Genetic-based, differential susceptibility to paraquat neurotoxicity in mice

Lina Yin [a], Lu Lu [b,c], Kavita Prasad [d], Eric K. Richfield [d], Erica L. Unger [e], Jialin Xu [f], Byron C. Jones [a,g,*]

[a] Intercollege Graduate Degree Program in Neuroscience, The Pennsylvania State University, University Park, PA 16802, USA
[b] Department of Anatomy and Neurobiology, University of Tennessee College of Medicine, Memphis, TN 38163, USA
[c] Jiangsu Key Laboratory of Neuroregeneration, Nantong University, Nantong 226001, People's Republic of China
[d] Environmental & Occupational Health Sciences Institute, Robert Wood Johnson Medical School, Rutgers, The State University of New Jersey, Piscataway, NJ 08854, USA
[e] Department of Nutritional Sciences, The Pennsylvania State University, University Park, PA 16802, USA
[f] Department of Statistics, The Pennsylvania State University, University Park, PA 16802, USA
[g] Department of Biobehavioral Health, The Pennsylvania State University, University Park, PA 16802, USA

ARTICLE INFO

ABSTRACT

Paraquat (PQ) is an herbicide used extensively in agriculture. This agent is also suspected to be a risk factor for Parkinson's disease (PD) by harming nigro-striatal dopamine neurons. There is likely, genetic-based, individual variability in susceptibility to PQ neurotoxicity related PD. In this study, we measured the delivery of PQ to the brain after three weekly injections of PQ at 5 mg kg^{-1}, PQ-related neural toxicity after three weekly injections of PQ at 1 mg kg^{-1} or 5 mg kg^{-1}, PQ-related iron accumulation and PQ-related gene expression in midbrain of DBA/2J (D2) and C57BL/6J (B6) inbred mouse strains after a single injection of PQ at 15 mg kg^{-1} and 10 mg kg^{-1}, respectively. Results showed that compared to controls, PQ-treated B6 mice lost greater numbers of dopaminergic neurons in the substantia nigra pars compacta than D2 mice; however, distribution of PQ to the midbrain was equal between the strains. PQ also significantly increased iron concentration in the midbrain of B6 but not D2 mice. Microarray analysis of the ventral midbrain showed greater PQ-induced changes in gene expression in B6 compared to D2 mice. This is the first study to report genetically-based differences in susceptibility to PQ neurotoxicity and to understanding individual differences in vulnerability to PQ neurotoxicity and its relation to PD in humans.

© 2011 Elsevier Inc. All rights reserved.

1. Introduction

Paraquat (PQ) is an herbicide used world-wide (Bromilow, 2004). Besides its target effect of toxicity to plants in agriculture, PQ is toxic to animals and can cause damage to lungs and brain, among other tissues. Notably, PQ has been reported to increase the risk for sporadic Parkinson's disease (sPD) via its toxicity to dopamine neurons in the substantia nigra. sPD is believed to be the result of environmental risk factors acting on genetically susceptible individuals during aging (Barbeau et al., 1985; Gwinn-Hardy, 2002). Current epidemiologic data show that PQ-associated risk for sPD is an estimated 4–7 times greater than for other environmental factors such as heavy metal exposure or rural residency (Hertzman et al., 1990; Liou et al., 1997; Landrigan et al., 2005). In the laboratory, mice treated with PQ developed neurochemical and behavioral signs consistent with Parkinsonism, including specific dopaminergic (DA) neuron loss and α-synuclein containing protein aggregation in the substantia nigra

pars compacta (SNpc) (Manning-Bog et al., 2002; McCormack et al., 2002; Prasad et al., 2007, 2009), specific dopamine depletion in striatum (Shimizu et al., 2003), and decrease of ambulatory activity (Brooks et al., 1999; Fernagut et al., 2007). PQ-induced cell death may be the result of oxidative stress (McCormack et al., 2005; Prasad et al., 2007), and possible intracellular signal pathways involved c-Jun N-terminal protein kinases (JNK) and caspase-3 (Peng et al., 2004). Thus, PQ is considered to be a strong risk factor for sPD, even though the exact biochemical mechanism of PQ neurotoxicity is unknown.

Different genetic backgrounds very likely predispose individuals to resistance or to susceptibility to environmental toxicants, such as PQ, that are implicated in sPD. In humans, not every individual exposed to PQ develops sPD. In mice, Smeyne and colleagues have reported strain-dependent susceptibility to another Parkinson's disease related neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydro-pyridine (MPTP), C57BL/6J mice more vulnerable to Swiss–Webster (SW) mice (Hamre et al., 1999). Later they identified a single QTL, Mptp1, for susceptibility to MPTP neurotoxicity in mice (Cook et al., 2003). Based on these data, certain genetic backgrounds likely contribute to differential susceptibility to PQ neurotoxicity. Identifying the specific genes involved may ultimately reveal the underlying molecular mechanisms for PQ neurotoxicity and likely other environmental

risk factors for sPD. It is also likely that PQ-related sPD is a complex trait, influenced by multiple genes acting in response to environmental conditions.

Because it is likely that sPD is a complex trait, modeling the etiology of the disease in animals is best accomplished using a genetic reference population. Inbred strains of rodents can be helpful. One such group of genetically-defined mice is the panel of BXD recombinant inbred (RI) mice. This panel has proven to be an important resource for dissecting the molecular architecture of complex traits and the genetic study of gene-environment interaction (Churchill et al., 2004). BXD RI mice were generated by crossing C57BL/6J (B6) and DBA/2J (D2) inbred strains. Currently there are 80 BXD RI strains all of which have been genotyped at more than 13,000 unique markers. This will enable fine mapping of chromosome and more precise identification of chromosome loci that contain genes underlying differential strain susceptibility to this environmental toxicant (Darvasi and Soller, 1997; Peirce et al., 2004). Genetic reference populations are useful in identifying genetic variants associated with phenotypes, genetic correlations among phenotypes and if available, gene expression data that can be used in describing mechanisms. For example, GeneNetwork (http://www.genenetwork.org) is the online database containing the sequencing information for a number of genetic reference populations, including the BXD RI strains. It also enables quantitative trait loci (QTL) mapping by using strain means of a certain trait/phenotype as the index for association analysis to locate the chromosome regions of which the genotype variations cause the phenotype variation among strains. In this report, we show that between the parental strains of BXD RI mice B6 mice are more sensitive to PQ neurotoxicity than D2 mice, which established the basis for using the larger panel of BXD RI strains of mice to measure the phenotype variation in terms of different susceptibility to PQ neurotoxicity, and to pinpoint the underlying genes. This may shed light to the genetics of sPD.

2. Materials and methods

2.1. Animals

Two inbred strains of mice, C57BL/6J and DBA/2J, obtained from our vivarium and from the University of Tennessee Health Sciences Center, were used in this study. The mice were maintained under a constant light–dark cycle (06:00–18:00, on–off rotation), controlled temperature (21 ± 2 °C) and humidity (40%). Food (standard Purina rodent diet# 5010) and water were provided *ad libitum*. All experimental protocols were approved by the Penn State Institutional Animal Care and Use Committee and were conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

2.2. Experiments

Male and female mice between the ages of 2.5–4 months were used for three experiments: measurement of brain PQ concentration (experiment I), quantification of dopaminergic neurons in the SNpc (experiment II) and identification of ventral midbrain transcriptome (experiment III). Mice aged 120 ± 10 days were used for measure-

ment of ventral midbrain iron concentration (experiment IV), as shown in Table 1. To avoid litter specific effects, for every experiment, mice within a litter were assigned to different treatment groups, *i.e.*, different doses of PQ or saline control.

2.3. PQ treatments

PQ dichloride trihydrate (Sigma Chemicals, St. Louis, MO) solution was made fresh in saline and administered by intraperitoneal (i.p.) injection. In experiment I (Table 1), PQ (5 mg kg^{-1}) was injected once weekly for 3 weeks and brain tissue (ventral midbrain (VMB) and cerebellum(CB)) harvested 24 h after the last injection. 5 male and 5 female mice of each strain were used. This is the highest dose used in our toxicology study and required to show equal distribution of PQ to the VMB. In experiment II, one of two doses of PQ (1 mg kg^{-1} or 5 mg kg^{-1}) *vs.* saline was injected once weekly for 3 weeks and brain tissue was harvested 24 h after the last injection. These doses and dosing regimen were based on the work of McCormack et al. (2002). Sample sizes of 4–7 male and 4–7 female mice of each strain were used for every treatment. In experiment III, PQ (10 mg kg^{-1}) or saline was injected once and brain tissue (ventral midbrain) was harvested at three different time points (24 h, 48 h, and 7 days) after the injection. The choice of this dose was to maximize possible effects on gene expression and minimize possible acute toxicity. We administered one dose to capture the acute kinetics of gene expression. Samples consisting of 2–4 male mice of each strain were used for every time point. In experiment IV, PQ (15 mg kg^{-1}) or saline was injected once and the brain tissue (ventral midbrain and caudate putamen) was harvested 24 h later from 2–4 male and 3–9 female mice of each strain. In this case, the animals were sacrificed within 24 h, because we wanted to maximize possible effects on acute iron accumulation. The mice were killed by CO_2 in experiments I and II, or cervical dislocation in experiments III and IV. Brain samples were stored at −80 °C until analysis.

2.4. Brain PQ determinations

Brain PQ was quantified by HPLC MS as described previously (Winnik et al., 2009). Briefly, tissue samples weighing 10–15 mg were placed into a 1.5-ml centrifuge tube, mixed with 150 μl 12% acetic acid and sonicated for 20 min. The centrifuge tubes were then placed in high-pressure microwaveable Teflon extraction vessels (CEM HP500; CEM Corp., Matthews, NC) and heated in a microwave digestion/extraction system (CEM MARS; CEM Corp.) for 30 min at 50% power. The samples were then centrifuged using a 10-kDa filter (Nanosep 10 k Omega Pall Trincor; Omega Pall Corp., Exton, PA) and the filtrate was transferred to HPLC/autosample vials for storage at −30 °C until analysis. The PQ separation was carried out on ZORBAX RX-C8, 4.6 mm × 15 cm, 5-μm column (Agilent Technologies, Santa Clara, CA) with reverse-phase column guard 4.3 mm × 1 cm. PQ was eluted at flow rate 0.3 ml/min with a retention time of 5 min monitored by both ultraviolet detector and mass spectrometer (Waters 996 photodiode array detector; Waters Corp., Milford, MA). A 50-μl aliquot was injected. Gradient elution was established with three-solvent system consisting of 0.1% formic acid in water (A), 0.1% formic acid in methanol (B), and 0.1% formic acid in acetonitrile (C). Each calibration

Table 1
PQ dosing regimen and sample size.

Exp.	Dose mg kg^{-1}	Sample size	Mice age	Dosing regimen (ip)	Measurements
I	5	5 M and 5 F	2.5–4 months	Once weekly ×3 weeks, killed 24 h later	PQ tissue (VMB, CB) concentration
II	1, 5	4–7 M and 4–7 F for each dose	2.5–4 months	Once weekly ×3 weeks, killed 24 h later	The number of DA neurons in the SNpc
III	10	2–4 M	2.5–4 months	Single injection, killed 24 h, 48 h, or 7 days later	VMB transcriptome
IV	15	2–4 M and 3–9 F	120 ± 10 days	Single injection, killed 24 h later	Iron concentrations in VMB and CP

Abbreviation: VMB: ventral midbrain, CB: cerebellum, DA: dopaminergic neurons, SNpc: substantia nigra pars compacta, CP: caudate putamen, h: hour.

curve was prepared separately for each experiment and composed of PQ standards (Sigma Chemicals) with a concentration range of 0–12 ng. As a part of each experiment matrix blanks and spikes were prepared. Matrix blanks were prepared by adding 150 µl of the extraction solvent to untreated mouse brain tissue. The linear range was usually 0–2 ng/µl. Quantification was then carried out using an LCQ ion trap mass spectrometer (Finnigan, San Jose, CA) equipped with an electrospray ionization source (ESI) and operated using Xcalibur 1.3 software (ThermoFischer Scientific Waltham, MA). Quantification of PQ was then based on integrated peak areas of the m/z 186 ion in selective ion monitoring (SIM) mode with isolation width m/z.

2.5. Immunohistochemistry

Immediately after sacrifice, mice were perfused with heparanized saline (0.15 M, about 15 ml) followed by fresh cold 4% paraformaldehyde solution (in 0.1 M PB, pH 7.4, about 100 ml). Brains were removed and postfixed in the same fixative solution at 4 °C for 24 h and then transferred to increasing concentrations of sucrose (10%, 20% and 30%, in 0.1 M PB, pH 7.4) for 24 h each for cryoprotection. Brains were then frozen in isopentane cooled by dry ice and stored at −80 °C until sectioning. The frozen fixed brains were cut using a cryostat (Leica CM-3050-S, chamber temperature −20 °C) into 40 µm serial coronal sections containing the entire SNpc; every fourth section was free-floating stained. Staining was performed by washing 3 times in PB (0.1 M, pH7.4). Non-specific binding was blocked by immersion in10% normal goat serum (NGS) for 1.5 h in carrier solution (1% NGS, 0.3% triton X-100 and 1%BSA in 0.1 M PB) at room temperature. Next was a 48 h incubation with rabbit anti-Tyrosine Hydroxylase (TH) primary antibody (Chemicon AB152, 1:4000) at 4 °C, followed by washing 3 times with carrier solution, and finally, incubation with biotinylated goat anti-rabbit IgG (H + L) antibody (Vector BA-1000, 1:400) for 24 h at 4 °C. The color was developed by using avidin–biotinylated enzyme complex (Vector PK-6100, Burlingame, CA). After drying overnight, the sections were counterstained using Cresyl violet.

2.6. Stereology

The number of TH positive (TH+) and TH negative (TH−) neurons in the SNpc were counted using the optical fractionator (Stereo Investigator, MBF bioscience, Williston, VT, USA), with a counting frame measuring 61 µm(x)×61 µm (y), grid size measuring 200 µm (x)×100 µm (y), and with 0.5 µm top and bottom guard zones. Images captured at low power (40×) were used to evaluate the staining and tissue quality, reorder the brain sections from rostral to caudal and to trace the region of interest (SNpc). Images captured at high power (1000×) were used for cell counting. Cell counting was performed on one side of each section (showing the best tissue quality) and by counting every other section. In total, four sections were subjected for each brain. Cell counting followed the criterion described by Thiruchelvam et al. (2004). Within one counting frame, TH+ neurons counted must show both brown TH staining in the cell body and blue nissl staining in the nucleus, and the nucleus does not touch or cross the red avoidance lines of the counting frame; TH− neurons counted must show blue nissl staining without cytoplasmic brown TH staining. Plus, the TH− neurons must have round to oval shape with smooth nuclear membrane, one macronucleolus with more than one small nucleoli, equal size with the nuclei of TH+ neurons, and the nucleus does not touch or cross the red avoidance lines of the counting frame. The total numbers of TH+ and TH− neurons in the SNpc of one brain hemisphere were estimated by the software.

2.7. Microarray analysis

The VMB was dissected and placed in RNAlater (Ambion AM7020, Foster City, CA) immediately after cervical dislocation. Total RNA was isolated using RNeasy Lipid Tissue MiniKit (Qiagen 74804, Valencia, CA). The quantity and purity of RNA were evaluated using a Nanodrop ND-1000 spectrophotometer ((NanoDrop Technologies Inc, Wilmington, USA). Only samples with an OD 260/280 ratio of 1.8 to 2 and an OD 260/230 ratio of 1.8 or greater were used for microarray analysis. RNA integrity was assessed using an Agilent Bioanalyzer 2100 (Agilent Technologies, USA). The RNA from each sample was processed using the Illumina TotalPrep RNA amplification kit (Ambion, Austin, TX, USA) to generate biotinylated, amplified cRNA. The biotin-labeled cRNA was then evaluated using a NanoDrop ND-1000. Those samples with OD 260/280 ratios between 2.0 and 2.3 were immediately hybridized to an Illumina Sentrix Mouse WG-6V1 oligomer BeadChip slide (Illumina, San Diego, CA, USA) following standard Illumina protocols. A total of 24 ventral midbrain samples were processed using 4 Illumina slides. Each slide contained both PQ-treated and saline treated samples and from both strains, assigned at random. The array consists of 46,632 unique probe sequences, each 50 nucleotides in length. This particular data set was processed using the Illumina Rank Invariant method. Values were log2 transformed, and variance of each array was stabilized to 4 units (SD of 2 units) and re-centered to a mean of 8.

2.8. Brain iron analysis

Each mouse brain was dissected into ventral midbrain (VMB) and caudate putamen (CP) immediately after death as described by Boone et al. (1997). Total iron concentrations in VMB and CP were determined according to the modified procedures of Erikson et al. (1997). Briefly, brain tissue was weighted and combined with ultrapure nitric acid (VMB = 400 µl, CP = 200 µl) (JT Baker 9598–00, Phillipsburg, NJ) in a 1.5 ml polypropylene centrifuge tube. All tissues were digested for at least 24 h at 60 °C and then re-suspended with an equivalent amount of nanopure water. Each sample was further diluted 1:50 with 0.2% ultrapure nitric acid and immediately analyzed for iron by atomic absorption spectrophotometry (Perkin Elmer AAnalyst 600, Perkin Elmer, Norwalk, CT). Standards were prepared by diluting a Perkin Elmer iron standard (PE# N9300126) in 0.2% ultrapure nitric acid and blanks prepared with digesting and diluting reagents to control for possible contamination.

2.9. Data analysis

Brain PQ and iron concentrations were compared by strain and by sex using ANOVA for a between-subjects variables experiment. For the numbers of neurons (TH+ and TH−), the main effects of strain and sex were compared using ANOVA. Post-hoc comparisons between treatment and control means for each strain were made using one-tailed Dunnett's t-tests, and strain differences for treatment effects were analyzed using Contrast (Kutner et al., 2004). Microarray data were analyzed using Nexus Expression (BioDiscovery Inc, El Segundo CA).

3. Results

3.1. PQ concentration in ventral midbrain and cerebellum

There were no strain, sex, or brain region differences in PQ concentrations in the VMB or CB (Fig. 1).
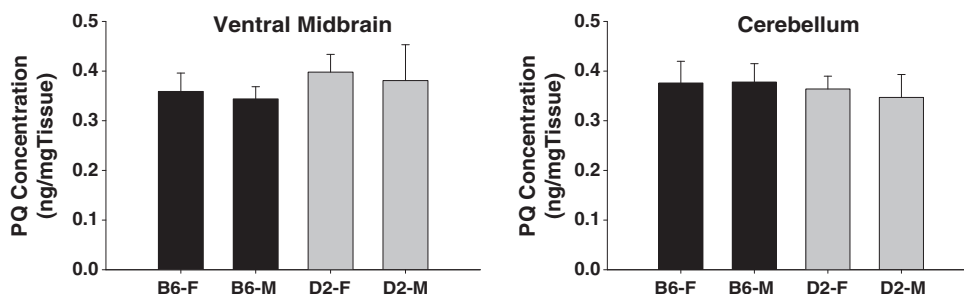
Fig. 1. PQ concentration in ventral midbrain and cerebellum. Black bars represent strain C57BL/6J females and males (B6-F and B6-M); gray bars represent strain DBA/2J females and males (D2-F and D2-M). Tissue PQ concentrations are equal between the two strains, two brain regions, and sexes, after 3 weekly ip injections at 5 mg kg^{-1}. Data are presented as means ± SEM.

3.2. Effects of PQ on TH+ neurons in the SNpc

The main effects for strain and sex were not significant ($F_{1,51} < 1$ for both). The main effect of treatment was significant ($F_{2,51} = 38.58$, $p < 0.001$) and accounted for 54% of total variance by est. ω^2. There was also a significant strain by treatment interaction ($F_{2,51} = 3.25$, $p < 0.05$). At 1 mg kg^{-1}, PQ produced significant TH+ neuron loss in B6 ($p < 0.05$) but not in D2 mice (Fig. 2). 5 mg kg^{-1} produced significant TH+ neuron loss in D2 mice (17.6%, $p < 0.001$) and in B6 mice (31.4%, $p < 0.0001$). Moreover the TH+ neuron loss at 5 mg kg^{-1} in B6 was significantly greater than in D2 mice ($p < 0.01$). When we analyzed data of each sex separately, in males, PQ induced TH+ neuron loss showed the same pattern as pooled sexes in both strains.

In females however, only 5 mg kg^{-1} produced significant TH+ loss in D2 ($p < 0.05$) and in B6 ($p < 0.001$), and there was no strain difference at this dose (data not shown). We also quantified the number of TH negative (TH−) neurons in the SNpc (data not shown). In D2 mice, significant TH− neuron loss was observed at 1 mg kg^{-1} and at 5 mg kg^{-1} ($p < 0.05$). In B6 mice, no significant TH− neuron loss was observed at either dose.

3.3. Effect of PQ on iron concentration in VMB

Compared to saline, we observed a 25% PQ-related increase in VMB iron concentration in B6 ($p < 0.05$) but not in D2s. PQ had no effect on iron in the CP in either strain (Fig. 3).
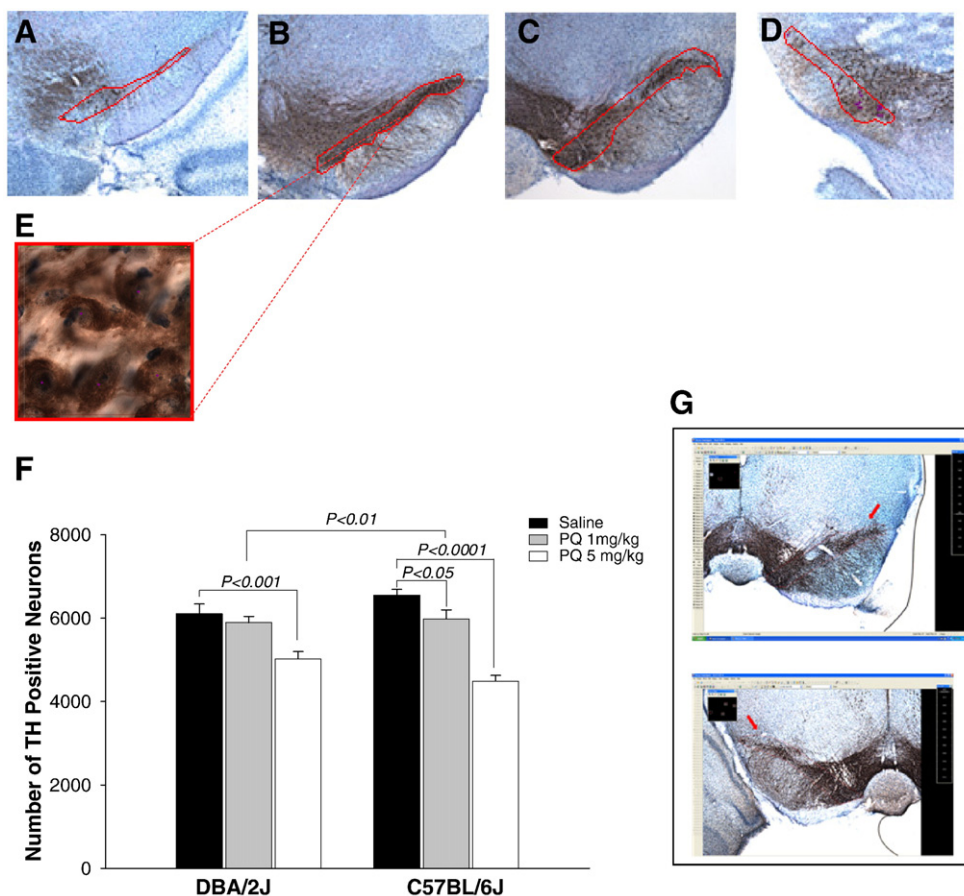

Fig. 2. Stereology of cell counting. A–D, micrographs taken at 40× magnification. The four sections represent every 8th section (1st, 9th, 17th, and 25th) of a brain sample containing the whole SNpc. Brown color indicates tyrosine hydroxylase (TH) positive staining, and blue color was from Nissl staining. SNpc lies within the red line. E, TH+ neurons under 1000× magnification. F, differential strain susceptibility to PQ neurotoxicity. In both strains, 5 mg kg^{-1} PQ induced significant loss of TH+ neurons compared to controls. C57BL/6J mice lost more TH+ than DBA/2J. Data are presented as means ± SEM. G, micrographs captured at 40× magnification. The top figure is from a saline treated male C57BL/6J mouse, and the bottom is from a PQ treated male C57BL/6J mouse. The red arrows point to the SNpc.
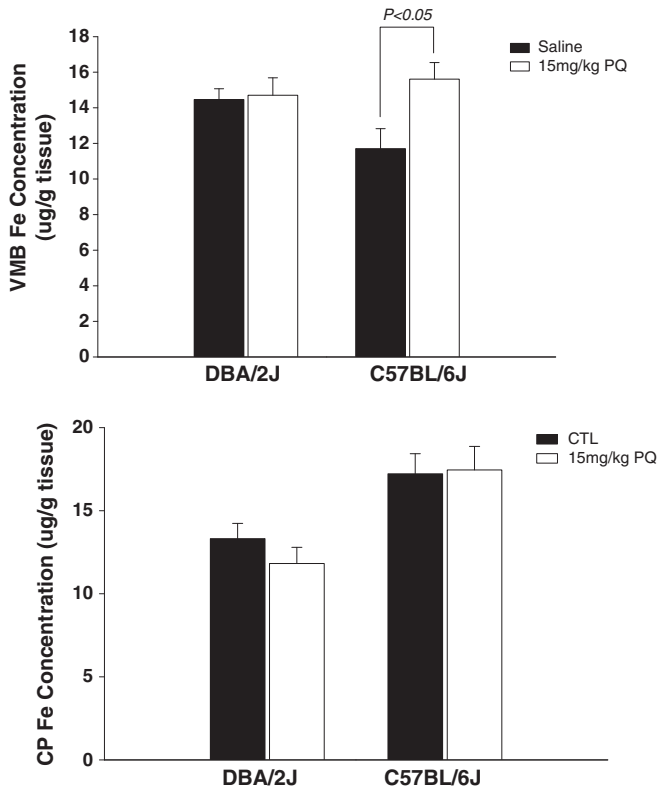
Fig. 3. PQ enhances ventral midbrain iron concentration. A 25% increase in ventral midbrain iron concentration was observed in C57BL/6J mice injected with a single ip injection of 15 mg kg^{-1} PQ. No such change was observed in the DBA/2J mice. Data are presented as means $\pm$ SEM.

3.4. Effect of PQ on gene expression in the VMB

Genes showing more than a 1.5-fold change (increase or decrease) in response to PQ and p-value less than 0.05 in paired-*t*-test (PQ *vs.* saline) were defined as differentially expressed genes. B6 mice evinced greater numbers of differentially expressed genes than D2 mice at all time points (Fig. 4). 24 h after PQ injection, the numbers of the differentially expressed genes in B6 and D2 were 5 (4− decreased, 1+ increased) and 1+, respectively. 48 h after PQ injection, the numbers of differentially expressed genes in B6 and D2 were 204 (82−, 122+) and 12 (5−, 7+), respectively. 7 days after PQ injection, the numbers of differentially expressed genes in B6 and D2 were 358 (225−, 133+) and 7 (2−, 5+), respectively, as shown in Fig. 4B. In summary, the numbers of differentially expressed genes kept increasing from 24 h to 48 h to 7 days after PQ treatment in B6 mice (numbers of differentially expressed genes = 5, 204 and 358, respectively) while the pattern was different in strain D2 mice, peaking at 48 h and then decreasing at 7 days (numbers of differentially expressed genes = 1, 12 and 7).

We performed gene enrichment analysis using Nexus Expression to group differentially expressed genes *by molecular function,* using gene ontology terms. As shown in Table 2 in the Supplementary data, the top three most significantly over represented groups at 48 h in B6 mice in order were iron ion binding, aromatic-ʟ-amino-acid decarboxylase activity which is involved in dopamine synthesis, and thiol–disulfide exchange intermediate activity (1 gene) which is related to redox homeostasis. At 7 days, the top three were iron ion binding, selenium binding, and the NADH dehydrogenase (ubiquinone) activity. The molecular functions of the differential expressed genes in other treatment groups are presented in Table 2c of the Supplementary data.

4. Discussion

4.1. Strain differences in PQ neurotoxicity

Our primary hypothesis that B6 and D2 mice evince differential, genetically-based susceptibility to PQ neurotoxicity was confirmed. Compared to D2 mice, B6 mice lost greater numbers of dopaminergic neurons in the SNpc, gained more VMB iron, and showed greater numbers of differentially expressed genes after PQ treatments (*vs.* saline control). Moreover, we also found no change in the number of non-dopaminergic (tyrosine hydroxylase negative, TH−) neurons in the SNpc in B6 mice after the PQ treatment as well, which confirmed the specific toxic effect of PQ on striatal dopaminergic system. However, D2 mice did show significant TH− neuron loss in the SNpc after the same treatment. This might indicate strain specific biological change, or a challenge to the TH and Nissl double staining strategy. A recent paper reported the advantage of using neuron specific nuclear antigen (NeuN) to specifically recognize neuronal nuclei, overcoming Nissl staining which universally stains all neuronal RNA (Prasad and Richfield, 2010). By using this staining technique in future studies, we might be able to answer whether the loss of TH− neurons shown in D2 mice was due to PQ toxicity or the staining technique. PQ has been reported to decrease striatal dopamine in mice (Shimizu et al., 2003). PQ in combined with Maneb has been reported to reduce tyrosine hydroxylase (TH) immunoreactivity in the dorsal striatum in mice, however, PQ alone did not show this effect (Thiruchelvam et al., 2000). In our study, the significant loss of TH positive neurons in both strains was not accompanied by a reciprocal increase in TH negative neurons which indicated that TH *per se* was not downregulated (Prasad and Richfield, 2008).

4.2. PQ neurotoxicity is not related to distribution

Under our dosing regimen, we also found that the differential strain susceptibility to PQ neurotoxicity was not related to distribution of PQ to the brain. Indeed, the seemingly equal distribution between ventral midbrain and cerebellum might indicate that unlike MPTP whose neurotoxic effects rely on being transported into DA neurons by dopamine transporter (DAT), PQ neurotoxicity may not depend on similar transport. We cannot be sure on this point at this time because we tested the entire ventral midbrain. The mechanism underlying why DA neurons are more vulnerable to PQ neurotoxicity than other types of neurons is not clear. One possibility is the interaction between iron and dopamine. Dopamine metabolism yields reactive oxygen species (ROS) which may accumulate in DA neurons with aging and make the neurons more sensitive than other types of neurons to neurotoxins such as PQ. Moreover, iron *per se* can produce ROS.

4.3. Role of iron in PQ neurotoxicity

Iron plays a central role in oxidative stress (Dauer and Przedborski, 2003) and is reported to induce the formation of α-synuclein protofibrils in brain (Golts et al., 2002; Rhodes and Ritz, 2008). Iron has been shown to exacerbate the neurotoxic effect of PQ as well (Peng et al., 2007). In this study, we found, for the first time, that PQ can increase iron in VMB, an effect that is strain-dependent. Furthermore, gene expression and enrichment analysis revealed that iron ion binding was the most overrepresented molecular function among the differentially expressed genes after PQ treatment in B6 mice. The pattern of numbers of DA neuron loss, elevated VMB iron, and differentially expressed iron-related genes shown in B6 (*vs.* D2) mice supports the involvement of iron in PQ neurotoxicity, and implies that the differential strain capabilities to regulate VMB iron homeostasis may well contribute to the differential strain susceptibility to PQ neurotoxicity. In addition, we found that caudate putamen iron concentration was refractory to PQ treatment. These support the hypothesis of specificity of PQ toxicity in DA neurons in the VMB
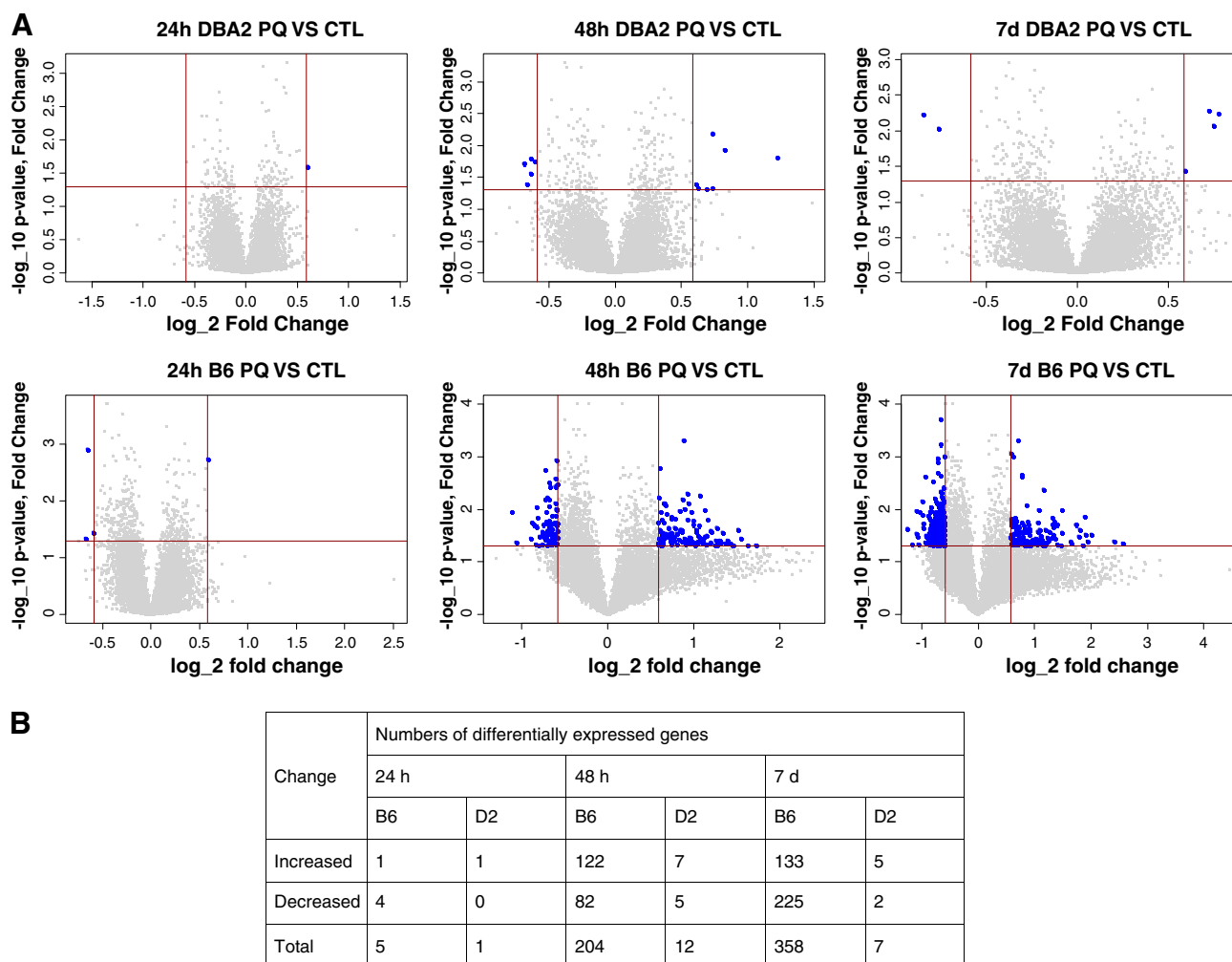
A

B

Change	Numbers of differentially expressed genes					
	24 h		48 h		7 d	
	B6	D2	B6	D2	B6	D2
Increased	1	1	122	7	133	5
Decreased	4	0	82	5	225	2
Total	5	1	204	12	358	7

Fig. 4. Gene expression change after paraquat treatment. A, Volcano plot of differentially expressed genes (blue colored) between experiment (PQ) and saline control groups, in each strain and at each time point. The Y-axis displays the –$\log_{10}$ of p values from paired t-tests, the x-axis displays the $\log_2$ of the fold change of gene expression level (experiment/control). The dark red lines correspond to p-value of 0.05 and fold changes of ($\pm$) 1.5. Genes with p<0.05, and fold change more than 1.5, were identified as differentially expressed and are highlighted in blue. B, the numbers of differentially expressed genes (p<0.05, 1.5< fold change <−1.5).

(including SNpc) and may implicate local regulation of iron homeostasis in VMB DA neurons. In humans, Iron accumulation in the VMB has been reported in Parkinson's disease patients, and the more accumulated VMB iron the more severe the disease (Bartzokis et al., 1999; Berg et al., 2001; Sofic et al., 1991). Thus, genes regulating VMB iron homeostasis might be novel therapeutic targets to prevent PQ induced sPD.

4.4. Gene expression

Our most salient finding in gene expression was PQ-related change in iron ion binding protein genes in the B6 strain. Alternatively, other than DOPA decarboxylase, no genes directly related to dopamine changed expression in response to PQ. Exactly why this is the case is unclear; however the former finding may help strengthen our understanding of the relationship between iron and dopamine. Mitochondrial dysfunction has been intensively studied as the basis for PQ neurotoxicity. The results are mixed as to whether PQ causes mitochondrial oxidative damage, and whether mitochondrial complex I is the major site of PQ-induced ROS production in the brain (Castello et al., 2007; Choi et al., 2008; Cochemé and Murphy, 2008; Richardson et al., 2005). Our study does suggest mitochondrial involvement in PQ neurotoxicity. Mitochondrial genes (Ndufs7, Ndufc2, Ndufv2, Grx2, and Sdhd) were differentially expressed based on the transcriptome data. The first 4 of these play important

roles in maintaining mitochondrial complex I activity and their expression was increased after PQ treatment. Sdhd is a subunit of mitochondrial complex II and its expression was increased after PQ treatment as well. Real-time PCR verification of these gene expression changes will help to elucidate their biological role in response to PQ.

In the D2 strain, no iron or dopamine related genes were responsive to PQ. Most of the genes that did respond were related to intermediate metabolism and reactive oxygen species (ROS).

Other than iron homeostasis and mitochondrial response, several other biological processes were affected by PQ, based on our transcriptome data. These include the ubiquitin proteosome system, lipid metabolism, catecholamine biosynthesis, and inflammatory response. This shows that PQ neurotoxicity is a highly complex trait. Thus, we believe using systematic genetic analysis will lead to better understanding the mechanism of PQ neurotoxicity and its role in the etiology of sPD.

4.5. Stereology of cell counting

On a methodological note, Schmitz and Hof (2005) reviewed the literature concerning stereological methods and sources of bias in selecting sections. In their view, when comparing between regions or animals (strain and sex), the sections to be counted for features should be picked at random in order to avoid bias. In the present situation, we chose which sections to count *a priori* and to be the same

for all animals regardless of strain or treatment. In our case, if we were making comparisons strictly between the strains on number of TH+ neurons in the SNpc, the potential for bias would be of great concern. Our interest, however, was to make comparisons between treatment conditions within strain. Thus, for our purposes it was important to compare the same sections between animals in different dose groups. Error in this case should be random error and not bias. Inbred strains are remarkably similar in the morphology of most structures, including the brain. Is it possible, however, that between the strains, we chose non comparable areas so that had we picked another area in the SNpc, there would be no difference in sensitivity to PQ between B6 and D2 mice? This is indeed possible. On inspection of all of the sections, the morphology of the SNpc is remarkably similar between the strains, so we think bias in this case is unlikely. As for sampling sites within the sections, the Stereo Investigator™ software assigns these at random.

5. Conclusion

In this work, we have shown that the extent of PQ neurotoxicity is influenced by genetic background and may be related to brain iron content or homeostasis. The genetic influence is supported by the stereology and gene expression work and the involvement of iron by the influence of PQ on iron content in the midbrain and that many of the genes that show change in response to PQ are related to iron homeostasis. This research sets the stage for more extensive work in genetic reference populations, such as the BXD recombinant inbred strains so that we may identify the genes and gene networks that underlie individual differences in susceptibility to PQ toxicity in humans. This is possible because the genomes in mouse and humans are 90% syntenic.

Conflict of interest statement

On behalf of all authors, I hereby certify that there exist no conflicts of interest in presenting this research.

Acknowledgements

Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.ntt.2011.02.012.

References

Barbeau A, Cloutier T, Roy M, Plasse L, Paris S, Poirier J. Ecogenetics of Parkinson's disease: 4-hydroxylation of debrisoquine. Lancet 1985;2:1213–6.

Bartzokis G, Cummings JL, Markham CH, Marmarelis PZ, Treciokas LJ, Tishler TA, et al. MRI evaluation of brain iron in earlier- and later-onset Parkinson's disease and normal subjects. Magn Reson Imaging 1999;17:213–22.

Berg D, Gerlach M, Youdim MB, Double KL, Zecca L, Riederer P, et al. Brain iron pathways and their relevance to Parkinson's disease. J Neurochem 2001;79:225–36.

Boone EM, Cook MN, Hou X, Jones BC. Sex and strain influence the effect of ethanol on central monoamines. J Stud Alcohol 1997;58:590–9.

Bromilow RH. Paraquat and sustainable agriculture. Pest Manag Sci 2004;60:340–9.

Brooks AI, Chadwick CA, Gelbard HA, Cory-Slechta DA, Federoff HJ. PQ elicited neurobehavioral syndrome caused by dopaminergic neuron loss. Brain Res 1999;27:1-10.

Castello PR, Drechsel DA, Patel M. Mitochondria are a major source of PQ-induced reactive oxygen species production in the brain. J Biol Chem 2007;282:14186–93.

Choi WS, Kruse SE, Palmiter RD, Xia Z. Mitochondrial complex I inhibition is not required for dopaminergic neuron death induced by rotenone, MPP+, or PQ. Proc Natl Acad Sci U S A 2008;105:15136–41.

Churchill GA, Airey DC, Allayee H, Angel JM, Attie AD, Beatty J, et al. The Collaborative Cross, a community resource for the genetic analysis of complex traits. Nat Genet 2004;36:1133–7.

Cochemé HM, Murphy MP. Complex I is the major site of mitochondrial superoxide production by PQ. J Biol Chem 2008;283:1786–98.

Cook R, Lu L, Gu J, Williams RW, Smeyne RJ. Identification of a single QTL, Mptp1, for susceptibility to MPTP-induced substantia nigra pars compacta neuron loss in mice. Brain Res Mol Brain Res 2003;110:279–88.

Darvasi A, Soller M. A simple method to calculate resolving power and confidence interval of QTL map location. Behav Genet 1997;27:125–32.

Dauer W, Przedborski S. Parkinson's disease: mechanisms and models. Neuron 2003;39:889–909.

Erikson KM, Pinero DJ, Connor JR, Beard JL. Regional brain iron, ferritin and transferrin concentrations during iron deficiency and iron repletion in developing rats. J Nutr 1997;127:2030–8.

Fernagut PO, Hutson CB, Fleming SM, Tetreaut NA, Salcedo J, Masliah E, et al. Behavioral and histopathological consequences of PQ intoxication in mice: effects of alpha-synuclein over-expression. Synapse 2007;61:991-1001.

Golts N, Snyder H, Frasier M, Theisler C, Choi P, Wolozin B. Magnesium inhibits spontaneous and iron-induced aggregation of alpha-synuclein. J Biol Chem 2002;277:16116–23.

Gwinn-Hardy K. Genetics of parkinsonism. Mov Disord 2002;17:645–56.

Hamre K, Tharp R, Poon K, Xiong X, Smeyne RJ. Differential strain susceptibility following 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP; administration acts in an autosomal dominant fashion: quantitative analysis in seven strains of Mus musculus. Brain Res 1999;15:91-103.

Hertzman C, Wiens M, Bowering D, Snow B, Calne D. Parkinson's disease: a case–control study of occupational and environmental risk factors. Am J Ind Med 1990;17: 349–55.

Kutner M, Nachtsheim C, Neter J, Li W. Applied linear statistical models. 5th edition. McGraw-Hill; 2004.

Landrigan PJ, Sonawane B, Butler RN, Trasande L, Callan R, Droller D. Early environmental origins of neurodegenerative disease in later life. Environ Health Perspect 2005;113:1230–3.

Liou HH, Tsai MC, Chen CJ, Jeng JS, Chang YC, Chen SY, et al. Environmental risk factors and Parkinson's disease: a case–control study in Taiwan. Neurology 1997;48: 1583–8.

Manning-Bog AB, McCormack AL, Li J, Uversky VN, Fink AL, Di Monte DA. The herbicide PQ causes up-regulation and aggregation of alpha-synuclein in mice: PQ and alpha-synuclein. J Biol Chem 2002;277:1641–4.

McCormack AL, Thiruchelvam M, Manning-Bog AB, Thiffault C, Langston JW, Cory-Slechta DA, et al. Environmental risk factors and Parkinson's disease: selective degeneration of nigral dopaminergic neurons caused by the herbicide PQ. Neurobiol Dis 2002;10:119–27.

McCormack AL, Atienza JG, Johnston LC, Andersen JK, Vu S, Di Monte DA. Role of oxidative stress in PQ-induced dopaminergic cell degeneration. J Neurochem 2005;93:1030–7.

Peirce JL, Lu L, Gu J, Silve LM, Williams RW. A new set of BXD recombinant inbred lines from advanced intercross populations in mice. BMC Genet 2004;29:5–7.

Peng J, Mao XO, Stevenson FF, Hsu M, Andersen JK. The herbicide PQ induces dopaminergic nigral apoptosis through sustained activation of the JNK pathway. J Biol Chem 2004;279:32626–32.

Peng J, Peng L, Stevenson FF, Doctrow SR, Andersen JK. Iron and PQ as synergistic environmental risk factors in sporadic Parkinson's disease accelerate age-related neurodegeneration. J Neurosci 2007;27:6914–22.

Prasad K, Richfield EK. Sporadic midbrain dopamine neuron abnormalities in laboratory mice. Neurobiol Dis 2008;32:262–72.

Prasad K, Richfield EK. Number and nuclear morphology of TH+ and TH− neurons in the mouse ventral midbrain using epifluorescence stereology. Exp Neurol 2010;225:328–40.

Prasad K, Winnik B, Thiruchelvam MJ, Buckley B, Mirochnitchenko O, Richfield EK. Prolonged toxicokinetics and toxicodynamics of PQ in mouse brain. Environ Health Perspect 2007;115:1448–53.

Prasad K, Tarasewicz E, Mathew J, Strickland PA, Buckley B, Richardson JR, et al. Toxicokinetics and toxicodynamics of PQ accumulation in mouse brain. Exp Neurol 2009;215:358–67.

Rhodes SL, Ritz B. Genetics of iron regulation and the possible role of iron in Parkinson's disease. Neurobiol Dis 2008;32:183–95.

Richardson JR, Quan Y, Sherer TB, Greenamyre JT, Miller GW. PQ neurotoxicity is distinct from that of MPTP and rotenone. Toxicol Sci 2005;88:193–201.

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

Schmitz C, Hof PR. Design-based stereology in neuroscience. Neuroscience 2005;130: 813–31.

Sofic E, Paulus W, Jellinger K, Riederer P, Youdim MB. Selective increase of iron in substantia nigra zona compacta of parkinsonian brains. J Neurochem 1991;56: 978–82.

Thiruchelvam M, Richfield EK, Baggs RB, Tank AW, Cory-Slechta DA. The nigrostriatal dopaminergic system as a preferential target of repeated exposures to combined paraquat and maneb: implications for Parkinson's disease. J Neurosci 2000;20: 9208–14.

Thiruchelvam MJ, Powers JM, Cory-Slechta DA, Richfield EK. Risk factors for dopaminergic neuron loss in human alpha-synuclein transgenic mice. Eur J Neurosci 2004;19:845–54.

Winnik B, Barr DB, Thiruchelvam M, Montesano MA, Richfield EK, Buckley B. Quantification of paraquat, MPTP, and MPP+ in brain tissue using microwave assisted solvent extraction (MASE) and high performance liquid chromatography-mass spectrometry. Anal Bioanal Chem 2009;395:195–201.