The impact of methylenetetrahydrofolate reductase (MTHFR) deficiency in a paraquat mouse model of Parkinson's disease

By

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

Master of Science

in

Neuroscience

Carleton University

Ottawa, Ontario

Abstract

The cause of Parkinson's disease (PD) remains unknown. Environmental toxicants such as paraquat have been linked to the characteristic dopaminergic (DA) neurodegeneration in the substantia nigra (SN); nutrition may also play a role. A common polymorphism (677C→T) in methylenetetrahydrofolate reductase (MTHFR), a folic acid metabolism enzyme, is associated with increased PD incidence. Using a mouse model that mimics this polymorphism, this study aimed to determine whether MTHFR deficiency leads to enhanced degeneration in a paraquat PD model. Male 3-month-old *Mthfr*$^{+/+}$ and *Mthfr*$^{+/-}$ mice received paraquat or saline injections. *Mthfr*$^{+/-}$ mice demonstrated motor and a trend for memory impairment compared to *Mthfr*$^{+/+}$ mice. No differences in SN DA neuron numbers or antioxidant activity were seen, however, increased oxidative stress and antioxidant activity were observed within the dorsal striatum of *Mthfr*$^{+/-}$ mice. These results suggest potential enhanced vulnerability to paraquat due to MTHFR deficiency through changes in such processes within this region.

Acknowledgments

I would like to thank Drs. Patrice Smith and Nafisa Jadavji for their continued support and guidance over the course of this thesis. Their expertise, advice, and encouragement have been invaluable to me both in the completion of this project and as inspiration for future scientific endeavours. I am tremendously grateful for the opportunities I have been fortunate to have while with the Smith lab, without which I am certain many of my accomplishments would not have been possible. Thanks also to members of the Hayley lab for their knowledge, generosity, and time spent providing me with training on techniques and methodology critical to the completion of this thesis.

Finally, I would also like to thank the faculty, staff, and students of the Department of Neuroscience for providing a positive and supportive environment, and for both sharing and continuing to further a love for the study of neuroscience.

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List of Abbreviations

5-HT – serotonin
6-OHDA – 6-hydroxydopamine
AAV – adeno-associated virus
AD – Alzheimer's disease
ANOVA – analysis of variance
BHMT – betaine homocysteine methyltransferase
BSA – bovine serum albumin
CD68 – cluster of differentiation 68
CI – confidence interval
DA – dopaminergic
DAB – diaminobenzidine
DAPI – 4',6-diamidino-2-phenylindole
DHF – dihydrofolate
dTMP – deoxythymidine monophosphate
dUMP – deoxyuridine monophosphate
GAPDH – glyceraldehyde 3-phosphate dehydrogenase
Gp91 – $gp91^{phox}$ subunit of NADPH oxidase
HPLC – high-performance liquid chromatography
HRP – horseradish peroxidase
Iba1 – ionized calcium-binding adapter molecule 1
i.p. – intraperitoneal
LC – locus coeruleus
L-DOPA – levodopa
LPS – lipopolysaccharide
LRRK2 – leucine-rich repeat kinase 2
MAT2A – methionine adenosyltransferase 2A
mPFC – medial prefrontal cortex
MPP^+ – 1-methyl-4-phenylpyridinium
MPTP – 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MS – methionine synthase
MTHFR – methylenetetrahydrofolate reductase
NADPH– nicotinamide adenine dinucleotide phosphate
NE – norepinephrine
NGS – normal goat serum
OR – odds ratio
PBS – phosphate buffered saline
PD – Parkinson's disease
PFC – prefrontal cortex
PHOX – $p47^{phox-/-}$
PINK1 – PTEN-induced putative kinase 1
PVN – paraventricular nucleus
ROS – reactive oxygen species
SAM – *S*-adenosylmethionine
SAH – *S*-adenosylhomocysteine

SEM – standard error of the mean
SHMT – serine hydroxymethyltransferase
SOD – superoxide dismutase
SN – substantia nigra
SNc – substantia nigra pars compacta
TH – tyrosine hydroxylase
THF – tetrahydrofolate
TS – thymidylate synthase
VTA – ventral tegmental area

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Introduction

As the second most common of the neurodegenerative disorders (Hirtz et al., 2007; Wirdefeldt, Adami, Cole, Trichopoulos, & Mandel, 2011), Parkinson's disease (PD) continues to be a critical area of research focus. Approximately 100,000 Canadians are currently affected by this disease (Bray & Huggett, 2016). Notably, symptoms typically appear later in life, at an average age of 64, with a diagnosis of Parkinson's typically occurring approximately 2 years later at age 66 (Wong, Gilmour, & Ramage-Morin, 2014). Due to an increasingly aging population (Statistics Canada, 2015; United Nations Department of Economic and Social Affairs, 2013), the number of those affected by PD is expected to grow substantially in the coming years, with some estimates suggesting an increase of 65% for those over age 40 by 2031 (Bray & Huggett, 2016) and others for the amount of affected individuals to double by 2050 (Bach, Ziegler, Deuschl, Dodel, & Doblhammer-Reiter, 2011). PD is a burden not only to those living with the disease, but also places considerable strain on caregivers and economic resources. When surveyed in a Canadian study, 84% of those receiving help due to PD relied on assistance from family, friends, and/or neighbours (Wong et al., 2014). Patients are also often required to pay out of pocket for assistive devices, rehabilitation therapy, and home support services (Wong et al., 2014). In 2011, indirect economic costs associated with PD in Canada were estimated at approximately $259 million, with a projected increase to $305 million by 2031 (Bray & Huggett, 2016). Such estimates underline the importance of further research to better understand and more effectively treat this disease.

PD is characterized by the progressive degeneration of dopaminergic (DA) neurons within the substantia nigra pars compacta (SNc) region of the midbrain. This degeneration is specific, as mesolimbic DA neurons of the ventral tegmental area (VTA) remain largely

unaffected. The death of the SNc DA neuronal population results in a depletion of striatal dopamine, which in turn leads to a range of motor impairments. At the onset of symptoms, approximately 60% of SNc DA neurons are generally believed to have already been lost (Dauer & Przedborski, 2003). As a result, individuals experience rigidity of movement, tremors at rest, and a slowing or even absence of voluntary movement (Dauer & Przedborski, 2003). These impairments affect daily tasks such as walking, writing, dressing, and eating, all of which contribute to an overall negative impact on quality of life. Using a measure incorporating eight functional categories, including cognition, emotion, speech, vision, mobility, dexterity, pain, and discomfort, people living with PD were found to have a significantly reduced quality of life, which translated to moderate to severe disability in 82% of individuals (Bray & Huggett, 2016). Because PD is chronic and progressive, symptoms also deteriorate further over time, leading to greater detriments to quality of life for patients.

Additional behavioural effects beyond those that affect movement often occur with PD. Effects on mood, including depression and changes in affect, are frequently experienced by those with PD (Bray & Huggett, 2016; Dauer & Przedborski, 2003). In some cases, cognitive symptoms may also occur. In a national study focusing on Canadians living with neurological conditions, 40% of individuals with PD reported changes in cognition such as limitations in thinking, problem-solving, and memory (Bray & Huggett, 2016). Research has also suggested potential impairment in spatial memory, particularly when individuals are required to develop their own strategies for organizing and memorizing spatial information or during driving navigational tasks (Aksan, Anderson, Dawson, Uc, & Rizzo, 2015; Foster, Black, Antenor-Dorsey, Perlmutter, & Hershey, 2008; Pillon et al., 1996, 1998; Uc et al., 2007; Yao et al., 2016). Mood and cognitive effects may also be mediated by alterations in other neurotransmitter

systems, such as norepinephrine (NE) and serotonin (5-HT), which also appear to be affected in PD (reviewed in Halliday, Leverenz, Schneider, & Adler, 2014).

In addition to these cognitive effects, those with PD have shown significantly greater risk for developing dementia compared to controls (Aarsland et al., 2001; Hobson & Meara, 2004). When dementia occurs after over a year of PD diagnosis, it is diagnosed as PD dementia. This form of dementia can be characterized by dysfunction in executive and visuospatial abilities, attention, and memory, with the latter typically less affected than seen in Alzheimer's disease (AD) (Dubois et al., 2007; Poewe et al., 2008). A link is also suspected between another type of dementia, known as Lewy body dementia, and PD. In this form, symptoms of dementia occur prior to or in conjunction with motor impairment (Zupancic, Mahajan, & Handa, 2011). Cytoplasmic inclusions called Lewy bodies, consisting primarily of α-synuclein protein, can be found within brain tissue in PD, PD dementia, and Lewy body dementia, and may contribute to cognitive symptoms (Baba et al., 1998; Spillantini, Crowther, Jakes, Hasegawa, & Goedert, 1998). Though Lewy bodies are observed in other neurological conditions and are therefore not specific to PD, they are commonly found in patients with PD and are considered a pathological feature of the disease (Spillantini et al., 1998).

Research dedicated to PD continues to be of critical importance as the precise cause of the neurodegeneration affecting DA neurons remains unknown. As a result, current methods of treatment fail to target a cause, and instead treat only the identifiable symptoms. For instance, the widely used drug levodopa (L-DOPA) helps replenish lost striatal dopamine but fails to address the underlying cell death occurring within the SNc. Despite its ubiquitous use in the treatment of PD, L-DOPA can also have limited effectiveness, wear off over time, and elicit undesirable side effects (Stacy et al., 2005). Consequently, the importance of understanding all

of the factors, both genetic and environmental, at play and their involvement in the degenerative process is clear, in order to develop more effective and targeted treatments for those with PD. Such knowledge could also lead to significant improvements in quality of life for those affected by PD.

Etiology of PD: contributions of genetic vs. environmental factors

PD can broadly be divided into two categories based on etiology: familial, in which genetic correlates have been identified; and sporadic, which is by far the more common and appears to have a more complex, multifactorial etiology involving factors such as age and environmental exposure, in addition to genetic changes (Sulzer, 2007). Familial PD makes up only about 10% of cases and is typically directly linked to mutations in individual identifiable genes. Mutations in the genes encoding for the α-synuclein protein, a major component of Lewy body cytoplasmic inclusions, have been identified in some cases of familial PD (Polymeropoulos et al., 1997). Other genes, including *Parkin* (Kitada et al., 1998), *PTEN-induced putative kinase 1* (*PINK1*) (Valente et al., 2004) and *Leucine-rich repeat kinase 2* (*LRRK2*) (Zimprich et al., 2004), have also been identified. Although only a minority of cases involve individual mutations like those seen in familial PD, studies of such cases have provided a basis for investigating possible common mechanisms and pathways that may be involved in the pathology of sporadic PD. In this way, they have helped to promote a greater understanding of the pathological features shared by all of those with PD.

In contrast to familial PD, less is known regarding the factors directly involved in the development of sporadic PD, although possible links to exposure to several substances within the environment have been proposed. Environmental factors that have been identified include

chemicals used as pesticides and herbicides. Contact with such substances has been associated with increased risk of developing PD in multiple epidemiological studies for those in rural and agricultural communities, as well as occupations that would experience greater probabilities of exposure. For instance, a cohort study in Alberta, Canada found that PD risk was elevated in rural regions of the province (Svenson, Piatt, & Woodhead, 1993). In a study also focusing on rural regions but in Quebec, Canada, researchers found PD prevalence was not uniform across all of the rural areas under study, but rather was linked to agricultural and commercial gardening activity (Barbeau, Roy, Bernier, Campanella, & Paris, 1987). Prevalence was significantly correlated with pesticide use within the regions. Prevalence ratios also increased with the amount of years of exposure to pesticides in a cohort consisting of mostly orchardists in the state of Washington in the United States (Engel et al., 2001). In Denmark, farmers and those involved in agriculture and horticulture had significantly higher risk of hospitalization for PD than expected based on incidence ratios for men and women of the cohort's age distribution (Tüchsen & Jensen, 2000). A case-control study involving populations in Scotland, Sweden, Malta, Italy, and Romania found significantly increased odds ratios (OR) for PD and Parkinsonism for those who had experienced exposure to pesticides (low vs. high exposure, OR = 1.13, 95% confidence interval (CI): 0.82 to 1.57, high vs. no exposure, OR = 1.41, 95% CI: 1.06 to 1.88) (Dick et al., 2007). A recent systematic review and meta-analysis, which critically analyzed the design of all included studies, found an increased risk of PD with occupational exposure to pesticides (Van Maele-Fabry, Hoet, Vilain, & Lison, 2012). The greatest significance was seen for studies with the best design, such as those that required neurologist-confirmed PD diagnoses. Together, these findings support a link between pesticide exposure and the development of PD.

In addition to examining the effects of all pesticides as a group, the potential role of specific pesticides has also been investigated. The non-specific herbicide paraquat (1,1′-dimethyl-4,4′-bipyridine), in particular, has been associated with greater PD incidence in several epidemiological studies (Costello, Cockburn, Bronstein, Zhang, & Ritz, 2009; Liou et al., 1997; Tanner et al., 2011), a relationship that has also been supported by laboratory evidence (Bové & Perier, 2012; Cannon & Greenamyre, 2010).

Paraquat and PD

Paraquat, first marketed as an herbicide in the 1960s, remains in widespread use worldwide for weed control in a range of crops, including soybeans, tea, sugarcane, cotton, and many varieties of fruit (Bromilow, 2003; Vaccari, Dib, & Camargo, 2017). Although it has been banned in 32 countries and faces substantial restrictions in others, paraquat remains the herbicide of choice in many regions. In the United States, where paraquat is restricted to commercial use only by those certified for its correct handling and application, data from the Environmental Protection Agency indicate an increase in use from 1-5 million tonnes of active ingredient in 2009 to 2-6 million tonnes in 2012 (Atwood & Paisley-Jones, 2017). Such figures underline the continued relevance of this substance to human populations.

Paraquat exerts its herbicidal effects through acting on photosystem I of chloroplasts, where it promotes the production of free radicals and superoxides through redox cycling (Figure 1) (Patterson, Small, & Scaiano, 1977). These reactive oxygen species (ROS) damage cell membranes, allowing water to escape and causing desiccation of foliage (Bromilow, 2003). Paraquat is fast-acting, and is effective even if rain occurs within 15-30 minutes of application

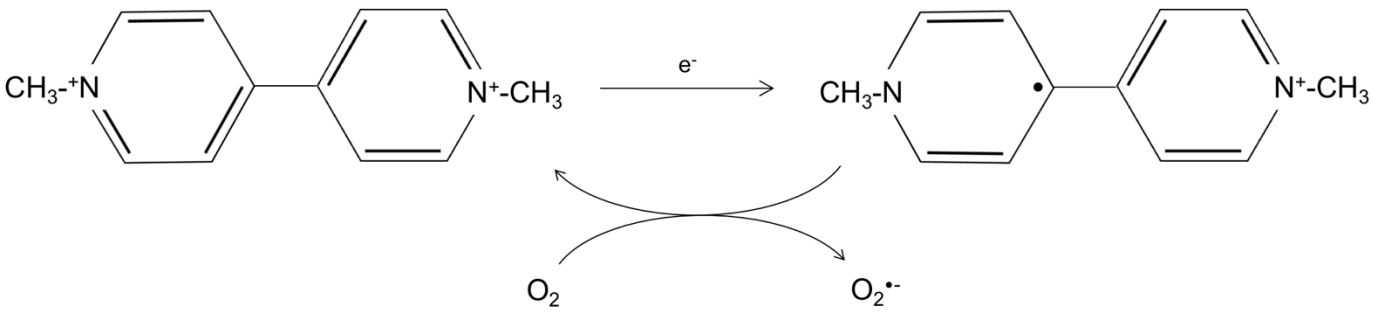

Figure 1. Redox cycling of paraquat.

(Sagar, 1987). This feature makes it particularly attractive for agricultural uses in countries with extensive rainfall during growing seasons.

Connections between paraquat and the development of PD were first suggested following observations of its structural similarity to 1-methyl-4-phenylpyridinium (MPP^+), the metabolized form of the synthetic neurotoxicant 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Snyder & D'Amato, 1985). This substance causes Parkinson-like characteristics in both humans and animals in such a way that closely resembles those with PD (Langston, Ballard, Tetrud, & Irwin, 1983). It infamously triggered users of a synthetic heroin compound to develop Parkinsonian symptoms, including rigidity, tremor, and flat affect, after accidental contamination with this by-product (Langston et al., 1983). In the years since, MPTP has been widely utilized as a model of PD in animal studies, and its mechanism of action has been shown to involve inhibition of mitochondrial complex I. Subsequent epidemiological studies have therefore investigated paraquat exposure in PD disease development. Tanner et al. (2011) examined residents of Iowa and North Carolina in a case-control study and found an association with use of paraquat and development of PD (OR = 2.5; 95% CI: 1.4 to 4.7). A case-control study in California found an increased risk of developing PD for those exposed within 500 metres of the home to paraquat and the fungicide maneb in combination (OR = 1.75, 95% CI: 1.13 to 2.73) (Costello et al., 2009). Exposure at an earlier timepoint, during the childhood, adolescence, or young adulthood of the individuals, to paraquat alone also increased risk of PD (OR = 2.27, 95% CI: 0.91 to 5.70). In a study in Taiwan, also case-control, use of paraquat suggested an increased risk for the development of PD (OR = 3.22; 95% CI: 2.41 to 4.31) (Liou et al., 1997). This relationship also followed a dose-response relationship, with those who used paraquat for more than 20 years showing the greatest risk (OR = 6.44; 95% CI: 2.41 to 17.20).

In addition to positive associations being found between individuals exposed to paraquat and subsequent development of PD, several *in vitro* and animal studies support the suggestion that paraquat may be involved in or is relevant to PD disease progression. Such research has demonstrated that paraquat can elicit damage to DA cells, a key pathological feature of PD. In primary cultures of DA neurons, paraquat reduced DA cell survival as assessed by counts of tyrosine hydroxylase (TH)-positive cells (Choi, Abel, Klintworth, Flavell, & Xia, 2010). As the enzyme involved in the rate-limiting step of dopamine production, TH is commonly employed as a marker for dopamine-producing cells. Higher doses of paraquat were associated with greater reductions in cell survival, thereby demonstrating a dose-dependent relationship. Cultures of rat ventral mesencephalic cells have shown similar results, with DA neurons being lost while non-DA neurons were spared (Cicchetti et al., 2005).

In vivo experiments using mouse models have shown reduced numbers of DA neurons within the SNc, as well as reductions in the number of terminals in the striatum, following paraquat exposure (Brooks, Chadwick, Gelbard, Cory-Slechta, & Federoff, 1999; Jiao, Lu, Williams, & Smeyne, 2012; McCormack et al., 2002). McCormack et al. (2002) also observed a dose-dependent reduction in DA neurons, with 10, 18, and 28% fewer cells induced by 1, 5, and 10 mg/kg of paraquat respectively. When counts of Nissl stained cells were compared to TH+ cell counts, the same reduced numbers of cells were seen, suggesting that the lost cells were TH immunoreactive cells. Reduced cell counts were not observed in regions outside the SNc, such as the pars reticulata or hippocampus, again supporting a preferential targeting of DA SNc neurons (McCormack et al., 2002). Mice also showed behavioural effects in terms of impaired locomotor activity (Brooks et al., 1999). Both behavioural and histological effects showed a dose-dependent relationship with paraquat administration (Brooks et al., 1999; McCormack et

al., 2002). Similar reductions in DA cells, of approximately 25-30%, have been observed in more recent studies (Mangano et al., 2011, 2012; Mangano & Hayley, 2009). Such decreases can also be enhanced by priming using the endotoxin lipopolysaccharide (LPS) prior to paraquat treatment (Mangano et al., 2011).

Reductions in DA cell numbers and density have also been observed in rats. Kuter et al. (2007) found 22 and 24% reductions in the numbers and density, respectively, of SNc TH-immunoreactive cells using stereological counts. An initial reduction in DA metabolites, followed by an increase in DA was also observed (Kuter et al., 2007). A loss of nigral DA neurons was also observed by Cicchetti et al. (2005), in conjunction with increases in microglial activation.

In addition to effects on DA neurons, paraquat can effectively model other aspects of PD, including changes in several neurotransmitter systems. It has been shown to alter NE signalling in the locus coeruleus (LC), paraventricular nucleus (PVN) of the hypothalamus, and hippocampus (Litteljohn, Mangano, Shukla, & Hayley, 2009; Litteljohn, Nelson, Bethune, & Hayley, 2011; Rudyk, Litteljohn, Syed, Dwyer, & Hayley, 2015). Decreased 5-HT in the medial prefrontal cortex (mPFC) and dorsal hippocampus has been demonstrated following paraquat treatment (Litteljohn et al., 2009), as well as reductions in glucocorticoid receptors in the hippocampus (Rudyk et al., 2017).

The herbicide has also been shown to trigger the formation of α-synuclein cytoplasmic inclusions (Manning-Bog et al., 2002), similar to the Lewy bodies seen in some cases of PD. In this study, researchers observed a dose-dependent increase in α-synuclein fibril formation with increasing paraquat administration. Paraquat is one of very few models of PD that replicates this characteristic of the disease. For example, the 6-hydroxydopamine (6-OHDA) model of PD

lacks the production of such inclusions (Dauer & Przedborski, 2003). As a result, paraquat is particularly useful for investigating the role α-synuclein may play in both the DA neurodegeneration and cognitive impairments seen with PD.

Paraquat's contributions to oxidative stress are also important to consider. Increases in oxidative stress within the SNc, as assessed via lipid peroxidation, have been observed in mice (McCormack et al., 2005). In this study, the onset of DA neurodegeneration was preceded by marked increases in the expression of lipid peroxidation markers, suggesting a possible relationship between oxidative stress and the degenerative process. Additionally, mice with knockouts in copper/zinc (Cu/Zn) superoxide dismutase (SOD) are more susceptible to paraquat, with all $SOD^{-/-}$ mice dying within 5 days of exposure, while wild-type and heterozygous mice survived (Y. Ho et al., 1998). Mice with reduced amounts of SOD2 have also been shown to be more sensitive to paraquat. Approximately 30% of $SOD2^{+/-}$ mice, which had 50% of normal levels of SOD2, died within 4 days of paraquat treatment (Van Remmen et al., 2003). In addition, overexpression of SOD2 has been shown to increase cell viability as well as animal survival following paraquat, suggesting an increased resistance to paraquat with higher SOD2 levels (Jang et al., 2009). Increased levels of intracellular ROS have also been observed in rats following paraquat exposure (Kuter, Nowak, Golembiowska, & Ossowska, 2010). In addition, it has been proposed that oxidative damage can predispose α-synuclein to misfolding and aggregation, so this mechanism may also be involved in the pathological α-synuclein accumulation component of the disease. Taken together, these findings provide supporting evidence for oxidative stress as a contributing factor in the degeneration-inducing effects of paraquat.

Paraquat is a useful model for PD for several reasons. It appears to elicit preferential degeneration of DA neurons within the SNc, much like PD (Brooks et al., 1999; Choi et al., 2010; Cicchetti et al., 2005; Kuter et al., 2007; McCormack et al., 2002). It also produces another key pathological feature of PD, inclusions of α-synuclein within cell cytoplasm. Paraquat consequently provides a means of examining the role of α-synuclein in the DA degeneration and behavioural impairments seen in PD. Epidemiological work has also demonstrated a strong potential for paraquat to play a role in the development of PD in those exposed to it. Because it remains in such widespread use in many regions, including North America, the potential for environmental exposure persists. As a result, this model is also clinically relevant.

Dietary factors: folate and homocysteine

Patients with PD consistently show elevated levels of homocysteine compared to individuals without PD (Camicioli, Bouchard, & Somerville, 2009; dos Santos et al., 2009; Levin, Giese, & Lorenzl, 2010; Rodriguez-Oroz et al., 2009). The reason for this remains unclear. Homocysteine is a non-protein amino acid that at high levels has several potentially toxic effects within cells, including DNA damage, oxidative stress, mitochondrial dysfunction, and apoptosis (Duan et al., 2002; Imamura, Takeshima, Nakaso, & Nakashima, 2007; Kruman et al., 2000). It is generated during the metabolism of methionine and is a component of the folate metabolic cycle (Figure 2).

Emerging evidence has indicated there may be a role for dietary factors, such as folate, in PD development. Folate is a B vitamin, found naturally in foods such as leafy green vegetables and liver. Folic acid is a synthetic form of folate and is found within vitamin supplements. It is

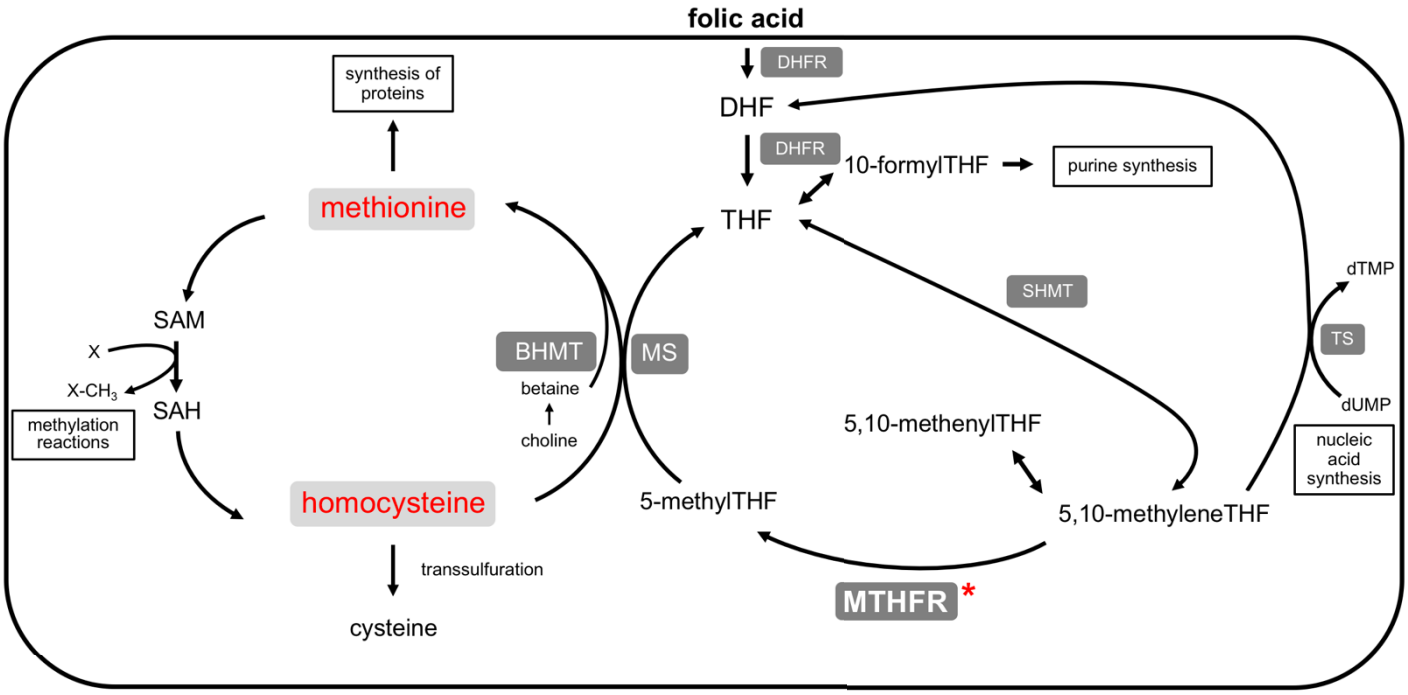

Figure 2. Summary of folate metabolism and related processes within the cell. DHFR: dihydrofolate reductase; DHF: dihydrofolate; THF: tetrahydrofolate; BHMT: betaine homocysteine methyltransferase; MS: methionine synthase; SHMT: serine hydroxymethyltransferase; dUMP: deoxyuridine monophosphate; dTMP: deoxythymidine monophosphate; SAM: *S*-adenosylmethionine; SAH: *S*-adenosylhomocysteine; TS: thymidylate synthase; MTHFR: methylenetetrahydrofolate reductase. *Modified from Jadavji et al., 2015*.

often prescribed to women for use both prior to and during pregnancy, because of its role in ensuring proper neural tube closure. In this way, folic acid acts as a preventative measure against neural tube defects (Ray et al., 2002). Because of this well-established protective effect, and due to numerous studies concluding a significant percentage of women of childbearing age have suboptimal levels of folate, several countries have elected to fortify milled grains with folic acid, thereby providing an additional dietary source of the vitamin.

Folate has significant roles in multiple cellular processes. Within cells, folate is converted to dihydrofolate (DHF), and then to tetrahydrofolate (THF). From this point, THF can follow several alternative pathways. Conversion into 10-formylTHF leads to the production of purines. The action of serine hydroxymethyltransferase (SHMT) results in 5,10-methyleneTHF, which then acts as a substrate for thymidylate synthase (TS). TS converts 5,10-methyleneTHF and deoxyuridine monophosphate (dUMP) into DHF and deoxythymidine monophosphate (dTMP). Through such conversions, folate is significantly involved in and important for nucleotide synthesis and DNA repair. 5,10-methyleneTHF can also act as the substrate for the enzyme methylenetetrahydrofolate reductase (MTHFR), and result in the production of 5-methylTHF. This is the biologically active form of folate that is used by methionine synthase (MS) to remethylate homocysteine into methionine, a reaction that also produces THF. From here, methionine can be converted by methionine adenosyltransferase 2A (MAT2A) to *S*-adenosylmethionine (SAM), which can then act as a methyl donor for methylation reactions. Such reactions provide methyl groups for DNA, RNA, and proteins. After losing its methyl group, SAM becomes *S*-adenosylhomocysteine (SAH), which can then be metabolized further into homocysteine. Folate is therefore a cofactor in the metabolism of homocysteine, through its conversion to 5-methylTHF, which allows for its remethylation into methionine.

Though its role is not yet well understood (Hannibal & Blom, 2017), increased levels of homocysteine have been associated with the development of several pathological conditions, including cardiovascular disease, mild cognitive impairment, and PD (Bots, Launer, Lindemans, Hofman, & Grobbee, 1997; Rodriguez-Oroz et al., 2009; Seshadri et al., 2002). It has also been linked to more negative outcomes in ischemic stroke (X.-Q. Wu et al., 2013). Interestingly, patients with PD have consistently been shown to have elevated levels of homocysteine compared to controls (Camicioli et al., 2009; dos Santos et al., 2009; Levin et al., 2010; Obeid et al., 2009; Rodriguez-Oroz et al., 2009).

An important caveat associated with these findings is that L-DOPA can itself contribute to elevated homocysteine levels. L-DOPA can increase amounts of homocysteine in circulation by receiving a methyl group from SAM, leaving SAH to be converted into homocysteine (Brosnan, Jacobs, Stead, & Brosnan, 2004; Liu, Wilson, & Charlton, 2000). Because L-DOPA is the most widely used treatment for PD, this would no doubt have an impact on measurements taken from PD patient populations. Indeed, several studies have found that homocysteine levels were significantly higher in PD patients treated with L-DOPA compared to non-treated individuals (Miller et al., 2003; Yuan et al., 2009). However, it may not be the only factor. Religa et al. (2006) examined patients on different dosages of L-DOPA in comparison to controls, including non-treated patients, and found that while PD patients had elevated levels of homocysteine, levels did not depend on the dosage of L-DOPA patients were receiving. Also observed was a trend toward higher homocysteine levels in non-treated PD patients compared to controls (Religa et al., 2006). Other studies have also found no impact of L-DOPA on increased homocysteine levels (dos Santos et al., 2009), but suggest L-DOPA may be interacting with other factors, such as folate deficiency or the duration of the disease, to elicit such effects. This

theory is supported by additional research, which has proposed that the extent of elevated homocysteine in PD patients can be influenced by their folate status (Miller et al., 2003). These findings suggest that while treatment with L-DOPA likely has an impact on homocysteine levels, it cannot be entirely ruled out that PD may itself contribute to observed elevations.

There are several mechanisms through which homocysteine may become elevated above normal concentrations. Homocysteine levels naturally increase with age (Brattström, Lindgren, Israelsson, Andersson, & Hultberg, 1994). Because the typical age of onset of PD is later in life, and prevalence continues to increase significantly with age (Dauer & Przedborski, 2003; Pringsheim, Jette, Frolkis, & Steeves, 2014), this may suggest a potential interactive effect between these two factors in the development of the disease. Indeed, it has been suggested that age interacts with genetic and environmental factors to increase susceptibility to PD (Zhou, Huang, Tong, & Xia, 2011).

Dietary factors can also play a role. Levels of folate and homocysteine generally show an inverse relationship, with low amounts of folate associated with elevations in homocysteine. As a cofactor in the metabolism of homocysteine, low levels of folate result in a reduction in the amount of 5-methylTHF. Lower levels of 5-methylTHF mean a decline in the amount of homocysteine undergoing reconversion to methionine, leaving homocysteine to accumulate as a result. Therefore, a failure to obtain adequate levels of folate within the diet can be associated with high concentrations of homocysteine in blood plasma.

Alternatively, homocysteine levels can become elevated due to impairments in other components of the folate metabolic pathway. Examples include mutations in genes encoding for enzymes such as MTHFR, which converts folate into 5-methylTHF, the substrate responsible for donating the methyl group necessary for the conversion of homocysteine into methionine.

Impairments in the MTHFR enzyme therefore also impact homocysteine reconversion, and lead to elevations in homocysteine levels. These effects are of particular relevance, as there is a common polymorphism in MTHFR, the prevalence of which varies slightly depending on demographic and region but that occurs in at least 5-15% of North American and European populations (Frosst et al., 1995; Schneider, Rees, Liu, & Clegg, 1998). This polymorphism involves a change from C to T at base pair 677, resulting in an amino acid change from alanine to valine (A222V) and 35-45% of normal enzymatic activity (Frosst et al., 1995; Gorgone et al., 2012; S.-S. Kang, Zhou, Wong, Kowalisyn, & Strokosch, 1988; Lievers et al., 2001). As a consequence of this reduced MTHFR activity, individuals with this polymorphism have significantly higher homocysteine levels than the general population (Gorgone et al., 2012; S.-S. Kang et al., 1988; Lievers et al., 2001; Yuan et al., 2009).

Such individuals have been shown to have a greater incidence for developing several pathological conditions, including PD. The 677 C→T polymorphism has also been observed at greater frequencies in PD patients than the general population. In a case-control study, Gorgone et al. (2012) found the 677TT polymorphism was significantly more frequent in PD patients than controls. Meta-analysis has also been employed to investigate this relationship, and has determined that there is a significant association between 677TT and PD in Europeans (OR = 1.17, 95% CI: 1.04 to 1.31) (Zhu, Zhu, He, Liu, & Liu, 2015). Another meta-analysis found an increased susceptibility associated with the T allele in European as well as Asian populations (Y. L. Wu, Ding, Sun, Yang, & Sun, 2013), though the latter was not seen in the study by Zhu et al. (2015).

A MTHFR-deficient mouse model that mimics the 677 C→T polymorphism has been developed. Heterozygous mice have reduced MTHFR enzymatic activity and elevated levels of

homocysteine comparable to their human counterparts with the 677 C→T conversion, but otherwise appear normal phenotypically (Chen, Karaplis, Ackerman, Pogribny, Melnyk, Lussier-Cacan, et al., 2001). As a result, these mice represent a highly relevant model for individuals carrying the 677 C→T polymorphism. Homozygous mice show even greater elevations in homocysteine (~10 times normal levels) as well as more severe impairment, including developmental delay and cerebellar abnormalities (Chen et al., 2001). These mice therefore act as a model of more deleterious mutations in MTHFR that result in an enzyme with very little to no activity. Such mutations are significantly more rare than the *MTHFR*677 C→T polymorphism (Chen, Karaplis, Ackerman, Pogribny, Melnyk, Lussier-Cacan, et al., 2001; Frosst et al., 1995; Schneider et al., 1998).

Folate deficiency and models of PD

In addition to the suggestion of increased vulnerability implied by human epidemiological work, *in vitro* and *in vivo* studies have been employed to examine the relationships between folate, homocysteine, and PD. Folate deficiency in cell cultures has been shown to elicit neurodegeneration and increase the amount of ROS production (P. I. Ho et al., 2003). Addition of homocysteine directly to cells also produced the same effects, while inhibition of homocysteine formation was effective at suppressing increases in ROS. In another study, homocysteine exposure in combination with the pesticide rotenone or iron led to increased membrane depolarization in the mitochondria of human DA cells (Duan et al., 2002). Mitochondrial ROS levels also increased with rotenone or iron exposure, were further exacerbated by homocysteine, and were suppressed by treatment with an antioxidant or an inhibitor of DNA damage.

In rodent studies, elevated homocysteine induced in mice using a folate-deficient diet has been shown to significantly reduce numbers of surviving DA neurons following MPTP exposure compared to a control diet (Duan et al., 2002). Directly infusing homocysteine into the SNc has also been shown to produce equivalent effects (Duan et al., 2002). These histological changes were also accompanied by increased motor impairment (Duan et al., 2002). Because these observations were seen only when MPTP was administered, and not in its absence, this suggests that folate deficiency and elevations in homocysteine may act to increase vulnerability to neurodegeneration, rather than triggering cell death directly. However, other experiments have found that homocysteine on its own at high levels can prove toxic for DA neurons (Imamura et al., 2007; Lee, Chen, Soliman, & Charlton, 2005). In a study investigating the effects on rat primary mesencephalic cells, homocysteine enhanced cell death in response to MPTP in a dose-dependent manner (Imamura et al., 2007). Cells with intracellular dopamine were also more vulnerable to homocysteine's toxic effects than other cells. Similarly, Lee et al. (2005) found that homocysteine decreased TH immunoreactivity as well as locomotor activity in mice. These inconsistent findings underline the need for further investigation in this area.

It is important to note that the precise role that homocysteine may play in conditions such as PD remains unclear. *In vitro* evidence of toxicity is typically the result of homocysteine concentrations much higher than physiological levels of ~13-15 μM (Camicioli et al., 2009; Hannibal & Blom, 2017). In addition, the elevations seen in pathological states may be indirect and the result of disease, rather than the cause.

Rationale for the present study

While studies have been conducted examining the effect of folate deficiency in other models of PD, there is a current lack of investigations exploring the potential effects using a paraquat model of the disease. Because so little is still known regarding the mechanisms leading to PD pathology, employing a model that reproduces many aspects of the disease is particularly desirable. The use of paraquat over other models of PD, due to its ability to produce both SNc DA degeneration and α-synuclein inclusions (Brooks et al., 1999; Choi et al., 2010; Cicchetti et al., 2005; Kuter et al., 2010; Manning-Bog et al., 2002; McCormack et al., 2002), as well as its environmental relevance, make it an especially useful animal model for experimental study. Because there are individuals within the population that have experienced paraquat exposure that have gone on to develop PD, and because paraquat use and therefore the risk of contact remains prevalent, the potential for directly related human cases remains high. Other synthetic models, such as 6-OHDA, are much less likely to be encountered environmentally. 6-OHDA also does not produce inclusions of α-synuclein (Dauer & Przedborski, 2003), and therefore the role of such inclusions in both the degenerative and cognitive effects of PD cannot be explored. Paraquat is able to incorporate both key pathological features of the disease while also allowing for clinical relevance in terms of potential human exposure.

Epidemiological data as well as animal models suggest an increased vulnerability to neurological conditions, including PD, associated with folate deficiencies and elevated levels of homocysteine (Rodriguez-Oroz et al., 2009). The former have indicated an increased risk for developing PD for those with the 677 C→T MTHFR polymorphism, which produces a less efficient enzyme and increases homocysteine levels (Gorgone et al., 2012; S.-S. Kang et al., 1988; Lievers et al., 2001; Y. L. Wu et al., 2013; Zhu et al., 2015). Animal studies using folate

deficient diets or direct homocysteine administration have shown increased SNc DA neurodegeneration and behavioural impairment in response to MPTP PD models, also supporting this idea (Duan et al., 2002; Lee et al., 2005).

In addition, deficiencies in folate and increased levels of homocysteine have been shown to exacerbate oxidative stress through increasing ROS production (P. I. Ho et al., 2003). Because paraquat is known to act on cells via redox cycling and the production of ROS (Kuter et al., 2010; McCormack et al., 2005; Patterson et al., 1977), this provides a potential opportunity for interaction between these factors.

The mouse model of MTHFR deficiency provides a relevant model for individuals with the 677 C→T conversion. Mice show comparable enzymatic deficiency and elevations in homocysteine, making for an ideal model to study the impact of this polymorphism in folate metabolism on PD pathogenesis (Chen, Karaplis, Ackerman, Pogribny, Melnyk, Lussier-Cacan, et al., 2001). While an increased susceptibility to DA neurodegeneration has been suggested for folate deficiency based on diet and using some models of PD, it remains unknown whether a genetic deficiency in folate metabolism caused by a polymorphism in the MTHFR enzyme leads to exacerbated degeneration in a paraquat mouse model of PD. The aim of this thesis is therefore to investigate whether MTHFR deficiency interacts with paraquat exposure to exacerbate dopaminergic neurodegeneration in a mouse model, which is clinically relevant for those with the 677C→T polymorphism.

Research Objectives and Hypotheses:

Objective 1: To assess motor and cognitive function in *Mthfr*$^{+/+}$ and *Mthfr*$^{+/-}$ mice following paraquat administration.

Hypothesis: Mice deficient in MTHFR will show greater impairment in motor and cognitive ability compared to wild-type mice following paraquat exposure.

Objective 2: To determine the extent of dopaminergic neurodegeneration within the substantia nigra pars compacta (SNc) in *Mthfr*$^{+/+}$ and *Mthfr*$^{+/-}$ mice after paraquat administration.

Hypothesis: MTHFR deficient mice will show reduced numbers of dopaminergic cells within the SNc compared to wild-type mice.

Objective 3: To investigate the mechanisms involved in vulnerability to paraquat, including oxidative stress, antioxidant activity, and neuroinflammation, in *Mthfr*$^{+/+}$ and *Mthfr*$^{+/-}$ mice.

Hypothesis: MTHFR deficient mice will show elevated levels of oxidative stress, antioxidant activity, and microglial activation in response to paraquat as compared to wild-type mice.

Materials and Methods

Animals

All experiments were performed in accordance with the guidelines of the Canadian Council on Animal Care (CCAC) and with approval of the Carleton University Animal Care Committee. Three-month-old *Mthfr*$^{+/+}$ (n=27) and *Mthfr*$^{+/-}$ (n=28) mice were divided into two cohorts (Table 1A and B) for this study. They were housed with a 12-hour light/dark cycle in standard caging conditions, with *ad libitum* standard mouse chow and water.

Table 1A. Number of mice in each experimental group in Cohort 1. Brain tissue was used for immunohistochemistry and immunofluorescence experiments and blood was collected for plasma homocysteine analysis.

		Group	
		Saline	Paraquat
Genotype	*Mthfr*$^{+/+}$	7	6
	Mthfr$^{+/-}$	7	7

Table 1B. Number of mice in each experimental group in Cohort 2, which underwent behavioural testing. Dissected brain tissue was used for Western blot experiments.

		Group	
		Saline	Paraquat
Genotype	*Mthfr*$^{+/+}$	6	8
	Mthfr$^{+/-}$	7	7

Experimental design

An experimental timeline summarizes the study design (Figure 2). At 3 months of age, MTHFR deficient (*Mthfr*$^{+/-}$) and wild-type (*Mthfr*$^{+/+}$) mice received either saline or paraquat (10mg/kg) intraperitoneal (i.p.) injections twice a week for 3 weeks for a total of 6 injections overall, as has been described previously (Mangano et al., 2011, 2012; Mangano & Hayley, 2009). In the week following the final injections, mice underwent a series of behavioural tests, one per day, to assess cognitive and motor function. Three days following the final test, mice were euthanized via either perfusion for immunohistochemistry and blood collection or live decapitation (Mangano et al., 2011; Mangano & Hayley, 2009) and snap frozen for Western blot analysis.

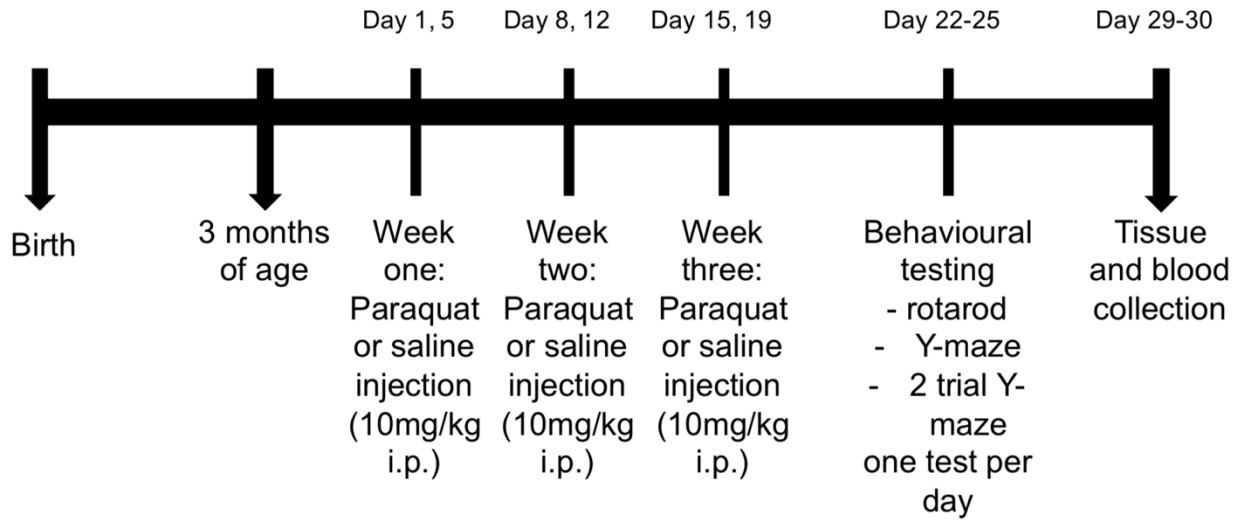

Figure 3. Experimental timeline for the study. In the first week of the experiment, 3-month-old male *Mthfr*$^{+/+}$ and *Mthfr*$^{+/-}$ mice received injections of either saline or paraquat (10mg/kg i.p.) on Day 1 and 5 (Monday and Friday). This was then repeated for the second and third weeks of the study, for a total of 6 injections. The following week, mice underwent behavioural testing with one test per day for the rotarod, spontaneous alternation Y-maze, and 2-trial Y-maze. In the week following testing, mice were euthanized and tissue and blood collected for analysis.

Behavioural tests

Rotarod

To test motor function, animals were assessed using a standard accelerating rotarod (Omnitech Electronic Inc.) consisting of a metal cylinder 3.0 cm in diameter and 6 cm wide, positioned at 30 cm above the ground using a plexiglass container. A rubber belt and small motor caused the movement of the rotarod. Animals were tested as the rotarod accelerated from 4 to 60 rpm over three 8-minute trials in a single day with an inter-trial interval of 5 minutes. Latency to fall was recorded.

Y-maze

To assess short-term spatial memory, animals were tested on the Y-maze (Jadavji et al., 2012; Sarter, Bodewitz, & Stephens, 1988). The maze consists of three identical arms of gray plastic. Each mouse was placed in the end of one arm and given 8 minutes to explore the maze. The number of arm entries for each arm was recorded. Arm entries require the complete entry of the hind paws of the mouse within the arm to be counted. The number and percentage of alternations (times the mouse visits each arm in turn without returning to an arm just visited) made by each mouse were then calculated. This task is structured around the innate preference shown by mice to novel stimuli and environments. As such, mice without cognitive impairment would perform more alternations than mice experiencing impairments in memory for arms they have just visited.

2-trial Y-maze

A variation on the typical Y-maze, this task consists of two trials in order to assess short-term spatial memory. In the first trial, one of the three arms was blocked, allowing the mouse to explore only two arms of the maze (Dellu, Contarino, Simon, Koob, & Gold, 2000). During the second trial, which took place after 60 minutes, the blockade was removed, and the mouse was able to explore all three arms. The amount and percentage of time spent in novel as opposed to familiar arms was then calculated. Similar to the Y-maze, it is expected that mice will spend more time in the novel arm than in familiar arms, and deviations from this expected norm would be indicative of potential cognitive impairment. By varying the inter-trial interval, different lengths of memory can also be evaluated.

Tyrosine hydroxylase (TH) immunohistochemistry

Frozen brains were sectioned coronally in a rostral to caudal direction at 40µm using a cryostat (ThermoScientific). Every third SNc section was collected for free-floating staining techniques in a well plate, with remaining sections mounted on superfrost plus slides for use in immunofluorescence analysis. Free-floating sections were stored in 0.01M PBS at 4ºC, while slide-mounted sections were stored at -20ºC until staining.

For TH staining, free-floating sections were blocked in 0.3% H_2O_2 for 30 minutes. Mouse TH primary antibody (1:2000; ImmunoStar) was diluted in 2% bovine serum albumin (BSA) and a blocking solution consisting of 5% normal goat serum (NGS), 10% Triton-X, and phosphate buffered saline (PBS). Sections were incubated at room temperature with primary antibody overnight. The following day, the secondary antibody, anti-mouse horseradish peroxidase (HRP) (1:200; Sigma-Aldrich), was diluted in 2% BSA, 5% NGS, 10% Triton-X, and

PBS and sections were incubated at room temperature for 4 hours. Sections were then incubated further with diaminobenzidine (DAB) for 5 minutes, at which time 0.3% H_2O_2 was added and incubation continued for an additional 10 minutes. After rinses in PBS, sections were mounted on superfrost plus slides and dried before being dehydrated with serial ethanol and xylene washes. Sections were then coverslipped using Permount (Fisher Scientific).

Assessment of TH positive cells within substantia nigra (SN)

Numbers of surviving dopaminergic cells were quantified using stereology-like analysis of TH+ cells. Serial sections were analyzed for 6-7 animals per group using Stereo Investigator (MBF Biosciences), which systematically and randomly selected a sample of fields for each section in which the number of TH+ cells were counted under a 60X oil immersion objective using a Zeiss AxioImager M2 microscope. Cells were counted within sections from multiple bregma levels (-2.70 to -3.80) for each animal. Sections from each bregma level were then compared across treatment groups to determine if any differences existed due to genotype or injection type. Analysis was carried out by an investigator blind to experimental groups.

Optical density of TH fibers

Striatal fibres were stained using TH immunofluorescence, as discussed in the section on immunofluorescence that follows. The optical density of TH fibers within the striatum was assessed using images obtained with a Zeiss AxioObserver D1 microscope. ImageJ (National Institutes of Health) was employed to trace the striatum and obtain optical density measurements, which were normalized to cortex. The experimenter remained blind to experimental groups.

Western blot

Western blot was used to investigate potential effects on antioxidant activity and levels of oxidative stress, homocysteine, α-synuclein, and methylation. Methionine adenosyltransferase 2A (MAT2A) produces the methyl donor *S*-adenosylmethionine (SAM) through the conversion of methionine, and therefore can provide an indication of methylation potential. Tissue from dorsal striatum (~20mg), SNc (~10mg), hippocampus (~20mg), and prefrontal cortex (PFC) (~40mg) was homogenized in RIPA buffer containing protease and phosphatase inhibitor (Cell Signalling). Samples were then incubated on ice for 20 minutes, after which they were centrifuged at 13,000 rpm for 15 minutes at 4ºC. BCA assays were then employed for protein quantification. SDS-PAGE was used to separate proteins, which were then transferred to nitrocellulose membranes.

After blocking in 5% milk, membranes were incubated with primary antibody overnight at 4ºC. Primary antibodies used include superoxide dismutase 2 (SOD2) (1:500; Life Technologies, expected molecular weight of ~26kDa), $gp91^{phox}$ (Gp91) (1:5000; AbCam, ~60-65 kDa), homocysteine (1:500; AbCam, ~135 Da), α-synuclein (1:4000; BD Biosciences, ~19kDa) MAT2A (1:2000; AbCam, ~44kDa), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:10 000; Cell Signalling, ~37kDa). The next day, membranes were incubated for 1 hour in secondary antibody, anti-rabbit or anti-mouse IgG (1:10 000, 800 Channel; LiCor Biosciences). Imaging was performed using Near Infrared (NIR) detection (Li-Cor Biosciences). Quantification of bands was determined using densitometry via ImageJ (National Institutes of Health) and normalized to GAPDH by an individual blind to the treatment groups.

Plasma homocysteine concentration

Cardiac blood samples were obtained at the time of euthanization. Samples were then processed and measured according to manufacturer's instructions, using the Advia Centaur Homocysteine kit and Centaur XP platform (Siemen's Canada), respectively.

Immunofluorescence: tyrosine hydroxylase and neuroinflammation

Immunofluorescence staining was performed to investigate both TH density and neuroinflammation mechanisms in this experimental model. Primary antibodies for anti-mouse TH (1:100, Immunostar), anti-mouse ionized calcium-binding adapter molecule 1 (Iba1) (1:100, AbCam), and anti-mouse cluster of differentiation 68 (CD68) (1:500, BioRad), the latter two of which act as markers of microglial activation, were diluted in 0.5% Triton-X. TH staining in the striatum was used to assess TH fiber density. Iba1 and CD68 staining in the SN and striatum was used to measure inflammation. After addition of primary antibody, sections were incubated at 4ºC overnight. The following day, sections were incubated with secondary antibody, either anti-rat or anti-rabbit Alexa Fluor 488 or 555 (Cell Signalling), for 2 hours at room temperature. Sections were then incubated with 4',6-diamidino-2-phenylindole (DAPI) (1:10 000) for 5 minutes and cover slipped using fluoromount (Sigma).

Iba1 and CD68 staining was visualized at 20X magnification using a Zeiss AxioImager M2 microscope. Iba1 positive cells that were co-labelled with CD68 were counted as positive cells using Fiji (National Institutes of Health). One to three brain sections were analyzed per animal, with 1-2 fields per section. Average numbers of positive cells were then determined for each animal. Cell counts were carried out by an individual blinded to the experimental groups.

Statistical analysis

Data was analyzed using IBM SPSS (version 20.0.0) and GraphPad Prism (version 7.0). Two-way analysis of variance (ANOVA) was used to assess numbers of TH+ cells, behavioural data, Western blot data, immunofluorescence cell counts, and plasma homocysteine concentrations. The two factors were genotype (*Mthfr*$^{+/+}$ and *Mthfr*$^{+/-}$) and injection treatment (saline and paraquat). In all analyses, a p-value of less than or equal to 0.05 was considered significant. Significant results were followed up using Tukey's pairwise comparisons where appropriate. Data are presented using mean ± standard error of the mean (SEM).

Results

Behavioural testing

Accelerating rotarod

In the week following the final saline or paraquat injection, the coordination of *Mthfr*$^{+/+}$ and *Mthfr*$^{+/-}$ mice was tested on the accelerating rotarod. A significant genotype effect was observed (Figure 4; $F_{(1, 26)}=5.385$, $p<0.05$), with Tukey's pairwise comparison ($p<0.05$) indicating *Mthfr*$^{+/-}$ paraquat mice had a significantly shorter latency to fall compared to *Mthfr*$^{+/+}$ saline mice.

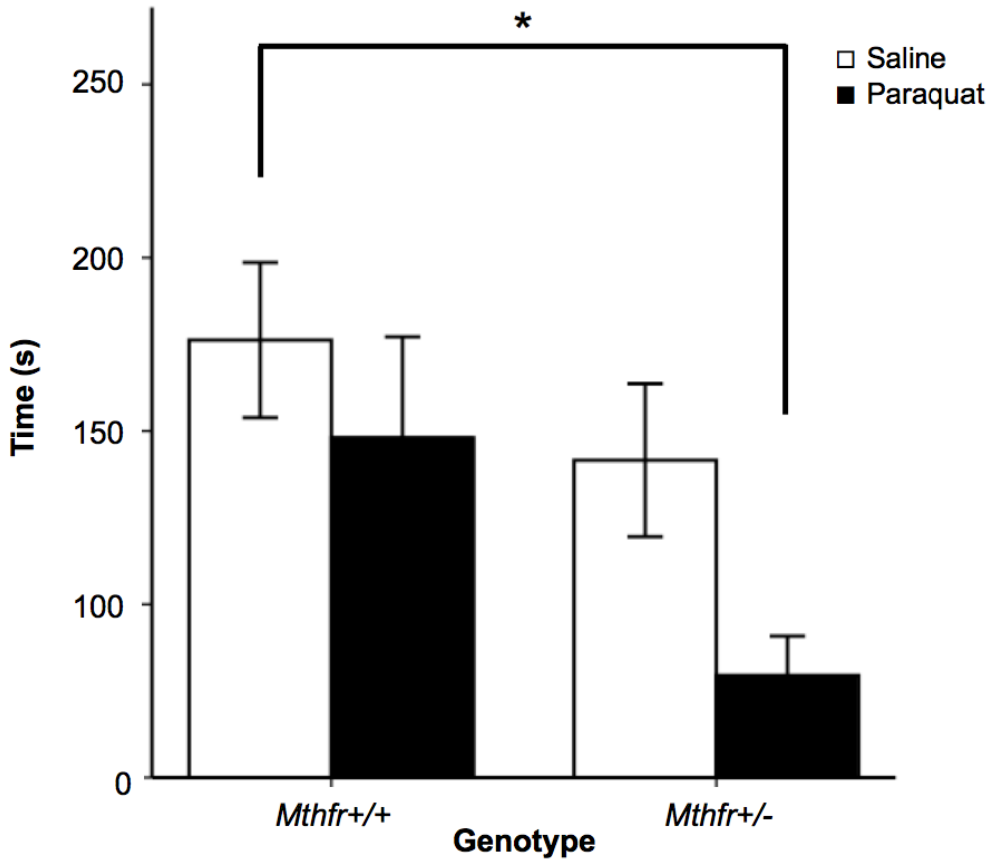

Figure 4: Impact of paraquat and MTHFR deficiency on coordination on the accelerating rotarod. Bars represent mean ± SEM of 5-7 mice per group. * $p<0.05$ for genotype effect in Tukey's pairwise comparison between *Mthfr*$^{+/+}$ saline mice and *Mthfr*$^{+/-}$ paraquat mice.

Spontaneous alternation and 2-trial Y-maze

Short-term spatial memory was assessed in mice using the spontaneous alternation Y maze and 2-trial Y maze. No differences in % alternations (Figure 5A; $p>0.05$) or number of entries (Figure 5B; $p>0.05$) were observed in the spontaneous alternation Y-maze. A trend toward a genotype effect for % time spent in the novel arm during the first minute of the second trial of the 2-trial Y-maze was observed, with *Mthfr*$^{+/-}$ mice spending less time than *Mthfr*$^{+/+}$ mice in the novel arm, but did not reach significance (Figure 5C; $F_{(1, 19)}=3.155$, $p=0.09$).

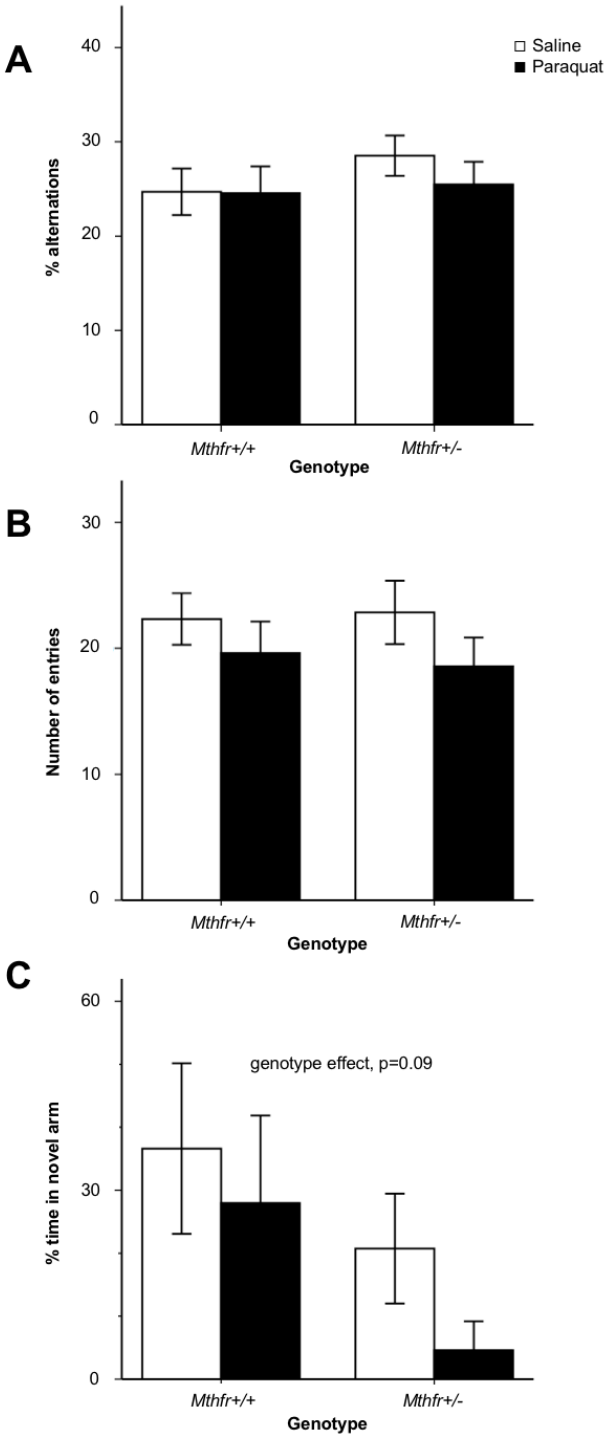

Figure 5: Impact of paraquat and MTHFR deficiency on short-term spatial memory. Percent alternations (A) and number of entries (B) were assessed in the spontaneous Y-maze task. Percent time spent in the novel arm during the first minute of the second trial was determined in the 2-trial Y maze (C). Bars represent mean ± SEM of 5-7 mice per group.

Neurodegeneration of dopaminergic neurons: tyrosine hydroxylase (TH) staining

TH staining was used to assess the numbers of remaining dopaminergic (DA) neurons. Representative images of TH staining in the substantia nigra pars compacta (SNc) are shown in Figure 6A. No significant differences in the numbers of TH positive cells in SNc were observed (Figure 6B, $p>0.05$). Additionally, there was no significant difference in the optical density of TH fibers in the striatum between groups (Figure 6C, $p>0.05$).

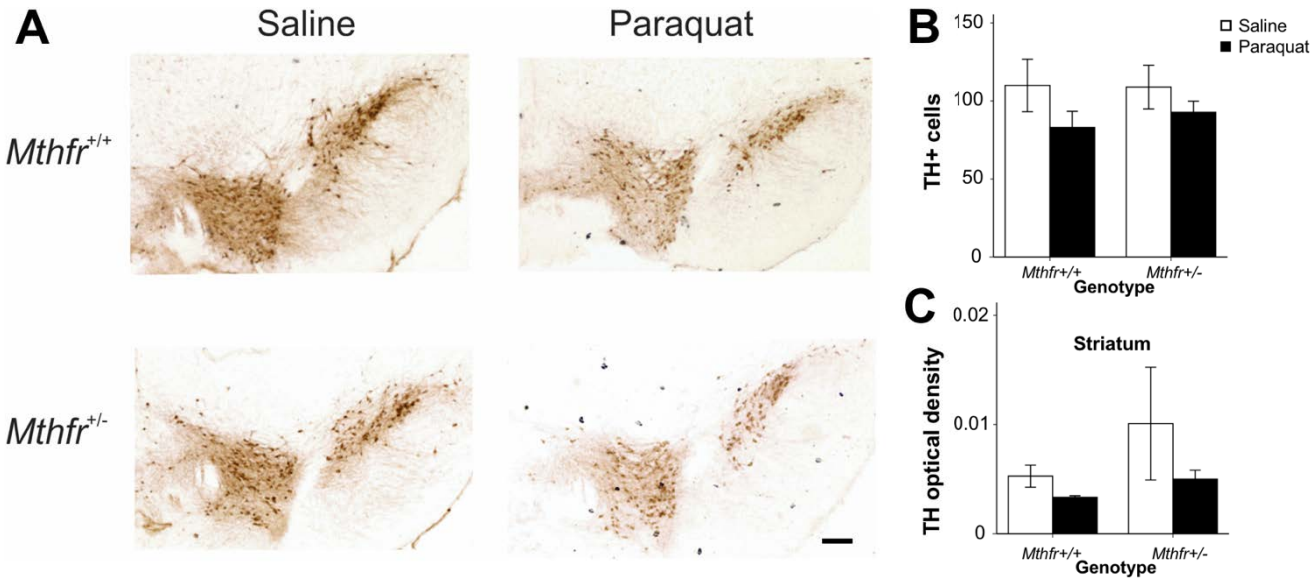

Figure 6: Effect of paraquat and MTHFR deficiency on neurodegeneration in the substantia nigra pars compacta (SNc) and striatum. Representative images show tyrosine hydroxylase (TH) staining in the SNc (A); magnification 2.5X, scale bar = 200µm. Quantification of TH positive cells in the SNc (B). Quantification of TH fiber optical density in the striatum (C). Bars represent mean ± SEM of 6-8 mice per group.

Neuroinflammation

Cluster of differentiation 68 (CD68) and ionized calcium-binding adapter molecule 1 (Iba1) are markers of macrophages and activated microglia (Korzhevskii & Kirik 2016). As such, they can provide an indication as to the level of neuroinflammation in brain regions of interest. The extent of CD68 and Iba1 expression was assessed via immunofluorescence staining in the substantia nigra (SN) and striatum. Cells with co-localization of CD68+Iba1 were quantified. Representative images of staining in SN are shown in Figure 7A. In the SN, significantly more cells were identified in paraquat treated mice compared to saline treated mice (Figure 7B; $F_{(1, 11)}=5.467$, $p<0.05$, group effect). Representative images of staining in the striatum can be seen in Figure 7C. In the striatum, no significant difference was observed (Figure 7D, $p>0.05$).

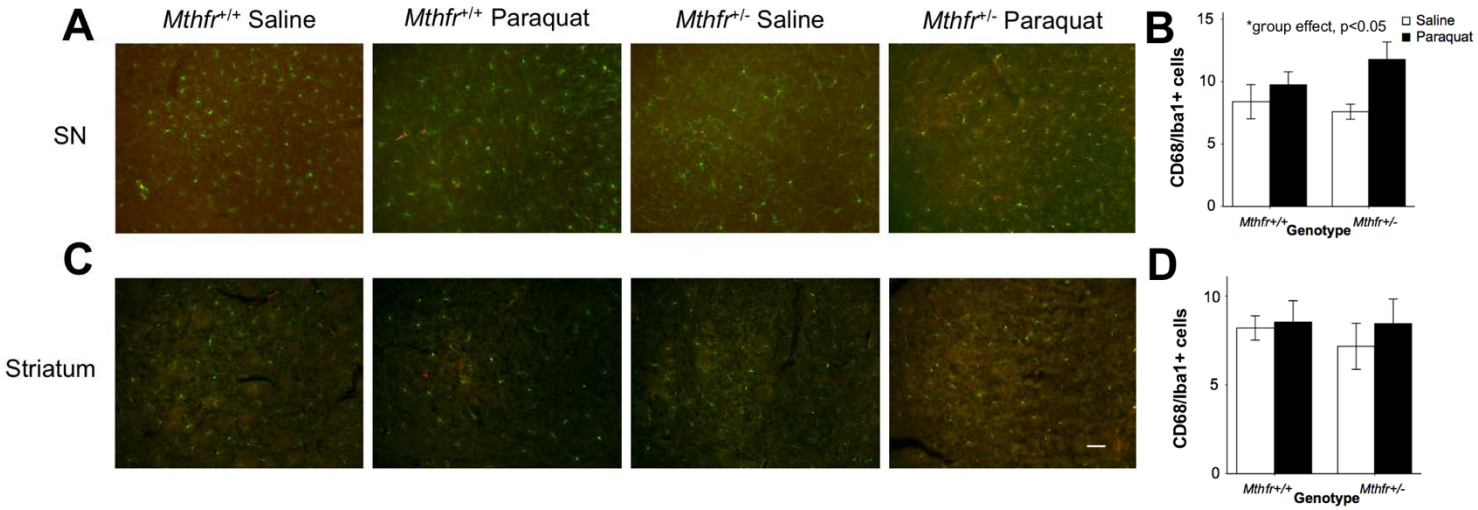

Figure 7: Effect of paraquat and MTHFR deficiency on cluster of differentiation 68 (CD68) and ionized calcium-binding adapter molecule 1 (Iba1) immunofluorescence staining in the substantia nigra (SN) and dorsal striatum. Representative images in the SN (A) show staining in *Mthfr*$^{+/+}$ and *Mthfr*$^{+/-}$ mice; magnification 20X, scale bar = 50µm. Quantification of the number of cells with CD68+Iba1 co-localization (B) was completed for both groups. Representative images in the striatum (C). Striatal CD68+Iba1 positive cells were similarly quantified (D). Data are expressed as mean ± SEM of 4 mice per group. * $p<0.05$ for group effect in 2-way ANOVA.

Homocysteine: plasma concentrations and levels in the brain

Elevated concentrations of homocysteine are a key feature of MTHFR deficiency (Chen et al., 2001). Plasma homocysteine concentration was measured in *Mthfr*$^{+/+}$ and *Mthfr*$^{+/-}$ mice treated with paraquat or saline. A significant genotype effect was found, with *Mthfr*$^{+/-}$ mice demonstrating higher levels of plasma homocysteine than *Mthfr*$^{+/+}$ mice (Figure 8A; $F_{(1, 22)}=19.811$, $p<0.001$).

Western blot analysis in the SN revealed no significant differences between groups (Figure 8B, $p>0.05$). Analysis of the dorsal striatum showed higher levels of homocysteine in *Mthfr*$^{+/-}$ mice compared to *Mthfr*$^{+/+}$ mice (Figure 8C; $F_{(1, 20)}=5.743$, $p<0.05$, genotype effect).

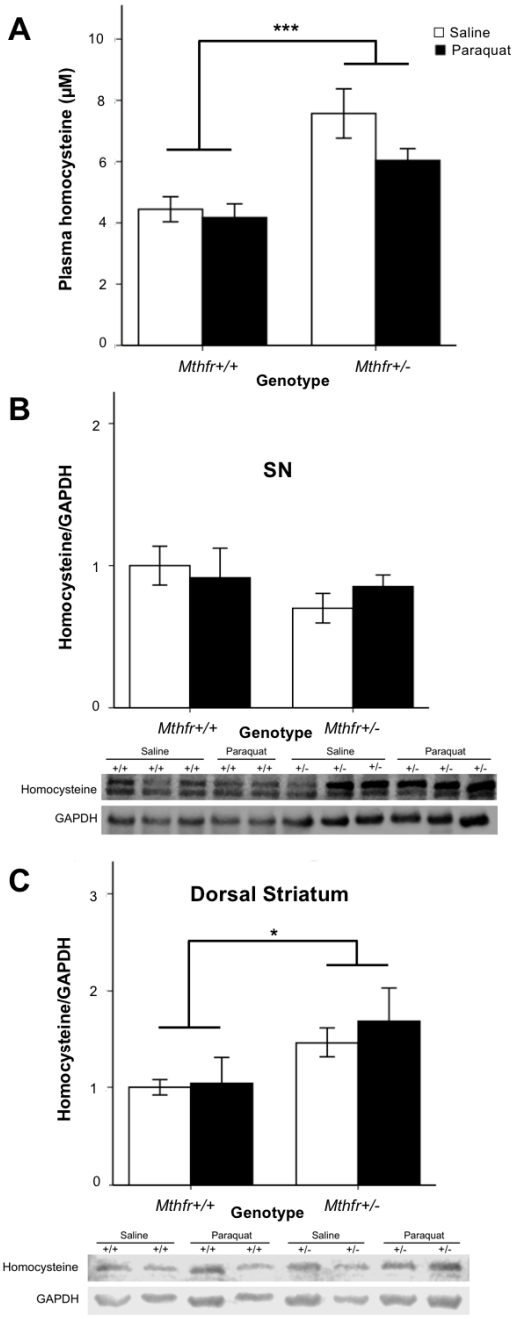

Figure 8: Effect of paraquat and MTHFR deficiency on homocysteine levels in plasma and brain tissue. Plasma concentrations of homocysteine (A). Amino acid levels of homocysteine in the substantia nigra (SN) (B) and dorsal striatum (C). Bars represent mean ± SEM of 4-7 mice per group. Representative images of bands are shown beneath each graph. * $p<0.05$ and *** $p<0.001$, genotype effect in 2-way ANOVA.

Antioxidant activity

Gp91

Both paraquat and MTHFR deficiency have been shown to contribute to increased oxidative stress through redox cycling and the activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, which produces superoxide (Dinis-Oliveira et al., 2006; Duan et al., 2002; Imamura et al., 2007; Purisai et al., 2007). Levels of Gp91, the catalytic subunit of NADPH oxidase (Yu et al., 1998), were therefore assessed in the SN and dorsal striatum. No significant differences were observed in the SN (Figure 9A, $p>0.05$). In the striatum, *Mthfr*$^{+/-}$ mice showed significantly greater levels of Gp91 than *Mthfr*$^{+/+}$ mice (Figure 9B; $F_{(1, 12)}=6.169$, $p<0.05$).

SOD2

Levels of the mitochondrial antioxidant superoxide dismutase 2 (SOD2), which breaks down superoxides such as those produced by paraquat (Cocheme & Murphy, 2008; Zelko et al., 2002), were similarly assessed to determine the level of response to oxidative stress. In the SN, no significant differences were seen (Figure 9C, $p>0.05$). In the striatum, *Mthfr*$^{+/-}$ mice had significantly greater levels of SOD2 compared to *Mthfr*$^{+/+}$ mice (Figure 9D; $F_{(1, 14)}=10.797$, $p<0.01$).

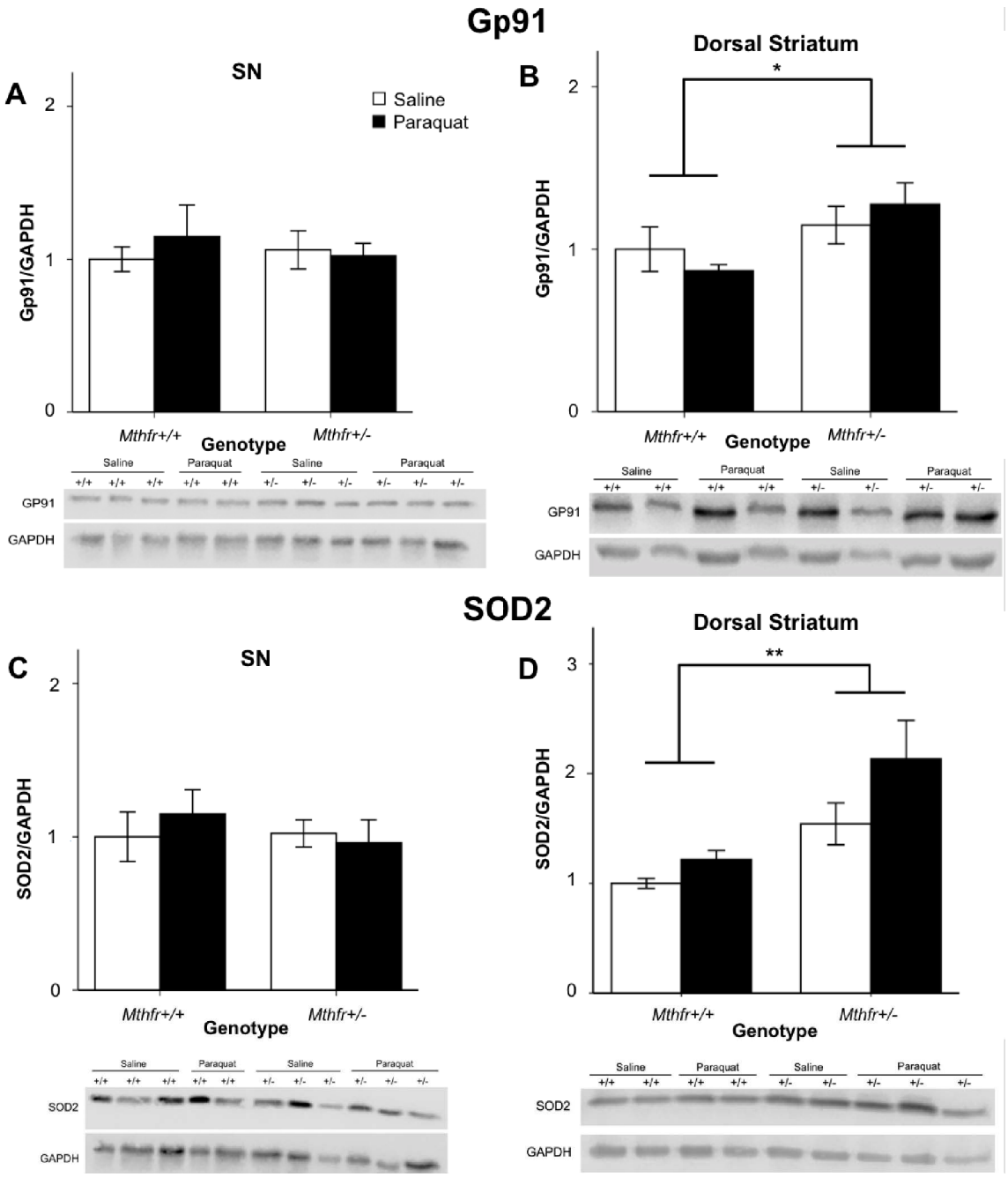

Figure 9: Impact of paraquat and MTHFR deficiency on antioxidant activity in substantia nigra (SN) and dorsal striatum. Protein levels of Gp91 in the SN (A) and dorsal striatum (B). Levels of SOD2 in the SN (C) and dorsal striatum (D). Bars represent mean ± SEM of 4-6 mice per group. Representative images of bands are shown beneath each graph.

* $p<0.05$ and ** $p<0.01$ for genotype effect in 2-way ANOVA.

Protein levels of α-synuclein

Increased aggregation of α-synuclein in the form of Lewy bodies has been characterized as a pathological feature of PD and has been shown to be produced in paraquat animal models (Baba et al., 1998; Manning-Bog et al., 2002; Spillantini, Crowther, Jakes, Hasegawa, & Goedert, 1998). Total levels of α-synuclein were determined and compared across groups in dorsal striatum (Figure 10A, $p>0.05$), hippocampus (Figure 10B, $p>0.05$), and prefrontal cortex (PFC) (Figure 10C, $p>0.05$). No significant differences were observed between groups.

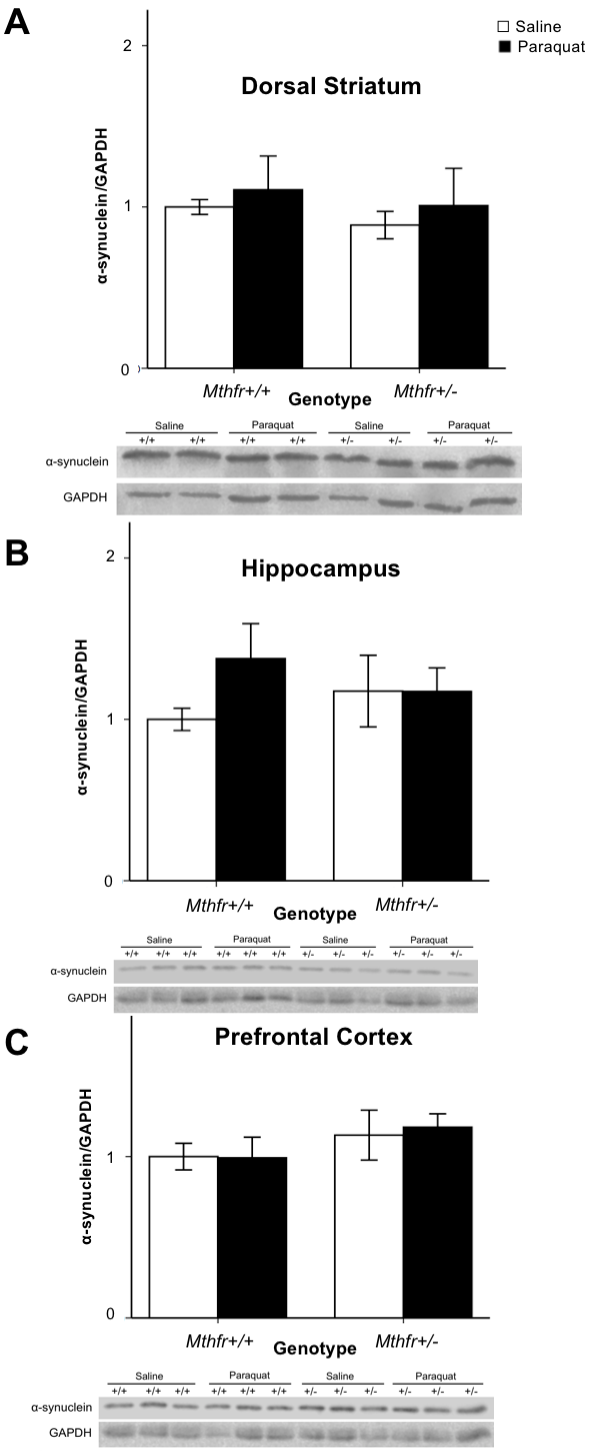

Figure 10: Effect of paraquat and MTHFR deficiency on α-synuclein levels in brain tissue. Protein levels in dorsal striatum (A), hippocampus (B), and prefrontal cortex (C). Bars represent mean ± SEM of 6 mice per group. Representative images of bands are shown beneath each respective graph.

Methylation

Methionine adenosyltransferase 2A (MAT2A) is an enzyme that converts methionine into the methyl donor *S*-adenosylmethionine (SAM) (Mato et al., 1997). Levels of MAT2A were assessed in the dorsal striatum. No significant differences in expression were observed across groups (Figure 11; $p>0.05$).

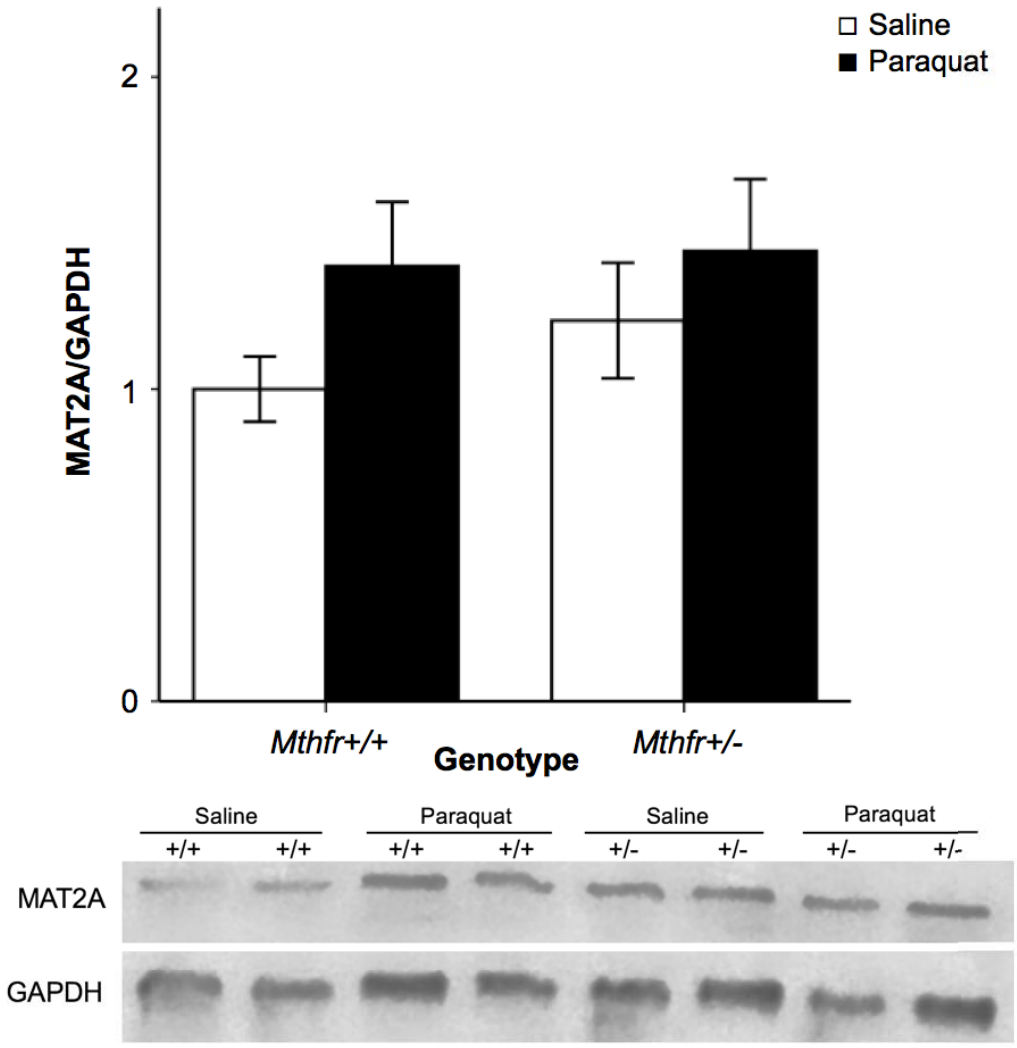

Figure 11: Effect of paraquat and MTHFR deficiency on methionine adenosyltransferase 2A (MAT2A) in dorsal striatum. Data represent mean ± SEM of 4 mice per group. Representative images of bands are shown beneath the graph.

Discussion

In this study, the impact of methylenetetrahydrofolate reductase (MTHFR) deficiency and paraquat was investigated in a mouse model that mimics the human 677C→T polymorphism in *MTHFR*. *Mthfr*$^{+/-}$ mice treated with paraquat showed impairments in coordination on the accelerating rotarod. These mice also showed a trend toward impairment in short-term spatial memory. Increased levels of reactive microgliosis were observed in the substantia nigra (SN) of mice treated with paraquat, while higher levels of homocysteine, gp91^{phox} (Gp91), and superoxide dismutase 2 (SOD2) were observed in the dorsal striatum of *Mthfr*$^{+/-}$ mice. Intriguingly, and contrary to the hypotheses of this study, significant dopaminergic (DA) neurodegeneration was not observed in the SN or striatum. No significant changes in α-synuclein or methionine adenosyltransferase 2A (MAT2A) levels were detected.

Increased oxidative stress and homocysteine levels in striatum may provide information regarding timing of disease progression

Most of the significant effects observed in this study occurred within the dorsal striatum rather than the SN, the region traditionally associated with Parkinson's disease (PD) pathology. There are many factors that may have contributed to such findings. Firstly, the lack of significant loss of DA neurons in the SN may be the consequence of the paraquat injection protocol used in this study. Many previous studies have used 9 injections of paraquat over a 3-week period (M. J. Kang, Gil, Lee, & Koh, 2010; Litteljohn et al., 2009; McCormack et al., 2002). Initially, this study planned to utilize the same procedure. However, due to increased animal mortality in pilot experiments, the protocol was modified to 6 injections. It is therefore possible that this injection protocol did not have the potency required to induce a significant

amount of degeneration. However, a similar 6 injection procedure has been employed by other studies, and has been able to elicit significant neurodegenerative effects (Jiao et al., 2012; Rudyk et al., 2017; Thiruchelvam et al., 2003), allowing for the possibility that it is other factors that are responsible for this difference.

It is conceivable, for instance, that the combination of paraquat and MTHFR deficiency, which this study hypothesized would interact to exacerbate DA degeneration, was not sufficient to cause degenerative effects. Multiple researchers have employed PD modeling methods based on a "multi-hit" hypothesis with regard to PD (discussed, for example, in Sulzer, 2007). In such cases, more than one factor associated with PD development is used in combination in order to trigger Parkinsonian degenerative effects. Examples of such models include the use of lipopolysaccharide (LPS) and paraquat (Mangano et al., 2011; Mangano & Hayley, 2009), maneb and paraquat (Kumar, Leinisch, Kadiiska, Corbett, & Mason, 2016; Wills et al., 2012), dopamine and paraquat (Feng & Maguire-Zeiss, 2011), and genetic models of α-synuclein accumulation with LPS (Gao et al., 2011), to name a few. Such multi-hit models are particularly popular in the case of paraquat, which typically shows relatively modest degenerative effects, reducing DA neurons by 20-30% (Kuter et al., 2007; Mangano et al., 2011, 2012; Mangano & Hayley, 2009; McCormack et al., 2002). It can prove advantageous when the aim is to determine the potential exacerbating effects of factors of interest and make it feasible to detect such differences. It is possible that, unlike these other models, MTHFR deficiency did not provide sufficient exacerbating effects. However, even if this was the case, it still would have been expected for paraquat to produce a modest amount of discernable degeneration.

An alternative explanation for the findings of this study relates to the theory that PD pathology first affects the striatal region of the brain, and that the characteristic SNc

degeneration occurs later in disease progression. Imaging studies on patients, reviewed and summarized by Cheng et al. (2010), suggest that more striatal terminals have been lost at the time of PD diagnosis than SNc cells (Cheng, Ulane, & Burke, 2010). Similarly, in a paraquat mouse model of PD, Mangano and Hayley (2009) observed greater effects of paraquat in the striatum in terms of loss of terminals than on cell bodies in the SNc. Such observations might suggest that terminals began degenerating at an earlier time, and thus more loss can be seen in this region. A study regarding patients with dementia with Lewy bodies provides a potential mechanism that may account for this difference, demonstrating that presynaptic accumulation of α-synuclein occurs in these patients (Kramer & Schulz-Schaeffer, 2007).

Experimental models have provided additional information concerning this process. Using an adeno-associated virus (AAV) vector to overexpress human α-synuclein in a rat model of PD, researchers found that changes in striatal axons and terminals were apparent prior to cell loss in the SNc (Decressac, Mattsson, Lundblad, Weikop, & Björklund, 2012). These changes were also accompanied by increases in α-synuclein aggregations. In another rat model of α-synuclein pathology, Stoica et al. (2012) observed that nerve terminals were first affected while the cell bodies of neurons were preserved. The timing of events seemed to begin with α-synuclein accumulation in presynaptic terminals, followed by axonal degeneration, with neurodegeneration as the end result (Stoica et al., 2012). Chung et al. (2009), also using an AAV α-synuclein overexpression model, detected pathology in striatal axons after 4 and 8 weeks, as well as reductions in transport proteins in these axons, but no SNc DA neuron loss.

Other studies involving animal models reviewed in Tagliaferro and Burke (2016) also demonstrate that first effects are often seen in axons and the striatum, followed by changes in the SNc at a later time point. In a review, Calo et al. (2016) propose that α-synuclein aggregation at

the synapse may spread in a retrograde fashion to the cell body, eventually leading to the death of the neuron through, for example, effects on SNARE proteins, vesicles, and neurotransmitter release. As such, the results observed in this study may represent an early stage of the disease, when most effects are limited to striatal regions. The higher levels of Gp91 and SOD2 observed in the dorsal striatum of *Mthfr*$^{+/-}$ mice suggest an increase in oxidative stress in this region. Homocysteine was also elevated in this area of the brain and may contribute to oxidative stress in models of PD (Duan et al., 2002; Imamura et al., 2007), though such studies typically use higher concentrations of homocysteine than would occur physiologically. Although we observed no change in total α-synuclein levels, it is possible that this increased oxidative stress elicited a change in the conformation of α-synuclein in the striatum at this point in time, and that such altered α-synuclein may then have been transported to the cell body in the SN. Such an effect of paraquat on the conformation of α-synuclein has been demonstrated, with paraquat increasing fibril formation (Uversky et al., 2001). If this study had followed mice for a longer period of time, more pronounced SN effects may have been observed.

However, it is worth noting that there was not a complete lack of observed effects in the SN in this study. Increased inflammation in terms of increased reactive microglia was seen in response to paraquat in this region. Such results are consistent with previous studies employing the paraquat model. For instance, Purisai et al. (2007) demonstrated that a single dose of paraquat induced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and activated microglia in the SNc, assessed by cells immunoreactive for Gp91. Other markers, such as CD11, have also shown paraquat to activate microglia (Mangano & Hayley, 2009; Mangano & Hayley, 2011). Research has demonstrated that the SN has a particularly dense and numerous population of microglia, more so than other brain areas (Kim et al., 2000; Lawson et al., 1990). Work by

Kim et al. (2000) has also shown that increased response to LPS in the SN is presumably due to the increased number of microglia present, rather than microglia from this region producing greater amounts of inflammatory factors. Therefore, it is possible that we observed a significant increase in microglial activation in this region due to the greater numbers of microglia present. This may explain why a significant increase was not also seen in the striatum.

There are several other mechanisms that may account for this observed effect. If we abide by the theory that increased oxidative stress in the dorsal striatum may be triggering the accumulation of small α-synuclein aggregates in presynaptic terminals, and that such aggregates are then transported back to the cell body in the SN, this may explain what might be causing the activation of microglia in the SN. In a cell culture study, Zhang et al. (2005) suggested that increased α-synuclein can activate microglia. Thus, the transported α-synuclein aggregates may be triggering the SN microglial activation, which may then exacerbate DA neurodegeneration. The plausibility of this theory is supported by the finding that increasing the percentage of microglia in culture has been shown to enhance neurotoxicity to DA cells (Zhang et al., 2005). In their study, Zhang et al. (2005) also suggested that microglial enhancement of degeneration was dependent on microglial phagocystosis of α-synuclein, the activation of NADPH oxidase, and its consequent production of ROS. $p47^{phox-/-}$ (PHOX) knockout mice were resistant to these effects, showed less pronounced increases in reactive oxygen species (ROS), and treatment with SOD helped to attenuate neurotoxicity, all pointing to a significant role for oxidative stress.

Other studies also implicate interactive effects between oxidative stress, microglia, and α-synuclein. Maneb and paraquat co-treatment has been shown to provoke α-synuclein radical formation and activated microglia, which seemed to be dependent at least in part on NADPH oxidase (Kumar et al., 2016). Gao et al. (2011), using a mouse model of α-synuclein

overexpression treated with LPS, demonstrated increased expression of multiple inflammatory markers in the SN and striatum of mutant mice, and found that inhibiting NADPH oxidase was effective at reducing these effects, as well as degeneration. These findings support a role for NADPH oxidase in the degenerative effects seen in such models, and the present study's observation of increased levels of Gp91, the NADPH oxidase subunit, in the striatum could be consistent with this. However, even if it is the case that α-synuclein is not at all affected in this study's MTHFR deficient paraquat model, the effects of the oxidative stress in the striatum are likely still impacting the SN to some degree. If it remains suspected that some process is occurring within SN cell bodies and that damage may be seen at a later time point, these SN neurons may still release some factor, either α-synuclein or another substance, that activates microglia and leads to progressive degeneration in this manner (Zhang et al. 2005). Though not statistically significant, there appeared to be a reduction in TH+ cells following paraquat treatment in this study that could imply some neuronal death occurring. It is possible that microglia were activated by this, in a reactive microgliosis response (Taetzsch and Block, 2013). This may also explain why the change in microglial activity showed a treatment effect such that it was seen in both groups treated with paraquat. Microglial activation in response to cell damage or death may also account for such effects being seen in the SN, the location of the cell bodies, and not the striatum. Again, this may have led to progressive effects on DA neurons if the study had continued and observed mice over a longer period of time.

No difference in total α-synuclein may not account for all possible changes in the protein

Following paraquat treatment in both wild-type and MTHFR deficient mice, no increases in total α-synuclein levels were seen. This result appears to be in contrast to previous studies

that have shown paraquat to elicit the formation of α-synuclein inclusions (Kumar et al., 2016; Manning-Bog et al., 2002; Manning-Bog et al., 2003; Wills et al., 2012), although some studies have also not demonstrated significant increases in α-synuclein following paraquat (Feng and Maguire-Zeiss, 2011).

What might explain such seemingly contradictory findings? Firstly, direct comparisons between studies may in some cases be difficult, as different researchers often are utilizing different measures of α-synuclein. The protein can be found in multiple conformational states: as a monomer, oligomer, protofibril or fibril. It can also be post-translationally modified via phosphorylation or nitrosylation. Such different forms can change the reactivity to assessment measures like antibodies. Landeck et al. (2016) summarize several different antibodies for α-synuclein and phosphorylated α-synuclein, for example. It is possible that, while total levels of α-synuclein were not significantly altered by paraquat and/or MTHFR deficiency, changes in the conformation of existing α-synuclein may have occurred that were not detected by the methods employed in this study. Utilizing markers for such different forms may have yielded different results. It has been suggested in past research that the percentage of conformers altered in PD is likely a small component of total α-synuclein and may be difficult to detect or differentiate from physiological α-synuclein with traditional immunohistochemical or Western blot methodology (Caughey & Lansbury, 2003; Feng and Maguire-Zeiss, 2011; Schulz-Schaeffer, 2010). It has also been demonstrated that α-synuclein changes conformation based on its environment (reviewed in Deleersnijder et al., 2013). Given that we did not employ additional markers for different conformers of α-synuclein, it is certainly possible that we may have missed a change in a specific type of α-synuclein.

In terms of looking specifically at the traditional inclusions observed in disease states, research indicates that post-translational modifications may differentiate them from physiological α-synuclein. In human patients of dementia with Lewy bodies, Fujiwara et al. (2002) demonstrated that α-synuclein is phosphorylated at serine 129 in lesions. The same study also suggested that this phosphorylated form increased the formation of fibrils *in vitro*. Another study using cells expressing human α-synuclein found that inclusions within cell bodies of SNc neurons contained α-synuclein phosphorylated at serine 129 of the protein (Decressac et al., 2012). It therefore may be useful to employ markers for phosphorylated α-synuclein to investigate changes in Lewy bodies explicitly.

Multiple studies have also claimed that other, less thoroughly investigated conformations of α-synuclein may be the factor that plays a significant role in PD pathology, rather than Lewy bodies per se. Such research has proposed that it is not Lewy bodies that are toxic to cells, but rather intermediate α-synuclein aggregations. In a study examining α-synuclein in dementia with Lewy bodies, researchers utilized a novel methodology to detect α-synuclein distribution that was not limited only to Lewy bodies (Kramer & Schulz-Schaeffer, 2007). They observed an accumulation of smaller α-synuclein aggregates within presynaptic terminals, which were much more numerous than the Lewy bodies; only a small fraction of the α-synuclein detected was within Lewy bodies (Kramer & Schulz-Schaeffer, 2007). These smaller aggregates may represent the intermediate stage in α-synuclein accumulation, which some researchers refer to as protofibrils (Caughey & Lansbury 2003, Milber et al., 2012). Winner et al. (2011) examined the effects of aggregation of this intermediate stage in comparison to fibril accumulation via mutations that promote either the former or latter *in vivo* in a rat model. The mutation that promoted the formation of protofibrils resulted in greater DA degeneration in the SNc (Winner et

al., 2011). These results support the theory that it is this stage, rather than Lewy bodies themselves, that can prove toxic to DA neurons. A potential mechanism for this was explored in a study conducted by Zhang et al. (2005), which found that intermediate α-synuclein aggregates activated microglia, increasing their production of superoxide, and were therefore more toxic to neurons than later stage fibrils. It is possible that such intermediate forms of α-synuclein had begun to form in this study, but were not distinguishable from physiological α-synuclein and were not detected as a result.

Additionally, it has been theorized that α-synuclein-containing Lewy body inclusions may not, in fact, be causative in the pathology of PD. Rather than eliciting detrimental effects to DA neurons, there is the possibility that they may actually be a neuroprotective response that limits the extent of damage. Support for such a purpose include the finding of no relationship between the density and distribution of Lewy bodies and the cell death of nigral neurons in PD patients (Parkkinen et al., 2011). If such inclusions were causative, one would expect increased density to mean greater cell death. Other researchers have also questioned a definitive link between cell death and Lewy bodies. Milber et al. (2012) observed that, in humans, motor symptoms and dysfunction of DA neurons preceded α-synuclein inclusion formation, while Manning-Bog et al. (2003) reported an increase in α-synuclein following paraquat treatment in mice, but no observed degeneration of cells. Tomkins and Hill (1997) looked at whether apoptosis occurred more in neurons with Lewy bodies or neurons without such inclusions in patients with Lewy body disorders, including PD. Signs of apoptosis were frequently – and, in fact, more often – seen in neurons without Lewy bodies, suggesting that it is possible for neurons to die before they form.

Supporting a potential protective role, McNaught et al. (2002) showed that Lewy bodies share characteristics with aggresomes, inclusions generated to protect cells by sequestering toxic material. Such inclusions have accumulated levels of proteasome proteins used to break down proteins that may otherwise lead to cytotoxic effects. This study also showed that inhibiting proteasomal function in culture resulted in the formation of aggresome and Lewy body-like inclusions (McNaught et al., 2002). Accumulation of other aggresome-related proteins in Lewy bodies has also been demonstrated (Miki et al., 2011). If Lewy bodies are, as some have suggested, an initially protective mechanism aimed at limiting the extent of degeneration, it could be that the lack of change seen in this study is related to the lack of significant degeneration that was also observed. It is possible that at a later time point, when degeneration may begin to take effect in a more robust way, increases in α-synuclein would occur as the Lewy body response increased. If this is the case, no increases might have been seen in the current study because there was no significant degeneration, as of yet, to respond to.

In summary, the assessment of total levels of α-synuclein as used in this study was likely not sensitive to all potential changes in the protein that could have conceivably occurred. As such, conclusions regarding the degree to which it was affected should be reserved for a time when further investigations employing more sensitive and specific methodology have been completed.

Effect of MTHFR deficiency and paraquat on methylation

MTHFR is necessary for many cellular functions, beyond maintaining homocysteine concentrations. This study therefore attempted to assess methylation, in order to determine if it was elevations in homocysteine that were likely causing the observed effects in this study, or if

MTHFR's roles in other processes, such as methylation, were also involved. MAT2A was used as a marker for methylation, as it converts methionine into the methyl donor *S*-adenosylmethionine (SAM) (Mato et al., 1997). It was hypothesized that, with higher levels of homocysteine, reduced levels of methionine might lead to reduced SAM and therefore a reduced methylation potential. SAM has been shown to inhibit MAT2A levels (Garcia-Trevijano et al., 2000; Lu et al., 2000; Schrötter et al., 2012), meaning an increase in MAT2A might have been expected if this was the case. However, no significant differences in MAT2A were observed in the dorsal striatum.

This may indicate that methylation is not significantly affected by MTHFR deficiency and paraquat in this study. It is also possible that measuring only MAT2A levels was not sufficient to detect changes in methylation and methylation potential. Perhaps, while protein levels of MAT2A were not significantly altered, the activity of the enzyme was affected. In this case, enzyme assays such as those used by Pegg et al. (2011) might prove useful. In addition, assessment of other players in the methylation process would be beneficial in order to more accurately determine any changes in such processes. For example, measuring levels of SAM and SAH directly, via methods such as high-performance liquid chromatography (HPLC), could provide more definitive information about the levels of such substances and the methylation potential. This method has proven useful in previous studies (Eto & Kimura 2002; Pegg et al., 2011; Zhao et al., 2001). Additionally, methodology such as the cytosine-extension assay method has been used by Chen et al. (2001) to assess global DNA hypomethylation changes in MTHFR deficient mice and could also provide useful data regarding methylation changes in this model.

Overall, further investigation is warranted before definitive claims regarding the impact of MTHFR deficiency and paraquat on methylation can be made.

Conclusions

The findings of this study provide for an intriguing array of possible conclusions. MTHFR deficient mice showed an increased vulnerability to paraquat in terms of impairment in coordination. Deficient mice also experienced a trend toward impairment in short-term spatial memory, in conjunction with increases in homocysteine and oxidative stress in the dorsal striatum. Increased inflammation in the SN was seen in response to paraquat. These changes occurred in the absence of observable neurodegeneration or changes in α-synuclein. Most of these results point toward a "dying back" mechanism of disease development, with the striatum showing the initial effects of increased oxidative stress, likely due to the combined effects of environmental exposure to paraquat and elevated homocysteine due to genetics. Potentially because this study ended only ~10 days after final injections, limited effects were observed in the SN, including a lack of significant neurodegeneration. It remains possible that more pronounced effects would have been seen at later time points, which should be investigated in future experiments. The timing of events, as well as specific changes in α-synuclein and methylation, should also be points of focus for future investigations, particularly given the fact that many of the involved processes can trigger and/or exacerbate one another. Such research would be useful for determining if the events of this study might be explained by increased oxidative stress in the striatum increasing the amount of small α-synuclein protofibril aggregations presynaptically, which may have been transported to cell bodies in the SN where they triggered the activation of microglia, in a process that may have eventually led to neurodegenerative effects. Elucidating

the timing and interplay between these mechanisms may prove useful in the development of more effective treatment strategies for PD, which might target the initial mechanism responsible for this cascade of effects. These results also underline the significance of interaction between environmental and genetic factors in PD, particularly how one's genetic background can influence susceptibility to further environmental risk factors, and highlight the need to consider such interactions when faced with individuals in a clinical setting.

Future directions

This study resulted in several interesting findings that may provide multiple avenues for future research. Firstly, as mentioned previously, it would be particularly worthwhile to perform this experiment over longer time periods. This would help narrow the potential explanations for the results of this study in its present form; specifically, it would determine if MTHFR deficiency will produce significant DA degeneration at a certain point following paraquat injections, or if the combination of these two factors is insufficient to produce effects at the level of neurodegeneration. The former could indicate that the results observed in this study represent a model of an early form of the disease, preceding degenerative effects that follow at later stages. Examining brain tissue at multiple time points could provide additional information regarding the timing of paraquat-induced changes in the regions and measures assessed in this study. For example, it could help determine which changes occur first, the increases in homocysteine and Gp91 in the striatum, or the microglial activation in the SN. Such knowledge would help to elucidate the mechanisms driving neurodegeneration in this model and indicate whether a "dying back" process that is initiated in the striatum may be occurring.

The timing of such changes is important to determine as many factors assessed in this study can have reciprocal, perpetuating effects on one another. For example, oxidative stress may cause α-synuclein misfolding and the degeneration of neurons, which themselves may then release α-synuclein aggregates that can then activate microglia and damage neurons further (Taetzsch & Block, 2013; Zhang et al., 2005; Kumar et al., 2016). Activated microglia can themselves increase oxidative stress through NADPH oxidase-mediated ROS production (Gao et al., 2011; Zhang et al., 2005). Identifying the first phase may be critical in designing the most effective means of treatment. Depending on where and what the initial site and sign of damage is, treatment methods could be developed that target that specific region and the change associated with it. For example, if enhanced oxidative stress in the striatum due to paraquat and homocysteine is occurring first, treatments that augment antioxidant activity may prove useful. If microglial activation in the SN is the primary feature of this model, anti-inflammatory measures that target this region might be more effective. Assessing changes over a prolonged period could also assist in demonstrating if this model recapitulates the progressive nature of PD, with increasing degeneration occurring over time. There is also precedent for more long-term studies of pesticide PD models. Thiruchelvam et al. (2003) saw progressive degeneration of tyrosine hydroxylase (TH) positive cells in response to paraquat 2 weeks and 3 months following the end of treatment, and some studies using 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) models suggest that the time when the greatest DA cell loss can be observed is 3-4 weeks after treatment is complete (Meredith and Rademacher, 2011). Such studies provide rationale for this longer timeline.

Future studies should also aim to further investigate the contributions of α-synuclein and the impact on methylation by employing a range of measures that might detect differences that

may have been present but not readily apparent in this study. As discussed, it is possible that changes in α-synuclein conformation and/or methylation did occur and were involved in the observed results of this study. Because many of the measures in this study can reciprocally affect one another, having accurate assessments of such potential changes is critical to better understand the timing of the interplay between these processes.

Another worthwhile future direction would involve looking at effects of aging in this model. Because the majority of PD cases are diagnosed after age 65 and the likelihood of developing PD increases with age (Wong et al., 2014), the effects of this factor are important to consider in an animal model. It may be that degeneration is more readily produced in older mice than the 3-month-old young adults used in this study. Indeed, Thiruchelvam et al. (2003) examined the effects of paraquat or maneb alone or in combination on 5- and 18-month-old mice. The 18-month-old mice showed motor impairment in response to paraquat alone, while younger 5-month-old mice had to be treated with paraquat and maneb together in order to see similar effects (Thiruchelvam et al., 2003). 18-month-old mice also showed the greatest reductions in TH+ cells when assessed after 2 weeks and 3 months following treatment (Thiruchelvam et al., 2003). It would be interesting to see if MTHFR deficient mice would react similarly. In addition, research has suggested some features of PD may be modulated by aging, such as changes in α-synuclein conformers and axonal degeneration processes (Bobela et al., 2015; Salvadores et al., 2017). Thus, aging may provide an additional "hit" to mice in this model and affect the progression of damage. A study conducted on aged mice has also observed unexpected effects of chronic low dose paraquat treatment, suggesting such a treatment regimen may trigger different, perhaps even adaptive, processes in these mice (Rudyk et al., 2017).

Therefore, age certainly remains a factor that is of critical importance to explore in further detail in future research.

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