HOST SUSCEPTIBILITY TO ENVIRONMENTAL TOXICANTS

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by
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Abstract

Heavy metals, pesticides and herbicides are suspected risk factors for neurological disorders including Parkinson’s disease (PD). Nevertheless, their role is not cut-cut, as there are inconsistencies in epidemiological and preclinical research. One reason may be related to individual differences in susceptibility, differences that can be traced to the genetic constitution of those humans and animals exposed. Taking into account the role of host susceptibility and individual differences in the study of complex traits such as pesticide toxicity will result in better understanding the etiology and pathways involved in the development of complex neurological disorders such as Parkinson’s Disease (PD). Newer epidemiological and animal methods address the role of genes, the environment and their interaction as key. Increased sensitivity to paraquat toxicity, a broadly used herbicide, in C57B6 mice compared to D2 is an example that highlights the importance of genetic constitution and gene-environment interaction. In this thesis, we report a broad response variation to MPTP neurotoxicity in 9-10 strains of BXD recombinant male and female mice. MPTP is a dopaminergic neurotoxin that induces parkinsonism symptoms in humans and animals who have been systemically exposed to it. MPTP neurotoxicity results in a significant reduction of dopamine (DA) concentration in the striatum in humans, primates and mice. C57B6 Mice exposed to a single injection of 30mg/kg MPTP show a 40-50% reduction of the striatal DA concentration. Using genetic reference families such as BXD RI mice with known genetic constitution, allows us to investigate the importance of host susceptibility in response to neurotoxicants including, but not limited to MPTP. Striatal dopamine (DA) and its metabolites, DOPAC and HVA, and serotonin and its metabolite, 5-HIAAA, were analyzed by HPLC. DA turnover was assessed using DOPAC/DA and HVA/DA ratios. Striatal tyrosine hydroxylase (TH), glial fibrilary acidic protein (GFAP), and iron content in ventral midbrain were quantified. All dopamine measures, as well as TH and GFAP, demonstrated wide, genotype-dependent differences in response to MPTP in both sexes. A significant correlation between DA related phenotypes with the exception of DOPAC was observed between males and females, indicating the involvement of
multiple factors in dopaminergic neurotoxicity and probably neurological disorders such as PD. Moreover, our data show that the association between MPP\(^+\) levels, the active metabolite of MPTP, and the striatal dopamine compromise caused by MPTP treatment is weak and not statistically significant. The results of this thesis support the complex nature of host susceptibility to neurotoxicants in which multiple genes and possibly multiple environmental factors are involved. This systemic approach to the study of environmental neurotoxicants in genetic reference populations of mice is likely to elucidate genetic factors underlying individual differences in developing neurodegenerative diseases such as PD.
# Table of Contents

List of Figures ix

List of Tables xvii

Acknowledgments xix

Chapter 1
Neurodegenerative Diseases and Pesticide Exposure 1
1.1 Introduction ........................................ 1
1.2 The Pathology of Parkinson’s Disease .................. 3
1.3 Epidemiological Studies .................................. 3
1.4 Animal Studies ........................................ 6
1.5 The Importance of Host Susceptibility in pesticide neurotoxicity 8
1.6 Use of BXD Recombinant Inbred Mice to Study the role of Individual Differences to Neurotoxicity 9

Chapter 2
Systems Analysis of Genetic Variation in MPTP Neurotoxicity in Mice 11
2.1 Abstract ............................................. 11
2.2 Introduction .......................................... 12
2.3 Material and methods .................................. 14
  2.3.1 Animals ............................................. 14
  2.3.2 MPTP and reagents ................................. 14
  2.3.3 Drug Treatment and brain dissection ................. 14
  2.3.4 Biochemical assays ................................ 15
2.4 Data analysis .......................................... 16
  2.4.1 Results ............................................. 16
  2.4.2 Effects of MPTP on tyrosine hydroxylase in caudate-putamen 16
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.4.3 Effects of MPTP on dopamine neurochemistry in caudate-putamen</td>
<td>18</td>
</tr>
<tr>
<td>2.4.4 Effects of MPTP on GFAP in caudate-putamen</td>
<td>19</td>
</tr>
<tr>
<td>2.4.5 Effects of MPTP on iron accumulation in ventral midbrain (VMB)</td>
<td>21</td>
</tr>
<tr>
<td>2.4.6 Effects of MPTP on serotonin (5-HT) neurochemistry in caudate-putamen</td>
<td>22</td>
</tr>
<tr>
<td>2.4.7 Systems genetic analysis of MPTP effects and correlation with gene expression in the striatum</td>
<td>24</td>
</tr>
<tr>
<td>2.5 Discussion</td>
<td>28</td>
</tr>
<tr>
<td>2.6 Conclusion</td>
<td>30</td>
</tr>
<tr>
<td>Chapter 3</td>
<td></td>
</tr>
<tr>
<td>MPTP Neurotoxicity is Highly Concordant Between the Sexes</td>
<td>31</td>
</tr>
<tr>
<td>in BXD Recombinant Inbred Mouse Strains</td>
<td></td>
</tr>
<tr>
<td>3.1 Abstract</td>
<td>31</td>
</tr>
<tr>
<td>3.2 Introduction</td>
<td>32</td>
</tr>
<tr>
<td>3.3 Materials and methods</td>
<td>33</td>
</tr>
<tr>
<td>3.3.1 Animals</td>
<td>33</td>
</tr>
<tr>
<td>3.3.2 MPTP and Reagents</td>
<td>34</td>
</tr>
<tr>
<td>3.3.3 MPTP Treatment and brain dissection</td>
<td>34</td>
</tr>
<tr>
<td>3.3.4 Biochemical Assays</td>
<td>34</td>
</tr>
<tr>
<td>3.3.5 Data Analysis</td>
<td>35</td>
</tr>
<tr>
<td>3.4 Results</td>
<td>36</td>
</tr>
<tr>
<td>3.4.1 Effects of MPTP on Dopamine Neurochemistry in the Striatum</td>
<td>36</td>
</tr>
<tr>
<td>3.4.1.1 Effects of MPTP on Dopamine in the Striatum</td>
<td>36</td>
</tr>
<tr>
<td>3.4.1.2 Effects of MPTP on DOPAC in the Striatum</td>
<td>36</td>
</tr>
<tr>
<td>3.4.1.3 Effects of MPTP on HVA in the Striatum</td>
<td>36</td>
</tr>
<tr>
<td>3.4.1.4 Effects of MPTP on Dopamine turnover Ratio(DOPAC/DA)</td>
<td>37</td>
</tr>
<tr>
<td>3.4.1.5 Effects of MPTP on Dopamine Turnover (HVA/DA)</td>
<td>40</td>
</tr>
<tr>
<td>3.4.2 Effects of MPTP on Serotonin (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA)</td>
<td>41</td>
</tr>
<tr>
<td>3.4.3 Effects of MPTP on Tyrosine Hydroxylase (TH)</td>
<td>42</td>
</tr>
<tr>
<td>3.4.4 Effects of MPTP on GFAP in the Striatum</td>
<td>42</td>
</tr>
<tr>
<td>3.4.5 Correlational analysis of MPTP effects between the sexes</td>
<td>42</td>
</tr>
<tr>
<td>3.4.6 Principal Component Mapping</td>
<td>43</td>
</tr>
<tr>
<td>3.5 Discussion</td>
<td>44</td>
</tr>
<tr>
<td>3.6 Conclusion</td>
<td>46</td>
</tr>
</tbody>
</table>
Chapter 4
Genetic Correlational Analysis Reveals no Association between MPP+ and the Severity of Striatal Dopaminergic Damage Following MPTP Treatment in BXD Mouse Strains 47
4.1 Abstract ................................................................. 47
4.2 Introduction .......................................................... 48
4.3 Materials and methods ............................................. 49
4.4 Results ................................................................. 50
4.5 Discussion ............................................................. 50

Chapter 5
Critical Role of Individual Differences in Susceptibility to Paraquat Toxicity 52
5.1 Abstract ................................................................. 52
5.2 Introduction .......................................................... 53
5.3 Pathology of PD ....................................................... 54
5.3.1 Lewy Bodies ....................................................... 54
5.4 Risk Factors for PD ................................................... 55
5.5 Environmental Factors and PQ .................................... 56
5.6 The Importance of Gene-Environmental Interaction in PQ Toxicity 57
5.7 Potential Mechanisms of Paraquat Toxicity ....................... 59
5.8 Conclusion ............................................................. 63
5.9 Gaps in the Literature ............................................... 64
5.10 Future Directions .................................................... 65

Chapter 6
Is Paraquat a Dopaminergic Neurotoxicant? An Experiment on Paraquat Neurotoxicity in BXD Mice 67
6.1 Introduction .......................................................... 67
6.2 Materials and methods ............................................. 68
6.2.1 Animals ............................................................ 68
6.2.2 PQ Treatment ..................................................... 68
6.2.3 Biochemical Assays .............................................. 68
6.2.4 Data Analysis ..................................................... 69
6.3 Results ................................................................. 69
6.3.1 Effects of PQ on Dopamine-neurochemistry in the striatum 69
6.3.2 Effect of PQ on Serotonin-neurochemistry .................... 72
6.3.3 Effect of PQ on Tyrosine Hydroxylase in the striatum ........ 74
6.3.4 Effects of PQ on GFAP in the striatum ....................... 75
6.4 Discussion ............................................................. 76
Chapter 7
Toxicogenetics: In Search of Host Susceptibility to Environmental Toxicants

7.1 Abstract ................................................................. 80
7.2 Epidemiological Studies ............................................. 80
7.3 Genome-Wide Association Studies ............................... 82
7.4 Animal Models in Toxicogenetics-Two Complementary Approaches .......................... 83
    7.4.1 Forward Genetic Analysis of Toxicity as a Complex Trait .............................. 84
    7.4.2 What about Reverse Genetic Analysis ......................................................... 87
7.5 Putting it All Together ................................................ 87
7.6 General Comments Concerning Rodents in Toxicology .................. 88
7.7 Conclusion ................................................................. 89

Chapter 8
Conclusion ........................................................................ 90

Appendix A
Author’s Contribution in chapters 2-7 ..................................... 93

Appendix B
ANOVA Tables .................................................................... 95

Bibliography .......................................................................... 103
List of Figures

2.1 Effect of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) on concentration of tyrosine hydroxylase (TH) in the caudate-putamen of 10 BXD recombinant inbred mouse strains. Male mice were injected with 12.5 mg/kg of the pro-neurotoxin, MPTP (vs. saline) and sacrificed 48 h later. TH levels were determined by ELISA. Experimental and control values (upper panel), normalized to total protein, are expressed as means ± s.e.m. The lower panel presents the effect of MPTP expressed as % control. ANOVA revealed significant effects for strain, MPTP and their interaction (F_{9,78} = 9.97; F_{1,78} = 257.62; F_{9,78} = 6.03, respectively; all p < 0.001).

2.2 Effect of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) on dopamine (DA) concentration in the caudate-putamen in 10 BXD recombinant inbred mouse strains. Male mice were injected with 12.5 mg/kg of the pro-neurotoxin, MPTP (vs. saline) and sacrificed 48 h later. DA was assayed from tissue homogenates by HPLC. Experimental and control values (upper panel), normalized to tissue weights, are expressed as means ± s.e.m. The lower panel presents the effect of MPTP expressed as % control. ANOVA revealed a significant main effect of strain, MPTP and a significant interaction (F_{9,77} = 11.25; F_{1,77} = 445.61; F_{9,77} = 9.05, respectively; all p < 0.001).
2.3 Effect of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) on dihydroxyphenylacetic acid (DOPAC) concentration in the caudate-putamen in 10 BXD recombinant inbred mouse strains. Male mice were injected with 12.5 mg/kg of the pro-neurotoxin, MPTP (vs. saline) and sacrificed 48 h later. DOPAC was assayed from tissue homogenates by HPLC. Experimental and control values (upper panel), normalized to tissue weights, are expressed as means ± s.e.m. The lower panel presents the effect of MPTP expressed as % control. ANOVA confirmed significant main and interaction effects for Strain and MPTP (F$_{9,76}$ = 6.45; F$_{1,76}$ = 148.54; F$_{9,76}$ = 4.21; respectively; all p < 0.001).

2.4 Effect of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) on homovanillic acid (HVA) concentration in the caudate-putamen in 10 BXD recombinant inbred mouse strains. Male mice were injected with 12.5 mg/kg of the pro-neurotoxin, MPTP (vs. saline) and sacrificed 48 h later. HVA was assayed from tissue homogenates by HPLC. Experimental and control values (upper panel), normalized to tissue weights, are expressed as means ± s.e.m. The lower panel presents the effect of MPTP expressed as % control. ANOVA indicated significant main and interaction effects for Strain and MPTP (F$_{9,77}$ = 5.80; F$_{1,77}$ = 41.76; F$_{9,77}$ = 3.48, respectively; all p < 0.01).

2.5 Effect of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) on 3-methoxytyramine (3-MT) concentration in the caudate-putamen in 10 BXD recombinant inbred mouse strains. Male mice were injected with 12.5 mg/kg of the pro-neurotoxin, MPTP (vs. saline) and sacrificed 48 h later. 3-MT was assayed from tissue homogenates by HPLC. Experimental and control values (upper panel), normalized to tissue weights, are expressed as means ± s.e.m. The lower panel presents the effect of MPTP expressed as % control. ANOVA showed significant main effects for Strain and MPTP but no interaction (F$_{9,77}$ = 2.27, p < 0.03; F$_{1,77}$ = 21.90, p < 0.0001; F$_{9,77}$ = 1.90, p < 0.07, respectively).
2.6 Effect of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) dopamine turnover as measured by DOPAC/DA in the caudate-putamen in 10 BXD recombinant inbred mouse strains. Male mice were injected with 12.5 mg/kg of the pro-neurotoxin, MPTP (vs. saline) and sacrificed 48 h later. Experimental and control values (upper panel), normalized to tissue weights, are expressed as means ± s.e.m. The lower panel presents the effect of MPTP expressed as % control. ANOVA revealed no effect of treatment on this measure of turnover but a significant effect of strain on the effect of MPTP as well as the interaction between strain and MPTP ($F_{1,76} = 2.7$ p>0.05; $F_{9,76} = 11.01$ , $F_{9,76} = 7.52$, p<0.001 for both). . . . . . . . . . . . . . . 23

2.7 Effect of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) dopamine turnover as measured by HVA/DA in the caudate-putamen in 10 BXD recombinant inbred mouse strains. Male mice were injected with 12.5 mg/kg of the pro-neurotoxin, MPTP (vs. saline) and sacrificed 48 h later. Experimental and control values (upper panel), normalized to tissue weights, are expressed as means ± s.e.m. The lower panel presents the effect of MPTP expressed as % control. ANOVA indicated a significant effect of strain and MPTP treatment on this measure of turnover as well as an interaction ($F_{9,77} = 21.94$; $F_{1,77} = 65.13$; $F_{9,77} = 15.44$, respectively; all p < 0.001). . . . . . . . . . . . . . . . 24

2.8 Effect of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) on the concentration of glial fibrilary acidic protein (GFAP) in the caudate-putamen of 10 BXD recombinant inbred mouse strains. Male mice were injected with 12.5 mg/kg of the pro-neurotoxin, MPTP (vs. saline) and sacrificed 48 h later. GFAP levels in the caudate-putamen were determined by ELISA. Experimental and control values (upper panel), normalized to total protein, are expressed as means ± s.e.m. The lower panel presents the effect of MPTP expressed as % control. ANOVA revealed significant main effects of strain, MPTP treatment and their interaction ($F_{9,78} = 9.76$; $F_{1,78} = 145.80$; $F_{9,78} = 5.08$, respectively; all p < 0.001). . . . . . . . . . . . . . . . . . . . . . 25
2.9 Effect of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) on iron concentration in the ventral midbrain in 10 BXD recombinant inbred mouse strains. Male mice were injected with 12.5 mg/kg of the pro-neurotoxin, MPTP (vs. saline) and sacrificed 48 h later. Atomic absorption spectroscopy was used to determine iron concentration from tissue homogenates. Experimental and control values (upper panel), normalized to tissue weight, are expressed as means ± s.e.m. The lower panel presents the effect of MPTP expressed as % control. ANOVA indicated a significant effect of strain (F_{9,77} = 4.92, p < 0.001) and interaction between strain and MPTP treatment (F_{9,77} = 4.55, p < 0.001) but not MPTP treatment (F_{1,77} < 1).

2.10 Effect of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) on serotonin (5-HT) concentration in the caudate-putamen in 10 BXD recombinant inbred mouse strains. Male mice were injected with 12.5 mg/kg of the pro-neurotoxin, MPTP (vs. saline) and sacrificed 48 h later. DA was assayed from tissue homogenates by HPLC. Experimental and control values (upper panel), normalized to tissue weights, are expressed as means ± s.e.m. The lower panel presents the effect of MPTP expressed as % control. ANOVA revealed a significant main effect of strain, MPTP and a significant interaction (F_{9,77} = 6.15, p < 0.001; F_{1,77} = 9.64, p < 0.005; F_{9,77} = 2.63, p < 0.02, respectively).

2.11 System network graph for striatal GFAP following MPTP (label 15159), MPTP neurotoxicity index (PC01), and transcript abundance for genes Kansl1l, Lncrl1 and Mtap2. This is a graphical representation of the data presented in Table 2.1.

3.1 Top panel. Effect of MPTP on DA concentration in the striatum in 9 strains of BXD recombinant inbred (RI) female mice. The mice were injected with 12.5 mg/kg MPTP (vs. saline) and sacrificed 48 h later. DA content in striatum was determined by HPLC, normalized to tissue wet weights and expressed as mean ± s.e.m. Bottom panel. Effect of MPTP in BXD mice expressed as % control, for both sexes. BXD40 showed the greatest MPTP-related DA loss for both sexes. ANOVA revealed a significant main effect of strain, sex, treatment (F_{8,140}=17.21, F_{1,140}= 7.77 , F_{1,140}=849.86 respectively, p<0.01 for each) and significant strain × sex, strain × treatment and strain × sex × treatment interaction (F_{8,140}=5.49, F_{8,140}=17.37 respectively, p<0.01 for both, p<0.01, F_{8,140}= 2.2, p<0.05).
3.2 Top panel. Effect of MPTP on striatal DOPAC in females. Mice were injected with 12.5mg/kg MPTP (vs. saline) and sacrificed 48 h later. DOPAC content in striatum was determined by HPLC, normalized to tissue wet weights and expressed as mean ± s.e.m. Bottom panel. The effect of MPTP on DOPAC expressed as % control for both sexes. BXD40 showed the greatest MPTP-related DA loss for both sexes. ANOVA revealed a significant main effect of strain, sex, treatment (F_{8,139}=42.42, F_{1,139}=125.81, F_{1,139}=142.24, respectively, p<0.01 for each) and significant strain × sex interaction (F_{8,139}=24.39, p<0.01) and strain × treatment interaction (F_{8,139}=2.38, p<0.05).

3.3 Top panel. Effect of MPTP on Homovanillic acid (HVA) concentration in the striatum in females. The mice were injected with 12.5mg/kg MPTP or saline and sacrificed 48 h later. HVA content in striatum was determined by HPLC, normalized to tissue wet weights and expressed as mean ± s.e.m. BXD40 showed the highest reduction in both sexes. Bottom panel. The effect of MPTP on HVA expressed as % control in both sexes. ANOVA revealed a significant main effect of strain and treatment (F_{8,140}=11.49, F_{1,140}=111.26 respectively, p<0.01 for each). ANOVA revealed significant strain × treatment interaction (F_{8,140}=6.70, p<0.01).

3.4 Top panel. Effect of MPTP on DA turnover as determined by the ratio of DOPAC/DA in the striatum in female BXD mice. Mice were injected with 12.5mg/kg MPTP (vs. saline) and sacrificed 48 h later. Experimental and control values normalized to tissue wet weights are expressed as mean ± s.e.m. Bottom panel. The effect of MPTP on DOPAC/DA expressed as % control for both sexes. ANOVA revealed significant main effects for strain, sex and treatment (F_{8,140}=4.42, F_{1,140}=13.11, F_{1,140}=6.98 respectively, p<0.01 for each) and significant effect for strain × sex interaction (F_{8,140}=3.62, p<0.01).
3.5 Top panel. Effect of MPTP on DA turnover as determined by the ratio of HVA/DA in the striatum of female BXD RI mice. Female mice were injected with 12.5mg/kg MPTP (vs. saline) and sacrificed 48 h later. Experimental and control values normalized to tissue weights are expressed as mean ± s.e.m. Bottom panel. The effect of MPTP on HVA/DA expressed as % control in both sexes. ANOVA revealed significant main effects for strain, sex and treatment (F\textsubscript{8,140}=24.48, F\textsubscript{1,140}=8.53, F\textsubscript{8,140}=128.89 respectively, p<0.01 for each). The \textit{sex × strain, strain × treatment}, and \textit{strain × sex × treatment} interactions were significant (F\textsubscript{8,140}=8.99, F\textsubscript{8,140}=19.97, F\textsubscript{8,140}=5.33, p<0.01 for each). The \textit{sex × treatment} interaction was also significant (F\textsubscript{1,140}=4.64, p<0.05).

3.6 Top panel. The effects of MPTP on tyrosine hydroxylase (TH) concentration in the striatum in females. Mice were injected with 12.5mg/kg MPTP (vs. saline) and sacrificed 48 h later. TH content was determined by ELISA according to the method of O’Callaghan et al. (1990) and normalized to total protein. Data are expressed as mean ± s.e.m. Bottom panel. Effect of MPTP on TH expressed as % control in both sexes. ANOVA revealed significant main effect of strain and treatment (F\textsubscript{8,141}=14.65, F\textsubscript{1,141}=627.63, p<0.01 for each). \textit{Sex × strain, strain × treatment} and \textit{sex × treatment} interactions were all significant (F\textsubscript{8,141}=10.74, F\textsubscript{8,141}=9.88, F\textsubscript{1,141}=8.12, p<0.01 for each). The \textit{sex × treatment × strain} interaction was also significant (F\textsubscript{8,141}= 2.28, p<0.05).

3.7 Top panel. Effect of MPTP on the concentration of glial fibrillary acidic protein (GFAP) in the striatum in females. The mice were injected with 12.5mg/kg MPTP (vs. saline) and sacrificed 48 h later. GFAP content was determined by ELISA (O’Callaghan, 1991). GFAP concentrations were normalized to total protein and expressed as mean ± s.e.m. Bottom panel. Effects of MPTP on striatal GFAP expressed as % control in both sexes. ANOVA revealed significant main effects for strain and treatment and their interaction (F\textsubscript{8,138}= 20.19, F\textsubscript{1,138}= 243.09, F\textsubscript{8,138}=10.65, respectively, p<0.01 for each). .
6.1 Effect of paraquat (PQ) on dopamine (DA) concentration in the caudate-putamen in 19 BXD recombinant inbred mouse strains. Male and female mice were treated with three weekly injections of 10 mg/kg of PQ (vs. saline) and sacrificed seven days after the last injection. DA was assayed from tissue homogenates, using HPLC. Experimental and control values, normalized to tissue weights, are expressed as means ± s.e.m. ANOVA revealed no significant main effect for treatment ($F_{1,90}<1; p> 0.05$).

6.2 Effect of paraquat (PQ) on dopamine metabolite, DOPAC, concentration in the caudate-putamen in 19 BXD recombinant inbred mouse strains. Male and female mice were treated with three weekly injections of 10 mg/kg of PQ (vs. saline) and sacrificed seven days after the last injection. DOPAC was analyzed from tissue homogenates, using HPLC. Experimental and control values, normalized to tissue weights, are expressed as means ± s.e.m. ANOVA revealed no significant main effect of treatment ($F_{1,88}<1; p> 0.05$).

6.3 Effect of paraquat (PQ) on dopamine metabolite, HVA, concentration in the caudate-putamen in 19 BXD recombinant inbred mouse strains. Male and female mice were treated with three weekly injections of 10 mg/kg of PQ (vs. saline) and sacrificed seven days after the last injection. HVA was analyzed from tissue homogenates, using HPLC. Experimental and control values, normalized to tissue weights, are expressed as means ± s.e.m. ANOVA revealed no significant main effect of treatment ($F_{1,90}<1; p> 0.05$).

6.4 Effect of paraquat (PQ) on serotonin (5-HT), concentration in the caudate-putamen in 19 BXD recombinant inbred mouse strains. Male and female mice were treated with three weekly injections of 10 mg/kg of PQ (vs. saline) and sacrificed seven days after the last injection. 5-HT was analyzed from tissue homogenates, using HPLC. Experimental and control values, normalized to tissue weights, are expressed as means ± s.e.m. ANOVA revealed no significant main effect of treatment ($F_{1,90}=3.03; p> 0.05$).

6.5 Effect of paraquat (PQ) on serotonin (5-HIAA), concentration in the caudate-putamen in 19 BXD recombinant inbred mouse strains. Male and female mice were treated with three weekly injections of 10 mg/kg of PQ (vs. saline) and sacrificed seven days after the last injection. 5-HIAA was analyzed from tissue homogenates, using HPLC. Experimental and control values, normalized to tissue weights, are expressed as means ± s.e.m. ANOVA revealed no significant main effect of treatment ($F_{1,90}<1; p> 0.05$).
6.6 Effect of paraquat (PQ) on Tyrosine Hydroxylase (TH) concentration in the caudate-putamen in 10 BXD recombinant inbred mouse strains. Male and female mice were treated with three weekly injections of 10 mg/kg of PQ (vs. saline) and sacrificed seven days after the last injection. TH was analyzed from tissue homogenates, using ELISA. Experimental and control values, normalized to total protein are expressed as means ± s.e.m. ANOVA revealed no significant main effect of treatment ($F_{1,67}<1; p> 0.05$). . . . . . . 75

6.7 Effect of paraquat (PQ) on glial fibrillary acidic protein, a marker of astrocyte toxicity, concentration in the caudate-putamen in 10 BXD recombinant inbred mouse strains. Male and female mice were treated with three weekly injections of 10 mg/kg of PQ (vs. saline) and sacrificed 7 days after the last injection. TH was analyzed from tissue homogenates by ELISA. Experimental and control values, normalized to total protein are expressed as means ± s.e.m. ANOVA revealed no significant main effect of treatment ($F_{1,67}<1; p> 0.05$). 76

7.1 This is the same figure presented in chapter 2 (Fig 2.2). Effect of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) on dopamine (DA) concentration in the caudate-putamen in 10 BXD recombinant inbred mouse strains. Male mice were injected with 12.5 mg/kg of the pro-neurotoxin, MPTP (vs. saline) and sacrificed 48 h later. DA was assayed from tissue homogenates by HPLC. Experimental and control values (upper panel), normalized to tissue weights, are expressed as means ± s.e.m. The lower panel presents the effect of MPTP expressed as % control. ANOVA revealed a significant main effect of strain, MPTP and a significant interaction ($F_{9,77} = 11.25; F_{1,77} = 445.61; F_{9,77} = 9.05$, respectively; all $p < 0.001$). . . . . . . 85
List of Tables

2.1 Correlation matrix for the graphical representation of the system analysis presented in Figure 2.11 Correlation presented are Pearson r.  27

3.1 Pearson product-moment correlations, males vs. females for difference scores, saline minus MPTP (GFAP is MPTP-saline). . . . . . . 42

4.1 Correlation matrix for the graphical representation of the system analysis presented in Figure 2.11 Correlation presented are Pearson r.  50

B.1 DA concentration in the striatum, male data . . . . . . . . . . . . . 95
B.2 DOPAC concentration in the striatum, male data . . . . . . . . . . . 95
B.3 HVA concentration in the striatum, male data . . . . . . . . . . . . 96
B.4 3-MT concentration in the striatum, male data . . . . . . . . . . . . 96
B.5 TH concentration in the striatum, male data . . . . . . . . . . . . . 96
B.6 5-HT concentration in the striatum, male data . . . . . . . . . . . . 96
B.7 DOPAC/DA % in the striatum, male data . . . . . . . . . . . . . . 97
B.8 HVA/DA % in the striatum, male data . . . . . . . . . . . . . . . . 97
B.9 Fe concentration in the Ventral Midbrain, male data . . . . . . . . 97
B.10 GFAP concentration in the striatum, male data . . . . . . . . . . . 97
B.11 DA concentration in the striatum, sexes combined data . . . . . . 98
B.12 DOPAC concentration in the striatum, sexes combined data . . . . 98
B.13 HVA concentration in the striatum, sexes combined data . . . . . 99
B.14 DOPAC/DA% in the striatum, sexes combined data . . . . . . . . . 99
B.15 HVA/DA% in the striatum, sexes combined data . . . . . . . . . . . 100
B.16 TH concentration in the striatum, sexes combined data . . . . . . 100
B.17 GFAP concentration in the striatum, sexes combined data . . . . . 101
B.18 DA concentration in the striatum, PQ data . . . . . . . . . . . . . 101
B.19 DOPAC concentration in the striatum, PQ data . . . . . . . . . . . 101
B.20 HVA concentration in the striatum, PQ data . . . . . . . . . . . . . 102
B.21 TH concentration in the striatum, PQ data . . . . . . . . . . . . . 102
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Dedication

I dedicate my dissertation to my mother and sister. Their love and support and the great memories we share together is what makes life meaningful to me.
1.1 Introduction

Pesticides, herbicides and heavy metals are a threat to human health. Acute and chronic exposure to these toxicants can result in long term negative health outcomes. A vast body of epidemiological and animal studies support the association between pesticide exposure and a variety of complex clinical conditions such as cancer, reproductive health problems and multiple neurological disorders including behavioral performance impairments and a range of neurodegenerative diseases such as Alzheimer’s disease (AD), Multiple Sclerosis (MS), Amyotrophic Lateral Sclerosis (ALS) and Parkinson’s disease (PD) [1].

The relation between neurodegenerative diseases and pesticide exposure have been vastly studied, both in epidemiological and animal studies, however inconsistencies exist regarding the role of pesticides in the development of neurodegenerative diseases including sporadic PD. Sporadic PD (sPD) which is often observed in individuals over 50 years of age or older is believed to be the result of interactions between multiple genes and environmental factors including, but not limited to pesticide exposure. Variation in response to pesticide neurotoxicity may be a key factor affecting the results and therefore explaining the existing discrepancies in the literature. The main question to be asked in this dissertation is whether everyone is equally susceptible to neurotoxicity and why. The answer to that question will have significant implications in explaining the etiology of complex neurological disorders.
and help researchers to detect the susceptible populations in order to implement appropriate interventions to prevent the prevalence of such diseases.

In the proposed thesis I will examine the role of host susceptibility to neurotoxicants including 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and paraquat (PQ) using mouse models of BXD recombinant inbred (RI) to detect response variation and response range across the strains. The use of BXD RI mice allows the study of complex traits including host susceptibility from a genetic standpoint, potentially resulting in identification of candidate genes involved in these polygenic traits. The result of this work will highlight the importance of genetic background and gene-environment interactions in response to neurotoxicants and potentially in the etiology of complex neurological disorders including sPD. The result of this dissertation will pave the way for further investigating the underlying mechanisms of sPD as the animal models used throughout our studies are considered robust animal models for sPD. Therefore sPD is one important application in which the result of our work may have significant influence.

The rest of the chapter explains the pathology of PD. Epidemiological and experimental studies in which pesticide exposure had been linked to the development of PD disease are also discussed.

In the second chapter we will present the result of our first preliminary study in which we examined genetic variation in response to the neurotoxicant MPTP in male BXD RI mice.

The third chapter is an extension of the first study in which we investigate the correlation of response variation to MPTP among male and female BXD RI mice, investigating the consistencies and inconsistencies between sexes.

In chapter four we discuss the role of the striatal MPP$^+$ concentration in the severity of MPTP neurotoxicity.

Chapter five is a short review of the epidemiological and animal studies regarding PQ toxicity with explanations of potential mechanism of PQ neurotoxicity.

In chapter six we present the result of our preliminary study concerning PQ neurotoxicity from a neurochemical standpoint.

Chapter seven is a short review of the importance of host susceptibility and possible models to study the phenomenon.

In chapter eight we make an overall conclusion of the whole thesis and propose questions that need to be addressed in the future.
1.2 The Pathology of Parkinson’s Disease

Parkinson’s disease (PD) is the second most common neurodegenerative disease especially among the elderly in which the individual suffers from motor impairments. Common symptoms of PD include Bradykinesia, resting tremors, and posture instability; in advanced stages of the disease, cognitive impairments and psychiatric complications may also be present [2]. Nigrostriatal dopamine degeneration is a pathological hallmark of PD [3]. Selective loss of DA neurons located in the substantia nigra pars compacta (SNpc) results in depigmentation of this brain region, as they contain high concentration of neuromelanin [3]. Beginning of clinical symptoms of PD coincides with 60 percent loss of DA neurons and 80 percent depletion of DA in dorsolateral putamen, which is the main projection site for nigrostriatal dopaminergic cells [4,5].

Another hallmark of PD is formation of Lewy bodies. Lewy bodies are cytoplasmic protein aggregates compose of parkin, ubiquitin, neurophylaments and alpha synuclein which are found in affected regions of the brain [6]. Substantia nigra, locus coeruleus, raphe nuclei and cerebral cortex are some of the brain regions in which Lewy bodies are detected [7]. Morphologically, Lewy bodies consist of several spherical bodies surrounded by a halo ring. The peripheral ring around Lewy bodies makes them distinguishable from other inclusions and axonal swellings in the cell [8]. Lewy neurites are another type of protein inclusions found in dendrites rather than the soma of the cells. Lewy bodies and Lewy neuritis are not specific to PD and are found in a number of other neurodegenerative diseases like Alzheimer’s, dementia with LBs and also in healthy individuals of old age [8].

1.3 Epidemiological Studies

Multiple factors encouraged researchers to study the association between pesticide exposure and neurological disorders such as sPD. Examination of the relationship between pesticide exposure and sPD was first triggered by the description of parkinsonism symptoms in drug users exposed to MPTP [9]. A review paper by Barbaeu [10], in which he thoroughly discusses the ground breaking work of
Cotzias regarding sPD and manganese toxicity has also played an important role in inspiring the study of pesticide exposures and sPD. In this review, Barbaeu (1984) discusses the role of MPTP and manganese toxicity in the development of PD and also suggests other trigger factors which may have similar neurotoxic effects; he mentions that chemicals structurally and mechanistically similar to MPTP and manganese may induce the same neurotoxicity and therefore be a part of the unknown etiology of sPD, specially for people who have been chronically exposed to them [10].

There are more than 40 epidemiological studies addressing the association between pesticide exposure and sPD. Using 19 case-control studies, Priyadarshi and colleagues obtained an odds ratio of 1.94 (95 percent CI, 1.49-2.53) for sPD risk in those exposed to pesticides [11]. Three other review papers conducting meta analysis on case-control and cohort studies have mentioned the linkage between increased risk of sPD and pesticide exposure, although a significant dose-response relationship has not been confirmed in the majority of the studies [12–14]. Twelve out of 20 papers reviewed by Couteur and colleagues (1999), mentioned a 1.6 -7 fold increased risk of sPD after pesticide exposure [15]. Thirteen out of 23 case-control studies, reviewed by Freire and colleagues (2012) found a risk estimate of 1.1-2.4 in people exposed to pesticides [16]. Engel and colleagues (2001), found a significant association between long term pesticide exposure and increased risk of PD in people with the average age of 69 working in agricultural fields [17]. Two studies conducted by Abbott and colleagues (2003) and Petrovitch and colleagues (2002) are other examples in which the association between long term pesticide exposure and sPD is examined. Findings of these two longitudinal studies revealed that chronic pesticide exposure for more than 10 years increases the risk for developing sPD especially in non cigarette smokers and non coffee drinkers among immigrant Asian populations who work on sugarcane and pineapple fields of Hawaii [18,19]. On another study conducted on a French cohort of elderly farmers, increased relative risk (RR) of 5.6 of sPD with exposure to pesticides was reported in men, however a similar association for women was not detected.

All of the aforementioned studies use a general category of pesticides as the source of exposure without separating herbicides, fungicides, and insecticides from each other, which prevents the researchers from making an association between a special type of pesticide and risk for sPD. Some epidemiological studies have
narrowed the category of exposure to more specific groups such as herbicides, fungicides and insecticides. In the majority of these studies a positive association between herbicide exposure and increased risk for PD was observed [20–24]. Rural living, farming and well water drinking are considered means of pesticide exposure in the majority of these studies [12].

Most of the epidemiological studies can be problematic in that people are often exposed to a combination of pesticides, making it hard to associate a particular pesticide to increase risk of sPD. There are only a few number of studies in which the relationship between a particular herbicide or heavy metal and increased risk for PD has been studied [24,25]. In a case-control study conducted by Gorell and colleagues (1997), increased risk of sPD was linked to occupational exposure to copper, manganese (OR=2.49, OR=10.61 respectively) and a combination of lead-copper (OR=5.24), lead-iron (OR=2.83), and iron-copper (3.69) [25]. Another study conducted by the same researchers, supports a significant association between herbicide and insecticide exposure with sPD (OR=4.10, OR=2.79 respectively) [24].

Several epidemiological studies have linked paraquat, a broadly used herbicide with significant structural similarities to MPTP, to increased risk for sPD [23,26], especially when the duration of exposure surpasses 20 years [23]. There are only few epidemiological studies in which the effect of exposure duration have been significantly related to increased risk for sPD [22–24]; in other words, risk for sPD is significantly increased in people who are exposed to pesticides for more than 10 or 20 years.

A combination of PQ and maneb has also been associated with increased risk for PD especially when exposure to these chemicals occurs at a younger age. People exposed to both maneb and PQ within 500 meters of their home showed 75% higher risk to develop PD [?]. Tanner and colleagues have investigated the association between pesticides and PD from a mechanistic point of view. They concluded that PD is positively associated with two groups of pesticides; those that disrupt mitochondrial function such as rotenone and those that induce oxidative stress such as PQ [27].
1.4 Animal Studies

Epidemiological studies do not provide sufficient evidence to draw a causality relationship between pesticide exposure and sPD. Animal models of PD have been used extensively to examine the effects of multiple pesticides on health and their contribution in the development of multiple neurodegenerative diseases such as sPD. One of the first neurotoxins reported to induce parkinsonism symptoms in humans [9, 28, 29] and also other species such as mice [30] is the prototoxin N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). The effect of MPTP along with the results of epidemiological studies encouraged the research in examining the role of pesticides and chemicals similar to MPTP in PD.

A number of factors should be considered to confirm the contribution of different chemicals in the development of PD in animal studies. 1- Effects of the chemical on the striatal dopamine concentration; there should be a decrease in dopamine concentration in the striatum induced by these chemicals. 2- Effects of the chemical on dopaminergic cells of substantia nigra (SN); there should be a reduction in the number of dopaminergic cells of SN after exposure to these chemicals, and finally 3- the mechanistic effects such as alpha-synuclein inclusions induced by the chemical. Numerous chemicals have been used to study the mechanistic pathology of PD to determine potential factors that may be involved in the etiology of the disease. The MPTP mouse model is one of the most broadly used PD animal models featuring all of the PD characteristics with the exception of Lewy-body inclusions. MPTP is a neurotoxicant that induces striatal dopamine depletion and degeneration of dopaminergic cells in the SN in mouse, making it a suitable model to study PD. Moreover, the replicability and robustness of the effect of MPTP on dopaminergic cells have made MPTP neurotoxicity a standard basis to evaluate the neurotoxic effect of other toxicants.

Dopaminergic degeneration in the SN and subsequent striatal DA depletion are two hallmarks of PD used as indices to examine the ability of multiple chemicals to induce neuronal toxicity. To consider a chemical as a neurotoxicant, the systemic used of the chemical should result in dopaminergic degeneration in the substantia nigra and striatal dopamine depletion. An herbicide that has been under scrutiny as a dopaminergic neurotoxicant and risk factor for sPD is paraquat (PQ). PQ is an herbicide with a high pulmonary toxicity [31] that bears structural similarities
to 1-methyl-4-phenylpyridinium (MPP\textsuperscript{+}), the active metabolite of MPTP. There are large number of animal studies in which the effect of paraquat and its potential mechanisms of action on dopaminergic cells have been examined. The results are inconclusive and controversy exists regarding PQ neurotoxicity. One issue contributing to the ambiguity of neuronal toxicity induced by paraquat is its weak ability to pass the blood brain barrier (BBB), as paraquat is a highly charged molecule. Studies in which PQ is directly injected to the brain (intracerebral, intranigral) provide strong evidence of neurotoxicity (Dopamine degeneration in the SN and subsequent striatal dopamine depletion is observed) [32]; however, biotransformation and the importance of PQ ability to cross the BBB is not taken into consideration in these studies.

Different routs of PQ administration are also suggested to affect the neuronal toxicity induced by PQ, another factor which may play a part in the complexity of PQ neurotoxicity. For example, based on some studies, subcutaneous administration of paraquat only poses a threat to the structures lying out side of the BBB such as pineal glands, hypothalamus and anterior portions of olfactory bulb [33]. On the other hand, there are other studies in which it has been suggested that PQ when used intraperitonealy or subcutaneously, passes the intact BBB and can reach multiple structures of the brain by the use of a neutral amino acid transporter, followed by a Na\textsuperscript{+} dependent transportation to the nigrostriatal pathway [31,34,35].

There are several studies indicating degeneration of dopamine cells of the substantia nigra after systemic use of paraquat in mice [36,37]. Aggregation and up-regulation of the protein alpha synuclein, a feature of PD, have been also observed in the substantia nigra of mice treated with PQ [38]. Most of these studies have indicated a 25-60% loss of dopamine cells, however, striatal dopamine depletion has not been observed in all of these studies [39]. The results of these studies is in contrasts with at least one study conducted by Brook and colleagues in which high does of PQ (10mg/kg) induced 61% dopaminergic degeneration in the SN and 94% reduction in the density of the striatal dopaminergic terminals [40].

There is evidence supporting the synergistic effects of PQ in combination with other pesticides such as maneb, regarding dopaminergic neurotoxicity. The combination of (PQ + maneb) treatment in mice results in more severe dopaminergic degeneration in the SN and a suggestive trend of DA depletion seven days after the last (PQ + maneb) injection. Neurobehavioral changes such as reduced locomotor
activity are also observed in mice treated with (PQ+ maneb) [41]. It is also shown that developmental exposure to (PQ + maneb) in mice changes the nigrostriatal system, making it more sensitive to subsequent environmental insults, resulting in more susceptible populations, in the adulthood. Developmental exposure to paraquat and maneb in C57BL/6 results in significant striatal dopamine depletion and loss of dopaminergic cells in the substantia nigra of these mice if rechallenged by pesticide exposure in their adulthood [42]. Research has been conducted on the oral exposure of low and high doses of PQ during the critical times of fast brain growth in C57B6 mice. Developmental PQ exposure in these mice results in permanent neurochemical changes of the striatum and reduced DA concentration and its metabolites in the adulthood, making PQ early exposure one of the risk factors for sPD.

1.5 The Importance of Host Susceptibility in pesticide neurotoxicity

Genetic profile significantly influences normal and pathological states of an individual. Individual susceptibility or resistance to particular type of disease or insult may be rooted to some extent in the genetic structure of an individual; environmental neurotoxicity is no exception. An example is polymorphisms of debrisoquine hydroxylase in cytochrome P450D6 (CYP2D6). CYP2D6 is an enzyme involved in the metabolism of multiple toxicants such as pesticides, herbicide and also MPTP; the activity of this enzyme is genetically determined and genetic polymorphisms influence the efficiency of the enzyme in metabolizing toxicants [43,44]. Five-10% of white populations are poor metabolizers of these toxicants with undetectable enzyme activity, which puts them in a category of susceptible populations with two fold increased risk for sPD associated with pesticide exposure [45].

Glutathione S-transferase polymorphisms of M1(GSTM1) and T1(GSTT1), are additional important examples affecting the risk for sPD. These are detoxifying enzymes which provide cellular protection in the liver and brain against oxidative stress induced by electrophilic xenobiotics such as carcinogens and environmental pollutants. Oxidative stress is a key pathological mechanism in PD development [46]. Goldman and colleagues (2012) have demonstrated that people with homozygote
deletions of T1 (GSTT1*0) have higher risk for Developing sPD with an odds ratio of 11.1 compared to people with GSTT1 genotype with an odds ratio of 1.5 [47].

Numerous animal studies have also highlighted the importance of gene environment interactions in the development of sPD. Based on the results of these studies, a combination of a dysfunctional neuron-protective gene and exposure to environmental toxicants is needed for the development of the disease. Deletion, mutation or even partial dysfunction of neuron-protective genes such as DJ-1, PINK1 or parkin, sensitizes the dopaminergic neurons of substantia nigra to neurotoxicants such as MPTP and paraquat [48–53]. Gene-induced individual susceptibility to paraquat and MPTP neurotoxicity have been reported in different strains of mice. Higher dopaminergic degeneration in the SN followed by higher iron concentrations in the ventral midbrain is observed in B6 mice compared to D2 mice after PQ and MPTP treatment [37]. B6 mice with 63% striatal dopamine depletion are also considered the more sensitive strains to MPTP compared to Swiss-Webster mice with only 14% striatal dopamine depletion [54].

It is also suggested that early exposure to paraquat changes the homeostasis of dopaminergic neurons in the substantia nigra which when combined with the temporal silencing of the neuron-protective gene PINK1, results in striatal dopamine depletion and loss of dopaminergic neurons in the substantia nigra [55].

Additionally, developmental exposure to paraquat or a combination of paraquat and the fungicide mane, may permanently change the nigrostraital system. Early exposure may reduce the threshold for dopaminergic degeneration in the substantia nigra in case of an additional insult or stressor later in life such as repeated pesticide exposure in the adulthood [42].

1.6 Use of BXD Recombinant Inbred Mice to Study the role of Individual Differences to Neurotoxicity

Neurodegenerative diseases and the study of host susceptibility to environmental toxicants are considered complex traits as there is no single gene responsible for the phenotype of interest and there is a continuous range of response variation across populations; not all the people exposed to environmental stressors develop parkinsonism symptoms nor does everyone show the same level of toxicity. Host
susceptibility and gene-environment interactions are considered important factors that can partially explain the basis for the existing phenotypic variation. In this thesis we have used BXD RI mice as a tool to investigate the importance of the polygenic trait of host susceptibility to environmental toxicants. Investigating individual differences in response to neurotoxicants may help researchers better understand the etiology of complex neurological disorders.

The study of host susceptibility and gene-environment interactions in humans is challenging; most of the time there is no control over environmental situations and not enough information is provided regarding the genetic constitution of the population under study. The use of genetic reference families with known genetic composition such as recombinant inbred mice (RI) in which environmental conditions are monitored, makes it possible to study gene environmental interactions and host susceptibility. One example of RI mice that has been extensively used to map complex traits is BXD RI mice. BXD RI mice are derived from breeding the two parental progeny of D2 and B6 and subsequently using the F1 offsprings to interbreed for more than 20 generations which results in segregation of allelic differences among the parents in a large and reproducible line of mice [56]. This procedure enables the researchers to use multiple mice from the same strain as replications, reducing the environmental and technical errors present in the experiment. D2 and B6 mice differ at approximately 4.8 million SNPs; additionally, more than 13000 markers associated with the genomic polymorphism among D2 and B6 mice have been mapped which can be used in quantitative trait analysis (QTL) studies associating the observed phenotypic variation in the population to a genomic polymorphism in multiple strains of mice [57].

In the current thesis we have used 9-10 strains of BXD mice to address the role of host susceptibility in response to environmental toxicants. The results of the research conducted on BXD RI mice adds to the knowledge from other experiments on the same strains of BXD, as these strains are genetically identical and reproducible, making them a great source to study gene environmental interactions. The genetic variability in BXD RI mice mimics to some extent the genetic variation observed in human populations and the results will pave the way for future studies concerning host susceptibility and individual differences in response to environmental toxicants in humans as mice and human genomes are 90% syntenic.
Chapter 2  
Systems Analysis of Genetic Variation in MPTP Neurotoxicity in Mice

2.1 Abstract

We analyzed genetic variation in severity of neuronal damage using the known dopaminergic neurotoxicant, MPTP, as a prototypical chemical denervation agent. Male mice from ten members of the BXD family of recombinant inbred strains received 12.5 mg/kg MPTP s.c. (vs. saline) and 48 h later brains were taken for multiple related biochemical analyses. Striatal dopamine (DA) and its metabolites, DOPAC and HVA, and serotonin (5-HT) and its metabolite, 5-HIAAA, were analyzed by HPLC. DA turnover was assessed using DOPAC/DA and HVA/DA ratios. Striatal tyrosine hydroxylase (TH), glial fibrilary acidic protein (GFAP), and iron content in ventral midbrain were quantified. All dopamine measures, as well as TH and GFAP, demonstrated wide, genotype-dependent differences in response to MPTP. Serotonin was largely unaffected. Principal components analysis (PC) on difference values, saline minus MPTP, for DA, DOPAC, HVA, and TH, yielded a dominant principal component. The PC trait residuals for each genotype were compared against complementary expression data for striatum of the same strains. Three transcripts representing Mtap2, Lancl1, and Kansl1 were highly correlated with the PC, as was the difference score, MPTP minus saline for GFAP. This systems approach to the study of environmental neurotoxicants holds promise to
define individual genetic differences that contribute to variability in susceptibility to risk factors for diseases such as Parkinson’s disease.

2.2 Introduction

Exposure to various industrial and agricultural chemicals, especially pesticides, has been implicated as conferring risk for multiple diseases, including neurodegenerative disorders. Several of the substances implicated include rotenone, maneb, paraquat, carbamate and organophosphorus insecticides.

Interactions among multiple risk factors, including gene variants and environmental exposure are thought to underlie differential vulnerability to neurological disease [58]. Although these factors and their interactions have not been defined, exposure to pesticides usually associated with rural living has been implicated as one key environmental cofactor. Epidemiological studies have been equivocal, and some have failed to find consistent associations between pesticide exposure and idiopathic or sporadic Parkinson’s disease (sPD) [13,59]. If gene environment interactions (GXE) are fundamental to understanding the etiology of diseases such as sPD, then ascertaining the right set of informative probands becomes problematic. Through the use of reference families of genetically diverse lines of mice, we can address the problems of the complex etiology of sPD and other environmentally related neurodegenerative diseases. This approach addresses the gene environment interaction framework in which to assess models of disease sensitivity and severity. As proof of concept, the administration of model toxicants to cases with precisely defined genomes has proved to be highly useful [60]. One example is recombinant inbred (RI) rodents. RI strains typically are derived from two parental inbred strains by first making an F1 cross and then inbreeding their offspring by many completely independent full sibling matings for 20 or more generations. This process redistributes (randomly segregates) allelic differences between the parents among a potentially large number of independent but genetically related progeny lines [56]. The aim is to develop a genetically diverse group of animals that can be used to model individual differences in genomes seen in genetically segregating populations, such as humans, and then to relate these genomic differences to phenotypic differences observed in nearly any normal or pathological biobehavioral domain. The use of such a genetic reference population confers several advantages. First, in contrast
to single gene mutant studies, the range of the phenotype of interest is revealed and thus some indication of individual differences. Second, the use of multiple strains together with multiple measures in one or more domains provides a powerful tool to examine experimental treatments from a systems biology perspective. Third, when genotyped, the reference population of inbred strains can be queried for polymorphisms that are associated with the phenotypes of interest and may lead to the identification of candidate genes that influence the trait.

As a model neurotoxicant, the proneurotoxin, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is a useful denervation tool because it damages the nigro-striatal dopaminergic pathway uniquely and selective antagonists are available to manipulate its neurotoxic effects. This has led to the use of MPTP in animal models of Parkinson’s disease (PD); for our purposes, MPTP can be used to reproducibly damage a specific neuronal pathway, absent the influence of other factors, such as blood borne cytokines entering through a damaged blood brain barrier (BBB) [61]. MPTP is readily distributed to the brain without damaging the BBB where it is metabolized by monoamine oxidase-B by glial cells to 1-methyl-4-phenylpyridinium (MPP+) taken up into dopamine (DA) neurons via the DA transporter. MPP+ then disrupts the mitochondrial complex I of the electron transport chain, leading to the accumulation of free radicals which in turn destroy the neuron.

The primary objective of this research was to determine differences in susceptibility on a genetic basis, specifically, based in gene environment interaction. There is evidence in mice for genetically based differences in MPTP neurotoxicity [62] and these authors reported a significantly associated marker (QTL) on chromosome 1 near the telomere. Others have identified QTL related to genetic differences in MPTP toxicity in mice on chromosomes 13 and 15 [63]. In this study, we report genetic differences in the effect of MPTP on multiple neurochemical indices related to dopamine in the caudate putamen in a random sample of 10 of the BXD family of RI strains derived from C57BL/6J and DBA/2J parental inbred strains. This is the first study of its kind to investigate strain-related differences in MPTP neurotoxicity in a panel of RI strains and using multiple outcome measures to begin to assemble a systems level perspective of MPTP neurotoxicity. Previously mentioned studies report effects on single measures and used F2 or backcross techniques.
2.3 Material and methods

2.3.1 Animals

Male mice from 10 of the BXD RI strains were used in this study. The animals ranged in age from 2 to 8 months and were reared in the vivarium at UTHSC. Ten days prior to being treated with MPTP, the animals were shipped to the CDC-NIOSH laboratory in Morgantown. The animals had free access to food and water at all times and were maintained on a 12 h:12 h light cycle. All procedures were conducted according to protocols approved by the institutional Animal Care and Use Committee and in accordance with the NRC Guide for the Care and Use of Laboratory Animals (National Academy Press, 1996).

2.3.2 MPTP and reagents

MPTP-HCl was purchased from Aldrich (Milwaukee, WI, USA). Mouse anti-rat tyrosine hydroxylase (TH) monoclonal antibody was purchased from Sigma (St. Louis, MO) and rabbit anti-rat TH polyclonal antibody was purchased from Calbiochem (San Diego, CA). Antibodies to GFAP are described by O’Callaghan (2002) [64].

2.3.3 Drug Treatment and brain dissection

Following the protocol of O’Callaghan et al. (1990), all animals were injected s.c. with 12.5 mg/kg MPTP, a dose that has been shown previously to cause pronounced effects on dopamine neurochemistry in the caudate-putamen while showing minimal damage to the DA perikarya residing in the SNc [61]. The primary goal of this experiment is to analyze the response variation to MPTP in multiple strains of BXD mice rather than the effect of MPTP on dopamine neurons in the substantial nigra. Using this dose and treatment allows us to only focus on the effect of MPTP on the striatal dopamine levels without damaging the dopaminergic cells in the substantial nigra. Forty-eight hours after the injection, the animals were killed by decapitation and the brains were removed and dissected freehand to yield the caudate-putamen and ventral midbrain, containing the ventral tegmentum and SNc. DA depletion is often stabilized 24 hours after last injection of MPTP and
most of the MPTP is metabolized and excreted from the body after 24 hours. For all strains, except BXD32 the numbers of animals treated were 5 each for saline and MPTP. For BXD32 the numbers were 4 each for saline and MPTP.

2.3.4 Biochemical assays

Using caudate-putamen samples from one side of the brain, dopamine (DA) and its metabolites, dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA) and 3 methoxytyramine (3-MT) and serotonin (5-HT) and its metabolite, 5-hydroxyindoleacetic (5-HIAAA) acid were analyzed for content as described below. Values obtained were normalized to tissue wet weight. DA, DOPAC, HVA and other neurotransmitter substances were analyzed by HPLC with electrochemical detection using the following system: tissue homogenates were prepared by sonication (Kontes Micro ultrasonicator/cell disruptor) on ice using a 30-s pulse in 0.2 M perchloric acid, containing 3,4-dihydroxybenzylamine 1 \( \mu \)M as internal standard. The homogenate was centrifuged at 10,000 \( \times \) \( g \) for 15 min, and the resulting supernatant immediately injected using an autosampler described below. The striatum was prepared in a standard volume (0.3 ml) and results were expressed as \( \mu \)g/g original tissue weight. Sample (10\( \mu \)l) was injected using a temperature controlled 4 °C Waters 717 Plus Autosampler (Waters, Milford, MA, USA) connected to a Waters 515 HPLC pump. The sample was passed over a reversed-phase C18 column (Waters Symmetry, 250 \( \times \) 4.6 mm, 5\( \mu \)m, 100Å). Analytes were detected using the Waters 464 pulsed electrochemical detector (range 10 nA, potential 700 mV) connected by means of the Waters bus SAT/IN module to a computer using Millenium Software 32. The mobile phase consisted of 75 mM sodium dihydrogenphosphate, 1.7 mM 1-octanesulfonic acid, 25 \( \mu \)mol ethylenediaminetetraacetic acid and 10% (v/v) acetonitrile. All components were adjusted to a pH of 3.0 with phosphoric acid, pumped at a flow rate of 1 ml/min. Under these conditions the average run time is 30 min with representative retention times (in min) for NE (5.99), 4-dihydroxybenzylamine (DHBA, internal standard, 8.24), DOPAC (8.93), DA (11.28), 5-HIAA (13.57), HVA (19.77), 5-HT (26.1). Quantitation was achieved by the use of the internal standard (10 pmol DHBA per injection) method using daily standard curves of each analyte (0.5-25 pmol per injection). The limit of detection is 0.5 pmol per injection, interassay variation is ±3%. Caudate-putamen
samples from the other side of the brain were homogenized in 1% (w/v) SDS heated to 80 – 90°C. The concentrations of TH and GFAP in the SDS-total homogenates were analyzed by ELISA (O’Callaghan, 1991, O’Callaghan, 2002 and Sriram et al., 2004). Values obtained were normalized to total homogenate protein assayed by BCA [65].

Ventral midbrain iron concentrations were measured according to the modified procedures of Erikson et al. (1997). Briefly, the ventral midbrain was dissected as described by Boone et al. (2007) weighed and combined with 200 µL of ultrapure nitric acid (OmiTrace, EM Science, NXO407-1) in a 0.5 mL polypropylene microcentrifuge tube. Brain regions were digested for 48 h in a 60°C sand bath and then re-suspended to 400 µL with nanopure water. Each sample was further diluted 1:50 (1:100 for ventral midbrain) with 0.2% ultrapure nitric acid and immediately analyzed for iron by graphite furnace atomic absorption spectrophotometry (Perkin Elmer Analyst 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.

2.4 Data analysis

Main and interaction effects for strain and treatment (MPTP vs. saline) were evaluated by analysis of variance (ANOVA) for a 2 between-subjects variables experiment. Post-hoc pairwise comparisons between saline and MPTP for each strain were made using the Bonferroni t test with α set at 0.05, two tailed. Pairwise comparisons for strain differences under saline treatment for each phenotype were made using the Tukey HSD test.

2.4.1 Results

2.4.2 Effects of MPTP on tyrosine hydroxylase in caudate-putamen

For TH we observed large strain differences in abundance, both in control and in response to MPTP (Fig. 2.1). Strains 29 and 84 showed the lowest basal levels while the highest was found in strain 9. Strains 29 and 62 were nearly refractory to
this dose of MPTP, while strains BXD40, BXD48 and BXD60 showed dramatic reductions in TH abundance.

Figure 2.1: Effect of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) on concentration of tyrosine hydroxylase (TH) in the caudate-putamen of 10 BXD recombinant inbred mouse strains. Male mice were injected with 12.5 mg/kg of the pro-neurotoxin, MPTP (vs. saline) and sacrificed 48 h later. TH levels were determined by ELISA. Experimental and control values (upper panel), normalized to total protein, are expressed as means ± s.e.m. The lower panel presents the effect of MPTP expressed as % control. ANOVA revealed significant effects for strain, MPTP and their interaction ($F_{9,78} = 9.97$; $F_{1,78} = 257.62$; $F_{9,78} = 6.03$, respectively; all $p < 0.001$).
2.4.3 Effects of MPTP on dopamine neurochemistry in caudate-putamen

Large and significant strain differences were observed for the basal levels of DA as well as the effect of MPTP (Fig. 2.2) with strain 29 showing the lowest and strain 9 the highest level (p < 0.01). All of the strains except strain BXD29 showed significant decrease of striatal DA concentration at p<0.01.

Figure 2.2: Effect of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) on dopamine (DA) concentration in the caudate-putamen in 10 BXD recombinant inbred mouse strains. Male mice were injected with 12.5 mg/kg of the pro-neurotoxin, MPTP (vs. saline) and sacrificed 48 h later. DA was assayed from tissue homogenates by HPLC. Experimental and control values (upper panel), normalized to tissue weights, are expressed as means ± s.e.m. The lower panel presents the effect of MPTP expressed as % control. ANOVA revealed a significant main effect of strain, MPTP and a significant interaction (F_{9,77} = 11.25; F_{1,77} = 445.61; F_{9,77} = 9.05, respectively; all p < 0.001).

The DA metabolites, DOPAC (Fig. 2.3), HVA (Fig. 2.4), and 3-MT (Fig. 2.5)
were also significantly affected by genotype and MPTP treatment. Strains BXD9, BXD27, BXD29, BXD40 and BXD 69 displayed nearly identical basal levels of DOPAC while the lowest was found in BXD48 and BXD94. The differences among the genotypes, however, did not reach statistical significance. In strains BXD9, BXD27, BXD29, BXD40, BXD48 and BXD60 MPTP treatment produced significant decreases in this metabolite (p < 0.01, all) while for strains BXD32, BXD62, BXD69 and BXD84 the decreases observed did not reach significance.

Genotype and treatment also affected HVA levels in striatum (Fig. 2.4). For this measure, we found the highest basal level in strains BXD9, BXD40, BXD48, BXD69 and the lowest in BXD32 (BXD69 vs. BXD32, p < 0.01). Following MPTP treatment significant decreases were observed for BXD9, BXD40, (p < 0.01), BXD48, BXD60 and BXD69 (p < 0.05) but not BXD27, BXD29, BXD32, BXD62 and BXD84.

Striatal levels of 3-MT were significantly affected by genotype and treatment but no interaction was detected (Fig. 2.5). As with DOPAC, there were no pairwise differences among the strain means. Furthermore, pairwise comparison showed that only strain BXD40 showed a significant reduction (p < 0.01) by MPTP of this DA metabolite.

Dopamine turnover ratios were also impacted by genotype and treatment. The basal level of DOPAC/DA turnover ratios (Fig. 2.6), an index reflecting primarily presynaptic processes, was affected by strain with BXD29 showing the highest and BXD 9 and BXD49 the lowest levels (p < 0.01 BXD29 vs. BXD49). MPTP decreased this index in BXD40 only (p < 0.05).

HVA/DA (Fig. 2.7) is thought to reflect primarily postsynaptic processes with BXD32 showing an extremely low basal level. None of the pairwise comparisons for basal levels reached significance. In general, MPTP treatment non-significantly increased this index in most of the strains but it was profoundly and significantly increased (~4.5-fold greater than saline control) in BXD40.

2.4.4 Effects of MPTP on GFAP in caudate-putamen

Genotype and MPTP treatment affected the level of striatal GFAP, an intermediate filament protein and astrocyte marker (Fig. 2.8), with the lowest basal level found in BXD32 and BXD69 and the highest in BXD9 (p < 0.01, BXD9 vs. BXD69).
Figure 2.3: Effect of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) on dihydroxyphenylacetic acid (DOPAC) concentration in the caudate-putamen in 10 BXD recombinant inbred mouse strains. Male mice were injected with 12.5 mg/kg of the pro-neurotoxin, MPTP (vs. saline) and sacrificed 48 h later. DOPAC was assayed from tissue homogenates by HPLC. Experimental and control values (upper panel), normalized to tissue weights, are expressed as means ± s.e.m. The lower panel presents the effect of MPTP expressed as % control. ANOVA confirmed significant main and interaction effects for Strain and MPTP (F\(_{9,76}\) = 6.45; F\(_{1,76}\) = 148.54; F\(_{9,76}\) = 4.21; respectively; all p < 0.001).

MPTP treatment markedly and significantly increased this protein in BXD9, BXD27, BXD40, BXD48, and BXD69 (p < 0.01 for all).
Figure 2.4: Effect of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) on homovanillic acid (HVA) concentration in the caudate-putamen in 10 BXD recombinant inbred mouse strains. Male mice were injected with 12.5 mg/kg of the pro-neurotoxin, MPTP (vs. saline) and sacrificed 48 h later. HVA was assayed from tissue homogenates by HPLC. Experimental and control values (upper panel), normalized to tissue weights, are expressed as means ± s.e.m. The lower panel presents the effect of MPTP expressed as % control. ANOVA indicated significant main and interaction effects for Strain and MPTP (F\(_{9,77}\) = 5.80; F\(_{1,77}\) = 41.76; F\(_{9,77}\) = 3.48, respectively; all p < 0.01).

2.4.5 Effects of MPTP on iron accumulation in ventral midbrain (VMB)

The accumulation of iron in the VMB was affected by strain but not by MPTP treatment (Fig. 2.9). Only BXD40 showed a significant (p < 0.01) increase in iron accumulation (nearly a 40% increase).
2.4.6 Effects of MPTP on serotonin (5-HT) neurochemistry in caudate-putamen

Large and significant strain differences were observed among basal levels of 5-HT (Fig. 2.10). BXD48 showed the highest concentration and BXD27 showed the lowest (p < 0.01). Only two strains-BXD32 and BXD62—showed significant reductions in
Figure 2.6: Effect of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) dopamine turnover as measured by DOPAC/DA in the caudate-putamen in 10 BXD recombinant inbred mouse strains. Male mice were injected with 12.5 mg/kg of the pro-neurotoxin, MPTP (vs. saline) and sacrificed 48 h later. Experimental and control values (upper panel), normalized to tissue weights, are expressed as means ± s.e.m. The lower panel presents the effect of MPTP expressed as % control. ANOVA revealed no effect of treatment on this measure of turnover but a significant effect of strain on the effect of MPTP as well as the interaction between strain and MPTP (F$_{1,76} = 2.7$ p>0.05; F$_{9,76} = 11.01$, ; F$_{9,76} = 7.52$, p<0.001 for both).

5-HT following MPTP treatment (p < 0.01 for both).
Figure 2.7: Effect of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) dopamine turnover as measured by HVA/DA in the caudate-putamen in 10 BXD recombinant inbred mouse strains. Male mice were injected with 12.5 mg/kg of the pro-neurotoxin, MPTP (vs. saline) and sacrificed 48 h later. Experimental and control values (upper panel), normalized to tissue weights, are expressed as means ± s.e.m. The lower panel presents the effect of MPTP expressed as % control. ANOVA indicated a significant effect of strain and MPTP treatment on this measure of turnover as well as an interaction (F<sub>9,77</sub> = 21.94; F<sub>1,77</sub> = 65.13; F<sub>9,77</sub> = 15.44, respectively; all p < 0.001).

2.4.7 Systems genetic analysis of MPTP effects and correlation with gene expression in the striatum

We combined the difference scores, saline minus MPTP on DA, DOPAC, HVA, and TH (all highly correlated) and used the strain residuals of the first principal component as our index for MPTP neurotoxicity. We then performed a correlation analysis with several large whole transcriptome expression data sets for the same BXD strains [66]. These data (accession number 285) are available on
Figure 2.8: Effect of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) on the concentration of glial fibrillary acidic protein (GFAP) in the caudate-putamen of 10 BXD recombinant inbred mouse strains. Male mice were injected with 12.5 mg/kg of the pro-neurotoxin, MPTP (vs. saline) and sacrificed 48 h later. GFAP levels in the caudate-putamen were determined by ELISA. Experimental and control values (upper panel), normalized to total protein, are expressed as means ± s.e.m. The lower panel presents the effect of MPTP expressed as % control. ANOVA revealed significant main effects of strain, MPTP treatment and their interaction ($F_{9,78} = 9.76; F_{1,78} = 145.80; F_{9,78} = 5.08$, respectively; all $p < 0.001$).

www.GeneNetwork.org. The data are from the HQF BXD Striatum Illumina Mouse-6.1 November 2007 Rank Invariant Data Set and corrected (December 2010) for a batch effect due to the hybridization of different strains on different dates. Our index showed high, statistically significant correlations with transcript abundance for many transcripts. Our interest however is in those transcripts with expression that is correlated with our index and with each other. Transcripts generated from
Figure 2.9: Effect of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) on iron concentration in the ventral midbrain in 10 BXD recombinant inbred mouse strains. Male mice were injected with 12.5 mg/kg of the pro-neurotoxin, MPTP (vs. saline) and sacrificed 48 h later. Atomic absorption spectroscopy was used to determine iron concentration from tissue homogenates. Experimental and control values (upper panel), normalized to tissue weight, are expressed as means ± s.e.m. The lower panel presents the effect of MPTP expressed as % control. ANOVA indicated a significant effect of strain (F_{9,77} = 4.92, p < 0.001) and interaction between strain and MPTP treatment (F_{9,77} = 4.55, p < 0.001) but not MPTP treatment (F_{1,77} < 1).

Three genes met both criteria and include Mtap2 (microtubule associated protein 2), Lancl (lantibiotic synthetase component C-like 1 (bacterial) 1) and Kansl1l (KAT8 regulatory NSL complex subunit 1-like). The systems diagram is presented in Fig. 2.11 and the values for the correlations are presented in Table 2.1.
Figure 2.10: Effect of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) on serotonin (5-HT) concentration in the caudate-putamen in 10 BXD recombinant inbred mouse strains. Male mice were injected with 12.5 mg/kg of the pro-neurotoxin, MPTP (vs. saline) and sacrificed 48 h later. DA was assayed from tissue homogenates by HPLC. Experimental and control values (upper panel), normalized to tissue weights, are expressed as means ± s.e.m. The lower panel presents the effect of MPTP expressed as % control. ANOVA revealed a significant main effect of strain, MPTP and a significant interaction (F_{9,77} = 6.15, p < 0.001; F_{1,77} = 9.64, p < 0.005; F_{9,77} = 2.63, p < 0.02, respectively).

<table>
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<th>Lancl1</th>
<th>Mtap2</th>
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<td>-0.93^{b,}_</td>
<td>-0.87^{b,}_</td>
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^{a} p < 0.05, ^{b} p < 0.01

Table 2.1: Correlation matrix for the graphical representation of the system analysis presented in Figure 2.11. Correlation presented are Pearson r.
2.5 Discussion

This is the first study to report the neurotoxic effects of MPTP in a panel of inbred mouse strains. MPTP at a dose of 12.5 mg/kg (s.c.) produced large variations in multiple indices of neurotoxicity across the 10 BXD RI strains. Moreover, the distribution of the strain differences in all measures is continuous, indicating the influence of multiple genes. MPTP neurotoxicity, thus, is considered to be a complex trait.

As MPTP is a known dopaminergic neurotoxicant it was to be expected for dopamine neurochemistry to be affected to a far greater degree overall than was serotonin. Interestingly, in strain BXD40, MPTP produced the greatest loss of DA from the striatum and was the only strain to show a significant increase of iron in the ventral midbrain. Iron is considered to be a risk factor in sPD per se, [67] and recently we showed that it co-operates with paraquat in neurotoxicity [37]. This strain is also quite sensitive to disruption in iron regulation as a result of being fed an iron-poor diet [68]. That this strain is also among the most susceptible to MPTP toxicity and exactly how iron and MPTP toxicity in this strain are
related remains to be seen. One hypothesis is that for BXD40, the 12.5 mg/kg dose does indeed damage DA neurons in the SNc by way of iron homeostasis dysregulation. Previously, we showed that paraquat neurotoxicity is likely related to its dysregulation of iron homeostasis in the ventral midbrain [37]. MPTP may produce the same effect, with individual differences. If true, then higher doses of MPTP should involve SNc damage and increase iron influx in more strains.

This study was designed as proof-of-concept to show that not all inbred mouse strains (hence humans) are equally susceptible to MPTP neurotoxicity, and likely other toxicants as well. This study also demonstrates the power of systems genetic and systems biology analysis. We did not measure the production of MPP\(^+\) and the degree to which individual differences in MPTP neurotoxicity is related to differences in the production of this neurotoxic metabolite will need to be addressed in future studies.

Now that we have demonstrated large genetically controlled differences in sensitivity to MPTP, the next step is to find gene variants that underlie these differences. In this prelude, we have used only 10 of \(\sim\)150 BXD lines. QTL mapping with only 10 strains is risky; nevertheless, when we performed PCA on mean differences, saline-MPTP on TH, DA, DOPAC and HVA and mapped the first principal component, we obtained an intriguing and nominally significant QTL (LOD = 4.3) at \(\sim\)60 \(\pm\) 10 Mb on chromosome 1. Interestingly, the three genes that we identified as correlated with our measure of dopamine neurotoxicity are all cis-regulated with eQTL at the same locus (\(\sim\)66 Mb on chromosome 1) as the dopamine-related QTL. This QTL will require verification by testing many more of the BXD strains. QTL analysis coupled with gene expression data is a very powerful technique for the nomination of candidate genes. In order to identify genes and mechanisms underlying individual differences in MPTP neurotoxicity, gene expression studies would have to be conducted in the SNc and caudate-putamen. The SNc is where TH, dopamine transporter and dopamine autoreceptors are synthesized and transported to the caudate-putamen. Gene expression studies in the latter tissue could help us understand the “collateral damage” and its contribution to sPD by an impaired DA transmission system. What about the genes that we identified whose expression correlates highly with our index of MPTP neurotoxicity? Lcn11 binds glutathione and is important in oxidative stress and related diseases [69]. Mtap2 is known to bind to TWIK-related potassium channels [70], and may be involved in maintaining
the integrity of dendritic microtubules, thus playing an important role in signal transduction (as reviewed by Goldstein and Yang, 2000) [71]. Kansl11 is also known as human KAT8 K(lysine) acetyltransferase 8 and acetylates histones in gene regulation. Presumably, the function will be the same in mouse. The association between MPTP neurotoxicity and expression of these genes presents new information on possible mechanisms underlying host susceptibility characteristics and sets the stage for more extensive study in a large panel of BXD RI (or other) mouse strains.

Finally, the systems analysis revealed important interrelationships among our phenotypes and the expression of genes that likely underlie individual differences in sensitivity to MPTP, and perhaps other toxicants, neurotoxicity.

2.6 Conclusion

We have shown wide, genetic variation in response to MPTP among 10 BXD genotypes across multiple MPTP-related phenotypes. Multivariate analysis has shown the rich landscape of associations among these indices and increasing the number of strains and doses will prove invaluable in elucidating the gene-environment underpinnings of sPD and will pave the way for similar study of similar diseases.
Chapter 3
MPTP Neurotoxicity is Highly Concordant Between the Sexes in BXD Recombinant Inbred Mouse Strains

3.1 Abstract

Continuing our previous work in which we showed wide-ranging strain differences in MPTP neurotoxicity in male mice among ten BXD recombinant inbred strains, we replicated our work in females from nine of the same strains. Mice received a single s.c. injection of 12.5 mg/kg MPTP or saline. Forty-eight hours later the striatum were dissected for neurochemical analysis. Striatal dopamine (DA) and its metabolites, DOPAC and HVA, striatal serotonin (5-HT) and its metabolite, 5-HIAA, were analyzed using HPLC. Tyrosine hydroxylase (TH) and glial fibrillary acidic protein (GFAP) were measured using ELISA. There were wide genetic variations in the DA, DOPAC, TH and GFAP responses to MPTP. We also performed principal component analysis (PCA) on the difference values, saline minus MPTP, for DA, DOPAC, HVA and TH and mapped the dominant principal component to a suggestive QTL on chromosome 1 at the same location that we observed previously for males. Moreover, there were significant correlations between the sexes for the effect of MPTP on DA, HVA, and TH. Our findings suggest that the systems genetic approach as utilized here can help researchers understand
the role of sex in individual differences. The same approach can pave the way to understand and pinpoint the genetic bases for individual differences in pathology attributable to toxicants. Such systems genetics approach has broad implications for elucidating gene-environment contributions to neurodegenerative diseases.

3.2 Introduction

Heavy metals, multiple pesticides and herbicides are considered to be risk factors for human health as acute and chronic exposure to them is linked to an increased risk for development of neurodegenerative diseases such as Parkinson’s disease (PD) [23, 26, 27, 72, 73]. The picture concerning exposure to pesticides and neurodegenerative disease however, is not clear as there are several conflicting reports regarding these agents as increasing risk for sPD [74–76]. Differential vulnerability to complex neurological disorders, including PD is thought to be related at least in part to host susceptibility (i.e., susceptible genotypes exposed to environmental risk factors).

There are a number of approaches to assess how the host responds to a toxic insult. One involves epidemiological studies in which subpopulations carrying potential susceptible genotypes are recruited and examined for responses to the toxicant. Identifying such individuals is challenging, but possible [47]. Another is the use of genetically-defined animal models that mimic the individual differences observed in humans [60]. There are several possible genetic reference populations of animals, especially mice. One such genetic reference population or model is recombinant inbred (RI) strains. RI mice are derived from two parental inbred strains by first making an F1 generation of the parents, and from the F1 population, making F2 and more advanced intercrosses, making families and inbreeding by brother-sister matings within the families for 20 generations or more. In this particular model the allelic differences between two or more parental lines segregate among a large population of independently derived, but related lines of progeny [56]. This approach enables the researchers to relate genomic polymorphisms to phenotypic variations in normal or pathological states and therefore assess the genetic basis for individual differences in phenotypes. Using this approach, we can assess the genetic basis for individual differences and more importantly, gene-environment and gene-gene interactions underlying these differences. Thus RI strains of rodents constitute an invaluable research tool to aid in identifying the underlying causes of major diseases, including
neurodegenerative diseases. In chapter two we reported large strain differences in the dopaminergic neurotoxicity of 1-methyl-4-phenyl-1,2,3,6-tetrahydro-pyridine (MPTP) in BXD RI mice derived from C57BL/6J and DBA/2J parental strains [77]. MPTP is a prototypical pro-neurotoxicant that targets dopaminergic cells of the nigro-striatal pathway of the brain and is broadly used in animals to investigate the factors contributing to the vulnerability of the nigro-striatal pathway to toxic insult [61].

Others have addressed genetic-based difference in susceptibility to MPTP neurotoxicity by evaluating the effects of this agent in different strains of mice, usually males, and have nominated candidate genes on chromosomes 1 [62], 13 and 15 [63]. The use of male animals to investigate host susceptibility to MPTP and other neurotoxicants, is short-sighted as we see gender differences in many neurological disorders. For example the prevalence of PD is higher in men compared to women; moreover, the progression of Alzheimer’s disease is slower in women receiving hormone replacement therapy [78]. There are experimental studies showing the protective effect of estrogen on nigrostriatal pathway and dopaminergic cells regarding the neurotoxicity of MPTP and methamphetamine in C57BL/6J mice [79, 80]. Therefore, in order to more fully elucidate the role of host susceptibility both sexes should be evaluated. The main purpose of this study was to expand on our previous study in which we reported differences in genetic-based susceptibility to MPTP in male BXD RI mice [77]. Here, we report strain differences in MPTP neurotoxicity in females and the sexes combined to show where they agree and where they might differ regarding MPTP neurotoxicity. Our data show a remarkable concordance between the female and male BXD mice for dopamine-based MPTP neurotoxicity.

### 3.3 Materials and methods

#### 3.3.1 Animals

Female mice from nine of the ten BXD strains which we reported the results in males in chapter two were used [77]. One strain, BXD27 is a poor breeder and no animals were available. All mice ranged in age from 2 to 8 months. Ten days prior to MPTP treatment, all of the animals were transferred from the University
of Tennessee Health Science Center to the Centers for Disease Control, National Institute of Occupational Safety and Health (NIOSH) laboratory in Morgantown, West Virginia. The animals were maintained on a 12:12 hour light and dark cycle and had free access to food and water. All procedures conducted on the animals were approved by the NIOSH Institutional Animal Care and use Committee and in accordance with the NRC guide for the Care and Use of Laboratory Animals (National Academy Press, 1996). NIOSH is an AAALAC accredited institution.

3.3.2 MPTP and Reagents

MPTP-HCl used in the study was provided by Aldrich (Milwaukee, WI, USA). Mouse anti-rat tyrosine hydroxylase (TH) monoclonal antibody and rabbit anti-rat TH polyclonal antibody were purchased from Sigma (St. Louis, MO) and Calbiochem (San Diego, CA) respectively. Details regarding the GFAP antibodies used in the study can be found in O’Callaghan (2002) [64].

3.3.3 MPTP Treatment and brain dissection

All mice were injected s.c. with 12.5 mg/kg MPTP or saline [61]. We have previously shown this dosing regimen to have a significant effect on the striatum as evidenced by decrease in DA and its metabolites, a loss in TH protein and an increase in GFAP indicating an astrogliosis in response to injury. Also the regimen produces minimal to no damage to DA cell bodies in the substantia nigra pars compacta (SNc). All of the animals were decapitated forty-eight hours after injection and the striatum dissected for neurochemical analysis.

3.3.4 Biochemical Assays

HPLC with electrochemical detection was used to determine the concentration of dopamine (DA) and its metabolites, dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA) as well as serotonin (5-HT) and its metabolite 5-hydroxyindoleacetic acid (5-HIAA) from one side of the brain. Analyte concentrations were normalized to tissue wet weight. Tissue homogenates used for HPLC assays were prepared by sonication (Kontes Micro ultrasonicator/cell disruptor) on ice using a 30-s pulse in 0.2 M perchloric acid, containing 3,4-dihydroxybenzylamine (1 μM) as internal
standard. The homogenate was centrifuged at $10,000 \times g$ for 15 min, and the resulting supernatant immediately injected using an autosampler described below. The striatum was prepared in a standard volume (0.3 ml) and results were expressed as $\mu$/g original tissue wet weight. Sample (10 $\mu$l) was injected using a temperature controlled (4°C) Waters 717 Plus Autosampler (Waters, Milford, MA, USA) connected to a Waters 515 HPLC pump. The sample was passed over a reversed-phase C18 column (Waters Symmetry, 250×4.6 mm, 5 $\mu$m, 100Å). Analytes were detected using the Waters 464 pulsed electrochemical detector (range 10 nA, potential 700 mV) connected by means of the Waters bus SAT/IN module to a computer using Millenium Software 32. The mobile phase consisted of 75 $\mu$M sodium dihydrogenphosphate, 1.7 mM 1-octanesulfonic acid, 25$\mu$mol ethylenediaminetetraacetic acid and 10% (v/v) acetonitrile. All components were adjusted to a pH of 3.0 with phosphoric acid, at a flow rate of 1 ml/min. Under these conditions the average run time is 30 min with representative retention times (in min) for NE (5.99), 4-dihydroxybenzylamine (DHBA, internal standard, 8.24), DOPAC (8.93), DA (11.28), 5-HIAA (13.57), HVA (19.77), 5-HT (26.1). Quantitation was achieved by the use of the internal standard (10 pmol DHBA per injection) method using daily standard curves of each analyte (0.5-25 pmol per injection). The limit of detection is 0.5 pmol per injection, inter-assay variation is ±3%. Striatum samples from the other side of the brain were homogenized in 1% (w/v) SDS heated to 80 – 90°C for determination of TH and GFAP by ELISA [61,64,81]. Values obtained were normalized to total homogenate protein assayed by BCA [65].

### 3.3.5 Data Analysis

Three between-subjects factor (strain, sex, treatment) analysis of variance (ANOVA) was used to evaluate the data using SAS statistical software. Pairwise comparisons were made using the Tukey HSD test with $\alpha$ set at 0.05. The male data reported here are from Jones et al. (2013) [77].
3.4 Results

3.4.1 Effects of MPTP on Dopamine Neurochemistry in the Striatum

3.4.1.1 Effects of MPTP on Dopamine in the Striatum

ANOVA revealed a significant main effect for strain with BXD32 and BXD48 having the lowest and highest DA concentrations respectively (Fig. 3.1, top panel). There were also significant main effects for treatment and sex. The strain $\times$ sex, treatment $\times$ strain and strain $\times$ sex $\times$ treatment interactions were also significant.

The percent of control (MPTP vs. saline) for DA in the striatum by sex is shown in Fig. 3.1, bottom panel. BXD40 showed the highest DA loss in the striatum in both sexes, BXD84 showed the least loss in females and BXD29 the least loss in males.

3.4.1.2 Effects of MPTP on DOPAC in the Striatum

ANOVA revealed significant main effects of strain, sex and treatment. Basal levels of DOPAC differed among the females with BXD32 and BXD48 showing the lowest and highest DOPAC levels respectively. Significant strain $\times$ sex and strain $\times$ treatment interactions were also observed. Treatment $\times$ sex interaction and strain $\times$ sex $\times$ treatment interactions were not significant. The percent of control (MPTP vs. saline) for DOPAC in the striatum by sex is shown in Fig. 3.2, bottom panel. BXD40 and BXD84 showed the highest and lowest DOPAC loss in the striatum in both sexes.

3.4.1.3 Effects of MPTP on HVA in the Striatum

ANOVA revealed significant main effects for strain and treatment. Significant differences in basal levels of HVA among females was observed with BXD9 and BXD32 having the highest and lowest HVA concentration respectively (Fig. 3.3 top panel). The main effect for sex was not significant. The strain $\times$ treatment interaction was significant. The Strain $\times$ sex, treatment $\times$ sex and strain $\times$ sex $\times$ treatment interactions were not significant. The percent of control (MPTP vs.
Figure 3.1: Top panel. Effect of MPTP on DA concentration in the striatum in 9 strains of BXD recombinant inbred (RI) female mice. The mice were injected with 12.5mg/kg MPTP (vs. saline) and sacrificed 48 h later. DA content in striatum was determined by HPLC, normalized to tissue wet weights and expressed as mean ± s.e.m. Bottom panel. Effect of MPTP in BXD mice expressed as % control, for both sexes. BXD40 showed the greatest MPTP-related DA loss for both sexes. ANOVA revealed a significant main effect of strain, sex, treatment (F\(_{8,140}=17.21\), F\(_{1,140}=7.77\), F\(_{1,140}=849.86\) respectively, p<0.01 for each) and significant strain \(\times\) sex, strain \(\times\) treatment and strain \(\times\) sex \(\times\) treatment interaction (F\(_{8,140}=5.49\), F\(_{8,140}=17.37\) respectively, p<0.01 for both, p<0.01, F\(_{8,140}=2.2\), p<0.05).

Saline) for HVA in the striatum in both sexes is shown in Fig. 3.3, bottom panel. Strain BXD40 has the highest HVA loss in both sexes.

### 3.4.1.4 Effects of MPTP on Dopamine turnover Ratio (DOPAC/DA)

ANOVA revealed significant main effects for strain with BXD48 and BXD69 having the lowest and highest basal levels of DOPAC/DA in females respectively.
Figure 3.2: Top panel. Effect of MPTP on striatal DOPAC in females. Mice were injected with 12.5mg/kg MPTP (vs. saline) and sacrificed 48 h later. DOPAC content in striatum was determined by HPLC, normalized to tissue wet weights and expressed as mean ± s.e.m. bottom panel. The effect of MPTP on DOPAC expressed as % control for both sexes. BXD40 showed the greatest MPTP-related DA loss for both sexes. ANOVA revealed a significant main effect of strain, sex, treatment (F<sub>8,139</sub>=42.42, F<sub>1,139</sub>=125.81, F<sub>1,139</sub>=142.24, respectively, p<0.01 for each) and significant strain × sex interaction (F<sub>8,139</sub>=24.39, p<0.01) and strain × treatment interaction (F<sub>8,139</sub>=2.38, p<0.05).

(Fig. 3.4, top panel). ANOVA revealed significant effects for sex and treatment on DOPAC/DA. DOPAC is one of two major metabolites, the other being homovanillic acid (HVA). Dopamine turnover can thus be estimated by calculating the ratio of either metabolite to the amine. DOPAC/DA is considered to reflect presynaptic process as DOPAC is produced by monoamine oxidase and HVA/DA is thought to reflect postsynaptic process as it is produced by catechol-O-methyltransferase. Changes in these indices can indicate pharmacological treatments, disease-relevant
Figure 3.3: Top panel. Effect of MPTP on Homovanillic acid (HVA) concentration in the striatum in females. The mice were injected with 12.5 mg/kg MPTP or saline and sacrificed 48 h later. HVA content in striatum was determined by HPLC, normalized to tissue wet weights and expressed as mean ± s.e.m. BXD40 showed the highest reduction in both sexes. Bottom panel. The effect of MPTP on HVA expressed as % control in both sexes. ANOVA revealed a significant main effect of strain and treatment (F_{8,140} = 11.49, F_{1,140} = 111.26 respectively, p<0.01 for each). ANOVA revealed significant strain × treatment interaction (F_{8,140} = 6.70, p<0.01).

Damage or compensation. Here, post hoc test revealed no differences between saline and MPTP groups across the strains. Strain × sex interaction was significant. Strain × treatment, sex × treatment and strain × sex × treatment interactions were not significant. The percent of control (MPTP vs. saline) of striatal HVA/DA for both sexes is shown in Fig. 3.4, bottom panel.
Figure 3.4: Top panel. Effect of MPTP on DA turnover as determined by the ratio of DOPAC/DA in the striatum in female BXD mice. Mice were injected with 12.5mg/kg MPTP (vs. saline) and sacrificed 48 h later. Experimental and control values normalized to tissue wet weights are expressed as mean ± s.e.m. Bottom panel. The effect of MPTP on DOPAC/DA expressed as % control for both sexes. ANOVA revealed significant main effects for strain, sex and treatment ($F_{8,140}=4.42$, $F_{1,140}=13.11$, $F_{1,140}=6.98$ respectively, $p<0.01$ for each) and significant effect for strain × sex interaction ($F_{8,140}=3.62$, $p<0.01$).

3.4.1.5 Effects of MPTP on Dopamine Turnover (HVA/DA)

ANOVA revealed significant main effects for strain, sex and treatment (Fig. 3.5 top panel). BXD69 and BXD9 show the lowest and highest basal levels for HVA/DA in females respectively (Fig. 3.5 top panel). The sex × strain, strain × treatment, treatment × sex and strain × sex × treatment interactions were also significant. BXD40 and BXD84 showed the highest and the lowest HVA/DA change after MPTP treatment respectively. The percent of control (MPTP vs. saline) for HVA/DA in the striatum in both sexes is shown in Fig. 3.5.
Figure 3.5: Top panel. Effect of MPTP on DA turnover as determined by the ratio of HVA/DA in the striatum of female BXD RI mice. Female mice were injected with 12.5mg/kg MPTP (vs. saline) and sacrificed 48 h later. Experimental and control values normalized to tissue weights are expressed as mean ± s.e.m. Bottom panel. The effect of MPTP on HVA/DA expressed as % control in both sexes. ANOVA revealed significant main effects for strain, sex and treatment (F$^{8,140}$=24.48, F$_{1,140}$=8.53, F$_{1,140}$=128.89 respectively, p<0.01 for each). The sex × strain, strain × treatment, and strain × sex × treatment interactions were significant (F$^{8,140}$=8.99, F$_{8,140}$=19.97, F$_{8,140}$=5.33, p<0.01 for each). The sex × treatment interaction was also significant (F$_{1,140}$=4.64, p<0.05).

### 3.4.2 Effects of MPTP on Serotonin (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA)

We observed small but significant effects by omnibus F for treatment, strain and sex on 5HT; however, post hoc tests showed no differences between saline and MPTP across the strains. There was no significant effect of treatment (MPTP) on 5-HIAA
concentrations compared to saline group.

3.4.3 Effects of MPTP on Tyrosine Hydroxylase (TH)

We observed significant main effects for strain and treatment (Fig. 3.6, top panel). There were significant differences in the basal level of TH across 9 strains of BXD mice with strain 9 showing the highest and strain 29 showing the lowest striatal TH levels. MPTP treatment significantly affected the striatal TH levels with strain 40 showing the highest and strain 62 showing the lowest striatal TH reduction. The main effect for sex was not significant. The Sex × strain, strain × treatment, sex × treatment and sex × treatment × strain interactions were all significant. Percent of control (MPTP-saline) for TH in both sexes is shown in the bottom panel.

3.4.4 Effects of MPTP on GFAP in the Striatum

Fig. 3.7 shows the effect of MPTP treatment on the astrocyte marker, GFAP. GFAP levels increase in response to damage. Strain and treatment significantly affected GFAP concentration in the striatum. Sex main effect was not significant. Strain × treatment interaction was significant; however, strain × sex, sex × treatment and sex × treatment × strain interactions were not significant. There were no differences among the strains in basal concentrations. strain 62 showed the lowest GFAP elevation in both sexes and strain 60 and 69 in females and males show the most GFAP elevation respectively.

3.4.5 Correlational analysis of MPTP effects between the sexes

We observed significant Pearson product-moment correlations between the sexes for GFAP, DA, HVA and TH (Table 1).

<table>
<thead>
<tr>
<th>DA</th>
<th>HVA</th>
<th>DOPAC</th>
<th>TH</th>
<th>5HT</th>
<th>5HIAA</th>
<th>GFAP</th>
</tr>
</thead>
<tbody>
<tr>
<td>r=0.80</td>
<td>r=0.66</td>
<td>r=0.05</td>
<td>r=0.79</td>
<td>r=0.03</td>
<td>r=0.52</td>
<td>r=0.2</td>
</tr>
<tr>
<td>p&lt;0.01</td>
<td>p&lt;0.5</td>
<td>n.s.</td>
<td>p&lt;0.01</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
</tbody>
</table>

Table 3.1: Pearson product-moment correlations, males vs. females for difference scores, saline minus MPTP (GFAP is MPTP-saline).
Figure 3.6: Top panel. The effects of MPTP on tyrosine hydroxylase (TH) concentration in the striatum in females. Mice were injected with 12.5mg/kg MPTP (vs. saline) and sacrificed 48 h later. TH content was determined by ELISA according to the method of O’Callaghan et al. (1990) and normalized to total protein. Data are expressed as mean ± s.e.m. Bottom panel. Effect of MPTP on TH expressed as % control in both sexes. ANOVA revealed significant main effect of strain and treatment (F_{8,141}=14.65, F_{1,141}=627.63, p<0.01 for each. Sex × strain, strain × treatment and sex × treatment interactions were all significant (F_{8,141}=10.74, F_{8,141}=9.88, F_{1,141}=8.12, p<0.01 for each). The sex × treatment × strain interaction was also significant (F_{8,141}= 2.28, p<0.05).

3.4.6 Principal Component Mapping

As we found earlier in males [77] the principal component QTL for the females mapped to chromosome 1 at about 60 Mb with a suggestive LOD of about 2.6 (suggestive limit was 2.49). When the data from the sexes were combined, the LOD reached significance at 5.
Figure 3.7: Top panel. Effect of MPTP on the concentration of glial fibrillary acidic protein (GFAP) in the striatum in females. The mice were injected with 12.5mg/kg MPTP (vs. saline) and sacrificed 48 h later. GFAP content was determined by ELISA (O’Callaghan, 1991). GFAP concentrations were normalized to total protein and expressed as mean ± s.e.m. Bottom panel. Effects of MPTP on striatal GFAP expressed as % control in both sexes. ANOVA revealed significant main effects for strain and treatment and their interaction (F8,138= 20.19, F1,138= 243.09, F8,138=10.65, respectively, p<0.01 for each).

3.5 Discussion

This is a follow up to our previous study [77], in which we examined the effect of host susceptibility to MPTP in male BXD mice. Genetic-induced response variation was examined in females from nine of the same strains previously evaluated in males (one of the strains BXD 27 was not available because of poor breeding). The correlation for GFAP, an astrocyte marker of injury, between males and females was r=0.2, leaving open the possibility for closer investigation of the role...
of sex in future studies. The agreement in some of the responses between the two sexes is remarkable; the DA measures, except for DOPAC, were highly correlated. Since only a limited number of strains were evaluated it remains to be seen if studying a greater number of strains should occur before concluding that gender does not play a role. Also encouraging, is the significant QTL obtained for the DA aggregate response to MPTP. Mapping with only 9-10 strains however, is risky and nomination of candidate genes should await replication with more strains. DA turnover as indicated by DOPAC/DA showed no evidence of MPTP treatment, whereas HVA/DA was generally increased by MPTP. While the exact mechanism for this is not possible to determine here, it may reflect a decrease in the efficacy of reuptake by the dopamine transporter.

MPTP neurotoxicity shows wide, genetic variability with responses showing continuous distributions, indicating the involvement of multiple genes to determine the susceptibility to MPTP toxicity, in other word, host susceptibility is considered to be a polygenic or complex trait, involving several genes that interact with the environment and likely with each other. Systems genetics analysis together with a forward genetics approach (starting with examining phenotypes followed by identifying candidate genes) is a powerful technique to identify underlying mechanisms. As shown here, the combination of several related phenotypes through principal components analysis strengthens the ability to locate and nominate candidate genes.

In the next chapter, by using systems genetics, we show that the extent of MPP$^+$ production is quantitatively unrelated to the extent of dopamine loss in the striatum and showing once again that MPTP neurotoxicity is highly complex and determined by multiple factors [82]. There is evidence showing the possible involvement of other enzymes such as mitochondrial cytochrome P450 2D6 (CYP2D6) in addition to monoamine oxidase B (MAOB) in the metabolism of MPTP to MPP$^+$ [83]. These researchers showed that the mitochondrial enzyme CYP2D6 located in the dopaminergic cells of substantia nigra is capable of metabolizing MPTP to MPP$^+$, almost with the same efficiency as MAOB. A future examination of CYP2D6 in BXD RI strains would provide additional insight into the factors controlling the neurotoxic response to MPTP in these strains. The existence of multiple enzymes for the metabolism of MPTP to the active neurotoxic agent MPP$^+$ indicates that MPTP neurotoxicity is a complex trait involving multiple pathways and
potentially multiple genes. Examining the effect of CYP2D6 polymorphisms along with other factors such as DA transporter polymorphisms and genetic differences in mitochondrial complex I players will likely shed light on the complex pathways involved in MPTP toxicity.

3.6 Conclusion

This study is a follow up to our previous study in males in which we examined the effect of MPTP female mice. We have shown that similar to males there is a broad range of response variation in the BXD strains in females. Moreover, there is remarkable similarity in the neurotoxic responses to MPTP between the sexes. Of course, there are some differences and whether these differences stem from measurement or methodological error or represent important biological processes remains to be seen. This work thus sets the stage for more extensive work in many more BXD strains (about 100 exist) or in similar genetic reference populations. Such work will allow gene mapping and gene expression resulting in the nomination and confirmation of candidate genes. Such studies will aid us in reaching our ultimate goal, to better understand how genes and the environment interact in the etiology of neurodegenerative diseases.
Chapter 4  
Genetic Correlational Analysis Reveals no Association between MPP$^+$ and the Severity of Striatal Dopaminergic Damage Following MPTP Treatment in BXD Mouse Strains

4.1 Abstract

1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is a pro-neurotoxicant that must be metabolized to 1-methyl-4-phenylpyridinium (MPP$^+$) and taken up into striatal dopaminergic neurons to produce neurodegeneration. Recently, we showed wide genetic variability in MPTP-associated neuronal damage in a panel of recombinant inbred mouse strains. Here we examined the amount of MPP$^+$ produced in the striatum in the same strains of inbred BXD mice. This allowed us to determine if the differences in the dopaminergic neurotoxicity and associated astrogliosis among the BXD mouse strains were due to differential metabolism of MPTP to MPP$^+$. Using the same BXD mouse strains examined previously [77], we found that the extent of the striatal damage produced following MPTP treatment is not correlated quantitatively with the production of MPP$^+$ in the striatum. Our findings also
extend those of others regarding strain differences in MPTP-induced dopaminergic neurotoxicity. Importantly, our finding suggests that additional factors influence the neurodegenerative response other than the presence and amount of the toxicant at the target site.

4.2 Introduction

Exposure to various industrial and agricultural chemicals, especially pesticides, has been implicated as conferring risk for multiple diseases, including neurodegenerative disorders. Several of the substances implicated include rotenone, maneb, paraquat, carbamate and organophosphorus insecticides.

MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) is a pro-neurotoxin that is able to enter the brain when given systemically where it is metabolized by astrocytes to produce 1-methyl-4-phenylpyridinium (MPP\(^+\)). MPP\(^+\) is taken up preferentially by the dopamine (DA) transporter resulting in striatal DA terminal degeneration and, at higher dosages, loss of DA neurons in the substantia nigra (SN) \[61,84\]. Treatment of mice with a single low dosage of MPTP serves as an experimental tool for studying factors that may impact striatal dopaminergic nerve terminal degeneration \[61,77,81,85\].

Recently, we reported large genetic variation in MPTP-induced dopaminergic neurotoxicity among ten BXD recombinant inbred mouse strains \[77\]. Male mice were treated with a single s.c. injection of 12.5 mg/kg MPTP and the striatum was harvested 48 h later \[77\]. This dosage regimen damages striatal dopaminergic nerve terminals but does not damage SN. Striatal dopaminergic neurotoxicity was apparent as evidenced by decreases in DA and its metabolites, homovanillic acid (HVA) and 3,4-dihydroxyphenylacetic acid (DOPAC), as well as in levels of tyrosine hydroxylase (TH) protein, a measure of DA terminal loss. Furthermore, this dopaminergic damage was accompanied by elevation in glial fibrillary acidic protein (GFAP), a hallmark of the astroglial response to underlying neural injury (e.g. see O’Callaghan et al., 2014) \[85\]. Nearly all of these indicators of striatal dopaminergic neurotoxicity were affected in our inbred mouse strains, especially striatal DA content, as measured by HPLC in tissue homogenates and reported in Jones et al. (2013) \[77\]. Because this reduction showed large differences across strains, i.e., between 20 and 90%, the question addressed in this short communication
was whether there are differences in metabolism of MPTP to MPP\(^+\) across the strains. In this work we determined the amount of MPP\(^+\) produced by each BXD strain following the same single dosage of MPTP used previously \[77\].

### 4.3 Materials and methods

Following the protocol of Jones et al. (2013) \[77\] we obtained male mice from nine of the original ten BXD strains; sufficient numbers of mice from one BXD strain (27) were not available for the determination of MPP\(^+\) in this paper due to poor reproduction. Mice (n = 5-6 per group) received 12.5 mg/kg MPTP s.c. and were killed 60 min later. The peak level of MPP\(^+\) in the brain is reached after 60 minutes MPTP post injection. The brains were removed and the striatum was dissected free hand and weighed prior to analysis for MPP\(^+\) by HPLC using a modification of the method of Miller et al. (1998) \[80\]. Briefly, the striatum was sonified in 200\(\mu\)l of 5\% trichloroacetic acid containing a known amount of the internal standard 1-methyl-3-phenyl pyridinium iodide and the supernatant collected following centrifugation twice at 14,000 g for 10 min. The supernatants were analyzed using a Waters Associates 616 pump, C18 RP column and 474 fluorescence detector (Milford, MA) with a mobile phase of 0.15 M triethanolamine/HCl, 0.1 M acetic acid (17.4 M), pH 2.3 with formic acid, and 9\% acetonitrile. Excitation and emission wavelengths of 290 and 370 nm, respectively, were used to detect MPP\(^+\) with a retention time of 10 min. Chromatograms were recorded and integrated using Millennium 32 software (Waters Associates, Milford, MA).

We determined the degree of association between the amount of MPP\(^+\) produced in this study and MPTP-related striatal dopaminergic neurotoxicity as well as the associated astrogliosis reported in the Jones et al. (2013) \[77\] study. GFAP is an astrogliosis marker (see O’Callaghan et al., 2014 and O’Callaghan and Sriram, 2005) \[85, 86\]. The associations were estimated by Pearson correlation using the BXD strain means for the principal component (PC) analysis-derived composite of MPTP-based reductions in striatal DA, DOPAC, HVA and TH protein from Jones et al. (2013) \[77\]. We also determined the correlation between the GFAP increases induced by MPTP and the degree of dopaminergic neurotoxicity (PCs) as well as MPP\(^+\) levels.
4.4 Results

Table 1 presents PC values (z-scores) for the dopaminergic neurotoxicity composite, the striatal MPP$^+$ (mg/g tissue) levels determined in this study and the striatal GFAP increases (µg/mg tissue) from Jones et al. (2013) [77]. We quantified the MPP$^+$ levels in the striatum but not SN as this dosage regimen normally does not damage SN in the C57BL6J. It remains an question as to whether the BXD strains that show the most damage in the striatum would have SN damage.

<table>
<thead>
<tr>
<th>BXD Strain</th>
<th>Mean Z score 1st principal component (PC) (a)</th>
<th>Mean±sem MPP$^+$mg/g striatum (b)</th>
<th>Mean±sem increase in GFAP µg/mg striatum (c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>-1.75</td>
<td>8.43 ± 1.10</td>
<td>0.44 ± 0.10</td>
</tr>
<tr>
<td>27</td>
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<td>0.23 ± 0.05</td>
</tr>
<tr>
<td>29</td>
<td>1.53</td>
<td>9.19 ± 1.03</td>
<td>0.08 ± 0.02</td>
</tr>
<tr>
<td>32</td>
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</tr>
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<tr>
<td>60</td>
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</tr>
<tr>
<td>62</td>
<td>1.92</td>
<td>15.95 ± 0.46</td>
<td>0.10 ± 0.06</td>
</tr>
<tr>
<td>69</td>
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<td>6.43 ± 0.51</td>
<td>0.22 ± 0.03</td>
</tr>
<tr>
<td>84</td>
<td>1.92</td>
<td>6.09 ± 1.27</td>
<td>0.14 ± 0.03</td>
</tr>
</tbody>
</table>

Table 4.1: Correlation matrix for the graphical representation of the system analysis presented in Figure 2.11 Correlation presented are Pearson r.

Our data show that the association between MPP$^+$ levels and the striatal dopamine compromise caused by MPTP treatment is weak and not statistically significant as measured by Pearson’s r. The association between MPP$^+$ and GFAP, a marker of astrocyte activation in response to neural injury, was also weak. However, there was a strong and significant relationship between the degree of dopaminergic neurotoxicity (PC) and GFAP ($r = 0.69$, $p < 0.03$).

4.5 Discussion

Others have reported strain differences in the response to MPTP in the parental strains of the BXDs [54]. Our observation that the association between MPP$^+$
production and a reduction in measures of striatal DA terminal integrity was weak or nonexistent was unexpected. But it should be noted that others also have reported strain differences in MPTP neurotoxicity that were not related to MPP$^+$ levels [87]. We also found little to no association between MPP$^+$ levels and the GFAP response to striatal dopaminergic terminal damage in the BXD strains, i.e., the strains with the most damage did not have the highest levels of MPP$^+$. Nonetheless, the MPTP-associated dopaminergic damage (decreased DA, its metabolites and TH protein) was significantly correlated with the increase in GFAP, as expected. It is well-established that MPTP must be converted to MPP$^+$ in the brain and its neurotoxicity is assumed to reflect “dose to target” levels of MPP$^+$. However, our clear strain differences in dopaminergic neurotoxicity and the lack of association with the MPP$^+$ levels indicate that other factors play a role in the degree of damage induced by MPP$^+$ in striatal DA nerve terminals. These factors could include but are not limited to a differential susceptibility of the DA terminal to damage or some other aspect of terminal function. The DA transporter (DAT) is responsible for entry of MPP$^+$ into the nerve terminal ultimately resulting in sufficient concentration of the toxicant to cause damage. The differential susceptibility of the BXD mice to MPP$^+$ could be conferred by strain differences in the DAT levels themselves or in their DA reuptake capabilities. Certainly, a continued investigation into the differential vulnerability of the BXD strains to MPTP is a worthy endeavor as it would help to broaden our understanding of the factors contributing to the susceptibility of the nigral striatal system to neurodegeneration.
Chapter 5  
Critical Role of Individual Differences in Susceptibility to Paraquat Toxicity

5.1 Abstract

Parkinson’s disease is the second most common neurodegenerative disorder with a prevalence of 1% especially among elderly. PD is divided to two types of familial and sporadic PD. Familial PD, mainly associated with multiple gene mutations, is generally manifested in early stages of life. Sporadic Parkinson disease is a complex neurodegenerative disease with unknown etiology in which gene-environment interactions are assumed to be the main factors explaining the disease. Rural-living, exposure to multiple pesticides and herbicides are two environmental elements which when accompanied by susceptible genotypes, increase the risk of PD. Paraquat is one of the herbicides associated with increased risk for PD. Pulmonary toxicity by paraquat has been approved by multiple studies; however there are equivocal reports regarding the involvement of PQ in increased risk for PD and its role in neurodegeneration. Overlooking genetically susceptible individuals and not taking into account the role and importance of multiple gene mutations in people exposed to paraquat may be one of the reasons of this discrepancy. Identifying genotypes sensitive to paraquat and other environmental stressors is a challenging issue that needs to be considered in order to enable researchers to address the etiology of sporadic PD precisely. The aim of this review is to
examine the evidence supporting the importance of gene-environmental interactions in increased risk of PD among genetically susceptible individuals, using paraquat as the main environmental stressor.

5.2 Introduction

Parkinson’s disease (PD), which was first described in 1817 by Dr. James Parkinson as shaking palsy, is one of the most common neurodegenerative diseases especially among the elderly in which the individual suffers from motor impairments. Currently, the disease affects more than four million people worldwide; that number is expected to double over the next 30 years [88]. Common symptoms of PD include Bradykinesia, resting tremors, and posture instability. In advanced stages of the disease, cognitive impairments and psychiatric complications may also be present [88].

Multiple factors are proposed to increase the risk for PD. Age, MPTP exposure, well water drinking, multiple gene mutations, different types of pesticides and herbicides are associated with higher risk of PD [89]. The commonly used herbicide Paraquat has been strongly implicated as a risk for PD since its structure is comparable to MPTP, which is a known neurotoxic highly linked to increased risk for PD [90].

Paraquat is broadly used in farming and agriculture. It is sold in more than 130 countries around the world. More than 8000 tones a year of paraquat is produced in countries such as United States, Brazil, Japan and England. Vast usage of paraquat in farming and agriculture especially in United States has triggered researchers to study paraquat neurotoxicity in numerous animal models, and the implications of its toxicity in public health and prevention strategies regarding PD. Therefore, further investigation is needed to investigate possible mechanism of paraquat neurotoxicity in addition to the role of genetics in individuals who may be more susceptible to the neurotoxic effects of paraquat. Additional research is warranted in order to address the importance of gene-environmental interactions in the complex etiology of sporadic PD. The aim of this review is to address the connection between paraquat neurotoxicity and PD, using epidemiological studies. In addition, the potential mechanisms by which paraquat induces its toxicity are discussed, using the result of numerous animal models for PD. The role of genetic
factors as mediators of the association between PD and paraquat neurotoxicity will also be discussed in the paper.

5.3 Pathology of PD

Nigrostriatal dopamine degeneration is a pathological hallmark of PD [91]. Selective loss of DA neurons located in the substantia nigra pars compacta (SNpc) results in depigmentation of this brain region, as they contain high concentration of neuromelanin [91]. Beginning of clinical symptoms of PD coincides with 60% loss of DA neurons and 80% depletion of DA in dorsolateral putamen, which is the main projection site for nigrostriatal dopaminergic cells [4,5].

Two hypotheses have been proposed by numerous research, explaining the sequence of dopamine cell degeneration in substantia nigra and DA depletion in striatum. The first hypothesis proposes a sequential degeneration, starting from degeneration of dopamine cells and resulting in DA depletion in striatum. Based on this theory, degeneration of dopamine cells located in SN precedes the DA depletion in striatum. On the other hand, the second theory suggests a retrograde degeneration. According to this “dying back” scenario, axonal terminals of dopamine cells are the primary target for degeneration, leading to DA depletion in the striatum and subsequent degeneration of DA cell bodies in SN [92].

Although dopaminergic loss in SNpc is a key component of PD, other brain regions may go through degeneration according to the age of the patient and severity of the disease. Degeneration of hippocampal structures and cholinergic systems, which are seen in the last stages of the disease in advanced age patients, may explain the dementia that accompanies PD in elderly patients. In some cases of PD, depression may precede motor symptoms, which may be an indication of the involvement of non-dopaminergic system pathways in PD [93].

5.3.1 Lewy Bodies

Another hallmark of PD is formation of Lewy bodies. Lewy bodies are cytoplasmic protein aggregates compose of parkin, ubiquitin, neurophylaments and alpha synuclein that are found in affected regions of the brain [93]. Substantia nigra, locus coeruleus, raphe nuclei and cerebral cortex are some of the brain regions in which
Lewy bodies are detected [94]. Morphologically, Lewy bodies consist of several spherical bodies surrounded by a halo ring. The peripheral ring around Lewy bodies makes them distinguishable from other inclusions and axonal swellings in the cell [8]. Lewy neurites are another type of protein inclusions found in dendrites rather than the soma of the cells [95]. Lewy bodies and Lewy neuritis are not specific to Parkinson disease and are found in a number of other neurodegenerative diseases such as Alzheimer’s, dementia with LBs and also in healthy individuals of old age [8].

5.4 Risk Factors for PD

Based on recent research, genetics, environment and age are three risk factors hypothesized for PD. How these factors interact remains a popular topic for debate, thus, further research is warranted [55]. While sporadic PD (sPD) is the common form of the disease, familial PD, which shares almost the same phenotypes as the sPD, is responsible for 15% of early onset (before the age of 50) cases. Multiple genes are linked to the development of familial PD [96]. Mutations in Alpha synuclein (SNCA), leucine-rich repeat kinase 2 (LRRK2), PINK1, DJ-1, and MAPT genes are implicated in the development of familial PD [96,97].

In most cases, the characteristics of familial PD are the same as sporadic PD with the exception of age of onset. Familial PD is manifested in younger ages compared to sPD. One of the gene investigated in the development of familial PD is the alpha-synuclein (SNCA). Alpha-synuclein (SNCA) gene codes for the alpha synuclein protein involved in the formation of Lewy bodies [98] and mutations in this gene are associated to Familial PD development. Two different forms of mutation, a single point mutation and a triplication of the region containing the gene have been linked to development of familial PD [98,99]. The average onset of the disease is 45 for the single mutation and 35 in case of triplication of the gene with 8-9 year survival in these two types of alpha synuclein gene mutations [98,100].

Although the involvement of alpha synuclein gene and protein have been confirmed in the familial PD, lack of alpha synuclein mutations in vast population of people with sporadic Parkinson’s disease indicates that additional mechanisms contribute in PD pathogenesis [101]. Interaction between the alpha synuclein gene and environmental risk factors such as the herbicide paraquat is a possible
mechanism responsible for the development of sporadic PD in people exposed to the herbicide.

It has been shown in an in vitro study that incubating alpha synuclein with paraquat increases the rate of alpha synuclein fibrillation [102]. Direct interaction of paraquat and alpha synuclein leads to conformational change of the protein, increasing the concentration of partially folded intermediate, alpha synuclein. Although the conformational change in alpha synuclein is slow, it precedes alpha synuclein aggregation [102]. Alpha synuclein inclusions lead to formation of amorphous and fibril deposits, which play a critical role in pathology and pathogenesis of Parkinson disease. Additionally, in vivo results supporting the role of paraquat in the formation of alpha synuclein inclusions are provided by the study conducted by Manning Bog and colleagues (2002) [38]. Based on this study, paraquat injection to mice results in aggregation of alpha synuclein in the brains of mice in a dose dependent manner. Paraquat increases alpha synuclein levels in substantia nigra and frontal cortex of mice, accompanied by elevation of alpha synuclein inclusions in these brain regions [38]. Over expression of alpha-synuclein and elevation in protein inclusions may be a neuronal response to the insult caused by paraquat toxicity. Paraquat causes oxidative stress in neurons via generating reactive oxygen species [103]. The oxidative stress imposed by paraquat may be involved in altering the biophysical properties of alpha synuclein, therefore impairing the ability of the protein to degrade properly, which results in protein aggregation.

5.5 Environmental Factors and PQ

Based on a set of diverse epidemiological studies, multiple environmental influences are considered risk factors for PD. Examples include but are not limited to pesticide and herbicide exposure, well water drinking, and heavy metal exposure [104]. Occupational exposure to these risk factors puts farmers, seasonal workers and even close residents in high risk category for PD.

Multiple case control studies show significant associations between pesticide exposure and increased risk for PD. A meta-analysis of 19 studies by Priyadarshi and colleagues (2001) showed a steady association between high pesticide use and increased risk for PD with a combined odds ratio of 2.15 for the US studies [12]. Farmers, people who lived close to the farms, or the ones exposed to the farming
animals were the populations at increased risk for PD. Additionally, a review of 38 case-control studies done by Brown and colleagues (2006) showed a robust relationship between long-term pesticide use and increased prevalence of PD [104].

A number of case control studies report incidence of PD following long-term exposure to pesticides and herbicides. For example, a crop duster pilot was diagnosed with PD after many acute and chronic pesticide intoxications. A 32-year-old man developed PD after working in a factory that made pesticides for more than 15 years [104]. Additionally, 38 case control studies were reviewed by Brown and colleagues (2006) and all of them showed a consistent increase in Parkinson disease linked to pesticide and herbicide exposure [104].

Several studies indicate that paraquat use is linked to PD incidence. A study by Liou and colleagues (1997) in Taiwan shows that chronic exposure to paraquat for more than 20 years is associated with increased risk of PD [23]. Individuals who are exposed to paraquat are at higher risk for developing PD compared to other types of herbicides and pesticides [105,106]. The effects of well water drinking and rural living as two risk factors for PD are only significant when accompanied by paraquat or other types of pesticide exposure [75]. Additional supporting evidence is provided in a study done by Ritz and colleague in specific regions of California, in which paraquat is extensively used. Regions with common paraquat use report higher mortality rates compared with paraquat free regions [107]. Numerous case control studies show a significant association between the extent of exposure to paraquat and the severity of the disease [108].

5.6 The Importance of Gene-Environmental Interaction in PQ Toxicity

Associations between PQ toxicity and PD, and also the involvement of multiple genes in the development of familial PD have sparked the interest to examine gene-environmental interactions in this regard. Recently, more research has begun to focus on the importance of gene-environmental interactions in increased risk of PD [37,47].

Multiple studies support the involvement of PQ as a risk factor for PD [23,26,27,72,73], however the result of both clinical and epidemiological studies concerning
paraquat neurotoxicity are controversial; not all of the epidemiological studies support the contribution of PQ in PD [74–76]. Although epidemiological studies are important tools to detect association between multiple factors, most of the time they fail to take into account the role of individual differences in subpopulations which may result in variant responses to the studied variables. One example is the polymorphisms of debrisoquine hydroxylase in cytochrome P450D6 (CYP2D6) [45, 109]. CYP2D6 is involved in metabolism of several xenobiotics including multiple pesticides and herbicides in addition to MPTP, which is structurally similar to paraquat. 5 to 10% of white populations are poor metabolizers (PM) of these xenobiotics, resulting in undetectable enzyme activity in these individuals. CYP2D6 activity is genetically determined; an autosomal recessive trait, which makes homozygotes with CYP2D6*4 genotype, slow metabolizers of numerous pesticides. A study by Elbaz and colleagues (2004) revealed a 2 fold increase in risk of PD in individuals with poor metabolizer genotype exposed to pesticides. The population included in the study were farmers or people frequently using pesticides for gardening purposes. Normal metabolizers exposed to pesticides had a slight increase in risk for PD compared to poor metabolizers without any pesticide exposure [45].

Mutations in two neuron-protective genes, Parkin and PINK also play a role in PD susceptibility [55,110,111]. Parkin and PINK are two neuroprotective genes involved in maintaining mitochondrial function and integrity, protecting the cells from harmful effects of multiple stressors including environmental toxicants [49, 53,112,113]. Different mutations in these two genes may result in mitochondrial impairment of the cells and subsequent decrease of ATP formation and increase of free radicals, inducing oxidative stress and energy deficiency. According to the reported results, deletion in these genes may result in increased sensitivity of dopaminergic cells of the substantia nigra to different neurotoxic substances such as paraquat [55]. On the other hand, early exposure to paraquat and similar pesticides may sensitize the dopaminergic cells of the brain to epigenetic modifications during life. Zhou and colleagues (2011) examined this new hypothesis using conditional transgenic RNAi in order to silence PINK1 gene in adult mice that were repeatedly exposed to paraquat beforehand. According to the results of the study, early paraquat exposure can change brain dopamine homeostasis, which in turn will reduce the threshold to develop PD in mice exposed to paraquat early in life [55].

Additional evidence to support the importance of host susceptibility is provided
by the results reported in the Goldman and colleagues study (2012) [47]. Glutathione s-transferases (GSTM1, GSTT1) are enzymes involved in numerous xenobiotix detoxifications in multiple tissues of the human body including, liver, guts and brain. These enzymes protect different cells of the body against the consequences of oxidative stress induced by multiple bio-reactions and also PD [46,114]. In 50% and 20% of caucasians homozygous deletions of M1 (GSTM1*0) and T1 (GSTT1*0) genotype is detected respectively [115]. The result of Goldman (2012) study shows that people exposed to paraquat with a homozygous deletion GSTT1*0 have an odds rations of 11.1 for increased risk of PD compared to people with GSTT1 with an OR of 1.5. No interaction between GSTM1 and exposure to PQ is reported in the study.

### 5.7 Potential Mechanisms of Paraquat Toxicity

Although previous literature supports the association between paraquat toxicity and increased risk of PD in the context of genetic susceptibility, the role and the mechanism by which paraquat can cause neurotoxicity remain unknown. Rodent models of paraquat exposure have provided some insight into these potential mechanisms. However, the results of animal models regarding paraquat toxicity are not consistent and the potential for paraquat to reduce striatal dopamine (DA) remains controversial. In a study conducted by Breckenridge and colleagues (2013), the toxicity effect of paraquat is examined by three consecutive weekly injections of paraquat to 2 month old C57BL/6J mice. Based on the reported results, no toxicity effect of paraquat was detected on nigrostriatal pathway. Silver staining did not show any significant reduction in the number of dopaminergic cells is the substantial nigra. Significant striatal DA depletion was not detected using HPIc [116]. The study By Breckenridge and colleagues (2013) is an example in which the results are surprisingly different from a vast literature addressing paraquat toxicity especially concerning the effect of PQ in degeneration of the dopaminergic cells of the substantia nigra [36,37,59].

While reductions in striatal dopamine levels have not been found in all paraquat treated animals [39], numerous animal studies in which paraquat was used systemically reported a 25-60% loss of dopaminergic neurons in the substantia nigra pars compacta [39–41,117]. Brooks and colleagues (1999) have shown significant
loss of nigrostriatal dopaminergic cells of 61% after 3 weekly injections of 10mg/kg paraquat accompanied by significant reduction of 94% in the density of dopamine terminals in the striatum. However, the results of multiple studies regarding the severity of dopaminergic cell degeneration in the substantia nigra followed by dopamine depletion in the striatum are equivocal. In the study by McCormack and colleagues (2002) not only paraquat toxicity resulted in a milder degree of dopaminergic cell loss in the substantia nigra but also no dopamine depletion was detected in the striatum. A compensatory mechanism in the surviving DA cells in the substantia nigra might be the reason for the lack of striatal dopamine depletion shown by the increased tyrosine hydroxyls activity in paraquat treated mice.

A study done by Kuter et al. (2007), shows consistent results with the McCormack study; 22% loss of dopaminergic cells in the substantia nigra was detected in male Wistar rats subchronically (5 days, one injection per day, 2-3 days of withdraw) treated with 10mg/kg paraquat treatment. Three days after the last paraquat injection an elevation in dopamine levels was discovered in the caudate-putamen, indicating a possible compensatory mechanism for dopamine cell loss also proposed in the McCormack study [118].

The results of aforementioned studies show that paraquat induces neurotoxicity in a dose dependent manner, resulting in dopaminergic loss in the substantia nigra. However, in none of these studies volume reduction of substantia nigra, which is a well-known marker of neuronal degeneration, was detected. One of the hypothesized reasons for the lack of shrinkage in substantia nigra is the duration of the paraquat treatment. Short paraquat treatment (3 weeks), might not be enough to cause the substantia nigra and dopaminergic cells to shrink, especially if compensatory mechanisms are active during this time window [119]. To resemble a chronic paraquat exposure, which is the main issue of farmers and farmworkers, Ossowska et al. (2005) treated rats with paraquat for multiple time durations of 4, 8, 12 and 24 weeks. The long period of paraquat treatment might reflect a more precise model of the chronic nature of PD and paraquat toxicity. The result of this study shows a significant reduction in the number of dopaminergic cells (37%) accompanied by a significant reduction in the volume of substantia nigra (24%) in rats treated with paraquat for 24 weeks [120].

Neurotoxicity effects of paraquat on nigrostriatal pathway are supported by vast amount of epidemiological and preclinical studies. Moreover, age of the animals is
considered a critical risk factor in PD and also the severity of PQ toxicity. The discrepancy between these studies with Breckenridge study may be due to the young age of the animals used in the later study, resulting in the null effects of PQ toxicity on nigrostriatal pathway. PD is a complicated disease in which age is considered one of the most important risk factors; not so many people of young age are diagnosed with sPD, therefore, using 2 month old mice that are transitioning from adolescence to adulthood can be misleading. The studies in which PQ neurotoxicity to nigrostriatal pathway is supported used older mice (4-10 month). Taking into account the importance of age makes these studies better animal models for PD.

Aforementioned studies show how paraquat may induce neurotoxicity, resulting in dopaminergic loss in the substantia nigra. Additionally, the epidemiological work of Goldman and colleagues (2012) and numerous animal models indicate the importance of host susceptibility and the age of the population exposed to paraquat, respectively. Additional studies have been done in this regard, supporting the existence of a host susceptibility factor to PD and PQ toxicity. Yin and colleagues (2012) examined individual differences to PQ toxicity using DBA/2J (D2) and C57BL/6J (B6) mice. Four month old mice were subjected to three weekly injections of 5 mg/kg PQ. Toxicity was measured by counting TH positive neurons, using stereology technique. Based on the reported results, B6 mice are more sensitive to the toxic effects of PQ and show greater dopaminergic cell loss in the SN accompanied with higher concentrations of iron in the ventral midbrain (VMB), compared to D2 and control mice. The higher sensitivity to PQ toxicity in B6 mice was not related to higher PQ brain concentration in this strain as HPLC showed equal PQ brain delivery in both strains of D2 and B6. Moreover, higher PQ-induced gene expression changes are detected in the VMB of B6 mice compared to D2 and control mice, using microarray analysis. The result indicates noticeable host differences to PQ neurotoxicity in animal models in which a disruption of iron regulation may be a potential mechanism underlying the neurotoxicity. Peng et al (2007) conducted a study in which the critical role of iron in mediating PQ toxicity in multiple age groups of mice was examined. According to the study (2007), the neurotoxic effects of PQ towards the dopaminergic cells increases when accompanied by iron; in other words, iron makes the dopaminergic cells more vulnerable to PQ toxicity. The synergistic effect of combined PQ and iron is ameliorated using a synthetic superoxide dismutase/catalase mimetic called EUK-
189, supporting the involvement of oxidative stress as a potential mechanism of PQ toxicity. Another study by Peng et al. (2009) indicates the involvement of microglia in increased toxicity of combined PQ and Iron. PQ and iron do not expose toxic effects on dopaminergic cells of SN unless microglia cells are present. This indicates that microglia cells have a significant mediating effect in PQ and iron toxicity to dopaminergic cells. Activation of microglia results in activation of NADPH oxidase and subsequent production of superoxide and other reactive oxygen species, all toxic to the cells. Therefore, iron and PQ induce their toxicity partly by activating microglia and the reduction cycle of microglia. These two studies (Peng et al. 2009, Yin et al. 2011) suggest that host susceptibility and iron concentration have noticeable impacts on PQ toxicity and subsequent dopaminergic cell loss of SN. Moreover, disruption of iron hemostasis via PQ toxicity is suggested based on Yin and colleagues (2011), results. However, how paraquat enters DA neurons and induces its neurotoxic effects remains a topic for further investigation [37,121,122].

One of the proposed hypotheses in this regard is the involvement of the DA transporter (DAT) in paraquat transportation into the DA cell. DAT is involved in induced DA toxicity of MPP⁺ [123]. Due to structural similarities between paraquat and MPP⁺, Rappold and colleagues (2011) hypothesized the involvement of DAT in paraquat DA neurotoxicity [117]. According to this study, paraquat is transported to dopaminergic neurons through the dopamine transporter. However, in order to be a substrate for DAT, paraquat needs to convert from its natively divalent cation nature (PQ⁺²) to monovalent cation of PQ⁺. This process is possible by an extracellular redox cycling via NADPH oxidase enzyme highly expressed in microglia. This is the stage in which iron availability is critical for microglia activation in order to convert PQ⁺² to PQ⁺, a suitable substrate for DAT. The reduction of paraquat resulting in oxidative stress mediates the extracellular neurotoxicity. Intracellular toxicity is mediated by DAT, responsible for PQ⁺ transport into DA cells. Inside the dopaminergic cells both paraquat and DA are involved in production of reactive oxygen species and subsequent neurotoxicity of dopaminergic cells. Blocking DAT, results in significant reduction of paraquat toxicity, indicating the involvement of DAT in this process. Furthermore, multiple case control studies have shown different susceptibilities to PQ toxicity associated with the genetic variants in DAT. In a study done by Ritz et al (2009), numerous variants of DAT and their association with PQ is examined. Based on the result of this study, individuals who
have one or more copies of the susceptible allele of DAT are at higher risk to develop PD after PQ exposure, suggesting a gene-environmental interaction and the role of host susceptibility in the etiology of complex neurodegenerative diseases such as PD. OCT3 is another transporter that mediates paraquat neurotoxicity and is plentiful in non-DA cells [124]. The result of the study shows that paraquat injection to both OCT3 null mice and wild type results in significant dopaminergic cell loss in SN (22%). However, there is a reduction in dopamine content of striatum exclusive to OCT3-/- mice, indicating the role of this enzyme in mediating paraquat toxicity. OCT3 is a bidirectional transporter, which buffers the amount of paraquat for DAT by carrying it to astrocytes and other types of non-dopaminergic cells in substantia nigra. Blocking OCT3 or using null OCT3 mice injected with paraquat results in more severe paraquat toxicity in addition to loss of DA content in striatum, indicating the mediating role of this enzyme in filtering the amount of paraquat available for DAT. There is an increased sensitivity to paraquat toxicity in mice with OCT3 deficiency which may be the result of increased PQ availability for DAT due to reduced buffering ability of non-DA cells.

5.8 Conclusion

Age, genetic and environment have been strongly associated with increased risk of Parkinson disease [55]. Several epidemiological studies link paraquat exposure to development of PD [23,26,72,73,75,76]. Although there have been multiple studies examining the effects of paraquat toxicity on PD progression, the mechanism by which these effects are implemented remains controversial and further investigation is needed.

Several different potential mechanisms have been proposed regarding paraquat induced toxicity. According to a study by Rappold and colleagues (2011), DAT is a mediator in paraquat toxicity to dopamine cells of substantia nigra. However, PQ2 needs to be converted to PQ+ in order to be taken up by DAT and implement its toxic effects on the nigrastral pathway. This conversion may happen by the help of reducing agents such as NADPH oxidase present on microglia cells which consequently results in increase intracellular concentration of PQ [117].

High iron concentration per se is considered a risk factor for PD [125,126]. Numerous studies have examined the role of iron in PQ toxicity, indicating a
synergistic effect between PQ and iron in inducing dopaminergic cell death in SN [121]. Additionally, the existence of genetic differences in iron regulation in mice is suggested by Yin and colleagues (2011). Similar genetic variances in iron regulation may exist in humans and therefore detecting those polymorphisms may pave the way to better understand the etiology of PD. It is worth mentioning that other polymorphisms have been link to increased risk for PD such as DAT and divalent metal transporter 1 gene [124,127]. The relevance of genetics in increasing the susceptibility to PQ toxicity has been examined in multiple studies. Transgenic animals have been developed in order to allow researchers to understand the role of genetic in development of PD. Alpha synuclein mutation or over expression is associated with increased risk of PD especially in the presence of an environmental stressor like paraquat exposure. According to multiple animal studies there is no sign of abnormal behavioral or histopathological alteration in the absence of the stressor in these muted animals which brings the importance of gene and environment interactions in order to fully understand the pathology of PD [128].

5.9 Gaps in the Literature

Animal studies have been helpful in shedding light on the different components and pathophysiology of PD. However, an adequate animal model of PD has not been developed yet. There is a continuous loss of DA cells in substantia nigra and consequent depletion of DA in the striatum in individuals suffering from PD. Unfortunately, an analogous animal model of PD that shows the same characteristics of the disease does not exist yet.

Most of the evidence regarding PQ neurotoxicity is based on animal models. It is not clear how these results will translate into humans and the effect of PQ on human brain is yet to be investigated. PQ neurotoxicity is a complex trait in which multiple factors and probably multiple genes are involved; using animals models with known genetic constitution such as BXD recombinant inbred mice will allow researchers to dissect multiple aspect of the trait; using BXD RI mice will help researchers to identify polymorphisms and candidate genes involved in the complex trait of PQ neurotoxicity. The result of these studies will shed light on potential underlying genes concerning PQ neurotoxicity in humans as mouse and human genomes are 90% syntenic.
Multiple studies have reported the loss of DA cells in substantia nigra due to PQ toxicity but almost none of them observed DA depletion in the striatum, which is an important hallmark of PD. Compensatory increase in tyrosine hydroxylase activity in the striatum, is one of the hypotheses being proposed to explain this discrepancy (Tieu K. 2011). More research is warranted to investigate possible explanations and mechanisms by which paraquat induces its toxicity without decreasing the DA content in the striatum.

5.10 Future Directions

PD is the second most common neurodegenerative disease in the whole world with almost 1% of the population suffering from the consequences of the disease. Any effort in trying to understand the mechanisms and etiology of PD using the right animal models and the right dosage of different toxins may help researchers to unravel the pathophysiology of the disease. Understanding the etiology and pathophysiology of the disease will have a huge impact on society, economy and also the quality of life of all individuals suffering from the disease.

Further research is warranted in regard to examining the effects of PQ on the DA levels of the striatum. Longitudinal studied are needed especially in areas where PQ exposure is high. PD is a complex and multifactorial disease; most of the evidence regarding PQ neurotoxicity is based on animal models. It is not clear how these results will translate into humans and the effect of PQ on human brain is yet to be investigated. PQ neurotoxicity is a complex trait in which multiple factors and probably multiple genes are involved. Using animals models with known genetic constitution such as BXD recombinant inbred mice will allow researchers to dissect multiple aspect of the trait. Using BXD RI mice will help researchers to identify polymorphisms and candidate genes involved in PQ neurotoxicity. The result of these studies will shed light on potential underlying genes concerning PQ neurotoxicity in humans as mouse and human genomes are 90% syntenic. Identifying candidate genes will have a great impact on understanding the etiology of multiple neurodegenerative diseases such as PD. Additionally, identifying the susceptible genotypes and populations will result in the development of more efficient interventions. Proper interventions can be implemented to prevent exposure to PQ and other risk factors especially for the high risk populations, using the right
animal and epidemiological data. This can also be public health/policy oriented with the fact that we may have to stop using PQ as a pesticide which may need government involvement.
Chapter 6

Is Paraquat a Dopaminergic Neurotoxicant? An Experiment on Paraquat Neurotoxicity in BXD Mice

6.1 Introduction

The herbicide paraquat (PQ) has long been under scrutiny of being involved in the development of neurodegenerative diseases such as PD. The association between PQ and increased risk for PD has been repeatedly mentioned in both epidemiological and experimental studies [26, 129, 130]. Most of the animal studies have shown the neurotoxic effects of paraquat by its involvement in dopaminergic cell degeneration in the substantia nigra, however striatal dopamine depletion, a hallmark of PD and a feature of dopaminergic toxicity, is not always observed in the studies investigating PQ neurotoxicity.

In the previous chapters we proposed using BXD RI mice as an invaluable tool to address the importance of host susceptibility to neurotoxicants. We used 9-10 strains of BXD RI mice to investigate the role of individual differences in the severity of MPTP neurotoxicity. As a follow up to our previous experiments, in this chapter we first examine PQ neurotoxicity in nineteen strains of BXD RI mice, using striatal DA and DA related phenotypes as indices for neurotoxicity. Subsequently, we investigate response variation to PQ neurotoxicity across 19 strains of BXD RI
6.2 Materials and methods

6.2.1 Animals

Nineteen BXD inbred strains of mice, male and female, obtained from our vivarium and from the University of Tennessee Health Sciences Center, were used in this study. Two weeks prior to paraquat treatment, animals were transferred to the animal center at The Pennsylvania State University. The mice were maintained under a 12:12 hour light dark cycle controlled temperature (21 ± 2 °C) and humidity (40%). Food (standard Purina rodent diet) and water were provided ad libitum. All experimental protocols were approved by the Pennsylvania State University Institutional Animal Care and were conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. Only 10 strains of BXD RI mice were used to measure striatal TH and GFAP concentrations.

6.2.2 PQ Treatment

19 male and female BXD RI strains of mice between the ages of 3-7 month were used in the study. Mice had three weekly i.p. injections of 10mg/kg of paraquat and were sacrificed by CO2 inhalation one week after the last injection to assess the prolonged effect of PQ. PQ has a long brain half life of almost 24 days, therefore repeated PQ treatment results in accumulation of PQ in the brain needed to induce toxicity as PQ cannot pass the BBB easily. Brains were dissected and striatum and ventral midbrain were isolated for biochemical assays. Paraquat dihydrochloride trihydrate was purchased from Sigma chemicals, St. Louis, MO and paraquat solution was freshly made each week.

6.2.3 Biochemical Assays

The striatum from one side of the brain was used to measure dopamine and its metabolites, dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (HVA), serotonin (5-HT) and its metabolite, 5-hydroxyindoleacetic acid (5-HIAA), using high performance liquid chromatography (HPLC), with analyte concentration
normalized to the tissue weight. To prepare the samples for the biochemical assays, first the homogenizing buffer containing protease inhibitor was added to the samples; for every gram of tissue 10 ml of homogenizing buffer was added to the samples. The samples were then homogenized with 10 up and down strokes and then sonicated (Knobes Micro Ultrasonicator/cell disrupter) on ice using a 30-s pulse sonicator. Fifty µl of 0.2 M perchloric acid, and 10µl 3,4-dihydroxybenzylamine 1µM as internal standard were added to the samples. The samples were centrifuged at 14000×g rpm at 4°C for 3 minutes and the supernatant was transferred into Costar 0.22µm filter tubes and centrifuged again for 1 minute at 14000×g rpm at 4°C. The supernatant was transferred into HPLC autosampler tubes and used for the assays. The other side of the brain was used to measure the concentration of Tyrosine hydroxylase (TH) and glial fibrillary acidic protein (GFAP). The concentrations of TH and GFAP in the SDS-total homogenates were analyzed by ELISA (O’Callaghan, 1991, O’Callaghan, 2002 and Sriram et al., 2004). Values obtained were normalized to total homogenate protein assayed by BCA [65].

6.2.4 Data Analysis

Two between-subject factors (strain and treatment) analysis of variance (ANOVA) was used to analyze the data. Pairwise comparisons were made using Tukey HSD test with α set at 0.05. SAS statistical package was used to analyze the data.

6.3 Results

6.3.1 Effects of PQ on Dopamine-neurochemistry in the striatum

ANOVA revealed no significant main effect for treatment. PQ did not change striatal dopamine concentration. Significant differences in basal levels of dopamine were observed with strain 87 and 89 having the lowest DA concentration and strain 69 having the highest DA concentration (Fig. 6.1). The interaction between strain and treatment was not significant.

PQ treatment did not significantly change the concentration of dopamine metabolite, DOPAC. However there was a main effect for strain; basal levels of
Figure 6.1: Effect of paraquat (PQ) on dopamine (DA) concentration in the caudate-putamen in 19 BXD recombinant inbred mouse strains. Male and female mice were treated with three weekly injections of 10 mg/kg of PQ (vs. saline) and sacrificed seven days after the last injection. DA was assayed from tissue homogenates, using HPLC. Experimental and control values, normalized to tissue weights, are expressed as means ± s.e.m. ANOVA revealed no significant main effect for treatment ($F_{1.90}<1; p> 0.05$).

DOPAC showed significant variation with strains 51 and 69 showing the lowest and highest striatal DOPAC concentration respectively. The strain treatment interaction was not significant(Fig. 6.2).
Figure 6.2: Effect of paraquat (PQ) on dopamine metabolite, DOPAC, concentration in the caudate-putamen in 19 BXD recombinant inbred mouse strains. Male and female mice were treated with three weekly injections of 10 mg/kg of PQ (vs. saline) and sacrificed seven days after the last injection. DOPAC was analyzed from tissue homogenates, using HPLC. Experimental and control values, normalized to tissue weights, are expressed as means ± s.e.m. ANOVA revealed no significant main effect of treatment ($F_{1,88}<1$; $p>0.05$).

Striatal HVA concentration was not significantly affected by PQ treatment and ANOVA revealed no main effects for treatment and the interaction between treatment and strain. Significant differences in the basal levels of HVA were observed with strains 60 and 86 having the lowest and highest striatal HVA concentration (Fig. 6.3).
Figure 6.3: Effect of paraquat (PQ) on dopamine metabolite, HVA, concentration in the caudate-putamen in 19 BXD recombinant inbred mouse strains. Male and female mice were treated with three weekly injections of 10 mg/kg of PQ (vs. saline) and sacrificed seven days after the last injection. HVA was analyzed from tissue homogenates, using HPLC. Experimental and control values, normalized to tissue weights, are expressed as means ± s.e.m. ANOVA revealed no significant main effect of treatment ($F_{1,90} < 1; \ p > 0.05$).

### 6.3.2 Effect of PQ on Serotonin-neurochemistry

ANOVA revealed no significant main effect for treatment on the concentration of serotonin (5-HT) in the striatum of BXD RI mice. Significant differences in the basal levels of 5-HT were observed with strains 100 and 32 having the lowest and highest 5-HT concentrations in the striatum, respectively (Fig. 6.4).
Figure 6.4: Effect of paraquat (PQ) on serotonin (5-HT), concentration in the caudate-putamen in 19 BXD recombinant inbred mouse strains. Male and female mice were treated with three weekly injections of 10 mg/kg of PQ (vs. saline) and sacrificed seven days after the last injection. 5-HT was analyzed from tissue homogenates, using HPLC. Experimental and control values, normalized to tissue weights, are expressed as means ± s.e.m. ANOVA revealed no significant main effect of treatment ($F_{1,90} = 3.03; p > 0.05$).

ANOVA revealed no significant effect of PQ on the striatal concentration of the serotonin metabolite 5-HIAA. Significant differences in the basal levels of 5-HIAA were observed with strains 60 and 80 having the lowest and highest striatal 5-HIAA concentration, respectively (Fig. 6.5).
Figure 6.5: Effect of paraquat (PQ) on serotonin (5-HIAA), concentration in the caudate-putamen in 19 BXD recombinant inbred mouse strains. Male and female mice were treated with three weekly injections of 10 mg/kg of PQ (vs. saline) and sacrificed seven days after the last injection. 5-HIAA was analyzed from tissue homogenates, using HPLC. Experimental and control values, normalized to tissue weights, are expressed as means ±s.e.m. ANOVA revealed no significant main effect of treatment ($F_{1,90}<1; p> 0.05$).

6.3.3 Effect of PQ on Tyrosine Hydroxylase in the striatum

The effect of PQ was examined on only 10 strains of the 19 BXD RI mice and ANOVA revealed no significant main effect for treatment. Significant differences in the basal level of striatal TH was detected in the 9 strains of BXD RI mice. Strains 77 and 24 showed the lowest and highest TH concentration in the striatum, respectively (Fig. 6.6).
Figure 6.6: Effect of paraquat (PQ) on Tyrosine Hydroxylase (TH) concentration in the caudate-putamen in 10 BXD recombinant inbred mouse strains. Male and female mice were treated with three weekly injections of 10 mg/kg of PQ (vs. saline) and sacrificed seven days after the last injection. TH was analyzed from tissue homogenates, using ELISA. Experimental and control values, normalized to total protein are expressed as means ± s.e.m. ANOVA revealed no significant main effect of treatment ($F_{1,67}<1$; $p>0.05$).

### 6.3.4 Effects of PQ on GFAP in the striatum

PQ treatment did not affect the levels of striatal GFAP, which is an intermediate protein used as a marker for astrocyte toxicity. Significant differences in the basal level of striatal GFAP was detected with strains 86 and 77 having the lowest and highest levels of GFAP, respectively (Fig. 6.7).
Figure 6.7: Effect of paraquat (PQ) on glial fibrillary acidic protein, a marker of astrocyte toxicity, concentration in the caudate-putamen in 10 BXD recombinant inbred mouse strains. Male and female mice were treated with three weekly injections of 10 mg/kg of PQ (vs. saline) and sacrificed 7 days after the last injection. TH was analyzed from tissue homogenates by ELISA. Experimental and control values, normalized to total protein are expressed as means ± s.e.m. ANOVA revealed no significant main effect of treatment ($F_{1,67}<1; p>0.05$).

6.4 Discussion

In the current study, we investigated PQ neurotoxicity on the nigrostriatal pathway, using striatal dopamine concentration and its metabolites as indices for PQ neurotoxicity. Our results did not show any significant effect of PQ on the concentration of DA-related phenotypes in the striatum. There was no significant change in the concentration of striatal TH. Elevation in GFAP concentration, a marker of astrocyte toxicity, was not detected in any of the 10 out of 19 strains of BXD RI mice used for the measurement.

The association between PQ toxicity and increased risk for PD has been reported in multiple epidemiological studies [23, 24, 26]. PQ is the only pesticides for which a dose-dependent relationship between its cumulative exposure and increased risk of PD has been reported [23]. Despite all of the supporting evidence linking PQ neurotoxicity to PD in epidemiological studies, the result of animal studies examining the neurotoxic effects of PQ are not consistent; although intranigral injection of PQ to male Wistar rats has been shown to have neurochemical, neuropathological and behavioral effects consistent with PD [32], systemic use of PQ in most of the
animal studies does not show the desirable consistency with PD neuropathological features; striatal dopamine depletion is not observed in most of the studies with systemic use of PQ even in the presence of nigrostriatal dopaminergic damage [39]. Moreover, there are numerous preclinical studies in which adequate supporting evidence regarding neuropathological and neurochemical damages due to PQ exposure is missing [33, 131]. The result of these studies are in contrast with at least one study conducted by Brooks and colleagues in which significant neurochemical and neuropathological effects of PQ on dopaminergic cells of substantia nigra were observed [40].

6.4.1 Explaining the Inconsistencies

6.4.1.1 PQ Penetration into the Brain

The structural similarities between MPP\(^+\), the active metabolite of MPTP, and PQ has been used as supporting evidence for the same neurotoxical effect suggesting a shared mechanism for toxicity and a common path for brain penetration between these two chemicals. MPTP easily crosses the BBB and reaches the microglia cells where it is metabolized to MPP\(^+\), and taken up by DAT into the dopaminergic cells, inducing its toxicity and damaging the nigrostriatal pathway. PQ on the other hand, is a highly polar chemical, which prevents it to efficiently cross the BBB via similar mechanism to MPTP [132]. There is evidence supporting the active passage of PQ through the BBB by the use of neutral amino acid transporter system into the brain and into the dopaminergic cells where PQ is believed to induce neurotoxicity in young rats [34]. Research has shown that PQ brain penetration is an age dependent phenomenon with neonatal rats (less than two weeks old) and old rats (between 12 to 24 months) having higher PQ concentrations in the brain 24 hours post-injection [131]. It has also been shown that neonatal exposure to PQ may permanently change the nigrostriatal structure, resulting in reduced striatal DA content later in life [133]. In the current study we did not measure the brain concentration of PQ to confirm that enough PQ is sequestered in the brain to induce dopaminergic toxicity. Measuring PQ concentration in the brain in future studies will help researchers to confirm or dispute the potential neurotoxicity of PQ on dopaminergic cells.
6.4.1.2 Mechanism of Action of PQ

Oxidative stress is the mechanism by which both PQ and MPTP induce neurotoxicity, however the biochemical steps leading to oxidative stress differs substantially between these two chemicals. Research has shown that MPP$^+$ induces oxidative stress by mitochondrial respiratory inhibition via the suppression of complex 1 of the electron chain transport, producing high concentrations of free radicals, subsequently inducing oxidative stress. PQ is not an efficient mitochondrial complex 1 inhibitor and induces its toxicity by cellular redox cycling in microglia, using NADPH as an electron donor [134]. The important question that needs to be addressed in future studies is whether there is enough PQ accumulation in dopaminergic cells of the substantia nigra to induce lethal oxidative stress that leads to dopaminergic cells degeneration and subsequent striatal dopamine depletion.

6.4.1.3 Host Susceptibility to PQ

Genetic background of the samples used in the study is an important factor affecting the results. Taking into account the genetic profile and the role it might play in the severity of PQ toxicity is an important issue which needs to be addressed. The importance of host susceptibility to environmental neurotoxicants has been shown in a work conducted by Goldman and colleagues [47]; people with homozygote mutations of glutathione S-transferase theta gene exposed to PQ have an odds ratio (OR) of 11.1 for developing PD compared to OR of 1.5 in people exposed to PQ who were not carrying the mutations. The importance of genetic constitution regarding PQ toxicity has been also shown in animal studies. For example B6 mice show higher dopaminergic neurodegeneration in the substantia nigra induced by PQ treatment compared to D2 mice [37]. Future studies with larger number of animals per strain, accompanied by large number of BXD RI strains will increase the power of the study and enable researchers to investigate the role of genetic composition in environmental neurotoxicity.

6.4.2 Conclusion

The result of the current study is consistent with the majority of the animal studies concerning PQ neurotoxicity in which striatal dopamine depletion is not detected even after DA degeneration in the substantia nigra. Evidence regarding PQ toxicity
on nigrostriatal pathway is controversial; striatal DA depletion is not detected in most of these studies even after a significant damage to the dopaminergic cells in the substantial nigra. Further animal studies with prolonged PQ exposure are warranted as there is evidence in which long PQ exposure for more than 30 injections has resulted in dopaminergic degeneration in the substantia nigra and subsequent striatal DA depletion [135]. More studies with longer exposure to PQ using different age groups of BXD RI mice may shed light on the existing ambiguity concerning the neuropathological and neurochemical effects of PQ on nigrostriatal pathway.

Additionally, evidence regarding the effect of PQ on nigrostriatal pathway is lacking in the epidemiological studies. Most of the epidemiological studies support the association between pesticide exposure and PD, however, no direct evidence is provided that suggests chronic pesticide exposure changes nigrostriatal pathway in humans. The use of imaging techniques to detect micro structural changes in the substantial nigra humans in some of these epidemiological studies may be the first step to detect a proper in vivo biomarker for people that may develop PD in near future [136].
7.1 Abstract

Heavy metals, various pesticide and herbicides are implicated as risk factors for human health. Paraquat, maneb, and rotenone, carbamate, and organophosphorous insecticides are examples of toxicants for which acute and chronic exposure are associated with multiple neurological disorders including Parkinson’s disease. Nevertheless, the role of pesticide exposure in neurodegenerative diseases is not clear-cut, as there are inconsistencies in both the epidemiological and preclinical research. The aim of this short review is to show that at least some of the inconsistencies are related to individual differences in susceptibility to the effects of neurotoxicants, individual differences that can be traced to the genetic constitution of the individuals and animals studies, i.e., host-based susceptibility.

7.2 Epidemiological Studies

For at least two major neurodegenerative diseases, Alzheimer’s and Parkinson’s, the age of onset suggests different mechanisms at play. Early onset disease (i.e., prior to age 50) is seen as less frequent than late onset and can often be tied to specific genetic factors [82]. The etiology of later onset disease is less clear and very
likely a result of genes interacting with the environment. Parkinson’s disease (PD) is characterized by a loss of dopamine (DA) neurons in the substantia nigra pars compacta and subsequent loss of DA function in the projection area, namely the striatum.

A number of researchers have reported the association between exposure to insecticides and herbicides as risk factors for developing PD [23, 26, 27, 72, 73]. In a meta-analysis of 19 studies, Priyadarshi et al. (2000) reported an association between high pesticide use and increased risk for PD with combined odds ratio of 2.15 among farmers, people living close to farms, and those exposed to farm animals [11]. Additionally, in a review of 38 case-control studies, Brown et al. (2006) showed a robust relationship between long-term pesticide use and increased risk for developing PD [104].

Epidemiological studies are problematic in that most of the subjects have been exposed to more than one agent, assessment of chronic exposure is based on recall and most such studies do not identify sub-populations that are at differential risk. Nevertheless on the first count, one pesticide, paraquat, an herbicide, is a major target of study. For example, Liou et al. (1997) showed chronic exposure to paraquat to be associated with increased risk for PD [23]. Moreover, individuals who are exposed to paraquat are at higher risk for developing PD compared to other herbicides and pesticides [27, 75, 105, 107]. Numerous case-control studies show a significant association between the extent of exposure to paraquat and the severity of the disease [108].

Results of both clinical and epidemiological studies concerning environmental toxicants are inconsistent; not all of the epidemiological studies support the contribution of the same toxicants in PD [74–76]. Gatto et al. (2009) reported that the increased risk for PD was associated not specifically to a single pesticide, but rather to a combination of several pesticides including organophosphorus compounds. In a case-control study conducted by Firestone et al. (2010), no association was found between exposure to industrial toxicants and risk for PD.

Some of the inconsistencies may derive from duration of exposure, diagnostic criteria, bias in case-control subject selection, and lack of control for other confounding factors [137, 138].

Although epidemiological studies are important tools for determining risk, they can be limited by failing to take into account the role of individual differences
reflected in sub-populations. Identifying sub-populations at different genetic-based risk is one way to improve the study design. Identifying individuals carrying such genotypes is challenging, but possible. One example involves polymorphisms cytochrome P450D6 (CYP2D6) [45,109]. CYP2D6 is involved in the metabolism of several drugs and toxicants, including insecticides and herbicides. One allele, CYP2D6∗4 is implicated in relatively slow metabolism of several pesticides, as an autosomal recessive trait. About 5-10% of white populations are homozygous for the allele and for them the enzyme activity is practically undetectable. Elbaz et al. (2004) revealed a twofold increase in risk for PD for those who were homozygous for the CYP2D6∗4 allele (i.e., poor metabolizers) and who were exposed to pesticides. The study population included farmers or people who used pesticides frequently for gardening. Alternatively, normal metabolizers exposed to pesticides showed a slight increase in risk for PD compared to poor metabolizers not exposed to pesticides. These results highlight the importance of gene-environment interactions relevant to neurotoxicology.

Additional evidence to support the importance of host susceptibility is provided by the results reported by Goldman et al. (2012) [47]. Glutathione S-transferases (GSTM1, GSTT1) are enzymes involved in detoxification of numerous agents in multiple tissues of the human body including, liver, gut, and brain. These enzymes protect different cells of the body against the consequences of oxidative stress induced by multiple bio-reactions and also PD [46,114]. Goldman et al. (2012) showed that individuals homozygous for GSTT1∗0 and exposed to paraquat had an odds ratio for PD risk of 11.1 compared to people with GSTT1 and exposed to paraquat with an OR of 1.5. No additional risk for GSTM1 or GSTM1∗0 and exposure to PQ was reported in the study. Genetic background of individuals exposed to pesticides has a significant effect on the severity of pesticide toxicity, making it an important influential factor that needs to be addressed in the studies concerning the effects of these chemicals on human health.

### 7.3 Genome-Wide Association Studies

Another approach to understanding individual differences in disease and that might have appeal here is the genome-wide association study approach, or GWAS. This approach compares 100s of 1000s or more polymorphic genomic markers in large
samples humans with variable phenotypes. This approach has been particularly useful for identifying genetic underpinnings of complex diseases such as restless leg syndrome [139], and familial PD [140]. Application of GWAS to toxicology can be illustrated by the work of Pierce et al. (2012) [141]. Arsenic contamination of water and soil has been a long-standing problem in Bangladesh and Pierce et al., reported increased signs of differential sensitivity to As poisoning (skin lesions) associated with polymorphisms in arsenite methyltransferase (As3MT) one gene known to code for a protein involved in arsenic metabolism. A nearby gene on the same chromosome indicated by the same study may in fact be a gene that regulates the expression of As3MT. Whether GWAS is a useful approach to the study of individual differences in response to other environmental toxicants remains to be seen. As stated by Zhou and Pearson (2013), GWAS applied to adverse drug reactions (and likely toxicology) may be problematic because of usually small sample sizes and also based on their observation that allelic variants associated with drug responses tend to be quite a bit fewer in number than allelic variants associated with common diseases [142]. The success of the Pierce et al., study is probably attributed to the rather large sample size, the widespread arsenic contamination and the involvement of one or more major genes. Thus, large samples, widespread exposure, well-defined phenotypes, and genes that influence the affected phenotype are important for GWAS studies. This also defines the limitations of GWAS studies in toxicogenetics as many of the effects are polygenic with small additive effects from each of the genes.

While including susceptible subpopulations in epidemiological studies is one way to refine our understanding of individual differences in susceptibility to toxicants, the underlying mechanisms are oftentimes difficult to assess. Complementary to epidemiological studies, animal models can help to elucidate the basis for genetic-based individual differences in susceptibility.

7.4 Animal Models in Toxicogenetics-Two Complementary Approaches

Genetic modification is used to create research animals either lacking in function or amplified function in one or more genes. Sometimes, the relevant phenotype
is known and sometimes left for discovery. Focusing on the gene initially is often called reverse genetic analysis. Alternatively, genetic analysis can focus on specific, well-defined phenotypes initially and then to a search for relevant genes. This is often termed, “forward genetic analysis”. An elegant description of both may be found in Alonso and Ecker (2006) [143].

7.4.1 Forward Genetic Analysis of Toxicity as a Complex Trait

Findings from epidemiological studies would implicate many if not most effects of environmental toxicants to be complex traits, i.e., effects influenced by several genes and their interaction with the environment. For example, consider our findings from on the effects of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) on striatal DA in BXD recombinant inbred mice presented in chapter 2 (Fig. 7.1). These 10 inbred strains (from among more than 100 such strains) were derived from C57BL/6J (B) and DBA/2J (D) parental inbred strains. F1 hybrid mice from these two strains were bred inter se to produce families that were inbred brother to sister for 20 or more generations in order to recombine and fix alleles. Allelic differences between the two parental strains are now distributed throughout these new strains, the BXD recombinant inbred strains [77].
Figure 7.1: This is the same figure presented in chapter 2 (Fig 2.2). Effect of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) on dopamine (DA) concentration in the caudate-putamen in 10 BXD recombinant inbred mouse strains. Male mice were injected with 12.5 mg/kg of the pro-neurotoxin, MPTP (vs. saline) and sacrificed 48 h later. DA was assayed from tissue homogenates by HPLC. Experimental and control values (upper panel), normalized to tissue weights, are expressed as means ± s.e.m. The lower panel presents the effect of MPTP expressed as % control. ANOVA revealed a significant main effect of strain, MPTP and a significant interaction (F$_{9,77}$ = 11.25; F$_{1,77}$ = 445.61; F$_{9,77}$ = 9.05, respectively; all p < 0.001).

The top panel of Figure 7.1 illustrates striatal DA concentrations in 10 BXD strains injected with saline (control) or 12.5 mg kg$^{-1}$ MPTP (s.c.) and the bottom panel illustrates the effect of MPTP on DA loss, expressed as percent of control. As can be seen in the left panel, there is about a 1.5 fold difference in DA concentration among saline-treated animals between the highest value (BXD 9) and the lowest (BXD 29). In the right panel, we see the extent of DA loss to be highly variable among the strains with BXD 40 being the most sensitive strain and BXD 29 being
MPTP neurotoxicity is achieved through its metabolism to 1-methyl-4-phenylpyridinium MPP$^+$ by astrocytes. MPP$^+$ is then taken up into neurons by the DA transporter where it then causes destruction of those neurons. Knowing that the production of MPP$^+$ is crucial to neurological destruction, we asked the question as to whether the extent of DA loss in the striatum is directly related to the amount of MPP$^+$ produced. Comparing the mean loss of DA with the mean production of MPP$^+$ the correlation was -0.15 and not significant at 7 degrees of freedom [82]. This illustrates one of the advantages of forward genetic analysis using a systems perspective.

The continuous variation in DA and DA loss from MPTP treatment shows that MPTP toxicity is polygenic, thus producing individual differences in susceptibility. Had there been only one significant gene, the results would have lined up against each allele from the two progenitor strains -i.e., shown Mendelian trait characteristic. Also, forward genetic analysis allowed us to compare several MPTP-related phenotypes across the same strains so that we could get a view of MPTP toxicity from a systems biology perspective [77]. Finally, using forward genetic analysis, we can compare our phenotypes against polymorphic markers in the mouse genome and also with gene expression in various tissues. Finally, forward genetic analysis is particularly well-suited for discovery of genes underlying complex phenotypes.

Recombinant inbred mouse strains are particularly useful in forward genetic analysis, and outbred stocks are valuable as well. The new resource available now is the Collaborative Cross which promises to deliver several hundred recombinant inbred strains derived from eight strains including wild-derived stocks [144]. These strains present a distinct advantage over extant recombinant strains by capturing more of the genetic variation found in mouse populations, compared to those recombinant strains derived from two inbred strains. The goal is to genotype all of these strains and to conduct and publish omnibus gene expression profiles in multiple tissues. Another resource, the Diversity Outbred mouse population is derived from the same eight strains, but not inbred [145]. The Collaborative Cross provides a unique platform for systems genetic analysis of complex traits and the Diversity Outbred offers precise mapping of complex traits.
7.4.2 What about Reverse Genetic Analysis

The distinction between forward and reverse genetics is somewhat arbitrary, but useful in focusing attention on what to examine first, phenotypes or genes. Gene modification can be particularly useful when working with well-established biochemical pathways and whose genes are known. A recent study by Choi et al. (2010) showed that repression of the gene that produces c-Jun-N-terminal kinase three reduced paraquat-and rotenone-related destruction of DA neurons—an example of specific gene targeting [146]. A recent review by Eastmond et al. (2013) presents evidence in the toxicogenetics of cancer that the use of genetically modified organisms may not be an efficient method for the detection of carcinogenic toxicants. We propose that genetically modified organisms may indeed be employed in the verification of candidate genes nominated via forward genetic analysis [147].

7.5 Putting it All Together

For the most part, we may consider responses to toxicants as complex traits; that is to say, for most individuals, targeted phenotypes are under the influence of multiple genes interacting with each other and with the environment. This means that there are possibly many genes with small and potentially additive effects involved in toxicity. Less commonly, we might expect to identify individuals who show a large effect produced by a rare genetic variant. Polygene identification in the former leads to difficulty in understanding which genes do what, relative to the phenotype, and in the latter case, sampling may miss those carrying the rare variant altogether. GWAS studies and forward genetics studies in animals can be complementary and informative. For example, Winkelmann et al. (2007) and Stefansson et al. (2007) each reported GWAS studies of individuals with restless legs syndrome and periodic limb movements that identified associated genetic markers near BTBD9 gene in humans [139,148]. Restless legs syndrome and periodic limb movements are associated with low iron in the substantia nigra and related DA dysfunction. When we conducted a study of iron concentration in ventral midbrain of mice [149], we noticed a weak QTL near Btbd9 in the mouse genome and remarked on this in a subsequent article [150]. DeAndrade et al. (2012) were able to produce mice with mutations in Btbd9 similar to those seen in humans and observed decreased
iron, sleep disturbances and abnormal movements similar to human RLS. In this case the mouse researchers were able to capitalize on findings from GWAS, partly confirm through forward genetics and then finally target the gene for manipulation and eventual identification of underlying mechanism [151].

7.6 General Comments Concerning Rodents in Toxicology

There are a number of valid criticisms about in vivo assessment of toxicants. Of utmost importance is whether the studies in animals provide useful information concerning humans. A recent article outlined the advantages and drawbacks of the two-year bioassay (standardized testing as developed more than 40 years ago) of proposed carcinogens in rodents [152]. In fact, the Marone article joins a number of others expressing some dissatisfaction with the assay, with criticisms including time, large numbers of animals, often single-endpoints without concern for the rest of the biological system and finally cost-effectiveness concerning informing human carcinogenesis. These problems lead some to question the value of animal studies in this effort altogether. Among ethical concerns about animal research in general and toxicology specifically, is the effort to refine, reduce and to replace (the three Rs). We propose that the use of genetically diverse animals (genetic reference populations of rats and mice) for initial screening for differences in response to toxicants can identify genes and biochemical pathways underlying the differences. Follow-up genetic manipulation studies can offer proof-of concept and a mechanistic explanation. This approach has the potential to refine methods and therefore reduce animal numbers. Moreover, a systems study of toxicity in rodents can further elucidate the impact of toxicant exposure. In our recent MPTP study, we performed principal components analysis on a number of DA-related measures and then were able to relate the composite index to a network of co-expressed genes and possible involved biochemical pathways [77].
7.7 Conclusion

Now that people are living longer, numerous chronic diseases that would be considered to be rare in earlier times are becoming more common. The remarkable increase in life expectancy over the past 100 years accompanied by longer exposures to environmental toxicants underscore the importance of toxicological research. Better identification of host characteristics in epidemiological and GWAS studies that affect toxicity to specific agents, coupled with carefully planned experiments in genetic reference populations in animals can lead to better prediction of individuals at risk and may even facilitate better prevention and treatment post exposure [153].
Chapter 8  Conclusion

Heavy metals, and pesticides herbicides are suspected risk factors for neurological disorders including Parkinson’s disease (PD). Nevertheless, their role is not clear-cut, as there are inconsistencies in epidemiological and preclinical research. One reason may be related to individual differences in susceptibility, differences that can be traced to the genetic constitution of humans and animals so exposed. Taking into account the role of host susceptibility and individual differences in the study of complex traits such as pesticide toxicity will result in better understanding the etiology and pathways involved in the development of complex neurological disorders such as PD. Newer epidemiological and animal methods address the role of genes, the environment and their interaction as key.

BXD RI are invaluable tools used to investigate complex or polygenic traits in which multiple factors, genes and the interaction between gene and environmental stressors are considered key elements in understanding the etiology of such complex traits. In this dissertation we investigated the role of host susceptibility to neurotoxicants, using BXD RI mice as valuable resources, allowing us to study complex traits such as host susceptibility. The result of our study will pave the way for the study of other polygenic traits, such as neurodegenerative disorders, in which multiple genes and environmental factors are involved.

In the first chapter we provided an overall introduction regarding environmental neurotoxicity and its association to the development of numerous neurological disorders including PD. Examples of multiple epidemiological and preclinical studies investigating environmental neurotoxicity and PD were provided. Investigating the mechanism underlying PD as a complex neurodegenerative disease, is a great application of the work presented in this dissertation. Animal models of PD used
in the studies along with the data presented here can be used to pave the way in understanding the etiology of PD in humans. The second chapter begins with empirical studies we conducted on male BXD RI mice to investigate the response variation to MPTP neurotoxicity in 10 strains of BXD mice. A broad range of response variation and individual differences concerning MPTP dopaminergic neurotoxicity in male BXD RI mice were observed.

In chapter three, we presented the result of our second empirical study, a follow up study to our male data, using female BXD RI mice to compare the results of these two studies to detect where they differ and where they converge. Similar to male data, female data showed broad response variation to MPTP neurotoxicity. The correlation between DA related phenotypes including the concentration of DA and its metabolites, DOPAC and HVA, as well as 5-HT and its metabolite 5-HIAA, TH and GFAP were measured. A significant correlation between DA related phenotypes with the exception of DOPAC was observed between males and females, indicating the involvement of multiple factors in dopaminergic neurotoxicity and probably neurological disorders such as PD. Although there are significant similarities concerning host susceptibility and response variation to MPTP toxicity between two sexes, not all the measures have high correlations with each other. Probably numerous factors are involved in determining host susceptibility, indicating the complex nature of our trait of interest.

In chapter four we measured the production of MPP$^+$ in all 10 strains of BXD RI of mice to investigate whether variation in response to MPTP toxicity would be partially explained by the striatal concentration of MPP$^+$, which is the active metabolite of MPTP that induces dopaminergic neurotoxicity. Interestingly, there was no significant correlation between MPP$^+$ production and the extend of MPTP neurotoxicity, which supports the complex nature of MPTP neurotoxicity and host susceptibility.

In chapters five and six we took a step further to investigate host susceptibility to PQ, an environmental toxicant with structural similarities to MPTP which has been linked to increased risk for PD. First, we provided a mini review regarding host susceptibility to PQ neurotoxicity and the association between PQ and PD, in chapter five. In chapter six, we presented our result concerning PQ neurotoxicity, using DA related phenotypes as indices for dopaminergic neurotoxicity. No significant change in striatal DA concentration, and its metabolites, DOPAC, HVA was
detected. TH, the rate limiting enzyme for DA production and GFAP, a marker for astrocyte toxicity, were not affected by PQ treatment. These result show that although PQ is structurally similar to MPP$^+$ the pharmacokinetics and pharmacodynamics of this chemical may substantially differ from MPTP. Additionally, the mechanism of action and the pathways by which PQ induces its toxicity is probably different from MPTP.

In chapter seven, we discussed the inconsistencies present in the field of host susceptibility to neurotoxicants and used evidence from our empirical studies to support the existence of host susceptibility as a potential reason explaining the discrepancies.

In our work we have used a systems genetics approach as a valuable tool to address the importance of host susceptibility to environmental neurotoxicants. We see the effects of neurotoxicants as complex traits, in which multiple genes that interact with each other and with the environment are involved. By the use of systems genetics such as BXD RI mice with identified genetic constitution we can provide a comprehensive picture of host susceptibility to neurotoxicants. We can use this information to study complex traits such as complex neurodegenerative diseases and track the underlying mechanisms.

As we have shown here, not all mouse strains and likely not all humans are equally susceptible to MPTP neurotoxicity. The addition of more strains to our work in the future (more than 40 strains) will allow us to map with good precision (2Mb) candidate genes underlying these differences, thus revealing important mechanisms. Gene manipulation studies, using knockout mice, will confirmed the role of the candidate genes obtained from QTL mapping. The systems genetics approach also allows us to investigate the role of other host characteristics, such as sex illustrated here. The approach has also shown us that although MPP$^+$ is the actual agent of toxicity, the extent of damage to the striatum is not quantitatively related to the amount of MPP$^+$ produced across the strains. Again, this shows us that MPTP toxicity and most likely other agent (e.g. paraquat) neurotoxicity are complex traits that involve a number of systems.
Appendix A
Author’s Contribution in chapters 2-7

- Chapter two is a published paper in which we explain the importance of genetic constitution in male BXD RI mice [77]. The data were obtained from our colleagues in NIOSH and the manuscript was written by Dr. Jones. The data analysis and the biochemical assays with the exception of TH and GFAP, is done by myself with additional help and supervision from Dr. Erica Unger.

- Chapter three is a submitted manuscript in which we follow up on the result of the second chapter by comparing MPTP toxicity in male and female BXD RI mice. Data used in chapter three were provided by our colleagues from NIOSH. Data analysis and writing the manuscript was done by myself and edited by Dr. Jones.

- Chapter four is a published paper in which we explained the association between MPTP toxicity and MPP+ production in the striatum [82]. Samples were provided by our colleagues from NIOSH. Biomedical assays were conducted by myself with the help and supervision of Dr. Erica Unger. Data analysis and writing the manuscript was done by Dr. Byron Jones.

- Chapter five is a short review about PQ toxicity and it’s association with PD. This paper is in progress for publication and was presented as part of my comprehensive exam.

- Chapter 6 is an experiment we conducted on BXD RI mice concerning PQ neurotoxicity. The whole experiment was conducted by myself. Data and
biochemical assays were done by myself. Data analysis and writing the manuscript was conducted by myself with Dr. Byron Jones supervision.

- Chapter 7 is a published short review addressing the importance of host susceptibility regarding neurotoxicity [?]. The manuscript was written by myself with the help and supervision of Dr. Byron Jones.
## Appendix B

### ANOVA Tables

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<th>Source</th>
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<th>Sum of Squares (Type III)</th>
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<th>F Value</th>
<th>Pr &gt; F</th>
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**Table B.1:** DA concentration in the striatum, male data

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**Table B.2:** DOPAC concentration in the striatum, male data
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<th>F Value</th>
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<td>3.48</td>
<td>0.0012</td>
</tr>
<tr>
<td>Residual</td>
<td>77</td>
<td>0.75</td>
<td>0.026</td>
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<td></td>
</tr>
</tbody>
</table>

Table B.3: HVA concentration in the striatum, male data

<table>
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<th>F Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strain</td>
<td>9</td>
<td>0.34</td>
<td>0.04</td>
<td>2.27</td>
<td>0.026</td>
</tr>
<tr>
<td>Treatment</td>
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<td>0.37</td>
<td>0.37</td>
<td>21.9</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Strain*Treatment</td>
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<td>0.03</td>
<td>0.04</td>
<td>1.9</td>
<td>0.06</td>
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<tr>
<td>Residual</td>
<td>77</td>
<td>1.29</td>
<td>0.02</td>
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</tr>
</tbody>
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Table B.4: 3-MT concentration in the striatum, male data

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<th>F Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
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<tr>
<td>Strain</td>
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<td>3.19</td>
<td>0.35</td>
<td>9.97</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Treatment</td>
<td>1</td>
<td>6.16</td>
<td>9.16</td>
<td>275.62</td>
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</tr>
<tr>
<td>Strain*Treatment</td>
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<td>1.9</td>
<td>0.2</td>
<td>6.03</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Residual</td>
<td>78</td>
<td>2.77</td>
<td>0.03</td>
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<td></td>
</tr>
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</table>

Table B.5: TH concentration in the striatum, male data

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<th>Mean Square</th>
<th>F Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strain</td>
<td>9</td>
<td>0.6</td>
<td>0.07</td>
<td>6.15</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Treatment</td>
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<td>0.11</td>
<td>0.11</td>
<td>9.64</td>
<td>0.0027</td>
</tr>
<tr>
<td>Strain*Treatment</td>
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<td>0.26</td>
<td>0.03</td>
<td>2.63</td>
<td>0.01</td>
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<tr>
<td>Residual</td>
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Table B.6: 5-HT concentration in the striatum, male data
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<th>F Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strain</td>
<td>9</td>
<td>65.68</td>
<td>7.29</td>
<td>11.01</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Treatment</td>
<td>1</td>
<td>1.79</td>
<td>1.79</td>
<td>2.7</td>
<td>0.10</td>
</tr>
<tr>
<td>Strain*Treatment</td>
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<td>44.85</td>
<td>4.98</td>
<td>7.52</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Residual</td>
<td>76</td>
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</tr>
</tbody>
</table>

Table B.7: DOPAC/DA % in the striatum, male data

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<th>Mean Square</th>
<th>F Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strain</td>
<td>9</td>
<td>1090.2</td>
<td>121.13</td>
<td>21.94</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Treatment</td>
<td>1</td>
<td>359.66</td>
<td>359.66</td>
<td>65.13</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Strain*Treatment</td>
<td>9</td>
<td>85.28</td>
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<td>15.44</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Residual</td>
<td>77</td>
<td>425.19</td>
<td>5.52</td>
<td></td>
<td></td>
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</table>

Table B.8: HVA/DA % in the striatum, male data

<table>
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<th>Mean Square</th>
<th>F Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strain</td>
<td>9</td>
<td>543.56</td>
<td>60.39</td>
<td>4.93</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Treatment</td>
<td>1</td>
<td>2.06</td>
<td>2.06</td>
<td>0.17</td>
<td>0.6</td>
</tr>
<tr>
<td>Strain*Treatment</td>
<td>9</td>
<td>501.88</td>
<td>55.76</td>
<td>4.55</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Residual</td>
<td>77</td>
<td>944.21</td>
<td>12.26</td>
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<td></td>
</tr>
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Table B.9: Fe concentration in the Ventral Midbrain, male data

<table>
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<th>F Value</th>
<th>Pr &gt; F</th>
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</thead>
<tbody>
<tr>
<td>Strain</td>
<td>9</td>
<td>0.60</td>
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<td>9.76</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Treatment</td>
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<td>145.80</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Strain*Treatment</td>
<td>9</td>
<td>0.31</td>
<td>0.03</td>
<td>5.08</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Residual</td>
<td>78</td>
<td>0.53</td>
<td>0.01</td>
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Table B.10: GFAP concentration in the striatum, male data
<table>
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<th>Mean Square</th>
<th>F Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strain</td>
<td>8</td>
<td>500.55</td>
<td>62.57</td>
<td>17.21</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Sex</td>
<td>1</td>
<td>28.26</td>
<td>28.26</td>
<td>7.77</td>
<td>0.006</td>
</tr>
<tr>
<td>Treatment</td>
<td>1</td>
<td>3089.96</td>
<td>3089.96</td>
<td>849.86</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Strain*Sex</td>
<td>8</td>
<td>159.69</td>
<td>19.96</td>
<td>5.49</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Strain*Treatment</td>
<td>8</td>
<td>505.27</td>
<td>63.16</td>
<td>117.37</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Sex*Treatment</td>
<td>1</td>
<td>11.17</td>
<td>11.17</td>
<td>3.07</td>
<td>0.08</td>
</tr>
<tr>
<td>Strain<em>Sex</em>Treatment</td>
<td>8</td>
<td>63.88</td>
<td>7.98</td>
<td>2.2</td>
<td>0.031</td>
</tr>
<tr>
<td>Residual</td>
<td>140</td>
<td>509.02</td>
<td>3.64</td>
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Table B.11: DA concentration in the striatum, sexes combined data

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<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strain</td>
<td>8</td>
<td>8.33</td>
<td>1.04</td>
<td>42.42</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Sex</td>
<td>1</td>
<td>3.09</td>
<td>3.09</td>
<td>125.81</td>
<td>0.006</td>
</tr>
<tr>
<td>Treatment</td>
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<td>3.49</td>
<td>3.49</td>
<td>142.14</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Strain*Sex</td>
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<td>0.6</td>
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<td>0.08</td>
<td>3.07</td>
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</tr>
<tr>
<td>Strain<em>Sex</em>Treatment</td>
<td>8</td>
<td>0.39</td>
<td>0.05</td>
<td>1.99</td>
<td>0.031</td>
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<tr>
<td>Residual</td>
<td>139</td>
<td>3.41</td>
<td>0.03</td>
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Table B.12: DOPAC concentration in the striatum, sexes combined data
<table>
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<th>Mean Square</th>
<th>F Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strain</td>
<td>8</td>
<td>1.67</td>
<td>0.21</td>
<td>11.49</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Sex</td>
<td>1</td>
<td>0.0035</td>
<td>0.0035</td>
<td>0.19</td>
<td>0.6</td>
</tr>
<tr>
<td>Treatment</td>
<td>1</td>
<td>2.02</td>
<td>2.02</td>
<td>111.25</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Strain*Sex</td>
<td>8</td>
<td>0.13</td>
<td>0.02</td>
<td>0.93</td>
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<td>Strain*Treatment</td>
<td>8</td>
<td>0.97</td>
<td>0.012</td>
<td>6.7</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Sex*Treatment</td>
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<td>0.0007</td>
<td>0.04</td>
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</tr>
<tr>
<td>Strain<em>Sex</em>Treatment</td>
<td>8</td>
<td>0.25</td>
<td>0.03</td>
<td>1.73</td>
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</tr>
<tr>
<td>Residual</td>
<td>140</td>
<td>2.54</td>
<td>0.02</td>
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Table B.13: HVA concentration in the striatum, sexes combined data

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<th>F Value</th>
<th>Pr &gt; F</th>
</tr>
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<tbody>
<tr>
<td>Strain</td>
<td>8</td>
<td>433.7</td>
<td>54.21</td>
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<td>Sex</td>
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<td>160.92</td>
<td>160.92</td>
<td>13.11</td>
<td>0.0004</td>
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<tr>
<td>Treatment</td>
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<td>85.69</td>
<td>6.98</td>
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<tr>
<td>Strain*Sex</td>
<td>8</td>
<td>354.93</td>
<td>44.37</td>
<td>3.62</td>
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<td>Strain*Treatment</td>
<td>8</td>
<td>154.25</td>
<td>19.28</td>
<td>1.57</td>
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<td>Sex*Treatment</td>
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<td>3.21</td>
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<tr>
<td>Strain<em>Sex</em>Treatment</td>
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<td>14.31</td>
<td>0.03</td>
<td>1.17</td>
<td>0.32</td>
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<td>140</td>
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</table>

Table B.14: DOPAC/DA% in the striatum, sexes combined data
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<th>Mean Square</th>
<th>F Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strain</td>
<td>8</td>
<td>826.43</td>
<td>103.3</td>
<td>24.48</td>
<td>&lt;0.0001</td>
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<tr>
<td>Sex</td>
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<td>36.01</td>
<td>8.53</td>
<td>0.004</td>
</tr>
<tr>
<td>Treatment</td>
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<td>543.99</td>
<td>128.89</td>
<td>6.98</td>
<td>&lt;0.0001</td>
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<tr>
<td>Strain*Sex</td>
<td>8</td>
<td>303.41</td>
<td>37.92</td>
<td>8.99</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Strain*Treatment</td>
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<td>674.43</td>
<td>84.3</td>
<td>19.97</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Sex*Treatment</td>
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<td>19.6</td>
<td>4.64</td>
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</tr>
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<td>8</td>
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<td>&lt;0.0001</td>
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<td>140</td>
<td>590.86</td>
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</table>

Table B.15: HVA/DA% in the striatum, sexes combined data

<table>
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<th>Mean Square</th>
<th>F Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strain</td>
<td>8</td>
<td>3.3</td>
<td>0.41</td>
<td>14.65</td>
<td>&lt;0.0001</td>
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<tr>
<td>Sex</td>
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<td>0.28</td>
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<tr>
<td>Treatment</td>
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<td>17.68</td>
<td>17.68</td>
<td>627.63</td>
<td>&lt;0.0001</td>
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<td>Strain*Sex</td>
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<td>&lt;0.0001</td>
</tr>
<tr>
<td>Strain*Treatment</td>
<td>8</td>
<td>2.22</td>
<td>0.27</td>
<td>9.88</td>
<td>&lt;0.0001</td>
</tr>
<tr>
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<td>0.22</td>
<td>8.12</td>
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<td>8</td>
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<td>0.06</td>
<td>2.28</td>
<td>0.02</td>
</tr>
<tr>
<td>Residual</td>
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<td>3.97</td>
<td>0.02</td>
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Table B.16: TH concentration in the striatum, sexes combined data
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<th>F Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strain</td>
<td>8</td>
<td>1.15</td>
<td>0.14</td>
<td>20.19</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Sex</td>
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<td>9.21</td>
<td>0.00</td>
<td>0.99</td>
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<tr>
<td>Treatment</td>
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<td>243.09</td>
<td>&lt;0.0001</td>
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<tr>
<td>Strain*Sex</td>
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<td>0.00</td>
<td>7.33</td>
<td>0.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Strain*Treatment</td>
<td>8</td>
<td>0.6</td>
<td>0.07</td>
<td>10.65</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Sex*Treatment</td>
<td>1</td>
<td>9.21</td>
<td>9.21</td>
<td>0.00</td>
<td>0.99</td>
</tr>
<tr>
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<td>8</td>
<td>0.0</td>
<td>7.33</td>
<td>0.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Residual</td>
<td>140</td>
<td>0.98</td>
<td>0.01</td>
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<td></td>
</tr>
</tbody>
</table>

Table B.17: GFAP concentration in the striatum, sexes combined data

<table>
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<th>Mean Square</th>
<th>F Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strain</td>
<td>18</td>
<td>17229</td>
<td>957.15</td>
<td>9.48</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Treatment</td>
<td>1</td>
<td>30.69</td>
<td>30.69</td>
<td>0.30</td>
<td>0.6</td>
</tr>
<tr>
<td>Strain*Treatment</td>
<td>18</td>
<td>1177.51</td>
<td>65.4</td>
<td>0.65</td>
<td>0.8</td>
</tr>
<tr>
<td>Residual</td>
<td>90</td>
<td>9087.11</td>
<td>100.96</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table B.18: DA concentration in the striatum, PQ data

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Sum of Squares (Type III)</th>
<th>Mean Square</th>
<th>F Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strain</td>
<td>18</td>
<td>1435.63</td>
<td>79.75</td>
<td>6.97</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Treatment</td>
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<td>6.26</td>
<td>0.55</td>
<td>0.46</td>
</tr>
<tr>
<td>Strain*Treatment</td>
<td>18</td>
<td>135.07</td>
<td>7.50</td>
<td>0.66</td>
<td>0.8</td>
</tr>
<tr>
<td>Residual</td>
<td>90</td>
<td>1006.85</td>
<td>11.44</td>
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<td></td>
</tr>
</tbody>
</table>

Table B.19: DOPAC concentration in the striatum, PQ data
### Table B.20: HVA concentration in the striatum, PQ data

<table>
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<th>DF</th>
<th>Sum of Squares (Type III)</th>
<th>Mean Square</th>
<th>F Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strain</td>
<td>18</td>
<td>209.9</td>
<td>11.66</td>
<td>15.98</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Treatment</td>
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<td>0.17</td>
<td>0.17</td>
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<td>0.6</td>
</tr>
<tr>
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<td>8.71</td>
<td>0.48</td>
<td>0.66</td>
<td>0.8</td>
</tr>
<tr>
<td>Residual</td>
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<td>65.69</td>
<td>0.72</td>
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</tbody>
</table>

### Table B.21: TH concentration in the striatum, PQ data

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<th>Source</th>
<th>DF</th>
<th>Sum of Squares (Type III)</th>
<th>Mean Square</th>
<th>F Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strain</td>
<td>9</td>
<td>0.11</td>
<td>0.012</td>
<td>15.98</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Treatment</td>
<td>1</td>
<td>0.01</td>
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<td>0.6</td>
</tr>
<tr>
<td>Strain*Treatment</td>
<td>18</td>
<td>0.06</td>
<td>0.01</td>
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<td>0.8</td>
</tr>
<tr>
<td>Residual</td>
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### Table B.22: GFAP concentration in the striatum, PQ data

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<th>DF</th>
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<th>Mean Square</th>
<th>F Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strain</td>
<td>9</td>
<td>2.49</td>
<td>0.27</td>
<td>2.34</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Treatment</td>
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<td>0.02</td>
<td>0.02</td>
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</tr>
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<td>0.58</td>
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<td>0.54</td>
<td>0.8</td>
</tr>
<tr>
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<td>67</td>
<td>7.94</td>
<td>0.11</td>
<td></td>
<td></td>
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</tbody>
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Bibliography


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111


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