AUSTRALIAN PESTICIDES AND VETERINARY MEDICINES AUTHORITY

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CHEMICAL REVIEW PROGRAM

REVIEW OF THE MAMMALIAN TOXICOLOGY

AND

METABOLISM/TOXICOKINETICS

OF

PARAQUAT

Supplement II: NEUROTOXICOLOGY

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This report was undertaken by the Office of Chemical Safety (OCS) at the request of the Australian Pesticides and Veterinary Medicines Authority (APVMA), under the Chemical Review Program. Paraquat is under review because of concerns over the potential risk to human health and the environment.

A draft toxicology review was submitted to APVMA in 2003 and was subsequently revised and updated in 2009. At that stage, the OCS identified published reports of neurotoxicity in mice following treatment with paraquat, where the histological lesions observed in the brains of mice had similarities to the neurological lesions observed in patients with Parkinson's disease.

In 2010, the APVMA requested that OCS prepare a supplementary report specifically considering the potential neurotoxicity of paraquat, up to and including the latest information, and including relevant published literature. This assessment was extended in 2013 to include new unpublished studies investigating potential neurological effects in mice,

In 2015, the complete review of the toxicology of paraquat was finalised and the report structured into three parts:

- Summary Report
  - *Comprises an overview of all relevant data available on paraquat relating to human health.*

- Supplement I: Toxicology
  - *Comprises a detailed technical report on paraquat toxicology (excluding neurotoxicity).*

- Supplement II: Neurotoxicity
  - *Comprises a detailed technical report on paraquat neurotoxicity [this report].*

These three reports should be considered together. This report 'Supplement II: Neurotoxicity', is a complete evaluation of the data pertaining to the neurotoxicity of paraquat provided to the OCS, together with new information available in the public domain.

1 ABBREVIATIONS

Time

d	Day
h	Hour
min	Minute
mo	Month
wk	Week
s	Second
yr	Year

Weight

bw	Body weight
g	Gram
kg	Kilogram
mg	Milligram
µg	Microgram
ng	Nanogram
wt	Weight

Length

cm	Centimetre
m	Metre
µm	Micrometre
mm	Millimetre
Nm	Nanometre

Dosing

id	Intradermal
im	Intramuscular
inh	Inhalation
ip	Intraperitoneal
iv	Intravenous
po	Oral
sc	Subcutaneous
mg/kg bw/d	mg/kg body weight/day

Volume

L	Litre
mL	Millilitre
µL	Microlitre

Concentration

M	Molar
ppb	Parts per billion
ppm	Parts per million

Brain - dopamine metabolites, enzymes and neurotransmitters

DA	Dopamine
DAT	Dopamine transporter
DOPAC	3,4-dihydroxyphentlacetic acid
3-MT	3-methoxytyramine
HVA	Homovanillic acid
5-HT	Serotonin
5-HIAA	5-hydroxyindoleacetic acid
GABA	γ-aminobutyric acid
GAD	Glutamic acid decarboxylase
PENK	Proenkephalin
NA	Noradrenaline
NMDA	N-methyl-D-aspartate
TH/TH+ or TH-	Tyrosine hydroxylase/ tyrosine hydroxylase positive or negative
TH-ir	Tyrosine hydroxylase immunoreactivity

Anatomy

BBB	Blood brain barrier
CNS	Central nervous system
CP	Caudate putamen
PFC	Prefrontal cortex
SN	Substantia nigra
SN pc	Substantia nigra pars compacta
VTA	Ventral tegmental area
VM	Ventral mesencephalic

Chemistry

Am Cu Ag	Amino Cupric Silver
DAPI	4',6'-diamidino-2-plenylindole
ECD	Electrochemical detection
ETC	Electron transport chain
GFAP	Glial fibrillary acidic protein
HPLC	High performance (or pressure) liquid chromatography
IBA-1	Ionised Calcium Binding Adaptor Molecule 1
IgG	Immunoglobulin G
LSC	Liquid scintillation counting
MS	Mass spectrometry
MPP+	1-methyl-4-phenyl-pyridinium ion
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahdyropyridine
NAD(PH)	Nicotinamide adenine dinucleotide (phosphate)
NM	Neuromelanin
PQ/PQ+	Paraquat/paraquat cation
RIA	Radioimmunoassay
TLC	Thin layer chromatography
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labelling

Terminology

AUC	Area under the curve
CI	Confidence interval
F	Female
GLP	Good Laboratory Practice
LC_{50}	Median lethal concentration
LD_{50}	Median lethal dose
LOEL	Lowest Observed Effect Level
LOAEL	Lowest Observed Adverse Effect Level
MTD	Maximum tolerated dose
M	Male
NOEL	No Observed Effect Level
NOAEL	No Observed Adverse Effect Level
PBPK	Physiologically-based pharmacokinetic
QA	Quality Assurance
RSD	Relative standard deviation
SD	Standard deviation or Sprague Dawley
SEM	Standard error of the mean

Organisations & Publications

APVMA	Australian Pesticides and Veterinary Medicines Authority
FAO	Food and Agriculture Organization (United Nations)
JMPR	The Joint FAO/WHO Meeting on Pesticide Residues
OCS	Office of Chemical Safety
OECD	Organization for Economic Co-operation and Development
WHO	World Health Organization

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2 EXECUTIVE SUMMARY

In a 2009 draft toxicology review report on paraquat, the OCS identified a potential issue regarding published reports of neurotoxicity in mice following treatment with paraquat where histological lesions in the brain had some similarities with neurological changes observed in patients with Parkinson's disease. Previous toxicological evaluations of paraquat have identified lesions in the lungs as the most sensitive toxicological endpoint, which has been used to establish health-based guidance values. This supplementary report examines the available data and provides conclusions based on a weight-of-evidence assessment of relevant studies. In this regard, the substantial unpublished toxicological database on experimental animals was augmented by recent developments in the methodology for observing/quantifying neurological damage, which has provided a much clearer picture of the neurotoxicity potential of paraquat.

Paraquat is structurally similar to the known dopaminergic neurotoxicant 1-methyl-4-phenyl-1,2,3,6-tetrahyropyridine (MPTP) and consequently, paraquat has previously been hypothesised as a possible etiological factor in Parkinson's disease. There are various epidemiology studies which investigated the potential link between the the use of paraquat or other pesticides and the development of Parkinson's disease. Two contemporary epidemiology studies have been examined in detail in this report. Although the studies lend weight to the suggestion that there is a positive association between adverse health effects and exposure to pesticides, the strength of that association is not considered robust. The studies were weakened to various degrees by issues such as case ascertainment, definition of exposure and determination of outcome. In addition, in a recent retrospective worker cohort study, there was no evidence of an increased risk of Parkinson's disease in workers involved in the manufacture of paraquat, and paraquat poisoning case studies have failed to demonstrate neurotoxic effects.

Some published studies have reported that when mice received paraquat by injection, significant losses of dopaminergic neurons in the substantia nigra pars compacta (SN pc) occurred and that histologically, this observation was similar to the hallmark pathology present in Parkinson's disease patients. However, the relevance of these findings to humans is limited by the route of administration and that similar observations have not been reproduced following oral dosing in more recent guideline studies. Additionally, some of the original studies reporting a positive association have since been withdrawn due to scientific misconduct.

With regard to a purported link between paraquat exposure and Parkinson's disease, *in vitro* and *in vivo* studies in animals, although supporting a mechanism of toxicity involving intracellular oxidative stress, do not support a mechanism of neurotoxicity consistent with MPTP and the known mode of action involved in the aetiology of Parkinson's disease

In conclusion, the overwhelming weight-of-evidence, taking into consideration the available database of studies on experimental animals, human epidemiology studies and poisoning cases, is that paraquat does not induce neurotoxicity via the oral, dermal or intranasal routes; routes that are of relevance to human exposure to this pesticide.

The OCS is therefore satisfied that exposure to paraquat, when used as a herbicide, does not pose a risk of neurotoxicity in humans and that lung damage remains the most sensitive and critical toxicological endpoint of concern in establishing health-based guidance values.

A. PART 1

3 INTRODUCTION

3.1 Report structure

This report only considers a single toxicity endpoint (neurotoxicity), and is not a comprehensive human health risk assessment. This report is 'Supplement II' to the 'Review of the mammalian toxicology and metabolism/toxicokinetics of paraquat: Summary report' whereas 'Supplement I: Toxicology' considers all other toxicology endpoints. The report structure for this supplement has been chosen to reflect the current understanding of the potential neurotoxicity of paraquat.

Part 1 provides a discussion of the overall database relating to the potential neurotoxicity of paraquat.

- Section 3 provides a summary of the existing knowledge on the toxicity of paraquat, and some background on Parkinson's disease.
- Section 4 provides a history of considerations of the neurotoxicity of paraquat, including published studies establishing a link between paraquat and neurotoxicity, and potentially Parkinson's disease, as well as those studies which do not demonstrate such an effect.
- Section 5 discusses research that may inform the assessment of the human relevance of the neurological findings in rodents.
- Section 6 includes an evaluation of two contemporary epidemiology studies which claimed a potential association between the development of Parkinson's disease with the use of paraquat or other pesticides.
- Section 7 provides details on the conclusions reached by the OCS.

Part 2 of the report contains additional detail on data discussed in Part 1.

- Section 8 provides a summary of neurotoxicity studies by different dose-routes, as well as mechanistic and epidemiology research findings.
- Sections 9 and 10 includes detailed evaluations of the most relevant studies cited in Part 1, including data on human occupational exposure and poisonings.
- Section 11 contains provides an evaluation of recent epidemiology studies.

3.2 Summary of paraquat toxicity

Paraquat is a non-selective contact herbicide belonging to the bipyridinium class of compounds which also includes the herbicide diquat. Both compounds share a similar mode of action which involves the inhibition of photosynthesis (specifically photosystem I). Plants die rapidly after treatment and exposure to light.

The mechanism of the mammalian toxicity of paraquat, like its mode of action in plants, is via the generation of highly reactive free radicals and consequent peroxidation of membrane lipids, leading to membrane damage and cell death. OCS has evaluated data which confirmed the high acute and chronic toxicity associated with paraquat, which was characterised by pulmonary lesions due to the preferential uptake of paraquat by the lungs. There was no evidence to indicate that paraquat is carcinogenic in chronic experimental animal studies. The weight-of-

evidence indicates that paraquat is non-mutagenic and therefore it is not considered to pose a significant genotoxic risk to humans. The appropriate standard studies showed no reproductive or developmental toxicity in experimental animals. Single oral doses of up to 84 mg paraquat ion/kg bw/d to rats did not demonstrate any clinical signs or neurotoxicity (Brammer, 2006). Additional detail on the toxicology of paraquat is included in the separate report ''Review of the mammalian toxicology and metabolism/toxicokinetics of paraquat. Supplement I: Toxicology".

3.3 Parkinson's disease

Parkinson's disease is characterised at the pathological level by the relatively selective loss of 40-80% of pigmented dopaminergic neurons (often only apparent at diagnosis) in that part of the human brain known as the substantia nigra pars compacta (SN pc) and a dramatic reduction of striatal dopamine (DA) levels (Lang & Lozano, 1998). The disease is progressive and may be present sub-clinically for some period before diagnosis is confirmed. An additional important pathological feature of Parkinson's disease is the presence of intracytoplasmic inclusions called Lewy bodies in the neuronal cell body and Lewy neurites in neuronal processes of the remaining dopaminergic neurons (Forno, 1996).

At the time of clinical diagnosis, the apparently selective neuronal deficient in the SN pc has been estimated at 40-80%. Subclinical loss of such a large proportion of SN pc neurons before the clinical manifestations of disease become apparent, demonstrates an innate capacity for the brain to compensate for the decreased DA levels. The affected regions of the brain include the basal ganglia, which is associated with a variety of functions, including motor control and learning. The main components of the basal ganglia are the striatum, palladium, SN, and subthalamic nucleus. The SN is located in the midbrain within the brainstem and plays an important role in cognition, motor activity, motivation, reward, mood, attention, and learning (Jones & Miller, 2008). The SN consists of two sub-regions, the pars compacta (pc) and pars reticulata (pr). Neurons in the SN pc are generally dopaminergic (i.e., produce the neurotransmitter DA) and are involved in a variety of central nervous system processes. In brief, alterations in DA transmission may affect a variety of neurological processes and lead to debilitating behavioural and movement disorders.

4 HISTORICAL BASIS FOR THE POTENTIAL LINK BETWEEN PARAQUAT AND NEUROTOXICITY

4.1 Structural similarity to MPTP

The hypothesis that paraquat may be associated with Parkinson's disease stems from its structural similarity to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, or MPTP, a contaminant in illicit heroin preparations that was identified as the causative agent for idiopathic Parkinson's disease in recreational drug users (Burns *et al*, 1983; Davis *et al*, 1979; Langston *et al*, 1983). The structural similarities of paraquat (Figure 1), MPTP (Figure 2) and the highly toxic metabolite MPP+ (Figure 3) were sufficient to initiate investigations of paraquat as a potential Parkinsonism-inducing agent (Langston & Ballard, 1983; Langston *et al*, 1983; Langston *et al*, 1983; Woolley *et al*, 1989; Snyder & D'Amato, 1985; Langston, 1996). Indications of association were described in early epidemiology studies and mechanistic studies which examined the influence of the blood brain barrier (BBB) and age on the passage of paraquat into the brain (see report summaries in Part 2 for Corasaniti *et al* (1991); Naylor *et al* (1995); Widdowson *et al* (1996a)).

Chemical structures of paraquat, MPTP and MPP+

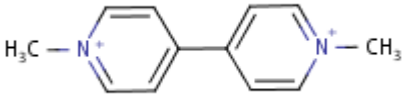

Figure 1. Paraquat

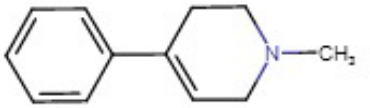

Figure 2. MPTP

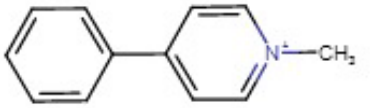

Figure 3. MPP+

4.2 Neurotoxic profile of paraquat in rodents

Animal studies which demonstrate that paraquat reproduces most of the hallmarks of dopaminergic pathogenesis of Parkinson's disease include studies where paraquat is injected directly into the brain (Liou *et al*, 1996) or by the intraperitoneal route (McCormack *et al*, 2002). Treatment of mice with paraquat has been shown in these studies to lead to a decreased number of DA neurons in the SN pc and development of pathological abnormalities similar to those characteristic of Parkinson's disease. Other research groups which have used this model of disease include: Brooks *et al* (1999); Shimizu *et al* (2003); Cicchetti *et al* (2005); Ossowska *et al* 2005a); Ossowska *et al* (2005b); Kuter *et al* (2007 and Fei *et al*, 2008).

A critical issue for some of these studies, including McCormack *et al* (2002), is that in 2012 the US Office of Research Integrity (ORI) found a study author (M. Thiruchelvam) engaged in research misconduct, by falsifying and fabricating data in a paper investigating an aspect of paraquat and Parkinson's disease (Thiruchelvam *et al*, 2005). Two of the papers published as a direct result of this fabricated data were withdrawn (ORI, 2012). A further 8 studies included in this report were co-authored by Thiruchelvam. Due to the uncertainty surrounding the integrity of the data subsequent to this authors fraudulent action, these studies have been deemed unsuitable for regulatory use by the OCS. Four of these studies are included in this neurotoxicity supplement (detailed in APPENDIX III). Reference to these studies within the text of this report has been retained to provide the complete history of the paraquat neurotoxicity review, but have been clearly marked by footnote.

In a study by McCormack *et al*, 2002, it was found that administration of paraquat to mice induced the death of dopaminergic neurons in the SN pc *via* apoptosis with an apparent LOEL of 1 mg/kg bw when administered by the intraperitoneal route (ip), once weekly for 3 weeks The study authors concluded that the loss of dopaminergic neurons in the SN pc was up to 28%, was dose dependent and statistically significant at all doses tested (1, 5 and 10 mg/kg bw ip). Of particular concern at first review by the OCS, was that in this study, microscopic lesions in the brain were induced by only 2-3 doses at weekly intervals. Although this study is no longer considered to be of regulatory value, it was a catalyst for subsequent studies on the effect of paraquat on the SN pc and is retained in this discussion for completeness.

The findings in the McCormack *et al* (2002) were not reproducible in more recent and well conducted studies by Beck (2012 a,c).

In the studies by Beck (2012a,c), C57BL/6J male mice received ip injections of paraquat at 0, 10, 15 or 25 mg/kg bw/dose (equivalent to 0, 7.2, 10.9, 18.1 mg paraquat ion/kg bw/dose), once weekly for 1, 2 or 3 injections. The lowest dose was chosen based on findings of the McCormack *et al* (2002) study. A positive control received MPTP. Extensive examination of the brain by microscopic examination was conducted including specialist staining techniques and measurement of neurotransmitters/metabolites. There was no evidence of neuronal damage to the brain or alterations to the levels of DA or its two metabolites; 3,4-dihydroxyphentlacetic acid (DOPAC) and homovanillic acid (HVA). A 10% decrease in total contour volume of the SN pc in mice treated with 15 and 25 mg/kg bw/dose was noted, but the toxicological relevance of this is unclear and may be artifactual (due to methodology). The positive control group demonstrated the validity of the study design, with damage noted in the SN and striatum, indicative of cell death, glial cell activation and an inflammatory response. In addition, the positive control group had a reduction in the number of TH+ (tyrosine hydroxylase; an enzyme involved in the formation of DA) neurons and decreased striatal DA, DOPAC and HVA levels and an increase in DA turnover. Unlike other published studies included in this review, experimental methodology and raw data for the Beck study was available to the OCS which adds to its value from a regulatory perspective. The detailed assessment and comprehensive methodology utilized in the study leads OCS to conclude that the Beck study casts significant doubt on the reproducibility or reliability of findings in other studies showing effects in the brain following ip injection of paraquat.

More relevant to a human exposure scenario, oral studies on laboratory animals have also been conducted. The OCS has evaluated a neurotoxicity study on paraquat performed according to OECD Guideline 424 (Chivers, 2006). In this study, rats were administered paraquat cation in the diet for 90 days up to 150 ppm (males 10.2 mg/kg bw/d, females 11.9 mg/kg bw/d). Treatment-related effects were limited to effects on body weight at the high dose only. No adverse effects were found in the histological examinations where the brain tissue was examined at 7 levels. As this study was conducted in accordance with OECD Guideline 424, the speciality studies conducted by Beck (both ip and oral studies) were not included in the protocol. However, this study demonstrated an absence of neurotoxicity in the parameters examined.

A study by Beck (2013) assessed the effect of dietary administration of up to 50 ppm paraquat dichloride (equivalent to 10.2 mg/kg bw/d paraquat cation in males and 15.6 mg/kg bw/d in females) on the dopaminergic neurons in the brains of C57BL/6J mice. Detailed examination of the brain through neuropathology with specialist staining showed paraquat exposure was not associated with any neuronal or glial changes in the SN or striatum. Stereology analysis of TH stained sections of the SN pc did not show any effects on the number of dopaminergic neuronal cells or total contour volume. There were also no effects on the levels of DA or its two metabolites (DOPAC and HVA) in the striatal tissues. The test system was validated by findings in a positive control group treated with ip injections of MPTP (Minnema *et al*, 2014).

A study by Rojo *et al* (2007) investigated if symptoms of Parkinson's could be produced in mice and rats following intranasal inoculation of paraquat (up to 30 mg/kg bw/d) daily for 30 days. Although this methodology is not a standard route of administration in animal studies, the nasal mucosa would be a site of potential contact and absorption in humans following occupational exposure. This study demonstrated that paraquat was only detected in the olfactory bulb (10 minutes after a single dose of 20 mg/kg bw) and not other parts of the brain following this route of exposure. Following 30 daily doses, there was no effect on DA or DOPAC levels, or the number of TH^+ neurons in the striatum or SN. The doses resulted in severe toxic effects (weight loss, lung damage, death, hypokinesia, truncal dystonia and evidence of effects on the vestibular system) and due to the lack of effects in the neurotoxic parameters tested, is likely a result of generalised systemic toxicity and not neurotoxicity. This study was validated by concurrent treatment of a group of animals with MPTP by the same route, where clear effects were noted in the striatum. While it is recognised that intranasal inoculation limits the site of absorption to the nasal cavity (vs absorption via lungs in an inhalation study), the study provides evidence that even at doses causing severe toxicity, there are no effects on dopaminergic neurons in both mice and rats.

Treatment of mice with paraquat at relatively high doses during pregnancy was reported to alter the chronology and magnitude of synaptic transmission in the developing mouse cerebellar cortex. Miranda-Contreras *et al* (2005) used a repeat-dose protocol with exposure at 10 mg/kg bw/dose, ip, over gestation days 12 to 20 at 2 day intervals.. Fetal resorptions, skeletal anomalies and maternal mortality have been reported when mouse dams received paraquat at 3.35 mg/kg bw/d, ip, over gestation days 8-14 (Bus *et al*, 1975). Therefore, it is likely that the effect on the cerebellar cortex are a secondary effect of maternotoxicity.

A single study was available to assess developmental neurotoxic effects by the oral route, however no effects on neuronal pathology were reported. In regulatory developmental studies

(presented in Supplement I: Toxicology) there is no evidence of developmental effects at non-maternotoxic doses when paraquat is administered by the oral route. Based on a weight-of-evidence approach, the OCS does not have concerns regarding developmental neurotoxicity by human relevant routes.

4.3 Toxicokinetics

There is a substantial database on the pharmacokinetics of paraquat, including repeat dose studies in mice, rats and rabbits and single dose studies in mice, rats, rabbits, dogs and monkeys.

Paraquat is absorbed poorly when received by the oral route with approximately 10% of an oral dose being absorbed in rats, rabbits and dogs with the remainder being eliminated in the faeces within 72 h. Plasma C_{max} following oral dosing was achieved from 15 min to 2 h and binding to plasma proteins appears to be low.

Paraquat distributes to most organs of the body with initial concentrations being distributed to the lungs and the kidneys. In rabbits which received high doses of paraquat (e.g., 30 mg/kg bw), a rebound in plasma levels was noted at 72 h after dosing together with signs of impaired renal function. Equivocal data indicates that paraquat may possibly accumulate to a measurable level in lung tissue from rats or rabbits, due to an active uptake mechanism. The rate of paraquat disposition into lung tissue from rats and rabbits was similar between these species and suggested that the extent of pulmonary exposure was not species specific. The deposition of paraquat in other rat tissues following oral dosing was generally low, with the exception of the pulmonary disposition following inhalational or intra-bronchial dosing.

Studies have shown that paraquat can distribute to the brain. In mice dosed two or three times a week with 6, 12, 18 or 24 doses of 10 mg/kg bw ip paraquat, it was demonstrated that the striatal level of paraquat plateaued at around 18 doses and the frequency of dosing did not affect steady-state levels. Following a single ip injection (10 mg/kg bw), the elimination half-life in the brain was found to vary with the strain of mouse from around 1 to 3 months (Prasad *et al*, 2009). Repeat oral administration of 5 mg/kg bw/d to rats for 14 days resulted in a 10-fold increase in brain concentration as compared to a single dose, while a 2 to 4 fold increase was measured in other tissues tested (Widdowson *et al*, 1996b). Distribution into the rat brain has been reported to be 40% higher after a single ip dose compared to the same dose administered orally. Although paraquat has been shown to cross the BBB (eg. Corasaniti *et al*, 1991; Widdowson *et al*, 1996b; Shimizu *et al*, 2001), other studies demonstrate the BBB impedes entry (Naylor *et al,* 1995) including an *in vivo* study in the rhesus monkey where only minimal uptake and retention was noted in the whole brain, and particularly no selective accumulation in the DA terminals (Bartlett *et al*, 2009). Distribution to the brain is likely to be hindered given that paraquat is a di-cation and would not readily cross biological membranes *via* passive diffusion, including the BBB. This is in contrast to MPTP which readily crosses the BBB (JMPR, 2003). In a study with a single administration via the intranasal route to mice and rats, paraquat was only detected in the olfactory bulb, whereas MPTP was detected in the olfactory bulb, the striatum and ventral midbrain (Rojo *et al*, 2007).

Paraquat is excreted largely unchanged in the urine (90-95%, 72 h) indicating that it does not undergo extensive metabolism *in vivo*. In rats, three uncharacterised urinary metabolites (~0.1-0.8% of the administered dose), have been detected but not identified following oral dosing.

In rats and monkeys, approx. 45% of an oral dose was excreted *via* the combined urinary and faecal routes within 48 h of treatment, which was consistent with other reports in rats, rabbits and dogs, where approximately 10% of an oral dose was absorbed and the remainder (90%) being eliminated in the faeces within 72 h. Elimination of orally ingested paraquat *via* the urine and faeces has been reported to be detectable for up to 21 d in monkeys

4.4 Toxicodynamics

The biological activity of paraquat following absorption and distribution is generally accepted to be due to the generation of reactive oxygen species. Interaction with cellular reducing systems such as NAD(P)H oxidases leads to one electron reduction to form the paraquat radical ($PQ^{\cdot -}$), which can then react with dissolved O_2 to form the superoxide anion ($O_2^{\cdot -}$) with regeneration to the paraquat cation (PQ^{+}) (Figure 4). It is also well-established that the former can then spontaneously and/or enzymatically, dismutate to hydrogen peroxide (H_2O_2), and then react *via* Haber-Weiss or iron-catalysed Fenton reactions, to form the highly reactive hydroxyl radical ($\cdot OH$) and hydroxide anion (^{-}OH). These reactive species have the capacity to cause oxidation of membrane lipids and formation of reactive aldehydes, oxidation of glutathionyl or protein sulphydryl groups and potentially to form cross-links and oxidatively modify DNA. It is hypothesised that these reactions may play a role in the potential neurotoxicity of paraquat, and this is discussed further in Section 5.3 on neuromelanin.

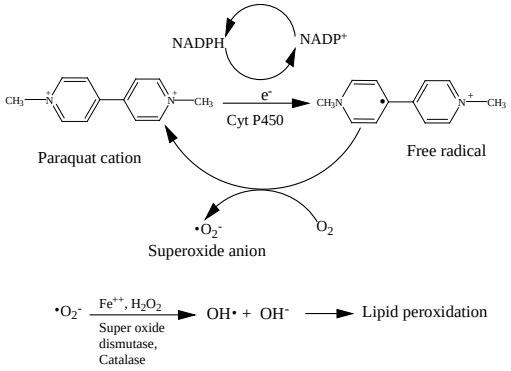

Figure 4. Oxidation-reduction of paraquat and formation of free radicals (Adapted from Bus & Gibson, (1984); Bismuth *et al* (1990) & Honroé *et al*, (1994

In order to prevent oxidative tissue damage, antioxidant defense systems have evolved to counter-act the reactive nature of these chemical species within lipid bilayer structures, soluble cellular fractions and the extracellular milieu. These systems include both chemical and enzymatic mechanisms (enzymatic e.g., catalase, glutathione peroxidase/ reductase and non-enzymatic, e.g., glutathione, ascorbic acid, tocopherols) and it is well established that free radical-dependent intracellular damage is generally delayed until the antioxidant defenses are overwhelmed or depleted. The antioxidant capacity of the neurons of the SN pc in mice or humans have not been thoroughly characterised, however, it has been suggested that this capacity is insufficient or impaired in the neurons of the SN pc in animal models of Parkinson's disease (Zecca *et al*, 2001, 2006). Although one study claimed to demonstrate that the over-

expression of superoxide dismutase or glutathione peroxidase offers protection against neuronal/ brain injury due to treatment with paraquat + maneb (Thiruchelvam *et al*, 2005[1]), this paper has been retracted as fraudulent (ORI 2012).

In mice which received paraquat, 4-OH-2-nonenal, a major product of lipid peroxidation (primarily from the oxidation of arachidonic acid) has been detected and localized to the SN pc using immunohistochemistry (McCormack *et al*, 2005). It is well established in the scientific literature on free radical research that this aldehyde reacts readily with reduced thiols such as glutathione or protein thiols and causes protein modification, cross-linking, aggregate formation and loss of thiol-dependent enzyme function and is associated with many disease pathologies.

In a study by Castello *et al* (2007), *in vitro* exposure of rat brain mitochondria to paraquat demonstrated production of hydrogen peroxide (H_20_2). It was proposed that mitochondrial H_2O_2 production induced by PQ^{2+} in the brain is an early event that may later initiate other cellular events, such as nonspecific electron transport chain (ETC) inactivation, microgliosis, and NADPH oxidase activation.

[1] This paper has been retracted as fraudulent (ORI, 2012) (further information in Appendix II)

4.5 Summary

Some observations of the effects of paraquat in mice and rats suggest that paraquat is capable of causing a selective injury to the SN pc TH^+ neurons leading to loss of cells and decreased DA production when administered by the intraperitoneal route. These observations are recognised hallmarks of the neuronal deficit described in Parkinson's disease. However, evidence of effects resulting from intraperitoneal injection of paraquat is challenged by compelling results from recent studies in mice. In one of these studies, no effect in the brain was found following up to 25 mg paraquat dichloride/kg bw/dose (equivalent to 18.1 mg paraquat ion/kg bw/dose) of up to 3 weekly injections (ip), despite evidence of systemic toxicity. This study was supported by evidence of neuronal damage in the brains in a positive control group (ip, MPTP). This study is of high regulatory value and casts doubt on the reliability/reproducibility of the previous studies. In this study, paraquat did not act like MPTP.

More importantly from a human health perspective, when paraquat dichloride was administered in the diet of mice for up to 90 days, there were no effects on the dopaminergic neurons at up to 50 ppm (equivalent to paraquat cation of 10.2 mg/kg bw/d (males) and 15.6 mg/kg bw/d (females)), the highest dose tested, despite evidence of damage to the brains of the positive control group (ip, MPTP). The oral route is more relevant to human exposure than intraperitoneal injection and hence this study is more applicable to the human health risk assessment. In addition, intranasal inoculation in mice and rats of up to 30 mg/kg bw/d for 30 days did not result in any evidence of neurotoxicity, despite severe systemic toxicity. Although this is not a standard route of administration in animal studies, the nasal mucosa is a potential site of contact and absorption in humans following inhalation of paraquat. While recognising that the absorption is limited to a specific area of the respiratory tract, the study provided no evidence that paraquat might lead to the development of PD in rats and mice. This conclusion was validated by concurrent treatment of a group of animals with MPTP by the same route, where clear effects were noted in the striatum.

Therefore, evidence for paraquat having a neurotoxic effect is limited mainly to studies in mice dosed via the intraperitoneal route (Brooks *et al*, 1999, Ossowaka *et al*, 2005b and McCormack *et al*, 2002[2]), and these findings were not reproducible in a more recent comprehensive study by the same route (Beck, 2012 a,c). The remainder of the database does not report any other evidence of neurotoxicity in rodents, rabbits or dogs, including any clinical signs (consistent with a neurotoxicant) or neuropathy.

[2] Not suitable for regulatory use following fraudulent actions by co-author (M.Thiruchelvam) on another paper published 2005 (see Appendix II).

5 THE RELEVANCE TO HUMANS OF THE FINDINGS IN RODENTS

5.1 Evidence of neurotoxicity from human exposure

Numerous poisoning cases have provided no clear indication that paraquat was neurotoxic to humans. Several case reports have described cerebral damage in subjects who died following paraquat poisoning (Grant *et al*, 1980; Hughes, 1988), however, this tissue injury appears to have been secondary to more severe effects on other organ systems and may have been indirectly due to hypoxia. Extensive case reports have been appended in Part 2 of this report.

Paraquat has been detected in the brains of poisoned humans at levels ranging from 0.08-0.35 μg/g tissue (Ameno *et al*, 1994) compared to up to 1.98 ng/mg brain tissue from mice which received 10 mg/kg bw paraquat (ip) for twice a week for 24 doses (Prasad *et al*, 2009). The OCS is not aware of reports of subsequent development of Parkinson-like symptoms or neurological signs and symptoms in survivors of poisoning events or evidence of accumulation of paraquat in brain tissue of paraquat-poisoned humans.

5.2 Comparative pharmacokinetics

A difficulty in determining the human relevance of the available animal studies which describe neurotoxicity findings has been that animal studies demonstrating neuronal injury have used exposure routes which are not relevant to the likely human exposure routes. Generally, animal studies have used the ip, sc or oral dietary routes whereas the most likely route of exposure for humans is *via* oral, inhalational or dermal routes.

5.3 Neuromelanin and oxidative stress

An innate property of neuromelanin (NM), which is present in the neurons of the SN, is the capacity to act as a molecular 'sink' for poorly regulated natural molecules/compounds and also to bind extraneous molecules such as pesticides, including paraquat (Lindquist *et al*, 1988). This binding activity by NM has led to an array of experimental evidence which suggests that the NM which has been isolated from autopsy samples of the SN of Parkinson's disease patients has molecular characteristics which suggest that the NM was modified and that those modifications were consistent with prior exposure to increased oxidative stress. The following discussion represents a contemporary opinion as to how these findings may relate to the hypothesised association between exposure to paraquat and the development of neuronal injury which has similarities to the pathology observed in Parkinson's disease patients.

Neuromelanin is broadly a member of the melanin pigment family (such as the peripheral melanins) but with distinct differences in origin and composition. NM is most abundant in human brain tissue, present in lesser amounts in non-human primates and appears to be absent from the brain of many lower order species, such as rodents (see review by Fedorow *et al*, 2005). The latter observation may represent a possible divergence between the mechanisms of action of paraquat in the mouse model of neuronal injury initiated by paraquat and the observations from end-stage disease brain tissue samples available from Parkinson's disease patients at autopsy.

Neuromelanin which has been isolated from autopsy tissue from Parkinson's disease patients differs from NM isolated from normal human brain tissue. Early studies reported the marked accumulation of iron in the SN neurons; this was thought to be associated with the severity of Parkinson's disease and that the disease may be related to a dysfunction in the regulation of iron storage in the SN pc neurons. The relevance of this observation to oxidative stress and tissue injury is that iron is a key catalyst of the Fenton-reaction which leads to the formation of the highly reactive hydroxyl radical which can initiate the oxidation of lipids, proteins and DNA. A pro-oxidant status of NM, i.e., promotion of oxidant reactions, is suggested by a report that the reduction/oxidation (or redox) activity of NM-aggregates isolated from Parkinsonian patients was significantly increased (69%) and was highest in patients with the most severe neuronal loss (Faucheux *et al,* 2003). High and low affinity iron binding sites have been described on normal NM. Therefore, it is conceivable that if there is high binding site vacancy, particularly of the high affinity binding sites as would be expected in normal neurons, then NM could have an anti-catalytic activity and suppress iron-mediated injury.

Parkinson's disease has been associated with descriptions of modified NM protein which would be consistent with the effects of oxidant stress. NM extracted from SN brain tissue from Parkinson's disease patients was found to be composed of highly cross-linked, protease resistant, lipoprotein material when compared to NM isolated from normal brain tissue (Aime *et al,* 2000, Halliday *et al,* 2005). Another early observation in the SN pc of Parkinson's disease patients is the presence of Lewy bodies as a hallmark of disease. Prior to Lewy body formation, intracellular changes in the optical density of NM were associated with a loss of NM-associated cholesterol and an aggregation of a lipoprotein later described as α-synuclein, to the NM lipid (see Halliday *et al,* 2005). The changes in the optical density of NM from Parkinson's disease patients were comparable to normal NM which had been treated with H_2O_2 (Faucheux *et al,* 2003) and may be an early biochemical event due to increased cellular oxidation and saturation of the iron-binding capacity in Parkinson's disease (Halliday *et al,* 2005).

α-Synuclein has been extracted from Lewy bodies and Lewy neurites from Parkinson's disease patients where it was present as insoluble β-sheet-rich amyloid filaments compared to the normal form of an unstructured, very soluble lipoprotein in an α-helix structure when in contact with membranes (Cole, 2008). No publications were found which might indicate that paraquat could modify α-synuclein directly to the form of α-synuclein which was found associated with NM lipid. There is evidence from other studies describing the formation of α-synuclein fibrils in the presence of iron (Fe^{2+}) , whereas reaction with aldehydes from lipid peroxidation (e.g., 4-hydroxy-2-nonenal), led to the formation of soluble oligomers rather than insoluble filaments (reviewed in Cole, 2008). The former share structural properties with amyloidogenic peptides/ proteins which are capable of forming membrane pores and permeabilising lipid bilayers, causing proteosome dysfunction, and may initiate α-synuclein filament formation. This area of research is in its early stages and is not considered relevant to paraquat-mediated neuronal injury and/or Parkinson's disease in humans.

6 RECENT EPIDEMIOLOGICAL STUDIES ON THE ASSOCIATIONS BETWEEN PARKINSON'S DISEASE AND EXPOSURE TO PARAQUAT

6.1 Introduction

Several epidemiology studies have been carried out to investigate whether there is a statistical association between developing Parkinson's disease following exposure to paraquat. Some studies have claimed to find a positive association specifically for paraquat (Barbeau *et al,* 1987; Hertzman *et al,* 1990; Liou *et al,* 1997), or an association between the occupational use of herbicides in general and the development of Parkinson's disease (Semchuk *et al,* 1992; Ritz & Yu, 2000), whereas other studies have concluded that there is no evidence to support a causal association between pesticide exposure and Parkinson's disease (Li *et al,* 2005).

The APVMA requested the OCS provide comment on the robustness of two contemporary epidemiology studies in which associations between Parkinson's disease and exposure to paraquat or pesticides were claimed by the study authors. The papers (Kamel *et al,* 2007; Costello *et al,* (2009) have been evaluated in regards to the relationship between pesticide exposure and presence of Parkinson's disease. In 2013, the OCS was asked to review an additional epidemiological study (Tomenson & Campbell, 2011). As this study was not included in the review of Kamel *et al* and Costello *et al,* this assessment is presented separately in Part 2, Section 10.1.

A comprehensive analysis of the robustness of these studies was conducted by Kamel *et al* (2007) and Costello *et al* (2009) (see Section 11).

6.2 Summary of the studies

The study by Kamel *et al* (2007) reported that the incidence of Parkinson's disease (newly diagnosed cases) was associated with cumulative days of pesticide use at enrolment for the highest quartile versus the lowest quartile, with an Odds Ratio (OR) of 2.3 (95% CI=1.2,4.5). The authors also claimed that the incidence of Parkinson's disease was associated with personally applying pesticides more than half the time but was not statistically significant. Exposure to pesticides was obtained by respondents' recall, which may have been biased due to the length of time since exposure, or having a Parkinson's disease diagnosis. The large number of respondents ensured that some associations were likely to be found, but the strengths of those associations were not compelling, and many were not statistically significant. The data at best indicated a trend towards a positive association between pesticide exposure and the presence of Parkinson's disease.

The study by Costello *et al* (2009) reported that exposure to paraquat and maneb within 500 m of the home increased the risk of Parkinson's disease by 75% above the background rate (OR=1.75, 95% CI=1.13, 2.73), and that persons <60 years at the time of diagnosis were at a higher risk of Parkinson's disease when exposed to either paraquat or maneb alone. These findings were statistically significant. In contrast, the study by Kamel *et al* (2007) indicated that persons aged ≥ 60 years were at greater risk for Parkinson's disease. The authors enrolled 368 incident Parkinson's disease cases who had lived for ≥ 5 years among a largely agricultural population. Controls came from the same population without a diagnosis of Parkinson's disease. Costello *et al* (2009) reported that respondents <60 years old showed over twice the

risk of a Parkinson's disease diagnosis, while those exposed to both paraquat and maneb were over four-times more likely to be diagnosed with Parkinson's disease. These data indicate that there was a statistical association between exposure to pesticides and Parkinson's disease diagnosis.

6.3 Summary analysis of methodology

In studies of exposure to environmental hazards relating to human disease, both the level of exposure and the ascertainment of disease are critical elements to the study design, and both exposure and end-points should be defined carefully. In the study by Kamel *et al* (2007), exposure was not well defined, and the presence of Parkinson's disease was at times loosely defined as having "a health professional", which could be a nurse, dentist or podiatrist, decide the diagnosis for the individual. Further, it was apparent that the respondents knew of the purpose of the study and that pesticide use was a factor of interest in the study. The study was further weakened by the authors' choice of controls. More than 99% of applicators had personally mixed or applied pesticides and 56% of spouses also had mixed or applied pesticides in the timeframe under discussion. Therefore, it appears that half the controls were exposed to pesticides. These shortcomings contributed to the findings of a weak association between pesticide use and a Parkinson's disease diagnosis.

In the study by Costello *et al* (2009), exposure was clearly defined but was still only a modeled estimate with pesticide application rates being averaged over specific time periods and weighted by the annual application rate. The presence of Parkinson's disease was documented by neurologists, while cases were also recruited from clinics, Health Maintenance Organisations (HMOs), Veteran's Administration newspapers and local radio stations. The study did not explain how 'caseness' was determined other than a neurologist's report, and this was considered to be a limitation of the study. Controls were appropriately selected from residents residing primarily in the designated counties under study for at least five years, without Parkinson's disease, and at least 45 years of age, with only one person per household permitted to enrol. The methodology in this study was more robust than the study by Kamel *et al* (2009), and the findings showed a more robust association between pesticide use and a diagnosis of Parkinson's disease.

6.4 Results and conclusions

In the study by Kamel *et al* (2007), the odds ratios were reported to be elevated for prevalent Parkinson's disease cases for the herbicides pendimethalin, paraquat, and cyanazine, and the fumigants carbon disulphide/carbon tetrachloride and ethylene dibromide. Odds ratios for incidence of Parkinson's disease associated with exposure were not statistically significant for dicamba, trifuralin, 2,4,5-trichlorophenoxyacetic acid, butylate, lindane, phorate, chlorothanolil, benomyl and methyl bromide. The exception was an association with cyanazine, where statistical significance was found.

Costello *et al* (2009) used a GIS model which allowed the estimation of the likely dosage of active ingredients. The authors also acknowledged that these quantities were not comparable across all pesticides and exposure could not be estimated with any degree of accuracy for individual pesticides. The study also lacked statistical power to perform extensive analysis as the sample cohort comprised of only three cases exposed to maneb alone with one control case.

The study by Costello *et al* (2009) also found that there was a positive relationship between Parkinson's disease and exposure to both paraquat and maneb from the earlier years of observation, 1974-1989, and 1974-1999, but not for later years of 1990-1999. The basis for this cannot be discerned from the data available as there would be many confounding factors to consider. It is possible that the lower association in recent years may be attributable to the disease requiring time to develop to a diagnosable condition. Alternatively, this association may only be apparent when exposures to both paraquat and maneb have occurred. An intriguing observation in the epidemiology analysis of Kamel *et al* (2007) was a 'protective effect' of education and, speculating from an epidemiological perspective, given that the same 'protective effect' of education was also present in the cohort surveyed by Costello *et al* (2009), then later users of pesticides may have been more cognisant of the risks of paraquat toxicity or more attentive to the requirements of protective clothing. This study lends some weight to the suggestion that there is a positive association between adverse health effects and exposure to pesticides (i.e., paraquat and maneb), but presently, the strength of that association does not extend to exposure to paraquat alone.

6.5 Conclusions

The examination of the Kamel *et al* (2007) and Costello *et al* (2009) studies revealed that neither of these studies made a robust case for an association between Parkinson's disease and the exposure to pesticides, in particular to paraquat. The findings by Costello *et al* (2009) were considered more robust as the authors went to greater lengths to define the exposure and the case ascertainment in a more stringent manner than in the study by Kamel *et al* (2007). Assignment of exposure (to paraquat or pesticides) was also assessed differently, and the outcome, Parkinson's disease, was assessed by a variety of inputs; as being diagnosed by a neurologist, or being assessed by a doctor, or a health professional. The latter definition broadened the catchment of cases, but was a weaker description of the disease.

Overall, the above findings from the two epidemiology surveys, particularly the study by Costello *et al* (2009), lend some weight to the suggestion that there is a positive association between adverse health effects and exposure to pesticides (including paraquat and maneb), but presently, the strength of that association would not be considered robust. An additional retrospective cohort study by Tomenson & Campbell (2011) reviewed by the OCS at a later date, showed no evidence of an increased incidence of Parkinson's disease, as compared to control populations.

7 OVERALL CONCLUSIONS

This supplementary report addresses the hypothesis that there may be sufficient new toxicological information to support a case that paraquat may have a previously unrecognized adverse effect on human health, specifically, investigations into whether paraquat may cause neurotoxicity in humans in the form of injury to the SN pc resulting in the loss of dopaminergic neurons and development of clinical signs consistent with Parkinson's disease.

This supplement discusses the pharmacokinetic profile of paraquat, relevant findings in laboratory animal studies and cases of human poisoning, relevant advances in the understanding of the possible role of neuromelanin in Parkinson's disease and its possible interaction with paraquat and comments on three recent epidemiology studies with differing conclusions on the association between exposure to pesticides, including paraquat, and the development of Parkinson's disease.

Early studies in mice showed that injections (ip and sc) of paraquat results in the loss of dopaminergic neurons in the SN pc with the LOEL for this effect only 1 mg/kg bw/wk (ip, weekly for 3 weeks). However, not only has one of these studies been determined to be fraudulent, a recent comprehensive and contemporary study demonstrated no effects on the dopaminergic neurons in the SN pc at up to 25 mg/kg bw/dose (ip, weekly for 1-3 weeks) over-riding the reliability of earlier studies. Critically for this human health risk assessment, new data demonstrated that when paraquat is administered orally to adult mice, a more relevant route of exposure to humans, no such effect was seen in the SN pc at doses up to 50 ppm paraquat dichloride (≈10 mg/kg bw/d paraquat ion).

Epidemiological studies do not demonstrate a robust statistical correlation between human exposure to pesticides, including paraquat, with Parkinson's disease.

The OCS is therefore satisfied that exposure to paraquat, when used as a herbicide, does not pose a risk for neurotoxicity in humans.

B. PART 2

8 SUMMARY OF NEUROTOXICITY STUDIES

8.1 Oral Administration

The acute neurotoxicity of paraquat in rats was investigated following single po gavage doses of 0, 8, 25, or 84 mg/kg bw paraquat ion/kg bw according to OECD Test Guideline 424 and included functional observational battery parameters of landing foot-splay, sensory perception and muscle weakness (Brammer, 2006). Mortalities and clinical signs of systemic toxicity were observed consistent with the high dose administered. A slight increased incidence of reduced foot-splay reflex was noted in male but not in female rats in addition to an increased incidence in female control animals. Histopathological examination did not include examination of brain / SN pc. Overall, on the parameters measured there was no evidence of neurotoxicity following the oral administration of a single dose of up to 84 mg paraquat ion/kg bw to rats.

A study by Beck (2013) was specifically designed to assess the effect of dietary administration of up to 50 ppm paraquat dichloride (equivalent to paraquat ion in males 10.2 mg/kg bw/d, females 15.6 mg/kg bw/d) on the dopaminergic neurons in the brains of C57BL/6J mice. Detailed examination of the brain through neuropathology with specialist staining showed paraquat exposure was not associated with any neuronal or glial changes in the SN or striatum. Stereology analysis of TH stained sections of the SN pc did not show any effects on the number of dopaminergic neuronal cells or total contour volume. There were no effects on the levels of dopamine or its two metabolites (HVA and DOPAC) in the striatal tissues. The test system was validated by findings in a positive control group treated with ip injections of MPTP. No LOEL was identified for neuronal cell loss in the striatum or SN pc, with no effects noted at the highest dose tested (≈ 10 mg/kg bw/d paraquat ion). Therefore, the NOEL for neuronal cell loss was 10 mg paraquat ion/kg bw/d (the highest dose tested). This study has since been published in Regulatory Toxicology and Pharmacology (Minnema *et al,* 2014).

The neurotoxicity of paraquat technical was investigated in a subchronic study where rats were administered paraquat cation in the diet for 90 days at 0, 15, 50 or 150 ppm or 0, 1.0, 3.4 or 10.2 mg/kg bw/d in males and 0, 1.1, 3.9 or 11.9 mg/kg bw/d in females (respectively) (Chivers, 2006). The study was performed according to OECD test Guideline 424 and included functional observational battery (FOB) parameters of landing foot-splay, sensory perception, grip strength, muscle weakness, and motor activity. Nervous system tissues from control and high dose animals were examined histologically, however, the SN pc was not included in the tissues selected for examination. Treatment-related clinical signs of toxicity were limited to changes in body weights at the high dose. There were no treatment-related FOB abnormalities and there were no treatment-related histological changes observed in the nervous system tissues selected for examination. As per Guideline, the SN pc was not specifically examined. The NOEL was 150 ppm or 10.2 and 11.9 mg/kg bw/d in male and female rats (respectively), the highest dose tested.

The effect of Gramoxone (a paraquat-containing formulation) on the concentration of various central monoamines and acetylcholine were determined in the cerebral cortex, midbrain and pons/medulla oblongata 12 days after po administration to male mice at 0, 3.12, 10.4 and 31.2 mg/kg bw/d (equivalent to 0, 2.25, 7.5 and 22.5 mg/kg bw paraquat cation) for 3 consecutive

days. There was a significant elevation in the concentration of choline in the cerebral cortex at 10.4 and 31.2 mg/kg bw/d, and in the midbrain and pons/medulla oblongata at all doses. The elevation in midbrain choline levels did not follow a dose-response pattern and therefore the toxicological significance of this finding was unclear. There was a dose-related depression in the concentration of DA and norepinephrine in the midbrain, with the effect statistically significant at the highest dose only. The concentration of serotonin (5-HT) in the midbrain was significantly lower than the control at every dose, but in the absence of a dose-response effect the toxicological significance of this finding was unclear. The concentration DOPAC in the midbrain and pons/medulla oblongata was significantly elevated at the highest dose. A significant depression in the concentration of 5-HT in the pons/medulla/oblongata occurred at the low and high dose. In the absence of a dose response effect these findings were not considered to be toxicologically relevant. No evidence was provided that these neurochemical effects were due to paraquat (eg measurement of paraquat levels in the brain) and it is thus conceivable that other Gramoxone components could have been responsible (Endo *et al,* 1988). Additional detail is presented in APPENDIX I.

A study was undertaken to investigate whether paraquat would affect the dopaminergic system and the behaviour of adult mice in a manner similar to MPTP when given to neonatal mice during the brain growth spurt. Paraquat was administered orally to male mice (aged 10-11 days) at 0, 0.07 or 0.36 mg/kg bw (equivalent to 0, 0.05 and 0.26 mg/kg bw paraquat cation). Two additional groups received MPTP orally at 0.3 or 20 mg/kg bw. No signs of acute toxicity or differences in body weight gain were observed in any of the paraquat- or MPTP-treated mice, although no supporting data were provided to substantiate these findings. The LOEL was 0.07 mg/kg bw (equivalent to 0.05 mg/kg bw paraquat cation), the lowest dose tested. Hypoactivity at 60 and 120 days of age, and a significant depression in striatal HVA at 125 days of age, were observed at 0.07 and 0.36 mg/kg bw (equivalent to 0.05 and 0.26 mg/kg bw paraquat cation). At 0.36 mg/kg bw, a significant depression in striatal DA and DOPAC occurred. Although the study authors concluded that the observed changes were permanent, no behavioural or neurochemical measurements were performed beyond 120 days (Fredriksson *et al,* 1993).

The neurotoxicity and tissue distribution of paraquat were examined in male rats following multiple po oral dosing at 0 or 5 mg/kg paraquat cation bw/d for 14 days. There were no treatment-related effects on behaviour, motor coordination, grip strength or total locomotor activity. Paraquat-treated rats showed a significantly higher striatal DA concentration compared to the control which the study authors suggested may have been due to a paraquat-induced stress response. In contrast, there was no treatment-related effect on the concentration of DA in the hypothalamus. There was no perturbation in the level of DOPAC in the striatum or hypothalamus. Noradrenaline appeared to be slightly elevated in the striatum, hypothalamus and frontal cortex, however none of these results were statistically significant. There was no treatment-related effect on the density of dopamineD1 and D2, muscarinic, NMDA or benzodiazepine receptors. Paraquat-treated rats showed no signs of neuronal cell damage or death. Collectively, these observations suggested that paraquat was not neurotoxic. Twenty-four hours after a single po dose, the lung had the highest concentration of [^{14}C]-paraquat followed by the kidney, liver, brain and plasma. Twenty-four hours after 14 daily po doses, the lung again showed the highest paraquat concentration followed by the brain, the kidney, liver and plasma. The brain exhibited an approximately 10-fold greater paraquat concentration after 14 repeat doses compared to a single dose while all other tissue only showed a 2- to 4-fold increase (Widdowson *et al,* 1996b).

8.2 Subcutaneous and Intravenous Administration

A study was undertaken to compare the effect of repeated sc injections of the maximum tolerated dose (MTD) of paraquat, reduced paraquat, MPTP (single dose only) and two MPTP analogues, on striatal levels of DA, DOPAC and HVA in mice. Paraquat was injected at a dose of 14.5 mg/kg bw (equivalent to 10.44 mg/kg bw paraquat cation) on 3 occasions, each separated by 3-day intervals. Reduced paraquat was given over 6 days at between 7.3 to 116.3 mg/kg bw/d. MTIQ (N-methyl-1,2,3,4-tetrahydroisoquinoline) was administered over 8 days at 40 to 160 mg/kg bw/d. MTHBC (2-methyl-1,2,3,4-tetrahydro-β-carboline) was administered at 40 mg/kg bw on a single day, and then 80 mg/kg bw/d for three consecutive days. A single injection of MPTP was administered at 40 mg/kg bw. Rapid weight loss and death were reported in paraquat-treated mice during preliminary experiments, but no clinical signs were reported in mice given reduced paraquat. Sedation, ataxia, tremors, convulsions or death were observed at the highest doses of MTIQ and MTHBC. MPTP-treated mice exhibited no clinical signs. One month after the last injection, striatal DA was significantly elevated in paraquat-treated mice, while DOPAC and HVA were unaffected. This effect on striatal DA was not considered to be toxicologically relevant. In contrast, MPTP caused a significant depression in striatal DA, DOPAC and HVA. Reduced paraquat, MTIQ or MTHBC had no effect on striatal levels of DA, DOPAC or HVA (Perry *et al,* 1986).

A range of experiments were performed to determine the effect of acute paraquat exposure on complex I activity (NADH: ubiquinone oxidoreductase), lipid peroxidation and catecholamines in the rat brain. A single iv injection was given to 8-week old male rats at 20 mg/kg bw/d for 5 days (equivalent to 14.4 mg/kg bw/d paraquat ion). A significant treatment-related depression in striatal DA occurred, while there was no effect on striatal norepinephrine. Lipid peroxidation was significantly elevated in the brain and liver but not the plasma. Complex I activity was significantly inhibited in the brain and liver of paraquat-treated rats. (Tawara *et al,* 1996).

Shimizu *et al* (2003) studied the *in vivo* toxic mechanism of paraquat to DA neurons. Paraquat dichloride (10 mg/kg bw sc, equal to 40 µmol) was given to male Wistar rats (8 weeks old, 210-260 g; SLC, Shizuoka, Japan) once a day sc for 5 days. Monoamine concentrations in eight regions of brain were determined 2 days after the 5-day treatment with paraquat. Paraquat affected DA and its acidic metabolites HVA and DOPAC in the striatum, midbrain and cortex. DA content in the striatum was reduced to 70% of that in control rats ($p < 0.05$). The HVA level was 80% of control value in the striatum ($p < 0.01$). Paraquat reduced DOPAC and HVA contents to 40-60% and 25-50% compared with the control in the midbrain and cortex ($p < 0.05$).

A study by Kuter *et al* (2007) examined whether short-term, daily, paraquat sc injections in rats produced neuropathological and neurochemical alterations, similar to those observed after long-term administration. Male Wistar rats (≈ 250 g) were treated with paraquat dichloride dissolved in sterile distilled water and administered sc at a dose of 10 mg/kg bw (7.2 mg paraquat ion/kg bw). Injections were made once a day for five consecutive days. Control animals received saline sc. At necropsy 2 and 3 days after the last injection, the animals were subjected to biochemical analyses and histopathological examination. The main finding was that a short-term paraquat administration induces the 22% loss of dopaminergic (TH-ir) neurons in the SN pc. Concomitantly with this neuropathological alteration, a moderate

decrease in the [^{3}H]GBR 12,935 binding to DAT and reductions of the levels of DA metabolites and DA turnover in the caudate-putamen, SN and prefrontal cortex (PFC), were observed, especially 2 days after withdrawal. Subchronic paraquat administration by subcutaneous injection triggers processes characteristic of early stages of degeneration of dopaminergic neurons, and activates compensatory mechanisms involving dopaminergic, noradrenergic, serotonergic and GABAnergic transmissions.

8.3 Intraperitoneal Administration

In a study by Beck (2012a,c), C57BL/6J male mice received ip injections of paraquat dichoride at 0, 10, 15 or 25 mg/kg bw/dose (equivalent to 7.2, 10.9, 18.1 mg paraquat ion/kg bw/dose), once weekly for 1, 2 or 3 injections. The lowest dose was chosen based on findings of the McCormack *et al* (2002) study. A positive control received MPTP. Extensive examination of the brain by microscopic examination was conducted including specialist staining techniques and measurement of neurotransmitters/metabolites. Decreased body weight gain was noted from 15 mg/kg bw/dose and reduced food consumption at 25 mg/kg bw/dose. There was a single mortality at the highest dose. There was no evidence of neuronal damage to the brain or alterations to the levels of DA or its two metabolites (DOPAC and HVA). A 10% decreased in total contour volume of the SN pc in mice treated with 15 and 25 mg/kg bw/dose was noted, but the toxicological relevance of this is unclear and may be artifactual (due to the methodology). The positive control group demonstrated the validity of the study design, with damage noted in the SN and striatum, indicative of cell death, glial cell activation and an inflammatory response. In addition, the positive control group had a reduction in the number of TH+ (dopamine) neurons and decreased striatal DA, DOPAC and HVA levels and an increase in DA turnover. The NOEL for neuronal cell loss was 18.1 mg paraquat ion/kg bw/ for 3 x weekly ip injections (the highest dose tested). Unlike other studies showing a possible neurotoxic effect following ip injection, the experimental methodology and raw data from the Beck study was available to the OCS which increases its value from a regulatory perspective. The comprehensive methodology utilised in the Beck (2012a,c) study leads OCS to conclude that the reproducibility and/or reliability of findings in other studies showing effects in the brain following injection of paraquat are questionable for regulatory purposes.

To compare the neurotoxicity of paraquat and MPTP, 3 ip injections of paraquat were given to adult male mice at 0, 5 or 10 mg/kg bw (equivalent to 0, 3.6 or 7.2 g/kg bw paraquat ion), whilst 4 ip injections of MPTP were given to separate groups of mice at 0, 10 or 30 mg/kg bw. Both compounds caused a significant loss of dopaminergic neurons at and above 5 and 10 mg/kg bw, respectively, as shown by the reduction of Fluoro-gold and/or TH^+ cells in the SN. Both compounds also caused a significant reduction in ambulatory locomotor activity, however, this finding was difficult to interpret due to the limited reporting of the results, the small group sizes and that paraquat and MPTP were each administered using different protocols (Brooks *et al*, 1999).

To investigate the possibility that opioid receptors are involved in paraquat-induced shaking behaviour in male rats, a single ip injection was given at 0, 30, 50 or 70 mg/kg bw (equivalent to 0, 21.6, 36 and 50.4 mg/kg bw paraquat cation, respectively), or a single intracerebroventricular injection was given at 12.9 or 25.7 μg/10 μL/rat (equivalent to 9.3 and 18.5 μg cation, respectively), in the presence and absence of morphine or naloxone (5 and 1.5 mg/kg bw, ip, respectively). When administered intraperitoneally, paraquat caused a significant

dose-related increase in the frequency of shaking behaviour at and above 30 mg/kg bw (equivalent to 21.6 mg/kg bw paraquat cation). Myoclonus was seen only at 70 mg/kg bw (equivalent to 50.6 mg/kg bw paraquat cation). Morphine but not naloxone pre-treatment significantly reduced paraquat-induced shaking behaviour suggesting that shaking behaviour was mediated by opioid receptors. Intracerebroventricular injection of paraquat induced tremor, but not shaking behaviour or myoclonus, at 23.7 μg/rat (equivalent to 18.5 μg paraquat cation). Although no neurohistopathological abnormalities were detected in any rats, fluorescein uptake by the brain was significantly elevated with paraquat treatment, an effect that was prevented by morphine. These later findings suggested that paraquat increased the vascular permeability of the brain (Hara *et al,* 1993).

Cicchetti *et al* (2005) provided evidence that paraquat alone and paraquat in combination with maneb is toxic to dopaminergic neurons of eight week old male Sprague Dawley rats both *in vitro* and *in vivo* and that the combination leads to Parkinson-like movement deficits. The rats were injected ip twice a week for four weeks with 10 mg/kg bw paraquat dichloride hydrate (7.2 mg paraquat ion/kg bw) or a combination of paraquat and maneb at 10 mg/kg bw and 30 mg/kg bw respectively. Evidence for the induction of Parkinson-like movement deficits by paraquat was limited.

Ossowska *et al* (2005a) examined whether paraquat affects DAT *in vivo* in rats. MPP^+ is an active metabolite of MPTP, a parkinsonism inducing compound which is taken into dopaminergic neurons by DAT. Paraquat dichloride was administered at a dose of 10 mg/kg bw (7.2 mg paraquat ion/kg bw) ip to male Wistar rats (250 g). Animals were examined at necropsy at 2 and 24 h or 7 days after the injection. The treatment induced a decrease in the specific binding of [^{3}H]GBR 12,935 to DAT in the dorsal and ventral caudate-putamen but not in the SN. The decreases were reversible and observed only 2 and 24 h, but not 7 days after paraquat injection. Paraquat did not significantly change the levels of DA, DOPAC and HVA in any structure examined and at any time point measured. However, paraquat increased the level of 3-MT at 24 h after the injection by 19.6% and 18.5% in the anterior and posterior caudate-putamen, respectively. The 3-MT/DA ratio was also significantly increased in the anterior (16%, $p < 0.05$) and posterior caudate-putamen (15%, $p < 0.05$) 24 h after administration. Paraquat also induced a slight influence on dopaminergic transmission with persistent but reversible effects on dopamine transporter (DAT) in the caudate-putamen, a finding supported by decreases in the binding of [^{3}H]GBR 12,935. The study did not ascertain whether paraquat, is actually transported into dopaminergic neurons via DAT.

Ossowska *et al* (2005b) examined long term paraquat administration (up to 24 weeks) in male Wistar rats (200-250 g) to determine if a slowly progressing and selective degeneration of nigrostriatal neurons leading to effects on dopaminergic transmission occurs. Various stages of this process were examined. Paraquat dichloride was administered at a dose of 10 mg/kg bw ip once a week for 4, 8, 12 or 24 weeks. The study showed that long-term ip paraquat administration induces slowly progressing loss of nigrostriatal neurons and a delayed deficit of striatal dopaminergic transmission in rats. These neuronal alterations in rats bear some similarities to pathological processes operating in early, pre-symptomatic, stages of Parkinson's disease.

8.4 Intracerebral/nigral/hippocampal Administration

Experiments were performed to investigate the behavioural and neuropathological effects of intrahippocampal and systemic injections of paraquat in rats. The effect of the muscarinic acetylcholine receptor antagonists, atropine and methylatropine, and the N-methyl-D-aspartate (NMDA) receptor antagonist, MK 801, on paraquat-induced neurotoxicity was also determined. A single injection of paraquat (unspecified purity) was administered intrahippocampally at 0, 10 nmol, 100 nmol or 1 μmol (equivalent to 0, 1.86, 18.6 and 186.3 μg, respectively or 0, 1.34, 13.4 and 134.1 μg paraquat cation, respectively). Additional groups were injected with 1 μmol (equivalent to 186.3 μg paraquat or 134.1 μg paraquat cation) paraquat and 5 or 50 nmol atropine. Another group received an ip injection of the NMDA antagonist MK 801 (0.3 mg/kg bw) 60 minutes before intracerebral injection of paraquat. Paraquat was also administered systemically to 5 rats/group by an ip injection at 5, 20 and 100 mg/kg bw (equivalent to 3.6, 14.4 and 72 mg/kg bw paraquat cation, respectively). Two additional groups were pre-treated with an ip injection of 150 mg/kg bw atropine or 5 mg/kg bw methylatropine 30 minutes prior to an ip injection of 100 mg/kg bw paraquat (equivalent to 72 mg/kg bw paraquat cation). No effect was seen at 10 nmol (equivalent to 1.86 μg or 1.34 μg paraquat cation) following a single intrahippocampal injection. Seizures, behavioural excitation, motor stimulation (ataxia, stereotyped jaw movements, clonus of the upper extremities, rapid ear movements, vibrissae) and the presence of multifocal brain damage (neural damage/degeneration in the CA1 region, dentate granule cell layer, hilus fascia dentata, and pyriform cortex) were seen at and above 100 nmol (equivalent to 18.6 μg paraquat or 13.4 μg paraquat cation). Atropine (50 nmol) and MK 801 (0.3 mg/kg, ip) prevented the occurrence of these treatment-related effects suggesting that the mechanism of neurotoxicity involved muscarinic and NMDA receptors. No effect was seen following a single ip injection of paraquat at 5 mg/kg bw/d (equivalent to 3.6 mg/kg bw paraquat cation). Behavioural and motor stimulation (muscle fasciculation, chromodacryorrhea, salivation, tremors, infrequent wet dog shakes, movement of the vibrissae, clonus of the forelimbs and rearing), seizures and brain damage (neural death in the pyriform cortex) were seen at and above 20 mg/kg bw (equivalent to 14.4 mg/kg bw paraquat cation). Pre-treatment with atropine, but not methylatropine, prevented these treatment-related effects. A dose-related increase in the concentration of paraquat in the medulla oblongata, hippocampus and pyriform cortex was observed 24 h after an ip injection, with a similar concentration of paraquat measured in each brain region (Bagetta *et al*, 1992).

The neurotoxicity of paraquat in rats was investigated following a single intracerebral injection at 0, 0.01 or 0.1 μmol (equivalent to 0, 1.86 and 18.6 μg paraquat, respectively, or 0, 1.34 and 13.4 μg paraquat cation, respectively), or a single subcutaneous injection at 0, 5.0 or 20 mg/kg bw (equivalent to 0, 3.6 and 14.4 mg/kg bw paraquat cation, respectively). Treatment-related effects were observed at 0.10 μmol (18.6 μg paraquat or 13.4 μg paraquat cation) and included ataxia, neuronal death and damage in the CA1 and CA3 pyramidal cell layers, the granule cell layer and hilus fascia dentata of the hippocampus, and the pyriform complex, behavioural stimulation (clonus of the upper extremities, movement of the ears and vibrissae) and generalised seizures. Following subcutaneous injection, no effects were seen at 5 mg/kg bw (equivalent to 3.6 mg/kg bw paraquat cation), but neuronal death and damage in the pyriform cortex, and abnormal behaviour (tremors, wet dog shakes, clonus and rearing) were seen at 20 mg/k bw (equivalent to 14.4 mg/kg bw paraquat cation). The occurrence of neuronal death in the pyriform cortex correlated with brain paraquat analysis which revealed the pyriform cortex

contained the highest concentration of paraquat followed by the hippocampus and the caudate (Corasaniti *et al*, 1992).

The effect of paraquat or MPP^+ on the SN of male rats was studied following direct unilateral intranigral injection of 0, 1, 2, 3 or 5 μg paraquat (equivalent to 0, 0.72, 1.44, 2.16 and 3.6 μg paraquat cation, respectively), or 8 μg MPP^+. At and above 1 μg paraquat (equivalent to 0.72 μg paraquat cation), a significant dose-related depression in striatal DA occurred. Neurohistopathological abnormalities (abnormal neurons, neuronal loss and astrocyte proliferation) and a statistically significant dose-related increase in apomorphine-induced contralateral circling were observed at and above 2 μg (equivalent to 1.44 μg paraquat cation). At 3 μg (equivalent to 2.16 μg paraquat cation), rotational behaviour in the direction of the lesion, asymmetrical posture or weak sniffing behaviour was exhibited, in addition to a significant depression in striatal DOPAC and HVA. At 5 μg, all rats died. Rats treated with 8 μg MPP^+ showed a significant depression in striatal DA, DOPAC and HVA, and neuronal loss, astrocyte proliferation and abnormal behaviour (Liou *et al*, 1996).

When paraquat was administered to the striatum through a microdialysis probe, a significant amount of paraquat was detected in the ipsilateral but not contralateral striata after a sequential 180-min washout with Ringer's solution. Treatment with 50 μM GBR-12909 (a selective DAT inhibitor) significantly inhibited the striatal uptake of paraquat ($p < 0.02$). It was concluded that paraquat stimulated glutamate efflux from neural cells or inhibited the glutamate uptake system, and initiated a cascade of excitotoxic reactions leading to damage of dopaminergic terminals. The mechanism involves glutamate induced activation of non-*N*-methyl *D*-aspartate (non-NMDA) receptors, resulting in activation of NMDA receptors. The activation of NMDA receptor channels results in a massive influx of Ca^{2+} into the cells. The entry of Ca^{2+} into cells stimulates nitric oxide synthase (NOS). In discussing their results Shimizu *et al* (2003) suggested that released nitric oxide (NO) would diffuse to dopaminergic terminals and induce mitochondrial dysfunction by the formation of peroxynitrite, resulting in continuous and long-lasting DA overflow. Paraquat might trigger production of reactive oxygen species (ROS) from the mitochondrial electron transport chain in DA neurons. The ROS easily react with NO to generate peroxynitrite, which could be a major substrate underlying paraquat neurotoxicity They speculated that paraquat could be considered an exogenous neurotoxin involved in the etiology of Parkinson's disease, or at least, exposure to low levels of paraquat for a long time would make dopaminergic neurons vulnerable to oxidative stress and cell death. This speculation could be applied to any agent capable of generating ROS and does not indicate that paraquat exposure through normal dietary, inhalational or dermal route would elicit the same responses (Shimizu *et al*, 2003).

8.5 Intranasal Administration

Rojo *et al* (2007) studied the induction of Parkinson's disease symptoms in mice and rats following the intranasal inoculation of 3, 10, 20 or 30 mg paraquat[3]/kg bw/day for 30 days. As a positive control, MPTP was administered at 10, 20, 30 or 60 mg/kg bw by the same route. Mice that received up to 20 mg/kg bw paraquat showed no behavioural changes early after dosing. After one week of dosing, mice receiving 20 mg/kg bw developed, strong hypokinesia in addition to curved position, agitated breathing, weight loss, cyanotic feet and mouth, and

[3] Referred to simply as 'paraquat'. Unspecified if paraquat dichloride or cation.

lung damage. Around a third of mice treated with 30 mg/kg bw died in the first week and another third showed truncal dystonia and fast rotation when held by the tail (indicative of vestibular dysfunction). MPTP-treated mice demonstrated piloerection, salivation, tremor and smooth rigidity shortly after administration of 30 or 60 mg/kg bw. In addition, motor activity was reduced, becoming statistically significant at the end of the treatment period. Mice receiving 20 mg/kg bw day showed no difference from controls in the levels of striatal DA or its major metabolite DOPAC. Mice treated with 30 or 60 mg/kg bw MPTP showed significant reductions in these levels.There was no difference in tyrosine hydroxylase immunoreactivity (TH-ir) in the striatum or SN in mice treated at 20 mg/kg bw/d paraquat, in contrast to MPTP-treated mice with 20-30% less TH^+ neurons than controls. Paraquat appeared then not to alter the nigrostrial system, and the authors attributed the motor deficit as to weakness due to systemic effects. Further exploration with a single dose of 20 mg/kg bw revealed that 10 minutes afterwards paraquat was detected only in the olfactory bulb (0.15 µg/g tissue) and not in the striatum or ventral mid-brain which suggested that it might use at least in part the olfactory nerve tract to reach the olfactory bulb. It appeared as though paraquat had a limited capacity to reach the nigrostriatal system in mice when the intranasal route of exposure is used. MPTP by contrast was detected in the olfactory bulb, striatum and SN. Rats subjected to the same paraquat dosing regime, also did not show any progressive nigrostriatal dopaminergic degeneration and paraquat was only detected in the olfactory bulb. Although this method is not a standard route of administration in animals studies, it demonstrates that absorption via the nasal mucosa- a potential point of contact for humans following inhalation- does not result in effects in the nigrostriatal system despite clear evidence of systemic toxicity.

8.6 Blood Brain Barrier (BBB) Mechanistic Studies

The localisation of paraquat in the lung and brain was immunohistochemically assessed in male rats following a single iv injection at 5 mg/kg bw (equivalent to 3.6 mg/kg bw paraquat cation). As expected, inflammatory cell infiltration, deposition of collagen fibres in interstitial spaces and thickening of the alveolar septum were observed, but no histopathological brain abnormalities were detected. In the lungs, paraquat was localised to blood vessel walls, histiocytes and bronchiolar epithelial cells. According to the study authors the localisation of paraquat to bronchiolar epithelial cells appeared to be associated with secretion of paraquat into the bronchiole. In brain slices, paraquat was detected in capillary walls and glial cells, but not in nerve cells (Nagao *et al,* 1991).

A series of studies investigated tissue distribution and elimination kinetics, regional brain distribution and neuropathological effects of paraquat in male rats following a single sc injection of 20 mg/kg bw (equivalent to 14.4 mg/kg bw paraquat cation). The highest concentrations of paraquat in the blood, brain, liver, and lungs were observed 30 minutes post-dose while the highest kidney levels were detected at one hour post-dose. With the exception of the lung, tissue paraquat levels declined rapidly over time. At 24 h post-dose, the highest paraquat concentration in the brain was detected in the forebrain while the lowest level was detected in the striatum. Relatively high concentrations of paraquat were found in brain regions which lack, or lie outside, the BBB such as the pineal gland, lining of the ventricles, anterior olfactory bulb, olfactory tubercle, area postrema of the medulla oblongata and hypothalamus. No evidence of any neuronal damage was observed in any part of the brain (Naylor *et al,* 1995).

In a study to further investigate the penetration of the BBB by systemic paraquat, anaethetised Rhesus macaques were subject to PET imaging of the brain following the administration of intravenous [^{11}C] paraquat. The study found minimal paraquat uptake and retention in the whole primate brain, being weakly sequestered by the pineal gland and lateral ventricles, but not the DA terminals of the caudate nucleus and putamen (Bartlett *et al*, 2009).

Experiments were performed to determine the extent of entry of a single median lethal dose of paraquat (20 mg/kg bw, sc; equivalent to 14.4 mg/kg bw paraquat cation) into the brain of neonatal, adult and aged rats. Paraquat entered the brain of all groups of rats of various ages in the absence of any neuronal damage. In contrast to adult and aged rats where brain paraquat levels fell over 24 h, paraquat levels in neonatal brains did not decrease over the study period and were significantly higher than adult and aged brains at 24 h post-dose. In all age groups, paraquat was initially located to regions outside the BBB (dorsal hypothalamus, area postrema, anterior olfactory bulb) but had moved into deeper brain regions by 24 h (striatum, hypothalamus and substantia). The elevation in paraquat uptake by neonatal brains was attributed to impaired BBB integrity (Widdowson *et al*, 1996a).

Widdowson *et al* (1996b) also demonstrated that paraquat can cross the BBB in a study where male rats received oral gavage doses of 0 or 5 mg/kg bw/d paraquat ion. In this study, 24-h following a single oral dose, levels of paraquat were highest in the lung, then the kidney, liver, brain and plasma, whereas following 14 daily oral doses the levels were highest in the lung, followed by the brain, kidney, liver and plasma. Levels in the brain were approximately 10-fold higher following repeated dosing as compared to a single dose, and only 2-4 fold higher in other tissues measured. Overall, this study demonstrated no evidence of neurotoxicity when paraquat is administered by oral gavage to Wistar-derived rats at 5 mg/kg bw/d paraquat cation for 14 days, despite evidence that paraquat could enter the brain.

An *in vivo* brain microdialysis technique was used to determine whether a single sc dose of paraquat at 5, 10 or 20 mg/kg bw (equivalent to 3.6, 7.2 and 14.4 mg/kg bw paraquat cation, respectively), or a single dose of MPP^+ at 10 mg/kg bw, could penetrate the BBB in rats, and the possible mechanism of any uptake. Paraquat crossed the BBB in a dose-dependent manner which appeared to be mediated by a neutral amino acid transporter as pre-treatment with L-valine (200 mg/kg bw, ip), but not L-lysine (200 mg/kg bw, ip) significantly reduced uptake. Entry into striatal cells appeared to require Na^+, but did not involve the polyamine transporter as 50 μM putrescine did not block uptake (Shimizu *et al*, 2001).

The effect of age on the permeability of the BBB to paraquat was investigated in 2-week, 3-, 12- and 24-month old male rats. Rats sacrificed 1 h after sc dosing at 1, 2.5 and 5.0 mg/kg bw (equivalent to 0.72, 1.8 and 3.6 mg/kg bw paraquat cation) had a dose-related increase in brain paraquat levels across all age groups. Paraquat was confined to brain tissue and not located in the cerebral circulation. There was an equivocal effect of age on paraquat uptake with the 2-week and 24-month old rats showing an elevation in brain paraquat levels relative to 3-and 12-month old rats. This result was difficult to interpret given the small group sizes and the absence of plasma paraquat levels to confirm changes in the integrity of the BBB (Corasaniti *et al*, 1991).

Rojo *et al* (2007) studied the induction of Parkinson's disease symptoms in mice and rats following the intranasal inoculation paraquat. A subset of this study assessed the presence of

both paraquat and the positive control MPTP in the brain. Ten minutes following a single intranasal dose of 20 mg paraquat/kg bw, paraquat was only detected in the olfactory bulb (0.15 µg/g tissue) and not in the striatum or ventral mid-brain, suggesting tracking along the olfactory nerve at least in part, to reach the olfactory bulb. It appeared as though paraquat had a limited capacity to reach the nigrostriatal system in mice when the intranasal route of exposure is used. MPTP by contrast was detected in the olfactory bulb, striatum and SN. The same findings were made in rats subject to the same dosing methodology.

8.7 Neurotoxicity Mechanisms

Miranda-Contreras *et al* (2005) studied the effects of prenatal exposure to paraquat on the development of amino acid synaptic transmission in mouse cerebellar cortex. Pregnant (10 days gestation) NMRI mice (University of Los Andes, Merida, Venezuela) were randomly assigned into four treatment groups (40/group), which were administered with either saline, paraquat, mancozeb or a combination of paraquat and mancozeb between gestation days 12 and 20 at intervals of 48 h between each dose, in a total of five doses. The dams were injected ip with either the vehicle (saline), paraquat at a dose of 10 mg/kg bw, mancozeb at a dose of 30 mg/kg bw or a combination of 10 mg/kg bw paraquat and 30 mg/kg bw mancozeb.. Only the results of paraquat treatment were considered here. Simultaneous assays of the neurotransmitter amino acids, glutamate, aspartate, glycine, taurine and GABA, were conducted using high performance liquid chromatography (HPLC) to assess affects on cerebellar cortex synaptic circuitry. Significant alterations in the normal chronology and magnitude of excitatory and inhibitory synaptic transmissions in the developing mouse cerebellar cortex caused by prenatal exposure of the mice to paraquat were observed in this study. Additional details are presented in APPENDIX I.

Richardson *et al* (2005) examined the role of the DAT and Complex I in the toxicity of paraquat in *in vitro* and *in vivo* studies, respectively. It was concluded that the toxicological effects of paraquat are not the result of transport by DAT or complex I inhibition.

Castello *et al* (2007) showed the involvement of brain mitochondria in PQ^{2+}-induced ROS production in an *in vitro* study. There was a robust and instantaneous production of H_2O_2 by mitochondria after exposure to PQ^{2+}. PQ^{2+} -induced H_2O_2 production by brain mitochondria is dependent on a constant electron flow provided by respiration substrates and a functional ETC. With the use of specific inhibitors of the ETC, complex III has been identified as a novel site of action for PQ^{2+} in the process of redox cycling to generate ROS. Further, the mechanism of induced ROS production is dependent on mitochondrial membrane potential. It was proposed that mitochondrial H_2O_2 production induced by PQ^{2+} in the brain is an early event that may later initiate other cellular events, such as nonspecific ETC inactivation, microgliosis, and NADPH oxidase activation.

Cicchetti *et al* (2005) provided evidence that paraquat alone and in combination with maneb is toxic to dopaminergic neurons of eight week old male Sprague Dawley rats both *in vitro* and *in vivo* and that the combination leads to Parkinson-like movement deficits. The rats were injected ip twice a week for four weeks with 10 mg/kg bw paraquat dichloride hydrate (7.2 mg paraquat ion/kg bw) or a combination of paraquat and maneb at 10 mg/kg bw and 30 mg/kg bw respectively. There was no significant evidence that paraquat alone induced Parkinson's like movements in rats. Activation of microglia in the SN pc of animals with a significant

decreases in TH-ir cells was indicative of inflammation in the area, however the role of inflammation in the overall process is unknown. Additional detail is presented in APPENDIX I.

Ramachandiran *et al* (2007) studied the molecular mechanism of paraquat toxicity in an *in vitro* study. Paraquat specifically oxidized the cytosolic form of thioredoxin and activated Jun N-terminal kinase (JNK), followed by caspase-3 activation. Conversely, 1-methyl-4-phenylpyridinium (MPP^+) and rotenone oxidized the mitochondrial form of thioredoxin but did not activate JNK-mitogen-activated protein kinase and caspase-3. Oxidative modification of cytosolic proteins is critical to paraquat toxicity, while oxidation of mitochondrial proteins is important for MPP^+ and rotenone toxicity.

Choi *et al* (2008) tested the hypothesis of the inhibition of mitochondrial complex I being one of the leading hypotheses for dopaminergic neuron death associated with Parkinson's disease. They used a mouse strain lacking functional Ndufs4, a gene encoding a subunit required for complete assembly and function of complex I. They determined that dopaminergic neuron death induced by treatment with rotenone, MPP (+), or paraquat is independent of complex I inhibition.

Fei *et al* (2008) identified a BAK-dependent cell death mechanism is required for paraquat-induced neurotoxicity in an *in vitro* study with SK-N-SH cell lines. Paraquat induced morphological and biochemical features that were consistent with apoptosis, including dose-dependent cytochrome *c* release, with subsequent caspase-3 and poly(ADP-ribose) polymerase cleavage. Changes in nuclear morphology and loss of viability were blocked by cycloheximide, caspase inhibitor, and Bcl-2 overexpression, Evaluation of Bcl-2 family members showed that paraquat induced high levels of BAK, Bid, BNip3, and Noxa. Small interfering RNA-mediated knockdown of BNip3, Noxa, and BAK each protected cells from paraquat, but Bax knockdown did not. The sensitivity of BAK-deficient mice was tested and they were found resistant to paraquat treatments that depleted TH immuno-positive neurons in the SN pc of wild-type mice.

To investigate the possibility that opioid receptors are involved in paraquat-induced shaking behaviour in male rats, a single ip injection was given at 0, 30, 50 or 70 mg/kg bw (equivalent to 0, 21.6, 36 and 50.4 mg/kg bw paraquat cation, respectively), or a single intracerebroventricular injection was given at 12.9 or 25.7 μg/10 μL/rat (equivalent to 9.3 and 18.5 μg cation, respectively), in the presence and absence of morphine or naloxone (5 and 1.5 mg/kg bw, ip, respectively). It was noted that morphine but not naloxone pre-treatment significantly reduced paraquat-induced shaking behaviour suggesting that shaking behaviour was mediated by opioid receptors. Although no neurohistopathological abnormalities were detected in any rats, fluorescein uptake by the brain was significantly elevated with paraquat treatment, an effect that was prevented by morphine. These later findings suggested that paraquat increased the vascular permeability of the brain (Hara *et al*, 1993).

Bagetta *et al* (1992) found that seizures, behavioural excitation, motor stimulation (ataxia, stereotyped jaw movements, clonus of the upper extremities, rapid ear movements, vibrissae) and the presence of multifocal brain damage (neural damage/degeneration in the CA1 region, dentate granule cell layer, hilus fascia dentata, and pyriform cortex) were seen at and above 100 nmol (equivalent to 18.6 μg paraquat or 13.4 μg paraquat cation) when administered by intrahippocampal injection. Atropine (50 nmol) and MK 801 (0.3 mg/kg, ip) prevented the

occurrence of these treatment-related effects suggesting that the mechanism of neurotoxicity involved muscarinic (acetylcholine) and NMDA (glutamate) receptors. Additional study detail presented in APPENDIX I.

8.8 Summary of key neurotoxicity endpoints and observations from evaluated studies

A tabulated summary of all the NOELs/LOELs for neurotoxicity endpoints is provided in Table 1 below. Table 2 includes other studies which didn't establish NOELs or LOELs, but were assessed as supporting information.

Table 1 - Summary of key neurotoxicity endpoints relevant to risk assessment†

Species	Dose route	Study duration	NOEL/LOEL Paraquat ion	Observations (reference)
Mouse	Oral	Single dose administered to neonatal rats at age 10-11 days	LOEL 0.05 mg/kg bw[b]	Hypoactivity (60 – 120 days of age) and decreased striatal homovanillic acid (HVA) (125 days of age). A significant decrease in striatal dopamine and 3,4-dihydroxyphentlacetic acid (DOPAC) was observed at a dose of 0.26 mg/kg bw. (Fredriksson et al, 1993).
		90 days	NOEL >10 mg/kg bw/d[a]	No neuronal cell loss up to the highest tested dose (Minnema, 2014).
	Intranasal	30 days	LOEL 20 mg/kg bw/day	Behavioural effects, foot, mouth and lung damage observed at dose of 20 mg/kg bw/day (Rojo et al, 2007).
Rat	Oral	Single dose	NOEL 84 mg/kg bw[a]	No evidence of neurotoxicity at highest dose of 84 mg/kg bw (Brammer, 2006).
		14 days	NOEL 5 mg/kg bw/d	No sign of neuronal damage cell damage or death at dose of 5 mg/kg bw/d (paraquat ion) (Widdowson et al, 1996b).
		90 days	NOEL 10.2 (males) mg/kg bw/d[a] NOEL 11.9 (females) mg/kg bw/d[a]	No effect on nervous system tissues (Chivers, 2006).

† Only dose-routes considered relevant to human exposure are included in this table

[a] Highest dose tested

[b] Lowest dose tested

Table 2 - Summary of observations from other studies evaluated in the neurotoxicity risk assessment

Species	Dose route	Study duration	Observations (reference)
Mouse	Subcutaneous	Single dose for 3 injections at 3 day intervals	Some neurochemical variations observed at doses of 10.44 mg/kg bw paraquat ion, but not deemed to be toxicologically relevant (Perry et al, 1986).
	Intraperitoneal	3 injections at weekly intervals	Some neurochemical variations observed at doses of 3.6 mg/kg bw paraquat ion (Brooks et al, 1999).
Rat	Subcutaneous	Single injection	No effects observed at dose of 3.6 mg/kg bw paraquat ion. Behavioural effects and neural damage observed at dose of 14.4 mg/kg bw paraquat ion (Corasaniti et al, 1992).
		5 days	Some neurochemical variations observed at a dose of 10 mg/kg bw/d (Shimizu et al, 2003).
			Some neurochemical variations observed at a dose of 10 mg/kg bw/d (Kuter et al, 2007).
	Intraperitoneal	Single injection	Increased shaking behaviour observed at doses ≥21.6 mg/kg bw paraquat ion (Hara et al, 1993).
			No effect observed at dose of 3.6 mg/kg bw. Behavioural effects and neural damage observed at doses at and above 14.4 mg/kg bw paraquat ion (Bagetta et al, 1992).
			Consistent but reversible influence on dopamine at a dose of 10 mg/kg bw (Ossowska, 2005a).
		1, 2 or 3 injections at weekly intervals	NOEL >25 mg/kg bw/3 weekly ip injections[a] No neuronal cell loss up to the highest tested dose (Beck, 2012a,c).
		Administered twice a week for 4 weeks	Toxic effects on dopaminergic neurons observed at dose of 10 mg/kg bw paraquat ion (Cicchetti et al, 2005).
		Once a week for 4, 8, 12 or 24 weeks	Slowly progressing neuronal effects observed at 10 mg/kg bw (Ossowska, 2005a).
	Intracerebroventricular	Single injection	Tremor induced at dose of 18.5 µg paraquat ion/rat (Hara et al, 1993).
	Intravenous	5 days	Some neurochemical variations observed at dose of 14.4 mg/kg bw/d paraquat ion (Tawara et al, 1996).
	Intrahippocampal	Single injection	Behavioural effects and neural damage observed at and above dose of 13.4 µg paraquat ion (Bagetta et al, 1992).
	Intracerebral		Behavioural effects and neural cell damage observed at dose of 13.4 µg/kg bw paraquat ion (Corasaniti et al, 1992).
	Intranigral		Neurochemical variations observed at dose of 0.72 µg paraquat ion. Neural damage observed at dose of 1.44 µg paraquat ion. Behavioural effects observed at dose of 2.16 µg paraquat ion (Liou et al, 1996).

[a] Highest dose tested

8.9 Epidemiology

Epidemiology studies by Kamel *et al* (2007) and Costello *et al* (2009) claimed an association between Parkinson's disease and exposure to paraquat or pesticides. A comprehensive analysis was conducted for these studies, in terms of robustness of survey methodology, and in terms

of the basis for the strength of the associations which were drawn in the respective reports (presented in detail in Section 11). It was concluded that although the data indicated a slight trend towards a positive statistical association between pesticide exposure and the presence of Parkinson's disease, the strength of this association was considered to be tenuous.

Tomenson and Campbell (2011) conducted a retrospective cohort study to assess the risk of Parkinson's disease among a UK workforce who manufactured paraquat. The study was based on an original cohort from Paddle *et al* (1991) assessing workers manufacturing paraquat in 4 plants from 1961-1985. It was considered feasible that exposure of these manufacturing workers was at least comparable to paraquat sprayers, based on static and personal monitoring data, although high exposure was only considered likely in workers from the 1960s. No evidence of an increase in Parkinson's was found as compared to control populations.

9 EVALUATIONS OF SELECTED ANIMAL STUDIES

9.1 General studies investigating the neurotoxicity of paraquat

Brammer, A (2006) Paraquat technical: Acute neurotoxicity study in rats. Report No: AR7536-REG; Study No: AR7536; Task No. T000344-05. Syngenta Central Toxicology Laboratory, Alderley Park, Macclesfield, Cheshire, UK. Study initiation 22 Dec, 2005, Study commencement: 3 Jan, 2006; Study termination: 6 April, 2006; Report date 8 June, 2006.

Guidelines & GLP: GLP and QA study. Study was conducted in compliance with the UK Principles of GLP (1999, 2004) and OECD principles of GLP, in accord with ENV/MC/CHEM(98)17 (1997); OECD Guideline 424 (1997), Neurotoxicity Study in Rodents; US EPA Health Effects Guidelines, OPPTS 870.6200 (1998) Neurotoxicity Screening Battery; Japanese Ministry of Agriculture, Forestry and Fisheries Test Guideline 2-1-12 (Sept 2001).

Materials & Methods: Technical grade paraquat (33.4% paraquat ion, 46.1% paraquat dichloride, batch P47, CTL ref No. Y12693/106, Huddersfield, *via* Syngenta Crop Protection, Munchwilen AG) was diluted in de-ionised water and administered at 10 mL/kg bw.

Rats (Alpk:AP_fSD; Wistar-derived, 10/sex/group, ≥ 42 days old at dosing, males 259-330 g, females 189-246 g on Day -1) received a single gavage dose of 0, 25, 75, or 250 mg paraquat technical/kg bw (or 0, 8, 25, or 84 mg/kg bw paraquat ion/kg bw, respectively) and were observed for 14 days. Dose selection was based on a preliminary neurotoxicity study (Brammer, 2005, not provided). Functional observational battery, including quantitative assessments of landing foot splay, sensory perception and muscle weakness, were performed in week -1, and days 1 (2 h post-dose), 8, and 15. Rat body weights and food consumption were measured weekly. At termination, rats (5/sex/group) were perfused *in situ* and selected nervous system tissues (brain, eye -with optic nerve and retina, spinal cord -including cervical and lumbar swellings, spinal nerve roots of cervical and lumbar swellings, dorsal root ganglia at cervical and lumbar swellings, proximal sciatic, proximal and distal tibial nerves and gastrocnemius muscle) were removed and processed for microscopy. Remaining animals were sacrificed and discarded.

Results: One high dose male died on day 5 (following reduced splayed reflex and pinched in flanks over days 1-4) and one high dose female was terminated *in extremis* on day 4 due to adverse clinical signs (irregular breathing, flaccidity, pinched in flanks, upward spinal curvature on days 2-4 and piloerection and ocular discharge on days 3-4). No other clinical signs of toxicity were reported and all remaining rats survived until scheduled termination.

Body weights were slightly variable in rats which received the high dose but without statistical significance on day 15. At the mid dose, male body weights were significantly lower only on day 8 (no effect in females), and in rats which received the low dose no difference from controls was observed. No significant changes in food consumption were observed.

Minor changes were noted in the functional observation battery parameters, but none were dose-related. An increased incidence of reduced splay reflex was generally observed in a

minority of treated animals. The observations in control females and absence in control males suggests that there may be gender differences in base-line data for this functional observation parameter and along with the lack of statistical analysis preclude an assignment of whether the observations are toxicologically relevant (Table 3 and Table 4). There were no treatment-related changes in landing foot-splay, time to tail-flick, and fore- and hind-limb grip strength measurements. Changes in motor activity measurements were sporadic and not associated with treatment.

Table 3. Cumulative observations –Reduced splay reflex

	Male				Female			
Dose*	0	25	75	250	0	25	75	250
No, events	0	4	3	11	9	5	13	17
No. rats affected	0	1	3	2	2	1	2	3
No, rats at Day 15	10	10	10	9	10	10	10	9
Day range		8 - 11	-7 - 15	-7 - 15	8 - 15	-7 - 15	1 - 15	1 - 15

*Dose = paraquat technical mg/kg bw.

Table 4. Summary of intergroup comparison of functional observation battery – Reduced splay reflex

	Male				Female			
Dose*	0	25	75	250	0	25	75	250
Day -7	0	0	1	1	0	1	0	0
Day 1	0	0	0	2	0	1	1	2
Day 8	0	1	0	1^{A}	1^{B}	0	2	2^{A}
Day 15	0	0	2	1^{A}	2	1	1	1^{A}

*Dose = paraquat technical mg/kg bw; ^{A} n=9; The severity of all events was graded as slight except as marked "^{B}" which was of moderate severity.

At necropsy, no treatment-related differences in (perfused) brain weights or macroscopic observations were noted. Microscopic examinations were reported for control and high dose groups only. Minimal demyelination/nerve fibre degeneration was observed in sciatic nerves but in both control and high dose animals. Demyelination/nerve fibre degeneration in proximal tibial nerves affected 4/5 control males and 4/5 high dose males compared to 2/5 control females and 4/5 high dose females. While group sizes examined (5/sex) were minimal, these observations appear to be without association to the functional observations and therefore may be incidental to treatment. Histopathological examination did not include examination of brain or SN pc.

Conclusions: Mortalities/unscheduled deaths (10%) were observed at the high dose (84 mg paraquat ion/kg bw) in males and females and was consistent with the po LD_{50} range of 100-249 mg/kg bw. Preceding clinical signs were consistent with paraquat toxicity. In surviving rats, slight changes in body weights in rats which received ≥25 mg paraquat ion/kg bw, were likely to be indicative of general systemic toxicity. Results regarding the reduced splay reflex suggested a slight effect in males but the data were inconclusive in females due to a higher incidence in the control females. Therefore, following a single oral dose of paraquat of up to 84 mg paraquat ion/kg bw to rats there was no clear evidence of neurotoxicity.

Chivers S (2006) Paraquat: Subchronic neurotoxicity study in the rat. Report No: PR1322-REG; Study No: PR1322; Task No. T000153-06. Syngenta Central Toxicology

Laboratory, Alderley Park, Macclesfield, Cheshire, UK. Study initiation 22 Dec, 2005, Study commencement: 15 March, 2005; Study termination: 2 September 2005; Report date 6 June 2006.

Guidelines & GLP: GLP and QA study. Conducted in compliance with the UK Principles of GLP (1999, 2004) and OECD principles of GLP, in accord with ENV/MC/CHEM(98)17 (1997) OECD Guideline 424 (1997), Neurotoxicity Study in Rodents; US EPA Health Effects Guidelines, OPPTS 870.6200 (1998) Neurotoxicity Screening Battery; Japanese Ministry of Agriculture, Forestry and Fisheries Test Guideline 2-1-12 (Sept 2001).

Materials & Methods:
Technical grade paraquat (33.4% paraquat ion, 45.1% paraquat dichloride, batch 216/ reference P47, CTL ref No. Y04517/015, Huddersfield, *via* Syngenta Crop Protection, Munchwilen, AG) was administered *via* the diet (based on CT1/RM3 diet).

Rats (Alpk:AP_fSD (Wistar-derived, 12/sex/group, ≥ 42 days old at dosing), received 0, 15, 50, or 150 ppm paraquat cation in the diet for 90 days. The dose equivalents (as paraquat cation) received by males was 0, 1.0, 3.4 or 10.2 mg/kg bw/d and by females 0, 1.1, 3.9 or 11.9 mg/kg bw/d, respectively. Dose selection was based on a preliminary 28 day dietary neurotoxicity study (100, 150 or 250 ppm, Chivers, 2006, not provided) and was adequately justified as follows: signs of lung pathology and decreased body weight were observed at 250 ppm, therefore this dose would preclude examination of neurotoxicity findings and was unsuitable for a subchronic dietary neurotoxicity study. No adverse effects were reported at 100 or 150 ppm paraquat cation in the preliminary study, however other reports have indicated signs of lung toxicity at 150 ppm. In the subchronic study (Chivers, 2006), functional observational battery (including quantitative assessments of landing foot splay, sensory perception, grip strength and muscle weakness) and motor activity were performed in weeks -1, 2, 5, 9 and 14. Rat body weights and food consumption were measured weekly. At termination, rats (5/sex/group) were perfused *in situ* with formal saline. Selected nervous system tissues from control and high dose animals were removed and processed for microscopy (brain at 7 levels, but specific location details/ orientation for each level were not specified [the OECD TG states that *"The areas examined should normally include: the forebrain, the centre of the cerebrum, including a section through the hippocampus, the midbrain, the cerebellum, the pons, the medulla oblongata"*], eye -with optic nerve and retina, spinal cord -including cervical and lumbar swellings, spinal nerve roots of cervical and lumbar swellings, dorsal root ganglia at cervical and lumbar swellings, proximal sciatic, proximal and distal tibial nerves and gastrocnemius muscle). Remaining animals were sacrificed and discarded.

Results:
One male rat which received the low dose died on day 22. At necropsy, clear exudate from the nasal passages, a dark partially inflated lung and unilateral pelvic dilation of the kidney were reported. The finding was not considered to be related to treatment. No other clinical signs of toxicity were reported and all remaining rats survived until scheduled termination.

Body weights for males which received the high dose were significantly lower than control male body weights only during week 2. This observation appeared to be a consequence of decreased food consumption in week 1 for this treatment group. No other effects on body weight or food consumption were observed. Minor changes were noted in the functional

observation battery parameters, but none were considered to be significant because the incidence and severity was similar between control and test animals. No macroscopic findings (all groups), changes in brain organ weights, ophthalmology changes (in control or high dose) or microscopic findings (control or high dose) which were related to treatment were reported.

The NOEL assigned for neurotoxic potential in rats as investigated in this study was 150 ppm (as paraquat cation) in the diet for 90 days, or 10.2 and 11.9 mg/kg bw/d for males and females, respectively. This was based on the absence of treatment-related toxicity findings in both male and female rats at the high dose.

9.2 Studies on brain distribution (including other tissues) of paraquat

Naylor JL, Widdowson PS, Simpson MG, Farnworth M, Ellis MK & Lock EA (1995) Further evidence that the blood/brain barrier impedes paraquat entry into the brain. ***Hum Exp Toxicol 14: 587-594.***

Male Wistar rats (250-300 g body weight, age unspecified; Alderley Park strain) were given a single sc injection of [^{14}C]-paraquat (93-103 mCi/mmol; Cambridge Research Biochemicals, Gadbrook Park, Northwich, Cheshire, UK) in sterile deionised water at 20 mg/kg bw; (equivalent to 14.4 mg/kg bw paraquat cation; 2 mCi/kg bw) which was the LD_{50} dose. The dose volume was 2 mL/kg bw. A separate group of rats were given a single tail vein injection of [^{14}C]-carboxyl-inulin (a polysaccharide which does not cross the BBB) in sterile deionised water at 16 µCi/kg bw. In both cases, the group sizes were unspecified. Rats were sacrificed at 0.5, 1, 2, 4, 8 and 24 h post treatment, and the amount of [^{14}C]-paraquat in the brain, lung, liver and kidney analysed by liquid scintillation counting (LSC). The amount of ^{14}C-paraquat and ^{14}C-inulin in whole blood was determined by sample oxidation. To determine whether any paraquat remaining in the brain after 24 h of treatment was confined to the cerebral circulation, a group of 5 paraquat treated rats were sacrificed by pentobarbitone overdose and intracardially perfused with saline.

In a separate experiment, the regional distribution of paraquat in the brain was determined following a single sc injection of [^{14}C]-paraquat at 20 mg/kg bw (n=4; equivalent to 14.4 mg/kg bw paraquat cation; 10 mCi/kg bw). Rats were sacrificed 30 minutes or 24 h later, their brains removed and processed for autoradiography. Autoradiograms were analysed by quantitative densitometry.

To investigate any treatment-related neuropathological effects, two additional groups of rats were treated with paraquat at 20 mg/kg bw (equivalent to 14.4 mg/kg bw paraquat cation; group size unspecified). Rats were sacrificed at 24 and 48 h post treatment. Two other groups were sacrificed at the same intervals and intracardially perfused with Karnovsky's fixative. The brain of each rat was histopathologically examined following staining with haematoxylin and eosin

Findings: The presence/absence of deaths and clinical signs were unreported. The highest concentration of [^{14}C]-paraquat in the blood, brain, liver and lung was detected at 0.5 h post-dose, while the highest kidney levels were detected at one hour (see Table 5 below). Paraquat levels in all tissue then declined rapidly over time, with the exception of the lung where approximately 25% of the 0.5 h concentration remained at 24 h. The study authors noted that little paraquat was lost from the brain between 8 to 24 h.

Table 5: Distribution of paraquat (nmol/g tissue) in different tissues

Time (h)	Whole blood	Plasma	Brain	Kidney	Lung	Liver
0.5	77.7 ± 0.9	158.2 ± 12.1	11.9 ± 5.6	508.4 ± 102.2	86.5 ± 4.5	44.2 ± 1.6
1	44.0 ± 0.8	94.2 ± 3.4	5.4 ± 0.4	752.7 ± 113.4	73.1 ± 7.2	44.2 ± 3.7
2	9.3 ± 0.3	18.8 ± 0.3	2.9 ± 0.3	203.2 ± 57.5	47.0 ± 1.2	24.6 ± 4.0
4	1.5 ± 0.1	2.2 ± 0.2	3.4 ± 0.9	37.1 ± 4.1	32.2 ± 2.7	11.2 ± 2.6
8	0.8 ± 0.1	0.9 ± 0.1	1.9 ± 0.0	22.9 ± 0.6	34.7 ± 2.6	6.8 ± 0.8
24	0.6 ± 0.0	0.3 ± 0.0	1.6 ± 0.1	12.4 ± 0.9	21.0 ± 1.1	4.2 ± 0.1

Results are expressed as mean ± 1 SEM (n=3)

Graphically presented data revealed that perfusion of rat's immediately after sacrifice at 0.5 and 4 h post-dose reduced the amount of [^{14}C]-paraquat in the brain by approximately 50%, suggesting that some paraquat was confined to the vascular compartment. However, this reduction was not statistically significant when compared with that of non-perfused brain tissue. At 24 h post treatment, no difference was seen between the concentration of ^{14}C-paraquat in perfused and non-perfused brain tissues.

Graphically presented data illustrated that the highest concentration of [^{14}C]-paraquat at 24 h post-dose was located in the rest of forebrain (~2.5 nmol/g wet weight), while the lowest level was found in the striatum (~1 nmol/g wet weight). The concentration of paraquat in other brain regions (parietal cortex, hippocampus, entorhinal cortex, cerebellum, midbrain, frontal cortex, medulla oblongata) was approximately 1-2 nmol/g of wet weight at 24 h post-dose.

Quantitative autoradiography revealed that relatively high concentrations of paraquat were found in the pineal gland, lining of the ventricles, anterior olfactory bulb, olfactory tubercle, area postrema of the medulla oblongata and hypothalamus (see Table 6 below). The pineal gland and linings of the cerebral ventricles lie outside the BBB, whilst the anterior olfactory bulb, hypothalamus and area postrema do not have a BBB. Little difference in paraquat distribution was observed between the remaining brain regions. Autoradiograms illustrated that paraquat did not accumulate in dopaminergic terminal regions in the striatum or in the neuromelanin-containing SN. The study authors attributed the increased concentration of [^{14}C]-paraquat in the anterior and posterior olfactory bulbs at 24 h to the gradual leaking of paraquat into some brain regions via adjacent regions lacking a BBB.

Table 6: [^{14}C]-paraquat levels (nmol/g tissue) in various regions of the rat brain at 30 minutes and 24 h after a single sc injection

Brain region	Paraquat Concentration (nmol/g of tissue)	
	30 minutes	24 h
Anterior olfactory bulb	5.8 ± 0.8	9.1 ± 0.6
Posterior olfactory bulb	2.9 ± 0.5	4.8 ± 0.4
Olfactory tubercle	5.8 ± 0.9	4.0 ± 0.3
Striatum	0.5 ± 0.0	2.2 ± 0.1
Nucleus accumbens	0.7 ± 0.1	2.8 ± 0.1
Parietal cortex (layers I-III)	2.0 ±0.2	1.9 ± 0.1
Parietal cortex (layers IV-VI)	0.9 ± 0.2	2.2 ± 0.1
Thalamus	2.0 ± 0.8	2.2 ± 0.1
Hypothalamus	4.1 ± 1.0	5.1 ± 0.2
Hippocampus	0.9 ± 0.2	2.8 ± 0.2
Lining of lateral ventricles	4.8 ± 1.0	4.6 ± 0.7
Substantia nigra	1.5 ± 0.4	3.2 ± 0.2
Superior colliculus	1.2 ± 0.1	2.8 ± 0.1
Inferior colliculus	1.1 ± 0.1	2.8 ± 0.1
Pons	4.2 ± 0.9	3.0 ± 0.1
Lining of fourth ventricle	4.3 ± 0.5	4.3 ± 0.2
Medulla oblongata	2.6 ± 0.4	2.8 ± 0.1
Cerebellum	1.2 ± 0.1	1.7 ± 0.0
Area postrema	6.3 ± 2.0	4.9 ± 0.7
Pineal gland	17.0 ± 2.6	8.5 ± 0.3

Data are expressed as means ± 1 SEM for 3-4 animals

The distribution of [^{14}C]-paraquat in different brain regions at 24 h closely matched the distribution of [^{14}C]-inulin at 20 minutes post-dose, indicating that the overall distribution of paraquat in the brain was correlated with blood volume (however, approximately 30-50% of [^{14}C]-inulin could not be removed from some brain regions by perfusion). The authors concluded that paraquat detected in the brain after 24 hours was related to the cerebral circulation, and there was limited entry of paraquat in to brain regions without a BBB (such as the pineal gland and linings of cerebral ventricles and hypothalamus) and was associated with elements of the cerebro-circulatory system. No evidence of any neuronal damage was seen in any part of the brain 24 or 48 hours following systemic administration of a toxic dose of paraquat.

Bartlett RM, Holden JE, Nickles RJ, Dhanabalan M, Barbee DL, Barnhart TE, Christian BT & DeJesus OT (2009) Paraquat is excluded by the blood brain barrier in rhesus macaque: An *in vivo* PET study. *Brain Res. 1259:74-79.*

This study aimed to clarify the issue of BBB penetration of systemic paraquat by assessing the uptake of [^{11}C] paraquat in the non-human primate brain *in vivo* using quantitative positron emission tomography (PET) imaging. Four adult rhesus monkeys were used for the PET studies. The animals were anaesthetised and heads immobolised to allow PET scanning parallel to the orbitomeatal (OM) line. [^{11}C] paraquat at a dose of 3.0-6.1 mCi (0.1-0.4 µg/kg) was injected into the saphenous vein. PET images were scanned concurrently with oral delivery of the radiotracer. Venous blood samples were collected throughout the PET imaging sequence.

The axial and sagittal views of monkey brains at early, mid and late time points after [^{11}C] paraquat injection provided visualisation of the pharmacokinetics of the radiotracer and [^{11}C]-intensity was related to perfusion.

The images and tissue time courses showed that there was minimal paraquat uptake and retention in the whole primate brain. Paraquat was weakly sequestered by specific brain structures, namely, the pineal gland and the lateral ventricles. The tissue time courses indicated no selective accumulation of paraquat in the DA terminals located at the caudate nucleus and putamen but rather close association with plasma radioactivity. This is consistent with rodent studies that reported the high correlation of cerebral distribution with cerebral blood volume of [^{14}C]-paraquat 24 h after systemic administration (Naylor *et al*, 1995).

Corasaniti MT, Defilippo R, Rodinò P, Nappi G & Nisticò G (1991) Evidence that paraquat is able to cross the blood-brain barrier to a different extent in rats of various age. *Funct Neurol 6 (4): 385-391.*

Male Wistar rats (body weight and source unspecified), aged 2 weeks, 3, 12 or 24 months, were given a single sc injection of paraquat dichloride (unspecified purity and batch no.; Sigma, USA) in double-distilled, pyrogen free water at 1, 2.5 or 5.0 mg/kg bw (6-8 rats/group; equivalent to 0.72, 1.8 or 3.6 mg/kg bw paraquat cation, respectively) in a dose volume of 1 mL/kg bw. Animals were sacrificed one hour after dosing (method unspecified) and their brains immediately removed, weighed and stored at -20°C until further analysis. To determine whether paraquat levels in cerebral vessels could affect the measurement of paraquat in brain tissue, 12 month-old rats were given a single sc injection of paraquat at 5 mg/kg bw (n=4; equivalent to 3.6 mg/kg bw paraquat cation) and 1 h later, intracardially perfused with saline prior to removal of the brain. Brain paraquat levels were analysed by ion-pair solid-phase extraction and HPLC.

There was a dose-related increase in brain paraquat levels across all age groups (see Table 7 below). Paraquat levels were significantly higher in 24-month old rats relative to 3-month old rats ($p<0.01$-0.05; t-test) while no significant difference occurred between 12- and 24-month old rats. At 2.5 mg/kg bw, a significant difference ($p<0.05$; t-test) in the concentration of paraquat was found between 12- and 3-month old rats, however, in the absence of a similar effect at the highest dose (ie 5 mg/kg bw) this result was not considered definitive in assessing the difference in paraquat levels between these two age groups. At and above 2.5 mg/kg bw, 2-week old rats had a significantly higher ($p<0.05$; t-test) level of brain paraquat compared to 3-month old rats. The study authors concluded that there was an age-related effect on paraquat uptake by the brain, with 2-week and 24-month old rats taking up more paraquat than 3- and 12-month old rats due to changes in BBB permeability. The reviewing toxicologist considered that this age-related effect was equivocal given the small group sizes and that plasma paraquat concentrations were not measured to enable changes in blood:brain ratio to be monitored. This study therefore has reduced regulatory value.

Table 7: Brain paraquat levels in rats of different ages after a single sc injection

Dose (mg/kg bw)	Age (weeks)			
	2	**13**	**52**	**104**
1.0	64	39	47	80*
2.5	118*	82	133*	171 *
5.0	334*	186	216	243*

Rounded data presented; ng/g wet weight; * cf. 13 week old rats at $p<0.05$ (t-test); ** cf. 13 week old rats at $p<0.01$ (t-test).

Brain paraquat levels in rats treated at 5 mg/kg bw and then intracardially perfused with saline prior to brain removal, were not significantly different from the levels found in non-perfused rats (194.7 ± 9.9 versus 216.0 ± 22.6 ng/g wet brain weight, respectively). This finding suggested that paraquat concentrations measured in the brain were not influenced by any located in the cerebral circulation.

Widdowson PS, Farnworth MJ, Simpson MG & Lock EA (1996a) Influence of age on the passage of paraquat through the blood-brain barrier in rats: A distribution and pathological examination. *Hum Exp Toxicol 15: 231-236.*

To examine the effect of age on brain exposure to paraquat, a single median lethal dose of [^{14}C]-paraquat of 20 mg/kg bw was administered via sc injection to neonatal male and female rats (10 day old), male adult (3 month old) and male elderly (18 month old) rats.

Experimental: Groups of between 6-8 male and female neonatal (10-day old), male adult (3-month old) and male aged rats (18-month old) (Alpk:Apfsd, Wistar-derived; body weight unspecified) were given a single sc injection of [^{14}C]-paraquat (103 mCi/mmol, > 98% purity; Cambridge Research Biochemicals, Gadbrook Park, Northwich, Cheshire, UK) at 20 mg/kg bw (equivalent to 14.4 mg/kg bw paraquat cation) in a dose volume of 2 mL/kg bw. Rats were observed periodically for any signs of toxicity or abnormal behaviour and sacrificed when deemed to be moderately stressed. At 0.5 and 24 h post-dose, rats were sacrificed with an overdose of sodium pentobarbitone and their brains, liver, lungs, kidneys and blood collected. Paraquat levels in plasma and tissues were determined by LSC. Paraquat levels in various regions of the brain were measured by quantitative autoradiography as described by Naylor *et al* (1995). To determine the amount of paraquat that was confined to the blood in each organ, groups of rats were perfused transcardially with saline at 0.5 and 24 h after dosing, prior to paraquat analysis. In a separate experiment, neonatal, adult and aged rats (4/group) were given a single sc injection of 20 mg/kg bw paraquat (equivalent to 14.4 mg/kg bw) or water (10 mL/kg bw, controls). Rats were sacrificed 24 and 48 h after dosing and their brains histopathologically examined following staining with haematoxylin and eosin.

Findings: The presence/absence of clinical signs or behavioural abnormalities were unreported. The brains of all paraquat-treated and control rats appeared normal and did not show any evidence of neuronal death. Graphically presented data illustrated that at 0.5 h after dosing, relatively low levels of paraquat were detected in the brain, liver and lung from all 3 ages of rats. While there was no difference in lung paraquat levels, brain and liver concentrations were significantly different across all age groups ($p<0.01$-0.001, ANOVA) with paraquat levels in the brains of aged rats significantly higher than neonatal and adult rats. Relatively high levels of paraquat were found in the kidney, with concentrations in adult and aged rats significantly higher than neonatal rats ($p<0.001$, ANOVA). Renal paraquat levels were comparable between adult and aged rats.

Graphically presented data revealed that concentrations of paraquat in the lung, liver and kidneys had fallen by 24 h. Renal levels had decreased to between 2-8% of the levels measured at 30 minutes in all age groups, and were not significantly different to each other. Lung paraquat levels had fallen by approximately 50% in neonates, while an 85% decrease was observed in both adult and aged rats. Liver paraquat concentrations had fallen by 60% in neonates, while an 80 and 90% reduction was seen in adult and aged rats, respectively. Neonatal lung and liver

paraquat levels were significantly higher than those in adult and aged rats ($p<0.05$-0.001, ANOVA). At 24 h, neonatal brain paraquat level had not decreased relative to the levels measured at 30 minutes, while in adult and aged rats, the concentration of paraquat in the brain had fallen to 14-16% of the 30 minute levels. Statistical analysis revealed that neonatal brains had significantly higher paraquat levels than adult and aged rats 24 h after dosing ($p<0.001$, ANOVA).

In the brain, liver and lungs of adult and aged rats, paraquat was not located in the blood of each organ as perfusion did not significantly reduce tissue paraquat concentrations at either 0.5 or 24 h post-dose. Perfusion of adult rats resulted in a 60% decrease in renal paraquat levels ($p<0.05$, t-test) at 0.5 but not 24 h post-dose. The later finding suggested that the majority of paraquat detected in the kidneys at 0.5 h is confined to the blood.

Quantitative autoradiography revealed that the highest concentration of radioactivity was detected in the anterior olfactory bulb, ventral hypothalamus and the area postrema of the medulla oblongata in all 3 age groups (see Table 8 below). The lowest concentrations were detected in the medulla adjacent to the area postrema and in deeper structures like the cerebral cortex, SN and medulla oblongata. From 0.5 to 24 h after dosing, radioactivity in these deeper lying regions, like the striatum, hypothalamus and SN increased in all age groups. Statistical analysis revealed no difference in levels between regions of adult and aged brains. In contrast, all regions of the neonatal brain had significantly higher levels of radioactivity than either adult or aged rats (up to 10-fold higher levels in some regions).

Table 8. [^{14}C]-paraquat concentrations (nmol/g tissue) in various regions of the brain of neonatal, adult and aged rats at 0.5 and 24 h after a single dose (20 mg/kg bw, sc)

Brain region	Neonates		Adults		Aged	
	0.5 h	24 h	0.5 h	24 h	0.5 h	24 h
Olfactory bulb						
Anterior portion	30.1 ± 3.7**	21.5 ± 1.5**	5.8 ± 0.8	9.1 ± 0.6	2.9 ± 0.1††	5.4 ± 1.0††
Posterior portion	19.1 ± 1.9**	10.2 ± 0.8**	2.9 ± 0.5	4.8 ± 0.4	2.8 ± 0.2††	5.5 ± 0.7††
Frontal cortex	10.2 ± 0.9**	14.3 ± 0.6**	1.5 ± 0.2	2.0 ± 0.1	2.8 ± 0.3††	6.5 ± 0.7††
Striatum	8.4 ± 0.5**	16.6 ± 0.5**	0.5 ± 0.2	2.2 ± 0.1	2.3 ± 0.2††	2.6 ± 0.1††
Hypothalamus						
Ventral region	20.6 ± 0.13**	35.3 ± 3.0**	4.1 ± 1.0	5.1 ± 0.2	5.0 ± 0.5††	9.2 ± 0.7††
Dorsal region	9.0 ± 0.7**	23.3 ± 1.7**	1.5 ± 0.6	2.3 ± 0.5	3.0 ± 0.2††	4.0 ± 0.2††
Ependymal lining of the lateral ventricles	19.2 ± 0.5**	46.5 ± 4.1**	4.8 ± 1.0	4.6 ± 0.7	4.3 ± 0.5††	7.2 ± 1.5††
Substantia nigra	10.7 ± 0.4**	16.5 ± 0.8**	1.5 ± 0.4	3.2 ± 0.2	2.6 ± 0.2††	3.7 ± 0.5††
Area postrema	24.9 ± 4.3	45.2 ± 3.4	6.3 ± 2.0	4.9 ± 0.7	5.5 ± 0.8†	7.9 ± 1.1†
Medulla oblongata	13.9 ± 1.6**	42.1 ± 2.6**	2.6 ± 0.4	1.7 ± 0.0	2.6 ± 0.1††	5.3 ± 0.5††

Data are expressed as means ± 1 SEM for 5 groups of brains measured in duplicate; ** $p<0.01$ compared to the concentration measured in the adult brain at the same time point; † $p<0.05$, †† $p<0.01$ compared to neonatal rats at the same time point (ANOVA, Bonferonni tests).

In summary, paraquat was shown to enter the brain of rats of various ages following a single sc injection in the absence of any neuronal damage. The observations that brain paraquat levels in neonates did not decrease over the study period, and that these levels were significantly higher than those in adult and aged rats, were attributed to impaired BBB integrity. There was no evidence of treatment related cell damage in the brain.

Widdowson PS, Farnworth MJ, Upton R & Simpson MG (1996b) No changes in behaviour, nigro-striatal system neurochemistry or neuronal cell death following toxic multiple oral paraquat administration to rats. *Hum Exp Toxicol 15: 583-591.*

Paraquat (33% paraquat cation; unspecified batch no.; ICI Chemicals and Polymers, Widnes, Cheshire, UK) in deionised water was administered by po gavage at 0 (control) or 5 mg/kg bw/d paraquat cation, in a dose volume of 1 mL/kg bw, to groups of 8 male AP (Alpk: Apfsd, Wistar-derived) rats (200-250 g body weight; age and source unspecified) for 14 days. All rats were weighed daily and observed for any clinical signs of toxicity. Locomotor activity was examined in an open field test on days 4 and 12 at 22°C. Over a 10 minute period, the total number of line crossings per box, line crossings in the centre of the box, line crossing in the periphery of the box and the total number of rearings were recorded by an observer sitting 2 metres from the box. Additionally, any abnormal movements (eg excessive grooming, licking etc) were recorded.

Motor coordination and grip strength were measured on days 4, 8 and 15. Front and rear leg grip strength was measured in 3 trials using strain gauges. Motor coordination was measured twice following the grip strength experiment by recording the time taken for each rat to remove their hind limbs out of 2 x 5 cm holes that were cut 2.5 cm apart in a perspex platform. Total locomotor activity was measured on day 15 using animal activity monitors (infrared motion detectors). Rats were sacrificed on day 15 by inhalation of CO_2 and their brains analysed for DA, DOPAC and noradrenaline by HPLC.

In a separate experiment, 6 rats/group received a daily po gavage of 0 or 5 mg/kg bw/d paraquat cation for 14 days in a dose volume of 1 mL/kg bw. Following sacrifice at day 15, brains were removed and examined for neurotransmitter receptor densities (dopamine D1 and D2 receptors, muscarinic receptors, N-methyl-D-aspartate receptors, benzodiazepine sites on $GABA_A$ receptors) using quantitative receptor autoradiography.

To determine the tissue distribution of paraquat, 4 rats/group received 5 mg/kg bw/d [^{14}C]-paraquat cation (98% purity, 103 mCi/mmol; batch no. unspecified; Cambridge Research Biologicals, Gadbrook Park, Northwich, Cheshire, UK) in water for one (106 μCi/mL) or 14 (20 μCi/mL) days in a dose volume of 1 mL/kg bw. Rats were sacrificed with an overdose of fluothane 24 h after the single dose or 24 h or after the last of the 14 daily doses. Blood was collected transcardially and the concentrations of paraquat in the plasma, liver, kidneys, brain and lung measured according to the method of Naylor *et al* (1995).

To determine whether paraquat could cause neuronal damage or death, 4 rats/group received 0 or 5 mg/kg bw/d paraquat cation in water at a dose volume of 1 mL/kg bw for 14 days. Rats were sacrificed with an overdose of Euthatal 24 h after the final dose and perfused intracardially with saline followed by a 4% paraformaldehyde solution. Brains were removed and processed for histopathological examination as previously described (Naylor *et al*, 1995).

Paraquat-treated rats showed no clinical signs of toxicity, however a slight depression in body weight gain was observed over 14 days (90 $\pm$ 5 g) compared to the control group (103 $\pm$ 4 g), a finding which was not statistically significant. The study authors concluded that the low

paraquat dose was toxic to the rats however there were no overt signs of toxicity reported, including any effects on behaviour or motor activity.

Twenty-four hours after a single oral dose, the lung had the highest concentration of [^{14}C]-paraquat (0.57 ± 0.12 nmol/mL), followed by the kidney (0.21 ± 0.02 nmol/mL), liver (0.11 ± 0.01 nmol/mL), brain (0.05 ± 0 nmol/mL) and plasma (0.02 ± 0.01 nmol/L). Twenty-four hours after 14 daily oral doses, the lung again showed the highest paraquat concentration (2.52 ± 0.37 nmol/mL), followed by the brain (0.47 ± 0.05 nmol/mL), the kidney (0.44 ± 0.02 nmol/mL), liver (0.34 ± 0.01 nmol/mL) and plasma (0.06 ± 0.01 nmol/mL). The brain exhibited an approximately 10-fold greater paraquat concentration after 14 repeat doses compared to a single dose while all other tissue only showed a 2 to 4-fold increase.

There was no treatment-related effect on behaviour, motor coordination, grip strength or total locomotor activity. Paraquat-treated rats showed a significantly higher (p=0.005; 2-sampled, 2-tailed t-test) striatal DA concentration (219.4 ± 10.2 ng/mg protein) compared to the control (157.4 ± 15.6 ng/mg protein) which the study authors suggested may have due to a paraquat-induced stress response. In contrast, there was no treatment-related effect on the concentration of DA in the hypothalamus. There was no perturbation in the level of DOPAC in the striatum or hypothalamus. Noradrenaline appeared to be slightly elevated in the striatum, hypothalamus and frontal cortex, however none of these results were statistically significant. There was no treatment-related effect on the density of dopamine D1, dopamine D2, muscarinic, N-methyl-D-aspartate or benzodiazepine receptors.

Photographic evidence illustrated that paraquat-treated rats showed no signs of neuronal cell damage or death in the laminar pars reticulata or the lamina pars compacta of the SN.

In summary, paraquat showed no evidence of neurotoxicity when administered by po gavage to Wistar-derived rats at 5 mg/kg bw/d paraquat cation for 14 days, despite evidence that paraquat could enter the brain. There was no treatment-related effect on behaviour, locomotor activity and nigrostriatal neurochemistry, and no evidence of neuropathology. Although all paraquat-treated rats received the same dose, measurements of tissue paraquat concentrations, neurotransmitter receptor densities and neurohistopathology were performed on separate groups of rats than the main experiment. In this study paraquat did not behave like MPP^+ (N-methyl-4-phenylpyridinium), the neurotoxic metabolite of MPTP.

Shimizu K, Ohtaki K, Matsubara K, Aoyama K, Uezono T, Saito O, Suno M, Ogawa K, Hayase N, Kimura K, Shiono H (2001) Carrier-mediated processes in blood-brain barrier penetration and neural uptake of paraquat. *Brain Res 906: 135-142.*

This study developed a microdialysis probe technique in stereotaxically implanted male rats. These authors also investigated paraquats' affect on the BBB and various brain uptake mechanisms.

Experimental: Male Wistar rats (8-weeks old, 210-260 g body weight; SLC Shizuoka, Japan) were stereotaxically implanted with 22-gauge cannulae, according to the co-ordinates of Paxinos & Watson, (1986) under sodium pentobarbital anaesthesia. Microdialysis probes (I-shaped), with a dialysis area of 3 mm length, were prepared according to the method of Nakahara *et al* (1989), inserted through the guide cannulae and connected to a microinfusion

pump. Brains were perfused with Ringer's solution at 2 μL/min for 4 h to allow for recovery after possible injury due to insertion of the probe. Following this recovery period, the dialysate was collected for 60 minutes and this was used as a blank sample.

Paraquat dichloride (unspecified purity and batch number; Tokyo Chemical Industry, Tokyo, Japan) in saline was injected sc into the back of the neck of 3-8 rats/group at 5, 10 or 20 mg/kg bw (equivalent to 3.6, 7.2 and 14.4 mg/kg bw paraquat cation, respectively). A separate group of 4 rats was given a single sc injection of 10 mg/kg bw MPP^+ iodide (unspecified purity and batch no.; Research Biochemicals International, Natick, MA, USA) to test the suitability of the brain microdialysis technique. Dose volumes were unspecified. The dialysate was collected at 60 minute intervals over 180 minutes. At the end of the collection period, blood was collected from each rat by cardiac puncture under deep pentobarbital anaesthesia. The amount of paraquat and MPP^+ in dialysate and blood samples were analysed by HPLC.

To determine whether paraquat might damage the BBB and thereby facilitate entry into the brain, MPP^+ (10 mg/kg bw, sc) was administered one hour after a single paraquat injection (20 mg/kg bw, sc), and then the amount of MPP^+ in the dialysate analysed. To determine whether paraquat was carried into the brain via a specific amino acid transporter, L-valine or L-lysine (200 mg/kg bw, ip) were injected 30 minutes prior to a sc injection of paraquat (20 mg/kg bw). To determine whether paraquat was taken up by striatal cells, paraquat (50 μM) or MPP^+ (10 μM) was administered directly into the striatum through the microdialysis tube for 60 minutes, followed by a sequential 180 minute washout with Ringer's solution. Rats were decapitated, the ipsi- and contra-lateral striata immediately removed, homogenised and the amount of paraquat and MPP^+ analysed by HPLC. To determine whether the uptake of paraquat was mediated by an ion exchange transporter, the above procedure was performed using Na^+-free Ringer's solution. To determine whether the polyamine transporter was involved in paraquat uptake by the brain (as is the case with lung paraquat uptake) this experiment was performed in the presence of 50 μM putrescine. Statistical differences were determined using a 1-factor ANOVA, followed by a post-hoc Dunnet's t-test.

Findings: A HPLC chromatogram illustrated that paraquat was detected in the dialysate at 60, 120 and 180 minutes after a single injection (20 mg/kg bw, sc) indicating that paraquat had crossed the BBB. Paraquat was also shown to be present in a blood sample collected 3 h after a sc injection. In contrast, a HPLC chromatogram showed that no MPP^+ was detected in the dialysate following a single injection (10 mg/kg bw, sc) and thus none had crossed the BBB. MPP^+ was, however, detected in the serum of rats 3 h after administration.

There was a dose-related increase in both the serum and extracellular striatal concentrations of paraquat, and a dose-related decrease in the ratio of extracellular striatal to serum paraquat concentrations (see Table 9 below). Graphically presented data confirmed that the concentration of paraquat in the striatal dialysate was dose-dependent. Paraquat was quickly eliminated from the striatal extracellular fluid. The elimination constant and half-life in rats injected with 20 mg/kg bw paraquat were 0.91/h and 0.76 h, respectively. No elimination constant or half-life data were provided for the other dose groups.

Table 9. The ratio of extracellular to serum paraquat or MPP+ concentrations 3 h after administration.

Treatment	n	Serum concentration (nmol/mL)	Striatal extracellular concentration (nmol/mL)	Ratio
5 mg/kg bw PQ	3	1.42 ± 1.50	0.28 ± 0.06	0.208 ± 0.064
10 mg/kg bw PQ	5	3.88 ± 0.79	0.36 ± 0.09	0.126 ± 0.031
20 mg/kg bw PQ	8	8.04 ± 2.41	0.49 ± 0.09	0.085 ± 0.023**
10 mg/kg bw MPP^+	4	11.62 ± 4.84*	Not detected	-

Data are expressed as means ± 1 SEM; PQ = paraquat; * = statistically different to the 10 mg/kg bw PQ group at $p<0.01$; ** = statistically different to the 5 mg/kg bw paraquat group at $p<0.01$.

A HPLC chromatogram illustrated that pre-treatment of rats with paraquat (20 mg/kg bw, sc) prior to injection of MPP^+ (10 mg/kg bw, sc) did not result in any MPP^+ being detected in the striatal dialysate. This result indicated that the entry of paraquat into the brain had not resulted from damage to the BBB due to paraquat toxicity.

Pre-treatment of rats with L-valine (200 mg/kg bw, ip) prior to injection of paraquat (20 mg/kg bw, sc) resulted in a significantly lower concentration of extracellular striatal paraquat and a concomitant increase in serum paraquat relative to the control (see Table 10 below). In contrast, pre-treatment of rats with L-lysine (200 mg/kg bw, ip) did not affect the entry of paraquat into the brain. These findings suggested that paraquat was carried through the BBB by a neutral amino acid transporter.

Table 10. Effects of L-valine and L-lysine on entry of paraquat into the brain of rats

Treatment	Extracellular striatal PQ concentration (nmol/mL)			Serum PQ (3h)	Ratio
	0-1 h	1-2 h	2-3 h		
PQ (control)	4.50 ± 1.06	2.03 ± 0.38	0.74 ± 0.13	8.04 ± 2.41	0.085 ± 0.023
PQ + L-valine	1.32 ± 0.29**	0.71 ± 0.13*††	0.06 ± 0.23	11.74 ± 2.80	0.032 ± 0.006†
PQ + L-lysine	3.47 ± 0.09	1.90 ± 0.26	0.84 ± 0.26	7.59 ± 1.74	0.100 ± 0.028

Data are expressed as means ± 1 SEM (n=8); PQ = paraquat; * $p<0.05$; ** $p<0.01$; † $p<0.05$ (L-lysine); †† $p<0.01$ (L-lysine).

Paraquat (172.5 ± 53.9 pmol) and MPP^+ (23.1 ± 8.2 pmol) were detected in the ipsi-lateral but not the contra-lateral striata following direct administration of each compound (50 and 10 μM, respectively) into the striatum via the microdialysis probe. Paraquat uptake was significantly inhibited ($p<0.02$) when Na^+-free Ringer's solution was used (76.3 ± 6.8 pmol). These findings suggested that the uptake of paraquat by striatal cells was Na^+-dependent. Putrescine (50 μM) failed to affect the striatal uptake of paraquat (169.1 ± 60.5 pmol) which suggested that the polyamine transporter was not involved in the uptake of paraquat by striatal cells.

Nagao M, Takatori T, Wu B, Terazawa K, Gotouda H, Akabane H, Inoue K & Shimizu M (1991) Immunohistochemical localisation of paraquat in lung and brain. ***Med Sci Law 31 (1): 61-64.***

Male SD rats (200-250 g body weight; sample size, age and source unspecified) were given a single iv injection of paraquat dichloride (unspecified batch no., purity & dose volume; Aldrich Co, USA, purity unspecified) in saline at 0 or 5 mg/kg bw (equivalent to 3.6 mg/kg bw paraquat cation). Rats were sacrificed at 3, 12 and 24 h, and at 3, 7 and 10 days post-dose. Lung and brain tissues were immunohistochemically examined for paraquat using a polyclonal α-

paraquat antibody followed by biotin-streptavidin-peroxidase staining. Lung and brain tissues were also histologically examined following haematoxylin-eosin, and trichrome staining (to examine collagen generation in the lung).

Paraquat-treated rats exhibited a number of histopathological lung abnormalities consistent with paraquat toxicity including inflammatory cell infiltration, deposition of collagen fibres in interstitial spaces and thickening of the alveolar septum. No histopathological brain abnormalities were detected in any paraquat-treated rats. In the lungs of all treated rats, paraquat was localised to blood vessel walls, histiocytes and bronchiolar epithelial cells, irrespective of the time of sacrifice. The localisation of paraquat to bronchiolar epithelial cells appeared to be associated with secretion of paraquat into the bronchiole. In brain slices from treated rats, paraquat was detected in capillary walls and glial cells, but not in nerve cells. These positive reactions were completely inhibited when antisera were absorbed with sufficient paraquat before use. No positive findings were observed in lung and brain tissue obtained from control animals.

9.3 Studies on paraquat-induced neurodegenerative effects

The studies of Naylor *et al* (1995), Widdowson *et al* (1996b) discussed above did not detect any signs of neuronal damage in rats following subcutaneous administration of paraquat. Widdowson *et al* (1996b), also did not find any evidence that multiple oral dosing with paraquat can lead to changes in locomotor activity or grip strength, nor was there any neuropathology or any changes in neurochemistry. These authors reported the use of detailed histopathological studies in examining for neuronal cell damage. Brammer (2006) investigated the acute neurotoxicity following a single oral dose of up to 84 mg paraquat ion/kg bw in rats and found no clear treatment related clinical signs or neuropathology (histopathology of the brain was not conducted).

Chivers (2006) did not find any evidence of neurotoxicity in a standard guideline study when rats were administered paraquat in the diet, although the SN pc was not specifically examined. Beck (2012, a,c) did not find any evidence of damage to the brains of mice receiving up to 18.1 mg paraquat ion/kg bw/dose for up to 3 weekly doses by intraperitoneal injection, despite very specific testing for damage to these neurons (specialised staining, stereology and neurochemistry). In a later study, Beck (2013) administered up to 50 ppm paraquat (equivalent to 10.2 (males), 15.6 (females) mg paraquat cation/kg bw/d) in the diet for up to 90 days and did not detect any effect on the dopaminergic neurons, again using specific testing methodology. Both Beck studies were supported by a positive control group (ip MPTP) which demonstrated the validity of the study design and are presented below.

The following studies were considered pivotal in the evaluation of the neurotoxicity potential of paraquat in mice. On this basis, a higher level of methodological and reporting detail has been included relative to other studies in the report.

Beck MJ (2012a) A multi-time and multi-dose pathology study using paraquat dichloride in mice. Study No./Report No. WIL-639093. Task No.: TK0053121. Unpublished. Report date: January 10, 2012

Test Compound:	Paraquat dichloride (purity: 99.9%)
Batch:	ASJ10083-03 [WIL ID No. 09013F]
Test Species:	Male C57BL/6J mice, 35/group, age: approximately 9 weeks, bw: 18.2-25.1 g; source: The Jackson Laboratory, Bar Harbor, ME, USA
Study Duration:	November 16, 2010- May 11, 2011
Laboratories:	• WIL Research Laboratories, LLC, 1047 George Road, Ashland, OH 44805-8946 USA (performing laboratory) • Experimental Pathology Laboratories (EPL), Inc. PO Box 169, Sterling, VA 20166-2194 • Neuroscience Associates, Inc 10915 Lake Ridge Drive, Knoxville, TN 37934 USA • Tox Path Specialists, LLC 20140 Scholar Drive, Lab 109, Hagerstown, MD 21742 USA (neuropathology)
GLP & QA:	Yes
Guidelines:	Not applicable

Supplementary reports which were included in the appendices of this study (Beck, 2012a):

Neuropathology:

Butt MT (2011) Pathology report from: *a multi-time and multi-dose pathology study using paraquat dichloride in mice.* Study No./Report No. WIL-639093. Task No.: TK0053121. Unpublished, December 8, 2011

Analysis of dosing formulations:

Beck MJ (2012b) A *multi-time and multi-dose pathology study using paraquat dichloride in mice.* Analyses of dosing formulations. Supplemental report. Study No./Report No. WIL-639093. Task No.: TK0053121. Unpublished, Report date: January 10, 2012

NB. The findings of these specialist reports are incorporated into this study (Beck, 2012a).

Method

Male C57BL/6J mice (35/group) were administered paraquat dichloride at 10, 15 or 25 mg/kg bw/dose (equivalent to 7.2, 10.9, 18.1 paraquat ion) as 1, 2 or 3 once-weekly intraperitoneal injections to establish the time course of potential pathology in the brain. One set of mice were administered 10, 15 or 25 mg paraquat/kg bw/dose once (study day 14), another set were injected twice (study days 7 and 14) and another set were injected 3 times (study days 0, 7 and 14). The vehicle control group also received 3 injections (days 0, 7 and 14).

Mice treated with MPTP hydrochloride (4 × ip injections, 10 mg/kg bw/dose at approximately 2-h intervals on a day 14) were positive controls. The groups are summarised in Table 11 below.

Table 11. Summary of study group arrangements

Treatment	Group (n=35)	Dose level (mg/kg bw/dose)	Dose volume (mL/kg bw)	Total number of doses	Study day(s) of treatment
Vehicle control	1	0	0	3	0, 7, 14
Paraquat dichloride	2	10	1	1	14
	3	15	1.5	1	
	4	25	2.5	1	
	5	10	1	2	7, 14
	6	15	1.5	2	
	7	25	2.5	2	
	8	10	1	3	0, 7, 14
	9	15	1.5	3	
	10	25	2.5	3	
MPTP HCl	11	10	4	4	14

Mice were quarantined for approximately 3-5 weeks prior to study commencement and housed individually in standard laboratory conditions (21.3º -21.5ºC; relative humidity 41.9%-49.3%; 12 h light/dark photoperiod; minimum 10 fresh air changes/h). Feed and water were available *ad libitum*. Dosing formulations were analysed and found to be within the WIL Research SOP range (90-110% of target) and presented as a separate report (Beck, 2012b).

All animals were observed twice daily for mortality/moribundity and clinical observations were made weekly from study day 0. Additional observations were made approximately 1 h following dosing. Body weights and food consumption were recorded weekly, from 2 weeks prior to commencement of treatment, as well as on study days 0, 1, 2, 7, 8, 9, 14, 15, 16, 18 and 21. Food intake was calculated as g/animal/day.

A complete necropsy was performed on all animals found dead or sacrificed *in extremis*. Scheduled euthanasia was carried out on 5 mice/group (where possible) at 4, 8, 16, 24, 48, 96 or 168 h on study day 14. Anaesthesia by ip injection (sodium pentobarbital) and *in situ* perfusion were then undertaken.

Following perfusion, the head was removed, skinned and placed into paraformaldehyde. The brain (and olfactory bulbs) were removed, weighed and measured. Lungs and kidneys of all mice perfused at 168 hours following the last dose were preserved and retained for possible histopathological examination. Intact brains were sent to Experimental Pathology Laboratories, Inc (EPL) where the brains were separated into hemispheres, the left being retained at EPL and the right side sent to Neuroscience Associates, Inc.

At Neuroscience Associates, Inc., brains were trimmed, multiembedded and the area of the striatum (caudate/putamen area; basal nuclei) through the SN frozen sectioned at 30 µm in the coronal plane. Every 12th section was stained with one of the stains described in Table 12 below (note: TUNEL staining was performed in conjunction with Caspase 3 staining on a subpopulation of slides from the SN).

Table 12. Stains used for neuropathology examination, including purpose (detail from Butt, 2012)

Amino Cupric Silver (AmCuAg)	Identifies necrotic/disintegrating neurons, neuronal processes (axons/dendrites and synaptic terminals). Possible artifactual staining, staining of erythrocytes not removed during perfusion. Will non-selectively stain some normal neurons and processes (not as intensively as those undergoing necrosis/disintegration). **Increased staining indicates neuronal cell necrosis.**
Tyrosine hydroxylase (TH)	Identifies dopaminergic neurons and processes (tyrosine hydroxylase is a key enzyme in the production of DA). Neurons of the SN pc region stain selectively and consistently with this stain. In addition, projections from these neurons onto neurons in the striatum also stain consistently. **Decreased staining in SN pc indicates loss of dopaminergic neurons (verified by AmCuAg) or loss of tyrosine expression in neuronal population.** **Decreased staining in striatum indicates loss (absolute or functional) of dopaminergic neurons as expressed by a decreased amount of TH in the synaptic terminals in the striatum.**
Glial Fibrillary Acidic Protein (GFAP)	Selectively stains an intermediate filament that in the brain is mostly unique to astrocytes. Demonstrates an astrocyte response (indicative of parenchymal damage in all regions of the brain, but especially SN and striatum). CNS damage from different causes may result in an astrocytes response. **Increased staining indicates an astrocytic response.**
Ionised Calcium Binding Adaptor Molecule1 (IBA-1)	Indicates the presence of IBA-1 which is is a protein specific for cells of the mononuclear phagocyte system (MPS), includes macrophages and microglial cells. This protein is expressed when these cells are activated, eg. cellular damage and inflammation. **Increased staining indicates microglial activation.**
Caspase-3	Caspase-3 is involved in cell apoptosis. This stain can detect possible apoptotic cells and/or if apoptosis was involved in any necrosis detected by AmCuAg stain. **Increased staining indicates apoptosis.**
Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) or MicroApoTag	Detects DNA fragmentation from cellular apoptosis. Apoptosis should also label with TUNEL and Caspase-3, hence the stains support each other . **Increased staining indicates cell death due to apoptosis.**
Thionine	A general morphology stain to identify cell nuclei. Suited for thick frozen sections. **General morphology stain.**

The slides were prepared by Neuroscience Associates, Inc. and sent to Tox Path Specialists for interpretation. Results were subject to statistical analysis.

This was a non-Guideline study, although the 'in-life' portions of the study were well conducted and in keeping with OECD Guidelines (TG 424). The study was then deviated to specifically assess the parts of the brain of interest, as per OECD Guidance Document for Neurotoxicity Testing (No. 20).

Results

Clinical signs, mortality and moribundity: Two mice treated with paraquat (groups 7 and 10) were euthanised *in extremis* on study days 4 and 12 respectively (ie 4 and 5 days following the first dose, respectively). The group 7 mouse exhibited hunched posture, hypoactivity and a cool body on the day of euthanasia, and the group 10 mouse showed no clinical signs. The

euthanised mice had severe body weight loss (up to 25%) and/or reduced food consumption (≤ 0.2 g/ day). All other mice survived to scheduled necropsy.

No clinical signs were noted in any other mice, with the exception of one mouse (group 4, single 25 mg/kg bw/dose) which exhibited hypoactivity at the 1 h post-dose examination on day 14 only.

Positive control mice (MPTP) did not exhibit any treatment related clinical signs at any of the weekly examinations. However hypoactivity was noted an hour after dosing, increasing in incidence with subsequent injections (1, 4, 9 and 19 mice following the first, second, third and fourth injections, respectively). Also an hour post-dosing, rocking, lurching or swaying when walking was noted following the second, third and fourth administration (at an incidence of 1, 1 and 2 mice, respectively). Single occurrences of tremors and hunched posture were seen an hour after the fourth dose. Half-closed eyelids were noted 1 h after the 3rd and 4th dosing in two animals.

Body weight and food consumption: Body weight losses were noted in all paraquat treated groups. In most instances, injection with ip paraquat resulted in a body weight loss the following day. This loss was statistically significant for all 25 mg/kg bw/dose administrations (up to 12.7% lower than controls), half of the 15 mg/kg bw/dose administrations (up to 5.9% lower than controls), and only once for the 10 mg/kg bw/dose (up to 4.1% lower than controls). Increasing number of injections of paraquat increased the severity of the weight loss. Weight change over the study period day 0 to 14 was dose-relatedly reduced for mice receiving 2 or 3 injections of paraquat, however was statistically significantly reduced only for the mice which received 3 doses at 15 and 25 mg/kg bw. Overall weight change (from study day 0-21) was dose relatedly reduced for the mice receiving 3 injections of paraquat (1.3, 0.6 and 0.4 g for 10, 15 and 25 mg/kg bw/dose respectively) although the weight gained by the lowest dosed group was greater than the vehicle control group (0.7 g). Groups receiving one or two injections of paraquat did not show a dose-relationship in the overall day 0-21 weight gain. These results were not subject to statistical analysis. Overall, administration of paraquat dichloride by ip injection resulted in a treatment related reduction in body weight gain, or actual body weight loss at all doses tested. The severity was greater with increased dose and with increased frequency of dosing.

The positive control group showed a slight reduction in body weight following treatment (day 15, -3.5% from controls) followed by a statistically significant gain of 6.2 % greater than controls on day 18. Other fluctuations in body weight for paraquat-treated groups and MPTP-treated groups are considered sporadic due to the small magnitude of the change/ the direction of the change/ occurrence prior to dosing and/or lack of a dose relationship. Food consumption was dose-relatedly reduced for mice receiving 15 or 25 mg/kg bw paraquat and is considered treatment-related. Food intake was less than controls following the first dose administration, correlating with reduced body weight gain. These differences were generally statistically significant (with the exception of group 3, one injection of 15 mg/kg bw). Reduced food consumption persisted to the second day after administration for the 25 mg/kg bw/ dose groups (groups 4, 7 and 10), in most cases this difference was statistically significant.

Groups receiving 10 mg/kg bw/dose paraquat did not exhibit any effects on food consumption. The positive control group (Group 11) had statistically significant lower mean food consumption on days 14-15 only.

*Necropsy, macroscopic findings and brain weights:*The two males sacrificed *in extremis* on days 4 and 12 (25 mg/kg bw/dose, 3× and 2× respectively) showed no gross abnormalities at necropsy. Further detailed pathology examination was limited to the brain. The cause of death is unknown, but it cannot be ruled out as treatment related as the deaths occurred at the highest dose. At the scheduled necropsy, gross observations of "red" or "depressed" areas in the cerebral hemisphere or cerebellum were noted for several animals across all groups (including controls) and did not correlate to any microscopic findings. These are accepted to be artefacts of processing.

Brain weights and measurements were similar to control group values for both paraquat and MPTP–treated groups.

Table 13 - Summary of results

Dosing method	Vehicle	Dosing / observation period	Doses tested * (mg/kg bw/dose)	Results
ip injection	0.9% sodium chloride for injection	1-3 injections a week apart: 3× injection of 0, 10, 15 or 25 (study days 0, 7 and 14) or 2× injection of 10, 15 or 25 (study days 7 and 14) or 1× injection of 10, 15 or 25 (study day 14)	0, 10, 15, 25 (equivalent to 0, 7.2, 10.9, 18.1 paraquat ion)	25 mg/kg bw/dose (×2 or × 3): mortality. 10, 15 and 25 mg/kg bw/dose: reduced body weight gain/body weight loss. No neurological effects.
Positive control: ip injection		4 injections, approx. 2 hours apart (study day 14 only)	10 mg (free base) /kg/dose [4 mg/mL]) MPTP HCl	Evidence of effects in the brain: cell death in the SN pc and striatum, loss of TH^+ neurons, increased in size/number astrocytes, and microglial cells.

* Paraquat dichloride dose was based on the dichloride salt, the paraquat ion content was obtained by dividing by 1.38. MPTP dose was corrected using a factor of 1.21 based on the free base of MPTP.

Neuropathology

Microscopic findings:

AmCuAg Stain: Brain sections from groups treated with paraquat did not show any positive findings with this stain , although in a few animals there was some evidence of staining in the optic tracts. This was considered unrelated to treatment. Positive control mice showed variable staining of neurons in the SN pc, which peaked and then plateaued between 16 to 48 hours post-exposure. This staining indicates cell death. Staining was also common in the striatum, particularly in the terminals, with all 5 mice affected at the 24 h timepoint, although the most severe effects were noted at 48 h. This indicates synaptic terminal disintegration.

TH stain: Several findings in individual mice treated with paraquat lacked a dose response and were not considered treatment related. In addition, a few of these results were potentially not true positives, as the study author stated that reading of low severity effects were more prone to false positives. In positive control animals there was a decrease in TH staining of neurons in the striatum and SN pc, indicating a loss of TH+neurons.

GFAP stain: Brain sections from groups treated with paraquat did not show any positive findings with this stain. In the positive control group there were findings (increase in size or number of astrocytes) in the striatum and SN, being more consistent in the former. In the SN, the GFAP reaction was noted from 48-96 hours post-exposure, whereas in the striatum the reaction was noted earlier and persisting until the last sacrifice point (168 h).

IBA-1 stain: Brain sections from groups treated with paraquat did not show any positive findings with this stain. Positive results were noted in positive control animals, indicating an increase in size and/or number of microglial cells.

Caspase-3 stain: Brain sections from groups treated with paraquat did not show any positive findings with this stain. Only a single finding was noted for this stain (at 96 h post-exposure) in the positive control group. This was not considered treatment related as the TUNEL stain did not demonstrate any effects, suggestive that the Capsase-3-stain finding may have been a false positive. The lack of findings with Caspase-3 indicated that apoptosis is not involved in neuron death.

TUNEL and thionine stains: There were no positive findings with these two stains in either paraquat-treated groups or positive control animals. This indicates that apoptosis was not involved in neuron death (TUNEL).

Table 14. Microscopic findings

Incidence (average severity[#])		MPTP Group 11 (n=5)							Paraquat treated group as marked (n=5)
Stain	**Post-exposure timepoint (h)► ▼Area; finding**	**4**	**8**	**16**	**24**	**48**	**96**	**168**	**Variable as marked**
Amino Cu Ag	Striatum; Terminals, sliver+	3 (1.40)	3 (1.80)	4 (3.00)	5 (3.00)	4 (3.60)	2 (1.20)		
	Striatum; Axons, silver+					3 (1.20)	1 (0.40)		
	Striatum; Neurons, silver+					1 (0.40)			
	SN; Neurons, silver+		1 (0.20)	2 (1.60)	3 (1.20)	2 (1.60)	1 (0.20)		
	SN; Axons, silver+			2 (1.00)	3 (0.60)	2 (1.20)	1 (0.20)		
	SN/VTA; Terminals, silver+			1 (0.60)		1 (0.60)			
	Substantia reticulata; Axons/terminals, silver +			1 (0.40)					
	VTA; Neurons, silver+			2 (1.00)	1 (0.20)				
TH	Striatum; Decreased TH staining	1 (0.40)	2 (0.80)	5 (2.60)	5 (3.00)	5 (4.00)	5 (3.40)	4 (1.80)	**168 h, Grp 3**:1 (0.20)*
	SN pc; Decreased TH staining/decreased TH^+ neurons		1 (0.40)	2 (0.60)	1 (0.60)	3 (1.20)	2 (0.60)		**4 h, Grp 5**: 1 (0.20)* **8h, Grp 5**:1 (0.20)* **16 h, Grp 1 & Grp 6:** 1 (0.20)* **24 h, Grp 2:**1 (0.20)*
	Ventral tegmental area; Decreased TH^+ neurons						1 (0.40)		
GFAP	Striatum; Increased GFAP staining		3 (1.00)	5 (2.40)	4 (2.40	5 (4.80)	5 (4.60)	5 (4.00)	
	SN pc; Increased GFAP staining					2 (0.80)	2 (1.20)		
IBA-1	Striatum; Increased IBA-1 staining	3 (1.20)	4 (2.00)	5 (2.80)	5 (2.80)	5 (3.40)	2 (1.00)	1 (0.20)	
	SN pc; Increased IBA-1 staining		1 (0.20)	2 (0.80)	2 (1.20)	3 (1.60)	2 (1.40)		
	VTA; Increased IBA-1 staining			2 (0.80)					
	Hippocampus; Increased IBA-1 staining						1 (0.60)		
	SN, reticulata; Increased IBA-1 staining					1 (0.60)			
Casp-3	Lateral white matter tract; caspase + cells						1 (0.20)*		

VTA: ventral tegmental area. # Average severity is calculated as the sum of severity scores for a particular diagnosis divided by the number of animals in the group or the number of animals with a particular tissue examined. Severity ranks from 1 (slight), 2 (minimal), 3 (mild), 4 (moderate) and 5 (severe). * Considered an isolated finding and not attributed to treatment.

Conclusion

Administration of paraquat via the intraperitoneal route to male C57BL/6J mice over one, two or three once weekly injections of 0, 10, 15 or 25 mg/kg bw/dose (equivalent to 0, 7.2, 10.9, 18.1 paraquat ion), resulted in toxicity at all doses with possibly treatment related mortality at the highest dose and mean body weight losses and/or reduced mean body weight gains in all treated groups. Microscopic examination of brain tissue with targeted staining methods did not find any evidence of neuronal damage (or glial reaction) in the brain. The morphological appearance of the brains in paraquat-treated groups was the same as brains from vehicle control mice.

Findings in positive control animals (ip MPTP) validate the test system with damage detected in the SN (neuronal necrosis, glial reactions, decrease TH staining) and striatum (glial reactions, disintegration of synaptic terminals, and decreased TH staining). These indicate cell death, glial cell activation and an inflammatory response.

Beck MJ (2012c) A dose range-finding and method comparison stereology study using paraquat dichloride and MPTP in mice. Study No./Report No.: WIL-639092. EPL No.: 140-120. RTI No.: 0212823.004. Task No.: TK0053122. Unpublished, Report date: June 29, 2012

Test Compound:	Paraquat dichloride (purity: 99.9%)
Batch:	ASJ10083-03 [WIL ID No. 09013F]
Test Species:	Male C57BL/6J mice, stereological assessment: 105 mice (20-25 mice/group), neurochemistry assessment: 40 mice (10 mice/group), age: approximately 9 weeks, bw: 20.1-19.7 g; source: The Jackson Laboratory, Bar Harbor, ME, USA
Study Duration:	November 16, 2010- May 17, 2012
Laboratories:	• WIL Research Laboratories, LLC, 1047 George Road, Ashland, OH 44805-8946 USA (performing laboratory) • Experimental Pathology Laboratories (EPL), Inc. PO Box 169, Sterling, VA 20166-2194 • RTI International, 3040 Cornwallis Rd, Research Triangle Park, NC 27709-2194 USA
GLP & QA:	Yes (non-GLP neurochemistry analysis by RTI International and statistical analysis of stereology data by Sielken & Associates Consulting, Inc.)
Guidelines:	Not applicable

Supplementary reports which were included in the appendices of this study (Beck, 2012c):

Neurochemistry:

Matthews JM (2011) Determination of dopamine, dopamine metabolites, and serotonin in mouse striatal brain tissues (performed in support of: *A dose range-finding and method*

***comparison stereology study using paraquat and MPTP in mice* (WIL Study No. 639092)). RTI Report No.: 0121823.004. Unpublished, Report date: April 8, 2011**

Stereology:

Wolf JC (2012) Pathology report: *A dose range-finding and method comparison stereology study using paraquat and MPTP in mice* (WIL Study No. 639092)). EPL Project No.: 140-120. Unpublished, Report date: May 17, 2012

Sielken RJ (2012) Statistics report on *a dose range-finding and method comparison stereology study using paraquat and MPTP in mice*. WIL Study No.: WIL-639092. Unpublished, Report date: May 15, 2012

(Statistical analysis of the stereology *in situ* perfusion fixation data only)

Analysis of dosing formulations:

Beck MJ (2012d) *A dose range-finding and method comparison stereology study using paraquat and MPTP in mice* (WIL Study No. 639092)): Analyses of dosing formulations. Supplemental report. Unpublished, Report date: May 17, 2012

NB. The findings of these specialist reports are incorporated into this study (Beck, 2012c).

Dosing method	Vehicle	Dosing / observation period	Doses tested * (mg/kg bw/dose)	Results
ip injection	0.9% sodium chloride for injection	3 injections one week apart (study days 0, 7 and 14)	0, 10, 15, 25 (equivalent to 0, 7.2, 10.9, 18.1 paraquat ion)	No neurological effects in any group
Positive control: ip injection		4 injections, approx. 2 hours apart (study day 14 only)	10 mg (free base) /kg/dose [4 mg/mL]) 1-methyl-4-phenyl-1,2,3,6- tetrahydropyridine hydrochloride (MPTP HCl)	Decrease in the number of TH+ neurons, striatal DA, DOPAC and HVA concentrations and an increase in DA turnover

* Paraquat dichloride dose was based on the dichloride salt, the paraquat ion content was obtained by dividing by 1.38. MPTP dose was corrected using a factor of 1.21 based on the free base of MPTP.

Method

Male C57BL/6J mice were administered paraquat dichloride by three, once weekly intraperitoneal injections at 0, 10, 15 or 25 mg/kg bw/dose (equivalent to 0, 7.2, 10.9, 18.1 paraquat ion) to determine the effect on the number of TH^+ dopaminergic neurons in the SN pc. Brains were specially prepared and sent to different laboratories. The first assessed the number of dopaminergic neurons by stereological procedure using two staining techniques-chromogenic and fluorescent methods using antibodies directed at TH. The second quantified the concentrations of the neurotransmitter DA and its two metabolites (HVA and DOPAC), and 5-HT in the striatum. Mice treated with MPTP (4 ip doses, 10 mg/kg bw/dose at 4 mg/mL and dose volume of 2.5 mL/kg/dose, on a single day) served as positive controls. Mice were assigned to one of nine treatment groups (Table 15 below).

The C57BL/6J species and strain of mice were selected due to known sensitivity to neurotoxicants, specifically MPTP. The low dose selection for paraquat dichloride was based

on the dose reported by McCormack *et al* (2002[4]) that reduced the number of TH+ neurons in the SN pc. The high dose level was close to the maximum tolerated dose identified in a previous intraperitoneal study (Beck, 2010 WIL-639063 not submitted). The study author stated that the intraperitoneal route was selected as it is an accepted route of exposure for this study design and historically, has been used extensively.

Table 15. Summary of study groups (Beck 2012d)

Group No.*	Treatment	Dose level (ion) (mg/kg bw/dose)	Dose volume (mL/kg bw)	Concentration (mg/mL)	n
Stereology groups#					
1	Vehicle control	0	10	0	25
2	PQ-Cl_2	10 (7.2)	10	1	20
3	PQ-Cl_2	15 (10.9)	10	1.5	20
4	PQ-Cl_2	25 (18.1)	10	2.5	20
5	MPTP (positive control)	10	2.5	4	20
Neurochemistry groups					
6	Vehicle control	0	10	0	10
7	PQ-Cl_2	15 (10.9)	10	1.5	10
8	PQ-Cl_2	25 (18.1)	10	2.5	10
9	MPTP (positive control)	10	2.5	4	10

* Groups receiving paraquat dichloride or vehicle only received ip injections on study days 0, 7 and 14. Positive controls mice received ip injections x 4 on study day 14 (total dose 40 mg/kg bw).
# 10 mice/group were used for a chromogenic procedure, and another 10 mice/group for a fluorescent procedure. Five additional mice (assigned to group 1) were added as controls for calibration of stereology parameters.

Mice were acclimated for approximately 3-5 weeks prior to study commencement and housed individually in standard laboratory conditions (temp 21.3-21.5º C, 41.9-49.3 % humidity, 12-hour light/dark cycle, min 10 fresh air changes per hour). Feed was available *ad libitum*. Dosing formulations were prepared weekly and analysis showed the preparations to be within WIL Research SOP range (90-110% of target) (Beck, 2012d).

Animals were observed twice daily for mortality/moribundity and clinical observations were recorded weekly. Mice were also observed for signs of toxicity at approximately 1 hour following dose administration (study days 0, 7 and 14 for control and paraquat treated mice, and day 14 only for MPTP treated mice). Body weight and food consumption were recorded

[4] Not suitable for regulatory use following fraudulent actions by co-author (M.Thiruchelvam) on another paper published 2005 (see Appendix II).

weekly, beginning 2 weeks prior to the commencement of injections. These were also measured on study days 0, 1, 2, 7, 8, 9, 14, 15, 16 and 21. Food intake was calculated as g/animal/day.

A complete gross necropsy was performed on all animals found dead. The kidneys, lungs and gross lesions were preserved for possible future histopathology. Scheduled euthanasia occurred on study day 21 (7 days after the final dose). Euthanasia, necropsy, collection of brain tissue and subsequent processing of tissue samples were conducted differently for neurochemistry and stereology groups:

- Stereology groups (Groups 1-5)

Surviving mice were anaesthetised (ipsodium pentobarbital) and perfused *in situ*. The mice were flushed with buffered saline, followed by paraformaldehyde. The head was removed and the brain (including olfactory bulbs) removed from the skull, dissected and preserved. Lungs and kidneys were also collected and preserved for possible future histological examination. The brain tissue was sent to another laboratory (Experimental Pathology Laboratories, Inc.) where the midbrain region was isolated and fixed (paraformaldehyde (PFA)). The samples were transferred to sucrose solutions prior to flash freezing and stored until sectioning and staining.

Tissues were sectioned serially in the coronal plane (ie. transversely along the rostral-caudal axis) and every third section was stained. This was conducted differently for chromogenic and fluorescent staining techniques, as shown (Table 16 below):

Table 16. Stereology staining summary

Staining type (antibody used)	**Section thickness (µm)**	**Neurons identified and stain used**		
		TH^+	**TH^-**	**Total neurons**
Chromogenic (anti-TH antibody of rabbit origin + a biotinylated goat anti-rabbit immunoglobulin (IgG).	40	3,3-diaminobenzidine (DAB) (Neurons have dense cytoplasmic staining)	cresyl violet2 (Neurons have deep basophilic cytoplasmic staining of Nissl substance)	NA
Fluorescent (polyclonal anti-TH antibody of rabbit origin + secondary antibody (goat anti-rabbit IgG) labelled with a fluorescent dye)	30	ALEXA red flurophore (Neurons have dense cytoplasmic staining)	NA	4',6'-diamidino-2-plenylindole (DAPI) (Nuclei of ALL cells in section (eg TH^+, TH^-, glial))

Stained sections were counted for the number of TH^+ and TH- neurons by the optical fractionator approach and unbiased systematic random sample methodology. Using suitable equipment, virtual outlines of the right and left SN pc were drawn allowing for a measurement of contour volume. Cell bodies were counted in a subsample of each section, section thickness and section areas were measured and then extrapolated to provide estimates of total cell numbers per brain. Sections were also examined for the size of nuclei relative to other cell types such as glia, and/or presence of dendritic or axonal processes. For the fluorescent staining, the final stain stained the nuclei of all cells (not only TH neurons), such that TH^- cells were estimated by subtracting the number of TH^+ cells identified with the ALEXA stain from

the total number. Estimate of the number of TH^+ neurons, TH^- neurons or total neurons was performed by software.

Intra-study variability was assessed by chromogenic staining of sections from 5 control animals evaluated periodically and blindly, serving as internal controls. This was not conducted for the fluorescent stained sections as it was expected that emittance of the flurophore would change over time due to repeated light exposure with repeat assessment and artificially skew the results.

- Neurochemistry groups (Groups 6-9)

Surviving mice were euthanised (cervical dislocation) and decapitated. The brain was removed and the left and right striatum dissected, weighed together and flash frozen in liquid nitrogen. The remaining part of the brain was discarded. The frozen tissue was sent to RTI International for analysis of neurotransmitter levels (DA, DOPAC, HVA and 5-HT).

Preparation of the tissue involved homogenisation in solution, centrifugation and an aliquot of the supernatant run through a HPLC device coupled with ECD. DA turnover was estimated by calculating the ratio of the sum of DOPAC and HVA concentrations to DA concentration.

All results were subject to the appropriate statistical analysis. Body weight, body weight change and food consumption were subject to a one way ANOVA, and then subject to Dunnett's test or a two–sample t-test if an intergroup variance was found. Striatal neurotransmitter concentration data and stereology data were also subject to statistical analysis, the former conducted by WIL Research Laboratories, the latter by Sielken & Associates Consulting, Inc.

This was a non-Guideline study, although the live portions of the study were well conducted and in keeping with OECD Guidelines (TG 424). The study was then modified to specifically assess the parts of the brain of interest, as per OECD Guidance Document for Neurotoxicity Testing (No. 20).

Results

Clinical signs, morbidity and mortality: There was only one unscheduled death: one male in the 25 mg/kg bw/dose paraquat group (Group 4) was found dead on day 7, prior to the second injection. This mouse showed no clinical signs or effects on body weight or food consumption. There were no clinical findings in any mouse administered paraquat at any examination point.

Mice administered MPTP showed hunched posture, hypoactivity, and a body that was cool to touch approximately 1 h following each injection on day 14. This persisted in one male (Group 9) until the following morning.

Body weight and food consumption: Body weight gain was reduced in a dose-dependent manner on the day following dosing for all paraquat treated mice (i.e days 0-1, 7-8 and 14-15), however only statistically significantly reduced for most measurements at 15 mg/kg bw/dose and for all at 25 mg/kg bw/dose (Table 17 below). Following this reduction, a rebound increase in body weight gain was seen. These were mostly statistically significant. Body weight gain over the entire study (days 0-21) was dose dependently reduced, although only statistically significantly so for group 3 (15 mg/kg bw/dose) and groups 4 and 8 (25 mg/kg bw/dose). Actual body weight was reduced in 25 mg/kg bw/dose mice for up to 2 days post injection, with the greatest effect noted following the first dose (-14.1%, group 8) and these findings were mostly statistically significant. At 15 mg/kg bw/dose there was also a trend to reduced body weight following injection however this did not reach statistical significance. At 10 mg/kg bw/dose,

effect on body weight were not present. Effects on body weight are considered treatment related at 15 and 25 mg/kg bw/dose.

Variability was evident in results as comparing the groups treated in the same manner (ie groups 1 and 6, 3 and 7, 4 and 8) show differences in magnitude, although change is largely in the same direction. Increased variability was also noted groups 6-9, attributed to the smaller group sizes (10 *vs* 20). Sporadic fluctuations in body weight occurred also during the acclimation period (range of -4.4% to+ 2.5%).

Table 17. Summary of mean body weights

Mean bw (g) (% diff)	Dose (mg/kg bw/dose)								
Group	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Study Day	**0**	**10**	**15**	**25**	**MPTP**	**0**	**15**	**25**	**MPTP**
-14	20	20.4 (2.0)	20.5 (2.5)	20.5 (2.5)	20.3 (1.5)	20.5	19.6 (-4.4)	20.4 (-0.5)	20.6 (0.5)
-7	21.3	21.3 (0.0)	21.5 (0.9)	21.4 (0.5)	21.2 (-0.5)	21.4	20.8 (-2.80	21.2 (-0.9)	21.3 (-0.5)
0	**22.7**	**22.7 (0.0)**	**22.9 (0.9)**	**22.6 (-0.4)**	22.5 (-0.9)	**22.4**	**22.6 (0.9)**	**22.3 (-0.4)**	22.5 (0.4)
1	22.5	22.2 (-1.3)	22.0 (-2.2)	20.0* (-11.1)	22.5 (0)	22.7	22.2 (-2.2)	19.5* (-14.1)	22.8 (0.4)
2	22.7	22.6 (-0.4)	22.4 (-1.3)	21.2* (-6.6)	22.5 (-0.9)	22.7	22.5 (-0.9)	20.7* (-8.8)	22.7 (0)
7	**23.0**	**23.1 (0.4)**	**22.9 (-0.4)**	**22.6 (-1.7)**	22.9 (-0.4)	**23.3**	**23.2 (-0.4)**	**22.3 (-4.3)**	22.7 (-2.6)
8	22.8	22.7 (-0.4)	22.4 (-1.8)	21.6 (-5.3)	23.0 (0.9)	23.2	22.7 (-2.2)	21.2* (-8.6)	22.8 (-1.7)
9	23.2	23.0 (-0.9)	22.9 (-1.3)	22.3 (-3.9)	23.2 (0)	23.7	23.3 (-1.7)	22.1* (-6.8)	23.1 (-2.5)
14	**23.6**	**23.6 (0)**	**23.3 (-1.3)**	**23.0 (-2.5)**	**23.7 (0.4)**	**24.1**	**23.9 (-0.8)**	**23.0 (-4.6)**	**23.4 (-2.9)**
15	23.5	23.3 (-0.9)	23.0 (-2.1)	21.9 * (-6.8)	22.5** (-4.3)	24.1	23.4 (-2.9)	21.6* (-10.4)	22.1# (-8.3)
16	23.7	23.5 (-0.8)	23.5 (-0.8)	22.6 (-4.6)	23.3 (-1.7)	24.4	23.9 (-2.0)	22.5* (-7.8)	22.6** (-7.4)
21	24.3	24.2 (-0.4)	23.9 (-1.6)	23.5 (-3.3)	24.2 (-0.4)	24.9	24.4 (-2.0)	23.7 (-4.8)	23.9 (-4.0)

For statistical analyses, control group 1was compared to groups 2, 3 and 4; control group 1 was compared to positive control group 5. Control group 6 was compared to groups 7 and 8; control group 6 was compared to positive control group 9. **BOLD** figures indicate the day of dosing. Note MPTP groups were only dosed on Day 14.

* Statistically significantly different from control group 1 at 0.01 (Dunnett's test)

Statistically significantly different from control group 1 at 0.01 (two-sample t-test)

** Statistically significantly different from control group 1 at 0.05 (two-sample t-test)

Table 18. Summary of body weight changes

Mean bw change (g)	Dose (mg/kg bw/dose)								
Group	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Study period (day)	**0**	**10**	**15**	**25**	**MPTP**	**0**	**15**	**25**	**MPTP**
-14 to -7	1.3	0.9	1.0	0.8	0.9	0.8	1.2	0.8	0.7
-7 to 0	1.4	1.4	1.4	1.2	1.2	1.1	1.8##	1.1	1.2
0 to 1	-0.3	-0.5	-0.9*	-2.6*	0.0**	0.3	-0.4*	-2.8*	0.3
1-2	0.3	0.3	0.4	1.2*	0.1**	-0.1	0.4	1.2*	-0.1
2 to 7	0.3	0.5	0.5	1.4*	0.4	0.6	0.7	1.6*	0.0**
7 to 8	-0.2	-0.4	-0.5	-1.0*	0.0	-0.1	-0.6*	-1.1*	0.1
8 to 9	0.4	0.3	0.4	0.6##	0.2	0.5	0.7	0.8##	0.3
9 to 14	0.4	0.6	0.5	0.7	0.5	0.4	0.6	1.0*	0.3
14 to 15	-0.1	-0.3	-0.3	-1.0*	-1.2#	0.1	-0.5	-1.4*	-1.3#
15 to 16	0.2	0.2	0.4	0.7*	0.8#	0.3	0.6	0.9	0.5
16 to 21	0.6	0.7	0.4	0.9##	0.9**	0.5	0.5	1.2*	1.3**
0 to 21	1.6	1.5	1.0##	0.9##	1.8	2.5	1.8	1.4*	1.4#

For statistical analyses, control group 1was compared to groups 2, 3 and 4; control group 1 was compared to positive control group 5. Control group 6 was compared to groups 7 and 8; control group 6 was compared to positive control group 9.
Groups 1-4 and 6-8 were treated on days 0, 7 and 14. Groups 5 and 9 (MPTP) were only dosed on day 14.
* Statistically significantly different from vehicle control group at 0.01 (Dunnett's test)
# Statistically significantly different from vehicle control group at 0.01 (two-sample t-test)
** Statistically significantly different from vehicle control group at 0.05 (two-sample t-test)
Statistically significantly different from vehicle control group at 0.05 (Dunnett's test)

Body weight of positive control mice fluctuated prior to the day of dosing (study day 14). Following dosing, both groups lost body weight until the day of sacrifice (up to -8.3% on day 15), however this was only statistically significantly reduced for the first day or two. The overall body weight change (day 0-21) was statistically significantly reduced for one of the groups (group 9) only, however absolute body weight at day 21 was only slightly reduced and not statistically significantly different from control groups (-0.4% and -4%) (Table 18 above). Food consumption for mice treated with 10 or 15 mg/kg bw/dose were similar to control animals (Table 19 below). At 25 mg/kg bw/dose, food consumption was statistically significantly reduced following the first and second doses (group 4) or for the first dose only (group 8). This mirrors the findings in mean body weight losses. Overall intake for the entire treatment period was similar to control values. Positive control groups showed a statistically

significantly lower mean food consumption following treatment, corresponding to body weight loss for this period. The overall food consumption was similar to controls. A treatment-related effect on food consumption was noted at 25 mg/kg.0 bw/dose paraquat and 10 mg/kg bw/dose MPTP.

Table 19. Mean food consumption (g/animal/day)

Mean food consumption (g/animal/d)	Dose (mg/kg bw/dose)								
Group	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Study period (day)	**0**	**10**	**15**	**25**	**MPTP**	**0**	**15**	**25**	**MPTP**
-14 to -7	4.3	4.4	4.4	4.3	4.3	4.4	4.3	4.3	4.2
-7 to 0	5.6	5.4	5.0	5.3	5.2	4.9	5.5	5.4	4.8
0 to 1	4.5	4.8	4.6	3.2##	5.4**	4.4	4.9	2.1*	5.0
1-2	4.9	4.9	4.4	4.7	5.0	4.9	5.4	4.3	4.8
2 to 7	4.9	5.5	5.2	5.4	4.9	4.7	5.9	6.1##	4.7
7 to 8	4.6	4.5	4.0	3.3*	4.6	4.3	4.9	3.8	5.5**
8 to 9	5.0	4.8	4.3	4.6	4.9	4.8	4.3	4.8	5.0
9 to 14	5.0	5.2	4.8	4.8	5.0	4.8	5.4	5.4	4.9
14 to 15	4.9	4.6	4.3	4.0	2.3#	4.5	4.4	5.3	1.8#
15 to 16	4.7	4.9	4.6	3.9	5.2	5.0	5.3	4.5	4.3
16 to 21	5.3	5.6	4.9	5.6	5.4	5.3	6.1	5.5	5.1
0 to 21	4.9	5.2	4.8	4.9	4.9	4.8	5.5	5.2	4.8

For statistical analyses, control group 1was compared to groups 2,3 and 4; control group 1 was compared to positive control group 5. Control group 6 was compared to groups 7 and 8; control group 6 was compared to positive control group 9.
Groups 1-4 and 6-8 were treated on days 0, 7 and 14. Groups 5 and 9 (MPTP) were only dosed on day 14.
* Statistically significantly different from vehicle control group at 0.01 (Dunnett's test)
# Statistically significantly different from vehicle control group at 0.01 (two-sample t-test)
** Statistically significantly different from vehicle control group at 0.05 (two-sample t-test)
Statistically significantly different from vehicle control group at 0.05 (Dunnett's test)

*Necropsy, macroscopic examination and brain weights:*The mouse found dead on day 7 (group 4, 25 mg/kg bw/dose) was found to have multiple dark red areas on all lung lobes at necropsy. There were no other gross observations in this animal. This cannot be ruled out as treatment related with the available information, as paraquat is known to target lung tissue.

Following scheduled euthanasia of Groups 1-5 mice, there were no gross changes in the appearance of the brain. The brain was not examined in Groups 6-9. Mean brain striatum weights for groups 6-9 were similar to concurrent controls. The brains were not weighed for groups 1-5.

Stereology Assessment (groups 1-5): Not all brain sections were able to be evaluated. For chromogenic staining, 2 mice had total neuron counts adjusted for missing sections (1x group 1, 1x group 5) and 6 mice could not be assessed due to tissue artefacts precluding accurate counts (2x group 1, 1x group 2, 1x group 3, 2x group 4). For fluorescent staining, 1 mouse had a needle mark through the SN pc (1x group 1), 3 had insufficient number of sections recovered (2x group 4, 1x group 1) and 1 needed the total neuron counts adjusted for missing sections (1x group 5).

Chromogenic-stained sections demonstrated TH^+ neurons were slightly reduced in paraquat treated groups (5%, 14% and 12% less than controls for 10, 15 and 25 mg/kg bw/dose, respectively) (Table 20 below). For fluorescent-stained sections, the mean numbers of TH^+ sections were also reduced (2%, 12% and 13%). However, none of these values were statistically significant. The only statistically significant finding in paraquat treated mice was a 10% reduction in total contour volume (chromogenic-stained) for the 15 and 25 mg/kg bw/dose groups. However this was not dose-related and not evident in the fluorescent-stained sections. The significance of this finding is unclear and may be an artefact of the technique used in the determination of the individual contours. Comparing numbers between the two staining techniques, it is evident that greater TH^+ neurons and a greater total contour volume were detected in fluorescent stained sections. The study author commented this may be artefactual where chromogenic staining produces a higher degree of tissue shrinkage which may make it more difficult to identify individual cells due to overlapping or crowding. In this study, mean section shrinkage was 27% and 56% for fluorescent and chromogenic-stained sections, respectively.

Table 20. Stereological findings in the SN pc

Mean, (% of ctrl) Group	Chromogenic-stained sections			Fluorescent-stained sections		
	TH^+ neurons	Total neurons (TH^+&TH^-)	Total contour volume (μm^3)	TH^+ neurons	DAPI stained neurons (TH^+&TH^-)	Total contour volume (μm^3)
Vehicle control (Group1)	9 988 n= 8	16 463 n= 8	252 360 000 n= 8	16 615 n= 8	29 637 n= 8	312 227 125 n= 8
Paraquat 10 mg/kg bw/ dose (Group 2)	9 518 n= 9 (95%)	17 672 n= 9 (107%)	238 481 889 n= 9 (95%)	16 270 n= 10 (98%)	28 225 n= 10 (95%)	305 169 000 n= 10 (98%)
Paraquat 15 mg/kg bw/dose (Group 3)	8 631 n= 9 (86%)	17 571 n= 9 (107%)	227 367 222* n= 9 (90%)	14 541 n= 10 (88%)	26 852 n= 10 (91%)	294 151 900 n= 10 (94%)
Paraquat 25 mg/kg bw/dose (Group 4)	8 748 n= 7 (88%)	15 503 n= 7 (94%)	227 212 286* n= 7 (90%)	14 470 n= 8 (87%)	27 282 n= 8 (92%)	293 270 375 n= 8 (94%)
Positive control MPTP (Group 5)	8 595 n= 10 (86%)	15 384 n= 10 (93%)	208 991 400** n= 10 (83%)	13 633* n= 10 (82%)	25 611 n= 10 (86%)	278 525 950 n= 10 (89%)

*Statistically significant $p<0.05$, **$p<0.01$

The positive control group showed a reduction in the number of TH^+ neurons both in the chromogenic-stained sections (14% less than controls) and in the fluorescent-stained sections (18% less than controls), although this was only statistically significant in the latter. The study author suggested that the lack of significance in the chromogenic-stained section can be attributed to the small number of mice in this group, which reduces the power of the assay to detect low magnitude differences.

For the chromogenic staining, there was a statistically significant reduction in the total contour volume (17%) and in the fluorescent stained sections this difference was 11% but was not statistically significant. Total neurons were reduced by 7% and 14% for the two staining methods, chromogenic and fluorescent respectively, but neither was statistically significant. Left and right TH^+ counts did not differ statistically significantly. Positive results in this group of animals validates the study design for detecting effects on the number of TH^+-dopaminergic neurons in the SN pc.

There was some variation in the number of TH^+ or TH^- neurones detected with different section depths, which was explained by the study author as variability in staining uptake depending on the thickness of the section and damage to neurones.

Data from the Group 1 internal control animals evaluated periodically throughout the stereological assessment showed that Total Contour Volume was related to the magnitude of cell counts. In addition, most variability was noted when the same animal was counted very early and very late in the study, suggested that this may have been an artefact of the technician

being required to transition between creating contours for chromogenic and fluorescent sections throughout several months of evaluation, resulting in a degree of "drift".

Neurochemistry Assessment (groups 6-9)

Treatment with paraquat up to 3 x 25 mg/kg bw/dose showed no effect on the striatal concentrations of DA, DOPAC, HVA or DA turnover. Levels were not dose relatedly affected and/or lacked change in a toxicologically significant direction. There were statistically significant findings (Table 21 below).

Treatment with MPTP demonstrated statistically significant reductions in all three compounds (DA -74%, DOPAC -61%, HVA -44%) and a statistically significant increase in the rate of DA turnover ($^{+}$135%). This validates the study design to detect effects on the levels of these compounds in the striatum. Striatal concentrations in vehicle control groups were stated by the study author as being consistent with concentrations previously reported by the laboratory.

Striatal 5-HT concentrations could not be evaluated as the method used to assess this neurochemical failed to accurately quantify 5-HT in earlier validation studies. 5-HT measurements are therefore considered unreliable.

Table 21. Mean neurotransmitter concentrations (ng/mg tissue) in striatal tissues (n=10)

Group	6	7	8	9
Mean (% diff control) **Analysis:**	**Vehicle control**	**15 mg/kg bw/dose paraquat**	**25 mg/kg bw/dose paraquat**	**10 mg/kg bw/dose MPTP**
DA (ng/mg tissue)	19.2	19.92 (+4%)	19.85 (+3%)	4.91** (-74%)
DOPAC (ng/mg tissue)	2.18	1.84 (-16%)	1.99 (-9%)	0.84** (-61%)
HVA (ng/mg tissue)	1.61	1.59 (-1%)	1.59 (-1%)	0.90** (-44%)
5-HT^ (ng/mg tissue)	1.15	1.28 (+11%)	1.25 (+9%)	0.94 (-18%)
DA turnover#	0.20	0.17 (-12%)	0.18 (-7%)	0.47** (+135%)

** Statistically significantly different form the control group at $p<0.01$
^ 5-HT data is unreliable due to issues with assay variability
# Data not homogeneous using Bartlett's test

Conclusion

Paraquat administered to male C57BL/6J mice at 10, 15 or 25 mg/kg bw (equivalent to 0, 7.2, 10.9, 18.1 paraquat ion) once a week for 3 weeks by ip injection, resulted in decreased body weight gain from 15 mg/kg bw/dose and reduced food consumption at 25 mg/kg bw/dose (highest dose tested). At the high dose there was also a single mortality that may have been treatment related. Stereology assessment showed no statistically significant effect on TH^{+} (dopamine) neurons. There was a statistically significant decrease in the total contour volume of the SN pc in mice treated with 15 and 25 mg/kg bw/dose (10%) as detected by chromogenic staining only, however the toxicological relevance of this finding is unclear with the lack of effects in other endpoints and may be an artefact of the technique. Administration of paraquat did not affect the levels of neurotransmitter in the striatum (DA, DOPAC and HVA) or the turnover of DA.

A concurrent positive control (10 mg/kg bw of MPTP administered ip 4 x on a single day) demonstrated the validity of this study design. Mice in this group showed a decrease in the number of TH^+ neurons, although only one staining technique gave a statistically significant result (fluorescent). Total contour volume was reduced by 17% and 11% for chromogenic and fluorescent stained sections respectively, although only statistically significant for the former. This group showed a statistically significant decrease in striatal DA, DOPAC and HVA concentrations and an increase in DA turnover.

Beck MJ (2013) Subchronic (91-day) dietary study to assess the effects of paraquat dichloride on dopaminergic neurons in C57BL/6J mice. Study No./Report No.: WIL-639158. Task No.: TK0000969. Unpublished.\ Report date: January 24, 2013

Test Compound:	Paraquat dichloride (purity: 99.9%)
Batch:	ASJ10083-03 [WIL ID No. 110018]
Test Species:	C57BL/6J mice, **stereological assessment**: 20/sex/dose, **pathological assessment**: 15 sex/dose (5/sex/dose positive control), **neurochemistry assessment:** 6/ sex/dose, age: approximately 10 weeks (positive control group were 22 weeks at time of dosing), bw: 18.0 – 26.8 g for males and 14.9 – 21.1 g for females; source: The Jackson Laboratory, Bar Harbor, ME, USA
Study Duration:	August 1, 2011 – January 17, 2013
Laboratory:	• WIL Research Laboratories, LLC, 1047 George Road, Ashland, OH 44805-8946 USA (performing laboratory)
Consultant laboratories*:	• Experimental Pathology Laboratories (EPL), 45600 Terminal Drive, Sterling, VA 20166 (stereology) • Sielken & Associates Consulting, Inc. 3833 Texas Av, Suite 230, Bryan, TX 77802 USA (statistical analyses of stereology data) • Tox Path Specialists, LLC, 8420 Gas House Pike, Suite G, Frederick, MD 21701 (formerly Lab 109, 20140 Scholar Drive, Hagerstown, MD 21742) (neuropathology) • Neuroscience Associates, Inc. 10915 Lake Ridge Drive, Knoxville, TN 37934 USA (preparation and staining of slides for neuropathology) • RTI International, 3040 Cornwallis Rd, PO Box 12194, Research Triangle Park, NC 27709-2194 USA (neurochemistry analyses)
GLP & QA:	Yes (Non-GLP: statistical analyses of stereology data and neurochemistry analyses)
Guidelines:	Not applicable

*Full results from these specialist laboratories are presented separately (Stereology: Wolf (2013), pathology: Butt (2012) and neurochemistry: Matthews (2012) at APPENDIX II.

Table 22 - Study summary

Dosing method	Vehicle	Dosing / observation period	Doses tested (ppm)
Dietary	None	Continuously in the diet for 13-weeks	0, 10, 50 (*Calculated average paraquat dichloride*: Males: 0, 2.4, 14.1 mg/kg bw/d Females: 0, 3.7, 21.5 mg/kg bw/d) *(Calculated average paraquat ion*: Males: 0, 1.7, 10.2 mg/kg bw/d Females: 0, 2.7, 15.6 mg/kg bw/d)
Positive control: ip injection	0.9% sodium chloride for injection	4 injections, approx. 2 hours apart 7 days prior to scheduled euthanasia	10 mg (free base)/kg/dose [4 mg/mL]) 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine hydrochloride (MPTP HCl)

Method

Mice were administered paraquat dichloride in the diet for 91-95 days at concentrations of 0, 10 or 50 ppm, to determine the effect(s) on dopaminergic neurons in the brains of mice. The author indicated doses where selected based on previous studies, with the high dose not expected to exceed the maximally tolerated dose for mice of this strain. In addition, this strain (C57BL/6J) is susceptible to the effects of neurotoxicants, specifically the positive control substance MPTP hydrochloride (MPTP-HCL).

Mice were treated as four different groups (Table 23 below), including negative and positive control groups. Also shown in the table are the mean calculated quantities of paraquat dichloride/paraquat ion consumed. For the 10 and 50 ppm groups, the average paraquat dichloride consumed was 2.4 and 14.1 mg/kg bw/d (males) and 3.7 and 21.5 mg/kg bw/d (females), respectively (equivalent to paraquat cation of 1.7 and 10.2 mg/kg bw/d (males) and 2.7 and 15.6 mg/kg bw/d (females). The positive control groups received MPTP -HCL as 4 intraperitoneal injections (10 mg (free base)/kg/dose [4 mg/mL]) spaced approximately 2 hours apart, 7 days prior to euthanasia.

Each group was further divided into 3 subsets:

- Subset I (stereology) examined the potential effect of paraquat on dopaminergic neurons by determining the number of TH^+ dopaminergic neurons, the number of TH^- neurons, and total number of neurons (TH^+ plus TH^-) in the SN pc. TH is a key enzyme in the production of DA. The total contour volume of the SN pc was also calculated.
- Subset II (pathology) examined serial sections from the SN to the striatum using specialist staining techniques (including immunohistochemistry) to assess potential effects in this part of the brain, including necrosis, apoptosis, astrocyte reactions as well as an assessment of general morphology.
- Subset III (neurochemistry) was included to quantitate DA, the two metabolites; HVA and DOPAC and 5-HT in striatal tissues of treated mice.

These three subsets are summarised in this report and presented in detail in APPENDIX II- Stereology: Wolf (2013) p 149, Pathology: Butt (2012) p 153 and Neurochemistry: Matthews (2012) p 157.

Table 23. Summary of test substance consumption and animal test groups

Treatment	Dietary concentration (ppm)	Mean calculated test substance consumption # (mg/kg bw/d)		Number of animals (n/sex/dose)		
		paraquat dichloride	paraquat ion	Subset I Stereology	Subset II Pathology	Subset III Neurochemistry
Control	0	0	0	20	15	6
Paraquat dichloride	10	Male: 2.4 Female: 3.7	Male: 1.7 Female: 2.7	20	15	6
Paraquat dichloride	50	Male: 14.1 Female: 21.5	Male: 10.2 Female: 15.6	20	15	6
Positive control*	0	0	0	20	5	6
Time of scheduled euthanasia (day of study)				Day 91, 92, 93 or 94	5/sex on day 31, 59 or 94 (Positive control: all at wk 13)	All at day 95

Summation of mean test substance consumption/number of weeks assessed (13).
* Animals in the positive control group received untreated diet throughout the study. Seven days prior to scheduled euthanasia, they were administered 1-methyl-4-phenyl-1,2,3,6- tetrahydropyridine (MPTP)-HCl (4 ip. injections at 10 mg (free base)/kg/dose [4 mg/mL] delivered approximately 2 hours apart. Dose volume: 2.5 mL/kg/dose.

The study author stated that the number of mice selected for this study, specifically 20/sex/dose for the stereology subset, was calculated as the number required to detect an approximately 20% reduction in neuronal cells (based on results from previous stereology studies).

Mice were acclimated for a minimum 20 days and housed individually under standard laboratory conditions Two weeks prior to study commencement, mice were examined, weighed and assigned to study groups (Table 23 above), including three subsets of endpoints within each of the four groups (stereological assessment, pathological evaluation and neurochemistry assessment). Feed and water was offered *ad libitum*.

Animals were observed twice daily for mortality and moribity and clinical observations were recorded weekly. Animals receiving MPTP were observed for toxicity approximately 1 hour following each dose. Body weight data was collected weekly from 2 weeks prior to test substance administration from which mean body weights and body weight changes were calculated, including for the following intervals: study days 0-28, 0-56 and 0-91. Food consumption was recorded weekly, from 2 weeks prior to test substance administration, and food intake was calculated as g/animal/d for the corresponding body weight change intervals. Mean test substance consumption (mg/kg bw/d) was determined.

Test diets were prepared at concentrations of 10 and 50 ppm paraquat dichloride (the 10 ppm test diet was a dilution of the 50 ppm diet). Samples were collected in study weeks 0, 3, 7 and 12, with a repeat remix and analysis for the first 10 ppm diet due to out-of-specification results. The concentration of paraquat in the diet admix formulation was determined by a validated HPLC method using mass spectrometry (MS) detection. The method was validated, but extended in this study to included concentrations as low as 7.50 ppm. The analysis was

presented as a supplemental report (Beck, 2012e), providing detailed description of the process. The MPTP formulation was also analysed.

A complete necropsy was performed on all animals found dead or euthanised (by cervical dislocation) *in extremis*. Cranial, thoracic, abdominal and pelvic cavities were examined, with the following organs examined grossly: adrenal glands, eyes, kidneys, mammary gland, spinal cord, stomach, thyroids glands, brain, gall bladder, liver, pancreas, salivary gland (mandibular), seminal vesicles, trachea, epididymides, vagina, heart, ln (mesenteric), pituitary, skin, testes, urinary bladder, oesophagus, intestine, lungs, prostate, spleen, thymus, diaphragm. The kidneys, lungs and any gross lesions were retained for possible further histopathology.

I. Scheduled euthanasia was performed differently for each subset of mice: For stereology groups (20 sex/group), after 91, 92, 93 or 94 days of exposure mice were anaesthetised (ip sodium pentobarbital) and perfused *in situ*. Following perfusion, the head was removed and the brain (including olfactory bulbs) was removed from the skull and preserved (4% paraformaldehyde). The lungs and kidneys of mice in 0, 10 and 50 ppm groups were collected and preserved (10% neutral-buffered formalin) for possible future histopathological examination. The brains were sent to another laboratory for processing (Experimental Pathology Laboratories, Inc (EPL)), where the midbrain was isolated and subject to immersion-fixation and flash freezing until processed for stereological analysis. At EPL, the brains were frozen sectioned into 40 micron sections and treated with immunohistochemistry techniques to enable an assessment of the number of TH^+ (tyrosine hydroxlase) and TH^- neurons, and total number of neurons in the SN pc. Statistical analysis was conducted by another organisation (Sielken & Associates Consulting, Inc). Additional detail of methodology is included in APPENDIX II: Wolf, 2013.

II. For pathology groups 5 sex/group/time in the 0, 10 and 50 ppm groups were euthanised after 31, 59 or 94 days exposure and 5 sex/group in the positive control group were euthanised during study week 13 (7 days after MPTP treatment). Mice were anaesthetised (ip sodium pentobarbital) and perfused *in situ*. The head was then removed and skin removed for visual examination of the dorsal surface of the skull. The head (with calvaria intact) was placed in 4% paraformaldehyde for up to 23 h before the brain (including olfactory bulbs) was removed from the skull, weighed and measured. The intact brains were and sent to another laboratory (Neuroscience Associates, Inc.) for processing. The lungs and kidneys from all groups were also collected and preserved (10% neutral-buffered formalin) for possible future histopathological examination. The brains were then trimmed (Neuroscience Associates, Inc.) to the area of the SN to the striatum, multi-embedded and sectioned at 30 µm in the coronal plane and the serial sections stained using the following stains: TH stain, GFAP stain, IBA-1 stain, AM Cu Ag, Caspase 3, Thionine, TUNEL (refer to Beck, 2012a, Table 12 for a description of these stains).

   All slides from the SN were sent to Tox Path Specialists, LLC for examination. This included an analysis of the presence of necrotic cells, any glial reaction and the degree of dopaminergic neuron loss. Additional detail of methodology is included in APPENDIX II: Butt, 2012.

III. For the third subset of animals (neurochemistry), following 95 days of exposure, 6 sex/group were euthanised via cervical dislocation, followed by decapitation and brain removal. Brains were placed in a matrix, and coronal sections prepared. The left and right striatum were dissected free, weighed and flash frozen. The midbrain was also collected and the remaining brain discarded. Collected portions were sent to RTI International for neurotransmitter analysis by ECD of DA, DOPAC, HVA and 5-HT.

Striatal tissue from each mouse was homogenized, centrifuged and an aliquot of homogenate supernatant ran through an Electrochemical Detector (ECD). Striatal concentrations for each neurochemical was determined. Additional detail of methodology is included in APPENDIX II: Matthews, 2012

Results were subject to statistical analysis by an in-house system ('WTDMS'), with the exception of the stereology data. Analyses were conducted using 2-tailed tests (unless otherwise noted) for minimum significance of 1% and 5%, as compared to the control group. Statistical analysis of the stereology data was conducted by Sielken & Associates Consulting, Inc. The effects of treatment (paraquat or MPTP) were assessed using a one-sided t-test for dopaminergic (TH^+) neurons, total (TH^++ TH^-) neurons and contour volume. Differences in means were considered statistically significant at $p \leq 0.05$.

This was a non-Guideline study, although the 'in-life' portions of the study were well conducted and in keeping with OECD Guidelines (TG 424). The Guideline protocol was then deviated to specifically assess the effect of paraquat on dopaminergic neurons in the parts of the brain of interest, as per OECD Guidance Document for Neurotoxicity Testing (No. 20)."

For this reason, the absence of more general tests (eg. functional tests, ophthalmology or histological examination of the complete central and peripheral nervous system) as required in TG 424, is acceptable. It is noted that the study deviated from guidelines by only using two dose groups, whereas TG 424 states at least three should be used. This Guideline is supplemented by the OECD series on testing and assessment Number 20: '*Guidance document for neurotoxicity testing provided additional guidance on strategies and methods for testing of chemicals for potential neurotoxicity'*. This guidance document describes the use of neuropathology and neurochemistry methods which are more specialised to further identify effects on the nervous system.

A number of deviations from the protocol occurred, however are not expected to have affected the quality, integrity or outcome of the study.

Results

*Mortality, moribundity and clinical observations:*A total of 5 animals died prior to scheduled sacrifice. Three males in the 10 ppm group were found dead on study days 5, 14 and 42. In addition, one male in the 50 ppm group was found dead on day 84. These males showed no significant clinical or macroscopic findings and the cause of death was not determined. The deaths showed a lack of a dose response relationship and the deaths in the lower dose group occurred earlier than the single death at the higher dose, therefore the deaths were not considered treatment related. One female in the high dose group was euthanised *in extremis* on study day 77, following clinical findings of hunched posture, decreased defaecation on the day of euthanasia and 7.7 g body weight loss the preceding week. There were no relevant findings

at gross necropsy and the cause of death could not be determined, but was not considered by the study author to be treatment related.

All other mice survived to scheduled euthanasia. There were no clinical signs considered related to paraquat, with findings occurring at similar incidence in the control group, limited to single animals, not occurring in a dose-related manner and/or were considered common for laboratory mice of this age/ strain. The types of findings noted were hair loss on various parts of the body, scabbing of the ear(s) or other body part and hunched posture.

Positive control male mice showed hunched posture and/or hypoactivity, occurring at increasing incidence with subsequent doses (at the time of administration or one hour post – dosing). Piloerection was noted in 1 male at the time of the second dose administration and another 1 h later, and another male at the time of the third dose. Tremors were seen in 2, 3 and 4 mice 1 h following the second, third and fourth dose, respectively. For females administered MPTP, there were no significant clinical observations noted at the time of dosing, but an increasing incidence of hunched posture, piloerection and/or hypoactivity was noted, one hour following the second, third and/or fourth doses. A single female showed tremors 1 h after the fourth dose. None of these findings in male and female positive control mice were present in the examination one week following the final dose.

Body weight and food consumption: Body weight gain fluctuations were noted throughout the study. In males, during the first week (Day 0-7) body weight change was statistically significantly lower than controls for the 50 ppm group only. Significantly lower weight gains were also noted in the week of 70-77, however significantly higher weight gains were noted for an earlier interval (day 42-49). Males in the 10 ppm group showed fluctuations over different periods with both statistically significantly reduced or increased weight gain noted. The period of day 70-77 is the only time where both paraquat treated groups showed a reduced body weight gain compared to controls (0.3 g for both treated groups *vs* 0.5 g for controls ($p<0.05$)). However, overall for the entire study (day 0-91), males dosed with 10 or 50 ppm paraquat, did not show any statistically significant difference in mean body weight change (Table 24 below).

Females also showed fluctuation in body weight gain with the 50 ppm group having a statistically significantly reduced body weight gain for intervals day 7-14 and 49-56 (as compared to the control group). Statistically significantly increased body weight gain was noted for interval days 56-63 and 77-84. As for males at 10 ppm, females also showed a statistically significant reduced or increased body weight gain for different intervals throughout the treatment period (decreases day 28-35, 49-56, and 84-91 and increases days 35-42, 42-49, 56-63 and 77-84). Overall, for the entire study (day 0-91), females dosed with 10 or 50 ppm paraquat, did not show any statistically significant difference in mean body weight change. The Day 49-56 interval is the only period where both paraquat treated groups showed a reduced body weight gain compared to controls (0.0 g for both treated groups vs 0.4 g for controls ($p<0.01$)).

Considering the lack of dose relationship, or the direction of change being not toxicologically significant, the effects on body weight are not considered treatment related for males and females at 10 ppm. The initial reduction in body weight gain in males and females at 50 ppm is considered treatment related, but recovery was evident over the course of the study.

For the positive control group, after one week post-dosing, male mice had a statistically significant mean body weight loss of 0.4 g (*vs* 0.1 g loss in control males). In females, a

statistically significant mean body weight loss of 0.1 g was noted (*vs* 0.4 g body weight gain in control females). These were considered treatment related. Positive control males showed statistically significant fluctuations prior to treatment (decreases day 28-35, 70-77, increases day 42-49, 77-84) however, actual body weight loss was only noted the week following treatment. Similarly, in females statistically significant reductions in body weight gain occurred day 49-56 and increases day 42-49 and 77-84. These fluctuations are considered normal variation.

Table 24. Summary of mean body weight change and final mean body weights (g)

Mean body weight change (g)	Dose group (ppm)							
	Males				Females			
Study day interval:	**0**	**10**	**50**	**MPTP**	**0**	**10**	**50**	**MPTP**
0-28	1.8	2.0	1.5	2.1	2.1	2.2	2.0	2.1
0-56	3.3	3.8	**2.7***	3.4	3.6	3.5	3.4	3.9
Total: 0-91	**4.3 (n=31)**	**4.6 (n=30)**	**3.8 (n=30)**	**4.3 (n=31)**	**4.8 (n=31)**	**4.8 (n=31)**	**5.2 (n=30)**	**4.9 (n=31)**
Mean body weight (g) (% difference from control) (Day 91)	27.1 (n=31)	27.3 (+0.7%) (n=30)	26.4 (-2.6%) (n=30)	26.5 (-2.2%) (n=31)	22.2 (n=31)	21.7 (-2.3%) (n=31)	22.3 (0.5%) (n=30)	22.0 (-0.9%) (n=31)
Final mean body weight (g) (Day 95)	27.5 (n=6)	27.2 (n=5)	27.7 (n=5)	26.5 (n=6)	22.8 (n=6)	21.4 (n=6)	22.4 (n=5)	21.9 (n=6)

* Statistically significantly different from control group (0 ppm) at 0.05 using Dunnett's test

Mean food consumption for 10 and 50 ppm treated groups were similar to controls throughout the study, although there were sporadic statistically significant reductions. However, these were not considered test substance related due to the lack of a dose relationship and a similar occurrence in the pre-treatment period.

In the positive control mice, mean food consumption was slightly lower than controls (-0.9 g/animal/day for both males and females) during the week following administration. This difference was statistically significant in males only. Mean food consumption was however of a greater difference in the previous week i.e before MPTP administration (-1.6 g/animal/day M, and -2.6 g/animal/day F). This is therefore not considered to be treatment related.

Dietary analysis:

Diet admix prepared with 10 and 50 ppm paraquat dichloride met the protocol specified requirements for target concentrations and stability (over 12 days), with a few exceptions. The homogeneity did not meet the protocol-specified criteria. The analysed MPTP formulation was found to be within acceptable limits (90% to 110% of target).

Macroscopic findings:

*Unscheduled deaths:*The four males that were found dead (3 x 10 ppm, 1 x 50 ppm) and the one female killed *in extremis* (50 ppm) were subject to gross necropsy, which did not reveal any significant findings. The cause of death was not determined.

*Scheduled necropsy:*In subset I (stereology group), there was one male (50 ppm) noted to have a small left kidney, dilated renal pelvis and distended ureter. This was not considered treatment-related as it only occurred in a single animal.

In subset II (pathology), there were no gross findings in the brain considered treatment-related at any scheduled necropsies (weeks 4, 8 or 13). Findings in the brain (red areas and/or areas of red discolouration) did not demonstrate a dose relationship, were observed at levels similar to those in controls and were recorded during weeks 4 and 13 only (not week 8) (Table 25 below). The reviewing neuropathologist stated that these gross lesions did not correlate to any microscopic change and such findings are very common in the brain of animals, particularly those that are perfusion fixed. All gross lesions were attributed to artifactual changes related to death, intravascular perfusion, and/or necropsy.

Table 25. Incidence of macroscopic findings in the brain (Subset II)

Finding in brain (n=5)		Dose group (ppm)							
		Males				Females			
	Wk	0	10	50	MPTP	0	10	50	MPTP
Red area(s)	**4**	1	1	2	NA	2	3	2	NA
	8	0	0 (n=3)	0	NA	0	0	0	NA
	13	2	2	2	1	1	1	1	0
Red discolouration	**4**	1	4	4	NA	2	2	4	NA
	8	0	0	0	NA	0	0	0	NA
	13	0	1	2	2	1	0	1	1

NA- Not applicable, as not necropsied at this time

Mice treated with MPTP were not found to have any treatment-related changes to the gross appearance of the brain at the week 13 necropsy.

*Brain weights and measurements (Subset II):*Brain weights, length and width did not show a treatment-related effect at any scheduled necropsy, including positive control (MPTP) mice. There was no statistical difference with controls.

*Brain striatum weights (Subset III):*Mean brain striatum weights for both paraquat treated groups and the MPTP positive control group were similar to the control group. No statistically significant differences or dose response was noted.

Stereology assessment (Subset I)

In male and female mice treated with paraquat (10 or 50 ppm), there was no significant treatment-related effect. The number of TH^+ neurons, TH^- neurons, total neurons and total contour volumes of the SN pc were not statistically different from the controls.

In positive control male mice (MPTP), the number of TH^+ neurons was reduced (10%) and TH- neurons slightly increased (1%), but the total number of neurons (TH^+ + TH^-) was reduced (-6%), as compared to negative control animals (Table 26 below). However only the reduction in TH^+ neurons was statistically significant. The total contour volume was statistically significantly reduced (-9%) in positive control males as compared to negative controls.

In females in the positive control group (MPTP), the results reflected those in males with the number of TH^+ neurons reduced (-5%), the number of TH^- neurons increased (4%), the total number of neurons reduced (-3%) and the total contour volume reduced (-3%) as compared to the negative controls. However, in females none of the results were statistically significant.

Table 26. Significant stereological findings in the SN pc for MPTP

Group		Males				Females			
		TH^+ neurons	TH^- neurons	Total neurons (TH^+ & TH^-)	Total contour volume (μm^3)	TH^+ neurons	TH^- neurons	Total neurons (TH^+ & TH^-)	Total contour volume (μm^3)
0 ppm	**n**	20				20			
	Mean	15,173	7,510	22,683	345,661,900	14,813	6,024	20,838	339,634,150
	SD	2,447	2,039	3,197	27,303,337	2,657	1,442	2,937	29,853,427
MPTP	**n**	17[#]				20			
	Mean	13,675[*]	7,599	21,274	313,410,412[**]	14,004	6,298	20,302	329,448,500
	SD	2,298	1,816	3,449	32,778,538	2,344	1,420	2,692	34,881,823
	% change	-10	+1	-6	-9	-5	+4	-3	-3

*Significant $p \leq 0.05$, ** $p \leq 0.01$, [#] 3 mice from this group could not be evaluated due to irreparable tissue artifacts.

Neuropathology (Subset II)

*Microscopic pathology:*The use of several specialty staining techniques to assess the presence of necrotic cells, the character of the glial reaction and the degree of dopaminergic neuron loss showed there were no microscopic changes, in the parts of the brain examined, associated with 4, 8 or 13 weeks of administration of paraquat dichloride at 10 or 50 ppm to mice. The staining technique was verified by concurrent staining of positive controls.

The positive control mice showed treatment related neurohistopathology. The effects were considered by the study author as being consistent with direct toxicity to the dopaminergic neurons of the SN pc. Results are detailed in APPENDIX II: Butt, 2012. There was decreased TH staining in the striatum in all MPTP animals, and was due to a persistent decrease in dopaminergic synaptic terminals in this region. This finding was less consistent in the SN (3/5 males and not found in any females). Increase in GFAP (astrocyte stain) and IBA-1 (microglial) staining was seen consistently across all males and females in the striatum. An increase in staining in the SN was only detected in a single male and no females. These stains indicate microglial activation and an astrocyte response.

There were no apoptotic cells detected (via Caspase-3 or TUNEL stains). The study pathologist notes there were no findings in the Amino Cupric silver stained sections (to detect necrotic/disintegrating neurons and processes) and attributes this to the time frame of harvesting the tissues.

Neurochemistry Assessment (Subset III)

Mice treated with paraquat did not show a consistent effect on DA or its two metabolites, across both dose groups, with both increases and decreases in percentage difference from negative controls noted (Table 27 below). For males, this was particularly inconsistent. For females, DA was unchanged at 10 ppm and increased from controls by 20% at 50 ppm (positive control -33%) however the direction of change is not toxicologically significant. For DOPAC, levels in females were dose relatedly reduced (-43% and -49%) for paraquat treated groups (positive control reduced by -49%), and for HVA the levels were reduced, but not in a dose related manner (-14% and -2%, positive control -20%). The significance of the DOPAC levels being dose relatedly reduced in females is unclear, without corresponding findings in the DA levels, and findings in males, and is further complicated by unusually high levels in negative control females.

5-HT, as an estimate only, was increased from controls in all groups tested. For 10 ppm, 50 ppm and positive controls, the percentage increased from controls for males was + 44% and +22% (+21% for positive controls), and for females was +11% and +2.9% (+9.4% for positive controls), respectively. A dose response is therefore lacking and in addition, interpretation of the 5-HT levels are limited due to their lack of accuracy as the methodology did not pass the validation process.

The positive control group showed marked effects in the levels of these neurotransmitters. The percentage difference from controls for DA, DOPAC and HVA were -86%, -68% and -52% (males) and -33%, -49% and -20% (females) respectively. The mean DA turnover for male mice was +221% and for females was only +1.3%. It is evident from these figures that the effects in females were not as great as those for males, however the study author stated that MPTP has previously been demonstrated to induce greater neurotoxicity in male mice than females. Considering the higher than expected mean DOPAC levels detected in female negative control mice, this finding would have artificially increased the apparent difference in DOPAC concentrations in other groups, as well as the lower DA turnover in positive control females.

Table 27. Striatal neurotransmitter and metabolite levels (mean and percentage change from negative control)

Mean / % change from ctrl	Sex (n)	Striatal Neurotransmitter/Metabolite Levels (ng/mg tissue)				DA turnover*
Group		DA	DOPAC	HVA	5-HT#	
Negative control	M (6)	16.55	1.66	1.94	0.62	0.220
	F (6)	16.75	3.25	1.91	0.66	0.330
10 ppm paraquat	M (5)	16.16 -2.4%	1.70 +2.2%	2.16 +11.7%	0.90 +44.1%	0.248 +12.8%
	F (6)	16.75 0%	1.86 -42.7%	1.65 -13.8%	0.73 +11.2%	0.215 -34.9%
50 ppm paraquat	M (5)	18.43 +11.4%	1.35 -18.8%	1.94 +0.3%	0.76 +21.9%	0.180 -17.8%
	F (5)	20.14 +20.3%	1.66 -48.9%	1.87 -2.4%	0.68 +2.9%	0.176 -46.7%
Positive control (MPTP)	M (6)	2.32 -86.0%	0.53 -68.0%	0.93 -52.2%	0.75 +20.9%	0.706 +221.3%
	F (6)	11.23 -32.9%	1.66 -49.0%	1.53 -20.0%	0.72 +9.4%	0.326 -1.3%

* Estimated by dividing the sum of the DOPAC and HVA concentrations by the DA concentration, and the data is expressed as a ratio. # 5-HT levels are considered an estimate only.

Discussion

There were no treatment related effects on mortality or clinical signs in mice treated with paraquat. Mice treated with 50 ppm showed a treatment-related reduction in body weight gain for the first week of the study only, after which body weight was similar or greater than controls. The overall body weight gain of paraquat treated mice was not statistically significantly different from controls. Food consumption did not show any treatment-related effects. Mice receiving intraperitoneal injections of MPTP (positive control) showed clinical signs, body weight loss and reduced food consumption, as compared to the negative controls.

Following necropsy, the brain showed no abnormalities by gross examination or in size/weight. Neuropathology analysis concluded that paraquat dichloride did not cause any gross or microscopic changes in any of the brains regions examined. Specifically, paraquat was not associated with any neuronal or glial changes in the SN or the striatum. Mice treated with the positive control agent, MPTP, showed damage to the dopaminergic neurons in the SN pc within this test system, demonstrating its validity.

Stereology analysis of TH immunostained slides did not show any effects on the number of dopaminergic neuronal cells in the SN pc compared to untreated controls. Male mice treated with MPTP (positive control) showed a statistically significant reduction in the number of TH^+ neurons and in the total contour volume. Female mice from the positive control group did not show a statistically significant effect, although the pattern of change reflected that of males.

There was no paraquat-related effect on the levels of DA or its two metabolites (HVA and DOPAC) in striatal tissues of the brain, as compared to negative control tissues. Positive control male mice showed marked decreases in mean DA, DOPAC and HVA concentrations. Effects were not as marked in positive control female mice.

Conclusion

Paraquat at doses up to 50 ppm paraquat dichloride (males 10.2 mg/kg bw/d, females 15.6 mg/kg bw/d, paraquat ion) did not affect the dopaminergic neurons in the brains of male or female C57BL/6J mice when administered constantly in the feed for up to 13 weeks, under the conditions of this test system. This included an assessment of pathology (histopathological examination of neurons, astrocytes, microglia) and stereology (number of TH^+ cells in the SN pc) and neurochemistry (concentrations of DA and metabolites in the striatum). Findings in the positive control mice, treated by intraperitoneal injections of MPTP validate the test system.

Fredriksson A, Fredriksson M & Eriksson P (1993*)* Neonatal exposure to paraquat or MPTP induces permanent changes in striatum dopamine and behaviour in adult mice. *Toxicol Appl Pharmacol* 122: 258-264.

Fredriksson *et al* (1993) examined the effect of paraquat administered to neonatal mice during the brain growth spurt.

Paraquat (purity and batch no. unspecified; ICI Ltd., Alderley Park, Macclesfield, Cheshire, England) was administered to 12 male C57 black mice/group (age and body weights unspecified; ALAB, Sollentuna, Sweden) by po gavage in a 20% (w/w) fat emulsion vehicle (egg lecithin and peanut oil), when 10 and 11 days old, at doses of 0 (vehicle control), 0.07 or 0.36 mg/kg bw (equivalent to 0, 0.050 and 0.26 mg/kg bw paraquat cation, respectively) in a dose volume of 10 mL/kg bw. The dose selection was based on previous studies where paraquat was given up to 40 mg/kg bw in adult male C57 black mice as an MTD, and 0.36 mg/kg bw had been given to neonates with no evidence of clinical lung toxicity. Two additional groups (n=12) received MPTP (Aldrich Chemical Co. Milwaukee, WI, USA) orally at 0.3 or 20 mg/kg.

Spontaneous behaviour was tested once at both 60 and 120 days of age in 12 mice/group during 3 consecutive days as previously described (Eriksson *et al,* 1990; 1991a,b; 1992). A concurrent group of mice that received 0.36 mg/kg bw paraquat at 10 and 11 days of age were also tested for behaviour on day 18. Motor activity was measured over 3 x 20 minute intervals in an automated device as previously described (Eriksson *et al*, 1992). Eight of the 12 mice/group used for the behavioural studies were sacrificed by decapitation at 125 days of age, and striatal concentrations of DA, DOPAC, HVA, 5-HT and 5-HIAA were analysed by HPLC.

No signs of acute toxicity or differences in body weight gain were observed in any of the paraquat- or MPTP-treated mice, although no supporting data were provided to substantiate these findings. No treatment-related effect on spontaneous behaviour was observed at 18 days of age in mice given 0.36 mg/kg bw paraquat. At 60 days of age, marked hypoactivity was observed in mice at both 0.07 and 0.36 mg/kg bw paraquat (and 0.3 or 20 mg/kg bw MPTP). Paraquat-treated mice exhibited a statistically significant decrease ($p<0.01$, split-plot ANOVA) in locomotion, rearing and total activity during the first 20 minute period. During the second 20 minute period, locomotion ($p<0.05$-0.01, split-plot ANOVA) and total activity ($p< 0.01$, split-plot ANOVA), but not rearing, were still significantly lower than the control. During the third 20 minute period only total activity in the 0.36 mg/kg bw group was higher ($p<0.05$, split-plot ANOVA) than the control. This hypoactivity persisted and appeared more pronounced at 120 days with locomotion and total activity significantly lower ($p<0.01$, split-plot ANOVA) than the control over the first and second 20 minute periods for both doses of paraquat. Rearing

was significantly lower at both 0.07 and 0.36 mg/kg bw than the control ($p<0.01$, split-plot ANOVA) over the 3 x 20 minute periods.

There was a dose-related depression in striatal DA, DOPAC and HVA in paraquat-treated mice (see Table 28 below). At 0.07 mg/kg bw, only the depression in HVA was statistically lower than the control ($p<0.01$, 1-way ANOVA). At 0.36 mg/kg bw, the levels of DOPAC and HVA were statistically lower than the control ($p<0.01$, 1-way ANOVA), while the level of DA was statistically lower ($p<0.01$, 1-way ANOVA) than all other groups (including the control). There was no paraquat-related effect on the levels of 5-HT or 5-HIAA. MPTP also caused a concentration-related depression in striatal DA, DOPAC and HVA while 5-HT and 5-HIAA levels were unaffected by treatment. The concentration of DA was statistically lower than the control at both 0.30 and 20 mg/kg bw, while DOPAC and HVA were significantly lower than the control at 20 mg/kg bw.

Table 28. DA, 5-HT and metabolite levels in male mice neonatally exposed to paraquat or MPTP

Neurochemical	Control	Paraquat 0.07 mg/kg bw	Paraquat 0.36 mg/kg bw	MPTP 0.30 mg/kg bw	MPTP 20 mg/kg bw
DA	10.68 ± 0.45	9.90 ± 0.44	7.81 ± 0.25 *	8.71 ± 0.27 *	6.40 ± 0.25 *
DOPAC	2.16 ± 0.16	1.75 ± 0.11	1.55 ± 0.10 *	1.66 ± 0.08	1.16 ± 0.10 *
HVA	1.67 ± 0.06	1.04 ± 0.04 *	0.98 ± 0.04 *	1.48 ± 0.03	0.82 ± 0.06 *
5-HT	0.41 ± 0.02	0.44 ± 0.03	0.47 ± 0.05	0.44 ± 0.02	0.43 ± 0.04
5-HIAA	0.45 ± 0.01	0.50 ± 0.02	0.50 ± 0.03	0.48 ± 0.02	0.47 ± 0.01

Results expressed as the mean μg/wet weight ± SEM (n=8).

In summary, following neonatal exposure of male C57 black mice to paraquat, neurotoxicity was seen at 0.07 mg/kg bw (equivalent to 0.05 mg/kg bw paraquat cation), the lowest dose tested. Hypoactivity at both 60 and 120 days of age, and a significant depression in the striatal concentration of HVA measured at 125 days of age, were observed at 0.07 and 0.36 mg/kg bw (equivalent to 0.05 and 0.26 mg/kg bw paraquat cation, respectively). At 0.36 mg/kg bw, a significant depression in the striatal concentration of DA and DOPAC occurred. These observations suggested that neonatal exposure to paraquat induced lasting neurochemical and behavioural changes in mice. Although the study authors concluded that the observed changes were permanent, no behavioural or neurochemical measurements were performed beyond 120 days.

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

This study compared the neurotoxicity of paraquat and MPTP when administered by intraperitoneal injection.

Paraquat (unspecified purity & batch no.; RBI, Natick, MA, USA) in saline was administered as 3 ip injections to groups (n=6) of adult male C57BL/6J mice (unspecified body weight & source) at 0, 5 or 10 mg/kg bw (equivalent to 0, 3.6 and 7.2 mg/kg bw paraquat cation, respectively). Each injection was separated by one week and the dose volume was 0.3 mL. Separate groups of mice (n=6) were given 4 ip injections of MPTP (unspecified purity & batch no.; RBI, Natick, MA, USA) at 0, 10 or 30 mg/kg bw. The first 2 injections were separated by

16 h, and the 3rd and 4th injections were given 7 days after the first 2 and were also separated by 16 h. No rationale was given for the difference in dosing regimes between paraquat- and MPTP-treated mice. Housing and feeding conditions were unspecified. Unblinded behavioural assessments were performed at 2, 24 and 48 h, beginning one day after the final dose of paraquat and 1 week after the final dose of MPTP. Assessments were made in a sound-proof, dimly lit room and were compared to baseline measurements which had been taken 2 days prior to compound administration. Horizontal, vertical and ambulatory locomotor activities were measured at 5 minute intervals over 60 minutes on a single day.

Compound-related damage to neurons of the SN was assessed using a Fluoro-gold retrograde labelling method or by immunostaining for TH. Fluoro-gold (100 nL; Fluorochrome, Englewood, CO, USA) was stereotactically injected into the striatum of mice under halothane anaesthesia. Mice were allowed to recover for 5 days and then injected with paraquat or MPTP at the doses described above. Following behavioural testing, mice were anaesthetised, their brains removed and immunohistochemically stained for TH. Fluoro-gold and TH positive cells were visualised by fluorescence microscopy and quantified by densitometry. Seven to ten sections were analysed for each group, representing at least 3 mice/group. Cell counts were performed on 80 μm serial sections from each mouse brain within 24 h of immunohistochemical processing.

The presence/absence of mortalities and clinical signs were unreported. Graphically-presented data illustrated that paraquat or MPTP caused a dose-dependent decrease in Fluoro-gold-labelled cells indicating damage to neurons of the SN. A 36 and 61% reduction in Fluoro-gold positive cells were observed at 5 and 10 mg/kg bw paraquat, respectively, and these results were statistically significant ($p=0.0077$, 1-factor ANOVA) relative to the saline control group. Similarly, a 50 and 75% reduction in Fluoro-gold positive cells were observed at 10 and 30 mg/kg bw MPTP, respectively, with both results statistically significant ($p=0.0001$, 1-factor ANOVA).

Graphically-presented data showed that paraquat and MPTP caused damage to TH-containing terminals in the striatum. The density of TH-positive cells was significantly reduced by 87 and 94% following treatment with 10 or 20 mg/kg bw paraquat, respectively ($p<0.0001$, 1-factor ANOVA). Similarly, 76 and 98% reductions in TH-positive cells were observed in mice treated with 10 or 30 mg/kg bw MPTP, respectively, and this result was also statistically significant ($p<0.0002$ and 0.0001, respectively, 1-factor ANOVA).

Graphically-presented data revealed that all groups (including the control) exhibited a decline in ambulatory activity over the first 30 minutes of the assessment period. Over the remaining 30 minutes, control mice showed a relatively stable level of ambulatory activity suggesting that they had acclimatised to the test environment. Mice treated with either dose of paraquat or MPTP exhibited a decrease in ambulatory locomotor activity over the last 30 minutes of the assessment period. The effects of both paraquat and MPTP were significantly different to the control ($p=0.003$ and 0.0001, respectively, 1-factor ANOVA), with the effect of MPTP marginally greater than the effect of paraquat. Effects on horizontal or vertical (jumping, rearing) locomotor activities were not reported.

The major limitation of this study was the limited reporting of the results. Although behavioural measurements were made on three occasions over 48 h, only data for the last (ie 48 h) time

point was presented. Additionally, the sample size made interpretation of the behavioural findings difficult. The administration of each test compound via different protocols made it difficult to compare the effects of paraquat and MPTP.

Hara S, Iwata N, Kuriiwa F, Kano S, Kawaguchi N & Endo T (1993) Involvement of opioid receptors in shaking behaviour induced by paraquat in rats. ***Pharmacol Toxicol 73: 146-149.***

Paraquat dichloride (unspecified purity & batch no.; Sigma Chemical Co., St Louis, MO, USA) in sterile saline was injected intraperitoneally into 9 male SD rats/group (Clea Japan Co., Tokyo, Japan) at 0, 30, 50 or 70 mg/kg bw (equivalent to 0, 21.6, 36 and 50.4 mg/kg bw paraquat cation, respectively). Rats were 5-6 weeks old and weighed 170-250 g. In some experiments, 5-6 rats/group were injected with morphine (5 mg/kg bw, ip) or naloxone (1.5 mg/kg bw, ip) 30 and 15 minutes, respectively, prior to paraquat (70 mg/kg bw, ip). Separate groups of rats were given an intracerebroventricular injection of paraquat at 12.9 (n=3) or 25.7 μg /rat (n=9) in 10 μL (equivalent to 9.3 and 18.5 μg paraquat cation, respectively) over 50-60 seconds using a 26-gauge stainless steel cannula. Control rats (n=6) received an equivalent volume of saline. Rats had previously been stereotaxically implanted with polyethylene guide cannulae under pentobarbital anaesthesia according to the co-ordinates of Paxinos & Watson (1986). The dose selection for the intracerebroventricular injection was based on the study of De Gori *et al* (1988).

Immediately after dosing, rats were placed individually into polycarbonate boxes and observed for 60 minutes at 21-22°C. Shaking behaviour was scored as any episode of quick rotational movement of the head, thorax or entire body approximately the spinal axis. All other episodes of non-shaking behaviour were also recorded. Immediately following behavioural observations, rats treated with paraquat ± morphine, and the controls were sacrificed and their brains histopathologically examined. The effect of paraquat on brain microvascular permeability was determined by measuring fluorescein uptake according to the method of Koenig *et al* (1983). Briefly, 1% fluorescein (1 mL/kg bw, iv) was injected 45 minutes after paraquat (70 mg/kg bw, route unspecified) and brains removed 15 minutes later.

There was a statistically significant, dose-dependent increase in the frequency of shaking behaviour ($r=0.998$, $p<0.001$, Pearson's moment correlation coefficient) following a single ip injection of paraquat (see Table 29 below) which usually began 20-30 minutes after administration. Myoclonus was observed in 4/9 high-dose rats 60 minutes after paraquat administration but was not observed in any other animals.

Table 29. Effect of a single ip injection of paraquat on shaking behaviour in male SD rats

Dose (mg/kg bw)	Shaking behaviour	
	Frequency	Incidence
0	0.1 ± 0.1	1/9
30	10.4 ± 4.0*	8/9
50	16.6 ± 4.8*	8/9
70	25.2 ± 4.0*	9/9

Frequency data expressed as mean ± 1 SEM; *p<0.05 (1-way ANOVA, Duncan's multiple range test)

Pre-treatment of rats with morphine reduced both the frequency and incidence of paraquat-induced shaking behaviour, but only the effect on frequency was statistically significant

($p<0.01$) (see Table 30 below). There was also a reduction in the incidence of myoclonus following pre-treatment with morphine but this result was not statistically significant. Naloxone had no effect on the frequency or the incidence of paraquat-induced shaking behaviour, however the incidence of myoclonus was reduced. The frequency and incidence of paraquat-induced shaking behaviour, and incidence of myoclonus was not altered following combinational pre-treatment with morphine plus naloxone.

Table 30. Effects of morphine and naloxone on paraquat-induced shaking behaviour and myoclonus in male SD rats

Treatment	Shaking behaviour		Myoclonus
	Frequency	Incidence	Incidence
Control (saline)	0	0/6	0/6
PQ	24.5 ± 6.8**	6/6*	4/6*
PQ + morphine	2.2 ± 1.0[1]	3/6	2/6
PQ + naloxone	27.6 ± 5.2[2]	5/5	1/5
Paraquat + morphine + naloxone	25.4 ± 5.2[2]	5/5	3/5

Frequency data expressed as mean ± 1 SEM; PQ = paraquat; * $p<0.05$ (Fisher's exact probability test); ** $p<0.01$ (1-way ANOVA, Duncan's multiple range test); 1 = statistically different from the group treated with paraquat alone at $p<0.01$ (1-way ANOVA, Duncan's multiple range test); 2 = statistically different from the group pre-treated with paraquat plus morphine at $p<0.01$ (1-way ANOVA, Duncan's multiple range test).

Intracerebroventricular injection of paraquat at 11.9 or 23.7 μg/rat did not cause shaking behaviour or myoclonus. In contrast, tremor was observed in all nine animals given 23.7 μg/rat but not in any of those given 11.9 μg/rat or in the control. No histopathological abnormalities were detected in the brains of any treated or control rats. Fluorescein uptake was significantly increased ($p<0.05$) by paraquat and this effect was prevented by pre-treatment with morphine (see Table 31 below). Although this result suggested that paraquat had damaged the brain microvasculature, the toxicological significance of this finding was unclear as the magnitude of the effect was relatively small. Naloxone did not antagonise the action of morphine on fluorescein uptake.

Table 31. Effect of paraquat on the uptake of fluorescein into rat brain

Treatment	Fluorescein level (ng/g brain)
Control (saline)	9.54 ± 0.45*
PQ	12.83 ± 0.86
PQ + morphine	8.75 ± 1.07*
Paraquat + morphine + naloxone	9.70 ± 1.44*

Data expressed as mean ± 1 SEM; PQ = paraquat; * = significantly different from the group pre-treated with paraquat (1-way ANOVA, Duncan's multiple range test).

In summary, a single ip injection of paraquat given to male SD rats caused a significant dose-dependent increase in the frequency of shaking behaviour at and above 30 mg/kg bw (equivalent to 21.6 mg/kg bw paraquat cation). Myoclonus was seen only at 70 mg/kg bw (equivalent to 50.6 mg/kg bw paraquat cation). Morphine but not naloxone pre-treatment significantly reduced paraquat-induced shaking behaviour suggesting that shaking behaviour was mediated by opioid receptors. Intracerebroventricular injection of paraquat induced tremor, but not shaking behaviour or myoclonus, at 23.7 μg/rat (equivalent to 18.5 μg paraquat cation). Although no histopathological abnormalities were detected in the brains of any rats, fluorescein uptake was significantly increased by treatment and this was prevented by

morphine. The latter findings suggested that paraquat increased the vascular permeability of the rat brain.

Ossowska K, Wardas J, Smiałowska M, Kuter K, Lenda T, Wierońska JM, Zieba B, Nowak P, Dabrowska J, Bortel A, Kwieciński A & Wolfarth S (2005b). A slowly developing dysfunction of dopaminergic nigrostriatal neurons induced by long-term paraquat administration in rats: an animal model of preclinical stages of Parkinson's disease? *Eur J Neurosci.* 22(6):1294-304.

This study examined the effect of long-term (up to 24 weeks) intraperitoneal paraquat administration in rats, specifically, effects on nigrostriatal neurons and potential sequelae in dopaminergic transmission. The study also investigated the intermediate stages in this process by sampling at different time points.

Method: Male Wistar rats (200-250 g) were housed in in a controlled environment with feed and water provided *ad libitum*. Paraquat dichloride (Sigma-Aldrich, Germany) dissolved in saline was administered at a dose of 10 mg/kg/2 mL ip once a week for 4, 8, 12 or 24 weeks (approx. 7.2 mg/kg bw paraquat ion). Groups designated for biochemical or Western blot analysis were killed by decapitation 7 days after the last injection. Groups for histological examination were dosed only at 4, 8 or 24 weeks and perfused 7 days after the last injection. A vehicle control group received weekly ip injections

Following decapitation, brains were rapidly removed and the SN and caudate-putamen isolated. Levels of DA, DOPAC, 3-MT and HVA, 5-HT, 5-HIAA, and NA determined by HPLC and ECD. The binding of [^{3}H]1-2[2-[2-(diphenyl-methoxy)-ethyl]-4-(3-phenylpropyl)piperazine ([^{3}H]GBR 12,935) to DAT was estimated in the dorsal and ventral regions of the anterior caudate-putamen, expressed in Ci/mmol and converted to fmol/mg of tissue. Histological analyses of TH-ir neurons in the SN and TH-ir terminals in the caudate-putamen were conducted, including sterelogical counting of the TH-ir neurones

Results:

Number of TH-ir neurons in the SN and TH-ir immunoreactivity in the caudate-putamen (light microscopy): Stereology analysis of the number of TH-ir neurons at 4 or 8 weeks of treatment with ip paraquat (10 mg/kg bw) showed a dose related reduction in the number for TH-ir neurons in the SN (-17% and -28.5% difference from controls respectively), although this was not statistically significant. Following 24 weeks of ip injections, the reduction was significant at a loss of 37%.Manual counting showed that after 4 weeks the loss of TH-ir neurons was greater in the rostral region of the SN, after which the loss was evident in increasingly caudal parts, until the entire SN was affected at 24 weeks. The degree of staining for nigral neurons was greater after 8 weeks of treatment, but less so at 24 weeks. Surrounding areas showed a similar pattern of staining intensity.

There were no changes in the level of TH-immunoreactivity in the caudate-putamen at 4 or 8 weeks, but a decrease was evident at 24 weeks.

Volume of the SN: Treatment with paraquat resulted in a statisitically significant reduction in the volume of the SN as compared to controls (approx 24% less than controls) following 24 weeks of administration. Reduced periods of treatment resulted in no affects on SN volume.

DA levels and metabolism: In the caudate-putamen, levels of 3-MT and HVA were statistically significantly increased following 4 weeks of treatment, and at 8 weeks there was an increase in DA, DOPAC, 3-MT and HVA. At the 12 week sacrifice, DA and its metabolites were similar to control values and effects noted again at the 24 week sacrifice (DA and DOPAC concentrations dropped by 26-31% and 27-36%, respectively). An increase in the turnover rate (HVA : DA) was noted at 4-8 weeks of treatment, but not at12 weeks.

Differences were noted in the SN,with increased 3-MT levels only at 4 weeks. At 8 weeks, the levels of DA, DOPAC, 3-MT and HVA were increased but normal at12 weeks. There were no differences from controls in any neurotransmitter/metabolite at 24 weeks, although the ratio of 3-MT: DA was reduced.

5-HT and noradrenalin levels and metabolism: In the caudate-putamen at 4 weeks, there was an increase in 5-HIAA only which persisted until 12 weeks. At 8 and 12 weeks 5-HT levels were elevated. An increased 5-HIAA:5-HT was noted at 8 weeks only. No effects on these levels were present at 24 weeks.

In the SN, 5-HT levels were elevated at the 4, 8 and 12 week periods, but not at 24 weeks. 5-HIAA increased from 4 weeks, statistically significantly so at the 8 and 12 week periods. Serotonergic transmission was not significantly affected in either structure after 24 weeks of treatment with ip paraquat.

Noradrenalin levels were increased in the caudate-putamen only, present following 4, 8 and 12 weeks of treatment with paraquat.

[^{3}H]GBR 12,935 binding to DAT:
In the caudate-putamen, the binding of [^{3}H]GBR 12,935 was decreased only in the ventral region at 4 weeks, extending to include the dorsal region at 8 weeks. Values were similar to controls at 12 weeks and reduced at 24 weeks in both the dorsal and ventral regions (11% and 33%, respectively).

TH levels (Western blot analysis): No differences in TH levels in the striatum were noted after 4, 8 or 12 weeks of paraquat treatment. Following 24 weeks of treatment, the level of TH was reduced by 55% in the caudate-putamen. Levels were not significantly affected in the SN.

Conclusion:

Intraperitoneal administration of paraquat to rats, reduced the number of TH-ir neurons of the SN; after the 4-week treatment the reduction (17%, nonsignificant) was confined to the rostrocentral region of this structure but, after 24 weeks, had spread along its whole length and was 37%, significant). It induced a biphasic effect on dopaminergic transmission. First, levels of DA, its metabolites and turnover were elevated (4-8 weeks) in the caudate-putamen, then all these parameters returned to control values (12 weeks) and dropped by 25-30% after 24 weeks. The binding of [^{3}H]GBR 12,935 to DAT in the caudate-putamen was decreased after 4-8

weeks, then returned to control values after 12 weeks but was again decreased after 24 weeks. Twenty-four-week paraquat administration also decreased the level of TH in the caudate-putamen. In addition, paraquat activated 5-HT and noradrenaline transmission during the first 12 weeks of treatment but no decreases in levels of these neurotransmitters were observed after 24 weeks.

Kuter K, Smiałowska M, Wierońska J, Zieba B, Wardas J, Pietraszek M, Nowak P, Biedka I, Roczniak W, Konieczny J, Wolfarth S & Ossowska K (2007) Toxic influence of subchronic paraquat administration on dopaminergic neurons in rats. *Brain Res.* 25;1155:196-207.

Following on from the neuropathological and neurochemical effects noted in long-term administration of ip injections of paraquat in rats (weekly for 24 wks, Ossowska *et al*, 2005b), this study examined whether shorter periods (5 days) of daily sc injections of paraquat produced similar alterations.

This study measured the levels of DA and its metabolites in the SN, caudate-putamen and PFC, the number of TH-ir neurons of the SN pc, and the binding of [^{3}H]1-[2-(diphenyl-methoxy)-ethyl]-4-(3-phenylpropyl)piperazine ([^{3}H]GBR 12,935) to DAT in the caudate putamen (CP), as a marker of dopaminergic terminals.

Further, in order to examine non-dopaminergic compensatory processes developing in response to paraquat, the influence of paraquat on the levels of 5-HT, its metabolite 5-hydroxyindoleacetic acid (5-HIAA), and noradrenaline, as well as on levels of proenkephalin (PENK) mRNA and glutamic acid decarboxylase 67 (GAD67) mRNA, as markers of γ-aminobutyric acid (GABA)ergic striatal neurons, was also evaluated.

Method:

Male Wistar rats (approximately 250 g) were housed in standard laboratory conditions with food and water available *ad libitum*. Paraquat dichloride (Sigma-Aldrich, Germany) in sterile distilled water was administered sc at a dose of 10 mg/kg (approximately 7.2 mg paraquat ion/kg bw) daily for 5 days. Control animals received saline sc. Two and 3 days following the final injection, rats were either decapitated for biochemical analyses or deeply anaesthetised and perfused for histological analyses.

Brains were rapidly removed and the caudate-putamen (CP), SN and the region of the PFC of the right side of the brain were dissected and frozen for processing. Levels of DA and its metabolites: DOPAC, 3-MT and HVA, 5-HT and its metabolite 5-HIAA, and NA were assessed using HPLC with ECD. The neurotransmitter turnover rate were calculated as a ratio of the metabolite to neurotransmitter.

The left side of the brain was frozen sectioned for autoradiographic binding ([^{3}H]1-[2-(diphenyl-methoxy)-ethyl]-4-(3-phenylpropyl)piperazine ([^{3}H]GBR 12,935) binding to DAT) and *in situ* hybridization evaluations ([^{3}H]GBR 12,935 binding, PENK mRNA and GAD67 mRNA expression in regions of the central caudate-putamen).

Histological analysis was conducted on a subset of rats. The SN was dissected, frozen sectioned and incubated with an anti-TH antibody followed by an avidin-biotin peroxidase complex method. Stained sections were counted stereologically for TH-ir neurons on both sides of the brain. In addition, the total volume of the SN was estimated and the stained sections analysed for density of the TH-ir.

Results:
Number of neurons in the SN pc: Administration of paraquat resulted in a decrease in both the number and density of TH-ir neurons (22% and 24%, respectively) as compared to controls. The volume of the SN was similar to controls. In a previous study (Ossowska *et al*, 2005b) 24-week administration by the ip route resulted in a volume reduced by 24%. The study authors speculated that due to the shorter treatment period, the area of damage may not as yet be detectable. In addition, it was speculated that the region may be undergoing microglial activation, this reaction counteracting any perceivable shrinkage from loss of dopaminergic neurons. However, no methods of measuring this effect were utilized and this theory remains speculative only.

O*ptical density of TH-ir in the SN pc:* No changes in the optical density of TH immunoreactivity were noted in the SN pc. The study authors speculated that despite the loss of dopaminergic neurons, treatment with paraquat may increase the TH-ir in surrounding surviving neurons and therefore the optical density overall appears unaffected. In addition, it was stated that a subpopulation of dopaminergic neurons may be resistant to the toxicity of paraquat as described in McCormack *et al* (2006).

Levels and metabolism of DA in the caudate-putamen, SN and PFC: Following a 2-day withdrawal of paraquat, no significant effects in DA levels were found in any of the regions sampled. In the caudate-putamen, significant decreases (40-59%) were seen for DOPAC, HVA and 3-MT levels, as well as the ratios of DOPAC/DA, HVA/DA and 3-MT/DA. Similarly, in the SN, the DOPAC level was decreased by 19%, HVA by 59% and HVA/DA ratio by 49%. In the PFC, only DOPAC level and the ratios of DOPAC/DA and HVA/DA were reduced (31-42%).

Following a 3-day withdrawal, the level of DA in the caudate-putamen was significantly increased in comparison to both the control level and that measured after the 2-day withdrawal. In this region, DOPAC/DA, HVA/DA and 3-MT/DA ratios and levels of DOPAC, HVA, continued to be significantly reduced, but not 3-MT.

In the SN following a 3-day withdrawal, the previously decreased DOPAC and HVA levels were significantly increased as compared to controls. A trend to an increase in the DA level was also noted in comparison with the 2-day level. In the PFC, previously significant decreases in DOPAC, DOPAC/DA and HVA/DA ratios lost their statistical significance.

Effects on the binding of [^{3}H]GBR 12,935 to DAT in the caudate-putamen:
Following a 2-day withdrawal of paraquat, a reduction of 25-39% in the binding of [^{3}H]GBR 12,935 to DAT was detected in all regions of the caudate-putamen examined. Following a 3-day withdrawal, these levels had increased to be similar to controls in particular areas only (ventral area of the anterior caudate-putamen, and the dorsal and ventral areas of the central region). A statistically significant decrease in this binding persisted only in the dorsal part of

the anterior caudate-putamen in comparison to controls, although the level was slightly increased from the 2-day level.

Level and metabolism of 5-HT and level of noradrenaline in the caudate-putamen, SN and PFC: Following both the 2 and 3-day withdrawals from paraquat treatment, increased 5-HIAA levels and 5-HIAA/5-HT ratio was evident in the caudate-putamen. A similar increased was noted in the PFC following the 2-day period only, although the difference in the ratio was of borderline statistical signficance. There were no effects on the level of 5-HT level in either structure. Levels of 5-HT, 5-HIAA and the ratio of 5-HIAA/5-HT in the SN were not altered after the 2-day withdrawal, however following the 3-day withdrawal, 5-HT was significantly increased as compared to the 2-day levels and the level of 5-HIAA was also increased, although not statistically significantly.

Noradrenalin levels were increased at both time points in the CP only.

Proenkephalin (PENK) and GAD67 mRNAs expression in the caudate-putamen: Following a 3-day withdrawal from paraquat, PENK mRNA levels were significantly decreased (24-35%) in the ventral and dorsal regions of the anterior and central caudate-putamen. In the central caudate-putamen (dorsal and ventral regions) only a small, although statistically significant, decrease (5-7%) in GAD67 mRNA expression was noted.

Conclusion: This study demonstrated that short-term paraquat administration (daily for 5-days sc) was associated with a loss of dopaminergic (TH-ir) neurons in the SN pc (22%). In addition, a decrease in the [^{3}H]GBR 12,935 binding to DAT and reductions in the levels of DA metabolites and DA turnover in the caudate-putamen, SN and PFC, were observed, especially 2 days after withdrawal of paraquat treament. All these results indicate that short-term paraquat induces a toxic effect on dopaminergic neurons when administered by the sc injection route. The study demonstrated the occurance of compensatory mechanisms underway at 3 days after paraquat withdrawal, particularly in the SN. These mechanisms involve dopaminergic, noradrenergic, serotonergic and GABAergic transmissions.

9.4 Proposed mechanisms of paraquat neurodegeneration

Castello PR, Drechsel DA & Patel M (2007) Mitochondria are a major source of paraquat-induced reactive oxygen species production in the brain. *J Biol Chem 282(19):14186-93.*

Paraquat (PQ^{2+}) is a prototypic toxin known to exert injurious effects through oxidative stress and bears a structural similarity to the Parkinson disease toxicant, 1-methyl-4-phenylpyridinium. The cellular sources of PQ^{2+}-induced reactive oxygen species (ROS) production, specifically in neuronal tissue, remained to be identified. This study showed the involvement of brain mitochondria in PQ^{2+}-induced ROS production.

Highly purified rat brain mitochondria were obtained using a Percoll density gradient method. PQ^{2+}-induced hydrogen peroxide (H_2O_2) production was measured by fluorometric and polarographic methods. The production of H_20_2, was evaluated in the presence of inhibitors and modulators of the mitochondrial respiratory chain. The question of what cellular

components of the brain are involved in PQ^{2+} -induced ROS production required the analysis of H_2O_2 production in brain homogenate, cytosolic, and mitochondrial fractions after the addition of PQ^{2+}. Immunoblots for mitochondrial and cytosolic proteins were performed to confirm highly purified mitochondrial fractions void of any contamination from other cellular components. Rates of H_2O_2 production obtained from these traces under the conditions described are summarised in Table 32 below.

All rates were compared with the PQ^{2+}-dependent H_2O_2 production rate in mitochondria in the presence of malate and glutamate, which is expressed as 100%. All values in Table 32 below are expressed as a percentage of this.

Table 32: Rates of PQ^{2+}-induced H_2O_2 production in cellular fractions

Cellular fractions	Respiration substrate			
	Endogenous substrate	Succinate	Malate and pyruvate	Malate and glutamate
Homogenate	0.21 ± 0.02[a]	4.52 ± 0.19[a]	7.99 ± 0.34[a]	5.55 ± 0.17[a]
Cytosol	0.02 ± 0.003	0.06 ± 0.01	0.03 ± 0.01	0.05 ± 0.02
Mitochondria	0.98 ± 0.07[a,b]	81.48 ± 3.53[a,b]	143.83 ± 6.11[a,b]	100.00 ± 3.10[a,b]

[a] Significantly different from cytosolic fraction ($p < 0.05$) grouped by respiration substrate
[b] Significantly different from homogenate fraction ($p < 0.05$) grouped by respiration substrate

In the absence of exogenous substrates, mitochondria generated H_2O_2 at a rate 5 times that of whole brain homogenates, whereas the cytosol generated the least amount of H_2O_2. The addition of substrates feeding the tricarboxylic acid cycle and electron transport chain (ETC) at the level of complex I (malate + glutamate, malate + pyruvate) or at the level of complex II (succinate) did not have any effect on rates of H_2O_2 production from the cytosolic fraction. In the brain homogenate and mitochondrial fractions, the addition of respiration substrates significantly increased production in the range of 20- 40- and 83-145-fold, respectively. Mitochondrial rates of H_2O_2 production with substrates were greatest.

Subsequent experiments in this study showed that mitochondria are a major cellular component involved in ROS generation induced by PQ^{2+} in the rat brain. PQ^{2+} -induced H_2O_2 production by brain mitochondria is dependent on a constant electron flow provided by respiration substrates and a functional ETC. With the use of specific inhibitors of the ETC, complex III has been identified as a novel site of action for PQ^{2+} in the process of redox cycling to generate ROS. Further, the mechanism of induced ROS production is dependent on mitochondrial membrane potential.

The results demonstrated a robust and instantaneous production of H_2O_2 by mitochondria after exposure to PQ^{2+}. It was proposed that mitochondrial H_2O_2 production induced by PQ^{2+} in the brain is an early event that may later initiate other cellular events, such as nonspecific ETC inactivation, microgliosis, and NADPH oxidase activation.

Choi W-S, Kruse SE, Palmiter RD & Xia Z (2008) Mitochondrial complex I inhibition is not required for dopaminergic neuron death induced by rotenone, MPP^+, or paraquat. ***Proc Natl Acad Sci USA. 105(39):15136-15141.***

Inhibition of mitochondrial complex I is one of the leading hypotheses for dopaminergic neuron death associated with Parkinson's disease. These authors tested this hypothesis genetically using a mouse strain lacking functional Ndufs4, a gene encoding a subunit required for complete assembly and function of complex I. Deletion of the Ndufs4 gene abolished complex I activity in midbrain mesencephalic neurons cultured from embryonic day 14 mice, but did not affect the survival of dopaminergic neurons in culture. Although dopaminergic neurons were more sensitive than other neurons in these cultures to cell death induced by rotenone, MPP(+), or paraquat treatments, the absence of complex I activity did not protect the dopaminergic neurons, as would be expected if these compounds act by inhibiting complex 1.

These data suggest that dopaminergic neuron death induced by treatment with rotenone, MPP(+), or paraquat is independent of complex I inhibition.

McCormack AL, Atienza JG, Johnston LC, Andersen JK, Vu S & Di Monte DA (2005) Role of oxidative stress in paraquat-induced dopaminergic cell degeneration. ***Journal of Neurochem.*** **93:1030-1037.**

To elucidate the role of oxidative damage in possible paraquat neurotoxicity, the time-course of neurodegeneration was correlated to changes in 4-hydroxy-2-nonenal (4-HNE), a lipid peroxidation marker. When eight week old male C57BL/6 mice (Charles River, Hollister, CA, USA) were exposed to three weekly intraperitoneal injections of paraquat dichloride (Sigma, St Louis, MO, USA) at 10 mg/kg bw, no nigral dopaminergic cell loss was observed after the first administration, whereas a significant reduction of neurons followed the second exposure.

Changes in the number of nigral 4-HNE-positive neurons suggested a relationship between lipid peroxidation and neuronal death, since a dramatic increase in this number coincided with the onset and development of neurodegeneration after the second toxicant injection. The third paraquat administration did not cause any increase in 4-HNE-immunoreactive cells, nor did it produce any additional dopaminergic cell loss.

Ferritin transgenic mice which are characterized by a decreased susceptibility to oxidative stress, were completely resistant to the increase in 4-HNE-positive neurons and the cell death caused by paraquat. Thus, paraquat exposure yields a model that emphasizes the susceptibility of dopaminergic neurons to oxidative damage.

Richardson JR, Quan Y, Sherer TB, Greenamyre JT & Miller GW (2005) Paraquat neurotoxicity is distinct from that of MPTP and rotenone. ***Toxicological Sciences*** **88(1):193-201.**

Purpose: The purpose of this study was to determine the role of the DAT and Complex I in the toxicity of paraquat.

Background: MPTP and rotenone reproduce features of Parkinson's disease in experimental animals. Paraquat due to its structural similarity to MPTP is also considered to be a candidate

to reproduce some of these features. The exact mechanisms by which these compounds may damage the dopamine system are not firmly established, but selective damage to dopamine neurons and inhibition of complex I are thought to be involved. These authors have previously documented that the toxic metabolite of MPTP, MPP^+, is transported into dopamine neurons through DAT, while rotenone is not transported by DAT. They have also demonstrated that complex I inhibition and oxidative damage results in the dopaminergic neurodegeneration produced by rotenone. Based on structural similarity to MPP^+, it had been proposed that paraquat exerts selective dopaminergic toxicity through transport by the DAT and subsequent inhibition of mitochondrial complex I.

Findings: It was found *in vitro* that paraquat toxicity is independent of DAT expression. Paraquat is neither a substrate nor inhibitor of DAT. In assessing the ability of paraquat, MPP^+, and rotenone to compete with [^{3}H]-Dihydrorotenone for binding to complex I in crude mitochondrial preparations, it was also found that paraquat is a poor inhibitor of ^{3}H DHR Binding (IC_{50} values for rotenone, MPP^+ and paraquat 14 nM, 381 µM and 8.1 mM respectively) suggesting that paraquat does not accumulate in freshly isolated mitochondria as MPP^+ does. Inhibition of [^{3}H]-DHR Binding correlates well with the inhibition of NADH:Quinone Reductase (Complex I) activity and it was further established that paraquat is a poor inhibitor of mitochondrial Complex I activity.

It was also demonstrated that *in vivo* exposure to MPTP and rotenone, but not paraquat, inhibits binding of [^{3}H]-dihydrorotenone to complex I in brain mitochondria. Rotenone and MPP^+ were both effective inhibitors of complex I activity in isolated brain mitochondria, while paraquat exhibited weak inhibitory effects only at millimolar concentrations.

Conclusion: Despite the structural similarities between paraquat and MPP+, paraquat does not demonstrate similar *in vitro* mechanisms (inhibition, binding and transport). The neurological effects of paraquat were not the result of transport by DAT or complex I inhibition. The mechanism by which paraquat specifically targets dopamine neurons was unknown.

Ramachandiran S, Hansen JM, Jones DP, Richardson JR & Miller GW (2007) Divergent mechanisms of paraquat, MPP^+, and rotenone toxicity: Oxidation of thioredoxin and caspase-3 activation. *Toxicol. Sci. 95:163-171.*

Purpose: To further study the molecular mechanism of paraquat toxicity.

Background: Paraquat, MPTP and rotenone have been been investigated for their potential to reproduce Parkinson's disease like symptoms in animal and cell culture models. Molecular mechanisms for any potential effect of paraquat on DA are not well defined. These authors have previously shown that paraquat does not require functional DA transporter and does not inhibit mitochondrial complex I in order to mediate its toxic action (Richardson *et al,* 2005 above).

Findings: In this *in vitro* study, paraquat specifically oxidized the cytosolic form of thioredoxin and activated Jun N-terminal kinase (JNK), followed by caspase-3 activation. Conversely, 1-methyl-4-phenylpyridinium (MPP^+) and rotenone oxidized the mitochondrial form of thioredoxin but did not activate JNK-mitogen-activated protein kinase and caspase-

3. Loading cells with exogenous DA did not exacerbate the toxicity of any of these compounds.

Conclusion: Oxidative modification of cytosolic proteins is critical to paraquat toxicity, while oxidation of mitochondrial proteins is important for MPP^+ and rotenone toxicity. In addition, intracellular DA does not seem to exacerbate the toxicity of these dopaminergic neurotoxicants in this model.

Fei Q, McCormack AL, Di Monte DA & Ethell DW (2008) Paraquat neurotoxicity is mediated by a Bak-dependent mechanism. *Biol Chem* 8;283(6):3357-64.

The authors identified a Bak-dependent cell death mechanism that is required for paraquat-induced neurotoxicity. Paraquat induced morphological and biochemical features in SK-N-SH cell lines were consistent with apoptosis, including dose-dependent cytochrome *c* release, with subsequent caspase-3 and poly(ADP-ribose) polymerase cleavage.

Changes in nuclear morphology and loss of viability were blocked by cycloheximide, caspase inhibitor, and Bcl-2 overexpression. Evaluation of Bcl-2 family members showed that paraquat induced high levels of Bak, Bid, BNip3, and Noxa. Small interfering RNA-mediated knockdown of BNip3, Noxa, and Bak each protected cells from paraquat, but Bax knockdown did not.

The sensitivity of Bak-deficient mice was tested and they were found resistant to paraquat treatments that depleted TH immuno-positive neurons in the SN pc of wild-type mice.

9.5 The toxicokinetics and toxicodynamics of paraquat

Prasad K, Tarasewicz E, Matthew J, Strickland PA, Buckley B, Richardson JR & Richfield EK (2009) Toxicokinetics and toxicodynamics of paraquat accumulation in mouse brain. *J Exp Neurol.*, 215(2):358-367.

Male mice (C57BL/6J (B6J) and other strains (of 8-12 weeks of age) were used in experiments I, II, and IV. Mice up to 40 weeks of age were used in experiment III. Further details of the experiments are summarised in Table 33 below.

Table 33: Summary description of experiments

Experiment No	Route of exposure	Dose**	Total mouse sample size	Dosing frequency	Outcomes*	Paraquat samples
I	ip	10 mg/kg bw	70	2 or 3x/week for 6, 12, 18, or 24 doses	Paraquat, Locomotor activity	Individual
II	ip	10 mg/kg bw	55	3x and then 2x/week	Paraquat, HPLC	Pooled
III	Oral	0.03 mg/mL[1] 0.04 mg/mL[1] 0.05 mg/mL[1]	24	Daily	Paraquat	Pooled
IV	ip	10 mg/kg bw	80	Once	Paraquat	Pooled

* Mice were sacrificed 7 days after the last dose of paraquat for brain paraquat and metabolites estimations using HPLC-EC determinations or Western blot analysis **Dose as paraquat dichloride. 10 mg/kg bw ≈7.2 mg paraquat ion/kg bw. 0.03/0.04/0.05 mg ≈ 0.02/0.03/0.04 mg paraquat ion. [1] The OCS notes that these are the values generally provided in this published study. However, under 'PQ exposure in drinking water' it was stated that the mean concentrations of freshly made PQ were 0.30 mg/mL, 0.42 mg/mL and 0.47 mg/mL. This is assumed to be an error.

In experiment I, analyses were done using balanced samples (n=2-4) from the striatum of mice treated either 2x/week or 3x/week for 6, 12, 18, or 24 doses of 10 mg/kg bw. There were no differences in paraquat levels due to frequency of dosing at any dose number. In the 24 dose group, the values for the 2x/week (1.98 ± 0.99 ng/mg tissue, n=4) and 3x/week (1.83 ± 0.92 ng/mg tissue, n=4) were not significantly different (p = 0.73)

Experiment II demonstrated that the striatal level of paraquat reached a plateau after ≈ 18 doses.

Tissue from experiment II was used to measure the level of paraquat in frontal cortex, hippocampus, and cerebellum after the selected number of doses (18 and 36). Paraquat levels were: after 18 doses: frontal cortex (n=2) 0.91 ng/mg, hippocampus (n=4) 0.79 ng/mg, and cerebellum (n=4) 0.63 ng/mg. After 36 doses the paraquat level in the hippocampus (n=4) was 0.77 ± 0.09 ng/mg.

Experiment III tested if paraquat could safely and effectively be administered orally in drinking water at 0.03, 0.04 or 0.05 mg/mL for 8 or 12 weeks. Paraquat was stable in the drinking water for at least one week. No decline in the concentration of freshly made paraquat was seen after 3, 4 or 7 days in drinking water. Paraquat was measured in the striatum from each of the six groups and suggested that brain levels were related to the concentration of paraquat in the drinking water and the duration of exposure.

Experiment IV determined the half-life of paraquat in striatum following a single ip dose (10 mg/kg bw) to five different inbred strains (n=4 per strain and time point). The strains of mice were C57BL/6J, 129S1/EiJ, A/J, NOD/LtJ and PWK/PhJ. The striatal level of paraquat was measured after 4, 14, 28, and 56 days. The interaction between strain and time was significant (p = 0.0094). Strain NOD/LtJ differed significantly from all other strains (comparing to 129S1/EiJ, p = 0.0071; to A/J p = 0.0020; to C57BL/6J p = 0.0018; and to PWK/PhJ p = 0.0416). No other pair of strains differed significantly from one another. Elimination from brain was linear for each strain (C57BL/6J r^2=0.91, 129S1/EiJ r^2=0.99, A/J r^2=0.95, NOD/LtJ r^2=0.83 and PWK/PhJ r^2=0.90. The elimination half-life in brain was shortest in strain C57BL/6J (≈ 1 month) and longest in NOD/LtJ (≈ 3 months).

Striatal DA and its metabolites from Experiments I and II were measured 7 days after the last paraquat treatments. The ANOVA from Experiment I demonstrated a significant overall effect

of paraquat only on the level of striatal DA (≈ 75% of saline controls) ($p < 0.001$), after 24 doses. The levels of HVA – a metabolite were reduced to similar levels after 24 doses.

Following Experiment II, the ANOVA demonstrated a significant overall effect of paraquat on the level of striatal DA ($p < 0.001$). There were significantly lower levels of DA after paraquat treatments 18, 24, 30, and 36 compared to saline controls (all $p < 0.0001$, 71 to 80% of saline controls). There was also a significant overall effect of paraquat on the level of striatal DOPAC (3,4-Dihydroxyphenyl-acetic acid – a metabolite) ($p < 0.001$). There were significant decreases in DOPAC after the 30th and 36th dose (both $p < 0.0001$). There was also a significant overall effect of paraquat on the level of striatal HVA ($p < 0.001$). Significant decreases in HVA after the 24th, 30th and 36th doses (all $p < 0.05$) were noted. No significant paraquat-related changes occurred with 5-HT or 5-HIAA (5-hydroxyindoleacetic acid).

Conclusion

The toxicokinetics and toxicodynamics of paraquat in the brain of mice reflect the accumulation and plateau level of paraquat in brain after ip and are related to the long half-life. The toxicodynamic consequences are dependent on the paraquat brain level and the duration of its presence in the brain.

9.6 Summary of studies evaluated by route of administration

The data from studies reviewed in this report were grouped and considered here as prelude to determining the relevance of the experimental routes of administration used and the dose levels required to achieve particular brain levels of paraquat associated with neurological sequelae. The brain levels achieved (where reported) following various routes of administration associated with neuropathology are presented below. Overall, poor concordance was observed between dose and the pathology reported.

Oral administration

Table 34 - Oral administration

Reference	Dose administration details	Observations
Oral administration		
Brammer (2006)	Single oral doses of 0, 25, 75 or 250 mg paraquat technical/kg bw to rats (equivalent to 0, 8, 25 or 84 mg paraquat ion/kg bw).	No neurotoxicity or neuropathology noted (brain histopathology not conducted). Brain levels of paraquat not reported.
Fredriksson *et al* (1993)	Single doses of 0.07 or 0.36 mg/kg bw to neonate mice (equivalent to 0.050 and 0.26 mg paraquat ion/kg).	No neuropathology reported but hypoactivity and striatal depression of DA metabolites. Brain levels of paraquat not reported.
Widdowson *et al* (1996b)	5 mg paraquat cation/kg bw/day for 14 days to rats.	The brain (parts not specified): after a single oral dose: 0.05 ± 0.00 nmol/g tissue : after 14 oral doses:

Reference	Dose administration details	Observations
		0.47 ± 0.05 nmol/g tissue (9.6 fold increase); while all other tissue only showed a 2- to 4-fold. No neuropathology or adverse neurochemical changes observed.
Prasad *et al* (2009)	Administered in drinking water to mice at 0.03, 0.04 or 0.05 mg/mL for 8 or 12 weeks (equivalent to 0.02, 0.03 or 0.04 mg paraquat ion/mL).	Brain levels (not quantified) related to concentration of paraquat in water and the duration of exposure.
Chivers (2006)	Dietary administration of 0, 15, 50 or 150 ppm to rats for up to 90 days (males 0, 1.0, 3.4 or 10.2 mg/kg bw/d; females 0, 1.1, 3.9 or 11.9 mg/kg bw/d, respectively, as paraquat ion).	No neuropathology reported. Brain levels of paraquat not reported.
Beck (2013)	Dietary administration of 10 or 50 ppm to mice for up to 90 days (males 0, 1.7, 10.2 mg/kg bw/d; females 0, 2.7, 15.6 mg/kg bw/d, respectively, as paraquat ion)). Concurrent positive control (MPTP, 4x 10 mg/kg bw/dose ip injections, 2 h apart).	No neuropathology reported, including no effect on histology of the brain (specifically, no change to the SN or striatum), the number of dopaminergic neuronal cells (stereology analysis of tyrosine hydroxlase) or the levels of DA or its two metabolites. Positive control group showed clear effects on the brain, validating study methodology. Brain levels of paraquat not reported.
Intranasal administration		
Rojo et al (2007)	Intranasal inoculation: up to 30 mg/kg bw/d daily for 30 days, mice and rats. Positive control MPTP.	No neuropathology observed, including no effect on TH-ir concentration, striatial DA or DOPAC levels. Paraquat detected only in olfactory bulbs, 10 mins following a single intranasal inoculation of 20 mg/kg bw.
Subcutaneous administration		
Naylor et al (1995)	Subcutaneous: single injection of 20 mg/kg bw (14.4 mg paraquat ion/kg bw) in rats.	No neuropathology observed.

Table 35. Tissue concentration of paraquat following single sc injection of 20 mg/kg bw (14.4 mg paraquat ion/kg bw).

Brain region	Paraquat Concentration (nmol/g of tissue)	
	0.5 h	24 h
Anterior olfactory bulb	5.8 ± 0.8	9.1 ± 0.6
Posterior olfactory bulb	2.9 ± 0.5	4.8 ± 0.4
Olfactory tubercle	5.8 ± 0.9	4.0 ± 0.3
Striatum	0.5 ± 0.0	2.2 ± 0.1
Nucleus accumbens	0.7 ± 0.1	2.8 ± 0.1
Parietal cortex (layers I-III)	2.0 ±0.2	1.9 ± 0.1
Parietal cortex (layers IV-VI)	0.9 ± 0.2	2.2 ± 0.1
Thalamus	2.0 ± 0.8	2.2 ± 0.1
Hypothalamus	4.1 ± 1.0	5.1 ± 0.2
Hippocampus	0.9 ± 0.2	2.8 ± 0.2
Lining of lateral ventricles	4.8 ± 1.0	4.6 ± 0.7
SN	1.5 ± 0.4	3.2 ± 0.2
Superior colliculus	1.2 ± 0.1	2.8 ± 0.1
Inferior colliculus	1.1 ± 0.1	2.8 ± 0.1
Pons	4.2 ± 0.9	3.0 ± 0.1
Lining of fourth ventricle	4.3 ± 0.5	4.3 ± 0.2
Medulla oblongata	2.6 ± 0.4	2.8 ± 0.1
Cerebellum	1.2 ± 0.1	1.7 ± 0.0
Area postrema	6.3 ± 2.0	4.9 ± 0.7
Pineal gland	17.0 ± 2.6	8.5 ± 0.3

Corasaniti *et al* (1991)

Subcutaneous: Single injections of 1, 2.5 or 5.0 mg/kg bw (0.72, 1.8 or 3.6 mg/kg bw paraquat cation) in rats.
Brain parts not specified. Units of concentration: ng/g wet weight
Neuropathology not examined.

Table 36. Tissue concentration of paraquat following single sc injection of 1, 2.5 or 5 mg/kg bw (0.72, 1.8 or 3.6 mg/kg bw paraquat cation)

Dose (mg/kg bw)	Age of rat			
	2-week old	3-month old	12-month old	24-month old
1.0	64.0 ± 11.6	39.0 ± 6.6	47.1 ± 5.2	79.8 ± 16.3
2.5	117.6 ± 5.3	82.0 ± 12.1	132.6 ± 18.9	170.6 ± 13.2
5.0	334.0 ± 19.7	185.6 ± 17.5	216.0 ± 22.6	243.2 ± 16.8

Widdowson *et al* (1996a). Subcutaneous: Single injection of 20 mg/kg bw (14.4 mg paraquat ion/kg bw). Units of concentration: nmol/g tissue, Neuropathology not examined.

Table 37. Tissue concentration of paraquat following single sc injection of 20 mg/kg bw

Brain region	Neonates		Adults		Aged	
	0.5 h	24 h	0.5 h	24 h	0.5 h	24 h
Olfactory bulb						
Anterior portion	30.1 ± 3.7	21.5 ± 1.5	5.8 ± 0.8	9.1 ± 0.6	2.9 ± 0.1	5.4 ± 1.0
Posterior portion	19.1 ± 1.9	10.2 ± 0.8	2.9 ± 0.5	4.8 ± 0.4	2.8 ± 0.2	5.5 ± 0.7
Frontal cortex	10.2 ± 0.9	14.3 ± 0.6	1.5 ± 0.2	2.0 ± 0.1	2.8 ± 0.3	6.5 ± 0.7
Striatum	8.4 ± 0.5	16.6 ± 0.5	0.5 ± 0.2	2.2 ± 0.1	2.3 ± 0.2	2.6 ± 0.1
Hypothalamus						
Ventral region	20.6 ± 0.13	35.3 ± 3.0	4.1 ± 1.0	5.1 ± 0.2	5.0 ± 0.5	9.2 ± 0.7
Dorsal region	9.0 ± 0.7	23.3 ± 1.7	1.5 ± 0.6	2.3 ± 0.5	3.0 ± 0.2	4.0 ± 0.2
Ependymal lining of the lateral ventricles	19.2 ± 0.5	46.5 ± 4.1	4.8 ± 1.0	4.6 ± 0.7	4.3 ± 0.5	7.2 ± 1.5
SN	10.7 ± 0.4	16.5 ± 0.8	1.5 ± 0.4	3.2 ± 0.2	2.6 ± 0.2	3.7 ± 0.5
Area postrema	24.9 ± 4.3	45.2 ± 3.4	6.3 ± 2.0	4.9 ± 0.7	5.5 ± 0.8	7.9 ± 1.1
Medulla oblongata	13.9 ± 1.6	42.1 ± 2.6	2.6 ± 0.4	1.7 ± 0.0	2.6 ± 0.1	5.3 ± 0.5

Shimizu *et al* (2001). Subcutaneous: Single doses of 5, 10 or 20 mg/kg bw (equivalent to 3.6, 7.2 and 14.4 mg/kg bw paraquat cation).
Neuropathology not examined.

Table 38. Tissue concentration of paraquat following single sc injection of 5, 10 or 20 mg/kg bw (equivalent to 3.6, 7.2 and 14.4 mg/kg bw paraquat cation)

Treatment	n	Serum concentration (nmol/mL)	Striatal extracellular concentration (nmol/mL)	Ratio
5 mg/kg bw PQ	3	1.42 ± 1.50	0.28 ± 0.06	0.208 ± 0.064
10 mg/kg bw PQ	5	3.88 ± 0.79	0.36 ± 0.09	0.126 ± 0.031
20 mg/kg bw PQ	8	8.04 ± 2.41	0.49 ± 0.09	0.085 ± 0.023**

Kuter *et al* (2007)
Subcutaneous: 10 mg/kg bw/day (7.2 mg paraquat ion/kg bw/d) for 5 consecutive days in rats; necropsied 2or 3 days after cessation of treatment.
At 3 days after withdrawal: 22% decrease in dopaminergic neurons in the SN.
At 2 days after withdrawal: 20-60% decrease in DA turnover and DA metabolites.
At 3 days after withdrawal: level of DA increased, decrease in metabolites reversed.
Brain levels of paraquat not reported.

Intraperitoneal administration

Table 39 - Intraperitoneal administration

Reference	Dose administration details	Observations
Beck (2012a,c)	1, 2 or 3 weekly injections of 0, 10, 15 or 25 mg/kg bw/d (0, 7.2, 10.9, 18.1 mg paraquat ion/kg bw), concurrent positive control (MPTP, 4x 10 mg/kg bw/dose ip injections, 2 h apart).	Mortality and effects on body weight and/or food consumption noted. No neuropathology reported, including no effect on histology of the brain (specifically, no change to the SN or striatum), the number of dopaminergic neuronal cells (stereology analysis of tyrosine hydroxlase) or the levels of DA or its two metabolites. Reduced total contour volume of SN from 15 mg/kg bw/dose, but significance unclear. Positive control group showed clear effects on the brain, validating study methodology. Brain levels of paraquat not reported.
Brooks *et al* (1999)	Single doses of 5 or 10 mg/kg bw to mice (3.6 and 7.2 mg/kg bw paraquat cation, respectively).	Significant loss of dopaminergic neurons: 87% and 94% for each dose respectively. Brain levels of paraquat not reported.
Ossowska *et al* (2005b)	10 mg/kg bw/week (7.2 mg paraquat ion/kg bw/week) for 4, 8, 12 or 24 weeks (rats).	Slowly progressing loss of dopaminergic neurons; delayed deficit of striatal dopaminergic transmission. Brain levels of paraquat not reported.
Prasad *et al* (2009)	10 mg/kg bw for a total of 1, 3 (M-W-F) or 5 (M-W-F-M-W) in mice (7.2 mg paraquat ion/kg bw).	Ventral midbrain paraquat levels: 0.12 ± 0.04, 0.33 ± 0.03, 0.55 ± 0.04 ng/mg tissue per dose respectively at one week after the last dose. No neuropathology reported (toxicokinetic study).

10 HUMAN OCCUPATIONAL EXPOSURE/POISONING CASE INFORMATION

10.1 Occupational Exposure

Studies investigating exposure to paraquat in an occupational setting are included in a separate technical report 'Supplement I: Toxicology'. The studies reviewed do not indicate that paraquat is a neurotoxicant to humans in these exposure scenarios. Included here is an epidemiology study considering the relationship between mortality from Parkinson's disease (and other causes) among workers in a paraquat manufacturing plant.

Tomenson JA & Campbell C (2011) Mortality from Parkinson's disease and other causes among a workforce manufacturing paraquat: a retrospective cohort study. BMJ Open 2011;1:e000283. Doi:10. 1136/bmjopen-2011-000283

(Only the neurological aspects of this study are presented here)

Objective: This retrospective cohort study was conducted to assess the risk of Parkinson's disease among a UK workforce who manufactured paraquat. The study included an extension and an update of the cohort mortality study conducted by Paddle *et al* (1991) which investigated the mortality of employees in plants manufacturing 4,4'-bipyridyl or its subsequent conversion to paraquat or the packaging of paraquat solutions.

Method: The original cohort used in the Paddle *et al* study included all employees who had ever worked in one or more of 4 paraquat production plants in Widnes, UK between 1961 and 1983. The 4 plants used different processes to manufacture paraquat. The study was instigated to investigate skin lesions noted in several workers. Parkinson's was not investigated at this time and the only adverse health effect noted was a moderately increased incidence of lung cancer, further narrowed to the chemical diglyme and determined to be implausible in this exposure scenario. However it was recommended that a follow-up on the cohort be conducted.

This study included the same cohort used by Paddle *et al* (729 males, 32 females), with the addition of a further 217 male and 10 female employees. Twenty males from the original cohort were excluded due to either not being exposed to paraquat or having minimal identifying information. The final numbers were therefore 926 male and 42 female employees. The vital status of these workers were ascertained from the Medical Research Information Service of the National Health Service and of those that had died, the underlying cause of death and other causes of death mentioned on the death certificate were coded according to the International Classification of Diseases (ICD) by the Office of Population Censuses and Surveys.

Exposure to paraquat was ascertained by 1330 static monitoring results collected between 1979 and 1993 (summary information only pre-1987), and 100 personal monitoring results (1983-1993). Paddle *et al* performed a limited qualitative exposure assessment of male workers who would have been expected to have medium to high exposure only(approximately 300 of the 729 males). In addition, it was considered that exposure in the 1960s was likely to have been higher, and workers recruited after the time of the assessment were unlikely to have been exposed to medium or high levels of paraquat.

Following categorisation of deaths from selected causes, the observed number was compared with the expected number calculated on the basis of national and local age and period-specific mortalities. The standardised mortality ratio (SMR) was calculated as the ratio of the observed to the expected deaths, expressed as a percentage. Occupational cohort specific software (OCMAP-PLUS) was used to sum person-years within categories of age (5-year intervals) and calendar period (generally 5-year intervals to conform to changes in the ICD). Due to their small numbers (n=15), females were not included in this calculation, although their cause of death was noted. Mortality data for seven surrounding local districts and for England and Wales were used for comparison, for the time periods 1981-2008 and 1960-1980, respectively. Mortalities for Parkinson's disease were calculated using all certified causes of death listed on the death certificate (termed 'mentions'), as well as rates for Parkinson's as the underlying cause of death. Data on 'mentions' was not available pre-1993 and was conservatively estimated as the number of workers whose death certificate would be expected to mention Parkinson's disease (using mortalities for Parkinson's as an underlying cause of death pre-1993 and a mentioned cause of death from 1993).

Analyses were performed for workers who had worked a minimum of 3 months. Duration of employment and latency were taken into account (as time related variables) with values calculated for each person-year.

Results: Of the total workers included in the cohort, 118 were found to have worked in positions of high exposure to paraquat and 202 had positions with medium exposure. Of the total 926 male workers, 292 had died and of the 42 females, 15 had died. Ten workers had emigrated or joined the armed forces and 9 were lost to follow-up. The average age of males at first exposure to paraquat was 32.8 years, and there was a total of 28 963 person years of follow-up for males.

Static monitoring results (x1073) collected before 1987 had a mean exposure of 0.0120 mg of paraquat (PQ) ion/m^3 (range <0.002-1.005 mg PQ). Mean exposure from 6 personal monitoring results collected in a single month was 0.012 mg PQ ion/m^3 (max 0.4 mg PQ ion/m^3). Results collected in the period 1987-1993 showed a reduced exposure (257 x static monitoring: mean of 0.00328 mg PQ ion/m^3; 94 x personal monitoring: mean of 0.00258 mg PQ ion/m^3).

The standardised mortality ratios (SMRs) showed local mortalities for Parkinson's disease were similar to those for England and Wales (Table 40 below). No deceased females had a mention of Parkinson's on their death certificate. Only a single male worker was identified as Parkinson's disease being the underlying cause of death (1.8 expected) and this death certificate was the only mention of Parkinson's (3.3 expected; SMR=31). This worker was identified as having medium exposure to paraquat (1.1 expected) and no worker with high exposure had Parkinson's disease mentioned on their death certificate (0.5 expected).

Table 40. Observed numbers of deaths and standardised mortality ratios (SMR) for selected causes of death among males (taken from Tomenson & Campbell, 2011)

International Classification of Diseases-9	Cause of death category	Observed	England &Wales mortalities		Local mortalities	
			SMR^	(95% CI)	SMR^	(95% CI)
001-999	All causes of death	292	88*	(78-98)	76**	(68-86)
140-208	Malignant neoplasms	99	99	(81-121)	85	(69-104)
160-165	Respiratory system	30	91	(62-131)	72	(49-103)
162	Bronchus, trachea and lung	29	93	(62-133)	73	(49-105)
320-359	Neurological diseases	1	16*	(0-88)	16*	(0-88)
332.0	Parkinson's disease	1	55	(1-309)	61	(2-340)
332.0	Parkinson's disease (mentioned#)	1	31	(1-171)	32	(1-176)
390-398, 402, 404, 410-429	All heart disease	92	85	(68-104)	74**	(60-91)
430-438	Cerebrovascular disease	22	89	(56-135)	80	(50-121)
460-519	Non-malignant respiratory disease	28	76	(50-109)	59**	(39-85)
800-999	External causes of death	14	100	(55-168)	100	(55-168)

^SMR= standardised mortality ratio. Calculated as the ratio of the observed to expected deaths (%).
* $p<0.05$, ** $p<0.01$; SMR significantly different from 100.
# Mentioned cause of death (1993-2008); underlying cause of death (1960-1992).

Mortality from all neurological diseases (1 death) was lower than the England and Wales SMR (16), and according to the study authors there were no mentions of diseases secondary to Parkinson's disease and other movement disorders, or other neurological diseases on the death certificate of male and female employees. In fact, mortalities from all causes of death were significantly lower than expected, and deaths due specifically to all cancers, heart disease, cerebrovascular disease and non-malignant respiratory disease were lower than expected. This was similar in comparison to local mortalities. An examination of mortality patterns for the 320 workers who had ever held a job with high or medium exposure to paraquat (159 had died by the end of the follow-up period), showed lower mortality across most causes of death, although deaths due to malignant neoplasia was close to expected rates.

Discussion: In this retrospective cohort study, there was no evidence of increased mortality (underlying or mentioned cause) from Parkinson's disease in workers exposed to paraquat during its production. Only a single male worker, determined to have medium exposure levels to paraquat, had Parkinson's disease as an underlying cause of death written on the death certificate. This was lower than the general population. In fact, almost all of the causes of death examined showed a lower than expected incidence. This may be attributed to comparing a healthy population of workers with the general population, all of whom may not be fit for work. Although there was mention of selecting the control population based on age and period, the exact details are not clear.

It was advantageous that there were periods where both static and personal monitoring of paraquat exposure levels was recorded, albeit only up until 1993. The workers in this cohort were found to be exposed to levels below the UK occupational exposure limit of 0.08 mg/m^3 (8 h TWA, respirable fraction), and the authors assumed 10 m^3 of air are breathed during a shift

which equated to around 25.8 µg PQ ion inhaled. The study authors compare this to urine levels found in farm workers who mixed, loaded and sprayed paraquat of 3.0 µg PQ/24 h (Lee *et al*, 2009). Although the values cannot be directly compared, it is feasible that the exposure of a paraquat production worker on a daily basis is at least comparable with that of a paraquat sprayer, in fact, probably higher as sprayers are unlikely to be performing the activity daily. It was not clear from the report how long most workers in the manufacturing plant were employed for, although there is reference to a subcohort with a minimum 3 months exposure however, no data analysis within this subcohort was reported in the study. Therefore it is difficult to ascertain if exposure was of a chronic nature. Static and personal monitoring measurements showed levels of exposure continued to fall with time. Although a full quantitative exposure assessment was not conducted, this is not particularly important considering only one worker died of Parkinson's disease, which was much lower than expected levels.

One of the strengths of this study is due to its retrospective nature, there is no potential for recall bias, however it does rely on worker records and death certificates, which can change in time for accuracy and content for the purpose of this study. The rates for Parkinson's disease mentions on death certificates for example could only be calculated from 1993, and before this time had to use underlying cause of death rates. This resulted in an underestimate of the number of expected mentions of Parkinson's disease. The study authors quoted UK studies which commented on trends in mentions of Parkinson's on death certificates and modified the expected number of mentions from 3.3 to 3.5 (including 0.13 expected deaths before 1985), and suggest the true range is 3.6-3.7- based on approximately twice the number of mentions for the period 1985-2009 are expected for each case of Parkinson's disease as the underlying cause of death.

A few workers were lost to follow-up and not all workers in the cohort were able to be assessed for Parkinson's disease, as only death certificates were used. Another 616 males in the study were still alive at the time of the study. The study authors did not consider a morbidity study worthwhile considering the mortality study did not demonstrate an increased risk of Parkinson's.

The original Paddle *et al* study reported a modest increase in lung cancer deaths, although considered at the time to be unlikely to be occupational related. This follow-up study showed no increase in incidences of lung cancer, especially when compared to local mortalities.

Conclusion: This retrospective cohort study of workers involved in the manufacture of paraquat, showed no evidence of an increased incidence of Parkinson's disease based on mentions of Parkinson's on the death certificate, as compared to control populations.

10.2 Poisoning Incidents

Numerous poisoning incidents are reviewed and presented in Supplement I: Toxicology. The weight-of-evidence from these reports demonstrate accidental or deliberate oral ingestion of paraquat does not cause neurotoxicity, as do reports following dermal exposure.

11 EVALUATION OF SELECTED EPIDEMIOLOGY STUDIES

11.1 Epidemiological studies: Methodology

11.1.1 Case ascertainment

The manner in which cases are selected can influence the outcome of a study. In the study by Kamel *et al* (2007), prevalent cases were defined at enrolment of the cohort as those respondents who replied in the affirmative to the question "Has a doctor ever told you that you have been diagnosed with Parkinson's disease?" This question is clear, direct and clearly defines the group which constituted the prevalent cases. However, to ascertain incidence cases, i.e., newly diagnosed over the course of the study, the question for 'caseness' was broadened to "Has a doctor or other health professional ever told you that you have Parkinson's disease?" and also by self-report. This latter definition allowed more cases to be captured even if the respondents did not have a strict definition of Parkinson's disease. The risk associated with the use of this strategy to increase case capture, was that 'information bias' and 'differential recall bias' may be introduced into the study and lead to a null finding. Case and control subjects were recruited from a very large cohort of licensed private pesticide applicators and spouses who were participating in the US Agricultural Health Study. The number of cases at enrolment was 83, with almost 80,000 control subjects. At study follow-up, there were 78 cases with almost 56,000 controls.

In the study by Costello *et al* (2009), 368 cases from the Central Valley of California were recruited with the assistance of local neurologists in a case-control study. The authors concluded that prior to this study, insufficient (human) data were available to support any claim for specific pesticide to be associated with Parkinson's disease, largely because of challenges in the assessment of pesticide exposure. To address the latter impediment, the authors developed (and validated) an exposure assessment tool which was based on a geographic information systems (GIS) which integrated data from the Californian Pesticide Use reports and land-use maps to estimate the likely historical exposure to agricultural pesticides in residential environments.

11.1.2 Ascertainment of Controls

Characteristics of controls should be same as for cases in all respects but without the factor of interest, in this case, exposure to pesticides, and paraquat in particular. In the study by Kamel *et al* (2007), controls were spouses who are believed to have avoided exposure to pesticides, but have lived in the same area as applicators, and lived with similar lifestyles. Yet the authors also indicate that 56% of spouses had also personally mixed or applied pesticides. Therefore, it is not clear whether these spouses were considered to be controls or cases. In the study by Costello *et al* (2009), controls were aged 65 years and older and were recruited randomly from one of three counties in the study area. A total of 341 control respondents participated in the study.

11.1.3 Exposure Ascertainment

Exposure ascertainment can be problematic in studies attempting to find an association between pesticides and adverse health outcomes. An ideal study would ascertain the exposure to pesticide chemicals using independent sources such as tags or labels worn by workers, or hours and days worked from an employer, as well as dates, since seasonality may influence exposure variations. The study by Kamel *et al* (2007) relied on the self-reporting of exposure to pesticides and thus may have introduced recall bias. In addition, because respondents were selected based on screening using the prior diagnosis of Parkinson's disease, the recall 'capacity' of cases to prior exposures to pesticides may be more likely to be keen or 'primed' and perhaps recall events with undue positive bias.

The respondents were also asked, "As a result of using pesticides, how often have you seen a doctor or been hospitalised?" It is possible that respondents sought medical attention and at that time had no reason to suspect an association with exposure to environmental factors, and yet they were placed in the position of accepting or assuming an association on behaviours that had taken place well in the past. Similarly, the question "Have you ever had an incident or experience while using any type of pesticide which caused you high personal exposure?" allows for the insertion of positive recall bias when cases are more likely to "recall" exposure than non-cases.

In the study by Costello *et al* (2009), cases were also recruited when the authors estimated (from the GIS system) that pesticide exposures also occurred in the residential environment adjacent to agricultural crops to produce estimates of residential ambient or bystander exposures within a set distance of subjects' homes. Residential exposure to pesticides was calculated by the sum of exposures which occurred in a 500 m radius of the home for the relevant years of residence, normalised by the amount of pesticide applied and adjusted for the proportion of the subject's lifetime. This approach was considered to be a more robust method of ascertaining exposure than self-report of events which featured in earlier studies.

11.1.4 Outcome definition

The outcome definition ensures that the cases were assigned by a suitably qualified person, expert, or by an appropriate process. Some earlier studies used death from Parkinson's disease as an outcome, or signs and symptoms similar to Parkinson's disease as an outcome definition. Diagnosis of disease by a physician is considered to be the most robust indicator of this clinical endpoint. Death from the exposure-related disease, e.g., Parkinson's disease, may not be listed as a primary cause of death on the death certificate, and may not be mentioned as a secondary or tertiary contributor to death. This situation has the potential to decrease the strength of any associations drawn following the data analysis. In the study by Kamel *et al* (2007), prevalent cases were determined by a diagnosis from a doctor, and incident cases were based on information from either a doctor or a health professional. Other studies use death from Parkinson's disease as an outcome, or signs and symptoms similar to Parkinson's disease as an outcome. Death from Parkinson's disease may not be listed as a primary cause of death on the death certificate, and may not be mentioned at all even as a secondary or tertiary contributor to death. Similarly, in the study by Costello *et al* (2009), cases were confirmed to have Parkinson's disease by neurologists providing care for these patients. However, the study does not describe how subjects were recruited from sources other than *via* neurologists or health clinics (newspaper and radio announcements), were classified with Parkinson's disease.

11.1.5 Sample size

Sample size can influence study outcomes; when sample size is large, some associations and findings may occur serendipitously – the greater the number of respondents, the greater the chance of finding an association, even if that association were spurious. Conversely, small sample size numbers tend to introduce large variances and thus detract from the statistical significance of the study. As a rule, when the outcome, or disease of interest in a study, is low in prevalence, a large sample size is required. Therefore, it is interesting to note that the study by Kamel *et al* (2007) brought about statistically weak associations between pesticide exposure and a diagnosis of Parkinson's disease. The study by Costello *et al* (2009), with relatively few cases and even fewer controls, yielded robust associations of two and even four-fold risk of disease upon pesticide exposure.

11.1.6 Study type

A cross-sectional study and a case control study will provide information on the strength of an association between a dependent and an independent variable, and logistic regression will yield odds ratios as indicators of the strength of that association. The study by Kamel *et al* (2007) was a case control study. However, when the authors reported the odds ratios were ascertained at the beginning of the study, the authors neglected to report the relative risk when reporting the incident cases, or newly developed cases of Parkinson's disease as they emerged from the cohort or the prospective portion of the study.

The authors of the Costello *et al* (2009) study enrolled "368 incident Parkinson's disease cases". The use of the term "incident" is unfortunate as these cases would more appropriately be labelled as newly-diagnosed Parkinson's disease cases. If they were true incident cases, their diagnosis would have emerged during an observation period of a cohort which is known to have been exposed to a variable of interest. However, this is a case-control study which indicates a relationship and shows the strength of the relationship between a known exposure and the presence of the disease of interest.

Table 41. Comparison of exposure definition for two key epidemiology studies

Study	Kamel *et al* (2007)	Costello *et al* (2009)
Source of data:- Recall,	For the US AHS cohort*, established in 1993-1997, follow-up 1999-2003.	Recruited disease affected; recorded residence (1974-1999); estimated exposure; and matched to land-use data for both cases and controls; 88% respondents mapped with precision.
Pesticide use:- reporting.	Self reporting; apparent that the only data for exposure was question 10 which asked – "During your lifetime, have you ever personally mixed or applied any pesticide?" Information on events involving high personal pesticide exposure was collected by using the question: "Have you ever had an incident or experience while using any type of pesticide which caused you unusually high personal exposure?"	Pesticide Use Reporting data from California Department of Pesticide Regulation: name, poundage, crop, acreage, application method, date of application.
Duration data:- useful when duration of exposure affects disease.	Question 10b "How many days per year did respondent personally mix or apply pesticides?" Question 10c "What percent of time did respondent personally mix pesticides when mixing was required?" Question 10d "What percent of the application did respondent personally do?" Data on duration were not reported in the paper.	Between 1974 and 1999. Data able to be partitioned into exposure windows of 1974-1989 and 1990-1999. Duration of exposure adjusted for the period the participants were in residence in the test area and matched with pesticide application events.
Proximity or Distance:- to/from site.	Not addressed.	The time-specific total exposure at each location by pesticide, was derived through summation of exposures over a fixed 500m radius around the home for the relevant years of residence. Pesticide application rates were averaged over specific time periods and adjusted by application rates of pesticide applied pa.
Proximity and duration	Not reported in study.	As above. Data geo-coded using a geographic information system.
Wind direction	Not addressed.	Not addressed.
More than one source	Perhaps not applicable, unless someone lived on the edge of the area and someone lived in the middle of a few areas being sprayed.	As per Kamel *et al* (2007).

US AHS = United States Agriculture Health Survey; * persons applying for certification to use restricted-use pesticides in Iowa or North Carolina were enrolled in the study.

Table 42. Comparison of disease definition for two key epidemiology studies

Study	Kamel *et al* (2007)	Costello *et al* (2009)
Disease measured consistently:-	Prevalent Parkinson's disease was ascertained by: "Has a doctor ever told you that you have Parkinson's disease?" Note: report indicates that >99% of applicators* and 56% of spouses had personally mixed or applied pesticides. Therefore more than half of the control cohort had been occupationally exposed to pesticides.	Yes – all cases were receiving care from neurologists.
Did the researchers, health assessors, respondents know of the purpose of the study association?	At enrolment, the study was explained to potential participants. Consent and willingness to participate in the study was indicated by the return of questionnaires. At enrolment, cohort members were asked: "Has a doctor ever told you that you have been diagnosed with Parkinson's disease?" Information on pesticide-related medical attention was collected from applicators by the question: "As a result of using pesticides, how often have you seen a doctor or been hospitalised?"	Doctors/neurologists who were providing care were involved in the nomination/recruitment of cases to the study from 1998 to 2007. Collaboration was also solicited from Health Management Organisations, medical clinics and the VA, disease support groups, local newspapers and radio stations. It cannot be excluded that some respondents were aware of the hypothesis of the study and therefore more motivated to participate. This may have introduced positive bias.
Data sources differ between purpose collected, surveillance, registers. Blind to H_o ?	The US AHS collected the data "to investigate environmental factors, occupation and diet and their effects on an individual's health" (Enrollment Questionnaire, see : www.aghealth.nci.nih.gov/qnaires/enrol_pr.html).	Used California Pesticide Use Reporting (PUR) Data.
Health measurement free of bias, e.g. enzyme sand chromosomes	Some self report, for incident cases.	Health professional diagnosis.
Source of data:	Questionnaire/patient recall.	Medical specialist referral to the study, patient recall

US AHS = United States Agriculture Health Survey; * persons applying for certification to use restricted-use pesticides in Iowa or North Carolina were enrolled in the study; VA = Veterans Associations

11.2 Epidemiology studies: Results

11.2.1 Demographic Variables and Lifestyle

The study abstract of Kamel *et al* (2007) stated that incident Parkinson's disease (newly diagnosed cases) was associated with cumulative days of pesticide use at enrolment for the highest quartile versus the lowest quartile , with an OR of 2.3 (95% CI=1.2,4.5). The abstract also claimed that incident disease was associated with personally applying pesticides more than half the time (OR=1.9; 95% CI=0.7, 4.7) and some specific pesticides (OR ≥1.4) for pendimethalin, but the 95% CI=0.8 and 2.6, indicate that the relationship between pesticide use and Parkinson's disease was not statistically significant. Prevalent Parkinson's disease was not found to be associated with overall pesticide use.

The study by Kamel *et al* (2007) suggested that both prevalent and incident cases were associated with older age at enrolment with the relationship being stronger for prevalent cases. Incident disease was reported to be associated with greater age at follow-up. For both prevalent and incident cases, the risk of having a diagnosis of Parkinson's disease was lower in North Carolina, lower for spouses and higher for non-Caucasians. However, the results were not statistically significant. Enrolment data indicated that prevalent disease was inversely related to both former and current cigarette smoking, but incident disease showed no association with former or current smoking. The Kamel *et al* (2007) study indicated that having more than a high school education conferred a lower risk of disease among prevalent cases, however, the manner in which education conferred the effect, was not explored.

The study by Costello *et al* (2009) found a positive association between being likely to be exposed to pesticides through occupational use and Parkinson's disease (OR=1.52, CI=1.08, 2.14) and that an education of twelve years or more conferred a protective effect on the individual (OR=0.54, CI=0.37, 0.77). There was also an inverse relationship between Parkinson's disease and being a former smoker (OR=0.70, CI=0.52, 0.96) and being a current smoker (OR=0.48, CI=0.27, 0.86). There was also an inverse relationship for Parkinson's disease and subjects who smoked more heavily. These latter findings are intriguing as most studies indicate that smoking acerbates health status but in this study smoking had an inverse relationship.

The analysis also found that there was a positive relationship between Parkinson's disease and exposure to both paraquat and maneb from the earlier years of observation, 1974-1989, and 1974-1999, but not for later years of 1990-1999. The basis for this cannot be discerned from the data available. However, upon speculation if the same 'protective effect' of education which was observed by Kamel *et al*, (2007) was also present in the cohort surveyed by Costello *et al*, (2009), then later users of pesticides may have been more cognisant of the risks of paraquat toxicity and were more attentive to the requirements of protective clothing or followed improved OH&S precautions. There are no data available to indicate whether this trend continues to the present, from which it might be considered that occupational exposures to paraquat which occurred in 2000-2010 might lead to an even lower association with Parkinson's disease when analysed using the same definitions.

11.2.2 Symptoms of Parkinson's Disease

The study by Kamel *et al* (2007) indicated that at enrolment, all listed symptoms of Parkinson's disease were statistically associated with prevalent cases, but only shaking or trembling of hands was statistically significant in its association with incident cases. This may be related to the definition of being diagnosed with Parkinson's disease as at enrolment, prevalent cases were required to report a diagnosis from a doctor, but incident cases with unknown status at enrolment, could respond that they had been told they had Parkinson's disease by a doctor or a health professional, and thus possibly introducing error into the definition of Parkinson's disease and 'caseness'. In the Costello (2009) study, cases were recruited from practicing local neurologists who were providing care for the respondents, as well as from local clinics, newspapers and radio stations. Assessment of disease status of the latter recruits, especially those recruited through newspapers or radio stations, was not clear although the number of participants recruited by these pathways was not large.

11.2.3 All possible exposure to pesticides

In the study by Kamel *et al* (2007), it was indicated that when all the possible exposures to pesticides were considered, only the association between incident Parkinson's disease and a cumulative lifetime use of pesticides of 397 days or more was statistical significance.

The study by Costello *et al* (2009) estimated exposure to pesticides in the residential environment from applications to agricultural crops using a GIS-based system which combined pesticide-use data and land use data. The time-specific total exposure at each location, by pesticide, was derived by summing exposure over a fixed 500m radius around the home for the relevant years of residence. The application rate of pesticide per annum was summed for each residential buffer and weighted by the proportion of treated acreage in each buffer and weighted by the proportion of treated acreage in each buffer. The authors believed that this resulted in pesticide application rates that could be averaged over specific calendar periods of each subject's lifetime.

11.2.4 Exposure to specific chemicals

In the study by Kamel *et al* (2007), the odds ratios were reported to be elevated for prevalent Parkinson's disease cases for the herbicides pendimethalin, paraquat, and cyanazine, and the fumigants carbon disulphide/carbon tetrachloride and ethylene dibromide. Odds ratios for incident Parkinson's disease associated with exposure to dicamba, trifuralin, 2,4,5-trichlorophenoxyacetic acid, butylate, lindane, phorate, chlorothanolil, benomyl and methyl bromide were not statistically significant. The only statistically significant association found was for cyanazine.

Costello *et al* (2009) used a GIS model which allowed the estimation of the likely dosage of active ingredients. The authors also acknowledged that these quantities were not comparable across all pesticides and exposure could not be estimated with any degree of accuracy for individual pesticides. The study also lacked statistical power to perform extensive analysis when the sample cohort was comprised of only three cases exposed to maneb alone with one control case.

11.3 Epidemiology studies: Discussion

Understanding the association between environmental hazards and disease is important for preventive measures. In terms of epidemiological analysis, the first task is to determine whether there is a difference in health status between people with different exposures to environmental hazards. Most disease measurements are subject to some level of observer variation in diagnosis, assessment or reporting. The important question is whether disease is being measured consistently in exposed and unexposed people, or whether for any reason exposure status may affect the likelihood that people of equal health status being diagnosed as having the disease or not.

The authors Kamel *et al* (2007) relied on self-reporting by respondents in their questionnaire. The authors used only question 10 which asked "During your lifetime, have you ever personally mixed or applied any pesticide?" allowing for the possibility of recall bias. The authors also reported that more than 99% of applicators and 56% of spouses had personally mixed or applied pesticides. Therefore, it may be that more than half of the control cohort had been occupationally exposed to pesticides if these spouses were enrolled as controls. The paper does not clarify this point. In contrast, Costello *et al* (2009) estimated exposure of cases and controls using Pesticide Use reporting (PUR) data from the California Department of Pesticide regulation, matching land use data for both cases and control, and noting the name of the pesticide, crop, application rates, application method and date of application.

Additional bias in the assessment of the results may be attributable if, as appears to be the case, either the researcher, health assessor or the respondent, knew that a disease was being investigated and may be related to a certain exposure at the time when the health assessment was made. For example, the authors of a study may send a questionnaire to all members of a community to study the impact of an environmental hazard on health. If the questionnaire concerned health assessments on the more "subjective" end of the spectrum, for example, a headache and feelings of nausea, people who perceive themselves to have been exposed, may be more likely to report those symptoms. Further, people who are concerned by the possible effects of the exposure on their health may be more likely to return the questionnaire or phone call, compared to other potential participants, particularly if the respondent felt that their health had been impaired (Dolk, 2002).

Information on events involving high personal pesticide exposure was collected in the Kamel *et al* (2007) study by posing the question: "Have you ever had an incident or experience while using any type of pesticide which caused you unusually high personal exposure?" A question like this is likely to indicate to the respondent that their career activities may have medical consequences, and this would alert them to recall more acutely any exposure to pesticides and introduce another source of bias into the study. Incident cases were asked "has a doctor or a health professional told you (that) you have Parkinson's Disease?", where a 'health professional' was not defined or explained. However, it is assumed that the authors did not accept a diagnosis from an unrelated medial specialist.

Health conditions such as cancer and congenital anomalies are generally considered to be in the less subjective part of the spectrum. However, the manner in which information is gathered is still critic*al* For example, in a survey a toxic hazard and ascertaining cases of cancer or congenital anomalies in the population, if the authors compared the rate of disease to a disease

rate in data which was routinely collected in health statistic data bases, they may find a different rate in the community close to the toxic hazard due to differences in the completeness of case finding, or inclusion or exclusion criteria. Therefore, it is important that studies use the same data base or data source to measure disease in both exposed and unexposed populations. Studies based on disease registers, where cases are ascertained blind to the study hypothesis, have an advantage in this regard. The study by Kamel *et al* (2007) used data from the US AHS study, where exposure was self-reported, and thus was open to bias whereas Costello *et al* (2009) used data from the Pesticide Use Reporting data from the California Department of Pesticide Regulation.

Studies which measure exposure to a toxin or environmental hazard, in terms of a residence within a large area which contains the exposure factor, are in a weaker position to express an association between hazard and disease (Dolk, 2002). Such studies are improved when duration of exposure can be measured. Longer duration of exposure is generally considered to increase the risk of disease and thus respondents with the disease can be classified as to duration of exposure to determine if or to what extent duration of exposure may have affected disease outcome. These aspects were key elements in the Costello *et al* (2009) paper, where time-specific total exposure at each location and by pesticide, were derived through summing the exposures over a fixed 500 m radius around the home for the years of residence. Pesticide application rates were averaged over specific time periods and adjusted by application rates of pesticide applied per annum. In contrast, Kamel *et al* (2007) used questions about duration spent mixing or applying pesticides such as: "What percent of time did respondent personally mix pesticides when mixing was required?" and "What percent of the application did the respondent personally do?" The results of those questions on duration were not reported in the paper.

Studies based on distance of residence to a potential chemical hazard are often less expensive to conduct for a given sample size, as they can often use routinely collected data. When using only the distance to residence parameter, there are many causes of misclassification of relative degree of true exposure. These include the fact that exposure is not likely to radiate evenly in all directions from the hazard source, that people vary in their lifestyles and resulting probability of exposure (e.g., Do they stay home or work elsewhere? Do they engage in recreational activities near or far from the hazard source? Do they garden? In addition, people often move house between exposure events and disease diagnosis, and thus some of those classified as "exposed" may not have lived in the area at the relevant time and *vice versa*. The methodology of Costello *et al* (2009) measured exposure within 500 m of the respondent's residence and appear to take these issues into account, however, did not justify why the distance of 500 m was selected (compared to 750 m or 1 km) or how it may have affected the associations which were drawn from the data.

Predominant wind direction is a commonly quoted objection to studies using distance of residence as a surrogate of exposure. It is reasonable to anticipate that those who reside in the path of prevailing winds from agricultural areas would be exposed to more pesticides than those who reside further from the hazard source and that the "circle of exposure" should be replaced by a more elliptical shape or directional ellipse. Another problem in exposure classification is the situation of people living near more than one chemical source, e.g., living by farmlands would likely confer greater exposure compared to residing on the boundary of the treated areas. To be able to incorporate these issues when analysing retrospective or prospective survey data,

more exposure assessment research is needed. The recent establishment of the APVMA Operating Principles in Relation to Spray Drift Risk policy (July 2008) will lead to a greater understanding of the exposure to bystanders from pesticide spraying operations and therefore, in future epidemiological surveys, provide improved robustness of exposure (further details can be found at www.apvma.gov.au).

Misclassification or poor classification of exposure is often cited as a reason to disregard the results of studies which find evidence of an excess of disease near a toxic site. However, misclassification of exposure has been found to generally lead to an underestimate of exposure. Therefore, if an association has been made then it is likely to be attributable to a higher exposure than when originally classified. Exposure to the hazardous substance was well-defined in the study by Costello *et al* (2009) which may have contributed to the more robust associations between pesticide use and a diagnosis of Parkinson's disease. Measures of exposure in the paper by Kamel *et al* (2007) were less stringent and may have contributed to the findings of weaker associations.

The two studies under discussion show not only different results regarding the association between pesticide use and a diagnosis of Parkinson's disease, but also the use of different methodologies. A similar association was also reported by Ascherio *et al* (2007) between chemical exposure and the presence of Parkinson's disease. To more fully appreciate these associations, a literature review, or full desktop examination of these, and other studies on paraquat/pesticides and Parkinson's disease, could be conducted to outline the differences in case ascertainment, exposure methodology and outcome ascertainment and then examine the strengths of association under different circumstances. Such a study may identify the conditions under which the most robust findings are reported and could indicate if the trend (in association) is reproducible and if the trend is moving. While individual studies might show different results, in an overarching analysis, it is the preponderance of evidence which should guide decision-making in questions of health. The above findings, particularly the study by Costello *et al* (2009), lend weight to the suggestion that there is a positive association between adverse health effects and exposure to pesticides (i.e., paraquat and maneb), but presently, the strength of that association does not extend to exposure to paraquat alone.

An interesting trend which may be developing was the observation by Costello *et al* (2009) that recent occupational exposure (1990-1999) to paraquat and maneb had a lower association with prevalent Parkinson's disease cases when compared to occupational exposure from 1974-1989. While data are not available for the next decade (2000-2009), if this trend continues to decline in coming years through decreased exposure (and can be validated by appropriate epidemiology analysis), then the relatively higher risk of developing Parkinson's disease from exposures during the earlier period (i.e., 1974-1989) could be interpreted as being consistent with paraquat having the theoretical capacity of a contributory role in this disease in some individuals. However, it is unknown why those individuals were apparently susceptible to paraquat at the time of exposure and whether or not the actions of paraquat were to accelerate existing subclinical disease or initiated new disease.

Clinically, Parkinson's disease is characterised by a variety of clinical signs and symptoms which are usually progressive in nature with initial presentation as indeterminant or idiopathic disease before full diagnosis. The observation that the association in 1990-1999 was weaker, may align with decreased exposure through greater regulatory controls in the USA. A

theoretical possibility may exist that the current exposure (i.e., in 2010) to paraquat in the Central Valley of California, as surveyed by Costello *et al* (2009), may be insufficient to affect existing subclinical Parkinsonian-like disease or initiate neuronal injury in those susceptible individuals from the potential incident Parkinson's disease cohort.

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APPENDIX I ADDITIONAL STUDY DETAIL FROM PUBLISHED PAPERS DISCUSSED IN THIS SUPPLEMENT

Bagetta G, Corasaniti MT, Iannone M, Nistico G & Stephenson JD (1992) Production of limbic motor seizures and brain damage by systemic and intracerebral injections of paraquat in rats. *Pharmacol Toxicol* 71: 443-448.

Experiments were performed to investigate the behavioural and neuropathological effects of intrahippocampal and systemic injections of paraquat in rats. The effect of the muscarinic acetylcholine receptor antagonists, atropine and methylatropine, and the N-methyl-D-aspartate (NMDA) receptor antagonist, MK 801, on paraquat-induced neurotoxicity was also determined.

Twenty-five gauge stainless steel guide cannulae were implanted into the regio superior of the dorsal hippocampus of one hemisphere of adult male Wistar rats (280-300 g body weight; unspecified source) under chloral hydrate anaesthesia. Rats were allowed to recover for one week prior to compound administration. The study authors reported that implanted animals did not exhibit any overt alterations of posture or motor activity. Prior to treatment, rats were placed individually into transparent perspex cages and allowed to acclimatise for 10 minutes. Paraquat (unspecified purity, batch no. and source) in phosphate-buffered saline (pH 7.0) was administered as a single injection into the hippocampus using a 10 μL Hamilton syringe at 0 (n=6), 10 nmol (n=6), 100 nmol (n=12) or 1 μmol (n=12) (equivalent to 0, 1.86, 18.6 and 186.3 μg paraquat, respectively and equivalent to 0, 1.34, 13.4 and 134.1 μg paraquat cation, respectively) in a total volume of 1 μL.

Additional groups of rats (n=6) were similarly treated with 1 μmol paraquat (equivalent to 186.3 μg paraquat or 134.1 μg paraquat cation) and 5 or 50 nmol of the muscarinic acetylcholine receptor antagonist, atropine (at the same time as paraquat), or 0.3 mg/kg of the N-methyl-D-aspartate (NMDA) receptor antagonist, MK 801 (60 min before paraquat). Paraquat was also administered systemically to 5 rats/group by an ip injection at 5 (n=6), 20 (n=5) and 100 (n=10) mg/kg bw (equivalent to 3.6, 14.4 and 72 mg/kg bw paraquat cation, respectively) in an unspecified vehicle. Two additional groups were pre-treated with an ip injection of 150 mg/kg bw atropine (n=6) or 5 mg/kg bw methylatropine (n=5) 30 minutes prior to an ip injection of 100 mg/kg bw paraquat (equivalent to 72 mg/kg bw paraquat cation). All rats were observed continuously for 4 h following treatment. Postural and locomotor changes, and convulsive behaviours were recorded by 2 independent observers who were unaware of the treatment that the animals had received. Twenty-four hours after paraquat administration, all surviving animals were anaesthetised with chloral hydrate, their brains removed and histopathologically examined. Paraquat concentrations in the medulla oblongata, hippocampus and pyriform cortex were measured by HPLC according to the method of Corasaniti *et al* (1990).

There was a clear dose-related increase in the number of rats with seizures and the number of rats with multifocal brain damage following intrahippocampal injection of paraquat (see Table 43 below). No treatment-related effects were observed at 10 nmol. At 100 nmol, ataxia was observed 10-15 min after injection in all animals, followed by a 50-70 min period of behavioural excitation. Stereotyped jaw movements, clonus of the upper extremities, rapid ear

movements, vibrissae, and 2-4 seizures lasting up to 40 seconds each, were reported in 5/12 rats. At 1 μmol, more pronounced motor stimulation was reported with 8-13 seizures observed in all 12 rats, and one rat dying within 24 h. Brain damage occurred at and above 100 nmol, with neuronal damage or degeneration observed in the CA1 pyramidal cell layer of the hippocampus and in more distant ipsilateral structures (eg dentate granule cell layer, hilus fascia dentata, and pyriform cortex). The frequency of multifocal brain damage was less than the frequency of seizures at equivalent doses.

Administration of 50 nmol atropine with 1 μmol paraquat prevented seizures and multifocal brain damage, however the study authors reported sniffing, wet dog shakes and head nodding in 3/6 rats after 15 min. A 10-fold lower concentration of atropine did not protect against paraquat-induced seizures or brain damage. Pre-treatment with 0.3 mg/kg MK 801 also prevented seizures and brain damage. These observations suggested that the neurotoxicity of paraquat might involve muscarinic and NMDA receptors.

Table 43. Effect of intrahippocampal injection of paraquat on the incidence of seizures and brain damage in Wistar rats

Treatment	Amount	No. of rats with seizures	No. of rats with multifocal brain damage
Vehicle control	1 μL	0/6 (0%)	0/6 (0%)
Paraquat	10 nmol 100nmol 1 μmol	0/6 (0%) 5/12 (42%) 12/12 (100%)	0/6 (0%) 3/12 (25%) 6/11 (55%)
Paraquat + Atropine	1 μmol 5 nmol	6/6 (100%)	3/6 (50%)
Paraquat + Atropine	1 μmol 50 nmol	0/6 (0%)	0/6 (0%)
Paraquat + MK 801	1 μmol 0.3 mg/kg, ip	0/6 (0%)	0/6 (0%)

There was a clear dose-related increase in the number of rats with seizures and the number of rats with brain damage following ip injections of paraquat (see Table 44 below). There were no treatment-related effects at 5 mg/kg bw. At 20 mg/kg bw, paraquat caused seizures and brain damage in 2/5 rats. The study authors reported muscle fasciculation, chromodacryorrhea and salivation in 5/5 rats 20-25 min after administration, with these effects persisting for approximately 30 minutes. Tremors, infrequent wet dog shakes, movement of the vibrissae, clonus of the forelimbs and rearing were also reported in 2/5 rats. At 100 mg/kg bw, 8/10 rats had seizures and 7/10 exhibited multifocal brain damage. Behavioural abnormalities (tremors, wet dog shakes, movement of the vibrissae, clonus of the forelimbs, rearing) were observed 15-20 min after dosing and these persisted for the duration of the 4 hour observation period. It was reported that neuronal death was observed in the pyriform cortex at and above 20 mg/kg bw.

Table 44. Effect of an ip injection of paraquat on the incidence of seizures and brain damage

Treatment	Dose (mg/kg bw)	No. of rats with seizures	No. of rats with multifocal brain damage
Vehicle control	1 mL/kg bw	0/6 (0%)	0/6
Paraquat	5 20 100	0/6 (0%) 2/5 (40%) 8/10 (80%)	0/6 2/5 (40%) 7/10 (70%)
Paraquat + Atropine	100 150	0/6 (0%)	2/6 (33%)
Paraquat + Methylatropine	100 5	2/5 (40%)	3/5 (60%)

Pre-treatment of rats with an ip injection of atropine (150 mg/kg bw) prevented seizures, and reduced the incidence of brain damage by 37% (see Table 44 above) suggesting the involvement of muscarinic receptors during paraquat neurotoxicity. Photomicrographs illustrated that paraquat-induced neuronal cell damage in the pyriform cortex was prevented by atropine. Methylatropine failed to protect against paraquat-induced seizures and only slightly (~20%) reduced the incidence of brain damage.

A dose-related increase in the concentration of paraquat in the medulla oblongata, hippocampus and pyriform cortex was observed 24 h after a single ip injection. Concentrations of paraquat were similar in each brain region (see Table 45 below).

Table 45. Paraquat concentrations in different brain regions 24 h after an ip injection

Treatment (mg/kg bw)	Medulla oblongata	Hippocampus	Pyriform cortex
5 (n=3)	0.14 ± 0.02	0.11 ± 0.03	0.19 ± 0.06
20 (n=5)	0.56 ± 0.07	0.61 ± 0.13	0.82 ± 0.13
100 (n=5)	2.80 ± 0.18	2.04 ± 0.22	3.90 ± 0.26

Results expressed as mean μg/g wet weight ± 1 SEM

In summary, no neurotoxicity was observed following a single microinjection of paraquat into the hippocampus of rats at 10 nmol (equivalent to 1.86 μg paraquat or 1.34 μg paraquat cation). Seizures, behavioural excitation and the presence of multifocal brain damage were noted at and above 100 nmol (equivalent to 18.6 μg paraquat or 13.4 μg paraquat cation). Atropine (50 nmol) and MK 801 (0.3 mg/kg, ip) prevented the occurrence of these treatment-related effects implicating the involvement of muscarinic and NMDA receptors. Following a single ip injection of paraquat at 5 mg/kg bw (equivalent to 3.6 mg/kg bw paraquat cation), no effects were observed, while seizures and brain damage were noted at and above 20 mg/kg bw (equivalent to 14.4 mg/kg bw paraquat cation). Pre-treatment with atropine but not methylatropine prevented these effects.

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

Cicchetti *et al* (2005) investigated whether paraquat alone and paraquat in combination with maneb is toxic to dopaminergic neurons of eight week old male Sprague Dawley rats both *in vitro* and *in vivo* and if the combination leads to Parkinson-like movement deficits.

The rats were injected ip twice a week for four weeks with 10 mg/kg bw paraquat dichloride hydrate or a combination of paraquat and maneb at 10 mg/kg bw and 30 mg/kg bw respectively. However evidence for the induction of Parkinson-like movement deficits by paraquat was limited. Evidence for microglial activation was also found, possibly implicating neuroinflammation in the pathogenic mechanism associated with degeneration of TH-immunoreactive neurons. This study compared the effects of systemic paraquat or a combination of paraquat/maneb administration *in vitro* and *in vivo* in young adult rats and its relationship to inflammation by correlating this phenomenon to degenerative effects on the dopaminergic system.

Method:

In vitro analysis

Primary-ventral-mesencephalic neuron-glia cultures:

Primary ventral mesencephalic (VM) neuron-glia cultures were prepared by dissecting the VM from embryonic day 14 (E14) Wistar rats (Charles River, Wilmington, MA) followed by enzymatic digestion in hypsin and DNase in dissection medium (Ca^{2+}J Mg^{2+}-free Hanks solution with 10 mM HEPES and 0.6% glucose) at 37°C for 30 minutes. Cultures were treated with daily doses of saline (negative control), paraquat, maneb or paraquat/maneb for 7 days before fixation and staining.

Immunocytochemistry:

Cultures were fixed using a solution of 4% paraformaldehyde and 4% sucrose for 15 minutes at room temperature. After appropriate treatment and staining techniques, images were captured using a Leica DMIRE inverted microscope attached to a Retiga Exi monochrome camera and SimplePCl software and color-edited using Adobe Photoshop 7.0.

Cell number quantification:

Following immunostaining, every immunoreactive cell was counted in each well. These analyses were performed blindly by two independent investigators and processed for one-way ANOVA using the StatView program.

In vivo analysis

Animals and paraquat administration: Eight-week old young adult male Sprague-Dawley rats (Charles River Laboratories, Montreal, QC) were housed separately in a controlled environment with free access to food and water. Rats were injected ip twice a week (Tuesday-Friday) at the same time in the morning (9:00 am) for 4 weeks with either saline, paraquat dichloride hydrate (Sigma, St Louis MO; 10 mg/kg bw), or a combination of paraquat and maneb (10 mg/kg bw paraquat and 30 mg/kg bw maneb in separate injections. In total, 20 young adult rats paraquat (paraquat n = 8; paraquat/maneb n = 8) and vehicle (n = 4) were used in this study, monitored daily for weight variation, and ultimately sacrificed 24 hours following the last injection.

Behavioral analysis: Behavioral assessment comprised postural deficit, decrease in speed of overall movement and immobility. Each test session began by placing the animals in a new cage for a 15 min period of habituation. All animals were tested at eight different time points (days 2, 6, 9, 13, 16, 20, 23, and 27). The motor impairments were monitored 24 h after the ip injections in a 50 min session. All behavioral measures were performed blind by three independent investigators according to an impairment scale ranging from scores of 0 to 3.

Evaluations were carried out for 2 min every 6 min for 8 trials for a maximal disability score of 24.

Posture testing consisted of determining the degree of hunch back position (0: Normal; 1: Intermittent hunched back; 2: Permanent hunched back; 3: Hunched back-balance problems).

Speed testing consisted of determining at what speed animals conducted daily grooming, moving, and exploring in the cage (0: Normal; 1: Slow; 2: Very slow; 3: Very slow with freezing).

Mobility testing consisted of placing the animal on a metallic trellis of 45° angle to predominantly assess the degree of akinesia or bradykinesia (0: Normal; 1: Mild reduction; 2: Moderate reduction; 3: Severe reduction).

Post-mortem analysis: Animals were sacrificed via intracardiac infusion after which brains were collected, fixed and transferred for cryoprotection. Coronal brain sections of 35 pm thickness were cut on a freezing microtome. After overnight incubation with one of the primary antibodies, sections were washed and incubated. The bound antibodies were prepared for visualization. In some sections primary antibody was omitted from the incubation medium. These sections remained free of immunostaining and served as negative controls. Peripheral organ (liver, kidney, and lungs) abnormalities were assessed by hematoxylin and eosin (H&E) staining analysis.

Results

Effects of paraquat on VM DA cell cultures: E14 VM cultures were established containing both neurons and glial cells and treated with paraquat, maneb or paraquat/maneb for *7* days. Paraquat exposure resulted in a selective loss of TH-ir neurons in a dose-dependent manner *(p = 0.0001)*, with an ED_{50} of 1.0 µM, reflecting an increased sensitivity to paraquat to the non-TH-ir neuronal population (neuron-specific β1II-tubulin-immunoreactive). MB exposure resulted in a less robust but significant (p = 0.025) decrease in TH-ir neurons. However at any given dose of paraquat, maneb did not lead to further loss of DA neurons.

It was also observed that paraquat led to microglial activation as evidenced by increased presence of more amoeboid morphology among OX42-immunoreactive cells and increased microglial cell diameter measurements of up to 34.3%.

Maneb alone failed to induce morphological changes consistent with microglial activation nor did it add to the effects induced by paraquat.

Most of the rats treated with paraquat or a combination of paraquat and maneb progressively gained weight, albeit more slowly than vehicle-treated animals. Overall, only one paraquat treated rat and 2 paraquat/maneb treated rats demonstrated significant weight loss and could not reach the end of the experimental protocol (>20% of weight loss). The rapid weight loss prompted a general pathological examination, which revealed, in the 3 cases, acute lung involvement, alveolitis and bronchiolitis with no evidence of bronchitis, lymphoid aggregates, bronchiectasis, or fibrosis. Reduction of the alveolar airspace due to hyperplasia of the alveolar lining cells may have in part led to subsequent respiratory problems. All remaining animals displayed no evidence of lung pathology.

Degeneration of midbrain DA neurons: The average number of TH-ir in the SN pc of paraquat and paraquat/maneb treated rats was significantly lower than the vehicle treated animals *(p <* 0.01), and the remaining TH-ir neurons of the SN pc appeared qualitatively unhealthy, as indicated by the observation of a decrease in dendritic arborizations. Both treatment groups

also demonstrated significant decrease of Nurrl mRNA expression, which is specific to DA neurons, as evaluated by autoradiography of the SN pc *($p < 0.001$)*. Nissl staining using cresyl violet indicated a similar degree of neuronal loss in SN pc (15%-paraquat, 21% paraquat/maneb) as that observed with TH immunostaining, indicating that the loss of TH immunoreactivity was due at least in part to degeneration of DA neurons.

Motor impairments and parkinsonian behavior: Behavioral observations revealed no statistically significant differences in rats treated with paraquat/maneb compared with paraquat or vehicle (adjoining figure). There was a significant increase in hunch back posture ($p < 0.05$) and a decrease in speed ($p < 0.05$) in paraquat/maneb treated rats in relation to vehicle treated rats with a trend towards a decrease in the paraquat treated group. Although there was a trend towards a decrease in the paraquat/maneb group, the score for mobility did not reach significance in any of the treatment groups. Statistical analysis failed to reveal significant behavioral changes over time in treated rats for any of the locomotor measures.

These scores can be compared with those for rats treated with a combination of paraquat and maneb where the maximum respective scores for posture was 9 on day 9, 5 on day 9 and 1.3 on day 20; which in the case of posture and speed were statistically significantly different from both the scores of the vehicle group and the paraquat treated group ($p < 0.05$).

Neuroinflammatory response: Activation of microglia, was detected within the SN pc of treated animals where a significant decrease in TH-ir cells was observed. Control animals were all characterized by the absence of activated microglia in any brain region sampled for observation. Activated microglia in the paraquat treated rats were confined to the SN pc, whereas activated microglia were seen in the SN pc and VTA of paraquat/maneb treated rats.

Conclusion: This study provided evidence that paraquat alone and paraquat in combination with maneb is toxic to rat dopaminergic neurons both *in vitro* and *in vivo* and that the combination leads to Parkinson-like movement deficits. However evidence for the induction of Parkinson-like movement deficits by paraquat alone was limited.

Also shown here was DA degeneration, associated with markers of neuroinflammation, in response to paraquat and maneb VM DA cultures. *In vivo*, degeneration of DA neurons and inflammation in response to systemic administration in the rat of a combination of paraquat and maneb was observed. The importance of inflammation in the production of movement disorder remains to be elucidated.

Corasaniti MT, Bagetta G, Rodino P, Gratteri S & Nistico G (1992) Neurotoxic effects induced by intracerebral and systemic injection of paraquat in rats. *Hum Exp Toxicol* 11: 535-539.

The neurotoxicity of paraquat in rats was investigated following a single intracerebral or subcutaneous injection.

Paraquat (unspecified purity, batch no. and source) in double distilled pyrogen-free water was administered intracerebroventricularly to 6 adult male Wistar rats/group (280-300 g; unspecified source) at 0 (vehicle control), 0.01 or 0.1 μmol (equivalent to 0, 1.86 and 18.6 μg paraquat, or 0, 1.34 and 13.4 μg paraquat cation, respectively). Rats had previously been implanted with 25-gauge stainless steel guide cannulas under chloral hydrate anaesthesia and allowed to recover for one week prior to compound administration. A 5 μL Hamilton syringe

was used to inject 1 μL of paraquat over a minute. Separate groups of rats were administered a sc injection of paraquat at 0 (n=6), 5.0 (n=6) or 20 mg/kg bw (n=15) (equivalent to 0, 3.6 and 14.4 mg/kg bw paraquat cation, respectively. Electrocortical recordings (ECoG) were measured as described by Bagetta *et al* (1988). Rats were observed for any postural and locomotor changes, and convulsive behaviours under blind conditions over a 4 hour period. Twenty-four hours after paraquat administration, all surviving animals were anaesthetised, their brains removed and histopathologically examined. Paraquat concentrations in the caudate, hippocampus and pyriform cortex were measured by HPLC.

No behavioural abnormalities or neuropathology were reported following an intrahippocampal injection of 0.01 μmol paraquat. At 0.1 μmol, ataxia in an unspecified number of rats was reported within 10 minutes of injection followed by behavioural stimulation (clonus of the upper extremities, movement of the ears and vibrissae) which lasted for approximately 60 minutes. During the 4 hour observation period, 2-4 generalised seizures were observed in an unspecified number of rats treated with 0.1 μmol paraquat, and these seizures lasted for 20-50 seconds. Photomicrographs indicated that 24 h after an intrahippocampal injection of 0.1 μmol paraquat, neuronal death was observed in the CA1 and CA3 pyramidal cell layers, the granule cell layer, and hilus fascia dentata of the hippocampus, and the pyriform complex. The study authors reported that microinfusion of 0.2-0.4 μmol paraquat into the locus coeruleus or SN produced 'powerful stimulatory effects culminating in the high voltage ECoG spikes and motor seizures', however no evidence was provided to support these findings. It was reported that neuronal cell death occurred in the injected areas of these animals.

No behavioural abnormalities or brain damage were reported in rats administered 5.0 mg/kg bw paraquat sc. At 20 mg/kg bw, tremors, wet dog shake movements of the vibrissae, clonus of the forelimbs and rearing were observed in 10/15 rats within 15 minutes of injection. Photomicrographs illustrated that neuronal death occurred in the pyriform cortex of these rats. This correlated with brain paraquat analysis which revealed that the pyriform cortex contained the highest concentration of paraquat (208 $\pm$ 20 and 870 $\pm$ 289 ng/g wet tissue at 5 and 20 mg/kg bw, respectively) followed by the hippocampus (137 $\pm$ 9.0 and 700 $\pm$ 180 ng/g wet tissue at 5 and 20 mg/kg bw, respectively) and the caudate (85 $\pm$ 9.0 and 483 $\pm$ 180 ng/g wet tissue at 5 and 20 mg/kg bw, respectively).

In summary, intrahippocampal injection of paraquat into male Wistar rats resulted in neuronal death and damage to the pyriform cortex, ataxia, behavioural stimulation and generalised seizures at 0.10 μmol (equivalent to18.6 μg paraquat or 13.4 μg paraquat cation). A single sc injection of paraquat into male Wistar rats caused neuronal death and damage in the pyriform cortex, and abnormal behaviour (tremors, wet dog shakes, clonus and rearing) were seen at 20 mg/kg bw. The main limitations of this study were the use of 2 dose levels and the absence of quantitative data.

Endo T, Hara S, Kano S & Kuriiwa F (1988) Effects of a paraquat-containing herbicide, Gramoxone® on the central monoamines and acetylcholine in mice. *Res Commun Psychol Psych Behav* 13(4): 261-270.

The effect of Gramoxone on the concentration of various central monoamines and acetylcholine were determined in the cerebral cortex, midbrain and pons/medulla oblongata 12

days after po administration to male mice at 0, 3.12, 10.4 and 31.2 mg/kg bw/d (equivalent to 0, 2.25, 7.5 and 22.5 mg/kg bw paraquat cation) for 3 consecutive days.

Gramoxone (24% paraquat; unspecified batch no; Nihon Nohyaku Co., Tokyo, Japan) in distilled water was administered orally to male ICR-strain mice (22-24 g body weight; unspecified age and source) at 0, 3.12, 10.4 or 31.2 mg/kg bw/d (equivalent to 0, 2.25, 7.5 and 22.5 mg/kg bw/d cation, respectively) for 3 consecutive days. The exact method of po administration was unspecified. Mice were sacrificed 12 days after the last administration of Gramoxone, their brains removed and the cerebral cortex, midbrain and pons/medulla oblongata isolated. Tissues were homogenised and analysed for DA, DOPAC, HVA, norepinephrine, 5-HT and 5-hydroxyindoleacetic acid (5-HIAA) using HPLC. The concentration of acetylcholine and choline in homogenates of each of the 3 brain regions was determined using a commercially available acetylcholine assay kit (BAS Co., Tokyo, Japan). All data were analysed using a Student's t-test.

The presence/absence of clinical signs were unreported. There was no treatment-related effect on the concentration of DA, DOPAC, HVA, norepinephrine, 5-HT, 5-HIAA or acetylcholine in the cerebral cortex. There was however a statistically significant elevation ($p<0.05$) in the concentration of choline at the mid and high dose (261 $\pm$ 31 and 400 $\pm$ 88 nmoles/tissue, respectively) relative to the control (183 $\pm$ 16 nmoles/tissue).

There was a dose-related depression in the concentration of DA and norepinephrine in the midbrain, with the effect statistically significant ($p<0.05$) at the highest dose only (see Table 46 above). There was no treatment-related effect on the concentration of DOPAC or HVA. The concentration of 5-HT was significantly lower ($p<0.05$) than the control at every dose, however in the absence of a dose-response effect the toxicological significance of this finding was unclear. The concentration of 5-HIAA was significantly elevated at the highest dose ($p<0.05$). There was no treatment-related effect on the concentration of acetylcholine, while the concentration of choline was significantly elevated ($p<0.05$) at all doses. However, the elevation in choline levels did not follow a dose-response pattern and therefore the toxicological significance of this finding was unclear.

Table 46. Neurochemical effects of gramoxone in the mouse midbrain

Neurochemical	Dose of gramoxone (mg/kg bw/d)			
	0 (n=7)	**3.12 (n=9)**	**10.4 (n=8)**	**31.2 (n=8)**
DA	1207 $\pm$ 305	613 $\pm$ 146	491 $\pm$ 124	416 $\pm$ 87*
DOPAC	204 $\pm$ 37	181 $\pm$ 39	187 $\pm$ 42	230 $\pm$ 38
HVA	283 $\pm$ 38	195 $\pm$ 25	240 $\pm$ 40	240 $\pm$ 30
Norepinephrine	766 $\pm$ 64	649 $\pm$ 58	570 $\pm$ 51	519 $\pm$ 40*
5-HT	1196 $\pm$ 107	892 $\pm$ 51*	853 $\pm$ 48*	856 $\pm$ 45*
5-HIAA	1323 $\pm$ 100	1266 $\pm$ 34	1480 $\pm$ 80	1802 $\pm$ 133*
Choline (nmol/tissue)	361 $\pm$ 42	766 $\pm$ 108*	774 $\pm$ 96*	778 $\pm$ 100*

Result expressed as the mean ng/g tissue $\pm$ 1 SEM; * $p<0.05$

In the pons/medulla oblongata, there was a dose-related increase in the concentration of 5-HIAA (1002 $\pm$ 47, 971 $\pm$ 50, 1217 $\pm$ 151 and 1504 $\pm$ 170 ng/g tissue at 0, 3.12, 10.4 and 31.2 mg/kg bw/d, respectively) with the effect at the highest dose statistically significant ($p<0.05$). There was an incidental depression in DOPAC at the lowest dose ($p<0.05$) but in the absence

of an effect at the 2 higher doses this finding was not considered to be treatment-related. A significant depression in 5-HT ($p<0.05$) occurred at the low and high dose, however, in the absence of a dose response effect these findings were not considered to be toxicologically relevant. There was no treatment-related effect on the concentration of DA or norepinephrine. The study authors indicated that HVA levels were not measured as they were below the limit of quantitation. There was no treatment-related effect on the concentration of acetylcholine but there was a clear dose-related elevation in choline levels (307 $\pm$ 27, 456 $\pm$ 34, 656 $\pm$ 78 and 780 $\pm$ 73 nmol/tissue at 0, 3.12, 10.4 and 31.2 mg/kg bw/d, respectively, with every dose statistically higher ($p<0.05$) than the control.

In summary, daily po administration of Gramoxone to male ICR-strain mice for 3 days was shown to alter the neurochemistry of the cerebral cortex (increased choline), the midbrain (decreased DA and norepinephrine; increased 5-HIAA) and pons/medulla oblongata (increased 5-HIAA and choline). No evidence was provided that these neurochemical effects were due to paraquat (eg measurement of paraquat levels in the cerebral cortex, midbrain and pons/medulla oblongata) and it is possible that other Gramoxone components could have been responsible for the observed neurochemical abnormalities.

Liou H-H, Chen R-C, Tsai Y-F, Chen W-P, Chang Y-C & Tsai M-C (1996) Effects of paraquat on the substantia nigra of the Wistar rats: neurochemical, histological and behavioural studies. *Toxicol Appl Pharmacol* 137: 34-41.

The effect of paraquat or MPP^+ on the SN of male rats was studied following direct unilateral intranigral injection of 0, 1, 2, 3 or 5 μg paraquat (equivalent to 0, 0.72, 1.44, 2.16 and 3.6 μg paraquat cation, respectively), or 8 μg MPP^+.

Paraquat (unspecified purity and batch no.; Sigma, St Louis, MO, USA) in normal saline was stereotactically injected into the unilateral SN compacta of 3-month old male Wistar rats (250-300 g body weight; unspecified source) under pentobarbital sodium anaesthesia using the coordinates of Paxinos and Waston (1986). Twelve rats/group received 0 (saline), 1, 2, 3 or 5 (n=6) μg paraquat (equivalent to 0, 0.72, 1.44, 2.16 and 3.6 μg paraquat cation) in a total volume of 1 μL which was infused at a rate of 0.2 μL/min through a 30-gauge stainless steel needle. Two additional groups of rats received 3 μg of paraquat (n=30; equivalent to 2.16 μg paraquat cation) or 8 μg MPP^+ (N-methyl-4-phenyl-pyperidinium ion; RBI, Natick, MA, USA; n=12).

Spontaneous behaviour was observed throughout the study including immediately after paraquat administration, when rats were left undisturbed, and during handling. Six rats/group were studied for apomorphine-induced (0.5 mg/kg, sc) circling behaviour at 2, 4, 8 and 16 weeks after intranigral injection of paraquat, saline or MPP^+ using the automated rotometer bowls described by Ungerstedt and Arbuthnott (1970). All rats administered 1, 2 and 3 μg paraquat, and 6 rats treated with MPP^+, were terminated by decapitation 2 weeks after treatment. Six rats from the additional 3 μg paraquat group were decapitated at 4, 8 or 16 weeks after treatment. Brains were removed, homogenised and assayed for DA, DOPAC and HVA using HPLC. Two weeks after administration of 1, 2 or 3 μg paraquat, rats were anaesthetised with sodium pentobarbital, their brains removed and histopathologically examined. Data were

analysed by a repeated measures one-way ANOVA followed by a Scheffe's test for post-hoc comparison of means. Differences were considered to be statistically significant when $p<0.05$.

All rats (6/6) given 5 μg paraquat died within 4 days of injection. Rats administered 3 μg paraquat showed various spontaneous behavioural abnormalities compared to controls, while no behavioural abnormalities were observed at lower doses. Rotational behaviour in the direction of the lesion was reported when rats were stimulated. An asymmetrical posture was assumed when rotational behaviour ceased and continuous weak sniffing behaviour was exhibited. These behavioural anomalies ceased 3 days after microinjection but the tendency of the posture and movement toward the operated side remained.

Subcutaneous administration of 0.5 mg/kg bw apomorphine to rats 2 weeks after intranigral paraquat injection resulted in a dose-dependent increase in contralateral circling which was statistically significant ($p<0.01–0.5$) at and above 2 μg paraquat (frequencies of contralateral circling at 1, 2 and 3 μg paraquat were 18 ± 12, 34 ± 10 and 140 ± 31 turns/h, respectively compared with that for MPP^+ of 119 ± 23). Graphically presented data illustrated that apomorphine-induced contralateral behaviour began 2 weeks after intranigral injection of 3 μg paraquat or 8 μg MPP^+ and persisted for the 16-week study period.

There was a clear dose-related depression in the levels of striatal DA, DOPAC and HVA 2 weeks after intranigral injection of 1, 2 and 3 μg paraquat (see Table 47 below). DA levels were statistically lower than the control at all doses ($p<0.01–0.05$) while DOPAC and HVA levels were only statistically lower ($p<0.01$) than the control at the highest dose (3 μg). The ratios of DOPAC and HVA to DA were statistically higher ($p<0.01$-0.05) than the control only at 3 μg.

The study authors reported that the EC_{50} of paraquat after intranigral injection was approximately 9.5 nmol (equivalent to 1.77 μg paraquat; equivalent to 1.27 μg paraquat cation). Rats treated with 8 μg MPP^+ also showed a statistically significant depression in striatal DA, DOPAC and HVA levels, and an increase relative to the control group in the ratios of DOPAC and HVA to DA ($p<0.01–0.05$).

Table 47. Striatal concentrations of neurochemicals in rats 2 weeks after intranigral-administration of paraquat

Compound	Control	1 μg paraquat	2 μg paraquat	3 μg paraquat	8 μg MPP^+
DA	175.62 ± 12.40	128.82 ± 11.24**	69.29 ± 15.47 **	17.21 ± 9.15 **	31.07 + 9.13 **
DOPAC	26.67 ± 3.49	23.93 ± 4.27	18.04 ± 2.67	5.88 ± 3.06 **	11.71 ± 3.77 **
HVA	7.91 ± 1.08	6.77 ± 1.21	4.76 ± 0.81	4.01 ± 1.73 *	3.59 ± 1.32 **
DOPAC/ DA	0.15 ± 0.01	0.18 ± 0.02	0.29 ± 0.06	0.45 ± 0.06 *	0.46 ± 0.08 *
HVA/DA	0.04 ± 0.01	0.05 ± 0.01	0.07 ± 0.01	0.14 ± 0.01 *	0.11 ± 0.02 *

Data expressed as mean pg/μg ± SEM; n=6; * $p<0.05$; ** $p<0.01$

The magnitude of depression in the levels of striatal DA, DOPAC and HVA persisted for 16 weeks following administration of 3 μg paraquat, and this effect was statistically significant ($p<0.01$-0.05) at every sampling point (2, 4, 8 and 16 weeks). However, the interpretation of

these findings was complicated by the absence of a concurrent control group (ie 4-, 8- and 16-week paraquat samples were compared to a 2-week control sample).

The study authors reported no neurohistopathological abnormalities in rats treated with 1 μg paraquat. Photographic evidence illustrated a moderate loss of cells of the SN and abnormal neurons 2 weeks after microinjection of 2 μg paraquat, while a marked loss of neurons and prominent astrocyte proliferation were observed at 3 μg paraquat. The study authors also reported neuronal loss and astrocyte proliferation 2 weeks after injection of 8 μg MPP^+.

In summary, this study provided evidence that paraquat is neurotoxic when injected into the SN of male Wistar rats. At and above 1 μg paraquat (equivalent to 0.72 μg paraquat cation), a significant dose-related depression in striatal DA concentration occurred. Neurohistopathological abnormalities (abnormal neurons, neuronal loss and astrocyte proliferation) and a significant dose-related increase in apomorphine-induced contralateral circling were observed at and above 2 μg (equivalent to 1.44 μg paraquat cation). At 3 μg (equivalent to 2.16 μg paraquat cation), rotational behaviour in the direction of the lesion, asymmetrical posture or weak sniffing behaviour was exhibited in addition to a significant depression in DOPAC and HVA levels. At 5 μg (equivalent to 3.6 μg paraquat cation), all rats died. The major limitation of this study was that no concurrent control group was sampled over the 16 week experimental period.

Miranda-Contreras L, Davila-Ovalles R, Benitez-Diaz P, Pena-Contreras & Palacios-Pru E (2005) Effects of prenatal paraquat and mancozeb exposure on amino acid synaptic transmission in developing mouse cerebellar cortex. *Developmental Brain Research* 160:19-27.

This study involved paraquat, mancozeb and a combination of the two. Only findings for paraquat are reported here.

The purpose of this study was to determine the effects of prenatal exposure to paraquat on the development of amino acid synaptic transmission in mouse cerebellar cortex. This period is critical for the formation of the neuronal circuitry of the cerebellar cortex. During this stage, neurotransmitters may play important roles other than neurotransmission to control neural function. Evidence exists that neurotransmitters can modulate proliferation of neural stem cells, neuroblasts and glioblasts; regulate cell migration; and induce differentiation (Cameron *et al* 1998; Lauder 1993; Lipton & Kater 1989). Consequently, any toxicant that interferes with neurotransmission during development may cause permanent defects in the CNS (Lauder 1993; Ferguson 1996).

Study & observations: Pregnant (10 days gestation) NMRI mice (University of Los Andes, Merida, Venezuela) were used in this study. Mice were maintained in a controlled environment with food and water *ad libitum*. They were randomly assigned into four treatment groups (20/treatment group; 20/treatment control group), which were administered with either saline, paraquat, mancozeb or a combination of paraquat and mancozeb between gestation days 12 and 20 at intervals of 48 h between each dose, in a total of five doses. The dams were injected with either saline, paraquat at a dose of 10 mg/kg bw, mancozeb at a dose of 30 mg/kg bw or a combination of 10 mg/kg bw paraquat and 30 mg/kg bw mancozeb. These doses were consistent with previous studies. Paraquat and mancozeb were dissolved in saline and injected

ip. Body weight was used as the physical developmental parameter for the evaluation of the possible effects of pesticide exposure of the pups. All pups in control and experimental groups were weighed on post natal day 1 (P1), P3, P7, P14, P21 and P30.

The locomotor activity of pups was evaluated in an open- field arena, which was made of a wooden box, 40 x 40 x 35 cm, subdivided into 10 x 10 cm with infrared photosensors to quantify locomotor activity. A single pup from each litter was used for the test, and the locomotor activity test was performed on the same pups on P14, P21 and P30. Each mouse was placed in the centre of the arena, and the locomotor activity was assessed by recording the total number of photobeam breaks, corresponding to the number of times that the mouse ran a distance of 10 cm, during a period of 10 min. After each animal's motor activity evaluation, the testing surface was cleaned with water and alcohol and then dried.

Simultaneous assay of the neurotransmitter amino acids, glutamate, aspartate, glycine, taurine and GABA, were conducted using HPLC following derivatization with dansyl chloride. For this analysis, pups were obtained at random from the treatment group, and depending on the developmental age. Cerebellar cortical tissue samples were obtained from the treatment group at P 1, P3, P7, PI5 and P30 and were homogenized in 0.05 N perchloric acid. After taking aliquots for protein determination, the homogenates were spun at 4 ^{o}C for 15 min at 10.000 x *g.* The supernatant solutions were filtered through 0.45 pm Millipore filters, and aliquots were derivatized with dansyl chloride.

Dansylated amino acids were eluted at a flow rate of 0.6 ml/min. and absorbance detection was carried out at 215 nm. The levels of amino acid neurotransmitters were calculated by comparing peak areas with those of standards, and the values were expressed as µmol/100 mg proteins.

Findings: During the first week after birth, no significant differences in body weights were observed between control and experimental groups, however, on P30 the paraquat treated group showed a significant decrease (21%) in body weight ($p < 0.0001$).

With age, the control mice showed an increasing locomotor activity, whereas all mice in the treated group started to exhibit hyperactivity at P14 which moderated at P21. However a notable decrease with respect to control were observed by P30.

Excitatory amino acid neurotransmitters: During the first 2 weeks of birth, paraquat exposed pups showed significant changes in aspartate contents with respect to the saline-exposed control group (Table 48 below): a 53% reduction ($p < 0.0001$) at P3 (1.82 ± 0.38 cf 3.84 ± 0.91 for controls) and an increase of 37% ($p < 0.0001$) at P15 (8.47 ± 0.84 cf 6.19 ± 0.52 for controls): during the young adult stage. At P30, aspartate level was lower by 18% ($p < 0.0001$) (4.91 ± 0.98 cf 6.00 ± 0.62) than control mice. On the other hand, significant decreases in glutamate levels of paraquat exposed pups were observed during the first postnatal week: by 69% ($p < 0.0001$) at P3 and by 32% ($p < 0.0001$) at P7; thereafter, no important changes were observed until P30, when the glutamate level rose 39% ($p < 0.0001$) with respect to controls.

Table 48: Levels of aspartate and glutamate during mouse cerebellar cortex development in paraquat prenatally treated mice (µmol/100 mg proteins ± SD)

Age of neonate	Aspartate		Glutamate	
	Control	Treated	Control	Treated
P1	2.50 ± 0.67	2.40 ± 0.30	5.07 ± 0.76	5.03 ± 0.77
P3	3.84 ± 0.91	1.82 ± 0.38	13.88 ± 3.54	4.35 ± 0.64
P7	1.57 ± 0.21	2.14 ± 0.41	6.50 ± 0.87	4.42 ± 0.69
P11	2.84 ± 0.47	2.04 ± 0.43	6.01 ± 1.18	6.44 ± 0.48
P15	6.19 ± 0.52	8.47 ± 0.84	11.85 ± 1.75	12.45 ± 1.56
P30	6.00 ± 0.62	4.91 ± 0.98	6.57 ± 1.75	9.12 ± 0.99

Inhibitory amino acid neurotransmitters: The pattern of change of the inhibitory neurotransmitter GABA during cerebellar cortex development of paraquat exposed pups during late gestation showed a similar pattern to controls except for a significant decrease in GABA content at P3 (89%, $p < 0.0001$) (Table 49 below). Developmental variation in glycine levels, in paraquat exposed pups, notable changes were seen during the first postnatal week: lower glycine content than control at P1 (33%, $p < 0.0001$) and P3 (77%, $p < 0.0001$) followed by an increase of about 126% at P7 ($p < 0.0001$), thereafter, the values were similar to those of control group. Developmental changes in taurine content, in general, the treated group showed the same trend of variation as the control group, except for the decrease seen at P3 (59%, $p < 0.0001$) and at P11 (38%, $p < 0.0001$).

Table 49: Levels of GABA, glycine and taurine during mouse cerebellar cortex development in paraquat prenatally treated mice (µmol/100 mg proteins ± SD)

Age	GABA		Glycine		Taurine	
	Control	Treated	Control	Treated	Control	Treated
P1	1.02 ± 0.11	1.00 ± 0.19	2.41 ± 0.20	1.61 ± 0.25	19.65 ± 2.50	16.43 ± 2.00
P3	3.04 ± 0.35	0.33 ± 0.08	7.99 ± 1.74	1.87 ± 0.36	40.82 ± 8.29	16.54 ± 1.98
P7	2.60 ± 0.39	2.44 ± 0.47	2.72 ± 0.45	6.14 ± 1.39	25.78 ± 4.01	22.23 ± 3.36
P11	1.42 ± 0.23	1.34 ± 0.26	1.25 ± 0.29	1.43 ± 0.31	14.03 ± 2.56	8.76 ± 0.56
P15	1.91 ± 0.31	2.00 ± 0.33	1.63 ± 0.17	2.01 ± 0.43	7.07 ± 1.05	10.43 ± 2.15
P30	4.91 ± 0.69	5.44 ± 1.39	2.89 ± 0.56	2.67 ± 0.35	5.38 ± 0.86	6.09 ± 0.68

GABA: γ-amino butyric acid;

Significant alterations of cerebellar cortex synaptic circuitry caused by prenatal exposition of the animals to paraquat were observed in this study. Variations in the levels of excitatory neurotransmitters, aspartate and glutamate showed important changes in the magnitude of peak excitatory innervation periods of Purkinje cells: first, between P2-P5, when multiple climbing fibers from the inferior olivary complex innervate Purkinje cell dendritic trees, and second, between P 11-P15, when granule cell parallel fibers establish numerous synaptic contacts on Purkinje cell dendritic spines (Sotelo 2004; Welsh *et al,* 1995). Most notably affected was the period of excitatory innervation of the growing Purkinje cell dendrites by the afferent climbing fibres, which occurs around P3. The treated group showed significantly decreased levels of aspartate and glutamate with respect to the control group. These results indicated a marked degeneration of Purkinje cells which could have been induced by their exposure to the toxic effects of paraquat during the period when they are being generated (Sotelo 2004).

During the first postnatal week, the pattern of change in GABA levels of the paraquat exposed group indicated a 4-day delay in development of GABAergic neurons as compared with that of the controls.

The patterns of variation in glycine levels of paraquat exposed pups are similar to those observed in the case of GABA. There was a delay in glycine ontogenesis in paraquat exposed mice. While showing the same chronology as that of control, glycine levels were lower than control.

With respect to the inhibitory neurotransmitter taurine, high concentrations were detected during the first stages of postnatal development, both in the control and treated group. Significant reductions in taurine levels with respect to control were observed at P3 and P11 in paraquat exposed pups. During this period, deficiency in taurine could be affecting the growth of Purkinje cell dendritic trees and impairing granule cell migration from the external germinal layer to their final location at the internal granular layer. These events are two fundamental processes that define cerebellar morphogenesis. It has been documented that, when granule cell population is altered, the Purkinje cell dendritic tree shows abnormal morphology, indicating direct influence of granule cells on the growth of Purkinje cell dendrites.

Conclusion: Modulation of the basic cerebellar circuitry by the intrinsic neuronal elements, as well as those extrinsic to the cerebellum, occurred with prenatal exposure to paraquat administered to pregnant dams as 5 ip injections of 10 mg/kg bw every 48 hours between gestation days 12 and 20, causing alterations in the normal chronology and magnitude of excitatory and inhibitory synaptic transmissions in the developing mouse cerebellar cortex.

Perry TL, Yong VW, Wall RA & Jones K (1986) Paraquat and two endogenous analogues of the neurotoxic substance N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine do not damage dopaminergic nigrostriatal neurons in the mouse. Neurosci Lett 69: 285-289.

A study was undertaken to compare the effect of repeated sc injections of the MTD of paraquat, reduced paraquat, MPTP (N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, single dose only) and two MPTP analogues, on striatal levels of DA, DOPAC and HVA in mice.

Paraquat (unspecified purity and source) was injected sc into C57 black mice (sex, age, body weight and source unspecified) on 3 occasions, each separated by 3-day intervals, at doses of 0 (n=44) or 14.5 mg/kg bw (n=7) (equivalent to 10.44 mg/kg bw paraquat cation). The vehicle and dose volume were unspecified. The dose selection was based on the MTD of 14.5 mg/kg bw which was determined during preliminary experiments. A separate group of 10 mice were injected sc with between 7.3 to 116.3 mg/kg bw/d reduced paraquat (1,1'-dimethyl-1,1',2,2',3,3',6,6'-octahydro-4,4'bipyridine) over 6 days. Nine mice received a sc injection of 40 to 160 mg/kg bw/d MTIQ (N-methyl-1,2,3,4-tetrahydroisoquinoline) over 8 days. MTHBC (2-methyl-1,2,3,4-tetrahydro-β-carboline) was injected sc into 6 mice at 40 mg/kg bw on a single day, and then 80 mg/kg bw/d for the next 3 days. A group of 34 mice received a single 40 mg/kg bw sc injection of MPTP (unspecified source). Reduced paraquat, MTIQ and MTHBC were all synthesised on site. All surviving mice were sacrificed one month after the last injection by cervical dislocation. Striata were dissected out immediately and stored at -70°C until analysed. Striatal concentrations of DA, DOPAC and HVA were measured by HPLC.

During an undescribed preliminary experiment, paraquat caused rapid weight loss and death. In contrast, reduced paraquat did not cause any clinical signs or affect body weight. Mice injected with the highest dose of MTIQ (160 mg/kg bw) appeared 'markedly sedated' for 3 h after injection. Convulsions and death occurred in a single mouse within 15 minutes of an injection of 200 mg/kg bw MTIQ. No behavioural abnormalities were reported following a single injection of 40 mg/kg bw MTHBC, but sedation, ataxia, tremors and infrequent clonic convulsions (unspecified incidences) were observed at 80 mg/kg bw. There were no reported clinical signs following injection of 40 mg/kg bw MPTP.

There was a slight though statistically significant elevation ($p<0.001$, test unspecified) in striatal DA following paraquat administration, while DOPAC and HVA levels were unaffected (see Table 50 below). As the magnitude of this effect was small, and given that MPTP caused a large and statistically significant ($p<0.001$, test unspecified) depression in striatal DA, DOPAC and HVA levels, this finding was not considered to be toxicological significant. Reduced paraquat, MTIQ and MTHBC had no effect on striatal levels of DA, DOPAC or HVA.

Table 50. Striatal neurochemical concentrations (µg/g wet weight) in mice following sc injection of paraquat, MPTP or MPTP analogues

Treatment	n	Cumulative dose (mg/kg bw)	DA	DOPAC	HVA
Control	44		11.3 ± 0.4	1.0 ± 0	1.3 ± 0.1
MPTP	34	40	1.3 ± 0.1*	0.2 ± 0*	0.5 ± 0*
Paraquat	7	44	14.5 ± 0.4*	0.8 ± 0	1.3 ± 0.1
Reduced paraquat	10	342	12.7 ± 0.7	0.8 ± 0	1.3 ± 0.1
MTIQ	9	1040	14.3 ± 0.3	0.8 ± 0	1.2 ± 0.1
MTHBC	6	280	13.8 ± 0.8	0.8 ± 0.1	1.3 ± 0.1

Results expressed as mean ± 1 SEM; MTIQ = N-methyl-1,2,3,4-tetrahydroisoquinoline; MTHBC = 2-methyl-1,2,3,4-tetrahydro-β-carboline; * $p<0.001$

In summary, repeated sc injection of paraquat into C57 black mice at the MTD (14.5 mg/kg bw; equivalent to 10.44 mg/kg bw paraquat cation) did not perturb striatal levels of DA, DOPAC or HVA. In contrast, MPTP was found to significantly reduce all 3 neurochemicals. These findings suggested that paraquat did not damage nigrostriatal neurons in the mouse. The main shortcomings of this study were the uneven sample sizes, the use of a single dose, the lack of quantitative clinical signs and that the concentration of paraquat in the brain was not measured.

Rojo AI, Cavada C, de Sagarra MR & Cuadrado A (2007) Chronic inhalation of rotenone or paraquat does not induce Parkinson's disease symptoms in mice or rats. Exp Neurol. 208(1):120-6.

This paperinvestigated the induction of Parkinson's disease symptoms in mice and rats following the intranasal inoculation of paraquat, rotenone or MPTP daily for 30 days . The study authors stated this methodology provided a new model to test the parkinsonian potential of neurotoxins based on chronic inhalation. This review considers this study only in respect of paraquat and MPTP as a positive control

Part 1 Mice

Eight week old male C57BL/6 mice (Charles River Breeding Laboratories International Inc, MA) were housed in a controlled environment. Food and water were provided *ad libitum*. Solutions of paraquat in saline at concentrations of 25, 50 and 75 mg/mL to provide doses of 10, 20 and 30 mg/kg bw[5] respectively were used. Mice were inoculated intranasally. Briefly, animals were held by the neck and were laid upside down with a finger under the neck to limit liquid flow down the trachea. 10 µL of paraquat solution was slowly inoculated with a micropipette (about 15 s) in one nasal cavity. Afterwards, the animals were immobilized in this position for about 10 s by gently pulling the tail and after 2-3 min the same procedure was repeated in the other nostril. This procedure was repeated daily for 30 days. Other groups of mice were administered MPTP at 30 or 60 mg/kg bw/d[6] as positive controls, by the same method.

Results:

*Behavioural effects:*Ambulatory activity was examined weekly in an open field test. To avoid interference with the possible short-term effects of paraquat after inoculation, motor activity was analysed before administration of the daily dose.

Mice that received daily nasal inoculations of 10, 20 and 30 mg/kg bw paraquat did not show behavioral changes immediately after inoculation. However, after one week of daily infusions with 20 or 30 mg/kg paraquat mice developed strong hypokinesia. Loss of motor activity at 20 mg/kg/day was shown by the number of rears dropping from 35 on day 0 to 8 on day 30.

In addition these mice exhibited curved position, agitated breathing and weight loss, cyanotic feet and mouth, and lung damage. About one-third of the animals given 30 mg/kg bw/d paraquat died during the first week of treatment and another third developed truncal dystonia and fast rotation when they were suspended by the tail. This fast rotation is characteristic of vestibular dysfunction. It was speculated that paraquat might be reaching the inner ear though the Eustachian tube.

Mice receiving MPTP at 30 or 60 mg/kg bw/d exhibited piloerection, salivation, tremor and smooth rigidity shortly after administration, and reduced motor activity achieving statistical significance by the end of the treatment period.

*Striatal levels of DA and DOPAC:*Striatal levels of DA and DOPAC were determined by HPLC. Mice given 20 mg/kg bw/day intranasal inoculations of paraquat did not show a significant difference from saline inoculated control mice. MPTP by contrast resulted in a significant reduction in DA and DOPAC levels in the striatum at 30 or 60 mg/kg bw.

TH immunoreactivity: The levels of TH immunoreactivity, as a measurement of the number of dopaminergic neurons, was not significantly different in either the striatum or SN in mice

[6] The published report refers to 10, 20 or 30 mg/kg bw paraquat doses in the materials and methods section. This is assumed to be paraquat dichloride (ie. 7.2, 14.5 or 20.7 paraquat ion). It is noted in the results section only, a dose of 3 mg/kg paraquat is also referred to.

treated with 20 mg/kg bw/d paraquat. The brains of mice treated with MPTP had 20-30% less TH^+ neurons than controls, indicative of damage.

Concentration in olfactory bulb, striatum or ventral midbrain: Paraquat was detected only in the olfactory bulb, 10 minutes after a single intranasal dose of 20 mg/kg bw. MPTP (60 mg/kg bw) by contrast was detected in all three areas of the brain tested.

Conclusion: In this study, it was observed that paraquat produced strong hypokinesia in mice within less than one week of treatment. However, since there was no decrease in striatal DA or DOPAC nor in TH-ir, the motor deficit could not be correlated with nigrostriatal damage but rather with weakness due to systemic poisoning. Paraquat was detected only in the olfactory bulb and not other areas of the brain of mice shortly following a single intranasal administration, suggesting a limited ability to reach the nigostriatial system when admistered by this route.

Intranasal inoculation of paraquat does not result in significant dopaminergic damage at the doses used in this study.

Part 2 rats

Eight week old male Sprague-Dawley rats (Charles River Breeding Laboratories International Inc, MA) were investigated in the study. Solutions of paraquat in saline at concentrations of 25, 50 and 75 mg/mL to provide doses of 10, 20 and 30 mg/kg bw respectively were prepared for the study but results were reported only for the lower dose in rats. Rats were inoculated intranasally. Rats were anaesthetised (4% halothane and 40% O_2 and then lowered to 1.5 halothane and 40% O_2) and were laid upside down with a pillow under the neck to limit liquid flow down the trachea. Thirty µL paraquat solution was slowly inoculated in each nasal cavity in aliquots of 6 µL at 2 min intervals. Afterwards the rats were kept in this position for 10 min. This procedure was repeated every day for 30 days.

Results:

As in mice there was no progressive nigrostriatal dopaminergic degeneration following chronic nasal exposure to paraquat: Paraquat was detected only in the olfactory bulb. At 10 mg/kg bw/d, in some rats, paraquat provoked a strong diffuse peroxidase reaction in the olfactory bulb and frontal cortex but TH-specific staining was similar to control saline inoculated rats. Paraquat had no effect on brain acetylcholinesterase levels.

Conclusion: Intranasal inoculation of paraquat does not result in significant dopaminergic damage in rats at 10 mg/kg bw/d.

OCS note: Intranasal instillation is not a standard route of administration in animal studies. However, this study was not designed for regulatory purposes but to investigate whether paraquat might lead to development of PD when in contact with relevant sites of potential human absorption following exposure. The nasal mucosa would be a site of potential contact and absorption in humans following inhalation of paraquat. While recognising that the absorption is limited to a specific area of the respiratory tract, the study provided no evidence that paraquat might lead to the development of PD in rats and mice following absorption via

the nasal mucosa, even at dose levels producing severe systemic toxicity. The findings of this study are consistent with those seen in rodents following oral and dermal administration. Therefore although a non-standard study, paraquat did not induce neurotoxicity via the the intranasal instillation route of exposure in this animal model designed to investigate PD potential of chemicals.

Shimizu K, Matsubara K, Ohtaki K, Fujimaru S, Saito O, Shiono H (2003) Paraquat induces long-lasting dopamine overflow through the excitotoxic pathway in the striatum of freely moving rats. *Brain Res*. 27;976(2):243-52.

These authors had previously shown that paraquat penetrates the blood-brain barrier and is taken up by neural cells (Shimizu *et al*, 2001). In this study, the *in vivo* toxic mechanism of paraquat to DA neurons was examined. Ultimately it was suggested that paraquat activated the excitotoxic pathway to lead to a vulnerability of dopaminergic neurons and terminals in the midbrain and in the striatum of the brain, a mechanism which could be involved in the pathogenesis of Parkinson's disease.

Paraquat dichloride was obtained from Tokyo Chemical Industry (Tokyo, Japan). MPP + iodide, L-deprenyl and dizocilpine (MK-801) were purchased from Research Biochemicals International (Natick, MA, USA). 1-Octanesulfonic acid was obtained from Nacalai Tesque (Kyoto, Japan). GBR-12909, DA, norepinephrine, DOPAC, HVA, 5-HTAA, 5-HT were obtained from Sigma (St Louis, MO, USA). The other reagents were of analytical or HPLC grade, and were from Wako (Osaka, Japan).

Paraquat dichloride (10 mg/kg sc, equal to 40 µmol) was given to male Wistar rats (8 weeks old, 210-260 g; SLC, Shizuoka, Japan) once a day for 5 days. Monoamine concentrations in eight regions of brain were determined 2 days after the 5-day treatment with paraquat. Paraquat affected DA and its acidic metabolites in the striatum, midbrain and cortex. DA content in the striatum was reduced to 70% of that in control rats ($p < 0.05$). The HVA level was 80% of control value in the striatum ($p < 0.01$). Paraquat reduced DOPAC and HVA contents to 40-60% and 25-50% compared with the control in the midbrain and cortex ($p < 0.05$*).*

When paraquat was administered to the striatum of the brain through a microdialysis probe, a significant amount of paraquat was detected in the ipsilateral but not contralateral striata after a sequential 180-min washout with Ringer's solution. Treatment with 50 µM GBR-12909 (a selective DAT inhibitor) significantly inhibited the striatal uptake of paraquat ($p < 0.02$).

In measuring the glutamate efflux induced by paraquat it was observed that the perfusion of paraquat increased the extracellular levels of glutamate in the striatum in a dose-dependent manner ($p < 0.05$). The perfusion of 50 µM paraquat elevated the striatal levels of glutamate to 180% of the basal value. The extent of glutamate elevation was small, and the increased level returned to the basal level shortly after the termination of paraquat perfusion.

Paraquat (50 µM) increased the extracellular levels of NO_x^- in the striatum ($p < 0.01$). The extent of elevation of NO_x^- levels was small; however, this increase continued at least 3 h after termination of paraquat perfusion.

Paraquat increased the extracellular levels of DA in the striatum in a dose-dependent manner ($p < 0.01$). Although the extent of DA efflux induced by paraquat was small, it is noteworthy that DA overflow was continuously evoked more than 24 h after the paraquat perfusion. The perfusion with paraquat did not affect the extracellular DOPAC level.

Paraquat induced a long-lasting DA overflow that was observed at 24 h after its perfusion for 1 h. This effect of paraquat was inhibited by treatment with N^G-nitro-L-arginine methyl ester (L-NAME), dizocilpine (MK-801, 6,7-dinitroquinoxaline-2,3-dione (DNQX) and L-deprenyl ($p < 0.01$).

Conclusion:

Paraquat stimulated glutamate efflux from neural cells or inhibited the glutamate uptake system, and initiates a cascade of excitotoxic reactions leading to damage of dopaminergic terminals. The mechanism involves glutamate induced activation of non-NMDA receptors, resulting in activation of NMDA receptors. The activation of NMDA receptor channels results in a massive influx of Ca^{2+} into the cells. The entry of Ca^{2+} into cells stimulates NOS.

It was suggested that released NO would diffuse to dopaminergic terminals and induce mitochondrial dysfunction, causing continuous and long-lasting DA overflow.

The authors concluded that paraquat could be considered an exogenous neurotoxicant involved in the aetiology of Parkinson's disease, or at least, exposure to low levels of paraquat for a long time would make dopaminergic neurons vulnerable to oxidative stress and cell death.

Tawara T, Fukushima T, Hojo N, Isobe A, Shiwaku K, Setogawa T & Yamane Y (1996) Effects of paraquat on mitochondrial electron transport system and catecholamine contents in rat brain. ***Arch Toxicol*** **70: 585-589.**

A range of experiments were performed to determine the effect of a single intravenous dose of paraquat on complex I activity (NADH: ubiquinone oxidoreductase), lipid peroxidation and catecholamines in rat brain.

Paraquat (unspecified purity and batch no. Sigma, USA) in sterile physiological saline was injected into the tail vein of 8-week old male SD rats (unspecified body weight) Japan Clea, Tokyo, Japan) at 20 mg/kg bw/d for 5 days (equivalent to 14.4 mg/kg bw/d paraquat cation). Control animals were injected with an equivalent volume of sterile physiological saline. Rats were sacrificed at an unspecified time by microwave irradiation, their brains immediately removed and striata isolated according to the technique of Glowinski and Iversen (1966). The concentrations of DA and norepinephrine were measured by HPLC. Rats were sacrificed by decapitation after the last injection of paraquat and their brains, lungs and livers immediately removed. Lipid peroxidation was measured according to the method of malondialdehyde production (Ohkawa *et al,* 1979). Rats were sacrificed by decapitation after the last injection of paraquat and their brains, lungs and livers immediately removed. Submitochondrial particles were purified according to the method of Turrens and Boveris (1980) and assayed for complex I activity (ie NADH: ubiquinone oxidoreductase)) using the NADH:ferricyanide reaction (NFR assay) or the NADH:ubiquitone reaction (NQR assay).

Paraquat-treated mice had a significantly ($p<0.001$, t-test) lower concentration of striatal DA (6.14 + 0.81 μg/g tissue) than the control (8.76 ± 0.49 μg/g). There was no treatment-related effect on the concentration of striatal norepinephrine. Lipid peroxidation was significantly higher ($p<0.05$, t-test) in the brain (1.6-fold higher) and liver (1.8-fold higher), but not the plasma, of paraquat-treated rats compared to the control (see Table 51 below).

Table 51. Effect of 5 x 20 mg/kg bw/d iv injections of paraquat on lipid peroxidation (pmol/mg protein) in the brain, liver and serum of rats.

Group	Brain	Liver	Serum
Control (n=3)	40.1 ± 4.7	51.3 ± 7.1	2.47 ± 0.93
Paraquat (n=3)	63.7 ± 4.0*	92.2 ± 6.6*	2.10 + 0.44

Results expressed as mean ± 1 SD; * $p<0.05$

Complex I activity was significantly inhibited ($p<0.01$-0.05, t-test) in the brain and liver of paraquat-treated rats using either the NFR or NQR assay (see Table 52 below).

Table 52. Effect of 5 x 20 mg/kg bw/d iv injections of paraquat on complex I activity (IU/mg protein) in the brain and liver of rats.

	NFR assay	NQR assay
Brain		
Control	3.67 ± 0.29	2.06 ± 0.28
Paraquat	2.53 ± 0.20*	1.15 ± 0.09*
Liver		
Control	6.75 ± 0.43	2.42 ± 0.02
Paraquat	4.10 ± 0.17*	1.05 ± 0.16**

Results expressed as mean ± 1 SD; * $p<0.05$; ** $p<0.01$.

In summary, paraquat administered iv to male SD rats at 20 mg/kg bw/d (equivalent to 14.4 mg/kg bw/d paraquat cation) over 5 days reduced striatal DA levels, increased lipid peroxidation and inhibited complex I activity in the brain (and liver). Parallels can be drawn with the neurotoxicity of MPTP which reduces striatal DA and inhibits complex I activity.

APPENDIX II ADDITIONAL STUDY DETAIL FROM UNPUBLISHED PAPERS DISCUSSED IN THIS SUPPLEMENT

The following three studies are specialist reports conducted as part of:

"Beck MJ (2013) Subchronic (91-day) dietary study to assess the effects of paraquat dichloride on dopaminergic neurons in C57BL/6J mice. Study No./Report No.: WIL-639158. Task No.: TK0000969. Unpublished.\ Report date: January 24, 2013"

The findings are included in the Beck (2013) report, but additional details presented in this Appendix.

1) Stereology assessment of Beck (2013) study (Subset I):

Wolf JC (2013) Final stereology report: *Subchronic (91-day) dietary study to assess the effects of paraquat dichloride on dopaminergic neurons in C57BL/6J mice*. Study No.: WIL-639158. EPL Reference No.: 140-123. Unpublished, Report date: January 17, 2013

Test Compound*:	Paraquat dichloride (purity: 99.9%)
Batch:*	ASJ10083-03 [WIL ID No. 110018]
Test Species:*	C57BL/6J mice, **stereological assessment**: 20/sex/dose, age: approximately 10 weeks, bw: 18.0 – 26.8 g for males and 14.9 – 21.1 g for females; source: The Jackson Laboratory, Bar Harbor, ME, USA
Study Duration:*	July 12, 2011 – January 17, 2013
Laboratory:	Experimental Pathology Laboratories (EPL), 45600 Terminal Drive, Sterling, VA 20166 (stereology) Sielken &Associates Consulting, Inc. 3833 Texas Av, Suite 230, Bryan, TX 77802 USA (statistical analyses of stereology data)
GLP & QA:	Yes
Guidelines:	Not applicable

*In-life component conducted by WIL Research Laboratories, LLC, 1047 George Road, Ashland, OH 44805-8946 USA (performing laboratory)

Dosing method	Vehicle	Dosing / observation period	Doses tested (ppm)
Dietary	None	Continuously in the diet for 13-weeks	0, 10, 50 (*Calculated average paraquat dichloride:* Males: 0, 2.4, 14.1 mg/kg bw/d Females: 0, 3.7, 21.5 mg/kg bw/d) *Calculated average paraquat ion:* Males: 0, 1.7, 10.2 mg/kg bw/d Females: 0, 2.7, 15.6 mg/kg bw/d)
Positive control: ip injection	0.9% sodium chloride for injection	4 injections, approx. 2 hours apart 7 days prior to scheduled euthanasia	10 mg (free base)/kg/dose [4 mg/mL]) 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine hydrochloride (MPTP HCl)

Method

This report presents the stereology analysis findings only, from the study titled *Subchronic (91-day) dietary study to assess the effects of paraquat dichloride on dopaminergic neurons in C57BL/6J mice (Beck, 2013).* In this study, male and female mice were administered paraquat dichloride in the diet continuously for 91-95 days at concentrations of 0, 10 or 50 ppm, to determine the effect(s) on dopaminergic neurons in the brains of mice. A positive control group received four intraperitoneal injections of 1-methyl-4-phenyl-1,2,3,6- tetrahydropyridine hydrochloride (MPTP HCl), approximately two hours apart (4 x 10 mg/kg bw) seven days prior to scheduled euthanasia.

Further details of the method and study design can be found in Beck (2013). Stereology examined the potential effect of paraquat on the numbers of dopaminergic neurons in the SN pc by specific immunostaining for TH which is a key enzyme in the production of DA. The number of TH^+ dopaminergic neurons, the number of TH^- neurons, and a calculation of the total number of neurons (TH^+ plus TH^-) in the SN pc was determined. The total contour volume of the SN pc was also measured.

Necropsy and brain tissue preparation:
All necropsies and brain perfusions were performed at the original performing laboratory (WIL Research Laboratories). Whole brains from mice designated to Subset I were received at EPL for stereology analysis. Tissue was processed and cut into 40 µm serial thicknesses by frozen section. This was directed through the coronal plane (i.e. transversely along the rostral-caudal axis) and sections placed in wells with a matrix grid enabling every third section containing the SN pc to be selected.

Prior to staining, brain sections were suspended in phosphate buffered saline (PBS) free-floating mesh wells to maximise antibody penetration to both sides of the tissue. Following several PBS washes and quenching of endogenous peroxidase activity via immersion in hydrogen peroxide in PBS , non-specific binding of antibodies to targets was blocked by immersion in a solution of 10% normal goat serum in PBS and 1% bovine serum albumin.

Immunohistochemistry:

Immunostaining was conducted according to a standard ABC method. Sections were incubated with the primary antibody (polyclonal anti-TH antibody of rabbit origin, 1:1000) initially, followed by the secondary antibody (biotinylated goat anti-rabbit Ig G, 1:1000). Following incubationin an ABC solution, visualisation of the reaction was accomplished by immersion in 3,3-diaminobenzidine. Following a number of rinses and mounting onto slides, sections were counterstained (FD Cresyl Violet Solution).

Stereology assessment:

Stereology assessments were conducted on prepared slides at EPL. Counts were made to estimate the number of TH^+, TH^- neurons and the total number of neurons (TH^+ plus TH^-) in the SN pc. These estimations were made using the optical fractionator approach using a systematic random sample methodology. According to OECD Guideline 20, the use of an efficient unbiased sampling technique is critical to maximise the usefulness of this assessment.Virtual outlines of the right and left SN pc were drawn manually via a computer mouse. To determine the number of TH^+/TH^-, images were overlaid with a virtual counting frame grid and neuronal cell bodies counted. Section thickness was measured at each counting site and a mean thickness per section calculated. An estimation of the number of TH neurons in the left and right SN pc was performed by software and calculated as follows:

N_{obj}: 1/ssf x 1/asf x 1/tsf x ΣQ

(N_{obj}= number of objects
ssf(section sampling fraction) = the number of SN pc sections evaluated/total number of SN pc sections microtomed
asf (area sampling fraction) = counting frame size (microns)/counting frame interval (microns)
tsf (thickness sampling fraction) = dissector height (microns)/mean measure section thickness (microns)
ΣQ= the number of cells counted)

In addition to neuronal cell counts and other details (eg. estimates of total area and volume of the SN pc), coefficient of error values were calculated and intra-study variability was assessed by including chromogenic-stained sections from two internal control animals. Results were subject to statistical analysis by a consultant (Sielken & Associates Consulting). Analysis was performed on 4 endpoints (TH^+ neurons, TH^- neurons, total neurons and total contour volume) and determined the sample mean and standard deviation, with a significant of 5% (Sielken RL, 2013).

Results

Slide preparation results

Brains from 6 mice (4 M from positive control group and 2 F from the 50 ppm paraquat group) could not be counted for neuron numbers due to damage during the preparation of the brain and were not used.

General findings

An overall assessment, in terms of mean number of sections evaluated, mean section thickness, mean number of sampling sites, mean coefficient of error values for TH neurons and mean total contour volume, showed very similar results between male and females. The mean section shrinkage was also similar (61.5% males *vs* 60.5% females). As the mean coefficient of error

values for males and females were less than 0.1, the study author stated that the stereological sampling was sufficiently rigorous.

Two internal control animals were periodically recounted and it was found that the variation in counts was similar to that in control specimens.

There was a correlation with the number of $TH^{+/-}$ neurons with the thickness of the section (Z-depth), attributed to variable immunostain penetration for different thicknesses of tissue. For TH^+ neurons, the mean number increased for thinner sections, but then decreased, with a peak in neuron numbers at a section thickness of 4 µm (Z-depth). However, for TH^- neurons stained with cresyl violet, the mean number progressively increased over Z-depths of 2 to 5 µm.

Stereology findings

In male and female mice treated with paraquat (10 or 50 ppm), there was no significant treatment-related effects. The number of TH^+ neurons, TH^- neurons, total neurons and total contour volumes of the SN pc were not statistically different from controls.

In positive control male mice (MPTP), stereological analysis of TH immunostained brain sections showed a statistically significant lower number (-10%) of TH^+ neurons in the SN pc compared to controls . The number of TH^- neurons was slightly increased (1%) compared to controls, however the total number of neurons (TH^+ + TH^-) was reduced (-6%) but not statistically significantly so. The total contour volume was statistically significantly reduced (-9%).

In females in the positive control group (MPTP), the results reflected those in males with the number of TH^+ neurons reduced (-5%), the number of TH^- neurons increased (4%), the total number of neurons reduced (-3%) and the total contour volume reduced (-3%) as compared to the negative controls. However, in females none of the results were statistically significant.

Table 53.Significant stereological findings in the SN pc

Group		Males				Females			
		TH^+ neurons	**TH^- neurons**	**Total neurons (TH^+ & TH^-)**	**Total contour volume (μm^3)**	**TH^+ neurons**	**TH^- neurons**	**Total neurons (TH^+ & TH^-)**	**Total contour volume (μm^3)**
0 ppm	**n**	20				20			
	Mean	15,173	7,510	22,683	345,661,900	14,813	6,024	20,838	339,634,150
	SD	2,447	2,039	3,197	27,303,337	2,657	1,442	2,937	29,853,427
MPTP	**n**	17[#]				20			
	Mean	13,675[*]	7,599	21,274	313,410,412[**]	14,004	6,298	20,302	329,448,500
	SD	2,298	1,816	3,449	32,778,538	2,344	1,420	2,692	34,881,823
	% change	-10	+1	-6	-9	-5	+4	-3	-3

*Significant $p \leq 0.05$, ** $p \leq 0.01$, [#] 3 mice from this group could not be evaluated due to irreparable tissue artifacts.

Conclusion

There were no statistically significant differences in the number of TH^+ or TH^- neurons, total neurons or contour volume in male and female mice treated with 10 or 50 ppm paraquat dichloride, as compared to negative controls (calculated average paraquat ion intake was 1.7, 10.2 mg/kg bw/d (males) and 2.7, 15.6 mg/kg bw/d (females)). A positive control group

(MPTP) demonstrated the validity of the test system with effects noted in males only in this group. Positive control male mice showed a statistically significant decrease for TH^+ neurons and SN pc contour volume, but no significant finding for TH^- or total neurons. Female mice in the positive control group did not show any statistically significant differences from negative controls for any of the four parameters.

2) Pathology evaluation of Beck (2013) study (Subset II):

Butt MT (2012) Final pathology report: *Subchronic (91-day) dietary study to assess the effects of paraquat dichloride on dopaminergic neurons in C57BL/6J mice*. Testing Facility Study Reference No.: WIL-639158. Sponsor Study Reference No.: TK0000969. Unpublished, Report date: November 27, 2012

Test Compound*:	Paraquat dichloride (purity: 99.9%)
Batch:*	ASJ10083-03 [WIL ID No. 110018]
Test Species:*	C57BL/6J mice, **pathological assessment**: 15 sex/dose (5/sex/dose positive control), age: approximately 10 weeks, bw: 18.0 – 26.8 g for males and 14.9 – 21.1 g for females; source: The Jackson Laboratory, Bar Harbor, ME, USA
Study Duration:*	July 12, 2011 – January 17, 2013
Pathology Laboratory:	Tox Path Specialists, LLC, 8420 Gas House Pike, Suite G, Frederick, MD 21701 (formerly Room 109, 20140 Scholar Drive, Hagerstown, MD 21742) Neuroscience Associates, Inc. 10915 Lake Ridge Drive, Knoxville, TN 37934 USA (preparation and staining of slides for neuropathology)
GLP & QA:	Yes
Guidelines:	Not applicable

*In-life component conducted by WIL Research Laboratories, LLC, 1047 George Road, Ashland, OH 44805-8946 USA (performing laboratory)

Dosing method	Vehicle	Dosing / observation period	Doses tested (ppm)
Dietary	None	Continuously in the diet for 13-weeks	0, 10, 50 (*Calculated average paraquat dichloride:* Males: 0, 2.4, 14.1 mg/kg bw/d Females: 0, 3.7, 21.5 mg/kg bw/d) *Calculated average paraquat ion:* Males: 0, 1.7, 10.2 mg/kg bw/d Females: 0, 2.7, 15.6 mg/kg bw/d)
Positive control: ip injection	0.9% sodium chloride for injection	4 injections, approx. 2 hours apart 7 days prior to scheduled euthanasia	10 mg (free base)/kg/dose [4 mg/mL]) 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine hydrochloride (MPTP HCl)

Method

This report presents the neuropathology findings only, from the study titled *Subchronic (91-day) dietary study to assess the effects of paraquat dichloride on dopaminergic neurons in C57BL/6J mice (Beck, 2013).* In this study, male and female mice were administered paraquat dichloride in the diet continuously for 91-95 days at concentrations of 0, 10 or 50 ppm, to determine the effect(s) on dopaminergic neurons in the brains of mice. A positive control group was maintained on basal diet throughout the diet, and seven days prior to scheduled euthanasia received four intraperitoneal injections of MPTP HCl, approximately two hours apart (4 x 10 mg/kg bw).

Further details of the method and study design can be found in Beck (2013).

The neuropathology component examined serial sections from the SN to the striatum using specialist staining techniques (including immunohistochemistry) to assess potential effects in this part of the brain, including necrosis, apoptosis, astrocyte reactions as well as an assessment of general morphology.

Necropsy and brain tissue preparation:
The original performing laboratory performed all necropsies and initial brain tissue preparation on those mice designated to Subset II.

Slide preparation:
At Neuroscience Associates, Inc (NSA) the brains were trimmed and multiembedded and sectioned at 30 µm *via* frozen section in the coronal plane through the striatum to/through the SN. All sections were collected. Sections were stained with AmCuAg (Amino Cupric Silver), MicroApoTag (TUNEL), IBA-1 (Ionised calcium binding adapter molecule 1), GFAP (glial fibrillary acidic protein), TH, Caspase-3 and Thionine. The details of these stains are included in the main report (Beck, 2012a, Table 12). In addition, laboratory positive control slides (for TUNEL, AmCuAg and Caspase-3) were stained to verify the staining procedures. GFAP and IBA-1 stains did not require controls as brains contain an internal control.

Slide evaluation
Prepared slides were examined by TPS. The regions of the brain evaluated were categorised as:

- Brain, Level Frontal Cortex
- Brain, Level Striatum
- Brain, Level Thalamus/Hypothalamus
- Brain, Level Midbrain (including the SN)
- Brain, Pons

Not all levels were evaluated for all stains, based on knowledge of relevant structures/cells detected by each stain and location in the brain.

Results

General comments
The slide preparation and staining procedure was found to be good quality and consistent and the internal positive control slides verified the staining procedure.

Gross pathology
The laboratory performing the euthanasia and necropsy noted numerous gross lesions in all dose groups, noted as discolourations or areas of 'red'. None of these lesions correlated to any microscopic changes according to the TPS pathologist, and were noted as being very common findings in perfusion fixed animals. These gross findings are considered artifactual.

Microscopic pathology
There were no paraquat-related histological changes in the brain at any of the sacrifice intervals (4/8/13 weeks), in either males or females.

Microscopic changes were noted in the positive control group. Decreased TH staining in the striatum was noted in all MPTP animals, and attributed to a decrease in dopaminergic synaptic terminals. This finding was less consistent in the SN (3/5 males and not found in any females). The author stated that this was consistent with the effects of MPTP in this animal model but can also be attributed to the difficulty in interpreting this stain when neuron loss is not complete. It was stated that this change must be somewhat prominent to be able to be distinguished from normal. Increases in GFAP (astrocyte stain) and IBA-1 (microglial) staining were seen consistently across all males and females in the striatum. This indicates astrocytes responding to cellular injury. It was stated that as this area of the brain is large and easily sectioned, these stains will show even diffuse changes in glial cell number/size. An increase in staining in the SN was only detected in a single male and no females, for both stains.

There were no apoptotic cells detected (via Caspase-3 or TUNEL stains). The study pathologist noted there were no findings in the Amino Cupric silver stained sections (to detect necrotic/disintegrating neurons and processes) and attributed this to the time frame of harvesting the tissues 7 days after the administration of MPTP, such that active necrosis had already occurred and remnants removed. The study pathologist further addressed the timing of the positive control, and stated that if the brains were harvested at an earlier time point (e.g. 2-4 days following administration) disintegrating neurons (and synaptic terminals) would have been detected with the Amino Cupric Silver stain. The author stated that identifying changes in the brain likely to be persistent was more important.

Overall, the neuropathology in the positive control animals was indicative of cellular damage, specifically to the dopaminergic neurons in the SN pc.

Table 54. Biologically significant microscopic findings in positive control mice (MPTP)

Stain	Area; finding	Interpretation	Incidence (average severity#)	
			MPTP Males	MPTP Females
TH	Striatum; Decreased TH staining	Decreased TH staining due to a loss of synaptic terminals from dopaminergic neurons in the SN pc	5/5 (3.40)	5/5 (2.00)
	SN pc; Decreased TH staining/decreased TH^+ neurons		3/5 (1.20)	0/5
	Ventral tegmental area; Decreased TH^+ neurons		3/5 (1.20)	0/5
GFAP	Striatum; Increased GFAP staining	Increased GFAP staining due to an increase in reactive astrocytes responding to injury to synaptic terminals	5/5 (4.00)	5/5 (4.00)
	SN pc; Increased GFAP staining	Increased GFAP staining due to an increase in reactive astrocytes responding to injury to dopaminergic neurons	1/5 (0.60)	0/5
IBA-1	Striatum; Increased IBA-1 staining		5/5 (2.00)	5/5 (2.00)
	SN pc; Increased IBA-1 staining		1/5 (0.40)	0/5

TH- tyrosine hydroxylase, GFAP – glial fibrillary acidic protein, IBA-1- Ionised calcium binding adapter molecule, SN pc- Substantia nigra pars compacta.
Average severity is calculated as the sum of severity scores for a particular diagnosis divided by the number of animals in the group or the number of animals with a particular tissue examined. Severity ranks from 1 (slight), 2 (minimal), 3 (mild), 4(moderate) and 5 (severe).

Findings considered incidental included: Silver positive axons/terminals in a single block (considered artifactual) and increased IBA-1 staining in the striatum and frontal cortex (considered within normal variation). One positive control mouse was found to have a mild increase in silver positive axons in one side of the optic tract. The cause was not determined.

Conclusion

Neuropathological assessment showed that paraquat dichloride administered in the diet of mice at 10 or 50 ppm for up to 13 weeks did not cause any effects, either gross and histologically, to any of the brain regions examined (frontal cortex, striatum, or midbrain including the SN). More specifically, following specialty staining techniques, paraquat was not associated with any neuronal or glial changes in the SN or the striatum. Mice treated with the positive control agent, MPTP, showed damage to the dopaminergic neurons in the SN pc within this test system, demonstrating its validity.

3) Neurochemistry of Beck (2013) study (Subset III):

Matthews JM (2012) Determination of dopamine, dopamine metabolites, and serotonin in mouse striatal brain tissues. Performed in support of *Subchronic (91-day) dietary study to assess the effects of paraquat dichloride on dopaminergic neurons in C57BL/6J mice.* Study No./Report No.: WIL-639158. Syngenta Task No.: TK0000969. RTI Report No.: 0212823.008. Unpublished, Report date: December 3, 2012

Test Compound*:	Paraquat dichloride (purity: 99.9%)
Batch*:	ASJ10083-03 [WIL ID No. 110018]
Test Species:*	C57BL/6J mice, **neurochemistry assessment:** 6/ sex/dose, age: approximately 10 weeks, bw: 18.0 – 26.8 g for males and 14.9 – 21.1 g for females; source: The Jackson Laboratory, Bar Harbor, ME, USA
Study Duration:*	July 12, 2011 – January 17, 2013
Laboratory:	Drug Metabolism and Pharmacokinetics, Pharmacology & Toxicology, Discovery Sciences, RTI International, 3040 Cornwallis Road, Research Triangle Park, NC 27709-2194
GLP & QA:	Yes
Guidelines:	Not applicable

*In-life component conducted by WIL Research Laboratories, LLC, 1047 George Road, Ashland, OH 44805-8946 USA (performing laboratory)

Dosing method	Vehicle	Dosing / observation period	Doses tested (ppm)
Dietary	None	Continuously in the diet for 13-weeks	0, 10, 50 (*Calculated average paraquat dichloride:* Males: 0, 2.4, 14.1 mg/kg bw/d Females: 0, 3.7, 21.5 mg/kg bw/d) *Calculated average paraquat ion:* Males: 0, 1.7, 10.2 mg/kg bw/d Females: 0, 2.7, 15.6 mg/kg bw/d)
Positive control: ip injection	0.9% sodium chloride for injection	4 injections, approx. 2 hours apart 7 days prior to scheduled euthanasia	10 mg (free base) /kg/dose [4 mg/mL]) 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine hydrochloride (MPTP HCl)

Method

This report presents the neurochemical findings only from the study titled *Subchronic (91-day) dietary study to assess the effects of paraquat dichloride on dopaminergic neurons in C57BL/6J mice (Beck, 2013).* In this study, mice were administered paraquat dichloride in the diet continuously for 91-95 days at concentrations of 0, 10 or 50 ppm, to determine the effect(s) on dopaminergic neurons in the brains of mice. A positive control group received four intraperitoneal injections of MPTP HCl, approximately two hours apart (4 x 10 mg/kg bw),

seven days before scheduled euthanasia. Further details of the method and study design can be found in Beck (2013).

The aim of the neurochemistry component was to quantitate DA and the two DA metabolites –HVA and DOPAC, and 5-HT in striatal tissues using HPLC coupled with ECD.

Necropsy and brain tissue preparation:
The original performing laboratory performed all euthanasia and necropsies. Dissected left and right striatums from mice designated to Subset III were sent to RTI for for neurotransmitter analysis. RTI was also provided with the wet tissue weights.

HPLC analysis
Preparation of strial tissues involved homogenisation, centrifugation and collection of an aliquot of the supernatant for injection into an ECD. The striatal concentrations for each neurochemical were determined by comparison with standard curves prepared using the same homogenisation solution. The percentage recovery was also determined based on an internal standard, DHBA.

DA turnover for each tissue was expressed as a ratio of the sum of DOPAC and HVA concentrations to that of DA.

Results
Validation of the method for quantification of 5-HT did not pass, therefore these measurements were considered only an estimate. The study author stated that the striatal concentrations determined in the negative control mice (DA, DOPAC, HVA and 5-HT) were consistent with concentrations previously reported in this particular laboratory.

Treatment with paraquat did not cause a dose-related effect on DA or its two metabolites, across both dose groups, with both increases and decreases in percentage difference from negative controls noted . For males, this was particularly inconsistent. For females, DA was unchanged at 10 ppm and increased from controls by 20% at 50 ppm (positive control -33%). However the direction of change is not considered to be toxicologically significant.

For DOPAC, levels in females were dose-relatedly reduced (-43% and -49%) for 10 and 50 ppm paraquat treated groups respectively (positive control reduced by -49%), and for HVA the levels were reduced, but not dose relatedly so (-14% for 10 ppm and -2% for 50 ppm, positive control -20%). The significance of the DOPAC levels being dose relatedly reduced is unclear, without corresponding findings in the DA levels, and findings in males. A higher than expected mean DOPAC level in negative control females also skews these results.

5-HT, as an estimate only, was increased from controls in all groups tested. For 10 ppm, 50 ppm and positive controls, the percentage increased from controls for males was + 44% and +22% (+21% for positive controls), and for females was +11% and +2.9% (+9.4% for positive controls), for 10 ppm and 50 ppm groups respectively. This lacks a dose relationship however and in addition limited interpretation of the 5-HT levels can be made as the analytical method was not validated.

The positive control group showed marked effects in the levels of these neurotransmitters. The percentage difference from controls for DA, DOPAC and HVA were -86%, -68% and -52% (males) and -33%, -49% and -20% (females) respectively . The mean DA turnover for male mice was +221% and for females was only +1.3%. It is evident from these figures that the effects in females were not as great as those for males, however the study author stated that MPTP has previously been demonstrated to induce greater neurotoxicity in male mice than females. Considering the higher than expected mean DOPAC levels detected in female negative control mice, this finding would have artificially increased the apparent difference in DOPAC concentrations in other groups, as well as the lower DA turnover in positive control females.

Table 55. Striatal neurotransmitter and metabolite levels (mean and percentage change from negative control)

Mean / % change from ctrl		Striatal Neurotransmitter/Metabolite Levels (ng/mg tissue)				DA turnover*
Group	Sex (n)	DA	DOPAC	HVA	5-HT#	
Negative control	M (6)	16.55	1.66	1.94	0.62	0.220
	F (6)	16.75	3.25	1.91	0.66	0.330
10 ppm paraquat	M (5)	16.16 -2.4%	1.70 +2.2%	2.16 +11.7%	0.90 +44.1%	0.248 +12.8%
	F (6)	16.75 0%	1.86 -42.7%	1.65 -13.8%	0.73 +11.2%	0.215 -34.9%
50 ppm paraquat	M (5)	18.43 +11.4%	1.35 -18.8%	1.94 +0.3%	0.76 +21.9%	0.180 -17.8%
	F (5)	20.14 +20.3%	1.66 -48.9%	1.87 -2.4%	0.68 +2.9%	0.176 -46.7%
Positive control (MPTP)	M (6)	2.32 -86.0%	0.53 -68.0%	0.93 -52.2%	0.75 +20.9%	0.706 +221.3%
	F (6)	11.23 -32.9%	1.66 -49.0%	1.53 -20.0%	0.72 +9.4%	0.326 -1.3%

* Estimated by dividing the sum of the DOPAC and HVA concentrations by the DA concentration, and the data is expressed as a ratio. # 5-HT levels are considered an estimate only.

Conclusion

Paraquat dichloride administered to male and female mice in the diet at doses of 10 or 50 ppm for 13 weeks did not result in a treatment related effect on the levels of DA or its two metabolites (HVA and DOPAC) in striatal tissues of the brain, as compared to negative control tissues. Positive control male mice showed marked decreases in mean DA, DOPAC and HVA concentrations, which is consistent with the known neurotoxicity of MPTP. Effects were not as marked in positive control female mice.

APPENDIX III PUBLISHED STUDIES NOT RELIED ON

In 2012, the Office of Research Integrity (ORI) of the US Department of Health & Human services found that Dr. Mona Thiruchelvam "engaged in research misconduct by falsifying and fabricating cell count data that she claimed to have obtained through stereological methods in order to falsely report the effects of combined exposure of the pesticides paraquat and maneb on dopaminergic neuronal death and a neuroprotective role for estrogen in a murine model of Parkinson's disease. The respondent provided to the institution corrupted data files as the data for stereological cell counts of nigrostriatal neurons in brains of several mice and rats by copying a single data file from a previous experiment and renaming the copies to fit the description of 13 new experiments composed of 293 data files when stereological data collection was never performed for the questioned research".

"The fabricated data, falsified methodology, and false claims based on fabricated and falsified data were reported in two NIEHS, NIH, grant applications, two publications, a poster, and a manuscript in preparation".

The following two papers have been retracted:

- Rodriguez, V.M., Thiruchelvam, M., & Cory-Slechta, D.A. " Sustained Exposure to the Widely Used Herbicide, Atrazine: Altered Function and Loss of Neurons in Brain Monamine Systems." *Environ Health Perspect*. **113**(6):708-715, 2005.
- Thiruchelvam, M., Prokopenko, O., Cory-Slechta, D.A., Richfield, E.K., Buckley, B., & Mirochnitchenko, O. "Overexpression of Superoxide Dismutase or Glutathione Peroxidase Protects against the Paraquat + Maneb-induced Parkinson Disease Phenotype." *J. Biol. Chem.* **280**(23):22530-22539, 2005.

The latter paper was initially reviewed for this assessment of paraquat by the OCS, and is discussed in this neurotoxicity Supplement. Following the retraction, this paper was no longer relied on.

Source: http://ori.hhs.gov/content/case-summary-thiruchelvam-mona
(Federal Register Volume 77, Number 125 (Thursday, June 28, 2012) Pages 38632-38633. FR Doc No: 2012-15887. http://www.gpo.gov/)
Due to the fraudulent nature of Dr.Thiruchelvams's data in the publications listed above, uncertainty is cast on the reliability of other research papers involving Dr. Thiruchelvam's input. Therefore, the following papers are considered unsuitable for regulatory purposes:

McCormack AL, Thiruchelvam M, Manning-Bog AB, Thiffault C, Langston JW, Cory-Slechta DA & Di Monte DA (2002) Environmental risk factors and Parkinson's disease: selective degeneration of nigral dopaminergic neurons caused by the herbicide paraquat. *Neurobiol Dis* 10(2):119-127.

McCormack *et al* (2002) using doses of paraquat of 1, 5 and 10 mg/kg bw ip in mice ranging from 6 weeks to 18 months of age, demonstrated the apoptotic death of dopaminergic neurons in the SN of mouse brain which was dose and age dependent. The loss was confined to dopaminergic neurons of the *pars compacta* of the Sn and did not occur among the *pars reticulata* of the SN or the hippocampus.

Figure 2: Tyrosine hydroxylate positive neurons were counted using a stereological technique in the SN pc of mice treated with different paraquat doses (A), and animals of different ages injected with 10 mg/kg bw paraquat (B). Paraquat was administered once a week for three consecutive weeks, and mice were killed at 7 days after the last paraquat treatment. Data represent mean ± SEM ($n \geq 4$). $^{*}p < 0.05$ compared with the corresponding saline control. $^{\dagger}p < 0.05$ compared with all other treatment groups in B.

There was evidence of neurochemical compensatory production in surviving neurons. This loss of dopaminergic neurons was not matched by a significant occurrence of DA depletion. Measurements of striatal TH activity revealed a significant increase in all of three age groups injected with paraquat with the greatest effect in the 5 month old mice (70%) and the smallest in the 18 month old mice (28%). This increase suggested an enhancement of DA synthesis and a period of compensatory activity aimed restoring tissue levels of DA. A consequence of the compensatory activity may be that induced nigrostriatal injury may remain, at least initially, relatively silent from the neurochemical standpoint.

McCormack *et al* (2002) also noted a paraquat induced glial response in homogenates from the ventral mesencephalon, frontal cortex and cerebellum. The highest levels of glial proteins were observed at 2 days after the second and third of the 3 weekly injections.

As described at the beginning of this Supplement, this study is considered inconclusive and not suitable for regulatory purposes.

Prasad K, Winnik B, Thiruchelvam MJ, Buckley B, Mirochnitchenko O, Richfield EK (2007) Prolonged toxicokinetics and toxicodynamics of paraquat in mouse brain. *Environ Health Perspect* Oct;115(10):1448-53.

Prasad *et al* (2007) demonstrated that paraquat, following a single dose of 10 mg/kg bw ip, was persistent in the ventral midbrain (VM) and complete elimination did not occur after 4 weeks. When data from the longest time course (4 weeks) was plotted using a log scale, the elimination appeared linear, with a brain half-life of approximately 4 weeks ($r^2 = 0.76$ linear regression). [log scale values: -1.3, -2.1, -1.8, -1.9, -2.1, -2.8 at 0, 1, 4, 7, 14 and 28 days respectively (0.28, 0.12, 0.16, 0.13, 0.12, 0.06 ng/mg tissue) – mean data (n=4) extrapolated from curves produced by the authors; cf level in liver of 5.1 ng/mg tissue at 1 day which was eliminated by day 4].

When administered for a total of 1, 3 (M-W-F) or 5 doses (M-W-F-M-W) at 10 mg/kg bw ip per dose and measured in the VM one week after the last dose, there was a linear increase in

the amount of paraquat (r^2 = 0.99) with the number of doses. The concentration of paraquat was 0.12, 0.33 and 0.55 ng/mg tissue for the single, 3 dose and 5 dose groups respectively.

When paraquat was administered orally in single doses of 10, 20, or 50 mg/kg bw, a similar linear increase in paraquat levels was observed in the VM following an increasing dose (r^2 = 1.00). The concentration of paraquat was 0.07, 0.12 and 0.26 ng/mg tissue for each dose respectively.

Under the conditions of this study paraquat persisted in the VM of mice for a prolonged time with a half-life of approximately one month. The accumulation of paraquat in the ventral midbrain with repeated exposure was demonstrated. The reason for this persistence in brain in contrast to that in other organs was not known.

As described at the beginning of this Appendix, this study is considered inconclusive and not suitable for regulatory purposes.

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

A series of experiments were performed to determine the selectivity and sensitivity of a combination of paraquat and maneb (manganese ethylenebisdithiocarbamate) for the nigrostriatal system, the impact of exposure duration and the involvement of SN dopamine cell bodies. Male mice were injected intraperitoneally twice a week for 6 weeks with saline, 10 mg/kg bw paraquat (equivalent to 7.2 mg/kg bw paraquat cation), 30 mg/kg bw maneb or a combination of paraquat and maneb (as 2 separate injections). In the absence of any signs of toxicity (histopathological lung abnormalities or effects on body weight), repeated dosing of mice with paraquat/maneb resulted in significantly decreased locomotor activity, neurochemical alterations (increased striatal DA and DOPAC) and variations in brain protein levels (decreased TH and DAT, and increased glial fibrillary acidic protein in the dorsal striatum; reduced TH and cell counts in the SN), the effects of which were greater than either chemical on its own. Evidence of potentiation between paraquat and maneb was minimal with the majority of results suggesting an additive effect. The study authors however, concluded that paraquat and maneb had a synergistic effect. On its own, maneb was shown to cause a significant reversible decrease in motor activity, and to increase striatal DA and DOPAC. Paraquat had no effect on locomotor activity (except after a single ip injection of 15 mg/kg bw MPTP given after the 12th injection of paraquat) or brain protein levels, but caused a reversible increase in striatal DA. A single ip injection of 15 mg/kg bw MPTP potentiated the neurotoxic effect of paraquat, maneb and paraquat/maneb.

As described at the beginning of this Appendix, this study is considered inconclusive and not suitable for regulatory purposes.