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Pharmacokinetic, neurochemical, stereological and neuropathological studies on the potential effects of paraquat in the substantia nigra pars compacta and striatum of male C57BL/6J mice

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ABSTRACT

The pharmacokinetics and neurotoxicity of paraquat dichloride (PQ) were assessed following once weekly administration to C57BL/6J male mice by intraperitoneal injection for 1, 2 or 3 weeks at doses of 10, 15 or 25 mg/kg/week. Approximately 0.3% of the administered dose was taken up by the brain and was slowly eliminated, with a half-life of approximately 3 weeks. PQ did not alter the concentration of dopamine (DA), homovanillic acid (HVA) or 3,4-dihydroxyphenylacetic acid (DOPAC), or increase dopamine turnover in the striatum. There was inconsistent stereological evidence of a loss of DA neurons, as identified by chromogenic or fluorescent-tagged antibodies to tyrosine hydroxylase in the substantia nigra pars compacta (SNpc). There was no evidence that PQ induced neuronal degeneration in the SNpc or degenerating neuronal processes in the striatum, as indicated by the absence of uptake of silver stain or reduced immunolabeling of tyrosine-hydroxylase-positive (TH^+) neurons. There was no evidence of apoptotic cell death, which was evaluated using TUNEL or caspase 3 assays. Microglia (IBA-1 immunoreactivity) and astrocytes (GFAP immunoreactivity) were not activated in PQ-treated mice 4, 8, 16, 24, 48, 96 or 168 h after 1, 2 or 3 doses of PQ.

In contrast, mice dosed with the positive control substance, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP; 10 mg/kg/dose × 4 doses, 2 h apart), displayed significantly reduced DA and DOPAC concentrations and increased DA turnover in the striatum 7 days after dosing. The number of TH^+ neurons in the SNpc was reduced, and there were increased numbers of degenerating neurons and neuronal processes in the SNpc and striatum. MPTP-mediated cell death was not attributed to apoptosis. MPTP activated microglia and astrocytes within 4 h of the last dose, reaching a peak within 48 h. The microglial response ended by 96 h in the SNpc, but the astrocytic response continued through 168 h in the striatum.

These results bring into question previous published stereological studies that report loss of TH^+ neurons in the SNpc of PQ-treated mice. This study also suggests that even if the reduction in TH^+ neurons reported by others occurs in PQ-treated mice, this apparent phenotypic change is unaccompanied by neuronal cell death or by modification of dopamine levels in the striatum.

1. Introduction

Paraquat (1,1′-dimethyl-4,4′-bipyridinium dichloride, [PQ]) is a non-selective herbicide that interferes with photosynthesis (photosystem I) and damages plant membrane proteins by the production of oxygen free radicals. PQ is used pre-planting or pre-emergence on a variety of crops, and post-emergence around fruit

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trees, vegetables, vines, and sugar cane (Lock and Wilks, 2001). Although occupational exposure to PQ occurs mainly by the dermal route, PQ is poorly absorbed through intact human skin (0.03 μg/cm^2 over 24 h), with only ~0.3% of the applied dose absorbed within 24 h (Wester et al., 1984).

The association between pesticide use, rural living or living on a farm and the occurrence of Parkinson's disease (PD) has been reviewed (Wirdefeldt et al., 2011) and evaluated in several meta-analyses (Priyadarshi et al., 2000; Brown et al., 2006; Van der Mark et al., 2012) but reports of an association between PQ use and PD (Liou et al., 1997; Tanner et al., 2011) have been questioned (Berry et al., 2010; Mandel et al., 2012; Van Maele-Fabry et al., 2012). In addition, although PQ is frequently stated to be a structural analog of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a chemical frequently used as an animal model of PD (Jackson-Lewis and Smeyne, 2005; Jackson-Lewis et al., 1995, 2012; Jackson-Lewis and Przedborski, 2007), there is doubt that the reported effect of PQ on dopaminergic neurons could be mediated by the same mechanism as described for 1-methyl-4-phenylpyridinium (MPP$^+$), the active metabolite of MPTP (Miller, 2007; Richardson et al., 2007; Ramachandiran et al., 2007).

When PQ was administered by intraperitoneal (ip) injection to male C57BL/6J mice, a reduction in the number of tyrosine-hydroxylase-positive (TH$^+$) neurons in the substantia nigra pars compacta (SNpc) has been reported (Brooks et al., 1999; McCormack et al., 2002; Jiao et al., 2012). However, because the detection of dopaminergic neurons depends on TH$^+$ immunoreactivity, a reduction in the number of TH$^+$ neurons is not conclusive evidence that dopaminergic neurons have actually died. Although some authors have reported that the total number of neurons in the SNpc was reduced concomitantly with the reduction in the number of TH$^+$ neurons in Nissl-counterstained sections (McCormack et al., 2002), few investigators have assessed the effect of PQ on total neuronal count in the SNpc using Nissl-only stain. Furthermore, because the reduction in the number of TH$^+$ neurons has been reported to occur only after the second or third ip injection of PQ, but not after the first injection (McCormack et al., 2005), it is important to consider the timing of dose and the number of doses administered when assessing effects of PQ on the SNpc.

There is limited supporting evidence (McCormack et al., 2002, 2006; Purisai et al., 2007; Mitra et al., 2011) and some contradictory evidence (McIntosh et al., 2010) that dopaminergic neurons die following either PQ (McCormack et al., 2002, 2006) or PQ plus maneb (McIntosh et al., 2010) treatment, as indicated by the uptake of amino cupric silver stain and the activation of microglia and astrocytes in response to the appearance of dying and degenerating neurons. In one study, it was suggested that an observed reduction in TH$^+$ neurons in the SNpc in mice administered 10 doses of PQ (7 mg/kg/dose) over a 20-day period was due to cell death by an apoptotic mechanism (Peng et al., 2004).

The concentrations of dopamine (DA) and its metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), as well as DA turnover in the striatum of C57BL/6J mice, have been reported to be either unaffected (Woolley et al., 1989) or only marginally affected by PQ treatment (McCormack et al., 2002, 2005). Others have reported reductions in DA levels (Kang et al., 2009) and/or increases in DA turnover (Shepherd et al., 2006; Songin et al., 2011) in adult mice when the animals were exposed to PQ prenatally (Barlow et al., 2004), or when PQ was administered daily for 24 days prior to an evaluation which occurred 7 days after the last dose (Prasad et al., 2009).

There is indirect evidence that PQ may be transported across the blood–brain barrier *via* the neutral amino acid transporter (Shimizu et al., 2001; McCormack & Di Monte, 2003) or the organic cation transporters, OCT-2 and OCT-3 (Chen et al., 2007; Rappold et al., 2011). While it has been postulated that PQ is taken up into dopaminergic neurons by the dopamine transporter (DAT), the divalent paraquat cation does not appear to be a substrate for DAT (Foster et al., 2004; Richardson et al., 2005). Recent studies have suggested that the paraquat monovalent cation, which could form in a reduced anoxic environment, is transported into dopamine neuron terminals by DAT (Rappold et al., 2011) in a manner analogous to the transport of MPP$^+$ (Cui et al., 2009). However, direct measurement of [^{14}C] PQ in the brain has consistently shown low-level, homogenous labeling with no evidence of tissue localization, except in pigmented structures (Lindquist et al., 1988) or in structures outside the blood–brain barrier, such as the olfactory bulb and the tissues lining the lateral ventricles of the brain (Lindquist et al., 1988; Widdowson et al., 1996).

The pharmacokinetics of PQ in plasma and the brain have been described for male C57BL/6J mice (Prasad et al., 2007, 2009), the animal model that is most frequently used to assess the effect of PQ on dopaminergic neurons (McCormack et al., 2002). Prasad et al. (2009) reported that it took approximately 18 days of PQ treatment to reach a steady-state concentration in the frontal cortex, striatum, hippocampus and cerebellum of male C57BL/6J mice, and that PQ was slowly eliminated from the brain after the last dose.

The present series of studies were conducted to further investigate the mechanisms of action underlying the reported neurochemical and stereological effects of 1, 2 or 3 ip doses of 10 mg PQ/kg/occasion. Initial findings failed to confirm stereological results reported in the literature. In order to understand the basis for this, the potential dose-response was evaluated using an intermediate (15 mg PQ/kg/occasion) and a maximum-tolerated dose (25 mg PQ/kg/occasion) in young adult male C57BL/6J mice. In addition, the effects of highly-purified, analytical-grade PQ (supplied by Syngenta) were compared to the effects of PQ obtained from Sigma–Aldrich, a source of PQ commonly used by other investigators. Histopathological correlates of the reported loss of TH$^+$ stained dopaminergic neurons and neuronal processes in the SNpc and striatum were evaluated by conducting detailed, blinded, intensive time-series evaluation of indicators of cellular necrosis, apoptosis and glial activation in the midbrain and striatum. Pharmacokinetic parameters were determined in blood, plasma and brain to relate any effect of PQ on stereological or pathological endpoints to the concentration of PQ in the brain. A relatively low dose of MPTP (10 mg/kg administered 4 times in a single day with a 2 h inter-dose interval) was used as a positive control throughout this series of studies to determine if the methodology employed was sensitive enough to detect small changes in the number of TH$^+$ neurons in the SNpc.

2. Materials and methods

2.1. Study conduct

The in-life phase of studies reported herein were conducted in the AAALAC-accredited (Association for Assessment and Accreditation of Laboratory Animal Care International) laboratory of WIL Research Laboratories, LLC. The pharmacokinetic study (Study 7) was a non-GLP investigative study conducted at Syngenta's Central Toxicology Laboratory, UK. The other studies were conducted according to Good Laboratories Practices Regulations (US EPA, 1989; OECD, 1997) and the protocols for these studies were approved by the Institutional Animals Care and Use committee at WIL Research Laboratories. Brain neurochemistry investigations were performed by RTI International (Research Triangle Park, NC). Microscopic slides of brain sections for pathology evaluation were prepared by Neuroscience Associates Inc., Knoxville, TN and examined by a pathologist (Tox Path Specialists, Frederick, MD)

who was blinded to treatment group. Brain tissues for stereological investigations were prepared and evaluated by a stereologist at Experimental Pathology Laboratories (EPL, Sterling, VA) who was blinded to treatment group. Analyses of PQ ion concentration in samples of brain tissue were conducted by Charles River Laboratories, UK.

2.2. Test article/dose preparation

The positive control substance, MPTP hydrochloride, a 100% pure fine white powder (Lot Number 128K1549), was obtained from Sigma–Aldrich Inc., St. Louis, MO, and was stored at room temperature. The test chemical, PQ dichloride, a coarse, light-gray-to-white powder, was 99.9% pure and was supplied either by Syngenta (Lot Number ASJ10083-03 [SYN PQ]) or by Sigma–Aldrich (paraquat dichloride monohydrate, Lot Number SZE8163X [SIG PQ]). PQ was stored with a desiccant at a temperature of 2–8 °C. The vehicle control solution was 0.9% sodium chloride for injection, USP (physiological saline; Lot Numbers C775940 and C783100, manufactured by Baxter Healthcare Corporation, Deerfield, IL; and Lot Numbers 75-179-DK, 76-255-DK, and 76-432-DK, manufactured by Hospira Inc., Lake Forest, IL). All materials were used within the period defined by their respective expiration dates. Both MPTP and PQ were formulated as solutions in 0.9% sodium chloride at appropriate concentrations. All dose concentrations used throughout these studies were verified by HPLC to be homogeneous, stable and within ± 10% of the target concentration prior to dose administration.

2.3. Test animal/animal husbandry

Male C57BL/6J mice were supplied by the Jackson Laboratories, Bar Harbor, ME. Each study utilized a single batch of animals received at an approximate age of 6–7 weeks. Treatment with PQ or vehicle commenced at approximately 9–10 weeks of age, and MPTP administration occurred when the mice were approximately 11–12 weeks of age. The mice were housed in individual, stainless-steel, wire-mesh-floored cages. Food (PMI Nutrition International, LLC Certified Rodent LabDiet® 5002) and the municipal water supply were evaluated for potential contaminants.

During the 3–5 week quarantine period and subsequently, each animal was observed twice daily for mortality, as well as for changes in general appearance or behavior. At the end of the quarantine period, the mice were randomly assigned to control and treatment groups using a randomized block design, stratified by weight. Individual body weight and food consumption were recorded weekly for all animals beginning 2 weeks prior to the initiation of vehicle, MPTP or PQ administration. A 12 h light–dark photoperiod was maintained throughout each study; room temperature and relative humidity were maintained at 22 °C ± 3 °C and 50% ± 20%, respectively.

2.4. Dose administration

PQ was administered by ip injection, either 1, 2 or 3 times, with each dose separated by 1 week (Table 1). Doses evaluated in Studies 1 through 7 included 1, 10, 15, 25, 30 or 35 mg PQ salt/kg/occasion (Table 1). MPTP (expressed as free base) was administered every 2 h by ip injection at a dose of 10 mg/kg/occasion with a total of 4 doses being administered over an 8 h period. The dose volume was 10 ml/kg. The vehicle control (VC) group was administered 3 doses of vehicle, with each injection administered 1 week apart. The experimental designs for Studies 3 through 6 are shown in Table 2.

2.5. Concentration of PQ in forebrain and neurotransmitter concentrations in the striatum

Mice were euthanized by cervical dislocation, followed by decapitation, 7 days after the last scheduled dose of either MPTP or

Table 1
Experimental design of studies conducted on PQ and MPTP.

Study	Study title	MPTP dose[a]	PQ dose[b]	PQ conc[c]	Neurochem[d]	Stereology[e]	Pathology[f]
1	PQ dose tolerance	NE	20, 25, 30, 35 mg/kg × 3	NE	NE	NE	Lung, liver, kidney
2	Neurochemistry study[g]	10 mg//kg × 4	10 mg/kg × 3	NE	**Yes**	NE	NE
3	Pathology: 1, 2, 4, or 7 days after last dose[h]	10 mg/kg × 4	10 mg/kg × 1 or 2	NE	**Yes**	NE	**Yes**
4	Stereology: 7 days after last dose[g]	10 mg/kg × 4	**SIG vs. SYN PQ** 10 mg/kg × 3 **SYN PQ** 15 mg/kg × 1, 2 or 3	**Yes**	No	**Yes**	**Yes**
5	Stereology: 7 days after last dose[h]	10 mg/kg × 4	10 mg/kg × 3 15 mg/kg × 3 25 mg/kg × 3	No	**Yes**	**Yes**	No
6	Pathology: 4, 8, 16, 24, 48, 96 or 168 h after last dose[h]	10 mg/kg × 4	10 mg/kg × 1, 2 or 3 15 mg/kg × 1, 2 or 3 25 mg/kg × 1, 2 or 3	NE	NE	No	**Yes**
7	Pharmacokinetic: [^{14}C] PQ concentration in blood, plasma and brain 0.25, 0.5, 0.75, 1, 1.5, 4, 24, 72, 168 and 672 h after 1, 2 and 3 doses	NE	**Blood and plasma** 1 or 10 mg/kg × 1 dose **Brain** 1 or 10 mg/kg/dose × 1, 2 or 3 doses	**Yes**	NE	NE	NE

[a] MPTP administered by ip injection 4 times/day (2 h inter-dose interval) for a total dose of 40 mg/kg/day.
[b] Syngenta PQ or Sigma PQ (Study 4) administered by ip injection 1 time/day (7 day inter-dose interval) for a total of 1, 2 or 3 doses.
[c] PQ concentration was measured in the forebrain, anterior to the striatum.
[d] Striatal dopamine, DOPAC, HVA and dopamine turnover.
[e] Number of TH$^+$ neurons, Nissl-stained neurons and the total number on neurons determined in the SNpc.
[f] Cell death (cupric silver, TUNNEL, caspase 3), glial activation (IBA-1, GFAP), TH$^+$ and/Nissl staining in SNpc and striatum.
[g] Mice for stereology and/or neurochemistry killed by cervical dislocation and decapitation. DA, DOPAC, HVA and DA turnover evaluated in homogenates collected from the left and right striatum of male C57BL/6J mice administered either PQ or MPTP by ip injection according the to the dose regimen indicated.
[h] Mice for stereology and pathology killed by cardiac perfusion following anesthesia with sodium pentobarbital.
NE, not evaluated; VC, vehicle control; PQ, paraquat; WT, wild type; KO, knock out; SYN, Syngenta, SIG, Sigma–Aldrich.

Table 2
Experimental design for Studies 3, 4, 5 and 6.

Study 3: number of mice/group in the combined neurochemistry, stereology[a] and pathology study

Group	Treatment[b]	Neuropathology subsets				Stereology
		Subset A[c] (24 h)	Subset B[c] (48 h)	Subset C[c] (96 h)	Subset D[c] (168 h)	subset E[d] (day 14)
1-VC	Vehicle	5	5	5	5	5
2-Syn	PQ (1 × 10)	5	5	5	5	5
3-Syn	PQ (2 × 10)	5	5	5	5	5
4-Sig	MPTP (4 × 10)	5	5	5	5	5

Study 4: number of mice/group in the combined brain PQ, stereology and pathology study

Group	Treatment[b]	Neuropathology subsets				Stereology
		Subset A[c] (24 h)	Subset B[c] (48 h)	Subset C[c] (96 h)	Subset D[c] (168 h)	subset E[d] (day 14)
1-VC	Vehicle	5	5	5	5	10
2-Syn	PQ (3 × 10)	5	5	5	5	10
3-Syn	PQ (3 × 15)	0	5	0	5	10
4-Syn	PQ (2 × 15)	0	5	0	5	10
5-Syn	PQ (1 × 15)	0	5	0	5	10
6-Sig	PQ (3 × 10)	5	5	5	5	10
7-Sig	MPTP (4 × 10)	0	5	0	5	10

Study 5: number of mice/group in the combined neurochemistry and stereology study

Group	Treatment[b]	Stereology[d] chromogenic	Stereology[d] fluorescent	Neurochemistry[d]
1-VC	Vehicle	10	10	10
2-Syn	PQ (10 × 3)	10	10	10
3-Syn	PQ (15 × 3)	10	10	10
4-Syn	PQ (25 × 3)	10	10	10
5-Sig	MPTP (10 × 4)	10	10	10

Study 6: number of mice/group in the pathology study

Group	Treatment[b]	Subset A 4 h	Subset B 8 h	Subset C 16 h	Subset D 24 h	Subset E 48 h	Subset F 96 h	Subset G 168 h
1-VC	Vehicle	5	5	5	5	5	5	5
2-Syn	PQ (1 × 10)	5	5	5	5	5	5	5
3-Syn	PQ (1 × 15)	5	5	5	5	5	5	5
4-Syn	PQ (1 × 25)	5	5	5	5	5	5	5
5-Syn	PQ (2 × 10)	5	5	5	5	5	5	5
6-Syn	PQ (2 × 15)	5	5	5	5	5	5	5
7-Syn	PQ (2 × 25)	5	5	5	5	5	5	5
8-Syn	PQ (3 × 10)	5	5	5	5	5	5	5
9-Syn	PQ (3 × 15)	5	5	5	5	5	5	5
10-Syn	PQ (3 × 25)	5	5	5	5	5	5	5
11-Sig	MPTP (10 × 4)	5	5	5	5	5	5	5

[a] Tissues collected but the slides were inadequate to conduct stereology.
[b] Mice were administered either 2 (Study 3) or 3 doses (Studies 4–6) of the vehicle at a dose volume of 10 ml/kg or 1, 2 or 3 doses of 10, 15 or 25 mg PQ/kg/dose or 4 doses of MPTP on a single day at a dose of 10 mg/kg MPTP/dose (Total MPTP dose = 40 mg/kg).
[c] Mice were killed either 4, 8, 16, 24, 48, 96 or 168 h after the last dose of either PQ or MPTP.
[d] Mice were killed 7 days after the last dose of PQ or MPTP for stereology, neurochemistry (Studies 3 and 5) and brain chemistry (Study 4).
VC, vehicle control; PQ, paraquat; WT, wild type; KO, knock out; SYN, Syngenta, SIG, Sigma–Aldrich.

PQ. For animals designated for the analysis of PQ concentration in the forebrain (Study 4), the brain of each mouse was rapidly removed, placed in a mouse brain matrix, and a coronal block of tissue section rostral to the striatum was collected, weighed and then immediately flash-frozen in liquid nitrogen and stored frozen (at approximately −70 °C) until shipment to Charles River Laboratories, UK. The PQ concentration in the forebrain was determined by LC-MS-MS method (Charles River's Analytical Method Number 8936, version 2; limit of quantification [LOQ] = 0.5 ng PQ ion/g).

Brain samples collected for neurochemistry (Studies 2, 3 and 5) were obtained from the rostral to the caudal extent of the striatum. Concentrations of DA, DOPAC and HVA were measured *via* HPLC by injecting a 20-μL aliquot of the homogenate supernatant onto a Zorbax SB-C18, 4.6 mm × 250 mm column coupled to a Waters 464 Electrochemical Detector (time constant: 0.1 s; cell potential: 800 mV; cell current: 10 nA). The striatal concentration of each analyte was determined by comparing the area of each peak to the corresponding neurotransmitter peak area obtained from a standard curve. Dopamine turnover was calculated ([DOPAC + HVA]/DA]).

2.6. Pharmacokinetic study of PQ in blood, plasma and brain

Study 7 was conducted to characterize the uptake and clearance of [^{14}C]-paraquat ion ([^{14}C] PQ) from whole blood, plasma and brain of male C57BL/6J mice administered 1, 2 or 3 doses of 1 or 10 mg PQ/kg on days 0, 7 or 14 (Fig. 1B). [^{14}C] PQ was used because it had previously been established that PQ is not metabolized to a significant extent in rodents (Macpherson, 1995; JMPR, 2003). Blood/plasma samples were collected in heparinized tubes from groups of mice ($N = 3$) by cardiac puncture following anesthesia with isoflurane, from 0.25, 0.5, 0.75, 1, 1.5, 4, 24, 72, 168 or 672 h after a single dose of PQ (Fig. 1C). Brains from either perfused or non-perfused animals were dissected into 4 compartments (olfactory lobes, forebrain, midbrain and cerebellum), weighed and analyzed. Samples were oxidized (combustion efficiency ranged from 90.9 to 110%), and the $^{14}CO_2$ generated was absorbed and mixed with the scintillation cocktail, and then analyzed in a calibrated liquid scintillation counter (Packard Tricarb system). The number of disintegrations per minute (dpm) was calculated after correction

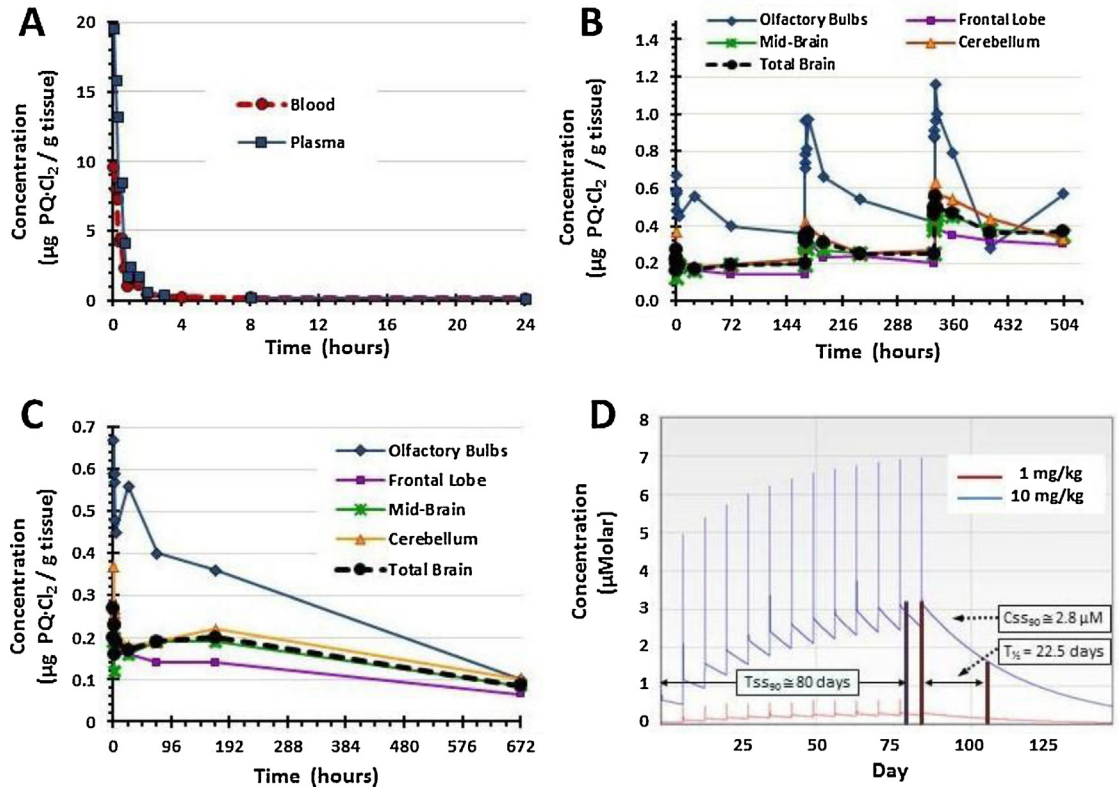

Fig. 1. Pharmacokinetics of [^{14}C] paraquat in the blood, plasma and brain of male C57BL/6J mice administered an ip dose of 1 or 10 mg/kg weekly for 1 (panels A and C), 3 (panel B) or 23 weeks (panel D – PBPK model predictions; Study 7).

for background and quenching. Paraquat ion concentration was expressed as μg/g of tissue.

A preliminary physiologically based pharmacokinetic (PBPK) model (not shown) was developed by The Hamner Institutes for Health Sciences, Research Triangle Park, NC, based upon the pharmacokinetic data obtained in Study 7, as well as on published literature (*e.g.*, Chui et al., 1988). The brain was described as two compartments, with one compartment representing the structures outside the blood–brain barrier (BBBo) and the other representing structures inside the blood–brain barrier (BBBi), based on differential kinetics between these two regions.

After fitting the PBPK model to time-course data for blood, plasma and brain (BBBo and BBBi) after 3 successive weekly ip doses in mice at 1 and 10 mg/kg (Fig. 1B), the model was used to estimate the terminal half-life in the BBBi. In classical pharmacokinetics, (Gibaldi and Perrier, 2007) the dosing time to reach a fraction of stable periodic behavior of a compound with repeated dosing is expressed as $N\tau = -3.32 \times t1/2 \times \log(1 - \text{fss})$ where N is the number of doses, τ is the interval between doses, $t1/2$ is the terminal half-life and fss is the fraction of stable periodic behavior (*i.e.*, steady state).

Based on this equation, it would take 3.32 half-lives to reach 90% of the steady state concentration and 6.64 half-lives to reach 99%. Under the assumption that uptake into the brain is much faster than clearance from brain, the approach-to-steady-state equation was used to calculate the time it would take to reach 90% of the steady state (T_{ss}) and the concentration of PQ in brain (C_{ss}) at T_{ss}. The model was also used to estimate the maximum concentration (C_{max}) of PQ in whole blood, plasma and brain after each daily dose of PQ, as well as the time needed to reach C_{max} (T_{max}). The terminal half-life of clearance from the brain was calculated as the duration of time in days for the brain concentration to be reduced by 50% after the cessation of treatment. An empirical method was also used to estimate the terminal steady state by estimating the rate of decline in the total brain concentration of PQ after a single dose of 10 mg/kg.

2.7. Brain sample collection and microtomy for stereologic investigation

Mice in Studies 4 and 5 designated for stereological investigations (Table 2) were deeply anaesthetized with sodium pentobarbital, perfused with fixative and brain tissues were processed and evaluated according to the procedures described in Supplementary Appendix 1.

2.8. Immunohistochemistry and counterstaining with cresyl violet

One in every 3rd brain section (40 μm thick) through the SNpc were prepared for immunostaining using the standard avidin–biotin complex (ABC) method (Study 4) or an immunofluorescence reactivity method (30 μm thick sections) (Study 5) as described in Supplementary Appendix 1. In Study 4, a second set of sections was stained with cresyl violet alone (CVO) to determine if the staining method had an effect on the total neuron count.

2.9. Stereological assessment

Unbiased estimates of the total number of TH$^+$ (DAB or Alexa red labeled neurons), TH$^-$ neurons (CV or DAPI-stained neurons minus TH$^+$ neurons) and total neurons (CVO or DAPI-stained neurons) were calculated using the optical fractionator probe of the Stereo Investigator software for design-based stereology, image analysis, and 2-dimensional anatomical mapping, version 8.11 (MBF Bioscience, Williston, VT, USA) as described in Supplementary Appendix 1. Stereological evaluation was performed blinded to treatment group. The operator was unaware of the treatment group to which individual mice belonged; the order

in which the animals were evaluated was determined by a block-randomized design. A needle track through one hemisphere permitted the operator to distinguish the left hemisphere from the right. Contours (virtual outlines) of the right and left SNpc were drawn manually using the computer mouse for each montage image. Contours were drawn around the SNpc in a manner consistent with the description provided by Baquet et al. (2009). Hence, contours were drawn to exclude the pars lateralis, pars reticulata and the ventral tegmental region. Rostrally, the contour of the SNpc began near the subthalamic nucleus, and it ultimately extended to the caudal aspect of the retrorubral field. The boundary for the posterior medial SNpc was the medial lemniscus, whereas the ventrolateral margin was indicated by the dorsal portion of the pars reticulata. The ventral tegmental area (VTA) defined the anterior medial border of the SNpc, and densely cellular populations of TH^+ neurons were used to approximate the mediodorsal extremity. The number of neurons in the right and the left SNpc were summed to provide an estimate of the total number of TH^+ or TH^- neurons in the SNpc.

Guard zones were established to prevent miscounting of neurons near the surface of the sections due to surface distortion artifacts (Boyce et al., 2011). Neuronal cell bodies were counted using the 100× oil microscope objective. A neuron was counted at the point where the nuclear membrane was in sharp relief while focusing up and down in the *z*-axis, and when the estimated center of the nucleus was within the counting frame and contour lines simultaneously, and also did not touch a red counting-frame line or contour line in the *xy*-axis.

The criteria governing the identification of neurons were established *a priori*, as follows:

(1) TH^+ neurons were identified by dense cytoplasmic immunolabeling (*i.e.*, detected with the DAB chromogen which appears as a dark brown reaction product within the neuron).
(2) In fluorescent-labeled sections, TH^+ neurons were identified by dense cytoplasmic immunostaining identified with the Alexa red fluorophore.
(3) TH^- neurons were identified in DAB-labeled sections by deep basophilic cytoplasmic CV staining of Nissl substance. TH^- nuclei, relative to other cell types such as glial cells, were generally smaller, and TH^- neurons had dendritic or axonal processes.
(4) In CVO- and DAPI-stained sections, TH^+ and TH^- neurons had CV- or DAPI-stained nuclei of all cells in the section (*e.g.*, TH^+ neurons, TH^- neurons and glial cells). DAPI-stained neurons (total neurons) were distinguished by their sizes, shapes and nucleolus appearances.

For the fluorescent-labeled sections, the number of TH^- neurons in the SNpc was calculated by subtracting the number of TH^+ neurons (Alexa red, immunolabeled neurons) from the total number of neurons (DAPI-stained neurons). Neurons stained with TH^+, TH^-, CVO and DAPI (see Fig. S1 for a representative photomicrograph) were tagged by applying virtual markers at the sites. Section thickness (μm) was measured at each counting frame site, and these measurements were averaged by the Stereo Investigator software to determine the mean thickness per section. Estimation of the total TH^+ and TH^- cell numbers in the SNpc of each mouse brain was performed by the software as described in Appendix 1.

To determine if the tissue processing method had an effect on total neuron count, which was calculated as the sum of TH^+ (chromogenic-labeled) and TH^- (CV-stained) neurons, a second set of sections was stained with cresyl-violet-only (CVO) staining (Study 4). In this case, the total number of neurons in the SNpc was calculated based upon the number of CVO-stained neurons. In Study 5, the total number of neurons was based upon the stereological count of fluorescent, DAPI-stained neurons; the number of TH^- neurons was calculated as the total number of neurons minus the number of TH^+ neurons.

Neuronal cell counts were recorded for each animal and other relevant details of the stereological analyses (*e.g.*, estimates of the total area and volume of the SNpc). Additionally, the Stereo Investigator software generated coefficient of error (CE) values for each of the total TH^+ and TH^- cell counts. The CE was used to determine if the number of neurons sampled stereologically was sufficient (*i.e.*, $CE < 0.1$). At least one animal in the MPTP and/or one animal in the vehicle control group was re-evaluated periodically and blindly within the randomized block in order to assess the intra-study variability of the stereological method.

Because there was an *a priori* hypothesis that PQ and MPTP would reduce both the number of TH^+ neurons in the SNpc and the contour area and volume of the SNpc, a one-sided two-sample *t*-test (Snedecor and Cochran, 1980) was performed on both TH^+ and total neuronal cell counts, as well as on contour volume. Statistical comparisons of neuronal cell counts between the hemispheres and across the depth of sections were two-sided, two sample *t*-tests.

2.10. Neuropathology

Time- and dose-intensive series of histopathological evaluations were performed on brain samples collected in Studies 3, 4 and 6. In Study 6, brains were collected from PQ and MPTP-treated mice from 4, 8, 16, 24, 48, 96 and 168 h after the last of 1, 2 or 3 doses of PQ (10, 15, or 25 mg/kg/doses), as well as after the last of 4 doses of MPTP administered on a single day (Table 2). Perfused-fixed brains from the pathology subsets of animals in Studies 3, 4 and 6 were shipped to Neuroscience Associates, Inc., for trimming, sectioning and staining. A maximum of 25 brains were embedded in a gelatinous matrix (Multibrain®) by Neuroscience Associates Inc. Twenty-five brains were assigned to the matrix in a block-random fashion, so that a block comprised of a vehicle control animal, an MPTP-treated animal and an animal from each PQ-treated group.

The brain (caudate/putamen area) was sectioned in the transverse plane from a level rostral to the striatum (caudate/putamen) caudally through the caudal extent of the SNpc at section thicknesses of 30 μm in the coronal plane. Seven serial sections were obtained at 12-section (360 μm) intervals. This block of tissue encompassed all of the structures defined as basal ganglia including the striatum, globus pallidus, SNpc, and subthalamic nuclei. Tissues adjacent to the basal ganglia, and in the plane of the section including the frontal cortex, hippocampus, VTA and rostral pons, were assessed separately.

The sections were processed as follows:

1. Tyrosine hydroxylase, an enzyme involved in the synthesis of DA, was immunohistochemically labeled (TH^+) to identify dopaminergic neurons and their neuronal processes (axons, dendrites and synaptic terminals).
2. Glial fibrillary acidic protein (GFAP) was immunohistochemically labeled to selectively identify protein filaments unique to activated astrocytes.
3. Ionized Calcium Binding Adaptor Molecule 1 (IBA-1), a protein expressed by activated microglia, was immunohistochemically labeled to detect microglial cell activation.
4. Amino Cupric Silver (Am Cu Ag) is a cytochemical stain that selectively stains the disintegrating elements of dead neuronal cell bodies and the neuronal processes (axons, dendrites and synaptic terminals). The dead cells/processes are labeled with black particles (silver) against a pale background, permitting the detection of single necrotic neurons.
5. Caspase 3 is an enzyme that is expressed by cells during apoptotic cell death. The caspase 3 cleavage product was immunochemically labeled to detect neurons that are in the

processes of dying *via* an apoptotic mechanism. Only SNpc sections were evaluated for caspase 3.
6. Thionine is a general morphological stain that detects Nissl substance, thereby revealing nuclear details within a variety of cell types.
7. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) is a method for detecting DNA fragmentation. When used in combination with cleaved caspase 3 immunoreactivity, it provides a sensitive indicator of apoptosis. Only sections of the SNpc were processed for TUNEL.

Slides for neuropathogical evaluation were prepared using standard procedures developed by Neuroscience Associates (http://www.neuroscienceassociates.com/stains.htm). The slides were evaluated by the study pathologist at Tox Path Specialists without knowledge of the treatment group (*i.e.*, "blinded") being evaluated. A qualitative scoring system was used to characterize the severity of the occurrence of necrotic cells, indicated by cellular uptake of amino cupric silver, the magnitude of the glial (microglia, astrocyte) response to treatment, the loss of staining for TH and an assessment of whether treatment triggered apoptotic mechanisms (caspase 3 expression and DNA fragmentation [TUNEL]) as described in Supplementary Appendix 1. An open, "unblinded" peer review was conducted for Study 6 by a second neuropathologist.

3. Results

PQ and MPTP solution concentrations were within ±10% of targeted concentrations in all studies, indicating that the intended doses were administered.

3.1. PQ and MPTP tolerability

Three out of 5 mice died or were sacrificed moribund after being administered the third of 3 doses of 35 mg PQ/kg (Study 1). These animals displayed perivascular edema with mixed inflammatory infiltrate in the lungs, endothelial hypertrophy of the pulmonary vasculature, tubular degeneration in the kidneys and vacuolation of the *zona fasciculata* of the adrenal gland.

There was no mortality in mice administered three doses of 10, 20, 25 or 30 mg PQ/kg. Clinical signs were minimal (hunched posture) at PQ doses of 10, 20, 25 or 30 mg/kg. PQ-treated mice in all groups displayed a dose-dependent reduction of food intake and body weight loss at all doses, with the largest effect (approximate 2 g weight loss) noted after the third dose (Supplementary Fig. S2). Effects were less pronounced at doses of 10 and 15 mg/kg/dose.

Only two out of 135 mice in these studies died when administered MPTP at ip doses of 10 mg/kg/dose, 4 times a day, 2 h apart. The 133 surviving mice did not display any clinical signs except for reduced food consumption and a transient reduction in body weight gain (Supplementary Fig. S2).

3.2. Pharmacokinetics (Study 7)

PQ was rapidly absorbed after ip injection. The time elapsed from injection to achieving peak blood and plasma concentrations (T_{max}) for the 10 mg PQ dose group ranged from 0.20 to 0.25 h (12–15 min). The concentration of [^{14}C] PQ quickly fell to below background levels within 4–8 h (Fig. 1A). Peak concentrations of [^{14}C] PQ (C_{max}) in whole blood and plasma tended to remain constant with each of the 3 successive doses (data not shown). C_{max} values calculated for whole blood and plasma were 0.070 and 138 μM [^{14}C] PQ, respectively, in the 10 mg PQ/kg dose group. C_{max} calculated for the 1 mg/kg dose group was approximately 10-fold lower.

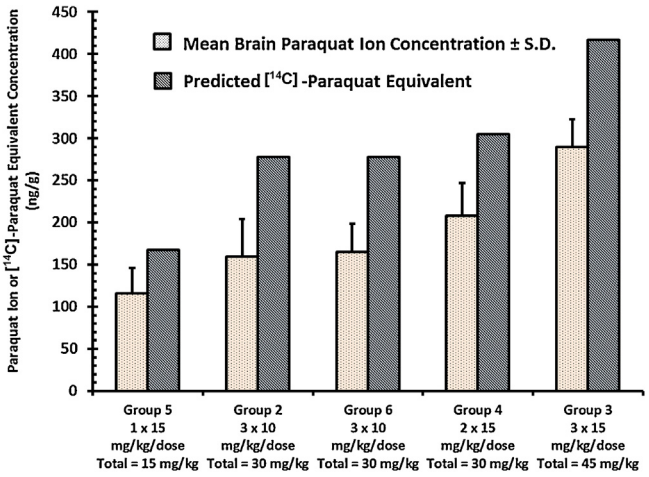

Fig. 2. Concentration of PQ in the forebrain (excluding the olfactory bulb) of male C57BL/6J mice 7 days after the last ip injection of 10 or 15 mg/kg PQ (Study 4).

The mean concentration of [^{14}C] PQ in the brain was similar at all time-points for the forebrain, midbrain and cerebellum and approximately 2-fold higher in the olfactory bulb (Fig. 1B and C) when compared to the rest of the brain. The T_{max} calculated for the brain (0.25 h) was only slightly longer than that calculated for plasma (0.2 h), indicating that PQ partitioned rapidly from blood to the brain. The concentration of PQ in the brain of animals that had been perfused by cardiac puncture, 90 and 240 min after dosing, were comparable to the brain concentrations in mice that had not been perfused (data not shown), suggesting that the [^{14}C] PQ found in the brain was not due to [^{14}C] PQ located in the brain's blood vessels.

The PBPK-modeled concentration of [^{14}C] PQ in the brain increased progressively with each successive dose of 10 mg PQ/dose based upon data from Study 7 (Fig. 1D). The duration of time (T_{ss}) needed to reach 90% of the steady state was ~80 days. The concentration (C_{ss}) in whole brain at T_{ss} was 2.8 μM [^{14}C] PQ (Fig. 1D). C_{ss} represented approximately 0.3% of the total daily dose of 10 mg/kg. The terminal half-life of elimination of PQ from the brain was 24 days, as determined by empirically fitting the data for PQ clearance from the brain after a single dose (Fig. 1C), or 22.5 days based upon modeling (Fig. 1D). A comparison of the C_{max} predicted by the PBPK model to an empirical fit of the data from the C57BL/6J mice treated with PQ indicated that the model overestimated the empirical data by approximately 2-fold.

The mean PQ concentration measured in the brain 7 days after the administration of PQ (Study 4) was dependent primarily on the total dose administered (Fig. 2). When the total dose administered was equivalent (*i.e.*, 10 mg PQ/kg × 3 *vs.* 15 mg PQ/kg × 2), the PQ brain concentration was marginally greater when the dose was administered over 2 weeks (*i.e.*, 15 × 2) than when it was administered over 3 weeks, indicating that although only a small fraction of the dose is taken up by the brain, it was slowly eliminated. Measured PQ concentration in the brain approximated the PBPK-estimate of [^{14}C] PQ (Fig. 2), although the model-predicted brain concentration of [^{14}C] PQ 7 days after the last dose was ~1.6-fold greater than the measured PQ concentration (Fig. 2).

3.3. Neurotransmitter concentration in the striatum (Studies 2, 3 and 5)

PQ, administered by the ip route at doses up to 25 mg/kg once a week for 3 weeks, had no consistent effect on the striatal concentration of DA, DOPAC or HVA, or on DA turnover (Studies 2, 3 and 5), as assessed 7 days after the last dose relative to the

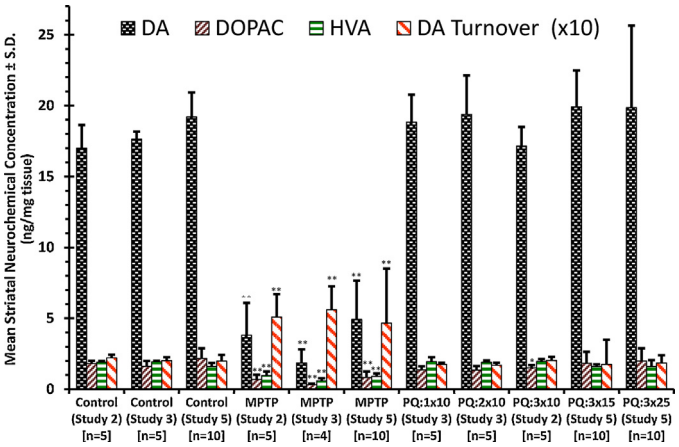

Fig. 3. Striatal dopamine, DOPAC, HVA concentration and dopamine turnover (percent of control mean) for C57BL/6J mice administered either PQ or MPTP.

control group (Fig. 3; Supplementary Table S1). In contrast, MPTP, at a dose of 10 mg/kg/dose administered 4 times in a single day, significantly suppressed DA (~75–90%), DOPAC (~60–80%) and HVA (~45–70%) levels, and significantly increased DA turnover (~130–180%) with respect to the control group (Fig. 3).

3.4. Stereology (Studies 4 and 5)

In Study 4, there was a statistically significant reduction in both the mean number of TH^+ neurons ($p = 0.001$) and in the mean number of TH^+ plus TH^- counter-stained CV neurons ($p < 0.023$) in mice that received 15 mg PQ/kg/dose × 3 doses, however, there was no effect of PQ on the total number of CVO-stained neurons ($p = 0.342$) in these mice (Fig. 4). There was no effect of PQ on the number of TH^+ neurons or the mean total number of CVO neurons in groups of mice administered either SYN PQ or SIG PQ at a dose of 10 mg PQ/kg/dose for 3 doses (Groups 2 and 6), or in groups of mice given 15 mg SYN PQ/kg/dose for either 1 (Group 5) or 2 (Group 4) doses.

In Study 5, there were no statistically significant effects of PQ administration on either chromogenic or fluorescent-labeled TH^+ neuronal cell counts (Fig. 5). The mean number of TH^+ neurons, however, trended downward, with progressively lower values obtained in the 3 × 15 and 3 × 25 mg/kg dose groups when compared to the 3 × 10 mg/kg dose group, for both chromogenic- and fluorescent-stained sections. There was no effect of PQ treatment on the mean total number of neurons assessed, in either CVO-stained sections or DAPI-labeled sections.

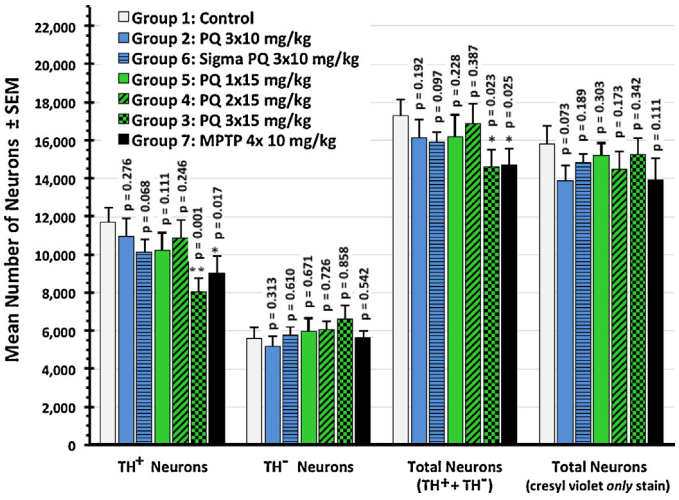

Fig. 4. Number of TH^+ (chromogenic-labeled neurons), TH^- (CVO-stained neurons) and the total number of neurons (chromogenic-labeled + CVO-stained neurons) (Mean ± SEM) in the SNpc of male, C57BL/6J mice 7 days after the last ip injection of either PQ or MPTP (Study 4).

The mean number of DAB-labeled, TH^+ neurons was significantly reduced ($p < 0.05$) in the MPTP-treated group in Study 4, but the mean total number of CVO neurons in the SNpc was not significantly reduced (Fig. 4). The mean number of fluorescent-labeled (Alexa red) TH^+ neurons was reduced ($p < 0.05$) in the MPTP group, whereas the mean number of DAB-labeled TH^+ neurons was reduced, but not significantly ($p = 0.06$; Fig. 5). The mean total number of neurons in the MPTP-treated groups (Study 4) was not significantly reduced when assessed using CVO-only staining ($p = 0.1$; Fig. 4) or DAPI staining ($p = 0.08$; Fig. 5; Study 5).

The mean cross-sectional area of the contours drawn blinded to dose group, and the calculated mean SNpc volume were significantly reduced in the MPTP-treated DAB-labeled sections, but not in Alexa red-labeled sections (Fig. 5; Study 5). Mean contour area and SNpc volume were also significantly reduced in the 15 mg PQ/kg × 3 dose group and the 25 mg PQ × 3 dose group for the DAB-labeled sections, but not for the fluorescent-labeled sections. There was no effect of PQ on mean contour area in the 10 mg PQ/kg × 3 dose group. There were no effects of PQ-treatment on SNpc contour area or volume in Study 4 (data not shown).

There were no significant differences between the left and right SNpc in the number of TH^+ neurons, TH^- neurons or the total number of neurons (Study 5 – data not shown). However, there were significant differences in neuronal counts through the depth of the thick section. These differences were more pronounced in the DAB-labeled than in the fluorescent-labeled sections (Supplementary Fig. S3).

Stain penetration artifact was not evident for either the CVO- or DAPI-labeled neurons (Fig. S3), although there were clear differences in cell density through the depth of DAB-labeled sections. The number of TH^+ Alexa red labeled neurons in the control group was approximately 66% greater than the mean number of TH^+ neurons determined by DAB labeling.

3.5. Neuropathology (Studies 3, 4 and 6)

PQ doses of 10, 15 or 25 mg/kg administered 1, 2 or 3 times, with each dose separated by 7 days (Studies 3, 4 and 6), did not induce neurodegenerative changes (degeneration or disintegration of neurons or neuronal processes including disintegrating synaptic terminals) in the SNpc or the striatum, as indicated by the absence of an increase in cupric silver staining (Supplementary Figs. S4 and S7; Photomicrographs: S10 and S11). The intensity of TH^+ immunoreactivity in PQ-treated animals was comparable to the controls (Supplementary Figs. S4 and S7; Photomicrographs: S16 and S17). There was no evidence of either increased labeling of the caspase 3 cleavage product or increased TUNEL in PQ-treated groups (data provided in Supplementary Table S2), indicating that apoptotic cell death was not triggered by the ip injection of PQ. Microglial cells and astrocytes were not activated by PQ treatment 4, 8, 16, 24, 48, 96 or 168 h after the last dose (Supplementary Figs. S5 and S8; Photomicrographs: S14 and S15), indicating that cell necrosis and cell death processes were not induced by PQ treatment. There was no evidence of neuronal cell death or microglial or astrocytic activation in the cerebral cortex, striatum, thalamus/hypothalamus, midbrain, SNpc, VTA or the pons (rostral hindbrain) based upon evaluations of brain sections that spanned the area from the striatum to the caudal midbrain. Numerical grading scores for Study 6 are presented in Supplementary Table S2.

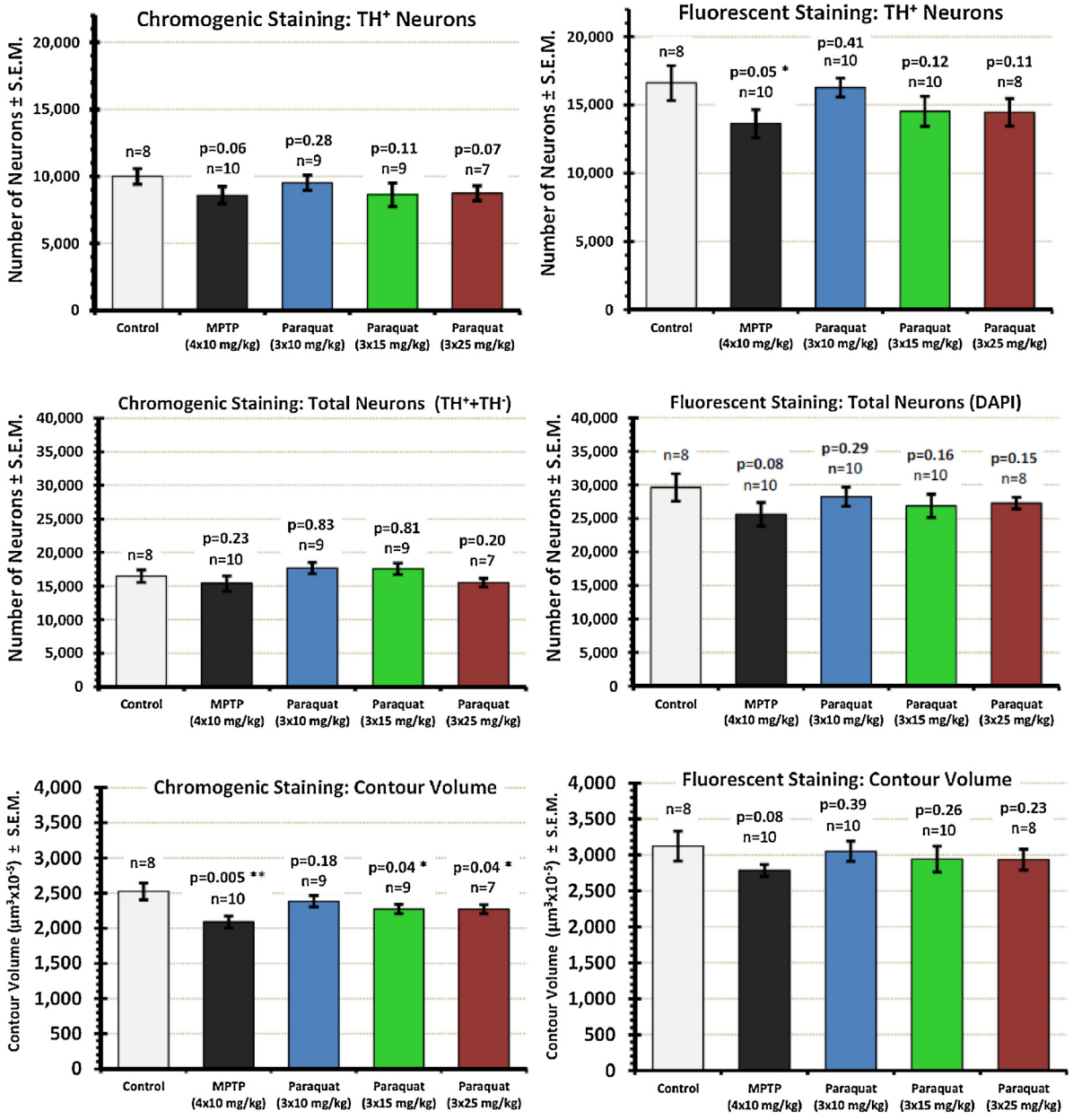

Fig. 5. Number of TH^+ (chromogenic- or fluorescent-stained neurons), TH^- (CVO-stained neurons) and total neurons (chromogenic- or fluorescent-labeled neurons + CVO-stained neurons) (Mean ± SEM) in the SNpc of male, C57BL/6J mice 7 days after the last ip injection of either PQ or MPTP (Study 5).

In contrast, MPTP-treated mice displayed degenerating/disintegrating neurons in the SNpc and degenerating neuronal processes (synaptic terminals) in the striatum, as indicated by the uptake of cupric silver stain (Figs. S4, S6, S7, and S9; see Fig. 6 for representative photomicrographs). A qualitative reduction in the intensity of TH labeling was present in the SNpc, but this was more pronounced in the striatum. There was no evidence of increased TUNEL or caspase 3 cleavage products in MPTP-treated mice, suggesting that MPTP-induced cell death in the SNpc was not mediated by apoptosis (Supplementary Table S2).

Accompanying the MPTP-induced neuronal degeneration were pronounced activation of microglia (IBA-1 immunoreactivity) and astrocytes (GFAP immunoreactivity) in the striatum, as well as moderate activation in the SNpc (Figs. S5, S6, S8, and S9; Fig. 6 displays representative photomicrographs of IBA-1 and GFAP staining in MPTP-treated mice). A plot of the time course of neurodegeneration and glial activation in MPTP-treated mice indicated that the intensity of neuronal degeneration increased earlier than either the reduction in TH^+ labeling or microglial and astrocyte activation (Figs. S6 and S9). In the striatum, all four processes peaked within 48 h of the last dose, but astrocyte activation persisted with greater severity in the striatum up to 168 h. In the SNpc, neuronal degeneration and glial responses were no longer detectable by 168 h post-treatment (Fig. S6).

4. Discussion

4.1. Pharmacokinetic studies

The pharmacokinetics of [^{14}C] PQ in the blood, plasma and brain following ip administration to C57BL/6J mice showed that [^{14}C] PQ was rapidly taken up after ip injection and that tissue concentration was linearly related to the dose. The initial half-life (rapid phase) of elimination from blood and plasma was short (17 and 18 min for the 1 and 10 mg PQ/kg doses respectively, calculated from the first hour of data). The concentration of [^{14}C] PQ in brain

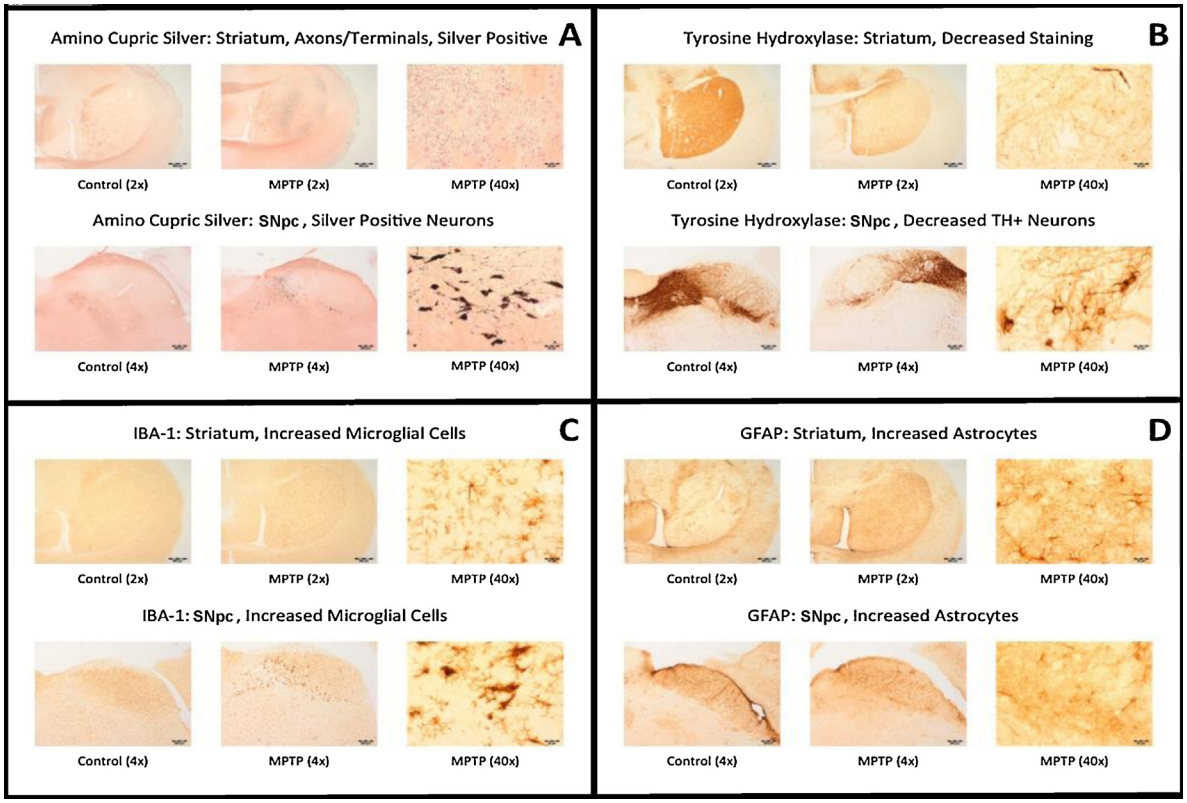

Fig. 6. Uptake of silver stain in degenerating neuronal processes in the striatum of a MPTP-treated mouse compared to a control (panel A); Decreased tyrosine hydroxylase staining in the striatum (panel B), increased staining for microglia (panel C) and astrocytes (panel D).

rapidly reached a peak (C_{max}) following each ip dose, but clearance from the brain was slow, with the terminal half-life estimated to be either 24 days, based on an empirical fit of the brain clearance data, or 22.5 days based on the PBPK model. The estimated terminal half-life is slightly shorter than that reported by others (28 days in Prasad et al., 2007 and 1 month in Prasad et al., 2009). The brain concentration of PQ increased with repeated PQ dosing (Fig. 1B), but the rate of elimination from the brain was independent of the number of doses administered.

The precise cellular localization of [^{14}C] PQ in the brain, as evaluated either by autoradiography (data not shown), or by radiometric counts in subsections of the brain, could not be determined in these experiments. However, approximately equal concentrations of [^{14}C] PQ were observed in all regions of the brain examined except for the olfactory bulb, which had an approximately 2-fold greater concentration (Fig. 1C). Previous work has demonstrated that PQ does not selectively accumulate in any specific region within the SNpc or the striatum of the rat, although the concentration of [^{14}C] PQ was greater in the olfactory bulb and pineal gland, including the lining of the lateral ventricle, the fourth ventricle, the area postrema and the hypothalamus, as compared to the rest of the brain (Naylor et al., 1995; Widdowson et al., 1996). In the rhesus monkey, PET imaging of [^{11}C] PQ indicated that PQ uptake into the brain was minimal and consistent, and dependent on the volume of blood distribution to substructures within the brain. The pineal gland and lateral ventricles were the only structures which showed evidence of significant PQ uptake (Bartlett et al., 2009). Overall, the pattern of PQ distribution in the brain is similar across species, with higher concentrations being found in areas of the brain not fully protected by the blood–brain barrier. These results are inconsistent with the proposal that monovalent PQ is taken up into dopaminergic neurons by the DAT transporter (Rappold et al., 2011).

Although PQ was slowly eliminated from brain, it is unknown whether PQ is bound to tissues or organelles within the brain, or whether specific neurons or other cells selectively retain PQ. It is also unknown whether PQ undergoes redox cycling in the intact brain to generate superoxide anions, as has been described for the lung (Bus et al., 1976; Smith, 1985). However, evidence of free radical production has been observed in neuronal cell cultures. Peng et al. (2004) reported that immortalized dopaminergic neurons (N27 cell line) exposed *in vitro* to 400 μM PQ for 18 h displayed an increased amount of the caspase 3 cleavage product. The authors suggested that the 50% reduction in viability after 24 h was in part due to an apoptotic mechanism triggered by increased free radical production, even though superoxide anion was not directly measured. Gonzalez-Polo et al. (2004) reported a 70% reduction in cerebellar granule cells' viability when exposed *in vitro* to 5 μM PQ for 24 h.

The PBPK model predicted that $C_{ss} \sim 2.8$ μM PQ in the brain after repeated daily ip dose of 10 mg PQ/day. This concentration was ~30- to 60-fold lower than the concentration that resulted in a 10% reduction in cell viability in the study by Peng et al. (2004), although it was similar to the concentration evaluated by Gonzalez-Polo et al. (2004). However, there was no evidence of either cellular necrosis or apoptotic cell death in mice administered high doses of PQ in the current investigation. These results are contrary to both the *in vitro* and the *in vivo* work reported by Peng et al. (2004). Based upon the pharmacokinetic data, it is unlikely that PQ exposure in the *in vitro* studies reflect the conditions of PQ exposure of the brain following systemic (ip) administration to C57BL/6J mice.

4.2. Neurochemistry

In contrast to the reports of reduced DA levels (Kang et al., 2009) and/or increased DA turnover in the striatum following PQ

treatment (Shepherd et al., 2006; Songin et al., 2011), our results show that weekly administration of PQ to C57BL/6J mice for 3 weeks at doses up to the maximum-tolerated dose had no effect. PQ doses up to 25 mg/kg administered by ip injection on 3 weekly occasions did not alter either striatal DA, DOPAC and HVA or the turnover of dopamine in three studies. In contrast, MPTP significantly reduced the level of dopamine and its metabolites and increased DA turnover, which is consistent with the known effects of MPTP (Heikkila et al., 1984a,b).

Researchers who have observed reductions in the number of TH^+ immunoreactive neurons in the SNpc without finding changes in striatal DA levels have suggested that the surviving SNpc neurons undergo a compensatory up-regulation of DA synthesis (McCormack et al., 2002). In our experiments, however, there was no evidence of dopaminergic neuronal cell death in the SNpc nor was DA turnover increased in the striatum. The most parsimonious interpretation of these results is that in PQ-treated mice, compensation of neurotransmitter synthesis in the striatum is not triggered because dopaminergic neurons in the SNpc have not died.

It is possible that repeated daily dosing of PQ for 24 days might reduce striatal DA concentrations without changing DA turnover as reported by Prasad et al. (2009). However, preliminary results from an ongoing study, in which PQ was administered in the diet of C57BL/6J mice for 13 weeks at doses of 14–22 mg/kg/day in males and females, respectively, indicate that neither striatal DA levels nor dopamine turnover were affected.

4.3. Stereological assessment of the number of dopaminergic neurons in the SNpc

Several authors have previously reported that ip administration of PQ to C57BL/6J male mice reduced the number of TH^+ dopaminergic neurons in the SNpc (Brooks et al., 1999; Thiruchelvam et al., 2000; McCormack et al., 2002; Peng et al., 2004; Jiao et al., 2012; Gollamudi et al., 2012). In our study, there was no consistent, statistically significant, stereological evidence of a loss of DAB-labeled TH^+ neurons in the SNpc following PQ treatment (Fig. 4). Although a statistically significant reduction in the number of TH^+ neurons was observed in the 15 mg PQ/kg $\times$ 3 dose group, 7 days after the last dose in Study 4, the total number of CVO-stained neurons in the SNpc was unaffected by treatment. Similarly, the total number of neurons, assessed using CVO or DAPI in Study 5, was also unaffected by PQ treatment. These results suggest that under some conditions, dopaminergic neurons may undergo a phenotypic reduction in TH expression following PQ treatment. Alternatively, PQ may have interfered with the epitope involved with the immune recognition of TH^+ neurons *in situ*.

The stereological finding of a reduced number of TH^+ neurons observed in Study 4 was not replicated in Study 5. Study 5 was more comprehensive than Study 4 because both DAB-labeled and Alexa red fluorescent-labeled TH^+ neurons were evaluated after 3 weekly doses of 10, 15 or 25 mg PQ/kg. Although the number of DAB labeled TH^+ neurons in the 15 and 25 mg PQ groups tended to be reduced compared to the control group (Fig. 5), this may have been due to the slightly, yet statistically significantly decreased contour areas and volumes for the 15 and 25 mg PQ-treated groups. This could occur if the intensity of TH^+ immunoreactivity was reduced in the high-dose PQ-treated groups, thereby making it more difficult to discern the precise margins of the SNpc. Mitra et al. (2011) reported a diminution in TH expression in the SNpc, hippocampus and frontal cortex in Swiss Webster mice given ip injections of 10 mg/kg PQ twice weekly for 4 weeks and a decreased number of TH^+ neurons in the SNpc of these mice; the effect was more pronounced in the 10 mg/kg group compared to mice administered lower (5 mg/kg) or higher (20, 40 or 80 mg/kg) of PQ.

The results from Studies 4 and 5 are inconsistent with those reported in older studies that used a similar dosing regimen (Brooks et al., 1999; Thiruchelvam et al., 2000; McCormack et al., 2002) and with more recent studies where PQ was administered more frequently by ip injection (Peng et al., 2004; Jiao et al., 2012) or continuously *via* an Alzmet pump (Gollamudi et al., 2012). The reasons for these discrepancies remain unclear. It is possible that in the more recent studies, the frequency of PQ administration may have resulted in different brain concentration kinetic profiles. However, it is unlikely that either ip injection or continuous subcutaneous administration of PQ is representative of potential human exposure encountered in the agricultural use of PQ (Lock and Wilks, 2001).

In Study 5, there were approximately 66% more TH^+ neurons in the SNpc in fluorescent-labeled compared to chromogenic-labeled sections ($\sim$10,000 neurons; Fig. 5), confirming the results of Prasad and Richfield (2010). This difference may in part reflect the greater penetration of fluorescent dye, as well as the greater ease of identifying TH^+ neurons with fluorescent labeling because of reduced shrinkage of the sections when aqueous mounts were employed for the fluorescent labeling method, compared to the air-dried mounting technique used for the DAB prepared sections. Although it has been reported that the absolute number of TH^+ neurons in the C57BL/6J male mice is $\sim$8300, based upon an evaluation of 10 μm paraffin-embedded serial sections throughout the entire SNpc (Baquet et al., 2009), it is likely that the variability in the number of TH^+ neurons between laboratories is attributed to different criteria used by investigators to draw contours around the SNpc and differences in the quality of immunochemical labeling of TH^+ neurons.

In Study 5, the mean number of DAB-immunostained TH^+ neurons in the vehicle control C57BL/6J male mice (Mean = 9988; SEM = 584; $N = 8$) was fever than some researchers have reported for DAB-immunostained TH^+ neurons (McCormack et al., 2002; Mean = 12,321; SEM = $\pm$118; $N \geq 5$) and greater than others as determined by serial reconstruction (Baquet et al., 2009; Mean = 8305; SEM = $\pm$539; $N = 4$) or using the optical fractionator (Baquet et al., 2009; Mean = 8716; SEM = $\pm$338; $N = 10$). It is likely that the serial reconstruction method of Baquet et al. (2009) provides the most accurate estimate of the number of TH^+ neurons in the SNpc.

Variability, as represented by the coefficient of variation (CV) in the number of DAB immunostained TH^+ neurons in Study 5 (CV = 0.165) was comparable to the variability reported by Baquet et al. (2009) based on serial reconstruction (CV = 0.130) or the optical fractionation method (CV = 0.123) and was much greater than the variability reported by McCormack et al. (2002; CV $\geq$ 0.021). The reason for the greater precision in the McCormack et al. (2002) study compared to Baquet et al. (2009) and the present investigation is unknown. Prasad and Richfield (2008), however, using a fluorescent method for detecting TH^+ neurons in the SNpc of male C57BL/6J, presented data indicating that 55 to 73% of untreated mice obtained from 5 different suppliers had abnormally low TH^+ staining and that these mice had on average 32% fewer TH^+ neurons than did "normal" TH^+ staining mice. Part of this variation was apparently due to inter-hemispheric differences, which Prasad and Richfield (2010) cautioned could confound the interpretation of stereology studies.

Furthermore, while few investigators have reported cell counts through the depth of sections, our results indicate that DAB-labeled sections are considerably more variable in TH^+ cell counts through z-depth than were TH^+ cell counts, based upon fluorescent-labeled TH^+ neurons (Alexa red) or DAPI-stained dopaminergic neurons. While varying cell count through the depth of section

is not ideal for stereological investigations (Boyce et al., 2011), the demonstration that there was no differential effect of PQ or MPTP treatment on this profile (Fig. S3) suggests that a relative effect of treatment would be detectable. This was confirmed by the results from the low-dose (10 mg/kg/dose × 4 doses) MPTP-treated group, where either a statistically significant or borderline statistically significant reduction in the number of TH^+ neurons was noted in the SNpc. Although some investigators have recommended that larger doses of MPTP be used (Jackson-Lewis and Przedborski, 2007), the low-dose regimen (four doses of 10 mg MPTP/kg administered 2 h apart) used in these experiments was deliberately employed to determine if the stereological methods would be sensitive enough to detect relatively small changes in the number of TH^+ neurons in the SNpc. The SEMs calculated for control groups using chromogenic-stained TH^+ neurons (SEM = 584) and Alexa red labeled TH^+ neurons (SEM = 1305) are greater than some researchers have reported (McCormack et al., 2002), but are in agreement with others (Jiao et al., 2012).

Using a relatively low dose of MPTP (10 mg/kg/dose × 4 doses 2 h apart), a reduction in DAB-labeled neurons ($p = 0.017$; Study 4 or $p = 0.06$; Study 5) or a statistically significant loss of Alexa red-labeled TH^+ neurons ($p = 0.05$; Study 5) was observed. There was clear evidence that this dose of MPTP resulted in the necrosis of dopaminergic neurons in the SNpc (Fig. S6), the activation of microglia, the activation of astrocytes and the depression of striatal DA concentration, with a corresponding increase in DA turnover (Fig. 3). Thus the failure to achieve a statistically significant loss in the number of DAB labeled TH^+ neurons in Study 4 was almost certainly due to a combination of using a relatively low dose of MPTP and high inter-animal variability in the number of TH^+ neurons (Figs. 4 and 5), leading to relatively large SEMs.

4.4. Neuropathological assessment of neuronal cell death in the SNpc and striatum

Histopathological changes were evaluated in the striatum, midbrain SNpc and rostral pons of C57BL/6J mice 4, 8, 16, 24, 48, 96 and 168 h after the last of 1, 2 or 3 doses of paraquat or MPTP treatment by a pathologist who was 'blinded' to the treatment groups of the individual animals. The pathologist identified every mouse that had been treated with MPTP based on clear evidence of cell damage/death, as indicated by amino cupric silver staining (Figs. S6 and S9), as well as by activation of microglia and astrocytes (Figs. S5 and S8). The pathological changes began in the striatum within 4 h of MPTP administration (Fig. S6). The effect of MPTP on the SNpc commenced within 8 h of dosing and returned to baseline within 4 days, although astrocytes remained activated for up to 7 days post-dosing (Fig. S6). There was no effect of PQ on any pathological endpoint in the striatum or SNpc at any time after 1, 2 or 3 doses of 10, 15 or 25 mg PQ.

There was no evidence of cell death in structures adjacent to the striatum, and the SNpc including the VTA, thalamic nuclei, hypothalamus, hippocampus and rostral pons, as indicated by no increase in the silver staining of neurons, indicators of apoptosis or evidence of glial or astrocytic activation based on blinded histopathological assessment of these structures. These results are in contrast to the work by Mitra et al. (2011), who reported evidence of a neuro-inflammatory response in the SNpc and hippocampus of PQ-treated Swiss Webster mice. Purisai et al. (2007) also reported increased counts of Mac-1 immunostained neurons in the SNpc of C57BL/6J mice 1, 2, 4 and 7 days after a single ip dose of 10 mg/kg PQ. The reasons for the differences between the work of Mitra et al. (2011), Purisai et al. (2007), and the present investigations is not known, although different staining and evaluation methods were used in each study.

5. Conclusions

When PQ was administered by ip injection to C57BL/6J mice, 0.3% of the dose entered the brain and it was then slowly eliminated. It is unknown whether PQ enters the neuron or if it undergoes reduction-oxidation cycling. However, PQ did not cause pathological or neurochemical changes in the brain when given in doses up to and including 3 × 25 mg PQ/kg.

These studies did not consistently detect a significant loss of TH^+-stained neurons of PQ-treated mice, as reported by others, nor was there any evidence of neuronal cell death in the SNpc, nor any effect on neurotransmitter levels in the striatum. However, the effect of PQ on the number of TH^+ neurons in the SNpc reported in the published literature cannot be dismissed. If PQ has an adverse effect in the brain of the C57BL/6J mice, it may be confined to reduced phenotypic expression of TH in dopaminergic neurons, which would explain the absence of pathology or any effect of PQ on DA turnover in the striatum. Reduced TH expression might also explain the statistically significant reduction in contour volume observed following dosing with MPTP or PQ since the construction of the contour is based, at least in part, on the immunolabeling of TH^+ neurons. What is clear is that the use of PQ to provoke a statistically significant reduction in TH^+ neurons in the SNpc of the mouse brain is a more fragile process than is currently characterized in the published literature. Additional studies are required to resolve the differences between our results and those reported by others, including the *in vivo* studies by Peng et al. (2004) and Jiao et al. (2012).

It is possible that the stereological methods used in our studies were inadequate to detect a PQ-induced reduction in the number of dopaminergic neurons reported by others. However, the totality of the evidence from this research program indicates there was no consistent stereological evidence of a loss of TH^+ staining in the SNpc of male C57BL/6J mice administered PQ at near-lethal doses. Neurochemical changes were always observed following MPTP treatment, but there were never any changes in DA levels or DA turnover in the striatum after PQ treatment. There was clear evidence of neuropathological changes in the SNpc and the striatum after MPTP treatment in three separate studies, but there was no evidence that PQ had any effect on a broad range of indicators of cell death, in both the SNpc and the striatum.

An important insight from this investigation was that the "blinded" neuropathological evaluation of the mouse brain was able to segregate with 100% accuracy the MPTP-treated mice from the control or PQ-treated animals. However, stereological assessment could only statistically separate the MPTP-treated mice from controls in two out of three studies. It is recognized that this may be a consequence of the variability in the stereological results, but the variation in these experiments was not markedly different from the results obtained by others who have conducted similar studies. This suggests that an unbiased assessment of neuronal cell loss in the SNpc must include carefully conducted pathological studies where the pathologist is "blinded" to the treatment of each experimental animal.

In conclusion, the use of stereology, pathology and brain neurochemistry may prove to be the appropriate combination of endpoints for determining whether neurons in the SNpc of treated mice are damaged following PQ treatment. If neurons in the SNpc died as a result of treatment, one would expect that there would be (1) a reduction in TH^+ neurons and a proportional reduction in total neuron count in the SNpc as determined by stereology; (2) evidence of cell death within the SNpc based upon pathological endpoints and (3) a reduction in DA levels and increased DA turnover in the striatum. This is precisely the constellation of effects seen following MPTP administration, but not after near-lethal doses of PQ.

Conflict of interest

These studies were funded by Syngenta Crop Protection, LLC, a registrant and basic manufacturer of PQ. All authors of this review either worked for the institutes where the research was conducted (DZ, JCW, JMM, MB and MB) or are employees of Syngenta, LLC (ARC, CBB, DM, KZT, LLS, MOT, NCS and PAB). ARC, KZT, LLS, NCS and PAB are employees or former employees of Syngenta Limited.

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Sielken and Associates Consulting Inc. (Bryan, TX) performed the statistical analyses of all the stereological data reported in this study. They also characterized the exponential decline of PQ from the brain after a single dose of 10 mg/kg to estimate the terminal half-life of PQ.

Dr. Bob Garmon (Consultants in Veterinary Pathology Inc., Murrysville, PA) conducted an open peer review of the pathology results from Study 6. The results of this assessment confirmed the findings of the reviewing pathologist at Tox Path Specialists.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.neuro.2013.03.005.

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