridinium dichloride; Sigma Aldrich, 1mg/kg) or an equivalent volume of physiological saline once per week for 16 consecutive weeks (n = 10-12/group). This dose was based on previous studies demonstrating no peripheral toxic effects and is believed to be below the concentration required to produce degeneration of DA neurons substantial enough to display motor disturbances (McCormack et al., 2002; Yin et al., 2011). Indeed, our primary goal was to evaluate the consequences of chronic low dose oxidative stress exposure in the context of advancing age and assess whether this gives rise to co-morbid PD symptoms and not to model the neurodegenerative effects seen in PD models.

The experimental behavioral procedures are outlined in Figure 1. In order to assess whether or not paraquat exposure influenced behavioral changes in "middle" vs. "advanced" aged mice, a variety of tests were performed throughout the paradigm assessing anxiety, cognitive, and depressive like behavior. All behaviors were performed between the hours of 8:00am and 4:00pm.

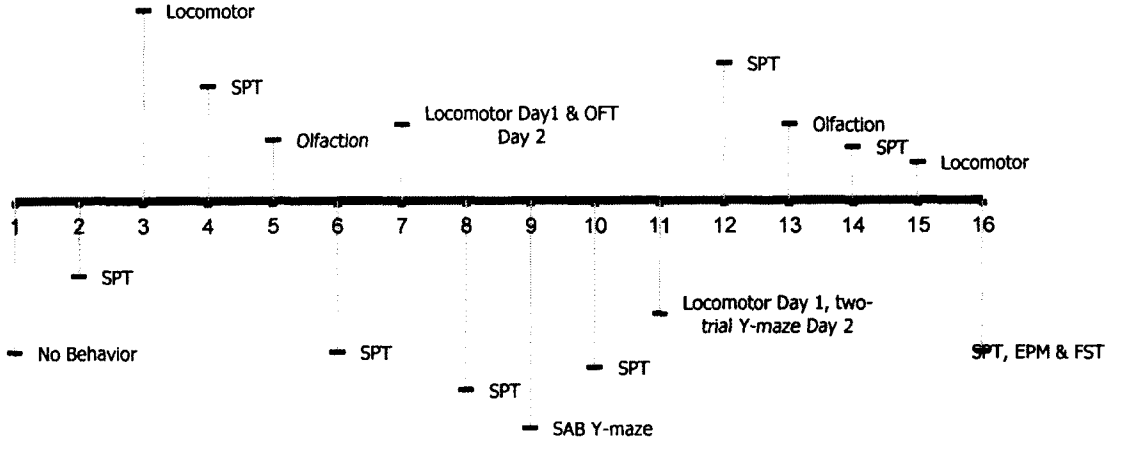

Fig. 1. *Behavioral Treatment schedule.* Weekly behavioral treatment schedule for "middle" and "advanced" aged mice chronically exposed to paraquat or saline. SPT: sucrose preference test; OFT: open field test; EPM: elevated plus maze; SAB: spontaneous alternation behavior; FST: forced swim test.

Behavioral tests

Home-cage locomotor activity. Spontaneous locomotor activity was measured over a complete 12 hour light/dark cycle using a Micromax (MMX) infrared beam-break apparatus (Accuscan Instruments, Columbus, OH, USA), as previously described (Litteljohn et al., 2008). The infrared beam-break apparatus is located external to the home-cage and spontaneous locomotor activity is measured via photobeam breaks. Briefly, following a 30 minute acclimation period in a behavioral testing room, measurements of 24 hour spontaneous home-cage locomotor activity occurred one hour after paraquat or saline exposure one time per month during the 16 week paradigm.

Open field test. In order to measure anxiety-like behavior the open field test was used, as previously described by Litteljohn et al. (2010). Briefly, a 40 cm^3 open white Plexiglas arena was used, and divided into pre-defined zones (outer, middle, and inner/center). Each animal was randomly placed in one of four corners and their exploratory behavior was recorded for a period of 15 minutes. The amount of time spent and frequency of entries into each zone was recorded using an automated videotracking system (EthoVision, Noldus, The Netherlands). Decreased time spent and entries into the center zone are indices of anxiety-like behavior. The open field test was conducted 24hr after injection on week 7 immediately after MMX locomotor testing. The arena was cleaned with 10% EtOH between trials.

Elevated plus maze. In order to further measure anxiety-like behavior the elevated plus maze was used, as previously described (Jacobson-Pick et al., 2011). Briefly, on the 16th week one day after the sucrose preference test, animals were individually placed in one of two closed arms in the four arm maze. Each arm was 24.8 cm long X 7cm wide with closed arms enclosed by 21 cm high walls elevated approximately 60cm off the floor. Behaviour was recorded on our camera for a period of 6 minutes and scored by an independent observer blind to the experiment. Recorded behaviours included the total number of entries into the closed or open arms (defined by all four paws being placed in the arm), time spent in closed or open arms, and number of head dips. Percent number of open-arm arm entries is believed to be a measure of anxiety-like behavior. All arms were cleaned with 10% EtOH between trials.

Olfactory discrimination. In order to determine whether low dose chronic paraquat exposure affects olfactory function in an age dependent manner, a modified version of the Tillerson et al. (2006) method was used. Briefly, animal bedding (approximately 1 g) was taken from an animal's own home-cage or from the cage of another male C57/BL6 mouse who was behaviorally naïve to the experiment. Bedding was individually placed in 50ml conical tubes that contained one 1.8 cm^3 block for a period of 12 hours. Animals were then presented with the block containing their own scent and the scent of another mouse in their home-cages for a period of 2 minutes. Blocks were randomly placed on either the left or right side approximately 5 cm from the wall. The time spent sniffing own versus novel scent was recorded for comparison. An animal was deemed to be sniffing his own or a novel scent when he was =/< 1cm away from the block and when the animal was in direct contact. Olfactory testing occurred on

weeks 5 and 13. In order to ensure that the animals own smell had no effects on this test, cages were changed 12hr prior to testing.

Spontaneous alternation behaviour (SAB) Y-maze. In order to test for working memory, a modified version of the Y-maze was used, as described by Wall and Messier (2001). Briefly, animals were individually placed in one of three enclosed arms at random for a period of 8 min. Arms were 40cm long X 3cm wide enclosed by 13cm high walls. All arms converged on an equilateral triangle in the centre. Same arm returns (SAR), alternate arm returns (AAR), and spontaneous alternation behavior (SAB) performance was recorded when each animal had placed all 4 paws in the arm runway. SAR is defined as an animal leaving an arm and then returning to the same arm, without 4 paw entry into another arm. AAR is defined as an animal returning to a previous arm after entry into another arm (for example arm C to B and then back to arm C). SAB is defined when an animal had sequentially entered all four paws into each arm without returning to the previous arm (for example arm C to B followed by arm B to A); it is believed that SAB is a measure of active spatial working memory. All scores were expressed as percentages in order to not bias any results which may be affected by number of arm entries according to the following equations: %SAB = total number of sequential alternations / total arm entries X 100, %AAR = alternate arm returns / total arm entries X 100, and %SAR = total number of same arm returns / total number of arm entries X 100. This version of the Y-maze was performed on week 15 immediately after MMX locomotor testing. All arms were cleaned with 10% EtOH between trials. All trials were recorded on our camera and later scored by an observer who was naïve to the experiment.

Two-trial Y-maze. In order to test working and spatial memory performance, the discreet two-trial Y-maze was used (Dellu et al., 2000). During this task animals were first individually placed into one of two arms in the Y-maze for a period of 5 minutes, with the third arm blocked by a divider at random. Following a 30 minute interval, the divider was removed and animals were allowed to freely explore all three arms of the maze for another 5 minutes. In order to analyze the task, the time spent exploring the novel versus familiar arms was calculated using the ratio [time spent in the novel arm/(time spent in the novel + adjacent arms)]×100. Time spent in novel arm is believed to be a measure of spatial memory and retention and was expressed in percentages in order to not bias any locomotor results (calculated as the number of arm entries during the acquisition trial). This task was held in our behavior room on week 11 of the behavioral paradigm immediately after MMX locomotor measurements were taken. All arms were cleaned with 10% EtOH between trials.

Sucrose preference test. In order to assess whether or not low dose chronic paraquat exposure is associated with depressive-like behaviours, a sucrose preference test was administered as a measure of anhedonia (Willner et al., 1987). Every other week, all mice were simultaneously exposed to two 200ml bottles containing 1% sucrose solution or tap water randomly placed approximately 1cm apart from each other one hour after paraquat or saline injection. Bottle locations were switched daily in order to eliminate animals that may have displayed any place preference. All mice received 5 days of baseline testing in which they were given 2 days of 2% sucrose solution followed by 3 days of a 1% sucrose solution. All solutions were made fresh each day and bottle weight measurements were taken before and 24hr after testing on day 2 in order to determine

sucrose preference. Preference for the sucrose solution was calculated according to the following formula: sucrose intake / (sucrose intake + water intake)*100.

Forced swim test. In order to assess depressive-like effects that may be induced by chronic paraquat exposure, a modified version of the Porsolt et al. (1977) forced swim test was used one day following the elevated plus maze. Here, mice were individually placed in a glass cylinder (20 cm diameter X 25 cm high) that contained a depth of approximately 20cm in temperature controlled water (22 ± 1 °C) for a period of 6 minutes. Time immobile (i.e. floating while making only necessary movements that require the animal to keep their head above the water) was recorded on our camera and scored by an independent observer blind to conditions of the experiment. Time immobile is believed to be a measure of behavioral despair in rodents. Immediately following the task, animals were dried off and placed in their home-cage and quickly transferred to necropsy where rapid decapitation was performed.

Brain dissection and plasma CORT collection

All mice were sacrificed five days following the last paraquat or saline injection. Five minutes after the final behavioural task (between 9:00am and 11:00am), mice were rapidly decapitated and brains and trunk blood were collected in order to evaluate plasma CORT and hippocampal protein expression (i.e. phosphorylated glucocorticoid receptors (GR), and BDNF) via radioimmunoassay and Western blot analyses respectively. Brains were excised and the hippocampal region was micro-punched from coronal brain sections and obtained using a chilled microdissecting block that contained slots (0.5mm apart) for single edged razor blades. The hippocampus was removed using standard microdissection needles. All brain punches were taken with reference to the mouse brain atlas of Franklin

& Paxinos (1997). Tissue was immediately frozen and stored at -80°C until processing. Trunk blood was centrifuged at 3,600 rpm (8 min.) in order to collect plasma and was stored at -80°C until CORT determination.

CORT determination

In order to determine concentration levels of CORT in plasma, a commercial radioimmunoassay kit (ICN Biomedicals Inc., USA) was used. Assays were performed in a single run to prevent inter-assay variability; intra-assay variability was <10%.

Western blots

Tissue punches were collected from the hippocampus to detect levels of BDNF and GR according to the methods as previously described by Litteljohn et al. (2011). Primary antibodies for BDNF (1:300, Abcam), and GR (1:500, Santa Cruz) were used and β-actin (1:200, Sigma) was applied as a loading control. Tissue punches were diluted in an extraction buffer (5.0 M NaCl, 0.5 M EDTA, 100 mM EGTA, 1, M DTT, 50% glycerol, 0.1 M PMSF, 10 mg/ml leupeptin, 5 ug/ml aproti-nin,1 M -glycerophosphate, 0.5 M NaF, and 100 mM Na-orthovanadate), sonicated, centrifuged at 14,000 rpm (15 min), and supernatant assessed for protein concentration using a BioRad protein assay. Hippocampal tissue was homogenized in ice-cold Radio Immuno Precipitation Assay (RIPA) buffer [50 mM Tris (pH 8.0), 150 mM sodium chloride, 0.1% sodium dodecyl sulphate (SDS), 0.5% sodium deoxycholate and 1% Triton X-100] mixed with 1 tablet of Complete Mini EDTA-free protease inhibitor (Roche Diagnostics, Laval, QC,) and then sonicated for 5 minutes in ice cold water. The lysate was then centrifuged at 5000x for 5 minutes at 4˚C. The protein concentration was determined using bicinchoninic acid (BCA) method (Thermo Scientific, CITY) and supernatants stored at -80˚C.

Ten μg of total protein was suspended in Laemmli sample buffer containing 5% β-mercaptoethanol (Bio-Rad, Hercules CA). The samples were heated in boiling water for 5 minutes and briefly centrifuged at room temperature at low speed. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using a 12.5% separating gel. The gel was run at 140 V for one hour in running buffer (25mM Tris base, 190 mM glycine, 3.5 mM SDS). The protein was then transferred overnight at 4°C and 180 mA in transfer buffer (25 mM Tris-base, 192 mM Glycine, 20% methanol), onto PVDF (Bio-Rad, Hercules, CA). The separation and transfer of protein was carried out using a Bio-Rad (Hercules, CA) mini-gel and transfer apparatus.

The membranes were blocked for 1 hour with gentle shaking in a solution of non-fat dry milk (5% w/v) dissolved in TBS-T buffer (10 mM Tris-base (pH 8.0), 150 mM sodium chloride, 0.5% Tween-20). The membranes were then incubated with a rabbit anti-BDNF, and anti-phospho-GR primary antibody (1:1000) diluted in blocking solution at room temperature for 1 hour. Any unbound antibody was removed using three 10 minute washes of 15 mL TBS-T at room temperature. Membranes were incubated on a shaker for 1 hour at room temperature with HRP (horseradish peroxidase) conjugated anti-rabbit (1:5000) secondary antibody and washed again with TBS-T. BDNF and phosphor-GR were then visualized using Western Lightning Plus chemiluminescent substrate (Perkin Elmer, Waltham, MA, cat#.NEL102001EA) and by exposure to Kodak X-OMAT film. The molecular weights of proteins were estimated using Precision Plus Protein Kaleidoscope Standards (Bio-Rad, Hercules, CA). Protein bands were quantified by densitometry using AlphaEase software (AlphaEase FC V.3.1.3., Alpha Innotech Co.,

San Leandro, CA) and normalized to actin. All chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

Statistical analysis

All data was analyzed using a 2(paraquat vs. saline treatment) X 2("middle" vs. "advanced" aged) between subjects Analysis of variance (ANOVA) design. Where there was an interaction effect, two-way ANOVA's were then followed by Fisher's planned comparisons ($p < 0.05$). Data is presented in the form of mean±standard error mean (mean±SEM). All data was analyzed using the statistical software StatView (version 6.0) or SPSS (version 19.0) and differences were considered statistically significant when $p < 0.05$.

RESULTS

Behavioral analysis

Spontaneous home-cage locomotor activity. Age related differences in home-cage locomotor activity were evident at week 3 as "advanced" aged mice (26964.75 ± 1561.38) displayed lower spontaneous home-cage locomotor activity levels ($F(1,36) = 11.87, p < .05$) when compared to "middle" aged mice (33042.10 ± 771.90) (Fig. 2A). A main effect of age was also evident at week 7 ($F(1,36) = 9.05, p < .01$) such that "advanced" aged mice (29186.45 ± 1623.92) continued to display lower total activity levels in comparison to their counterparts (35637.35 ± 1354.77) (Fig. 2B). However, exposure to paraquat treatment did not influence home-cage locomotor activity at any time point, as is evident by the lack of paraquat main effect(s) and interaction(s) with age. This finding suggests that or injection regimen was indeed below that required to cause nigrostriatal damage significant enough to affect motor behavior.

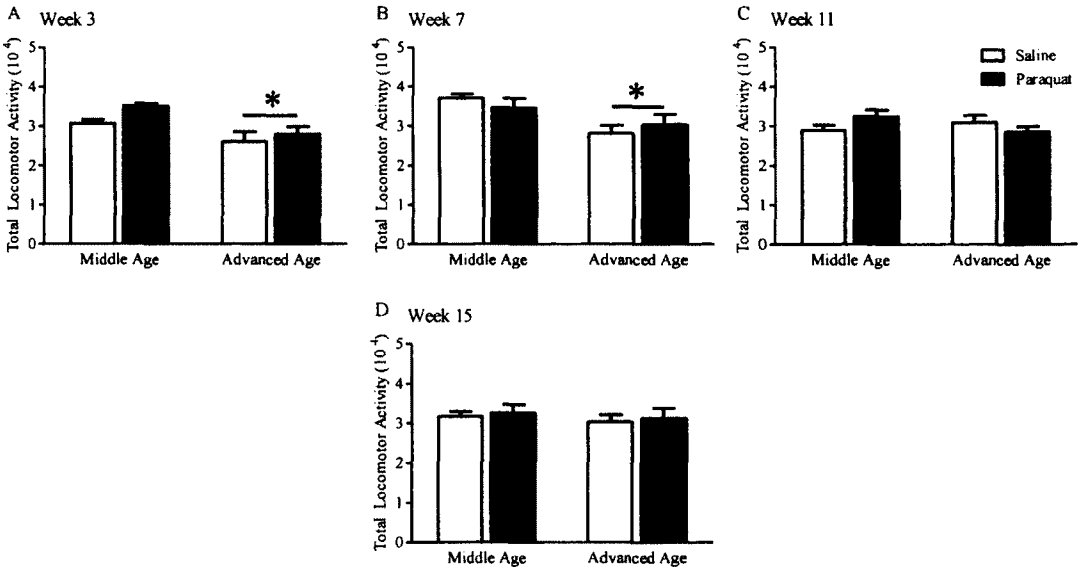

Fig. 2. *Spontaneous home-cage locomotor activity.* Irrespective of paraquat treatment, "advanced" aged mice had reduced total home-cage locomotor activity (expressed as total number of infared beam breaks (1.0 X 10^4) expressed over a complete 12hr light dark cycle) in comparison to "middle" aged mice at weeks 3(A) and 7(B). This effect was absent by weeks 11(C) and 15(D). All data is expressed as mean ± SEM. $*p < 0.05$ relative to "middle" aged mice (collapsed over paraquat treatment).

Anxiety-like behaviour in the open field test and elevated plus maze. In an open field arena, the "advanced" age animals made significantly fewer visits than "middle" aged animals to the centre zone ($F(1,37) = 27.86$, $p < .001$) (24.25 ± 2.74 vs. 42.62 ± 2.34), middle zone ($F(1, 37) = 31.36$, $p < .001$) (71.80 ± 6.97 vs. 119.24 ± 5.30), and outer zone ($F(1,37) = 5.23$, $p < .05$) (154.10 ± 9.91 vs. 179.24 ± 5.67) (Fig. 3A). Similarly, the older mice spent less time in both the middle zone ($F(1,37) = 4.43$, $p < .05$) (96.32 ± 12.06) and the center zone ($F(1,37) = 4.41$, $p < .05$) (48.10 ± 10.40) in comparison to younger animals (125.79 ± 7.50; 73.72 ± 6.64) (Fig 3B). Once again however, paraquat treatment had no effect on any of the open field parameters assessed (Table 1).

In order to assess whether mice displayed anxiety-like behavior after a cumulative 16 low-dose paraquat treatments, elevated plus maze activity was determined. In this regard, neither age nor paraquat treatment had any influence on the number of entries into the open or closed arms of the elevated plus maze (Table 1).

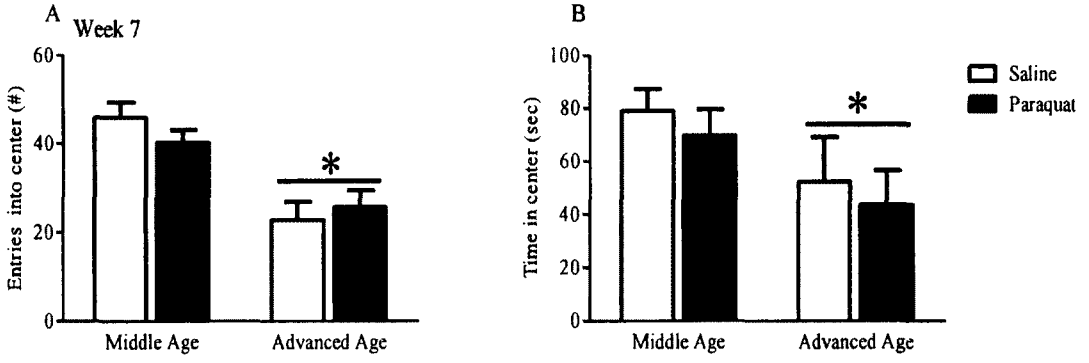

Fig. 3. *Anxiety-like behavior in the open field test.* Effect of paraquat treatment and age on anxiety-like behavior in the open field test. "Advanced" aged mice made fewer entries (A) and spent less time (B) in the center zone than "middle" aged mice irrespective of paraquat treatment. All data is expressed as mean ± SEM. $*p < 0.05$ relative to saline treated mice (collapsed over age).

	Middle Age		Advanced Age	
Elevated Plus	Saline	Paraquat	Saline	Paraquat
Closed arm entries	12.70 ± 1.48	11.67 ± 0.79	11.00 ± 1.62	10.86 ± 1.55
%Open arm entries	15.72 ± 4.13	10.65 ± 2.87	14.40 ± 3.64	12.16 ± 5.78
Open Field				
Visits to center zone	45.89 ± 3.42	40.17 ± 2.87	22.80 ± 4.15	25.70 ± 3.74
Visits to middle zone	127.89 ± 8.25	112.75 ± 6.31	67.10 ± 10.62	76.50 ± 9.33
Visits to outer zone	187.00 ± 8.60	173.42 ± 7.38	149.80 ± 18.76	158.40 ± 7.05
Time in center zone	78.98 ± 8.38	69.79 ± 9.92	52.49 ± 16.82	43.70 ± 13.00
Time in middle zone	132.75 ± 10.08	120.57 ± 10.84	97.23 ± 21.57	95.42 ± 12.18
Time in outer zone	491.54 ± 15.00	470.63 ± 19.73	463.71 ± 63.03	465.99 ± 38.31

TABLE 1. *Anxiety-like behavior in elevated plus maze and open field test.* Mean (±SEM) number of arm entries into closed arms, and percent arm entries into open arms in the elevated plus maze test, and number of visits and time spent in each zone in the open field test for "middle" or "advanced" aged mice exposed to a chronic dose of paraquat or saline.

Olfactory discrimination. In the olfactory test, we were interested in seeing whether age or paraquat exposure altered the ability of a mouse to discriminate between their own scent and the scent from another animal. In regards to time spent investigating the novel odor, no age, paraquat treatment, or age X paraquat treatment differences were seen at either of the time points (Table 2).

	Middle Age		Advanced Age	
Week 5	Saline	Paraquat	Saline	Paraquat
Own Scent	46.73 ± 1.56	42.21 ± 2.96	39.78 ± 2.48	42.67 ± 5.39
Novel Scent	37.92 ± 2.23	37.60 ± 2.31	42.27 ± 2.83	39.19 ± 3.08
Week 13				
Own Scent	39.38 ± 1.82	41.37 ± 2.04	38.73 ± 3.92	45.28 ± 2.71
Novel Scent	41.48 ± 2.20	41.64 ± 2.30	42.22 ± 3.81	38.28 ± 2.56

TABLE 2. *Olfactory test data.* Mean (±SEM) percent time spent exploring own scent versus novel scent for "middle" or "advanced" aged mice exposed to a chronic low-dose of paraquat or saline treatment.

Working memory in the SAB Y-maze. In order to assess whether spatial working memory is altered as a function of age or paraquat treatment, percent spontaneous alternation performance was analyzed over an 8-min period in which animals had free exploration to all arms of a Y-maze. A significant main effect was evident for paraquat treatment upon percent spontaneous alternations ($F(1,32) = 5.29$, $p < 0.05$). Although no significant age or age X paraquat treatment interaction was observed, it was clear that an age-dependent reduction in the number of spontaneous alterations was most apparent in the saline treated mice (Fig. 4). From another perspective, as shown in Fig 4, paraquat appeared to paradoxically enhance spontaneous alterations in the aged animals. In all cases, except the saline treated older mice, performance was statistically above chance (50%).

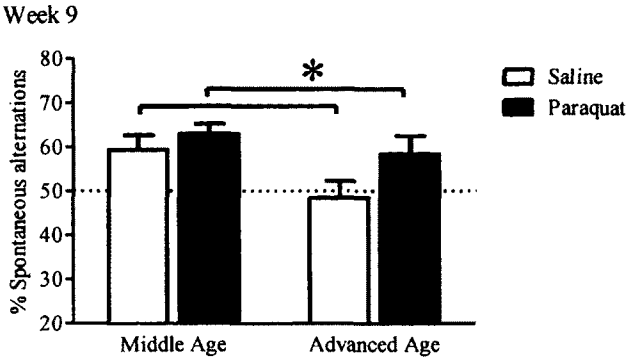

Fig. 4. *Spatial memory in the spontaneous alternation behavior Y-maze test.* Effect of age and paraquat treatment on spatial working memory in the SAB Y-maze test. Each mouse was allowed to explore the maze for 8 minutes. Irrespective of age, paraquat treatment increased spontaneous alternations. Dotted line indicates chance performance. All data is expressed as mean ± SEM. *$p < 0.05$ relative to saline treated mice (collapsed over age).

Spatial memory in the two-trial Y-maze. The two-trial Y-maze was used to test whether paraquat treatment or age altered spatial memory recall. After the 30 minute inter-trial interval, while age or paraquat treatment did not alter frequency of visits to the novel arm, mice treated with paraquat (33.38 ± 2.74) spent significantly less percent time ($F(1,33) = 4.42, p < 0.05$) exploring the novel arm during the 5 minute retrieval trial than those treated with saline (42.27 ± 2.98) (Fig. 5A). Follow up independent sample *t*-test analyses revealed that while "middle" or "advanced" aged mice exposed to paraquat did not perform better than chance (33.33%), "middle" and "advanced" aged saline exposed animals performed greater than chance level ($p < 0.05$). No age or age X paraquat treatment interaction was observed (Table 3). Furthermore, no significant differences were observed with regards to percent duration of time spent in familiar arms (Fig. 5B and 5C). In order to account for locomotor activity, total number of visits to the open arms across the 5-min acquisition trial shows that "advanced" aged mice (12.69 ± 1.01)

displayed significantly less ($F(1,33) = 4.75, p < .05$) locomotor activity than "middle" aged mice (15.86 ± 0.97). No paraquat treatment or age X paraquat treatment interaction was observed (table 3).

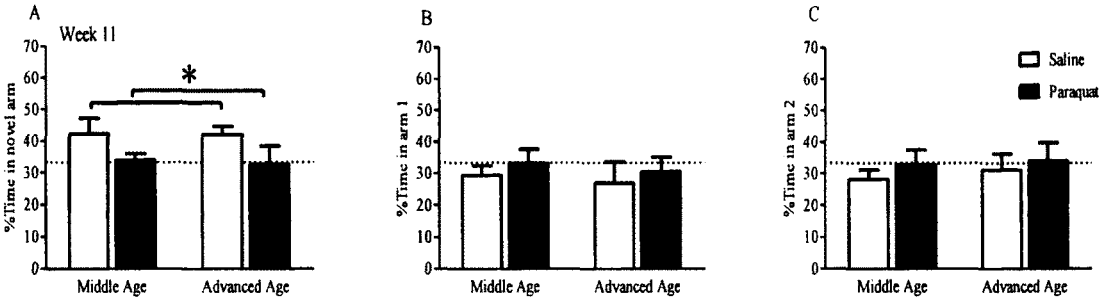

Fig. 5. *Spatial memory in the two-trial Y-maze.* Effect of paraquat treatment and age on mouse spatial memory performance in the two-trial Y-maze. While saline treated mice spent more time in the novel arm, mice treated with paraquat were no better than chance, irrespective of age (A). No significant difference in time spent exploring familiar arms was seen for any of the groups (B and C). All data is expressed as mean ± SEM. *$p <$ 0.05 relative to saline treated mice (collapsed over age).

	Middle Age		Advanced Age	
	Saline	Paraquat	Saline	Paraquat
Spontaneous Alternation				
Total Arm entries	28.80 ± 2.64	29.75 ± 1.40	23.89 ± 3.51	24.00 ± 3.56
%SAB	59.39 ± 3.31	63.04 ± 2.26	48.40 ± 3.88	58.49 ± 4.02
Discreet Trial				
Total arm visits trial 1	16.80 ± 1.41	15.00 ± 1.33	13.43 ± 1.70	12.11 ± 1.26
Visits to novel arm	33.48 ± 2.89	33.67 ± 1.72	38.09 ± 2.25	31.52 ± 3.77
%Time in novel arm	42.40 ± 4.94	33.97 ± 2.07	42.09 ± 2.71	32.67 ± 5.75
%Time in arm 1	29.40 ± 3.03	33.24 ± 4.47	26.86 ± 6.83	30.41 ± 4.71
%Time in arm 2	28.20 ± 2.30	32.79 ± 4.67	31.05 ± 5.21	33.93 ± 5.91

TABLE 3. *Spatial memory behavior data.* Spontaneous Alternation Behavior (SAB) Y-maze and Two-trial Y-maze tasks as measures of working and spatial memory performance. All data is expressed as mean ± SEM.

Anhedonia in the sucrose preference test. In order to test whether age or paraquat treatment induced depressive-like deficits, the sucrose preference test was administered once every other week. All animals showed a sucrose preference of at least 70% by the end of the baseline period. While one may argue that a relatively long interval elapsed between sucrose preference testing, preference was maintained across all groups two weeks after baseline measurements, suggesting that the solution was palatable enough to maintain sucrose preference. Results of the two-way ANOVA analyses at each time-point indicate that age or paraquat treatment alone did not induce a depressive-like deficit in motivate responding (Table 4). As shown in Figure 6 and confirmed by the

follow-up analyses, chronic paraquat exposure transiently augmented sucrose preference scores in "advanced" aged mice at weeks 10 (F (1,36) = 5.51, $p < .05$) and 16 (F (1, 33) = 4.81, $p < .05$). Specifically, at week 10, "advanced" aged mice exposed to paraquat had greater sucrose preference (83.56 ± 1.50) than mice exposed to saline at an "advanced" age (70.43 ± 4.53) ($p < .05$) (Fig 6A). At week 16, "advanced" aged mice chronically exposed to paraquat once again had higher sucrose preference (86.65 ± 2.49) than saline exposed mice of the same age (71.32 ± 2.44) ($p < 0.05$) (Fig. 6B).

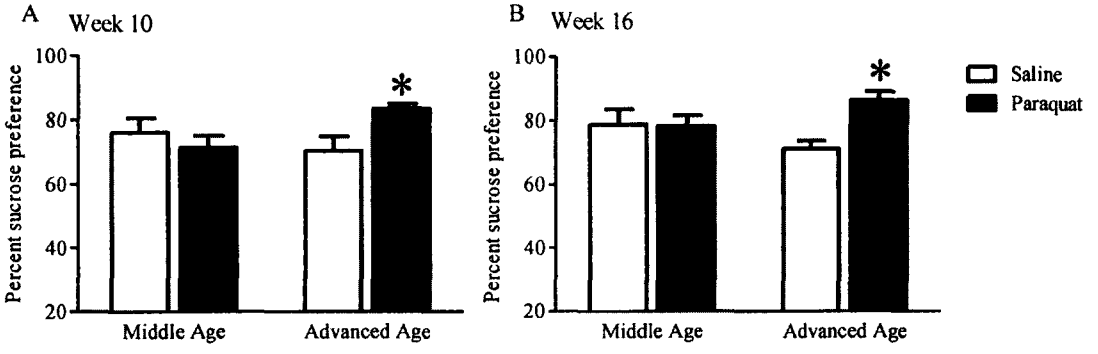

Fig. 6. *Sucrose preference in the sucrose preference test.* Effect of paraquat treatment and age on indices of anhedonia, as determined by a sucrose preference test. Chronic low dose paraquat treatment transiently augmented sucrose preference at week 10 (A), and week 16 (B) in "advanced" aged mice. *$p < 0.05$ relative to saline exposed "advanced" aged mice.

	Middle Age		Advanced Age	
Week	Saline	Paraquat	Saline	Paraquat
Baseline	71.94 ± 4.59	80.24 ± 2.08	70.76 ± 5.13	78.36 ± 6.75
2	81.68 ± 4.04	75.74 ± 2.89	74.01 ± 6.38	84.31 ± 5.90
4	72.31 ± 3.47	74.29 ± 4.56	67.78 ± 5.82	82.14 ± 1.87
6	74.23 ± 2.48	68.49 ± 4.40	75.06 ± 4.71	77.87 ± 6.01
8	81.82 ± 3.73	78.69 ± 3.30	89.00 ± 4.62	87.02 ± 4.32
10	76.07 ± 4.64	71.42 ± 3.74	70.43 ± 4.53	83.56 ± 1.50
12	83.44 ± 4.15	81.92 ± 2.28	75.58 ± 4.75	75.54 ± 7.67
14	80.55 ± 2.93	81.89 ± 2.41	86.52 ± 1.98	84.81 ± 3.34
16	78.84 ± 4.48	78.46 ± 3.53	71.32 ± 2.44	86.65 ± 2.49

TABLE 4. *Sucrose preference test data.* Sucrose preference mean score (±SEM) at baseline and during testing period among "middle" or "advanced" aged rats receiving saline or paraquat treatments.

Behavioral despair in the forced swim test. Behaviour despair in response to an acute stressor was measured in the forced swim test. The two-way ANOVA revealed a significant age X paraquat treatment interaction on duration animals spent immobile ($F(1,32) = 4.129, p = .05$). As shown in figure 7 and further confirmed by Fisher's planned comparisons, "advanced" aged mice given a cumulative low dose of paraquat treatment (83.83 ± 8.49) displayed a significant reduction in time immobile in comparison with their saline exposed "middle" (176.20 ± 10.23) and "advanced" aged (163.00 ± 16.56) counterparts, as well as those mice given cumulative exposure to the toxin during "middle" age (153.08 ± 13.45) ($p < .05$).

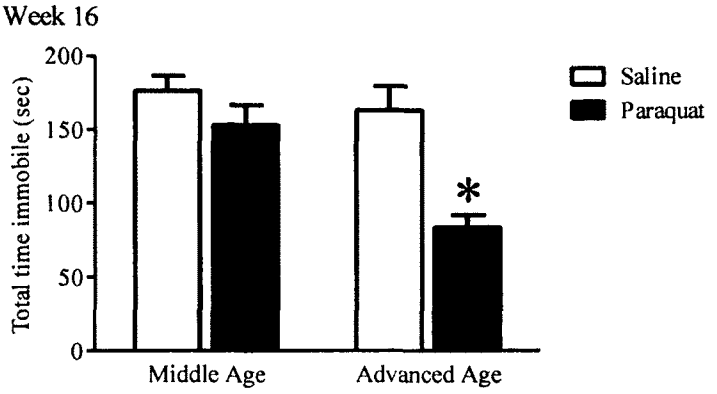

Fig. 7. *Immobility in the forced swim test.* Effect of paraquat treatment and age on immobility time in the forced swim test. Time spent immobile was determined by assessing the time mouse spent floating while making only the necessary movements required to keep their head above the water. "Advanced" age mice exposed to a cumulative low dose of paraquat spent significantly less time immobile than all other groups. $*p < 0.05$ relative to all other groups.

Plasma CORT. As shown in figure 8, a significant age X paraquat treatment interaction was evident with respect to CORT levels ($F(1,37) = 5.69, p < .05$). The follow up comparison indicated that CORT concentrations were significantly elevated in "advanced" aged mice in comparison to "middle" aged mice ($p < 0.05$). Furthermore, in "middle" aged mice, chronic low dose paraquat (36.71 ± 2.66) exposure robustly augmented CORT levels when compared to age matched controls ($p < 0.05$). While "middle aged" mice exposed to paraquat significantly differed from "advanced" age mice exposed to saline ($p < .05$), they do not differ from those mice exposed to paraquat at an "advanced" age.

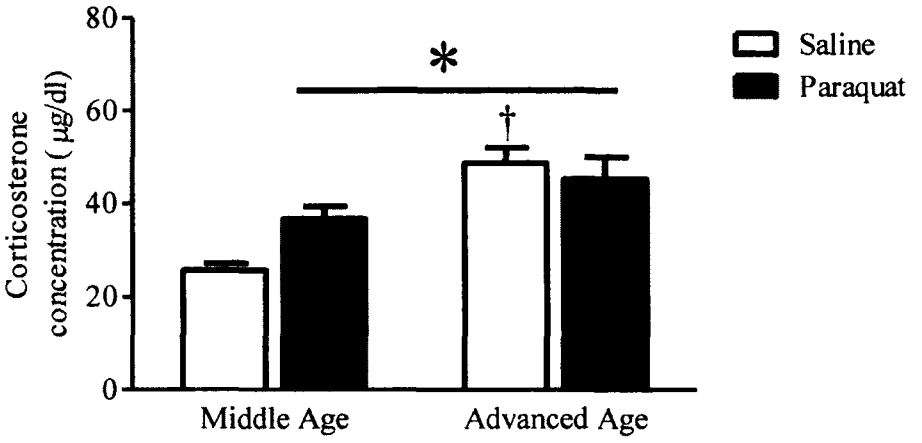

Figure 8. *Plasma corticosterone concentration.* Mean ± SEM plasma corticosterone (CORT) concentrations (µg/dl) in paraquat or saline exposed "middle" or "advanced" aged mice. Trunk blood was collected 5 minutes following the forced swim test. Clearly CORT levels were increased as a function of advanced age and paraquat treatment. However, no synergy was apparent between these variables. *$p < .05$ relative to "middle" aged saline exposed mice. †$p < .05$ relative to paraquat exposed "middle" aged mice.

Hippocampal GR and BDNF expression. Western blot analysis of hippocampal GR protein levels revealed a significant age X paraquat treatment interaction ($F(1,26) = 20.19, p < .01$) (Fig. 9A). Follow-up Fisher's planned comparisons revealed that mice cumulatively exposed to low-dose paraquat treatment during "middle" age had significantly augmented protein expression compared to their "middle" aged saline counterparts ($p < .05$). Saline or paraquat treated "middle" aged mice also had significantly lower GR protein expression than the respective "advanced" aged mice ($p < .05$). In contrast, BDNF levels in the hippocampus were not affected by age or paraquat treatment (Fig 9B).

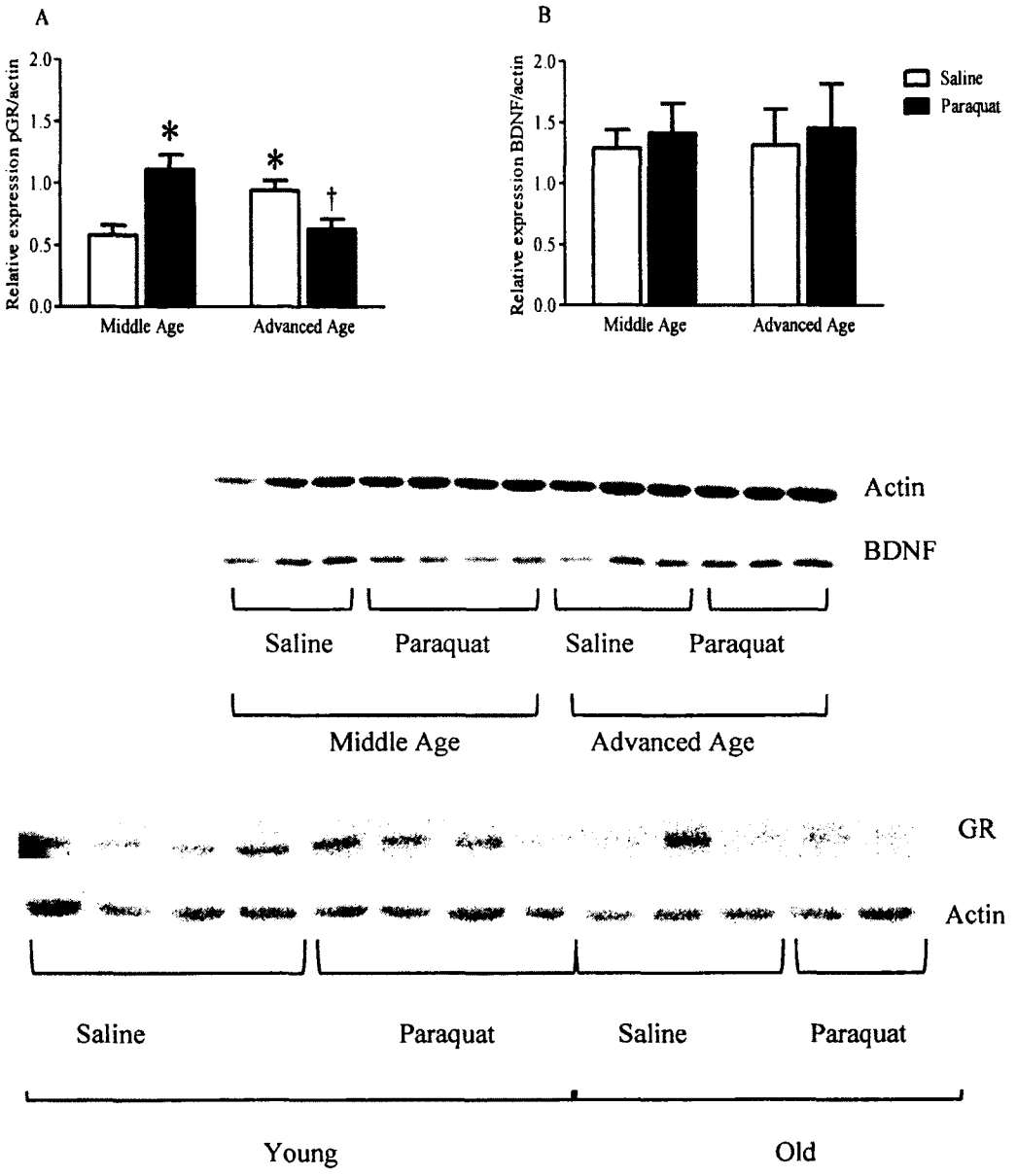

Fig. 9. *BDNF and phosphor-glucocorticoid receptor Western blots.* Hippocampal phosphorylated glucocorticoid receptor (GR) (A) and brain derived neurotrophic factor (BDNF) (B) protein expression in paraquat or saline treated "advanced" or "middle" aged mice following the forced swim test. Representative western blots are shown below the bar graph. *$p < 0.05$ relative to "middle" aged saline exposed mice, †$p < 0.05$ relative to "advanced" aged saline exposed mice and "middle" aged paraquat exposed mice.

Discussion

Background: Overview

Parkinson's disease (PD) is one of the most common and debilitating age-related neurodegenerative disorders, estimated to affect nearly 4 million people worldwide. The disorder is particularly prevalent in the elderly population, with a typical clinical onset after 60–65 years of age (Fahn, 2003). Notwithstanding the rare familial forms of PD that appear to have a strong genetic component, the vast majority of PD cases (upwards of 95%) are idiopathic and sporadic in nature (Olanow, 2007). In this regard, epidemiological evidence has increasingly identified environmental contaminants such as the commonly used pesticide, paraquat, as potential risk factors for PD (Wang et al., 2011). *In vivo* and *in vitro* studies reported that paraquat is transported across the blood brain barrier via a neutral amino acid transporter (Shimizu et al., 2001) and can recapitulate aspects of PD pathology, including damage to the nigrostriatal system that gives rise to motor impairment (Litteljohn et al., 2010b). Furthermore, paraquat can also reproduce many of the underlying pathological mechanisms implicated in PD (e.g. microglial activity, oxidative stress, and pro-inflammatory cytokine expression) (Litteljohn et al., 2010b).

While several animal studies have reported that paraquat only provokes degeneration and neuronal changes specific to the nigrostriatal pathway, more recent studies have demonstrated that exposure may, in fact, cause damage and dysfunction to other brain areas (Chaudhuri et al., 2006; Czerniczyniec et al., 2011). Indeed, mounting evidence suggests that PD is a multi-system disease that is characterized not only by motor disturbances but also other non-motor behavioral deficits (e.g. depression, anxiety,

cognitive deficits, olfactory dysfunction) (Chaudhuri et al., 2006; Czerniczyniec et al., 2011).

Oxidative and Inflammatory Pathways to Neurodegeneration: Influence of Age and Paraquat

It is well known that paraquat induces neurotoxicity and cellular dysfunction through oxidative stress (McCarthy et al., 2004). Indeed, several studies have found that administration of antioxidant enzymes can attenuate oxidative pathology among paraquat-exposed animals (He et al., 2012; McCarthy et al., 2004). Furthermore, pharmacological blockade or genetic ablation of endogenous antioxidant factors (e.g., SOD, catalase) enhanced the destructive effects of the toxin (Drechsel & Patel, 2008; Peng et al., 2005). One way that paraquat induces oxidative stress is through its oxidation-reduction interaction with NADPH oxidase on microglia (Rappold et al., 2011). This redox cycling produces a reduced form of paraquat, which due to its high affinity for the dopamine transporter (DAT) (as opposed to divalent paraquat), is then taken into DA neurons (Rappold et al., 2011). Upon entry into the neuron, paraquat can further participate in redox cycles, enter the mitochondria and inhibit different complexes within the electron transport chain (Bonneh-Barkay et al., 2005; Rappold et al., 2011). In addition, upon interacting with NADPH oxidase on microglial cells, extracellular superoxide (a potent free radical) is generated (Miller et al., 2009; Rappold et al., 2011). While itself possessing cytotoxic properties, superoxide can interact with other oxidative species in the brain microenvironment (e.g., NO) to form even more potent radicals; these moieties, such as peroxynitrate, can induce and propagate cellular damage (e.g. to lipids)

(Hald & Lotharius, 2005). Ultimately, paraquat administration is thought to lead to neuronal death through activation of oxidative and inflammatory-associated apoptotic mechanisms (Choi et al., 2010; Litteljohn et al., 2010b; Peng et al., 2004).

While evidence implicating environmental toxins as potential risk factors for PD is mounting, one clear undeniable risk factor that has been identified is advanced age (Schapira & Jenner, 2011). 'Normal' healthy aging is associated with numerous neurological changes over time, including alterations in neurotrophic and protective factors like BDNF (Chapman et al., 2012), and an increase in microglial activation (Jurgens & Johnson, 2010), oxidative stress (Pan, 2011) and BBB permeability (Blau et al., 2011). Paralleling these neurological changes, subtle cognitive and affective disturbances often also emerge over time (Bishop et al., 2010). Advancement in age has been suggested to prime microglia in the brain (Jurgens & Johnson, 2010), leading to a slow but progressive steady loss of neurons. Reasons for this include an age-dependent loss or decrease of endogenous anti-oxidant and protective mechanisms (Schapira & Jenner, 2011). Interestingly, while aging-related oxidative and inflammatory changes occur throughout the brain, certain neuronal populations appear to be more susceptible than others to age-related damage and decline (Surmeier et al., 2011). Not surprisingly, neurons that are more susceptible to oxidative stress (e.g., DA neurons in the SNc because of DA metabolism) are generally the ones most adversely affected and lost across time (Chung et al., 2011). Nonetheless, other areas including the hippocampus and basal forebrain have also been implicated, perhaps because of (at least in part) the higher metabolic energy costs associated with the relatively large axonal fields in these brain regions (Surmeier et al., 2011).

The BBB is also a structure that deteriorates during the aging process (Popescu et al., 2009). Normally, the BBB plays a critical role in protecting the brain from myriad potentially toxic substances in the peripheral circulation, including environmental toxins such as paraquat (Popescu et al., 2009). As one advances in age, however, the permeability of the BBB increases, which essentially allows for greater infiltration of potentially harmful substances (e.g., xenobiotics, circulating immune cells) (Popescu et al. 2009). Further, aging is associated with a decrease in the ability to clear these toxic substances, making the aged brain more susceptible to toxic insult (Schapira & Jenner, 2011). Indeed, most studies investigating the influence of age on toxin-induced PD-like pathology (e.g., MPTP, 6-OHDA models) have reported that aged animals are more adversely affected than their younger counterparts, displaying enhanced neurodegeneration within the SNc along with signs of heightened oxidative and inflammatory stress (Schapira & Jenner, 2011).

While multiple studies have reported that paraquat can induce motor disturbances and nigrostriatal DA dysfunction, and that such pathology is greater in advanced aged animals (McCormack et al., 2002; Peng et al., 2009; Purisai et al., 2007; Thiruchelvam et al., 2003), comparably little research has assessed the pesticide's potential involvement in the development of co-morbid neuropsychiatric behaviors. Moreover, to our knowledge no studies have yet been published concerning the potential interactional effects of advanced age and paraquat exposure on these non-motor outcomes. Hence, in the present study we assessed the impact of chronic low dose systemic paraquat exposure among "middle" and "advanced" aged mice on a range of motor and non-motor behavioural measures, as well as select physiological indices of hypothalamic-pituitary-adrenal

(HPA) functioning (the primary hormonal arm of stress responses). To this end, we used a paraquat dose that is $1/10^{th}$ of that normally associated with nigrostriatal degeneration and motor disturbances, but which may be relevant for more early-occurring "prodromal" aspects of PD (McCormack et al., 2002; Yin et al., 2011). In this regard, several retrospective studies have indicated that depression and other psychiatric symptoms commonly seen in PD patients might actually occur prior to motor pathology or PD diagnosis (Spiegel et al., 2006). This raises the possibility that the pathological mechanisms operative in PD might initially impact emotional regulatory brain regions long before "spreading" to the nigrostriatal circuit (Hawkes et al., 2010).

Thus, we were interested in whether chronic low dose paraquat exposure in aged mice would give rise to PD-like behavioral deficits (olfactory, cognitive, anxiety, depressive) not principally related to the nigrostriatal DA system. Moreover, we sought to determine whether stressor-like consequences that have been strongly linked to depressive- and- anxiety-like symptoms in rodents, namely alterations of HPA functioning and central BDNF expression, would accompany these prospective behavioural effects. In essence, since it is known that paraquat acts as a potent free radical inducer, we were interested in testing the hypothesis that advanced age and chronic oxidative stress would together augment the behavioral and hormonal effects of either insult alone.

PD and Co-morbid Anxiety/Depression

Neuropsychiatric disturbances (e.g. anxiety and depression) are estimated to occur in 40-50% of PD patients, severely impacting the quality of life for these individuals (Kano et al., 2011; McDonald et al., 2003; Visser et al., 2008). Furthermore, depressed

PD patients were reported to have more severe motor impairment and perform more poorly on cognitive tasks in relation to those PD patients without depression (Stefanova et al., 2006; Tan, 2012). This suggests that the presence of neuropsychiatric symptoms in PD may influence the actual progression of the disorder, and may even exacerbate the underling pathology associated with motor and non-motor behaviors (Stefanova et al., 2006).

Neuropsychiatric symptoms present in PD patients may arise directly from nigrostriatal damage, or alternatively could be the result of disturbances to other DAergic, 5-HTergic, and NAergic systems (Schrag, 2004; Vuckovic et al, 2008); these possibilities are not mutually exclusive. As previously mentioned, several retrospective studies have revealed that co-morbid psychiatric pathology, most notably anxiety and depression, occurred in PD patients prior to the onset of motor symptoms (Spiegel et al., 2006). These findings indicate that depression in PD is not uniformly secondary to the distress associated with disease diagnosis and/or loss of motor function, and suggest that brain regions outside of the nigrostriatal system are likely involved – and targeted early – in PD (Kano et al., 2011).

Accumulating evidence suggests that paraquat, in addition to causing PD-like nigrostriatal damage and motor pathology, can influence neuronal processes in stressor-sensitive brain regions that are important for cognitive and psychological functioning (e.g. PFC, hippocampus, locus coeruleus) (Chen et al., 2010; Chanyachukul et al., 2004; Czerniczyniec et al., 2011; Fernagut et al., 2007; Mitra et al., 2011). Indeed, our group previously demonstrated that paraquat provoked anxiety-like behaviors in mice as measured by time spent and number of entries into the center of the open field

environment (Litteljohn et al., 2008, 2009). This anxiety–like phenotype was associated with altered neurotransmitter activity in the hippocampus, PFC, and locus coeruleus (Litteljohn et al., 2008, 2009). Corroborating these findings, Czerniczyniec et al. (2011) also detected anxiety-like behavior in rats exposed to the pesticide as measured by the number of entries and time spent in the open-arms of the elevated plus maze; and these anxiety-like symptoms correlated with mitochondrial dysfunction and oxidative radical production in the PFC.

In contrast to these previous studies, we presently report that chronic exposure to low dose paraquat did not produce anxiety-like behavior in either the open field test or the elevated plus maze at any of the time points measured. It is likely the case that the dosing regimen used and/or time-points of measurement account for these differences. Indeed, in our previous work, paraquat was administered 3 times per week for 3 weeks at a dose of 10 mg/kg (cumulative dose: 90 mg/kg) (Litteljohn et al., 2008, 2009). Contrastingly, in the present study mice received 16 weekly paraquat injections at a dose of 1 mg/kg (cumulative dose: 16 mg/kg). Thus, although we did not assess brain regional neurotransmitter activity in the present investigation, it is conceivable that the low paraquat dose used did not sufficiently (or at all) disturb limbic neurotransmission and/or other relevant neural processes to a point of inducing an anxiety-like phenotype. Perhaps the rather protracted course of this dosing regimen facilitated the recruitment over time of compensatory and/or recovery mechanisms, which could have masked any subtle paraquat-induced brain neurotransmitter changes that might otherwise become manifest in behaviour. Yet, as will be discussed further in an ensuing section, we did not observe any changes in hippocampal BDNF as a function of paraquat treatment or advanced age.

Thus, any prospective neurocompensatory response to low dose paraquat is probably not dependent on this particular neuroplastic and pro-survival factor (although we of course cannot rule out BDNF changes in other brain regions and/or at earlier experimental time-points).

Previous studies using 6-OHDA to model PD pathology have reported depressive-like effects in the forced swim and sucrose preference tests (Branchi et al., 2008). To date, however, no research assessing the role of paraquat in depressive-like behavior has been reported. In this regard, the present investigation failed to detect a deficit in motivated responding following paraquat treatment, which is similar to what other groups have reported using the DA-targeting toxin, MPTP (Vuckovic et al., 2008). In fact, MPTP-treated mice failed to display depressive-like responses in the sucrose preference test, despite having reduced 5-HT and DA in brainstem, frontal, striatal and limbic structures (Vuckovic et al., 2008). Interestingly though, while the present data indicate that paraquat did not provoke an anhedonic-like deficit among mice of either age group, our data do suggest that advanced-aged mice receiving the chronic low dose paraquat treatment actually displayed a paradoxical enhancement of sucrose preference. Similarly, the advanced-aged but not middle-aged mice exposed to the low-dose paraquat regimen displayed markedly reduced immobility in the forced swim test (relative to age-matched saline-treated controls). Thus, chronic exposure to this low-grade oxidative stressor may differentially modify hedonic neurobehavioural processes, as well as those subserving behavioural despair, among middle and advanced aged mice.

We recently found that a comparatively much higher dose of paraquat (10 mg/kg) did, in fact, provoke alterations of motivated responding, suggesting that the putative

depressive-like effects of the pesticide are highly dose-dependent (Litteljohn, Rudyk and Hayley, unpublished results). Moreover, as depressive-like symptoms induced by this higher dose of paraquat corresponded to brain regional DAergic changes in the nucleus accumbens (Litteljohn, Rudyk et al., unpublished results), it is conceivable that neurotransmitter changes in this and potentially other limbic centers may have contributed to the observed behavioural effect(s) in the present study (but presumably in the opposite direction) .

As research continues to shed light on the effects of paraquat outside of the nigrostriatal pathway, it has become apparent that the pesticide has several brain and behaviour effects in common with traditional HPA-activating stressors (McEwen, 2007). For instance, both paraquat and chronic stressors have been found to provoke anxiety- and depressive-like symptoms in rodents, and to affect brain neurotransmitter (e.g., frontal and hippocampal 5-HT and NE), trophic (e.g., BDNF), and oxidative/inflammatory signalling (e.g., cytokines, microglia, COX-2) (Litteljohn et al., 2008, 2009; Silverman & Sternberg, 2012). An intriguing possibility, then, is that paraquat may too influence (other) important aspects of the stress response, most notably HPA axis functioning. In response to stress, CORT (cortisol in humans and corticosterone in rodents) release is controlled by the HPA axis in order to restore homeostatic functioning (Sapolsky et al., 2000). Upon release from the adrenal cortex, CORT acts on GRs expressed in diverse brain areas, including the PFC and hippocampus, acting to inhibit HPA functioning and subsequent cortisol release in what is known as a negative feedback loop mechanism (Jacobson & Sapolsky, 1991; Sapolsky et al., 2000; Silverman & Sternberg, 2012). However, persistent HPA axis and cortisol

activation is known to decrease GRs within cells in the hippocampus and PFC, which ultimately results in increased levels of this endocrine hormone and the consequent dysregulation of the stress turn-off mechanism (Sapolsky et al., 2000; Silverman & Sternberg, 2012).

The hippocampus has a particularly high level of GRs, and these receptors in conjunction with CORT, are known to influence not only the stress response, but also cognitive impairment and neuropsychiatric behaviors (McEwen, 1999; McEwen & Maarinos, 2001). In the present study, we are the first to report that chronic low dose paraquat influences plasma CORT and hippocampal GR signalling in an age dependent manner, with apparent consequences for behavioural function. Specifically, in "middle" aged mice, paraquat caused an increase in plasma CORT paralleled by augmented levels of GR in the hippocampus, in comparison to their saline exposed counterparts. Conversely, in "advanced" aged mice chronically exposed to the same low-dose of the toxin neither plasma CORT nor hippocampal GR levels were increased relative to age-matched controls (although CORT levels were augmented basally in the old mice compared to the younger ones). In fact, "advanced" aged mice exposed to paraquat actually had reduced levels of hippocampal GRs. These results suggest that paraquat (at least when administered chronically at a low dose) is capable of influencing HPA axis functioning in a manner that is reminiscent of more traditional psychological and social stressors. Moreover, it appears that the pesticide's interaction with elements of the stress response is governed, at least in part, by the aging process itself.

It is well known that increased levels of cortisol act as a protective mechanism against toxic or inflammatory insult (Clark, 2007; Silverman & Sternberg, 2012).

However, prolonged expression of the hormone is related to neurodegeneration as well as a host of other maladaptive disturbances (e.g. coronary; inflammatory and autoimmune disease, post-traumatic stress disorder; PTSD) (Silverman & Sternberg, 2012). Under basal conditions, CORT is believed to inhibit the expression of inflammatory and oxidative mediators such as proinflammatory cytokines and oxidative species through the activation of GRs (Silverman & Sternberg, 2012). For example, activated GRs in the presence of CORT translocate to the nucleus where they mediate the inhibition of the transcriptional factor NF-κB (Ros-Bernal et al., 2011; Silverman & Sternberg, 2012). NF-κB is highly associated with the production of potentially toxic species such as proinflammatory cytokines and oxidative radicals (Roman-Blas & Jimenez, 2006). Indeed, downregulation in GR expression results in an increase in inflammatory and oxidative factors and induces cell death not only in stress related brain regions such as the hippocampus, but also in the SNc (Ros-Bernal et al., 2011).

In the current study, then, it would be intriguing to say that chronic low dose paraquat exposure is altering glucocorticoid and GR signalling in an age dependent manner, such that it may be promoting protective like mechanisms in middle aged and perhaps invoking a pro-inflammatory and oxidative response in advanced age because of the reduced GR expression seen. While we can only speculate as to why the advanced-aged mice receiving paraquat failed to show a stressor-like CORT response (unlike their younger counterparts) and displaying reduced hippocampal GR activation, it is conceivable that an early-occurring *hyper-responsivity* of the HPA axis among old mice (featuring an exaggerated CORT response to paraquat) – which would be expected to drive the downregulation of hippocampal GRs – eventually gave way to *hypo-*

responsiveness of the stress system to low dose paraquat (presumably due to some aging-related defect) (McEwen, 2007). However, it warrants highlighting that the advanced-aged mice displayed markedly elevated basal CORT levels (irrespective of paraquat exposure). While consistent with earlier reports indicating that CORT increases with advancing age (McEwen, 2007), it is difficult to completely exclude that a ceiling in CORT effect occurred in the advanced aged mice (given that the animals has been exposed to forced swimming).

It should be underscored that we cannot speak to the influence that paraquat alone has on corticosteroid and GR signalling throughout the course of the experiment. All biological outcomes were taken 5 minutes after animals were exposed to the forced swim test and thus our interpretations of GC-GR signalling can only be made in the context of the behavioral response seen in the respective test. Accordingly, future research should address whether a low dose paraquat regimen alone affects HPA regulated processes in an age dependent manner and whether or not this may influence a proinflammatory and oxidative environment that gives rise to PD-related behaviors (Ros-Bernal et al., 2011).

In regards to the age-dependent decrease in immobility seen in the forced swim test, alterations of glucocorticoid receptor expression may explain the paradoxical effect observed in paraquat exposed aged animals. Studies have demonstrated that attenuating or blocking GR expression actually increased mobility in rodents regardless of whether they show a depressive-like phenotype (Korte et al., 1996; Ago et al., 2008). This is consistent with our results when comparing paraquat exposed advanced aged mice to their saline exposed counterparts, as the decreased glucocorticoid receptor expression is correlated with the increase in mobility seen. That being said, what one would expect is

an increase in time immobile in paraquat exposed middle aged mice as GR expression was increased in relation to their aged matched controls; however, this effect was not observed. One explanation for this inconsistency could be the interaction between CORT and GRs. In middle aged animals exposed to paraquat, an increase in CORT was seen relative to age matched controls, an effect which was not observed in "advanced" aged animals exposed to the low grade oxidative stressor. In addition, it may be that while ablating this receptor in the hippocampus results in increased mobility in the absence of a depressive-like deficit, increasing its expression may not result in the opposite result when no depressive-like deficit is observed.

Another mechanism known to increase mobility in the forced swim test includes the recruitment of hippocampal BDNF. BDNF is highly implicated as a mechanistic contributor to depression, and is vastly involved in a myriad of cellular processes including plasticity (for a review see Autry & Monteggia, 2012). For example in reference to the hippocampus, this trophic factor is known to decrease in the presence of chronic stress that gives rise to depressive-like behavior in rodents (Grønli et al., 2006). Furthermore studies administering antidepressant drugs have been shown to increase levels of BDNF suggesting that this factor is involved as a mechanistic contributor to depressive-like processes (Numakawa et al., 2011). Evidence has shown that prolonged CORT exposure decreases levels of BDNF in the hippocampus over time which results in an increase in depressive-like deficits in the forced swim test (Gourley et al., 2008). In addition, acute exposure to the trophic factor restores behavioral deficits, such that time spent immobile is decreased (Gourley et al., 2008). When taken together, this highly suggests that BDNF is involved in increased motivational responding, and thus its

expression may correlate with an increase in mobility in the forced swim test (Gourley et al., 2008). It is important to note however that in the current study, it appears that upregulation of this trophic factor was not observed in the hippocampus in any of the animals tested. Indeed recent evidence has shown that BDNF is not always required to increase mobility in the forced swim test as has been demonstrated by *bdnf* knockdown mice (Gourley et al., 2012; Adachi et al., 2008).

It may be then that other proteins and transcription factors promoted plasticity or cellular changes that resulted in this behavioral response. While paraquat is known to influence microglial activity and oxidative factors that ultimately result in damage and dysfunction, evidence has also shown that repeated exposures to low doses of the toxin actually promote the *protection* of cultured cells against exposure to a more potent oxidative stressor (Cysper & Johnson, 2002). In relation to these findings, we suggest that perhaps the low-dose paraquat regimen used in the current study primed the "advanced" aged mice overtime, such that it induced adaptive cellular mechanisms that promoted oxidative stress resistance (Hunt et al., 2011).

A possible cellular mechanism through which paraquat may have promoted this adaptive response is via activation of nuclear factor erythroid 2-related factor 2 (Nrf2). Nrf2 is a transcription factor that responds to oxidative stress by promoting an antioxidant response (Mizuno et al, 2011; Purdom-Dickson et al., 2007). Using 10mg/kg doses, paraquat is known to increase Nrf2 expression, albeit not to an extent whereby the antioxidant response mechanisms are sufficient to overcome the damage induced by this oxidizing agent (He et al., 2012; Mattson & Cheng, 2006). It may be, however, that the aged mice in the current study actually mounted an antioxidant response that was not

commensurate with the fairly mild oxidative challenge presented by chronic low dose paraquat. Such a disproportionate development of neurocompensatory mechanisms (possibly involving Nrf2 and/or other factors) in the face of a relatively low-grade oxidative stressor could conceivably have promoted the observed pattern of changes in motivated responding (He et al., 2012; Mattson and Cheng, 2006).

Thus, while at first glance the advanced-aged mice appear to have displayed paradoxically beneficial responses to low dose paraquat (i.e., enhanced hedonic responses, decreased behavioural despair), these behaviours may, in fact, reflect an underlying aberrant plasticity of antioxidant/anti-inflammatory systems. According to an "adaptive energy-conserving" reading of the data, the younger mice will presumably also mount a similar antioxidant defence against paraquat (involving analogous compensatory biomolecular processes), but only when the severity of oxidative challenge warrants it (perhaps the robust CORT response to paraquat offers sufficient protection in younger animals). Importantly, as the metabolic energy costs associated with mounting a sustained neurocompensatory response to low dose paraquat are probably quite high (and likely prohibitively so at a certain point), one could argue that the advanced aged mice are, in fact, responding maladaptively to low dose paraquat (a dose that normally does not provoke neurodegeneration). Notably, while the argument can be made that the advanced-aged mice may have *had* to respond more vigorously to low dose paraquat than their younger counterparts due to an aging-related increase in BBB permeability and presumably brain paraquat accumulation and oxidative stress, we failed to detect any hippocampal or PFC changes in the phophorylation status of key NAPDH oxidase subunits as a function of paraquat treatment or age (data not shown).

PD and Early Cognitive Impairment

It has now been well documented that cognitive deficits in working and spatial memory are a common characteristic observed in PD, perhaps even occurring before the onset of motor behavior disturbances (Ferrer, 2009). Similar to neuropsychiatric disturbances, the early cognitive deficits observed could be the direct result of damage to the nigrostriatal system, however they could also be the result of damage and dysfunction seen in other pathways (e.g. mesocorticolimbic) (Ferrer, 2009).

In the present study, we found that regardless of age of exposure, paraquat alters memory related processes in a time-dependent manner, improving spatial working memory performance at one time-point before impairing spatial memory and retention related processes at a later date. While it is difficult to decipher the possible underlying mechanics to explain this behavior in the absence of physiological evidence (as a five week time gap occurred before animals were sacrificed), a few explanations based on evidence from previous studies can be put forward.

Given our results, paraquat exposure seems to be altering functioning in the hippocampus as evidenced by differential GR expression. It is probable then that the cognitive alterations observed likely result from paraquat influencing hippocampal-dependent processes. While it is known that paraquat may cause damage to DAergic neurons in the nigrostriatal system via its ability to induce oxidative stress effects through reactions with NADPH and the toxins ability to enter the mitochondria (Bonneh-Barkay et al., 2005; Rappold et al., 2011), recent research has shown that paraquat exposure produces similar effects in the hippocampus (Sun et al., 2011). Furthermore, the increase

in oxidative stress coupled with a decrease in antioxidant enzymes in this limbic structure have been correlated with cognitive deficits (Sun et al., 2011).

Another underlying mechanism in the hippocampus that may explain these cognitive alterations includes the possibility that paraquat is affecting neurotrophic factors, albeit in a fluctuating and time-dependent fashion. Indeed, our lab previously showed that paraquat caused time-dependent alterations of hippocampal BDNF (Litteljohn et al., 2011; Mangano et al., 2011). Furthermore, it has been shown that this trophic factor and its receptor tyrosine-related kinase B (TrkB) play a role not only in PD, but also in memory related processes. For example, recent evidence has demonstrated that increased levels of hippocampal BDNF, TrkB, and receptor-mediated signalling pathways result in an increase in Y-maze performance (Shin et al., 2011), and ablation or knockdown of this trophic factor result in spatial memory impairment (Ma et al. 1998; Heldt et al. 2007).

Interestingly, our data show an increase in working memory performance in toxin exposed animals, which may suggest that paraquat is provoking mechanisms that stimulate the release of this trophic factor thus inducing plastic changes. Furthermore, because memory impairment was observed at a later time-point, this potential increase may have only been temporary, and increased exposure may have resulted in mechanisms that impair BDNF functioning. Alternatively, these cognitive alterations may be a result of paraquat induced toxicity on the nigrostriatal system. Paraquat exposure alters striatal functioning as a result of inducing damage to the SNc, which as a consequence influences transmitter release in the striatum. In this case, the damage to the nigrostriatal tract induced by paraquat exposure may promote transmitter dysregulation in frontostriatal

circuitry. The caudate nucleus (where SNc projection neurons terminate) connects with the dorsolateral PFC, an area associated with memory performance (Owen, 2004). It may be then, that paraquat is inducing disruptions to this circuitry to the extent that memory impairments are observed, even in the absence of motor impairments. In support of this notion, in a study done by Braga et al. (2005), researchers showed that after causing direct damage to the SNc via MPTP administration, cognitive impairments were evident as seen through altered Y-maze behavior. Furthermore, it was shown that the relatively small MPTP lesion induced to the SNc did not correlate with motor behavioral deficits in comparison to controls. Thus suggesting that marginal damage to the nigrostriatal system is enough to cause cognitive impairments.

While the current study showed transient sucrose preference, alterations in cognitive behavior, and increased motivational responding, we report that chronic exposure to the low grade oxidative stressor did not give rise to olfactory related deficits in the current paradigm. Furthermore, it is well documented that impaired memory and deficits in locomotor behavior are common features seen in both normal and pathological aging (Lalonde, 2002). The current study confirms these notions, however, these impairments are only temporal with absence of cognitive and locomotor differences seen between age groups after eleven weeks. Conceivably, one reason for the absence of differences at these time points includes the notion that younger mice had aged such that, similar biological characteristics emerged overtime giving rise to similar performance between age groups (Flurkey et al., 2006). Furthermore, the current experiment was admittedly composed of many behavioral tests. Theoretically, this repeated behavioral testing could have recruited neuroplastic processes that, in effect, masked any age-related

decline in working and/or spatial memory functioning. Indeed, both low intensity exercise and extensive behavioural testing have been linked to augmented BDNF, as well as increases in DA, NE, 5-HT and glutamate even in the face of advancing age and experimentally induced pathology (Costa et al., 2012; Fredriksson et al., 2011; Morishima et al. 2006; Waters et al. 2008). Future research should multiple sacrifice points to assess age related changes at the cellular level, as well as the effects of low-dose paraquat exposure on these changes, in both behavioral exposed and behaviorally naïve animals.

Conclusion

In conclusion, the widely used environmental toxin paraquat is known to promote a pro-inflammatory and pro-oxidative state (e.g. microglial activation, cytokine release, reactive oxygen species production) that, as demonstrated in this study, may give rise to transient behavioral outcomes (e.g. cognitive, motivated responding) not directly related to the nigrostriatal pathway implicated in motor disturbances. Specifically, in the absence of any motor deficits throughout the paradigm, the present study showed that paraquat exposure affected spatial memory, both enhancing and impairing performance in a time-dependent manner. These changes may result from paraquat affecting various neurotransmitters and trophic factors at different time-points. Furthermore, while the current investigation did not see any signs of anxiety or depressive-like outcomes (in the elevated plus maze, open field, sucrose preference test and forced swim tests), paraquat did produce a paradoxical increase in motivational responding in both the forced swim test and sucrose preference test in an age dependent manner.

The advanced aged increase in motivational responding seen in the forced swim test may have been a result of evidence of the toxins influence on the HPA axis and related brain structures (i.e. the hippocampus). Indeed, while all animals had increased corticosterone levels in response to the forced swim test, the chronic paraquat challenge significantly elevated plasma corticosterone levels among middle-aged but not advanced-aged mice in relation to their counterparts. Likewise, elevated GR levels were observed in within the hippocampus of the middle aged paraquat exposed mice. In fact, the advanced-aged mice displayed reduced hippocampal expression of activated GR; and all of these effects occurred in the absence of any BDNF change. Taken together, these data suggest that chronic low-dose paraquat exposure may age-dependently influence HPA functioning in the face of an acute stressor. Furthermore, should paraquat be altering HPA and related brain structure functioning this may have potential ramifications for cognitive and psychiatric outcomes in prodromal and later-stage PD.

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