THE EFFECTS OF OXIDATIVE STRESS ON CALCINEURIN ACTIVITY AND DJ-1
SUBCELLULAR LOCALIZATION

by

Diana Diec

A thesis submitted in conformity with the requirements
for the degree of Master of Science
Graduate Department of Physiology
University of Toronto

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Master of Science Thesis-2009
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University of Toronto

ABSTRACT

Oxidative stress and mutations in DJ-1, a redox sensitive protein, are linked to Parkinson's Disease. The protective mechanism of DJ-1 is unclear. I hypothesized that: 1) DJ-1 mediates protection by translocating to mitochondria after oxidative stress and, 2) when DJ-1 is downregulated, apoptotic pathways regulated by calcineurin are also downregulated. In PC12 cells and rat cortical neurons, oxidative stress resulted in the upregulation of DJ-1 and increased DJ-1 in the nucleus, but did not increase mitochondrial translocation of DJ-1. In cortical neurons and wildtype mouse embryonic fibroblasts, H₂O₂ induced cleavage of CnA into an inactive fragment. DJ-1 knockout fibroblasts had less nuclear localization of the transcription factor NFATc4, a substrate of calcineurin involved in apoptosis. H₂O₂ increased CnA cleavage in DJ-1 knockout fibroblasts, but NFATc4 localization was unchanged. These results suggest that the downregulation of apoptotic pathways regulated by calcineurin may be a compensatory response to the downregulation of DJ-1.
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### TABLE OF CONTENTS

ABSTRACT ....................................................................................................................... ii
ACKNOWLEDGEMENTS ............................................................................................... iii
LIST OF TABLES ............................................................................................................ ix
LIST OF FIGURES ......................................................................................................... x
LIST OF ABBREVIATIONS ............................................................................................. xii

**INTRODUCTION** ........................................................................................................ 1
  1.1. Mitochondria, ROS, and Neurodegenerative Diseases ........................................ 2
  1.2. Parkinson’s Disease ............................................................................................ 2
  1.3. Models of PD ........................................................................................................ 3
    1.3.1. Toxin-Induced Models .................................................................................... 4
      1.3.1.1. Toxin-Induced Models: 6-OHDA ............................................................... 4
      1.3.1.2. Toxin-Induced Models: MPTP ................................................................. 5
      1.3.1.3. Toxin-Induced Models: Rotenone ........................................................... 6
      1.3.1.4. Toxin-Induced Models: Paraquat ............................................................ 7
    1.3.2. Genetic Models ............................................................................................. 8
      1.3.2.1. Genetic Models: α-synuclein ................................................................. 8
      1.3.2.2. Genetic Models: parkin ......................................................................... 8
      1.3.2.3. Genetic Models: PINK1 ....................................................................... 8
    1.4. Genetic Models: DJ-1 ....................................................................................... 9
      1.4.1. DJ-1 as a cellular anti-oxidant/redox sensor ........................................... 9
      1.4.2. Sub-cellular localization of DJ-1 .............................................................. 12
      1.4.3. Potential mitochondrial translocation of DJ-1 in response to oxidative stress...13
  1.5. Mitochondria ....................................................................................................... 15
    1.5.1. Mitochondria and Ca^{2+} sequestration .................................................... 15
    1.5.2. Mitochondria and ROS Production ........................................................... 16
  1.6. Oxidative Stress and Antioxidant Defenses in the Brain .................................... 17
    1.6.1. Oxidative Damage ...................................................................................... 18
  1.7. Calcineurin .......................................................................................................... 18
    1.7.1. Cn and apoptosis ...................................................................................... 19
    1.7.2. Cn and neuroprotection in cerebral ischemia ............................................. 20
    1.7.3. Cn and neurodegenerative diseases ......................................................... 21
  1.8. RATIONALE ......................................................................................................... 24
  1.9. HYPOTHESES AND SPECIFIC AIMS ............................................................. 26

**MATERIALS AND METHODS** .................................................................................. 28
  2.1. PC12 cell culturing and differentiation ............................................................... 29
2.1.1. Culturing undifferentiated PC12 cells .................................................................29
2.1.2. Culturing differentiated PC12 cells ..................................................................29
2.2. Culturing of primary mouse embryonic fibroblasts .................................................30
  2.2.1. Culturing and re-plating of mouse embryonic fibroblasts .................................30
  2.2.2. Freezing of MEFs .........................................................................................31
  2.2.3. Thawing of MEFs .......................................................................................31
2.3. Rat cortical neuron culturing ..................................................................................31
  2.3.1. Substrate preparation and plate coating ...........................................................31
  2.3.2. Media preparation ........................................................................................32
  2.3.3. Dissection and plating of dissociated cultures .................................................32
2.4. Reagent preparation .............................................................................................33
2.5. MTT Reduction Assay .........................................................................................34
  2.5.1. Plating and treatment of PC12 cells ...................................................................34
  2.5.2. Plating and treatment of rat cortical neurons ..................................................34
  2.5.3. Plating and treatment of MEFs .......................................................................35
  2.5.4. MTT reduction assay protocol ......................................................................35
2.6. Flow cytometry ...................................................................................................35
  2.6.1. PC12 cell death analysis by flow cytometry .......................................................36
  2.6.2. MEF ROS analysis by flow cytometry ..............................................................36
2.7. Confocal fluorescence microscopy of ROS level and MTP ....................................37
  2.7.1. Confocal fluorescence microscopy of ROS levels in MEFs ...............................37
  2.7.2. Confocal fluorescence microscopy of MTP in MEFs ........................................37
2.8. Confocal fluorescence imaging of DJ-1 immunofluorescence ..............................38
  2.8.1. Optimization of cell fixing protocol ..................................................................38
  2.8.2. Optimization of immunofluorescence protocol ..............................................38
  2.8.3. Final optimized immunofluorescence protocol ................................................39
  2.8.4. Confocal fluorescence imaging .......................................................................41
2.9. Western blot analysis of CnA, DJ-1, NFATc4 protein expression .............................41
  2.9.1. Preparation of whole cell lysates .....................................................................41
  2.9.2. Preparation of subcellular fractions with digitonin buffer ....................................42
  2.9.3. Preparation of cytoplasmic, mitochondrial, and nuclear fractions of PC12 cells with Active Motif kits .................................................................42
  2.9.4. Preparation of cytoplasmic and nuclear fractions of rat cortical neurons and MEFs with an Active Motif kit .................................................................44
  2.9.5. Determination of total protein concentration in WCL and subcellular fractions ..................................................................................................................44
  2.9.6. SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) ....................................45
  2.9.7. Detection of CnA, DJ-1, NFATc4 .................................................................45
  2.9.8. Densitometry and Quantification of Results .....................................................47
2.10. Calcineurin Activity Assay ..................................................................................47
2.10.1. Sample Preparation..................................................................................47
2.10.2. Removal of free phosphate from the high speed supernatant.............49
2.10.3. Protein quantification in desalted lysates ........................................50
2.10.4. Cn Activity Assay..................................................................................50
2.10.5. Analysis of Cn activity ...........................................................................51
2.11. Statistical Analysis ..................................................................................51

RESULTS ........................................................................................................52
3.1. PQ reduced cell viability and increased cell death in PC12 cells .............53
3.2. H2O2 reduced cell viability in PC12 cells after 24h ...............................56
3.3. Menadione severely compromised cell viability in PC12 cells by 4h .........56
3.4. Oxidative stressors compromised cell viability in rat cortical neurons ....56
3.5. CnA levels do not change after short term PQ exposure in PC12 cells ......59
3.6. CnA levels in PC12 cells were decreased after a 24h or 48h exposure to PQ 63
3.7. CnA is cleaved after menadione exposure in PC12 cells .........................63
3.8. Cn activity did not change after PQ exposure in PC12 cells .................66
3.9. PQ treatment decreased CnA levels by 24h in rat cortical neurons ..........66
3.10. Menadione induced cleavage of CnA in rat cortical neurons .................70
3.11. H2O2 induced cleavage of CnA in rat cortical neurons .........................70
3.12. Cn activity did not change after PQ or H2O2 exposure in rat cortical neurons 74
3.13. Preliminary data: NFATc4 levels in the cytoplasm and nucleus are unchanged after H2O2 exposure in rat cortical neurons .........................74
3.14. Decreased cell viability after PQ exposure in MEFs was independent of DJ-1 KO ....................................................................................................76
3.15. H2O2-induced changes in cell viability in MEFs were independent of DJ-1 KO ....83
3.16. ROS levels were increased in MEF lines after 24h PQ with no effect of DJ-1 KO ....................................................................................................83
3.17. Confocal imaging indicated that ROS levels increased after 1h H2O2 in MEFs......86
3.18. Confocal imaging indicated that 24h PQ exposure did not alter MTP in MEFs ....86
3.19. Basal CnA levels were the same between Wt and DJ-1 KO MEFs .............90
3.20. Sub-lethal PQ did not alter CnA levels in Wt and DJ-1 KO MEFs ..........90
3.21. Sub-lethal 1h 100μM H2O2 induced CnA cleavage and had differential effects on Wt and DJ-1 KO MEFs .................................................................93
3.22. NFATc4 subcellular localization differed between Wt and DJ-1 KO MEFs under basal conditions .............................................................................95
3.23. Preliminary data: sub-lethal 2h 100μM H2O2 induced an increase in cytoplasmic NFATc4 in Wt MEFs, but not in DJ-1 KO MEFs .................................98
3.24. DJ-1 is localized to the cytoplasm, mitochondria, and nucleus under basal conditions in PC12 cells ........................................................................100
3.25. PQ did not induce translocation of DJ-1 to mitochondria in PC12 cells ....102
DISCUSSION ......................................................................................................................110

PART I: THE EFFECTS OF DJ-1 KO IN MEFs AFTER OXIDATIVE STRESS ........................................111

4.1.1. Introduction ...............................................................................................................111
4.1.2. DJ-1 KO does not decrease cell viability after oxidative stress .....................111
4.1.3. DJ-1 KO does not affect ROS levels in MEFs under basal conditions ...........112
4.1.4. DJ-1 KO increases ROS generation in MEFs after sustained PQ ..................113
4.1.5. Direct assessments of ROS after oxidative challenges in DJ-1 KO ..............114
4.1.6. The pitfalls of H2DCFDA ........................................................................................114
4.1.7. Rh123 as a fluorescence indicator for MTP .........................................................115
4.1.8. MTP is unchanged after sustained PQ exposure .............................................115
4.1.9. Microscopy versus flow cytometry for fluorescence measurements ............116
4.1.10. MEFs .....................................................................................................................116

PART II: EXPRESSION AND SUBCELLULAR TRANSLOCATION OF DJ-1 AFTER OXIDATIVE STRESS .................................................................................................................117

4.2.1. DJ-1 and subcellular translocation .......................................................................117
4.2.2. DJ-1 is localized to the cytoplasm, mitochondria, and nucleus .....................118
4.2.3. H2O2 does not increase mitochondrial translocation of DJ-1 .....................119
4.2.4. DJ-1 is upregulated after H2O2 .............................................................................120
4.2.5. PQ does not induce translocation of DJ-1 to mitochondria .........................121
4.2.6. Mitochondria and DJ-1 .......................................................................................121
4.2.7. The nucleus as a target of DJ-1 translocation .................................................122
4.2.8. Summary of DJ-1 translocation data ...............................................................123

PART III: THE EFFECTS OF OXIDATIVE STRESS ON Cn ACTIVITY AND CnA EXPRESSION .............................................................................................................................124

4.3.1. DJ-1 and Cn are potentially linked ....................................................................124
4.3.2. PQ, H2O2 and menadione affect CnA levels via distinct mechanisms ............124
  4.3.2.1. H2O2 decreases CnA levels via cleavage of CnA ...........................................124
  4.3.2.2. PQ decreases CnA levels without cleavage of CnA ......................................125
  4.3.2.3. CnA cleavage is also induced by menadione .............................................126
4.3.3. Oxidative stress decreases cell viability .............................................................126
4.3.4. Low dose menadione increases cell viability .....................................................127
4.3.5. Cn activity does not change after oxidative stress ........................................128
4.3.5.1. Cn is not inactivated after PQ exposure in PC12 cells and rat
cortical neurons or after H₂O₂ exposure in rat cortical neurons ...............128
4.3.5.2. Cn activity and CnA levels do not correlate after 24h PQ exposure
in rat cortical neurons ................................................................................129
4.3.6. Pitfalls of the Cn activity assay ..................................................................129
4.3.7. Cn downregulation mediates neuroprotection .......................................130
4.3.8. NFATc4 translocation in rat cortical neurons .........................................130
4.3.9. Pure nuclear fractions are difficult to obtain ..........................................131

PART IV: THE EFFECTS OF DJ-1 KO ON Cn AFTER OXIDATIVE
STRESS ...........................................................................................................131
4.4.1. DJ-1 KO does not affect basal CnA .......................................................131
4.4.2. CnA levels in DJ-1 KO MEFs after PQ ..................................................132
4.4.3. DJ-1 KO MEFs are more susceptible to Cn cleavage induced by H₂O₂....132
4.4.4. NFATc4 and DJ-1 KO ..............................................................................133
4.4.5. DJ-1 KO MEFs and NFATc4 under basal conditions .........................134
4.4.6. Wt MEFs and NFATc4 after H₂O₂ exposure ........................................134
4.4.7. DJ-1 KO MEFs do not display changes in subcellular NFATc4
localization after H₂O₂ exposure ...................................................................135
4.4.8. Summary and Working Model ..............................................................136
4.4.9. Ongoing Studies and Future Directions ...............................................141
4.4.10. Conclusions and Implications ............................................................141

REFERENCES .................................................................................................143
### LIST OF TABLES

| Table 1 | MTT reduction in Wt and DJ-1 KO MEFs after 24h 100μM PQ | 81 |
| Table 2 | ROS levels in MEFs after 2h 400μM PQ | 88 |
| Table 3 | ROS levels in MEFs after 1h 100μM H$_2$O$_2$ | 88 |
| Table 4 | MTP in MEFs after 2h 400 μM PQ | 91 |
LIST OF FIGURES

Figure 1  Cn and DJ-1 are redox-sensitive proteins that are involved in neurodegenerative pathways ........................................................................................................ 25
Figure 2  Optimization of primary DJ-1 antibodies for immunofluorescence ............ 40
Figure 3  Western blot illustrating the ability of both anti-CnA antibodies to detect cleaved CnA .......................................................................................................................... 48
Figure 4  Sample scanned x-ray film illustrating increases in band size and intensity with increasing total protein loading ................................................................. 48
Figure 5  MTT reduction assay: sustained PQ exposure decreased cell viability in differentiated PC12 cells ..................................................................................................................... 54
Figure 6  Flow cytometry: PI uptake revealed increased cell death in PC12 cells after sustained PQ exposure ................................................................. 55
Figure 7  MTT reduction assay: 24h H2O2 exposure decreased cell viability in differentiated PC12 cells ..................................................................................................................... 57
Figure 8  MTT reduction assay: 4h and 24h menadione exposure decreased cell viability in PC12 cells ................................................................. 57
Figure 9  MTT reduction assay: 24h PQ exposure decreased cell viability in rat cortical neurons ..................................................................................................................... 58
Figure 10 MTT reduction assay: 1h, 4h, and 24h H2O2 exposure decreased cell viability in rat cortical neurons ..................................................................................................................... 60
Figure 11 MTT reduction assay: 4h and 24h menadione exposure decreased cell viability in rat cortical neurons ..................................................................................................................... 61
Figure 12 Summary graph of differential cell viabilities of rat cortical neurons after exposure to PQ, H2O2, or menadione ................................................................. 62
Figure 13 Western blot: PQ exposure did not alter CnA levels in PC12 cells ................ 64
Figure 14 Western blot: sustained PQ exposure decreased CnA levels in PC12 cells .......................................................................................................................... 65
Figure 15 Western blot: 4h 10μM menadione exposure resulted in cleavage of CnA in PC12 cells .......................................................................................................................... 67
Figure 16 Cn activity was unchanged after 1h-24h PQ exposure in PC12 cells ......... 68
Figure 17 Western blot: CnA levels were decreased by 24h 10μM PQ exposure in rat cortical neurons ................................................................................................. 69
Figure 18 Western blot: CnA was cleaved by 10μM menadione exposure in rat cortical neurons .......................................................................................................................... 71
Figure 19 Representative Western blot of CnA in rat cortical neurons after 50μM H2O2 exposure .......................................................................................................................... 72
Figure 20 CnA was cleaved by 1h 50μM H2O2 exposure in rat cortical neurons ....... 73
Figure 21 Cn activity did not change after oxidative stress in rat cortical neurons.... 75
Figure 22 Western blot: Preliminary data indicate that NFATc4 levels in the cytoplasm and nucleus are unchanged after 1h – 24h 50μM H2O2 ................................. 77
Figure 23 Unpooled data: MTT reduction after 24h 100μM PQ showed no difference in cell viability between Wt and DJ-1 KO MEFs ................................................................. 78
Figure 24 Pooled data: MTT reduction after 24h 100μM PQ showed no difference
in cell viability between Wt and DJ-1 KO MEFs. 

**Figure 25**  MTT reduction assay: 48h 50µM PQ exposure decreased cell viability in MEFs, but no differential effects were observed between Wt and DJ-1 KO MEFs.

**Figure 26**  MTT reduction assay: 1h H₂O₂ exposure compromised cell viability in MEFs, but no differential effects were observed between Wt and DJ-1 KO MEFs.

**Figure 27**  Flow cytometry: ROS levels are increased after 24h PQ in Wt and DJ-1 KO MEFs.

**Figure 28**  Confocal fluorescence microscopy: ROS levels increased after 1h H₂O₂, but not after short term PQ, in Wt and DJ-1 KO MEFs.

**Figure 29**  Confocal fluorescence microscopy: Rh123 uptake showed that MTP did not change after 24h 100µM PQ exposure in MEFs.

**Figure 30**  Western blot: CnA expression is the same between Wt and DJ-1 KO MEFs.

**Figure 31**  Western blot: CnA expression did not change after sub-lethal PQ exposure in MEFs.

**Figure 32**  Western blot: 1h 100µM H₂O₂ cleaved CnA in both Wt and DJ-1 KO MEFs and decreased CnA levels in DJ-1 KO MEFs.

**Figure 33**  Preliminary data showed that NFATc4 subcellular localization differed between Wt and DJ-1 KO MEFs under basal conditions.

**Figure 34**  Western blot: Preliminary data indicated that 2h 100µM H₂O₂ changed NFATc4 levels in cytoplasmic fractions of Wt MEFs only.

**Figure 35**  DJ-1 is localized to the cytoplasm, mitochondria and nucleus under basal conditions in PC12 cells.

**Figure 36**  DJ-1 did not translocate to the mitochondria after oxidative stress with PQ in PC12 cells.

**Figure 37**  Immunofluorescence showed no increased translocation of DJ-1 to mitochondria after PQ or H₂O₂ exposure in PC12 cells.

**Figure 38**  Immunofluorescence showed that DJ-1 is upregulated after H₂O₂, but not PQ, exposure in PC12 cells.

**Figure 39**  DJ-1 did not translocate to the mitochondria, but increased in the nucleus after oxidative stress with H₂O₂ in PC12 cells.

**Figure 40**  Immunofluorescence showed that DJ-1 is upregulated after 3h 50µM H₂O₂ in rat cortical neurons.

**Figure 41**  Initial model of responses of DJ-1 and Cn after oxidative stress.

**Figure 42**  Revised model of the responses of DJ-1 and Cn after oxidative stress.

**Figure 43**  Working model of DJ-1 and Cn protective pathways.

**Figure 44**  Working model of protective pathways in DJ-1 KO cells.
**LIST OF ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>6-OHDA</td>
<td>6-hydroxydopamine</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimer disease</td>
</tr>
<tr>
<td>ARE</td>
<td>Antioxidant response element</td>
</tr>
<tr>
<td>ASK1</td>
<td>Apoptosis signal regulating kinase 1</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>A.U.</td>
<td>Arbitrary units</td>
</tr>
<tr>
<td>Bad</td>
<td>Bcl-2 antagonist of cell death</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>Basal-cell lymphoma 2 pro-survival protein</td>
</tr>
<tr>
<td>Bcl-X&lt;sub&gt;L&lt;/sub&gt;</td>
<td>Basal-cell lymphoma extra large pro-survival protein</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>Ca&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>Calcium ion</td>
</tr>
<tr>
<td>CaM</td>
<td>Calmodulin</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CCCP</td>
<td>Carbonyl cyanide m-chlorophenyl hydrazone</td>
</tr>
<tr>
<td>CM-H&lt;sub&gt;2&lt;/sub&gt;DCFDA</td>
<td>5-(and-6)chloromethyl-2',7' dichlorofluoresceindiacetate, acetyl ester</td>
</tr>
<tr>
<td>CO&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>Cn</td>
<td>Calcineurin</td>
</tr>
<tr>
<td>CnA</td>
<td>Catalytic subunit of calcineurin</td>
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<tr>
<td>CnB</td>
<td>Regulatory subunit of calcineurin</td>
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<tr>
<td>CNS</td>
<td>Central nervous system</td>
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<td>Copper zinc superoxide dismutase</td>
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<td>Death domain associated protein</td>
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<td>ddH&lt;sub&gt;2&lt;/sub&gt;O</td>
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<td>DMEM</td>
<td>Dulbecco's Modified Eagle Medium</td>
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<td>Dimethylsulfoxide</td>
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<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>Ethyleneglycoltetraacetic acid</td>
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<td>Electron transport chain</td>
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<tr>
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<td>Fetal bovine serum</td>
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<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
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<td>Goat serum</td>
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<td>Hank’s balanced salt solution</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>IMM</td>
<td>Inner mitochondrial membrane</td>
</tr>
<tr>
<td>IMS</td>
<td>Intermembrane space</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilo-Dalton</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>KO</td>
<td>Knockout</td>
</tr>
<tr>
<td>KRH</td>
<td>Krebs-Ringer-HEPES</td>
</tr>
<tr>
<td>L-DOPA</td>
<td>L-3,4-dihydroxyphenylalanine</td>
</tr>
<tr>
<td>MEF</td>
<td>Mouse embryonic fibroblast</td>
</tr>
<tr>
<td>MnSOD</td>
<td>Manganese superoxide dismutase</td>
</tr>
<tr>
<td>MPP⁺</td>
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<td>Mitochondrial</td>
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<td>mtDNA</td>
<td>Mitochondrial deoxyribonucleic acid</td>
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<td>Mitochondrial transmembrane potential</td>
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</tr>
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<td>Sodium ion</td>
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</tr>
<tr>
<td>NGF</td>
<td>Nerve growth factor</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartic acid</td>
</tr>
<tr>
<td>Nrf2</td>
<td>Nuclear factor erythroid 2-related factor</td>
</tr>
<tr>
<td>O₂</td>
<td>Molecular oxygen</td>
</tr>
<tr>
<td>OMM</td>
<td>Outer mitochondrial membrane</td>
</tr>
<tr>
<td>PARK2</td>
<td>Parkin gene</td>
</tr>
<tr>
<td>PARK7</td>
<td>DJ-1 gene</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PC12 cells</td>
<td>Pheochromocytoma cells</td>
</tr>
<tr>
<td>PD</td>
<td>Parkinson’s disease</td>
</tr>
<tr>
<td>pI</td>
<td>Isoelectric point</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
</tr>
<tr>
<td>PINK1</td>
<td>PTEN-induced putative kinase 1 gene</td>
</tr>
<tr>
<td>PLO</td>
<td>Poly-L-ornithine</td>
</tr>
<tr>
<td>PP1</td>
<td>Protein phosphatase 1</td>
</tr>
<tr>
<td>PQ</td>
<td>Paraquat dichloride (1,1’-dimethyl-4,4’-bipyridium dichloride)</td>
</tr>
<tr>
<td>PSF</td>
<td>Pyrimidine tract binding protein associated splicing factor</td>
</tr>
<tr>
<td>PUFA</td>
<td>Polyunsaturated fatty acid</td>
</tr>
<tr>
<td>Rh123</td>
<td>Rhodamine 123</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RT</td>
<td>Rotenone</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the means</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
</tr>
<tr>
<td>SNCA</td>
<td>α-synuclein gene</td>
</tr>
<tr>
<td>SNpc</td>
<td>Substantia nigra pars compacta</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>SOD1</td>
<td>Copper zinc superoxide dismutase</td>
</tr>
<tr>
<td>SOD2</td>
<td>Manganese superoxide dismutase</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
</tr>
<tr>
<td>TOM20</td>
<td>Translocase of outer membrane 20kDa</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>UPS</td>
<td>Ubiquitin proteasomal system</td>
</tr>
<tr>
<td>VDAC</td>
<td>Voltage-dependent anion channel</td>
</tr>
<tr>
<td>VK</td>
<td>Vitamin K₃</td>
</tr>
<tr>
<td>WCL</td>
<td>Whole cell lysate</td>
</tr>
<tr>
<td>Wt</td>
<td>Wildtype</td>
</tr>
<tr>
<td>Δ</td>
<td>Change</td>
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INTRODUCTION
1.1. Mitochondria, ROS, and Neurodegenerative Diseases

Mitochondria are a key regulator in cell survival and death pathways. Energy is produced in the form of adenosine triphosphate (ATP) through oxidative phosphorylation by the electron transport chain (ETC) located on the inner mitochondrial membrane (IMM). However, oxidative phosphorylation also produces deleterious reactive oxygen species (ROS) in the cell under basal conditions. Anti-oxidant defenses exist in the cell to combat ROS, but nonetheless, oxidative stress is produced when there is an imbalance between ROS production and ROS removal (reviewed in Fridovich, 1989). The brain is particularly susceptible to increased ROS production as it has high basal aerobic respiration activity in order to meet its high energy demands (reviewed in Halliwell, 1992). This imbalance is exacerbated in favour of ROS accumulation due to the weak anti-oxidant defenses in the brain in comparison to the rest of the body (Sinet et al., 1980). Furthermore, ROS accumulation triggers a feed forward effect where the ETC is slowed by ROS, resulting in increased ROS production due to enhanced electron transfer to molecular oxygen instead of down the ETC. Consequently, the proton gradient that drives ATP synthesis is weakened, leading to decreased ATP production. Ion homeostasis begins to fail due to deregulation of ATP-dependent channels and pumps on the plasma membrane and mitochondria (reviewed in Halliwell, 2006). As a result, mitochondrial function is disrupted by increases in intracellular Ca$^{2+}$, leading to further increase of ROS that ultimately culminates in oxidative damage to proteins, lipids, nuclear deoxyribonucleic acid (DNA) and mitochondrial DNA (mtDNA). Subtle changes in oxidative phosphorylation efficiency by the ETC can generate oxidative stress that lead to decreased neuronal survival and the development of neurodegenerative diseases.

1.2. Parkinson’s Disease

Parkinson’s Disease (PD) is a progressive neurodegenerative disorder that mainly affects the elderly population. It is characterized by bradykinesia, resting tremor, muscle rigidity, and postural instability. Pathologically, PD is characterized by progressive degeneration of dopaminergic (DAergic) neurons in the substantia nigra pars compacta (SNpc), noradrenergic neurons of the locus coeruleus, and other brainstem regions (Damier et al., 1999). Motor symptoms are not evident until 50% to 70% of DAergic neurons have degenerated (Orth & Schapira, 2002). DAergic neurons normally release dopamine (DA) to suppress over-activity of
the globus pallidus and subthalamic nucleus. Inhibitory effects on the subthalamic nucleus and globus pallidus are lost as DAergic neurons degenerate, causing hyperactivity in these regions and the classical motor symptoms of PD (Obeso et al., 2000). L-3,4-dihydroxyphenylalanine (L-DOPA), a precursor of DA, is prescribed to PD patients to combat motor symptoms caused by DA deficiency. However, this treatment only alleviates symptoms and does not halt the progression of disease (reviewed in Olanow et al., 2004 & Schapira, 2005). As the disease progresses and patients become non-responsive to L-DOPA, neurodegeneration spreads to other brain areas, giving rise to the autonomic disturbances and dementia that is seen in later stages of the disease (Terzioglu & Galter, 2008). In addition to neuronal loss, intracellular inclusions that are densely packed with α-synuclein, known as Lewy Bodies, are also characteristic of PD pathology (Spillantini et al., 1997).

Post-mortem analyses of the brains of PD patients have found free radical-mediated damage to lipids, proteins and DNA, as well as protein deficiencies in complex I of the mitochondrial respiratory chain (Schapira et al., 1989). Of the five complexes in the mitochondrial respiratory chain, complex I is most affected by endogenous oxidative stress (Keeney et al., 2006). Complex I defects result in decreased ATP production and increased free radical generation, leading to enhanced oxidative stress that may induce mitochondrial dysfunction. Subsequently, the cell becomes sensitized to pro-apoptotic signals, leading to neurodegeneration (Perier et al., 2005). Models of PD may recapitulate some or all of these mechanisms through use of toxins or genetic means.

1.3. Models of PD

A review of epidemiological literature reveals a consistent correlation between long-term pesticide exposure and the development of late-onset PD (Betarbet et al., 2000; Liou et al., 1997; McCormack et al., 2002). This has given rise to the environmental hypothesis of PD etiology, which suggests that environmental exposure to toxic chemical agents throughout the human lifespan selectively damage DAergic neurons, ultimately culminating in the development of PD in old age. The single commonality between all the environment agents that have been linked to PD is the fact that all of these chemicals generate cell-damaging oxidative stress. Most toxin-induced models of PD target mitochondria and produce oxidative stress in DAergic neurons. DAergic neurons are especially susceptible to mitochondrial dysfunction and
oxidative stress because DA metabolism gives rise to quinone and semiquinone metabolites that are capable of electron capture and generation of ROS, which results in considerable oxidative stress in the cell (Stokes et al., 1999). However, to date, no toxin-induced or genetic model of PD faithfully recapitulates all the symptoms of PD, but they have been nonetheless been valuable in our understanding of the progression of the disease.

The study of PD has proved to be difficult, as PD does not naturally occur in any animal species. Although familial forms of PD are rare, genetic manipulation of known causative genes in animal models has contributed to our understanding of the disease. Most of the causative genes identified to date display direct or indirect mitochondrial association, including, but not limited to, SNCA (α-synuclein), PARK2 (parkin), PINK1 (PTEN-induced putative kinase 1), and PARK7 (DJ-1). Genetic manipulation of these genes in mice or down/upregulation of these genes in cell culture have been valuable in our understanding of PD pathobiology. A brief summary of SNCA, PARK2, and PINK1 mutations will be provided, however, only the DJ-1 genetic model of PD will be discussed in further detail as a complete review of the literature on all of these genetic models is beyond the scope of this thesis.

1.3.1. Toxin-Induced Models

1.3.1.1. Toxin-Induced Models: 6-OHDA

6-hydroxydopamine (6-OHDA) was one of the first neurotoxins used in the study of PD. 6-OHDA is hydrophilic and cannot cross the blood-brain barrier; therefore, experiments utilizing 6-OHDA involve intracerebral injection (reviewed in Emborg, 2007). As a hydroxylated derivative of DA, 6-OHDA exploits the DA transport systems for cellular entry, namely the DA transporter (DAT) and the noradrenaline transporter (Luthman et al., 1989). 6-OHDA has been shown to induce cell death through the generation of superoxide and oxidative stress (Hasegawa et al., 1990). 6-OHDA administration results in selective and specific degeneration of DAergic neurons by oxidative damage.

The first models of PD using intracranial 6-OHDA involved bilateral injections in rats. Although this protocol successfully induced parkinsonian symptoms, the animals required extensive care throughout the duration of the experiment due to widespread neurotoxicity (Cenci et al., 2002; Jolicoeur et al., 1991). Currently, the more widely used model is the "hemiparkinsonian" model, in which 6-OHDA is unilaterally injected to destroy the ipsilateral
DAergic nigrostriatal pathway. This model has the advantages of an internal control and no debilitating side effects. The result is a reproducible, quantifiable rotational behaviour that responds to amphetamine or apomorphine and can be corrected by administration of L-DOPA (Perese et al., 1989; Schwarting & Huston, 1996). However, the relevance of rotational behaviour in the study of PD is disputed and parkinsonian symptoms occur only occasionally in the 6-OHDA hemiparkinsonian model (Lindner et al., 1999). In addition, other areas that are affected in PD, including the olfactory structures and lower brainstem region, are not sensitive to 6-OHDA toxicity. The relevance of 6-OHDA as a PD model is controversial, although it is still one of the most common models of PD used today.

1.3.1.2. Toxin-Induced Models: MPTP

In 1983, it was discovered that abusers of the illicit designer drug meperidine, which contained 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), developed parkinsonian-like symptoms (Langston et al., 1983). MPTP itself is not toxic and readily crosses the blood-brain barrier due to its lipophilic properties. Glial monoamine oxidase B converts MPTP into its highly toxic metabolite, the 1-methyl-4-phenyl-dihydropyridium ion (MPP⁺) (Chiba et al., 1984), which actively enters DAergic neurons through the DAT to selectively inhibit complex I of the mitochondrial respiratory chain (Gainetdinov et al., 1997; Krueger et al., 1990). This leads to free radical generation and subsequent DAergic neuronal loss by oxidative stress, resulting in parkinsonism (Fallo et al., 1997).

MPTP administration in animal models resulted in specific DAergic neuronal toxicity due to selectivity for DAT. Intracellular α-synuclein aggregates that are reminiscent of Lewy Bodies developed in DAergic neurons after infusion of MPTP in squirrel monkeys (McCormack et al., 2008). In vitro studies in SK-N-SH cells also suggested that MPP⁺ increased the expression of α-synuclein mRNA and resulted in the subsequent accumulation and aggregation of α-synuclein protein (Fan et al., 2006). Intravenous administration of MPTP to rhesus monkeys resulted in L-DOPA-responsive parkinsonian symptoms similar to those observed in humans, along with decreased DA levels in the striatum (Burns et al., 1983). Striatal injection of MPTP resulted in MPP⁺ accumulation in DAergic nerve terminals in both rats and mice; however, while there was significant DAergic neurodegeneration in mice, there was no DAergic neurodegeneration in rats, suggesting that rats are relatively resistant to MPTP toxicity.
(Sundstrom & Samuelsson, 1997). It has been reported that sensitivity of different mouse strains to MPTP toxicity vary, with C57BL/6 mice incurring the greatest decrease in neostriatal DA levels. This disparity is speculated to be due to differential monoamine oxidase B activity between strains (Sedelis et al., 2000). Taken together, the literature suggests that different animal models and different strains of mouse models respond differently to MPTP toxicity.

1.3.1.3. Toxin-Induced Models: Rotenone

Rotenone (RT) is a compound found in the roots and stems of tropical plants and is commonly used as a pesticide. Like MPTP, it specifically inhibits complex I of the mitochondrial respiratory chain by preventing electron transfer to ubiquinone to generate oxidative stress (Betarbet et al., 2000; Sherer et al., 2003). RT is not expected to confer DAergic neuron specific toxicity due to its highly lipophilic property that allows it to cross membranes with ease (Betarbet et al., 2000). Studies using RT as a PD model have yielded conflicting results regarding its specificity of action. Chronic RT exposure in rat midbrain organotypic slices resulted in specific destruction of SNpc neurons (Testa et al., 2005). Studies in mouse primary cultures have also found that DAergic neurons were more sensitive to long-term RT exposure than glia cells and non-DAergic neurons (Radad et al., 2008). In vivo studies using chronic low-dose infusion of RT in rats resulted in PD-like symptoms of akinesia and rigidity, along with increased ROS generation, enhanced lipid peroxidation in the striatum and a decreased threshold for apoptosis mediated by mitochondrial permeability transition (Bashkatova et al., 2004; Panov et al., 2005). Lewy Body-like intracellular α-synuclein aggregates were also present in nigral neurons after chronic systemic exposure of RT in rats (Betarbet et al., 2000). In addition, acidic forms of DJ-1 accumulated in the rat striatum, substantia nigra and olfactory bulb as a result of RT-induced oxidative stress (Betarbet et al., 2006). However, other reports suggested that the hypokinesia and dystonia observed in rats after RT exposure were due to digestive tract and general health problems because DAergic neurons were not lost when PD-like symptoms surfaced (Lapointe et al., 2004). In addition, many animals died from generalized free radical-induced acute toxicity that was unrelated to the central nervous system (Hoglinger et al., 2003). Reproducibility is poor between experiments, making RT an unfavourable model to use.
1.3.1.4. Toxin-Induced Models: Paraquat

Paraquat (PQ) is a redox-cycling synthetic herbicide that has been linked to the development of PD in farm workers (Hertzman et al., 1990; Liou et al., 1997). It is structurally similar to MPTP. Unlike MPTP and RT, PQ does not readily cross the blood-brain barrier because of its hydrophilic nature and relies on the neutral amino acid transporter for transport into the central nervous system (reviewed in Dinis-Oliveira et al., 2006). PQ entry has also been hypothesized to be correlated with age-related changes in the permeability of the blood-brain barrier that may permit accumulation of PQ in the brain with old age (Li & Sun, 1999).

Neuronal uptake of PQ occurs via Na$^+$-dependent transport, where it is reduced to the cationic PQ radical by accepting an electron from NADH (nicotinamide dinucleotide plus hydrogen) through complex I of the mitochondrial respiratory chain (Fukushima et al., 1995). In the presence of molecular oxygen (O$_2$), the cationic PQ radical is rapidly re-oxidized, regenerating the parent PQ compound while producing superoxide in the process. The high levels of superoxide generated in this manner are thought to inhibit mitochondrial complex I, leading to mitochondrial dysfunction (Fukushima et al., 1995). Inhibition of mitochondrial complexes III and IV have also been reported (Palmeira et al., 1995). The superoxide that is generated can also be converted into H$_2$O$_2$ and the hydroxyl radical. These in turn can cause lipid peroxidation of membranes that leads to dysregulation of the mitochondrial transition pore and subsequent cell death (reviewed in Dinis-Oliveira et al., 2006).

Animals exposed to PQ exhibit many of the pathobiological and motor symptoms of PD. Chronic systemic administration of PQ to mice and rats caused a decline in the number of DAergic neurons in the SNpc, a significant decrease in striatal DA levels and parkinsonian symptoms (decreased movement, increased rigidity and akinesia) (Brooks et al., 1999; Li et al., 2005; McCormack et al., 2002; Ossowska et al., 2005). The mechanism of PQ toxicity is thought to be mediated through oxidative stress, as increased ROS levels and decreased glutathione were detected in SH-SY5Y cells treated with PQ (Yang & Tiffany-Castiglioni, 2005). In addition, synthetic superoxide dismutase (SOD) and catalase mimetics protected against PQ toxicity in vitro (Peng et al., 2005). Intraneuronal Lewy Body-like α-synuclein aggregates were present after PQ exposure (Uversky et al., 2001). Although PQ induces generalized neurotoxicity, this is not seen as a drawback to its use because neurodegeneration in PD extends to brain regions beyond the SNpc and locus coeruleus (reviewed in Braak & Del Tredici., 2008).
1.3.2. Genetic Models

1.3.2.1. Genetic Models: α-synuclein

Missense and multiplication mutations in the SNCA gene coding for α-synuclein are linked to dominant early-onset PD (Chartier-Harlin et al., 2004; Ibanez et al., 2004; Polymeropoulos et al., 1997). α-synuclein is present throughout the brain and enriched in presynaptic nerve terminals (Solano et al., 2000). It is a prominent component of the Lewy Body, which is a characteristic feature of PD pathology (Spillantini et al., 1997). Overexpression of α-synuclein in PC12 cells resulted in decreased proteasome activity and increased sensitivity to mitochondria-dependent apoptosis (Tanaka et al., 2001). Lewy body-like intraneuronal inclusions, mitochondrial DNA damage and degeneration, and neurodegeneration of neocortical, brainstem, and motor neurons were evident in mice that overexpress α-synuclein (Martin et al., 2006). However, to date, the exact cellular function of α-synuclein under normal cellular conditions is still unclear.

1.3.2.2. Genetic Models: parkin

Parkin, encoded by the PARK2 gene, is a ubiquitously expressed ubiquitin E3 ligase that is a component of the ubiquitin-proteasomal system (Shimura et al., 2000). It is found in the mitochondria of proliferating cells, where it regulates transcription and replication of mitochondrial DNA (Kuroda et al., 2006). Drosophila with the PARK2 gene knocked out exhibited decreased lifespan, muscle degeneration accompanied by motor defects, male sterility, and altered mitochondrial morphology (Greene et al., 2003). In mice, parkin knockout (KO) resulted in decreased ETC activity and increased susceptibility to oxidative stress (Darios et al., 2003). A study in SH-SY5Y cells showed that parkin overexpression decreased cellular ROS and was protective against apoptosis (Jiang et al., 2004). Loss-of-function parkin mutations are linked to autosomal recessive early-onset PD and may account for up to half of familial PD cases (reviewed in Dekker et al., 2003).

1.3.2.3. Genetic Models: PINK1

PINK1 is a serine-threonine kinase located on the mitochondrial membrane. Homozygous loss-of-function mutations account of 1-7% of early onset PD (reviewed in
Downregulation of PINK1 by siRNA in SH-SY5Y cells reduced cell viability and increased apoptosis (Deng et al., 2005), while overexpression of PINK1 attenuated apoptosis (Petit et al., 2005). PINK1 KO may lead to neurodegeneration through induction of oxidative stress, as overexpression of SOD, an anti-oxidant enzyme, was able to protect against DAergic neuronal loss caused by inactivation of the PINK1 homolog in Drosophila (Wang et al., 2006). However, the physiological substrates of PINK1 are as yet unknown.

1.4. Genetic Models: DJ-1

DJ-1 is a highly conserved ubiquitously expressed 20kDa protein that is encoded by the nuclear PARK7 gene (Bonifati et al., 2003a; Taira et al., 2001). DJ-1 was first cloned as an oncogene in 1997 using a yeast two-hybrid screen, where it was found to interact with c-myc and possess the ability to transform NIH3T3 cells (Nagakubo et al., 1997). Since then, DJ-1 has been found to be upregulated in several cancers, including, but not limited to, pancreatic ductal adenocarcinoma, ovarian carcinoma, and prostate cancer (Davidson et al., 2008; Hod, 2004; Tian et al., 2008). DJ-1 has a ThiJ domain and is classified as part of the ThiJ/PfpI superfamily of proteins, which includes prokaryotic proteases, transcription factors, glutamine amidotransferase family proteins, bacterial catalases, and bacterial chaperones (reviewed in Bonifati et al., 2004). Some proposed activities of DJ-1 that have garnered experimental support include those as a cellular anti-oxidant/redox sensor, transcriptional regulator, and molecular chaperone, although its exact cellular function is still largely unknown. Single nucleotide substitutions and deletion mutations in DJ-1 resulting in loss-of-function have been linked to the development of PD (Bonifati et al., 2003b).

1.4.1. DJ-1 as a cellular anti-oxidant/redox sensor

Under unstressed conditions, DJ-1 is present as a homodimer with several isoforms of different isoelectric points (pI) (Bandopadhyay et al., 2004; Choi et al., 2006; Kinumi et al., 2004). Upon oxidative stress, DJ-1 is oxidized and a shift from pI 6.2 to the more acidic pI 5.8 is observed (Canet-Aviles et al., 2004). Canet-Aviles et al. (2004) specifically identified cysteine 106 (C106), which is oxidized to cysteine-sulfinic acid under oxidative conditions, as the critical residue for generating the acidic isoform of DJ-1. Post-mortem analysis of brains from PD patients indicated that the acidic form of DJ-1 was predominant, supporting a role of
oxidative stress and oxidized DJ-1 in PD pathology (Bandopadhyay et al., 2004; Choi et al., 2006). DJ-1 may be directly oxidized by free radicals, as the acidic isoform of DJ-1 increased with increasing levels of ROS in the cell (Mitsumoto et al., 2001). Reports that DJ-1 was upregulated and self-oxidized in the presence of H2O2 support its role as an anti-oxidant in cultured neurons, although whether DJ-1 actively eliminates ROS is still a subject of debate (Mitsumoto et al., 2001; Taira et al., 2004).

A role of DJ-1 in the transcriptional regulation of anti-oxidant genes has also been proposed. Nuclear factor erythroid 2-related factor (Nrf2) is a master regulator of the transcription of antioxidant response element (ARE) genes in the oxidative stress response. Such genes include those encoding for enzymes involved in glutathione synthesis, free iron management, and NADPH production (Clements et al., 2006). Under basal conditions, the inhibitor of Nrf2, Keap1, targets Nrf2 for degradation by ubiquitination (Furukawa & Xiong, 2005). This leads to decreased expression of ARE genes. After oxidative stress, DJ-1 binds to and stabilizes Nrf2, allowing Nrf2 to translocate to the nucleus, where it mediates increased expression of ARE genes to generate an oxidative stress response (Clements et al., 2006).

Studies investigating the role of DJ-1 as a cellular oxidant or redox sensor in relation to PD pathobiology have focused on four issues: 1) the effects of DJ-1 KO; 2) the effects of DJ-1 KO in the response to oxidative stress; 3) the effect of DJ-1 overexpression in preventing oxidative damage, and; 4) the effects of DJ-1 substitution mutations on the oxidative stress response. Studies that characterized DJ-1 KO mice reported little to no differences in motor and behavioural test performance in these mice when compared to wildtype (Wt) controls (Chen et al., 2005; Kim et al., 2005; Manning-Bog et al., 2007; Yamaguchi & Shen, 2007; Yang et al., 2007). Under normal conditions, the number of tyrosine hydroxylase (TH) positive neurons were similar between DJ-1 deficient mice and Wt mice, indicating that DJ-1 deficient mice did not exhibit DAergic neurodegeneration in the SNpc (Chandran et al., 2008; Chen et al., 2005; Kim et al., 2005; Manning-Bog et al., 2007; Yamaguchi & Shen, 2007). Furthermore, no oxidative damage was detected in DJ-1 KO mice, despite the proposed neuroprotective role of DJ-1 against oxidative stress (Chen et al., 2005; Yamaguchi & Shen, 2007).

Despite the lack of neurodegeneration in DJ-1 KO mice, it has been reported that the nigrostriatal pathway is dysfunctional, most likely due to aberrant DA and DAT regulation. Two studies reported increased striatal DA in DJ-1 KO mice versus Wt (Chen et al., 2005; Yang et al, 2007). In contrast, striatal DA levels and DAT expression were reported to be similar
between Wt and DJ-1 KO mice in three separate studies (Chandran et al., 2008; Kim et al., 2005; Yamaguchi & Shen, 2007). Manning-Bog et al. (2007) speculated that DAT distribution could be altered in DJ-1 KO mice, despite observations that DAT expression did not change. Through Western blotting of cytoplasmic and synaptosomal fractions, Manning-Bog et al. (2007) discovered that DAT redistributed into synaptosomal fractions, resulting in increased DA uptake. Since MPP⁺ enters neurons via DAT, redistribution of DAT into synaptosomal fractions may provide an explanation for the increased sensitivity of DJ-1 KO mice in response to MPTP exposure (Manning-Bog et al., 2007). Studies have reported decreased striatal DA in response to short-term PQ and MPTP exposure that was not accompanied by significant TH-positive neuronal loss (Manning-Bog et al., 2007; Yang et al., 2007). In contrast, chronic MPTP exposure in DJ-1 KO mice led to decreased immunostaining for TH-positive neurons and decreased striatal DA, suggesting that chronic oxidative stress in combination with DJ-1 deficiency are required for DAergic neurodegeneration (Kim et al., 2005).

In cellular models, DJ-1 knock-down by small interfering RNA (siRNA) resulted in increased cell death due to H₂O₂, MPP⁺, or 6-OHDA oxidative stress in SH-SY5Y cells (Taira et al., 2004). Similar studies performed in Neuro2A cells, N27 cells, and murine primary midbrain cultures also found increased cell death induced by H₂O₂ oxidative stress (Martinat et al., 2004; Yokota et al., 2003; Zhou & Freed, 2005). DJ-1 KO from embryonic stem cells increased susceptibility to H₂O₂, although initial ROS accumulation in these cells were no different from oxidatively stressed wildtype cells (Martinat et al., 2004). Similarly, knock-down of Drosophila DJ-1 homologs caused ROS accumulation and hypersensitivity to oxidative stress, as well as increased sensitivity to the oxidative stressors PQ and RT, leading to dysfunction and degeneration of DAergic neurons (Meulener et al., 2005; Yang et al., 2005). In zebrafish embryos, knock-down of DJ-1 by morpholinos resulted in increased loss of DAergic neurons after H₂O₂ exposure when compared to Wt controls (Baulac et al., 2009; Bretuad et al., 2007). This was reported to be a p53-dependent event, as pharmacological inhibition of p53 with pifithrin-alpha resulted in enhanced neuronal survival after treatment with MG132, a proteasome inhibitor. Furthermore, simultaneous knock-down of DJ-1 and mdm2, a negative regulator of p53, in zebrafish embryos resulted in increased DAergic neuronal loss in the absence of oxidative exposure (Bretuad et al., 2007), providing additional evidence that neuronal loss in DJ-1 deficient models are p53-dependent.
Numerous studies report protective effects of DJ-1 overexpression in response to oxidative stress. N27 cells, primary DAergic neurons, and SH-SY5Y cells were protected against H$_2$O$_2$ induced cell death with DJ-1 overexpression (Inden et al., 2006; Zhou & Freed, 2005). Mouse primary cortical neurons were protected against H$_2$O$_2$-induced apoptosis with overexpression of DJ-1 (Kim et al., 2005). Similarly, cell viability of DJ-1 KO embryonic stem cells after H$_2$O$_2$ exposure was rescued with DJ-1 transfection (Martinat et al., 2004). Overexpression of DJ-1 in wildtype and DJ-1 knock-down SK-N-BE cells was also found to be protective against oxidative stress induced by 6-OHDA and H$_2$O$_2$ (Batelli et al., 2008), which further supports a role of DJ-1 in anti-oxidant processes.

Studies investigating the effects of single nucleotide substitution mutations in DJ-1 have centered on the L166P mutant that was first identified in an Italian family with a history of familial PD. In this mutant, leucine residue 166 is substituted with proline, resulting in mutant DJ-1 protein that is highly unstable and rapidly degraded as a consequence of its inability to form homo-dimers due to the helix breaks at the C-terminal dimerization interface caused by the proline substitution (Miller et al., 2003). SH-SY5Y cells with the L166P mutation were more susceptible to cell death induced by H$_2$O$_2$, MPP$^+$, or 6-OHDA (Taira et al., 2004). The increased cell death may be attributed to other reports that suggest that the ability of the L166P mutant to eliminate exogenously added H$_2$O$_2$ is compromised (Takahashi-Niki et al., 2004). Furthermore, overexpression of the L166P mutant form was unable to rescue cell death caused by H$_2$O$_2$ oxidative stress in DJ-1 KO embryonic stem cells (Martinat et al., 2004). Later studies by Kim et al. (2005) and Inden et al. (2006) that exposed DJ-1 deficient mice primary cortical neurons and SH-SY5Y cells, respectively, to H$_2$O$_2$ or 6-OHDA also demonstrated the inability of the L166P mutant to protect against oxidative stress induced cell death (Inden et al., 2006; Kim et al., 2005). To summarize, all literature to date supports the neuroprotective role of DJ-1 against oxidative stress.

1.4.2. Sub-cellular localization of DJ-1

The vast majority of studies examining the subcellular localization of DJ-1 have utilized overexpression of exogenous and/or mutant DJ-1 in DAergic cell lines. Regardless of the cellular model used, a predominantly diffuse pattern of cytoplasmic staining of DJ-1 was consistently observed (Blackinton et al., 2005; Canet-Aviles et al., 2004; Junn et al., 2009; Lev
et al., 2008; Miller et al., 2003). Cytoplasmic localization of DJ-1 has been shown by immunolocalization and fluorescence microscopy in M17 cells (Blackinton et al., 2005; Canet-Aviles et al., 2004; Miller et al., 2003), SH-SY5Y cells (Lev et al., 2008), SK-N-BE(2)C cells (Junn et al., 2009), and COS7 cells (Miller et al., 2003). DJ-1 localization to the nucleus was detected by immunogold electron microscopy and subcellular fractionation of SH-SY5Y cells in one study (Zhang et al., 2005). In another study, strong nuclear localization of DJ-1 was detected by fluorescence microscopy and subcellular fractionation of SH-SY5Y cells and Neuro2A cells (Fan et al., 2008). As DJ-1 appears to have anti-oxidant functions, localization to the mitochondria is an attractive notion. Studies examining the mitochondrial localization of DJ-1 have generally yielded mixed results. Zhang et al. (2005) utilized immunogold scanning electron microscopy and reported that approximately 25% of wildtype DJ-1 was present in the mitochondria of SH-SY5Y cells, with specific localization to the intermembrane space and matrix. In contrast, a study by Fan et al. (2008) in SH-SY5Y cells and Neuro2A cells suggested that there was no endogenous DJ-1 localized to the mitochondria that was detectable by Western blot of subcellular fractions and fluorescence microscopy. Yet other groups have shown that wildtype DJ-1 did not specifically localize to mitochondria under unstressed conditions in M17 cells (Blackinton et al., 2005; Canet-Aviles et al., 2004; Miller et al., 2003) and SK-N-BE(2)C cells (Junn et al., 2009), but there was a small degree of overlap between DJ-1 and mitochondrial staining by MitoTracker (Blackinton et al., 2005; Miller et al., 2003) or VDAC (voltage-dependent anion channel) on the outer mitochondrial membrane (OMM) (Canet-Aviles et al., 2004) in a subset of cells, and some DJ-1 in mitochondrial subcellular fractions under basal conditions (Junn et al., 2009).

1.4.3. Potential mitochondrial translocation of DJ-1 in response to oxidative stress

The mechanism through which DJ-1 exerts cytoprotective effects are still as yet unknown. It is widely speculated that DJ-1 mediates its neuroprotective actions after oxidative stress through translocation to subcellular compartments, namely the mitochondria and the nucleus. Wildtype DJ-1 has been reported to translocate to mitochondria after PQ exposure in M17 cells (Blackinton et al., 2005; Canet-Aviles et al., 2004), and after RT and 6-OHDA exposure in SH-SY5Y cells (Blackinton et al., 2005; Canet-Aviles et al., 2004; Junn et al., 2009; Lev et al., 2008), as assessed by fluorescence microscopy and overlap of DJ-1 with mitochondrial markers. Western blot of subcellular fractions of SK-N-BE(2)C cells treated with
H₂O₂ also suggest mitochondrial translocation after oxidative stress (Junn et al., 2009). Limited trypsin digestion studies found that DJ-1 remained associated with the cytoplasmic side of the OMM after oxidative stress-induced translocation to the mitochondria in M17 cells and SK-N-BE(2)C cells (Canet-Aviles et al., 2004; Junn et al., 2009). The requirement of mitochondrial translocation for the neuroprotective capability of DJ-1 is controversial. In one study, the C106A DJ-1 mutant, in which the critical cysteine for oxidation is substituted with alanine, did not translocate to the mitochondria of M17 cells under oxidative conditions and displayed decreased cell viability when presented with oxidative stressors (Canet-Aviles et al., 2004). This finding supports the requirement of mitochondrial translocation for the protective effects of DJ-1. Conversely, a different study, also performed in M17 cells and COS-7 cells, reported that the highly unstable pathogenic L166P mutant of DJ-1 displays similar cellular distribution as wildtype DJ-1 (Miller et al., 2003), arguing against the neuroprotective effects of mitochondrial translocation. A recent study in SK-N-BE(2)C cells suggested that mutations at cysteine 106 or other cysteine residues of DJ-1 did not compromise the ability of DJ-1 to translocate to the mitochondria after H₂O₂ exposure. These C106A mutant cells still displayed decreased cell viability despite mitochondrial translocation, which further disputes the significance of mitochondrial translocation in the neuroprotective actions of DJ-1 (Junn et al., 2009). However, the same study reported contradictory findings of mitochondrially-targeted DJ-1 being more cytoprotective than wildtype DJ-1 against oxidative stress (Junn et al., 2009), which supports a role of mitochondrial translocation in the protective effects of DJ-1. To date, a clear link between mitochondrial translocation and the cytoprotective effects of DJ-1 have yet to be definitively elucidated.

Junn et al. (2009) found that the translocation of DJ-1 to subcellular compartments after oxidative stress was highly time-dependent, with mitochondrial localization increasing by 1h of H₂O₂ exposure and returning to basal levels by 12h, at which time nuclear localization of DJ-1 began to increase. The importance of nuclear translocation of DJ-1 in neuroprotection has not been widely studied, although DJ-1 has been reported to have the ability to sequester the death protein Daxx (death domain associated protein) in the nucleus and prevent it from activating ASK1 (apoptosis signal regulating kinase 1) in the cytoplasm of SH-SY5Y cells (Junn et al., 2005). In addition, it has been demonstrated that localization of endogenous DJ-1 in the nucleus of SH-SY5Y cells and Neuro2A cells influenced p53 transcriptional activity (Fan et al., 2008). Combined with the transcriptional regulator activity of DJ-1 in the expression of antiapoptotic
factors in SH-SY5Y cells (Xu et al., 2005), nuclear translocation of DJ-1 after oxidative stress may be vital in mediating neuroprotection.

1.5. Mitochondria

Mitochondria are the energy powerhouses of the cell. They produce the vast majority of cellular ATP, the primary energy currency in the cell, through oxidative phosphorylation. Mitochondria also take part in the metabolism of amino acids and lipids, Ca\(^{2+}\) homeostasis, and free radical scavenging. Structurally, mitochondria are dual membrane structures, with an outer mitochondrial membrane (OMM) and an inner mitochondrial membrane (IMM) that folds upon itself into cristae. The space between the two membranes is known as the intermembrane space (IMS), while the innermost cavity that the cristae protrude into is known as the matrix (reviewed in Bogaerts et al., 2008).

Oxidative phosphorylation is restricted to the IMM, where the ETC is located. The ETC, also known as the mitochondrial respiratory chain, is comprised of five electron carrier complexes: NADH ubiquinone oxidoreductase (complex I), succinate ubiquinone oxidoreductase (complex II), ubiquinone-cytochrome C reductase (complex III), cytochrome oxidase (complex IV), and ATP synthase (complex V). The energy released by electrons moving down the ETC chain is coupled by complexes I, III and IV to actively transport protons from the IMS into the matrix. This generates an electrochemical gradient across the IMM to produce the inner mitochondrial transmembrane potential (MTP), which is used to drive ATP synthesis (reviewed in Bogaerts et al., 2008).

1.5.1. Mitochondria and Ca\(^{2+}\) sequestration

Ca\(^{2+}\) is an important signalling molecule in the cell. Increases in intracellular Ca\(^{2+}\) levels, either via influx through ion channels located on the plasma membrane, or through increased release from sequestered stores in the mitochondria and/or endoplasmic reticulum, trigger a number of signalling cascades. MTP also drives sequestration of cytoplasmic Ca\(^{2+}\) into the mitochondrial matrix to maintain cytoplasmic Ca\(^{2+}\) levels at an acceptable level in resting cells. When the cytoplasmic concentration of Ca\(^{2+}\) increases above this setpoint, mitochondria actively sequester Ca\(^{2+}\) into the mitochondrial matrix through a Ca\(^{2+}\) uniporter located on the
IMM. Sequestered Ca\textsuperscript{2+} is retained in the matrix through binding with proteins and lipids. Small rises in intracellular Ca\textsuperscript{2+} levels trigger low level Ca\textsuperscript{2+} sequestration that is important in stimulating Ca\textsuperscript{2+}-sensitive dehydrogenase activity, leading to increased reducing agents being supplied to the ETC that ultimately results in enhanced ATP production. When cytoplasmic Ca\textsuperscript{2+} levels become too high, mitochondrial sequestration increases to a state where mitochondrial functions become compromised. As a result, MTP decreases, leading to uncoupling of oxidative phosphorylation and subsequent Ca\textsuperscript{2+}-induced apoptotic cytochrome c release. Therefore, the balance between cytoplasmic Ca\textsuperscript{2+} levels and mitochondrial Ca\textsuperscript{2+} buffering play an important role in neuronal viability, particularly after excitotoxic stress when large amounts of Ca\textsuperscript{2+} enter the cell through NMDA receptor channels (reviewed in Murphy et al., 1999).

1.5.2. Mitochondria and ROS Production

Although oxidative phosphorylation is a relatively efficient method of producing ATP for the cell, it is also the single major source of ROS under basal conditions. Since the ETC involves a series of single electron transfers, each complex is vulnerable to side reactions with O\textsubscript{2} instead of passing the electron onto the next electron carrier, thereby producing superoxide. The majority of superoxide is produced by complexes I, II, and III and released into the mitochondrial matrix. In addition to superoxide production on the IMM, monoamine oxidases located on the OMM catalyze oxidative deamination of biogenic amines, such as DA and epinephrine, generating H\textsubscript{2}O\textsubscript{2} that is released into the cytosol and mitochondrial matrix. H\textsubscript{2}O\textsubscript{2} can also be produced through enzymatic dismutase of superoxide. The subsequent reaction of H\textsubscript{2}O\textsubscript{2} with transition metals, such as iron or copper, in the Fenton reaction results in the generation of the hydroxyl radical, which is highly reactive and capable of widespread damage to biomolecules (reviewed in Halliwell, 2006).

Production of superoxide and H\textsubscript{2}O\textsubscript{2} is dependent on the mitochondrial metabolic state of the cell. Under conditions of slow respiration where ADP levels are low, the electron carrier complexes are in a more reduced state and generation of superoxide and H\textsubscript{2}O\textsubscript{2} is high. When oxygen intake and cellular ADP levels are high, respiration increases and the electron carrier complexes are more oxidized, resulting in lower superoxide and H\textsubscript{2}O\textsubscript{2} production (Boveris & Chance, 1973). The brain is particularly sensitive to mitochondrial ROS production, mostly
from complex I, due to its high $O_2$ consumption and ATP requirements (Kudin et al., 2005). Normal brain function depends heavily upon ATP-powered ion channels to maintain ion homeostasis. Hence, defects in ATP production caused by mitochondrial dysfunction result in rapid brain damage (reviewed in Halliwell, 1992). In addition, the brain contains an abundance of iron-containing proteins, which can release iron upon protein damage. These then undergo the Fenton reaction to generate deleterious hydroxyl radicals (Zecca et al., 2004).

1.6. Oxidative Stress and Antioxidant Defenses in the Brain

Oxidative stress is defined as an imbalance between ROS production and ROS removal. Under normal cellular conditions, ROS generated by mitochondria as a byproduct of oxidative phosphorylation is adequately removed by ROS scavenging systems in the cell. However, when there is an overload of ROS due to enhanced cellular production or exogenous agents, oxidative stress occurs. To combat oxidative conditions, the brain is enriched in low molecular mass antioxidants, such as glutathione and ascorbate, as well as a number of ROS scavenging enzymes. The regulation of some of these anti-oxidant enzymes involves DJ-1, which binds to and stabilizes Nrf2, resulting in activation of genes encoding proteins for glutathione synthesis, free iron management, etc (Clements et al., 2006). SOD, which reduces superoxide to $H_2O_2$, is present in different forms throughout the central nervous system (CNS) (reviewed in Fridovich, 1989). MnSOD (also known as SOD2), which contains manganese in its active site, is predominant in the mitochondrial matrix where superoxide levels are high. CuZnSOD (also known as SOD1) contains copper and zinc in its active site and can be found in the mitochondrial IMS and the rest of the cell. $H_2O_2$ is removed by glutathione peroxidase, which catalyzes the oxidation of glutathione by $H_2O_2$ to produce water (reviewed in Brigelius-Flohe, 1999). Peroxiredoxins have recently been touted as the most important $H_2O_2$-removing system in the brain (Rhee et al., 2005). They are present in all sub-cellular organelles, as well as in the cytosol, and are inactivated at high concentrations of $H_2O_2$ to allow $H_2O_2$ signaling functions to proceed (Rhee et al., 2005). In contrast, catalases are not significant in the antioxidant defenses of the brain and are not found in brain mitochondria (reviewed in Turrens, 2003), although they are important in ROS scavenging in the heart (reviewed in Cadenas & Davies, 2000).
1.6.1. Oxidative Damage

When subjected to oxidative stress, proteins can become oxidatively damaged and targeted for proteolytic digestion (Bence et al., 2001). Some oxidized proteins are digested by lysosomes. In the cytoplasm, nucleus and endoplasmic reticulum, the majority of oxidized soluble proteins are degraded by the ubiquitin-proteasomal system (UPS) that marks proteins for digestion by attachment of ubiquitin molecules (McNaught et al., 2001). Heavily oxidized proteins tend to resist proteolytic removal and form aggregates (Grune et al., 2004). Studies have suggested that the precipitation of such heavily oxidized proteins into insoluble clumps function to decrease their toxicity (Bence et al., 2001).

Iron and copper-containing proteins are abundant in the brain. When iron and copper are released, they are capable of participating in the Fenton reaction to form the highly toxic hydroxyl radical. The hydroxyl radical can cause lipid peroxidation of polyunsaturated fatty acid (PUFA) side chains in membrane lipids. The damage in membrane proteins and lipids result in decreased membrane fluidity and increased leakiness of membranes to substances that are normally restricted to transporters. In addition, receptors, enzymes, and ion channels in membranes are inactivated and there is extensive protein and DNA damage, eventually culminating in cell death (reviewed in Halliwell, 2006).

1.7. Calcineurin

Calcineurin (Cn) is the only known serine/threonine phosphatase that is directly regulated by Ca\(^{2+}\) and calmodulin (CaM) (Klee et al., 1979). It is ubiquitously expressed and selectively enriched in the brain, where it accounts for 1% of total brain protein and is ~10 times more concentrated in the brain than in other tissues, although expression in other tissues is also abundant (Wallace et al., 1980). Cn levels are particularly high in the cerebral cortex, substantia nigra, cerebellum, and hippocampus. Although the physiological function of high level Cn expression is still largely unknown, the brain regions of high Cn expression coincide with areas that are selectively vulnerable to cerebral ischemia, epilepsy and neurodegenerative diseases (Polli et al., 1991). Within the cell, Cn is present in the soma and dendrites and maintains close association with post-synaptic densities, the plasma membrane, and other membrane and cytoskeletal components (Wallace et al., 1980). Roles of Cn in T-cell activation (Dhein et al., 1995), cardiac hypertrophy (Graef et al., 2001), long term potentiation/depression (Malleret et
al., 2001), and apoptosis (Shioda et al., 2007) have been well-characterized. The exact cellular consequence of Cn in the development of neurodegenerative diseases have yet to be elucidated.

Cn is a heterodimer consisting of a catalytic A subunit (CnA) and a regulatory B subunit (CnB) that are tightly associated under cellular conditions and can only be dissociated with denaturants (Merat & Cheung, 1987). CnA contains the catalytic domain, along with three regulatory domains at the carboxy terminus: 1) a CnB binding domain; 2) a CaM binding domain, and; 3) an autoinhibitory domain that binds the substrate-binding cleft of Cn under resting cellular states. When intracellular Ca$^{2+}$ concentration increases, either through influx or release from sequestered mitochondrial and endoplasmic reticular stores, Ca$^{2+}$ binds CaM and the resulting Ca$^{2+}$/CaM complex associates with CnA at the CaM binding domain. The binding of CaM induces a conformational change in CnA that results in the dissociation of the autoinhibitory domain from the substrate binding cleft. Unlike similar protein phosphatases, CnA is inactive as a monomer and requires dimerization with CnB to exhibit phosphatase activity. CnB can bind four Ca$^{2+}$ with high affinity and is proposed to play a role in Cn activity through allosteric interactions with CnA that induce conformational changes in the latter (reviewed in Rusnak & Mertz, 2000). Constitutive activity of CnA can also be induced in the absence of CnB by permanent cleavage of the autoinhibitory domain to expose the substrate binding cleft or by deletion of the CaM binding domain to abolish Ca$^{2+}$ and CaM-dependency of Cn activity (Stemmer & Klee, 1994).

1.7.1. Cn and apoptosis

The role of Cn in apoptosis was first described in T lymphocytes, where Cn was discovered to mediate apoptosis through the Fas ligand-Fas receptor pathway by inducing de novo expression of Fas ligand (Dhein et al., 1995). In neurons, there exists a similar Cn-mediated apoptotic pathway that functions via activation of nuclear factor of activated T cells (NFAT). There are five NFAT isoforms, but the predominant isoform in the brain is NFATc4 (Bradley et al., 2005). Under resting cellular conditions, NFATc4 is maintained in a phosphorylated state by constitutively active kinases, resulting in predominantly cytoplasmic localization of NFATc4. Cn activation results in dephosphorylation of NFATc4 to uncover its nuclear localization sequence, leading to nuclear translocation and subsequent transcription of the Fas ligand gene. Upon Fas ligand binding to the Fas receptor on the cell membrane,
apoptotic death is triggered by caspase activation and DNA cleavage (Jayanthi et al., 2005). Cn maintains close association with cytoplasmic membranes. The pro-survival protein Bcl-2 (B-cell lymphoma 2) exerts an anti-apoptotic effect via complex formation with activated Cn at cytoplasmic membranes, thereby preventing Cn-activated nuclear translocation of NFATc4 and subsequent Fas ligand/Fas receptor mediated cell death (Shibasaki et al., 1997). Activation of NFATc4 by Cn has been linked to neuronal apoptosis after methamphetamine administration (Jayanthi et al., 2005) and ischemia (Shioda et al., 2007). The Cn-NFATc4-mediated apoptotic pathway is also part of normal developmental deafferentation of mouse anteroventral cochlear nucleus neurons. This same study reported that specific inhibition of NFATc4 activation by Cn is insufficient to completely rescue cell death, indicating that Cn induces NFATc4-independent apoptotic mechanisms as well (Luoma & Zirpel, 2008).

Studies have shown that Cn can induce apoptosis through interactions with pro-survival and pro-apoptotic factors from the Bcl-2 family. Under normal cellular conditions, the pro-apoptotic protein Bad (Bcl-2 antagonist of cell death) is phosphorylated and confined to the cytoplasm through interactions with the cytoplasmic 14-3-3 adaptor protein. When intracellular Ca$^{2+}$ is increased, activated Cn dephosphorylates Bad, allowing it to be released from the 14-3-3 adaptor protein to translocate to the mitochondria where it binds to the pro-survival protein Bcl-X<sub>L</sub> (basal cell lymphoma extra large) and hinders its anti-apoptotic functions. As a result, cytochrome c is released from the mitochondria and the apoptotic cascade is initiated (Wang et al., 1999).

1.7.2. Cn and neuroprotection in cerebral ischemia

Regions of the brain that are particularly susceptible to cerebral ischemia include CA1 of the hippocampus and the striatum (Polli et al., 1991). These are also the areas that express the highest levels of Cn in the brain. In ischemia-reperfusion injury, neurons are overloaded with intracellular Ca$^{2+}$ due to increased Ca$^{2+}$ influx through the NMDA (N-methyl-D-aspartic acid) receptor and other Ca$^{2+}$ channels. Mitochondria sequester Ca$^{2+}$ through a Ca$^{2+}$ uniporter in the IMM, using the MTP as a driving force to pump Ca$^{2+}$ into the matrix against its concentration gradient. During ischemia, mitochondria continue to buffer the high levels of intracellular Ca$^{2+}$, but at the bioenergetic cost of decreasing the rate of oxidative phosphorylation. Consequently, ATP production is decreased and oxidative stress is increased, resulting in loss of the MTP and
subsequent osmotic water influx into the mitochondrial matrix. Mitochondria swell until the OMM ruptures and releases pro-apoptotic proteins into the cytoplasm to trigger the apoptotic cascade. However, caspase-mediated apoptosis is an ATP-dependent event and ATP is scarce in neurons subject to ischemic injury. As a result, caspase-mediated apoptosis can only proceed if sufficient ATP is produced by anaerobic respiration or by remaining functional mitochondria in the cell. Otherwise, cell death occurs by necrosis, which occurs by an ATP-independent cell death mechanism (reviewed in Uchino et al., 2008).

It has been reported that Cn mediates delayed neuronal cell death after ischemia via NFATc4 transcriptional activation of Fas ligand or via dephosphorylation of Bad. Furthermore, increased intracellular Ca\(^{2+}\) in hippocampal CA1 pyramidal neurons subject to ischemia activates calpain, a Ca\(^{2+}\)-dependent protease, which cleaves the autoinhibitory domain from CnA to produce constitutively active Cn. As a result, NFATc4 is activated and subsequent apoptosis is induced (Shioda et al., 2006; Shioda et al., 2007). The role of Cn in delayed neuronal death after ischemia is further confirmed by studies reporting that the pharmacological inhibition of Cn is neuroprotective. Cyclosporin A (CsA) and FK506, both well-known immunosuppressants that inhibit Cn activity, have been shown to be neuroprotective against ischemic injury in animal models (Sharkey & Butcher, 1994; Uchino et al., 2008; Wang et al., 1999).

1.7.3. Cn and neurodegenerative diseases

Studies of Cn expression and/or activity have yielded conflicting results in models of aging and neurodegenerative diseases. Aging is associated with increased oxidative stress, especially in the brain where the energetic demands are high. While the vast majority of current literature supports redox sensitivity of Cn, a literature review shows that there is no consensus among reports regarding the exact direction of Cn expression and/or activity shift after exposure to oxidative stress (Agostinho et al., 2008; Celsi et al., 2007; See & Loeffler, 2001; Sommer et al., 2002). A study in aged rat brain reported decreased Cn expression and activity (Agbas et al., 2005), while another study reported increased Cn activity in aged hippocampal neurons (Norris et al., 2002). One group of data suggests that Cn is inactivated by ROS, most likely via oxidation of the metal centre, which results in less efficient positioning of water molecules for hydrolysis of the phosphoester bond (Namgaladze et al., 2002). In vitro studies indicate that
exposure of purified Cn to \( \text{H}_2\text{O}_2 \) or a xanthine/xanthine oxidase superoxide generating system resulted in inhibition of Cn activity, with \( \text{H}_2\text{O}_2 \) being more efficient; however, superoxide was found to be more efficient at inhibition of Cn activity in crude fibroblast lysates (Sommer et al., 2000; Sommer et al., 2002). These reports were further confirmed by findings that PQ inactivated Cn in neuroblastoma cells with no corresponding decrease in CnA and CnB expression (Ferri et al., 2000). One recent study proposed a novel method of Cn inactivation by proteolytic cleavage within the substrate binding cleft after \( \text{H}_2\text{O}_2 \) exposure in mouse primary cortical neurons (Lee et al., 2007). In contrast, Agbas et al. (2007) did not observe any changes in Cn activity after 1h PQ exposure in rat primary cortical neurons, despite increased levels of superoxide. Enhanced Cn activity has been reported in cerebellar granule neurons after a 15min \( \text{H}_2\text{O}_2 \) pulse (See & Loeffler, 2001) and after oxidative stress induced by \( \beta \)-amyloid (Agostinho et al., 2008). Further studies are required to elucidate whether Cn is responsive to selected oxidative stresses in unique manners.

Cn has been linked to the pathology of Alzheimer disease (AD), which is characterized by the presence of intracellular \( \beta \)-amyloid aggregates and neurofibrillary tangles of hyperphosphorylated tau protein. \( \beta \)-amyloid was found to generate oxidative stress that led to Cn inactivation in mouse cortical and hippocampal neurons (Celsi et al., 2007). In a separate study, \( \beta \)-amyloid exposure in mouse primary cortical neurons induced proteolytic cleavage of Cn, resulting in a 32kDa cleavage fragment that was inactive (Lee et al., 2007). Conversely, Cn activity in rat cortical neurons was reported to increase after \( \beta \)-amyloid exposure and was pivotal in predisposing cells to apoptosis via dephosphorylation of Bad (Agostinho et al., 2008). Studies indicate that tau is a direct substrate of Cn (Luo et al., 2008; Rahman et al., 2006; Yu et al., 2008). Treatment of mouse brain with Cn inhibitors resulted in the hyperphosphorylation of tau (Luo et al., 2008; Yu et al., 2006), and subsequent impaired spatial memory (Yu et al., 2006). Post-mortem analysis of brains from AD patients revealed decreased Cn activity, but no change in expression, in the frontal lobe, which correlates with neurofibrillary tangle pathology in this region (Ladner et al., 1996; Lian et al., 2001). Taken together, a role of Cn in Alzheimer pathology is well supported by current literature.

Although the involvement of Cn in AD pathology is well studied, research is just beginning to uncover links between Cn and PD. It has long been known that DA and cyclic adenosine monophosphate (cAMP)-regulated phosphoprotein 32 (DARPP-32) is a specific and
direct substrate of Cn. DARPP-32 is phosphorylated by cAMP-dependent protein kinase upon increases in cAMP that are triggered by DA binding to the D1-DA receptor. Phosphorylated DARPP-32 is a potent inhibitor of protein phosphatase 1 (PP1). Active PP1 diminishes the effects of DA. Decreases in DA that are characteristic of PD pathology result in decreased DARPP-32 activation by phosphorylation, leading to increased PP1 activity and subsequent nullification of any positive effects due to residual DA. Therefore, inhibitors of Cn may have therapeutic value in the treatment of PD through preventing dephosphorylation of DARPP-32 in order to amplify the DA response (Wera & Neyts, 1994). Recently, a study showed that overexpression of CnB led to a concomitant increase in DJ-1 expression, although the repercussions of this tandem increase were not probed (Wang et al., 2008). This is the first report that Cn expression may be intimately linked with the expression of a PD-associated gene.
1.8. RATIONALE

To date, only one report suggested potential association between Cn and DJ-1 (Wang et al., 2008). These two proteins appear to have common properties, although it is as yet unknown whether they bear any molecular significance. Both Cn and DJ-1 are redox-sensitive proteins and are involved in the response to oxidative stress (Ferri et al., 2000; Mitsumoto et al., 2001). Upon exposure to oxidative stress, DJ-1 was reported to translocate to the mitochondria and associate with the OMM to prevent its permeabilization, which induces cell death (Canet-Aviles et al., 2004). There are also reports of nuclear translocation of DJ-1 (Fan et al., 2008; Junn et al., 2009). Cn is involved in the regulation of pro-apoptotic and pro-survival proteins associated with the mitochondria, some of which regulate the permeabilization of the OMM in apoptosis (Jayanthi et al., 2005; Wang et al., 1999). Moreover, both proteins downregulated in neurodegenerative diseases (Bonifati et al., 2003; Celsi et al., 2007), although whether they share common pathways has yet to be elucidated. One recent cancer study by Wang et al. (2008) reported that DJ-1 was increased when Cn was upregulated, further suggesting a common pathway may exist between these two proteins. I hypothesize that DJ-1 and Cn pathways are activated after oxidative stress to mediate neuroprotection (Figure 1).
Figure 1: Cn and DJ-1 are redox-sensitive proteins that are involved in neurodegenerative pathways. Cn can be inactivated by ROS produced by mitochondria. DJ-1 can be oxidized after oxidative stress, which is thought to facilitate its translocation to the mitochondria. It has been proposed that DJ-1 may exert cytoprotective effects through interactions with Bcl-2 family proteins in the mitochondria. Similarly, Cn also interacts with pro- and anti-apoptotic proteins of the Bcl-2 family. Cn also plays a role in transcription factor activation and translocation to the nucleus. Both proteins are downregulated in neurodegenerative diseases. Wang et al. (2008) suggest a link between Cn and DJ-1 in a cancer model. However, to date, there are no published studies investigating intersecting pathways of Cn and DJ-1 with regards to neurodegenerative diseases.
1.9. HYPOTHESES AND SPECIFIC AIMS

**Hypothesis #1:** Oxidative stress modulates CnA expression and/or Cn activity through mechanisms that are cell-type specific, time-specific, and vary with types of oxidative stress.

**Specific Aims for Hypothesis #1:**

1. To establish models of sub-lethal oxidative stress using PQ, H$_2$O$_2$, and menadione in a neuronal cell line (PC12 cells) and rat cortical neurons. Rationale: PC12 cells are capable of DA synthesis and metabolism and have membrane receptors and transport systems that are similar to those of DAergic neurons in the midbrain (Hatanaka, 1981; Zhu et al., 1997). Rat cortical neurons more closely model conditions in the brain.

2. To determine if Cn activity and/or CnA expression and cleavage are differentially affected by PQ, H$_2$O$_2$, and menadione. Rationale: CnA cleavage has previously been reported only in response to H$_2$O$_2$ (Lee et al., 2007).

3. To determine if oxidative stress-induced changes in CnA expression and/or activity affect the cellular localization of NFATc4, a protein that translocates to the nucleus in response to activation by Cn. Rationale: Activation of NFATc4 is linked to the initiation of apoptosis (Jayanthi et al, 2005).

**Hypothesis #2:** DJ-1 KO increases sensitivity to oxidative stress and increases ROS-mediated inactivation of Cn. In the absence of DJ-1, Cn-regulated apoptotic pathways are downregulated.

**Specific Aims for Hypothesis #2:**

1. To determine whether Wt and DJ-1 KO mouse embryonic fibroblasts differ in cell viability, ROS production, and MTP after exposure to oxidative stress.

2. To determine if DJ-1 and Cn pathways are linked after oxidative stress, specifically, whether DJ-1 KO affects Cn activity and/or CnA expression and cleavage after exposure to PQ and H$_2$O$_2$.

3. To determine if Cn-regulated NFATc4 activation is decreased after oxidative stress in DJ-1 KO cells.
**Hypothesis #3:** Oxidative stress induces translocation of DJ-1 to the mitochondria or nucleus.

**Specific Aim for Hypothesis #3:**

1. To determine the subcellular distribution of DJ-1 under basal conditions in PC12 cells and rat cortical neurons.

2. To determine whether DJ-1 translocates to the mitochondria and/or nucleus under oxidative conditions using Western blot and immunofluorescence.
MATERIALS AND METHODS
2.1. PC12 cell culturing and differentiation

2.1.1. Culturing undifferentiated PC12 cells

A rat pheochromocytoma cell line (PC12) that stably expresses tetracycline-regulated mitochondrially-targeted GFP was previously developed in our lab (Sirk et al., 2003) to facilitate study of mitochondria protein import dynamics. As import processes were not the focus of this thesis, PC12 cells were permanently grown in the presence of tetracycline so as to maintain the mitochondrially targeted GFP gene in a transcriptionally inactive state. PC12 cells were grown in 100mm polystyrene plates (BD Falcon, #353003) and kept in a 5% CO₂/95% air humidified incubator at 37°C. Cells were maintained in RPMI 1640 medium (Invitrogen, #11875-093) supplemented with 10% horse serum (Invitrogen, #16050-122), 5% fetal bovine serum (FBS) (Invitrogen, #16000-044), and 0.5% penicillin-streptomycin (10,000 U/mL) (Gibco, #15240-062). To maintain transcriptional inactivity of the mitochondrially targeted GFP gene, medium was additionally supplemented with 50ng/mL tetracycline (Sigma, #T-7660). Medium was changed every other day by complete medium exchange. Cells were passaged once a week when cultures reached 80% confluency.

2.1.2. Culturing differentiated PC12 cells

Prior to re-plating, cells were cultured for 4 days in medium supplemented with nerve growth factor (NGF) (Harlan, #0005017) at a concentration of 25ng/mL to induce PC12 cell differentiation. One day prior to experimentation, cells were harvested using Ca²⁺- and Mg²⁺-free Hank's Balanced Salt Solution (HBSS) (Gibco, #14170), centrifuged at 1500rpm for 4 minutes to pellet cells, and re-suspended in medium (hereafter referred to as 1% serum with NGF) containing reduced serum (1% serum: 0.67% HS + 0.33% FBS), 25ng/mL NGF, and 50ng/mL tetracycline. Cells were re-plated at varying densities on 5% rat tail collagen coated or poly-L-ornithine (PLO) (Sigma, #P-4638) coated plates of varying sizes and materials depending on experimental need (see below). Unless otherwise specified, differentiated PC12 cells will be referred to as PC12 cells for the remainder of this thesis.
2.2. Culturing of primary mouse embryonic fibroblasts

2.2.1. Culturing and re-plating of mouse embryonic fibroblasts

MEF lines expressing Wt DJ-1 (Wt MEFs) and DJ-1 knockout MEFs (DJ-1 KO MEFs) were gifts from Dr. Howard Mount (University of Toronto). Briefly, E12-E17 embryos were dissected from pregnant mice and the head and internal organs were removed. The embryos were finely minced and incubated in 0.05% trypsin-EDTA for 30-45 min at 37°C in a 5% CO₂/95% air humidified incubator. The tissue was dissociated by repeated pipetting through a glass pipette. 100% fetal calf serum was added to inactivate the trypsin and the dissociated suspension was centrifuged to pellet cells. MEFs were grown in 100mm polystyrene plates (BD Falcon, #353003) and kept in a 5% CO₂/95% air humidified incubator at 37°C. Cells were maintained in medium containing Dulbecco's Modified Eagle Medium (DMEM) with high glucose (Invitrogen, #11965) supplemented with 10% FBS (Invitrogen, #16000-044), 1x MEM non-essential amino acid solution (Sigma, #M7145), 4.219 μg/mL β-mercaptoethanol (Sigma, #7522), and 1x penicillin-streptomycin (10.000 U/mL) (Invitrogen, #15240-062). Medium was changed every other day by complete medium exchange and cells were split once a week when confluency was greater than 80%. To detach cells from culture plates, medium was aspirated off, cells were rinsed briefly with sterile 1x phosphate buffered saline (PBS) (Sigma, #P4417) and treated for 3 minutes with 0.05% trypsin-EDTA (0.5% trypsin-EDTA diluted in HBSS) (Invitrogen, #25300). Trypsin was inactivated with double the volume of medium as trypsin-EDTA, and cells were sub-cultured into plates containing regular culture medium. MEFs were sub-cultured for a maximum of 20 passages, at which time they were discarded and MEFs of lower passage number were thawed for further experiments. Prior to experimentation, MEFs were re-plated at varying densities on uncoated or 10% rat tail collagen coated plates of varying sizes and materials, depending on the need of the experiment (see below). Wt and DJ-1 KO MEFs were matched to ± 2 passages for all experiments. 4 Wt MEF lines (Wt 5-6, Wt 5-5, Wt 4-3, Wt 4-3) and 5 DJ-1 KO MEF lines (KO 4-4, KO 5-2, KO 5-9, KO 4-11, KO 3-3) were used and the exact lines used for each experiment are indicated in all figures.
2.2.2. Freezing of MEFs

MEFs were harvested as previously described in section 2.2.1 and centrifuged at 1000rpm for 3 minutes to pellet cells. The cell pellet was resuspended in warm resuspension medium containing 80% DMEM, 20% FBS, and 1x MEM non-essential amino acid solution. An equal volume of ice-cold freezing medium containing 60% DMEM, 20% FBS, and 20% dimethylsulfoxide (DMSO) (Sigma, #154938) was added drop-wise to the cell suspension. 1mL of cell suspension was transferred to screw-top freezing vials (Bio Plas, #4204) for immediate freezing at -80°C.

2.2.3. Thawing of MEFs

Freezing vials were thawed in a 37°C water bath briefly and removed when only a small piece of ice remained. The outside of the vial was sprayed with 70% ethanol and the cell suspension was triturated once before it was transferred to a 15mL conical tube (BD Falcon, #352097). 10mL of MEF medium, as described in section 2.2.1, was added dropwise to the cell suspension. The resulting cell suspension was centrifuged at 1000rpm for 5min to pellet the cells and to remove the DMSO. The cell pellet was resuspended in 10mL fresh MEF medium, transferred to a 100mm polystyrene plate, and maintained in a 5% CO₂/95% air humidified incubator at 37°C. The contents of one freezing vial were thawed into one 100mm plate.

2.3. Rat cortical neuron culturing

2.3.1. Substrate preparation and plate coating

PLO (Sigma, #P-4638) was diluted in sterile double distilled water (ddH₂O) to yield a 10mg/mL stock solution that was kept frozen in 75μL aliquots at -20°C. PLO working solutions were prepared by diluting one 75μL PLO stock aliquot in 50mL of sterile ddH₂O along with 0.19g of Borax (Sigma, #221732) and adjusting the pH to 8.4. The PLO working solution was vacuum sterilized prior to use and was prepared fresh for each neuronal culture. One day prior to neuronal culture, plates were coated with varying volumes of PLO depending on size. In brief, 100mm polystyrene plates were coated with 5mL of PLO, 35mm glass bottom
plates (WillCo Wells, #HBST-3522) were coated with 2mL of PLO, and each well of 96-well plates (BD Falcon, #353072) were coated with 100µL of PLO. Plates were incubated in PLO at room temperature overnight. On the day of culture, PLO was aspirated from the plates and plates were rinsed twice with sterile ddH$_2$O and left to air dry in the biological hood for 1h.

2.3.2. Media preparation

Prior to dissection and neuronal culturing, media was prepared as follows:

**Dissecting medium:** 250mL HBSS without Ca$^{2+}$, Mg$^{2+}$ (Invitrogen, #14170-161), 5.6mL 1M HEPES buffer (Invitrogen, #15630-080), 1.25g sucrose (BDH, #B10274), 2.5g D-glucose (Sigma, #5767), and 100mL ddH$_2$O to adjust osmolarity to 310-320mOsm, with the final pH adjusted to 7.4.

**Plating medium:** Neurobasal medium (Invitrogen, #21103) supplemented with 2% B-27 with antioxidants (Invitrogen, #17504-044), 2mM Glutamax$^\text{TM}$ (Invitrogen, #35050-061), 1% FBS (Invitrogen, #16000-044), and 1x penicillin-streptomycin mixture (Invitrogen, #15240-062).

**Papain solution:** 2mg/mL papain (Worthington, #3119) diluted in plating medium and subsequently filtered through a 0.2µM filter attached to a 20cc syringe.

**Maintenance medium:** Neurobasal medium (Invitrogen, #21103) supplemented with 2% B-27 without antioxidants (Invitrogen, #10889-038), and 1x penicillin-streptomycin mixture (Invitrogen, #15240-062).

2.3.3. Dissection and plating of dissociated cultures

Wistar rats (Harlan and Charles River) were sacrificed on day 18 of pregnancy according to University Health Network guidelines for animal care. E18 rat pups were removed by dissection of the abdominal cavity and transferred to ice-cold sterile 1x PBS on ice. Fetuses were dissected from fetal membranes and placenta using fine forceps and scissors and individual fetuses were transferred to ice-cold dissecting medium on ice. Fetuses were transferred to another plate of ice-cold dissecting medium on ice after removal of residual fetal membranes.
The scull of the fetus from the snout to the back of the head was cut along the sagittal line with curved fine scissors and the brain was exposed by prying open the two sides and gently squeezing. The top third of both brain hemispheres was excised using curved fine scissors for cortical cultures and transferred to a 15mL conical tube containing 10mL of dissecting medium. 3-4 cortices were collected into each tube and centrifuged at 1000rpm for 3min to pellet cortices. The supernatant was removed by aspiration and 4mL of papain solution was added to each tube. Cortices were incubated in papain solution for 30min at 37°C on a orbital shaker, after which the cortices were pelleted by centrifugation at 1000rpm for 5min. The papain solution was removed by aspiration and 5mL plating medium was added per tube. The cortices were dissociated by repeated trituration through glass pipettes. Dissociated neuronal suspensions were pooled into the same 50mL conical tube, and the volume adjusted to 50mL with plating medium. Debris and undissociated chunks of cortical tissue were allowed to settle down to the bottom of the tube for 10min. Subsequently, cells in suspension were plated in a final volume of 10mL plating medium per 100mm polystyrene plate (BD Falcon, #353003) coated with PLO as described above. One litter of 10-12 pups were plated onto 20 100mm plates. Cortical neurons were maintained at 37°C in a 5% CO2/95% air humidified incubator and fed twice a week by half-medium exchange with maintenance medium.

2.4. Reagent preparation

Paraquat (PQ) (Paraquat dichloride x-hydrate PESTANAL) (Sigma, #36541) stock solution was made fresh prior to each experiment by dissolving in sterile ddH2O to make a 50mM stock solution.

Hydrogen peroxide (H2O2) (30% wt. solution in water) (Sigma, #216763) stock solution was made fresh prior to each experiment by serial dilutions in culture medium to a 76.6mM stock solution.

Menadione (2-methyl-1,4-naphthoquinone) (Sigma, #M57405) stock solution was made fresh prior to each experiment by dissolving in 95% ethanol to make a 50mM stock solution.
2.5. MTT Reduction Assay

The MTT reduction assay is commonly used to assess cell viability because the conversion of the yellow tetrazolium salt, r3-(4,5-demethylthiazol-2-yl)-2,5-dephennyltetrasolium bromide (MTT) (Sigma, #M2128), to purple formazan crystals is dependent upon reductases in living cells (Berridge et al., 2005).

2.5.1. Plating and treatment of PC12 cells

One day prior to experimentation, PC12 cells were plated at a density of 10,000 cells/well in 96 well plates (BD Falcon, #353072) coated with PLO. Cells were maintained in 100µL of 1% serum and NGF culture medium supplemented with tetracycline as previously described. All working solutions were prepared in 1% serum and NGF medium supplemented with tetracycline. Cells were treated with 10µM - 100µM PQ for 24h or 48h, 12.5µM - 5mM H₂O₂ for 24h, or 2µM - 50µM menadione for 4h or 24h. Controls for PQ and H₂O₂ experiments were treated with water and controls for menadione experiments were treated with 95% ethanol as a vehicle control. For experiments with menadione, a maximum of 0.4µL of 95% ethanol was added to 1mL of media as vehicle controls, depending on the greatest concentration of menadione stock used. Eight wells were used for each condition.

2.5.2. Plating and treatment of rat cortical neurons

Rat cortical neurons were plated as described in section 2.3.3 at a density of 20,000 cells/well in 96 well plates (BD Falcon, #353072) coated with PLO. All working solutions were prepared in maintenance medium. Cells were used for experiment on day 5 of culture. Cells were treated with 2.5µM - 30µM PQ for 24h, 12.5µM - 200µM H₂O₂ for 1h, 4h, or 4h, or 2µM - 50µM menadione for 4h or 24h. Controls for PQ and H₂O₂ experiments were treated with water and controls for menadione experiments were treated with 95% ethanol as a vehicle control, with a maximum of 0.4µL of 95% ethanol added to 1mL of media as vehicle controls for menadione. Eight wells were used for each condition.
2.5.3. Plating and treatment of MEFs

Wt and DJ-1 KO MEFs were plated at a density of 10,000 cells/well in 96 well plates (BD Falcon, #353072) coated with PLO and maintained in 100μL of MEF medium in each well. MEFs were exposed to 50μM PQ for 24h or 100μM H₂O₂ for 1h. Controls for PQ and H₂O₂ experiments were treated with water. Six wells were used for each condition.

2.5.4. MTT reduction assay protocol

After treatment of cells with the various oxidative stressors for the indicated periods of time in sections 2.5.1, 2.5.2, and 2.5.3, 10μL of 5mg/mL MTT stock was added to each well for every 100μL of medium. MTT stock was prepared fresh prior to addition to cell culture by dissolving MTT in the respective culture medium. Cells were incubated with MTT for 2h at 37°C. At the end of the 2h incubation, the medium was carefully aspirated from each well without disturbing the formazan crystals on the bottom of the well. DMSO was added to each well and the formazan crystals were dissolved by trituration and incubation for 5min on an orbital shaker. Absorbance was read on an ELISA plate reader at 570nm (measurement) and 630nm (reference). The highest and lowest measurements for each condition were excluded from final analysis. Absorbance values were converted to percentages of control.

2.6. Flow cytometry

The flow cytometer is able to analyze signal from thousands of individual cells in a matter of seconds. Cells for analysis may be dead or alive. Multiple cellular parameters, such as cell size and intracellular fluorescence, can be assessed based on differential scatter of light from a laser that are detected and analyzed by a computer. It is important that cells used for analysis are not clumped, as clumping may skew the signal that is produced.
2.6.1. PC12 cell death analysis by flow cytometry

PC12 cells were plated at a density of 350,000 cells/well of 5% collagen coated 6 well plates (BD Falcon, #353046). Cells were treated with 10μM- 100μM PQ for 24h or 48h. 1μL of 1mg/mL propidium iodide (PI) stock solution (Molecular Probes, #P-1304) was added per millilitre of media. Media from each well were collected into separate 15mL conical tubes and adherent cells were collected with ice-cold 1x PBS into the respective tubes. Suspensions were centrifuged at 1000rpm for 3min to pellet cells. The cell pellets were resuspended in ice-cold 1x PBS and transferred to 5mL polystyrene round bottom tubes (BD Falcon, #352052). Cells from 3 wells were analyzed per experimental condition. 10,000 cells from each sample were analyzed using the Fascan Flow Cytometer (Becton Dickinson, San Jose, CA). Cells treated with 70% ethanol for 3min displayed close to 100% cell death. Live cells were gated out based on this measurement.

2.6.2. MEF ROS analysis by flow cytometry

Wt and DJ-1 KO MEFs were plated at a density of 100,000 cells/well of uncoated 24 well plates (BD Falcon, #353047). Cells were treated with 50μM PQ to 1000μM PQ for 24h. 30μL DMSO was added to 1 vial (50μg) CM-H2DCFDA (5-(and-6)chloromethyl-2',7'dichlorohydrofluoresceindiacetate, acetyl ester) (Molecular Probes, #C6827) to make a 2.8mM stock solution. CM-H2DCFDA stock solution was diluted in serum-free DMEM to make a 4.6μM working solution. At 24h, media from each well was collected into separate 1.5mL Eppendorf tubes and kept on ice. 300μL CM-H2DCFDA were added to each well and incubated at 37°C for 30min. After incubation, media from each well was collected into the respective tube, wells were rinsed with ice-cold 1x PBS and cells were detached with 0.05% trypsin-EDTA by gently pipetting up and down. Trypsin was inactivated by FBS added directly into the Eppendorf tubes. Tubes were centrifuged at 9000rcf for 1min to pellet cells. The supernatant was aspirated and discarded. The cell pellet was resuspended in 300μL ice-cold 1x PBS, transferred to 5mL polystyrene round bottom tubes, and the tubes reserved on ice until flow cytometry analysis. 100μM H2O2 were added to cells just prior to DCF incubation and used as a positive control for ROS. Cells from three different wells were analyzed per experimental
condition. The flow cytometer analyzed 10,000 cells in each sample. Average DCF fluorescence for each sample was used for analysis.

2.7. Confocal fluorescence microscopy of ROS level and MTP

2.7.1. Confocal fluorescence microscopy of ROS levels in MEFs

Wt and DJ-1 KO MEFs were plated in 10% collagen-coated white-walled 96-well plates (Greiner, #655098) with 3 wells per experimental condition. On the day of experiment, 25µL DMSO was added to 1 vial (50µg) CM-H2DCFDA to make a 4mM stock. CM-H2DCFDA was diluted to a working concentration of 10µM in Krebs-Ringer-HEPES medium (KRH: 125mM NaCl, 5mM KCl, 25mM HEPES, 6mM glucose, 5mM NaHCO3, 1.2mM MgSO4, 1.2mM KH2PO4, 1.2mM CaCl2, 1% penicillin-streptomycin mixture). Medium was aspirated from the wells and 100µL CM-H2DCFDA working solution was added to each well and incubated at 37°C for 45min. After 45min, 100µL of 800µM PQ in KRH or 200µM H2O2 in KRH was added to each well with CM-H2DCFDA to give final concentration of 400µM PQ and 100µM H2O2, respectively. 100µL of KRH was added to control wells. Cells were treated with H2O2 for 1h and with PQ for 2h and then subjected to confocal fluorescence microscopy using an inverted Nikon scanning confocal microscope (Bio-Rad MRC-600) equipped with an argon-ion laser. The BHS (fluorescein) filter block was used for imaging ROS levels with a 20x fluor objective. SOM software (Bio-Rad) was used for imaging in PIC format and images were converted to TIFF format with Confocal Assistant v4.02 (Todd Clark Brelje). Only one image was taken per focal field to avoid photoactivation of DCF.

2.7.2. Confocal fluorescence microscopy of MTP in MEFs

Wt and DJ-1 KO MEFs were plated in 10% collagen-coated white-walled 96-well plates (Greiner, #655098) with 3 wells per experimental condition. The next day, media was aspirated and 100µL of 100µM PQ was added to each experimental well. MEF media was added to control wells. After 24h of PQ exposure, 50µL of media was removed from the 3 wells for each treatment and these were pooled in the one tube. Rh123 stock solution (0.5mM in ddH2O) was
diluted to a working concentration of 1000nM in the pooled media. 50µL of the media with Rh123 was added back to the respective wells to yield a final Rh123 concentration of 500nM in the wells. Cells were incubated in Rh123 for 45min at 37°C, then the cells were rinsed 3 times with MEF media and either control or PQ media was put back into each well for confocal fluorescence imaging. Cells were imaged using an inverted Nikon scanning confocal microscope (Bio-Rad MRC-600) equipped with an argon-ion laser. The GHS (rhodamine) filter block was used for imaging Rh123 labelled cells with a 20x fluor objective. SOM software (Bio-Rad) was used for imaging in PIC format and imaged were converted to TIFF format using Confocal Assistant v4.02. Only one image was taken per focal field.

2.8. Confocal fluorescence imaging of DJ-1 immunofluorescence

2.8.1. Optimization of cell fixing protocol

PC12 cells and MEFs were used for optimization of the immunofluorescence protocol. To plate PC12 cells and MEFs, 22mm square glass coverslips (VWR, #48366-067) were immersed in 90% ethyl alcohol overnight. The next day, coverslips were air dried and flame-sterilized under the biological hood. One coverslip was put into each well of a 6-well plate and coverslips were coated with PLO. PC12 cells and Wt and DJ-1 KO MEFs were plated onto the coverslips. To establish optimal fixation conditions, media was aspirated from the wells containing the coverslips the day after plating and cells were rinsed twice with 1x PBS. One set of PC12 cells and Wt and DJ-1 KO MEFs were fixed in 4% paraformaldehyde (BDH, #29447) in PBS for 6min at room temperature and subsequently permeabilized with 0.2% Triton X-100 (Sigma, #X-100) in PBS for 6min. Another set of PC12 cells and Wt and DJ-1 KO MEFs were fixed and permeabilized in -20°C methanol (Fischer Scientific, #A454-4) for 6min. After fixing with either method, cells were rinsed twice with 1x PBS and stored in 1x PBS at 4°C. Cells were typically processed for DJ-1 immunofluorescence within one month after fixation.

2.8.2. Optimization of immunofluorescence protocol

On the day of immunofluorescence processing, 1x PBS was aspirated off and cells were rinsed three times with fresh 1x PBS, then rinsed two times with 1x PBS containing 3% goat
serum (GS) (Wisent, #053-210) and 5% bovine serum albumin (BSA) (BioShop, #ALB001.50). Two primary anti-DJ-1 antibodies were assessed for immunofluorescence: 1) primary polyclonal anti-DJ-1 antibody from Covance (Covance, #SIG-39835), and 2) primary polyclonal anti-DJ-1 antibody from Abcam (Abcam, #ab18257). Both antibodies were assessed at dilutions of 1:100, 1:200 and 1:400 in 1x PBS containing 3% GS and 5% BSA. 100μL of one of the primary antibodies was added onto each coverslip so that there was one of each antibody dilution/fixing combination. After primary antibody incubation for 1h at room temperature, the coverslips were rinsed three times with 1x PBS, then rinsed two times with 1x PBS containing 3% GS and 5% BSA. 100μL of alexa fluor 488 F(ab’)2 goat anti-rabbit secondary antibody (Invitrogen, #A11070) (1:1000) diluted in 1x PBS with 3% GS and 5% BSA was added to each coverslip. The container was covered with aluminum foil and coverslips were incubated in secondary antibody for 1h at room temperature. After incubation, the coverslips were rinsed three times with 1x PBS. Coverslips were mounted to slides using GelTol Mounting Medium (Thermo, #484950) and left to dry overnight at room temperature covered with aluminum foil. Slides were stored at 4°C protected from light.

2.8.3. Final optimized immunofluorescence protocol

Optimization of DJ-1 immunostaining on PC12 cells, Wt and DJ-1 KO MEFs revealed that 4% paraformaldehyde yielded greater non-specific staining than methanol fixation. Use of the Covance anti-DJ-1 antibody resulted in pronounced non-specific staining throughout the cell in DJ-1 KO MEFs, while the Abcam anti-DJ-1 antibody produced faint non-specific staining in the nucleus only of DJ-1 KO MEFs. Figure 2A-F shows PC12 cells and Wt and DJ-1 KO MEFs fixed in -20°C methanol and treated with either the Covance or Abcam anti-DJ-1 antibody at a concentration of 1:200. Figure 2G shows that incubation of Wt MEFs in secondary antibody only produced no non-specific background fluorescence. The absence of DJ-1 was confirmed by Western blot of whole cells lysates of DJ-1 KO MEFs. As a result, for all subsequent DJ-1 localization experiments, cells were fixed in methanol and probed with polyclonal anti-DJ-1 antibody from Abcam.

Rat cortical neurons and PC12 cells were used in DJ-1 localization experiments. Rat cortical neurons were plated on PLO-coated 35mm glass bottomed plates (WillCo Wells,
Figure 2: Optimization of primary DJ-1 antibodies for immunofluorescence. PC12 cells (A), Wt MEFs (B) and DJ-1 KO MEFs (C) were incubated in primary anti-DJ-1 antibody from Covance at a concentration of 1:200. Note prominent nuclear staining in DJ-1 KO MEFs. PC12 cells (D), Wt MEFs (E) and DJ-1 KO MEFs (F) were incubated in primary DJ-1 antibody from Abcam at a concentration of 1:200. (G) Wt MEFs treated with alexa fluor 488 goat anti-rabbit secondary antibody only. (H) Phase image of Wt MEFs in (G). All cells were fixed with methanol. Secondary antibody concentration used was 1:1000. Images were taken with a 60x phase-fluor objective, 1x zoom.
#HBST-3522) as described in section 2.3 and PC12 cells were plated as described in section 2.8.1. Rat cortical neurons were treated with 50μM H₂O₂ for 3h or 24h and PC12 cells were treated with 40μM PQ, 80μMPQ, 50μM H₂O₂, or 100μM H₂O₂ for 3h, 6h, or 24h. Cells were fixed with -20°C methanol (see section 2.8.1). The primary polyclonal anti-DJ-1 antibody from Abcam (Abcam, #ab18257) was diluted to 1:200, which was found to be the optimal dilution, in 1x PBS containing 3% GS and 5% BSA. The protocol outlined in section 2.8.2 was followed for immunofluorescence processing. Rat cortical neurons in 35mm glass bottom plates were processed as described in section 2.8.2 as well, except all washing and antibody incubations were performed directly in the plate and a 25mm circle coverslip (Fisher Scientific, #12-545-102) was mounted directly over the glass bottom.

2.8.4. Confocal fluorescence imaging

An inverted Nikon scanning confocal microscope (Bio-Rad MRC-600) equipped with an argon-ion laser was used for fluorescence imaging of DJ-1. Slides were imaged with a 20x fluor and 60x planApo objectives. SOM software (Bio-Rad) was used for imaging in PIC format and imaged were converted to TIFF format using Confocal Assistant v4.02.

2.9. Western blot analysis of CnA, DJ-1, NFATc4 protein expression

2.9.1. Preparation of whole cell lysates

Protein expression after oxidative stress was analyzed in PC12 cells, rat cortical neurons, and MEFs by Western blot. PC12 cells were plated at a density of 2.2 x 10⁶ cells in 100mm polystyrene plates. Rat cortical neurons were plated as described above in section 2.3. Wt and DJ-1 KO MEFs were plated at a density of 1.2 x 10⁶ cells in 100mm polystyrene plates. After treatment with PQ, H₂O₂, or menadione, PC12 cells and rat cortical neurons were collected in ice-cold 1x PBS into 15mL tubes (BD Falcon, #352097). MEFs were collected by rinsing cells once with ice-cold 1x PBS and detaching cells with 0.05% trypsin-EDTA. Ice-cold 1x PBS was added to the plate, trypsin was inactivated by adding FBS, and cells were collected into 15mL tubes. Cell suspensions were centrifuged for 3min at 1000rpm. The supernatant was aspirated and the cell pellet was washed once with ice-cold 1x PBS and transferred to a 1.5mL Eppendorf
tube (Sorenson, #16130). The suspension was centrifuged for 1 min at 9300 rcf and then the cell pellet was lysed in 200µL-400µL of cell lysis buffer (50mM Tris-HCl pH 7.5, 150mM NaCl, 1% Nonidet P-400, 5% sodium deoxycholate, 1 protease inhibitor cocktail tablet (Roche, #11836153001)/25mL final volume). Cells were lysed by pipetting up and down and brief vortexing. Whole cell lysates (WCL) were frozen immediately at -80°C.

2.9.2. Preparation of subcellular fractions with digitonin buffer

PC12 cells were exposed to 4h or 24h 40µM PQ to obtain cytoplasmic and mitochondrial-enriched fractions. After PC12 cells were plated and collected as described in section 2.8.1, cell pellets were resuspended in 100µL-200µL of digitonin release buffer (250mM sucrose, 17mM MOPS, 2.5mM EDTA, 0.8mg/mL digitonin, 1 protease inhibitor cocktail tablet (Roche, #11836153001)/25mL final volume) and incubated for 1 min at room temperature. Cell suspensions were transferred to pre-cooled 2mL glass homogenizers and cell membranes were disrupted with 30 strokes of a size B glass pestle. Cell suspensions were transferred back into Eppendorf tubes and centrifuged at 1200g for 5 min at 4°C to remove nuclei and unbroken cells. The supernatant was transferred to a fresh tube and centrifuged at 21,000g for 30 min at 4°C to pellet the mitochondria. The supernatant was transferred to a fresh tube and labelled as the cytoplasmic fraction (cp). The mitochondria-enriched pellet was lysed in 50µL-100µL of cell lysis buffer by pipetting up and down and vortexing and labelled as the mitochondrial fraction (mt). Fractions were frozen immediately at -80°C.

2.9.3. Preparation of cytoplasmic, mitochondrial, and nuclear fractions of PC12 cells with Active Motif kits

As digitonin may disrupt the OMM and interfere with potential interactions between DJ-1 and the OMM, a digitonin-free fractionation protocol was also used to sub-fractionate for mitochondrial fractions. PC12 cells were plated at a density of 2.2 x 10⁶ cells in 100mm polystyrene plates. Cells were treated with 50µM H₂O₂ for 3h. Cytoplasmic, mitochondrial, and nuclear subcellular fractions were prepared from the same PC12 cell samples using the Mitochondrial Fractionation Kit (Active Motif, #40015) and the Nuclear Extract Kit (Active
Motif, #40010). In brief, cells were collected as described above in section 2.8.1. After the first centrifugation, cell pellets were resuspended in ice-cold 1x Cytosolic Buffer (Active Motif, #40015) to disrupt cell membranes and incubated on ice for 15min. Cell suspensions were transferred to pre-cooled glass homogenizers and cell membranes were broken by 80 strokes with a size B glass pestle on ice. Homogenized samples were assessed under the microscope to ensure that all cells were lysed. The supernatant was transferred to a fresh 1.5mL Eppendorf tube and centrifuged at 800g for 20min at 4°C. The supernatant (S1) contains the cytosol, including the mitochondria, whereas the pellet (P1) contains the nuclei, cellular debris, and intact cells. To prepare the mitochondrial fraction, the S1 supernatant was transferred to a fresh 1.5mL Eppendorf tube and centrifuged at 800g for 10min at 4°C to remove residual nuclei. The supernatant was transferred to a fresh 1.5mL Eppendorf tube and centrifuged at 10,000g for 20min at 4°C to pellet the mitochondria. The resulting supernatant (S2) is the cytosolic fraction and was transferred to a fresh 1.5mL Eppendorf tube and reserved on ice. The mitochondrial pellet was gently washed with 1x Cytosolic Buffer (Active Motif, #40015) and centrifuged at 10,000g for 10min at 4°C. The supernatant was discarded and the previous wash and spin was repeated. The supernatant was discarded and the mitochondrial pellet was lysed with Complete Mitochondria Buffer (Active Motif, #40015) by incubation on ice for 15min. After incubation, the mitochondrial fraction (mt) was vortexed for 10 seconds and frozen immediately at -80°C.

To prepare the cytoplasmic fraction, the S2 supernatant was centrifuged at 16,000g for 25min at 4°C to remove residual mitochondrial. The supernatant was transferred to a fresh 1.5mL Eppendorf tube and labelled as the cytoplasmic fraction (cp). Fractions were stored immediately at -80°C.

To prepare the nuclear fraction, the P1 pellet was resuspended in Complete Lysis Buffer (Active Motif, #40010) and vortexed for 10 seconds. The nuclear suspension was incubated on a rocking platform for 30min at 4°C and then vortexed for 30 seconds. The suspension was centrifuged at 14,000g for 10min at 4°C. The resulting supernatant was transferred to a fresh 1.5mL Eppendorf tube and labelled as the nuclear fraction (nc). Fractions were stored immediately at -80°C.
2.9.4. Preparation of cytoplasmic and nuclear fractions of rat cortical neurons and MEFs with an Active Motif kit

Rat cortical neurons were exposed to 1h, 4h or 24h of 50µM H₂O₂ and Wt and DJ-1 KO MEFs were exposed to 2h of 100µM H₂O₂. Both rat cortical neurons and MEFs were collected as in section 2.9.1 above, but in 1x PBS containing phosphatase inhibitors (Active Motif, #40010). After centrifugation at 1000g for 3min, the cell pellet was resuspended in 1x Hypotonic Buffer (Active Motif, #40010) to swell cell membranes and transferred to a fresh 1.5mL Eppendorf tube for incubation on ice for 15min. After the 15min incubation, detergent (Active Motif, #40010) was added to the suspensions and vortexed for 10 seconds to disrupt cell membranes. This procedure was sufficient to lyse rat cortical neurons; however, MEFs required additional douncing in a glass homogenizer with a size B pestle for 50 strokes on ice in order to lyse. Complete cell lysis was always assessed under the microscope prior to centrifugation. Suspensions were centrifuged in 1.5mL Eppendorf tubes at 14,000g for 30 seconds at 4°C. The supernatant was transferred to a fresh Eppendorf tube, labelled as the cytoplasmic fraction (cp), and frozen immediately at -80°C. The pellet was resuspended in Complete Lysis buffer (Active Motif, #40010) and vortexed for 10 seconds. The nuclear suspension was incubated on a rocking platform for 30min at 4°C and then vortexed for 30 seconds. The suspension was centrifuged at 14,000g for 10min at 4°C. The resulting supernatant was transferred to a fresh 1.5mL Eppendorf tube and labelled as the nuclear fraction (nc). Fractions were stored immediately at -80°C.

2.9.5. Determination of total protein concentration in WCL and subcellular fractions

The DC (detergent compatible) Protein Assay (BioRad, #500-0116), which is a modified version of the Lowry assay, was used for determination of protein concentration in WCL, cp, mt, and nc fractions. The quantification of protein concentration depends upon the reaction of the amino acids tyrosine, tryptophan, cystine, cysteine, and histidine in protein with an alkaline copper tartrate solution and Folin reagent, leading to blue colour development that is proportional to the amount of protein present. The absorbance ranges from 405nm to 750nm. The manufacturer's protocol for micro-plate assays was followed. A protein standard curve was generated by determining the absorbance of known concentrations of a protein standard
(BioRad, #500-0005). Protein concentrations for each WCL, cp, mt, and nc sample were determined by comparing their absorbances with the protein standard curve. Measurements for each protein standard and sample were performed in triplicates. Absorbance was read at 630nm using an ELISA plate reader.

2.9.6. SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Total protein load depended upon the protein to be detected. Typically, 50µg of total protein was loaded to detect CnA in all cell types and NFATc4 in rat cortical neurons. 20µg of total protein was loaded to detect DJ-1 in all cell types and NFATc4 in MEFs. The volume of lysate needed for the required protein load was determined using the protein concentration obtained as described in section 2.8.5. All samples were adjusted to the same volume with cell lysis buffer and a volume of 2x loading buffer (62.5mM Tris-HCl pH 6.8, 2% SDS, 0.00125% bromophenol blue, 10% glycerol, 715mM β-mercaptoethanol) equal to the largest total volume was added to all samples. All samples were boiled for 5min in a boiling water bath to denature the proteins. Mitochondrial fractions were loaded in triplicate in lieu of a mitochondrial loading control to control for equal protein loading, as oxidative stress may affect the levels of mitochondria proteins. The proteins in the samples were resolved on a 12% polyacrylamide gel at 30V through the stacking gel and at 70V through the running gel. The proteins were transferred to a PVDF membrane (PALL, #66543) overnight at 30V at 4°C. The next day, the membranes were washed with TBS-T (20mM Tris, 137mM NaCl, 3.8mM HCl, and 0.1% Tween-20, adjusted to pH 7.6) and then blocked for 1h at room temperature in 5% non-fat milk TBS-T to prevent non-specific antibody binding in the next step.

2.9.7. Detection of CnA, DJ-1, NFATc4

After blocking, membranes were incubated in primary antibodies overnight on an orbital shaker at 4°C. All primary antibodies were diluted in 5% non-fat milk TBS-T, except the anti-pan-calcineurin A antibody from Cell Signalling, which was diluted in 5% BSA TBS-T. The primary antibodies used are as follows:
- Anti-calcineurin (α-Subunit) mouse monoclonal antibody (Sigma, #C1956), 1:10,000 dilution, used in PC12 cells and rat cortical neurons

- Anti-pan-calcineurin A rabbit polyclonal antibody (Cell Signalling, #2614), 1:1000 dilution, used in all cell types

- Anti-DJ-1 rabbit polyclonal antibody (Covance, #SIG-39835), 1:5000 dilution, used in PC12 cells after PQ and MEFs

- Anti-DJ-1 rabbit polyclonal antibody (Abcam, #ab18257), 1:1000 dilution, used in PC12 cells after H$_2$O$_2$ and MEFs

- Anti-NFATc4 (H-74) rabbit polyclonal antibody (Santa Cruz, #sc-13036), 1:100 dilution, used in rat cortical neurons and MEFs

- Anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mouse monoclonal antibody (Calbiochem, #CB-1001), 1:15,000 dilution, used in all cell types

- Anti-TOM20 rabbit polyclonal antibody (Santa Cruz, #sc-11415), 1:2000 dilution, used in all cell types

- Anti-histone H3 rabbit polyclonal antibody (Cell Signalling, #9715), 1:1000 dilution, used in all cell types

- Anti-β-actin-peroxidase mouse monoclonal antibody (Sigma, #A3854), 1:50,000 dilution, used in all cell types

After washing 3 times with TBS-T for 10min each time, membranes were incubated in horseradish peroxidase (HRP)-linked secondary antibodies for 1h at room temperature on an orbital shaker. The secondary antibodies used are as follows:

- HRP-linked anti-rabbit donkey antibody (Amersham, #NA-934)
  - 1:2000 for anti-NFATc4, anti-histone H3
  - 1:5000 for anti-pan-calcineurin A, anti-TOM20, both anti-DJ-1

- HRP-linked anti-mouse sheep antibody (Amersham, #NA-931)
  - 1:2000 for anti-calcineurin (α-Subunit)
- 1:5000 for anti-GAPDH

The membranes were washed 3 times for 10min each with TBS-T. Membranes were rinsed with Western Lightning Chemiluminescence Reagent Plus (Perkin Elmer, #NEL-105) for 1min with shaking. Protein bands were then visualized through the recording of chemiluminescence onto X-ray film (Denville Scientific, #E-3018, 2012).

Figure 3 illustrates that both the primary anti-CnA antibodies from Sigma and Cell Signaling label the 32kDa cleavage fragment of CnA. The anti-CnA antibody from Cell Signaling was used for all subsequent experiments due to its strong signal, reusability, and lower cost.

**2.9.8. Densitometry and Quantification of Results**

Quantity One software (BioRad) was used to analyze scanned X-ray films. A box was drawn around the largest band on the film and the same box was copied and pasted around all other bands. To obtain background signal, the box was copied and pasted to an area above or below the protein band in the same lane. Figure 4 illustrates how bands are outlined in boxes for densitometry. Optical densities of all bands were measured in OD/mm². Final sample band density was calculated by subtracting background density from the measured protein band density. Bands from whole cell lysates and cytoplasmic fractions were normalized to the OD/mm² for GAPDH. Some cytosolic fractions were normalized to the OD/mm² for β-actin. TOM20 was used to check for purity of cytoplasmic and nuclear fractions and to confirm mitochondrial fractions. Triplicate band OD/mm² for mitochondrial fractions were averaged. Nuclear fractions were normalized to the OD/mm² for histone H3 where possible; in cases where histone H3 was not detectable, treatment bands were compared directly to control bands.

**2.10. Calcineurin Activity Assay**

**2.10.1. Sample Preparation**

The Calcineurin Cellular Assay Kit Plus (Biomol, #AK-816) was used to assess Cn activity. Free phosphate released due to Cn activity is complexed by Malachite green and
Figure 3: Western blot illustrating the ability of both anti-CnA antibodies to detect cleaved CnA. Rat cortical neurons were treated with menadione and Western blotting was performed on WCL. The anti-CnA antibodies from Sigma and Cell Signaling both detected cleaved CnA.

Figure 4: Sample scanned x-ray film illustrating increases in band size and intensity with increasing total protein loading. This Western blot was prepared by loading wells with 5μg, 10μg, or 20μg of total protein from WCL of PC12 cells. Band density in OD/mm² was quantified by drawing a box around the heaviest band and duplicating this box around all other bands. Background density was obtained by duplicating the box in each lane. Final sample density was determined by subtracting the corresponding background density from the band density for each lane.
absorbance is read at 630nm on an ELISA plate reader. For the assay, PC12 cells were plated at a density of 2.2 x 10^6 cells/plate and 2 plates were pooled per sample. Rat cortical neurons were plated as described in section 2.3 and 2 plates were also pooled per sample. Cells were collected in ice-cold tris-buffered saline (TBS) and centrifuged at 1000g for 3min to pellet cells. The supernatant was discarded and the cell pellet was washed with ice-cold TBS and transferred to a pre-cooled 1.5mL Eppendorf tube. The cell suspension was centrifuged at 9300rcf for 1min to pellet the cells. The resulting cell pellet was lysed with lysis buffer plus protease inhibitor by pipetting and vortexing. As a refrigerated microfuge with speeds of 100k x g to 200k x g was not available, the lysate was centrifuged at 21,000g for 3h at 4°C to obtain the high speed supernatant, which was transferred to a fresh tube and frozen immediately at -80°C. The pellet was discarded.

2.10.2. Removal of free phosphate from the high speed supernatant

Free phosphate and nucleotides have to be removed from the samples prior to performing the Cn activity assay because excess phosphate in cell lysates interfere with the activity assay. Desalting resin provided with the kit was rehydrated in ddH_2O overnight at 4°C. The next day, ddH_2O was decanted from the rehydrated resin and fresh ddH_2O was added in equal volume. The rehydrated resin was added to the desalting column to achieve a settled height of 5cm after ddH_2O had drained by gravity. To equilibrate the resin, 8mL lysis buffer was added and allowed to drain by gravity. The column with resin was fitted over a 15mL conical tube (BD Falcon, #352097) and centrifuged at 800g for 3min. The waste run-off was discarded. The column with resin was fitted over a fresh pre-cooled 15mL conical tube, 300μL of high speed supernatant was added to the resin, and everything was centrifuged at 800g for 3min. The run-off in the 15mL tube is the desalted phosphate-free lysate and was collected into a fresh 1.5mL Eppendorf tube and frozen immediately at -80°C. Resin was transferred back into rehydrating tube and rinsed with fresh ddH_2O between samples. The above procedure was repeated for all samples. To qualitatively assess if phosphate removal was complete, 100μL of Biomol Green reagent was added to 1μL of desalted lysate. Phosphate removal was deemed successful if the Biomol Green reagent-lysate mixture remained yellow after 30min at room temperature. If the mixture turned green, then phosphate removal was not complete and the procedure was repeated to remove residual phosphates. Typically, resin was used to desalt a
maximum of six samples and separate resin columns were used for control and experimental lysates.

2.10.3. Protein quantification in desalted lysates

Protein quantification was performed using the DC Protein Assay (BioRad, #500-0116) as described in section 2.8.5. Total protein of 5µg or more results in precipitation in the Cn Activity Assay, which interferes with absorbance readings. Hence, desalted lysates were diluted with lysis buffer so that no more than 5µg of total protein were used for each well of the assay.

2.10.4. Cn Activity Assay

All kit components and desalted lysates were thawed on ice. The assay was performed in a 96-well plate. 1x assay buffer was prepared by diluting 2x assay buffer in a 1:1 dilution with ddH$_2$O. Wells with phosphate standards were prepared by serial dilution of 80µM phosphate standard in 1x assay buffer to obtain 0.031-2nmol phosphate. CaM was diluted 1:50 in 2x assay buffer. Background wells contained 20µL ddH$_2$O and 25µL CaM assay buffer. Wells representing total phosphatase activity (total wells) contained 10µL ddH$_2$O and 25µL CaM assay buffer. Wells representing total phosphatase activity less Cn activity (EGTA wells) contained 10µL ddH$_2$O and 25µL 2x EGTA buffer; the lack of CaM in these wells, combined with the chelating of free Ca$^{2+}$ by EGTA, results in the inhibition of Cn activity. Positive control wells contained 10µL ddH$_2$O and 25µL CaM assay buffer. RII phosphopeptide, which is a Cn specific substrate, was reconstituted with 915µL ddH$_2$O per 1.5mg vial. 10µL RII phosphopeptide was added to all wells except background wells and phosphate standard wells and allowed to incubate at 30°C for 10min to equilibrate the samples to the reaction temperature. To initiate the Cn activity assay, 5µL of desalted sample lysate was added to background, total, and EGTA wells. Purified recombinant Cn was diluted to 8U/µL prior to addition into the positive control wells. The microplate was allowed to incubate at 30°C for 30min. After incubation, reactions were terminated by the addition of 100µL of Biomol Green™ reagent to all wells. The microplate was incubated at room temperature for 30min to
allow the colour reaction to occur completely. An ELISA plate reader was used to read the microplate at OD\textsubscript{620nm}.

2.10.5. Analysis of Cn activity

Absorbance from background wells was subtracted from the absorbances of total wells and EGTA wells. The resulting EGTA well absorbance was subtracted from the Total well absorbance to give a measure of absorbance due to Cn activity. Absorbances were converted to nmol of phosphate released using the phosphate standard curve. Phosphate released was normalized to total protein. The greater the amount of phosphate released, the greater the Cn activity. All values were expressed as nmol of phosphate released per microgram of protein in the lysate.

2.11. Statistical Analysis

The unpaired Student’s t-test or one-way ANOVA was used to assess statistical significance. The test used for each experiment is indicated under each Results section. Where data were found to be significant by one-way ANOVA, Bonferroni’s post-hoc comparison between groups was performed. Data were analyzed using Microsoft Office Excel equipped with the Data Analysis Toolpak and GraphPad Instat 3 software (La Jolla, CA). A p-value less than 0.05 was accepted to be statistically significant. In the case of MEFs, data were expressed as a percent of vehicle control of the respective MEF line unless otherwise stated.
RESULTS
3.1. PQ reduced cell viability and increased cell death in PC12 cells

PQ is a redox cycling compound that primarily generates oxidative stress through the production of superoxide, which is then dismutated by SOD to H$_2$O$_2$ (reviewed in Dinis-Oliveira et al., 2006). To establish a sub-lethal dose of PQ for PC12 cells, cells were differentiated for six days and then treated with 10µM-100µM PQ for 24h or 48h. Unless otherwise indicated, all experiments used PC12 cells differentiated for six days. Cell viability was assessed by the MTT reduction assay 24h and 48h post-PQ (Figure 5). The MTT reduction assay is a colorimetric assay in which yellow MTT is converted into purple formazan crystals by reductases in living cells only, and thus is commonly used as a measure of cell viability (Berridge et al., 2005). A 24h exposure to 10µM-100µM PQ did not reduce PC12 cell viability (PQ versus control, n = 5 experiments, 8 samples/experiment). By 48h, exposure to 80µM PQ and 100µM PQ significantly reduced viability to 52% ± 4% and 41% ± 5% versus controls, respectively (p < 0.001, PQ versus control, n = 4 experiments, 8 samples/experiment). Data were analyzed using one-way ANOVA with Bonferroni post-hoc analysis.

To assess cell death, PC12 cells were treated with 10µM-100µM PQ for 24h or 48h, and cells were labelled with PI. Controls received vehicle (water) and cell death was quantified by flow cytometry. Figure 6A shows representative flow cytometry traces of relative PI fluorescence signal plotted against cell count in control cells (left panel) and in cells treated with 100µM PQ (right panel) for 48h. After 24h, cell death in PQ-treated cultures was not different from control cultures (Figure 6B; PQ versus control, n = 3 experiments, 3 samples/experiment). By 48h, cell death increased from 16% ± 1% in controls to 44% ± 4% and 45% ± 4% in cells exposed to 80µM PQ and 100µM PQ, respectively (Figure 6B; p < 0.001, PQ versus control, n = 4 experiments, 3 samples/experiment). Data were analyzed using one-way ANOVA with Bonferroni post-hoc analysis. Collectively, these data indicate that concentrations of PQ up to 100µM for 24h and up to 40µM for 48h were sub-lethal, and consequently were used in all subsequent experiments on PC12 cells.
Figure 5: MTT reduction assay: sustained PQ exposure decreased cell viability in differentiated PC12 cells. (A) Representative graph of raw data obtained from one experiment using the MTT reduction assay, with absorbance at 620nm given as Arbitrary Units (A.U.). (B) By 48h, cell viability was reduced to 52% ± 4% and 41% ± 5% versus controls for doses of 80μM PQ and 100μM PQ, respectively. Values shown are the averages of 5 experiments (n = 5) for 24h and 4 experiments (n = 4) for 48h, with 8 samples per experiment. ** denotes p < 0.001. Error bars shown are ± SEM.
Figure 6: Flow cytometry: PI uptake revealed increased cell death in PC12 cells after sustained PQ exposure. (A) Representative flow cytometry traces of relative PI fluorescence signal plotted against cell count in control cells and in cells treated with 100μM PQ for 48h. Red bar indicates population of dead cells in the sample. (B) By 48h, cell death was significantly increased from 16% ± 1% in water-treated controls to 44% ± 4% and 45% ± 4% for doses of 80μM PQ and 100μM PQ, respectively. Values shown are the averages of 3 experiments (n = 3) for 24h and 4 experiments (n = 4) for 48h, with each sample performed in triplicate per experiment. * denotes p < 0.05 and ** denotes p < 0.001. Error bars shown are ± SEM.
3.2. H$_2$O$_2$ reduced cell viability in PC12 cells after 24h

H$_2$O$_2$ can readily cross biological membranes and was used to assess the effects of treatment with direct oxidative stress (reviewed in Armstrong & Whiteman, 2007). PC12 cell viability was assessed by the MTT reduction assay 24h after initial exposure to 12.5μM-5mM H$_2$O$_2$ (Figure 7). Preliminary experiments revealed that decreases in cell viability in response to H$_2$O$_2$ are cell density-dependent (data not shown) and that NGF is protective against H$_2$O$_2$-induced oxidative stress in differentiated PC12 cells (Satoh et al., 1996)). When plated at a density of 25,460 cells/cm$^2$, H$_2$O$_2$ caused a significant reduction in cell viability in the presence of NGF. After exposure to 200μM H$_2$O$_2$ or higher for 24h, MTT reduction values in H$_2$O$_2$ treated cells were significantly reduced (Figure 7; p < 0.05, n = 3 experiments, H$_2$O$_2$ versus control, 8 samples/experiment). Data were analyzed using one-way ANOVA with Bonferroni post-hoc analysis. Consequently, in subsequent experiments, concentrations up to 100μM for 24h were used as sub-lethal H$_2$O$_2$ doses in PC12 cells.

3.3. Menadione severely compromised cell viability in PC12 cells by 4h

Menadione, also known as vitamin K$_3$, is a superoxide-producing redox cycling compound that has been shown to exhibit anti-cancer effects through generation of oxidative stress (Kuriyama et al., 2005; Osada et al., 2001; Sasaki et al., 2008). To establish a sub-lethal dose of menadione, PC12 cells were treated with 2μM-50μM menadione and cell viability was assessed by the MTT reduction assay at 4h and 24h post-addition of menadione (Figure 8). Cell viability was significantly reduced by 4h and 24h exposures (p < 0.05, n = 7 experiments for 4h, n = 8 experiments for 24h, menadione versus control, 8 samples/experiment). Data were analyzed using one-way ANOVA with Bonferroni post-hoc analysis. Menadione concentrations ranging from 2μM to 10μM for 4h were used as sub-lethal doses in all subsequent experiments.

3.4. Oxidative stressors compromised cell viability in rat cortical neurons

Rat cortical neurons cultured for five days were treated with H$_2$O$_2$, PQ, or menadione to induce oxidative stress, and cell viability was assessed by the MTT reduction assay. Exposure to 5μM - 30μM PQ for 24h reduced neuronal viability (Figure 9; to 85% ± 3% of control for
Figure 7: MTT reduction assay: 24h H₂O₂ exposure decreased cell viability in differentiated PC12 cells. PC12 cells were plated at a density of 25,460 cells/cm² and exposed to 12.5μM - 5mM H₂O₂ for 24h in the presence of NGF. Cell viability was significantly decreased by 200μM H₂O₂ or greater. Values shown are the averages of 3 experiments (n = 3), with 8 samples per experiment. * denotes p < 0.05 and ** denotes p < 0.001. Error bars shown are ± SEM.

Figure 8: MTT reduction assay: 4h and 24h menadione exposure decreased cell viability in PC12 cells. Cell viability was significantly decreased at all menadione concentrations at 4h and 24h, with the exception of 24h 2μM menadione exposure, which incurred an increase versus ethanol-treated controls. Values shown are the averages of 8 experiments (n = 8), with 8 samples per experiment. * denotes p < 0.05 and ** denotes p < 0.001. Error bars shown are ± SEM.
Figure 9: MTT reduction assay: 24h PQ exposure decreased cell viability in rat cortical neurons. Cell viability was assessed in rat cortical neurons that had been exposed to 2.5μM - 30μM PQ for 24h. Cell viability was significantly decreased after 24h 5μM - 30μM PQ exposure versus water treated controls. Values shown are the averages of 7 experiments (n = 7), with 8 samples per experiment. ** denotes p < 0.001. Error bars shown are ± SEM.
5μM PQ; 63% ± 2% of control for 30μM PQ) (p < 0.001, PQ versus control, n = 7 experiments, 8 samples/experiment). Exposure of cortical neurons to 30μM PQ for 24h was found to significantly increase cell death, as assessed by fluorescence microscopy and PI (data not shown). Consequently, PQ concentrations up to 20μM applied for 24h were used as sub-lethal doses in all later experiments. Exposure to H₂O₂ reduced cell viability significantly at all time points (1h, 4h, 24h) for concentrations ranging from 25μM - 200μM (Figure 10; p < 0.05, H₂O₂ versus control, n = 3 experiments, 8 samples/experiment). For all subsequent experiments, 50μM H₂O₂ was used as a sub-lethal dose in rat cortical neurons (cell viability: 48% ± 6% of control at 24h). Menadione severely decreased cell viability at concentrations ranging from 5μM - 50μM for 4h or 24h; however, a 4h 2μM menadione exposure increased cell viability to 112% ± 4% relative to control (Figure 11; p < 0.05, menadione versus control, n = 4 experiments, 8 samples/experiment). 4h treatment of rat cortical neurons with 2μM to 10μM menadione were used as sub-lethal doses in all following experiments. As summarized in Figure 12, menadione was the most effective stressor in terms of decreasing cell viability. All data were analyzed using one-way ANOVA with Bonferroni post-hoc analysis.

3.5. CnA levels did not change after short term PQ exposure in PC12 cells

Cn has previously been shown to be sensitive to oxidative stress, although a literature search indicates that the exact effects of oxidative stress on Cn expression and activation are unclear (Lee et al., 2007; See & Loeffler, 2001). Cn activation has been linked to the initiation of apoptosis, while the inhibition of Cn activity has been reported to be protective against cell death (Asai et al., 1999; Jayanthi et al., 2005). Furthermore, CnA is reported to be susceptible to H₂O₂-induced inactivation by cleavage in mouse primary cortical neurons, leading to reduced activation of NFATc4 and subsequent anti-apoptotic effects (Lee et al., 2007). Therefore, the expression level and activity of Cn may be important in cell survival/death responses after short-term exposure to oxidative stress. To determine whether PQ, a redox cycling compound, induces changes in CnA levels and CnA cleavage, CnA protein levels in WCL of PC12 cells after short term exposure (30min to 4h) to 40μM or 80μM PQ were assessed by Western blot. Both anti-CnA antibodies were used to probe for CnA expression. Sub-lethal oxidative stress is not expected to alter the expression of GAPDH. Furthermore, the oxidative stressors, doses and
Figure 10: MTT reduction assay: 1h, 4h, and 24h H2O2 exposure decreased cell viability in rat cortical neurons. Rat cortical neurons were exposed to 12.5μM - 200μM H2O2 for 1h, 4h, or 24h. Cell viability was significantly decreased at H2O2 concentrations from 25μM - 200μM at all time points tested versus water treated controls. Values shown are the averages of 4 experiments (n = 4) for 1h and 4h and 3 experiments (n = 3) for 24h, with 8 samples per experiment. * denotes p < 0.05 and ** denotes p < 0.001. Error bars shown are ± SEM.
Figure 11: MTT reduction assay: 4h and 24h menadione exposure decreased cell viability in rat cortical neurons. Cell viability was significantly decreased by 4h 5μM - 50μM menadione exposure and remained compromised at 24h. 4h 2μM menadione treatment resulted in significantly increased cell viability to 112% ± 4% relative to ethanol treated controls. Values shown are the averages of 4 experiments (n = 4), with 8 samples per experiment. * denotes p < 0.05 and ** denotes p < 0.001. Error bars shown are ± SEM.
Figure 12: Summary graph of differential cell viabilities of rat cortical neurons after exposure to PQ, H$_2$O$_2$, or menadione. (A) Main graph shows MTT reduction as percent of control values plotted against concentration in µM. Trendlines were fitted to the scatter plots using exponential regression. Associated correlation coefficients are shown. The trendline for 24h PQ was extrapolated by 30 concentration units for clarity. (B) Graph in inset magnifies clustering in the main graph from 0µM-50µM. The trendline for 24h PQ was extrapolated by 20 concentration units.
treatment durations used in this thesis did not change GAPDH expression, thus GAPDH was a suitable loading control for the experiments outlined in this thesis. CnA levels were unchanged at all time points and PQ concentrations tested (see Figure 13A-B). In addition, no CnA cleavage fragments were detected after PQ exposure in PC12 cells (n = 4 experiments, 1 sample/experiment). Data were analyzed using one-way ANOVA with Bonferroni post-hoc analysis.

3.6. CnA levels in PC12 cells were decreased after a 24h or 48h exposure to PQ

Unlike H$_2$O$_2$, which induces immediate oxidative stress, PQ requires redox cycling to generate ROS. Therefore, ROS production after short-term PQ exposure may not have been sufficient to generate significant cellular effects. To assess if changes in CnA levels after PQ stress are time-dependent, PC12 cells were exposed to 10µM to 100µM PQ for sustained periods of 24h or 48h. CnA levels were assessed by Western blot of WCL with the primary anti-CnA antibody from Sigma (Figure 14A). On average, CnA levels were significantly reduced to 59% ± 9% and 61 ± 8% relative to time-matched controls after 24h 80 µM and 100 µM PQ exposure, respectively and this decline was sustained at 48h (see Figure 14B). It should be noted that although CnA levels for 48h 80µM and 100µM PQ treatment are shown, these two doses were toxic to PC12 cells at 48h. CnA levels after 48h 80µM and 100µM PQ were found to be significantly decreased to 41% ± 14% and 31% ± 11% versus control, respectively (p < 0.05, n = 4 experiments, 1 sample/experiment). Data were analyzed using one-way ANOVA with Bonferroni post-hoc analysis.

3.7. CnA is cleaved after menadione exposure in PC12 cells

Lee et al. (2007) showed that CnA was cleaved after oxidative stress from H$_2$O$_2$, whereas I found that oxidative stress induced by PQ decreased whole CnA levels after sustained exposure, but did not cleave CnA at all time points tested (see sections 3.5 and 3.6). To address whether CnA cleavage occurs after exposure to other redox cycling compounds, CnA levels and cleavage were examined in PC12 cells after exposure to menadione. Western blotting of WCL of PC12 cells exposed to 4h 2µM - 10µM menadione was used to assess CnA cleavage (Figure
Figure 13: Western blot: PQ exposure did not alter CnA levels in PC12 cells. (A) Representative Western blot of CnA expression in WCL of PC12 cells exposed to 40µM or 80µM PQ for 30min – 4h. GAPDH was used as a loading control. (B) CnA levels did not change relative to time-matched controls after short-term PQ exposure at these doses. Values shown are the averages of 4 experiments per treatment (n = 4). p > 0.05 for all comparisons. Error bars shown are ± SEM.
Figure 14: Western blot: sustained PQ exposure decreased CnA levels in PC12 cells. (A) Representative Western blot of CnA expression in WCL of PC12 cells exposed to 10μM - 100μM PQ for 24h or 48h. GAPDH was used as a loading control. (B) CnA levels decreased to 59% ± 9% and 61% ± 8% relative to time-matched controls after 24h exposure to 80μM and 100μM PQ, respectively. By 48h, CnA levels decreased to 41% ± 14% and 31% ± 11% versus controls for doses of 80μM and 100μM PQ, respectively. Values shown are the averages of 4 experiments per treatment (n = 4). * denotes p < 0.05. Error bars shown are ± SEM.
Primary anti-CnA antibody from Cell Signaling was used to probe for CnA. A 32kDa CnA cleavage fragment that corresponds to the inactive CnA fragment reported by Lee et al. (2007) was detected after 4h 10μM menadione exposure that was 3.5 ± 0.7 fold of control (Figure 15A-B; p < 0.05, n = 6 experiments, 1 sample/experiment). In this thesis, uncleaved, full-length 60kDa CnA will be referred to as “whole CnA”. Whole CnA levels were not decreased at all concentrations tested (Figure 15C). Control levels of the CnA cleavage fragment were negligible. Effects of long-term menadione exposure on CnA were not assessed due to high toxicity of menadione after sustained exposure. Data were analyzed using one-way ANOVA with Bonferroni post-hoc analysis.

3.8. Cn activity did not change after PQ exposure in PC12 cells

Whole CnA levels are not always reflective of Cn activity because Cn can be inactivated by oxidation of the metal centre in its substrate binding cleft instead of through proteolytic cleavage (Namgaladze et al., 2002). To address whether PQ induced inactivation of Cn that was independent of CnA cleavage, Cn activity was assessed using the Biomol Calcineurin Activity Assay Kit, which provides a measure of Cn activity based on the amount of free phosphate released. Cn activity was measured in PC12 cells exposed to 1h, 2h, 4h, or 24h of 40μM PQ and was found to be unchanged at all time points tested (Figure 16; n = 3 experiments, 1 sample/experiment). However, in the absence of a positive control, it is as yet unclear whether this assay can detect changes in Cn activity. Data were analyzed using one-way ANOVA with Bonferroni post-hoc analysis.

3.9. PQ treatment decreased CnA levels by 24h in rat cortical neurons

As oxidative stress induced by PQ did not cause cleavage of CnA in PC12 cells, rat primary cortical neurons were exposed to PQ to assess potential cell-type specific effects of PQ on CnA. Rat cortical neurons were exposed to 10μM or 20μM PQ for 4h to 24h and CnA levels in WCL were measured by Western blot (Figure 17A). The primary anti-CnA antibody from Sigma was used to probe for CnA. CnA levels remained unchanged at up to 16h of PQ exposure, but significantly decreased by 24h to 89% ± 3% and 47% ± 4% versus control at
Figure 15: Western blot: 4h 10μM menadione exposure resulted in cleavage of CnA in PC12 cells. (A) Representative Western blot of CnA expression and cleavage in WCL of PC12 cells exposed to 2.5μM - 10μM menadione for 4h. GAPDH was used as a loading control. CnA cleavage product is outlined in the black oval. (B) CnA cleavage was significant after 4h 10μM menadione and was 3.5 ± 0.7 fold of control. (C) Whole CnA levels did not change at all concentrations of menadione tested. Values shown are the averages of 6 experiments per treatment (n = 6). * denotes p < 0.05. Error bars shown are ± SEM.
Figure 16: Cn activity was unchanged after 1h-24h PQ exposure in PC12 cells. Cn activity was measured by the Biomol Green Calcineurin Activity Assay and is represented by nmol of phosphate released per µg of protein in the lysate analyzed. Cn activity did not change after 1h to 24h of 40µM PQ exposure in PC12 cells. Values shown are the averages of 3 experiments per treatment (n = 3). p > 0.05 for all comparisons. Error bars shown are ± SEM.
Figure 17: Western blot: CnA levels were decreased by 24h 10μM PQ exposure in rat cortical neurons. (A) Representative Western blot of CnA levels in WCL of rat cortical neurons that were exposed to 10μM or 20μM PQ for 4h – 24h. No CnA cleavage fragment was detected. GAPDH was used as a loading control. (B) 10μM and 20μM PQ decreased CnA levels to 89% ± 3% and 47% ± 4% of control by 24h. Values shown are the averages of 5 experiments (n = 5). * denotes p < 0.05. Error bars shown are ± SEM.
concentrations of 10μM and 20μM PQ, respectively (Figure 17B; p < 0.05, n = 5 experiments, 1 sample/experiment). Similar to results in PC12 cells, CnA cleavage fragments were not detected at any time point and concentration of PQ tested. CnA after shorter (1h - 3h) PQ exposures were also examined and no changes in CnA levels or cleavage were observed (data not shown), which is similar to the results observed in PC12 cells. Data were analyzed using one-way ANOVA with Bonferroni post-hoc analysis.

3.10. Menadione induced cleavage of CnA in rat cortical neurons

As discussed in sections 3.5 and 3.6, PQ did not cause CnA cleavage in PC12 cells. Furthermore, PQ did not cause cleavage of CnA in rat cortical neurons (see section 3.9). Menadione, another redox cycling compound, did induce CnA cleavage in PC12 cells (see section 3.7), consequently rat cortical neurons were treated with 2μM - 10μM menadione for 4h and CnA cleavage was examined by Western blot of WCL (Figure 18A). A prominent CnA cleavage fragment of approximately 32kDa was detected after 4h 10μM menadione (Figure 18B), however whole CnA levels were not decreased at all concentrations tested (Figure 18C; p < 0.05, n = 7 experiments, 1 sample/experiment). Control levels of the CnA cleavage fragment were negligible. Data were analyzed using one-way ANOVA with Bonferroni post-hoc analysis.

3.11. H₂O₂ induced cleavage of CnA in rat cortical neurons

A previous study showed that H₂O₂ exposure resulted in cleavage of CnA into an inactive 32kDa fragment in mouse primary cortical neurons (Lee et al., 2007). The use of H₂O₂ as an oxidative stressor provided a means of assessing the effects of direct ROS addition on CnA levels in rat cortical neurons. Figure 19A is a representative Western blot of CnA levels after H₂O₂ exposure in rat cortical neurons and Figure 19B shows the raw densitometry ratios of CnA normalized with GAPDH for the blot presented in Figure 19A. Western blot revealed a prominent 32kDa cleavage fragment of CnA after 1h, 4h, and 24h 50μM H₂O₂ exposure that was 7 ± 1.6 fold, 5.9 ± 1.5 fold, and 4.0 ± 0.7 fold of control, respectively (Figure 20A). This 32kDa cleavage product of CnA has previously been reported to be inactive and has no
Figure 18: Western blot: CnA was cleaved by 10µM menadione exposure in rat cortical neurons. (A) Representative Western blot of CnA levels in WCL of rat cortical neurons that were exposed to 2.5 - 10µM menadione for 4h. GAPDH was used as a loading control. CnA cleavage product is outlined in the black oval. (B) Cleaved CnA (32kDa) was detectable at 10µM menadione exposure at levels that were 7.2 ± 1.5 fold of control. (C) 2.5 - 10µM menadione did not decrease whole CnA levels after 4h exposure. Values shown are the averages of 7 experiments (n = 7). * denotes p < 0.05. Error bars shown are ± SEM.
Figure 19: Representative Western blot of CnA in rat cortical neurons after 50µM H$_2$O$_2$ exposure. (A) Representative Western blot of CnA levels in WCL of rat cortical neurons that were exposed to 50µM H$_2$O$_2$ for 1h – 24h. GAPDH was used as a loading control. CnA cleavage product is outlined in the black ovals. (B) Raw densitometry ratios of the Western blot presented in (A).
Figure 20: CnA was cleaved by 1h 50µM H₂O₂ exposure in rat cortical neurons. (A) Cleaved CnA (32kDa) was detectable by 1h 50µM H₂O₂ exposure and maintained at 24h. After 1h, 4h, and 24h 50µM H₂O₂ exposure, CnA cleavage was 7 ± 1.6 fold, 5.9 ± 1.5 fold, and 4.0 ± 0.7 fold of control, respectively. (B) 50µM H₂O₂ did not decrease whole CnA levels at all time points tested. Values shown are the averages of 9 experiments (n = 9) for 1h and 4h and 6 experiments (n = 6) for 24h. * denotes p < 0.05. Error bars shown are ± SEM.
dephosphorylative properties (Lee et al., 2007). The CnA cleavage fragment was still detectable by 24h 50μM H_2O_2, but was not statistically different from control. Negligible levels of CnA cleavage were detected in control cells. In summary, whole CnA levels were unchanged after H_2O_2 exposure (Figure 20B), whereas cleaved CnA levels were initially greatly elevated and gradually decreased over time. Data were analyzed using one-way ANOVA with Bonferroni post-hoc analysis.

3.12. Cn activity did not change after PQ or H_2O_2 exposure in rat cortical neurons

To evaluate the effects of CnA cleavage and decreased whole Cn protein levels on Cn activity, rat cortical neurons were exposed to PQ or H_2O_2 and Cn activity was assessed using the Biomol Calcineurin Activity Assay Kit, a colorimetric assay that measures Cn activity based on the amount of free phosphate released. Cn activity was measured after 4h and 24h of 10μM or 20μM PQ and was not found to be significantly different from control at both 4h and 24h exposure (Figure 21A; n = 3 experiments, 1 sample/experiment). As CnA cleavage was observed after 1h and 24h 50μM H_2O_2 exposure in rat cortical neurons, Cn activity was assessed at these two time points. 1h 50 μM H_2O_2 treatment did not result in decreased Cn activity (Figure 21B; n = 3 experiments, 1 sample/experiment), although CnA cleavage was most prominent at this time point (see Figure 19). 24h 50μM H_2O_2 exposure also did not result in decreased Cn activity (n = 3 experiments, 1 sample/experiment), which coincides with whole CnA levels being unchanged at this time point when compared to control. However, it’s worth noting that despite attempts to optimize the assay, there was considerable variability between experimental days. Data were analyzed using one-way ANOVA with Bonferroni post-hoc analysis.

3.13. Preliminary data: NFATc4 levels in the cytoplasm and nucleus are unchanged after H_2O_2 exposure in rat cortical neurons

NFATc4 is a substrate of Cn and its subcellular localization is dependent upon its phosphorylation status. Phosphorylated NFATc4 is inactive and resides in the cytoplasm (Loh et al., 1996). Upon dephosphorylation by Cn, NFATc4 translocates to the nucleus, where it
Figure 21: Cn activity did not change after oxidative stress in rat cortical neurons. (A) 10μM or 20μM PQ did not change Cn activity in rat cortical neurons at both 4h and 24h exposure. (B) Cn activity was unchanged in rat cortical neurons after exposure to 50μM H₂O₂ for 1h or 24h. Values shown are the averages of 3 experiments per treatment (n = 3). p > 0.05 for all comparisons. Error bars shown are ± SEM.
mediates changes in gene transcription (Jayanthi et al., 2005). The mobility of the NFATc4 band in nuclear fractions differs slightly from the mobility of the NFATc4 band in cytoplasmic fractions due to nuclear NFATc4 having been dephosphorylated on multiple serines prior to nuclear localization. As H$_2$O$_2$ exposure in rat cortical neurons altered CnA levels and Cn activity, downstream effects on NFATc4 phosphorylation and localization were investigated. Subcellular fractionation was performed on rat cortical neurons exposed to 1h, 4h, or 24h of 50µM H$_2$O$_2$. Western blotting of cytoplasmic and nuclear fractions was performed to assess NFATc4 localization and activation (Figure 22A). In two preliminary experiments analyzed to date, NFATc4 levels appeared to increase in the cytoplasm and decrease in the nucleus after 1h, 4h or 24h 50µM H$_2$O$_2$ exposure (Figure 22B). However, these experiments need to be repeated.

All samples were checked under the microscope to ensure complete cell lysis and isolation of nuclei. It should be noted that histone H3 was selected as a nuclear loading control; however, high levels of histone H3 were also present in cytoplasmic samples, suggesting nuclear contamination. It was revealed by the manufacturer that their nuclear extraction kit may extract histones into the cytoplasmic fraction (Brian Ricketts, Active Motif, oral communication, December 2008). The same amount of protein was loaded in each well; therefore, all experimental data from nuclear samples were compared to respective controls without normalization to a loading control. GAPDH present in nuclear samples also suggest a low level of cytoplasmic contamination in nuclear fractions.

3.14. Decreased cell viability after PQ exposure in MEFs was independent of DJ-1 KO

DJ-1 is a PD causative gene that has been shown to have anti-oxidative properties (Bonifati et al., 2003). PQ is an oxidative stressor linked to the development of PD and is associated with increased generation of superoxide through redox cycling mechanisms, ultimately leading to oxidative damage (reviewed in Dinis-Oliveira et al., 2006). To determine whether the elimination of DJ-1 alters cell vulnerability to oxidative stress from PQ, 4 Wt MEF lines and 5 DJ-1 KO MEF lines were treated with 100µM PQ for 24h and cell viability was assessed using the MTT reduction assay. Figure 23A shows the average raw absorbance values for one experiment. Cell viability after 24h of 100µM PQ from this single experiment was also expressed as a percent of cell viability in control cultures and this is presented in Figure 23B.
Figure 22: Western blot: Preliminary data indicate that NFATc4 levels in the cytoplasm and nucleus are unchanged after 1h – 24h 50μM H₂O₂. (A) Representative Western blot of NFATc4 expression in cytoplasmic and nuclear fractions of rat cortical neurons exposed to 50μM H₂O₂ for 1h – 24h. Histone H3 (H3) was used as a nuclear loading control and GAPDH was used as a cytoplasmic loading control. (B) Cytoplasmic and nuclear levels of NFATc4 in rat cortical neurons displayed upward and downward trends, respectively. Values shown are the averages of 2 experiments per treatment (n = 2).
Figure 23: Unpooled data: MTT reduction after 24h 100μM PQ showed no difference in cell viability between Wt and DJ-1 KO MEFs. (A) 4 Wt MEF lines and 5 DJ-1 KO MEF lines were exposed to 100μM PQ for 24h and MTT reduction was assessed. Raw absorbance values shown are the averages of 8 readings in one experiment. (B) Cell viability after 24h 100μM PQ exposure is presented as a percent of cell viability under control conditions for each MEF line.
From these data, DJ-1 KO did not appear to affect cell viability of MEFs in response to PQ-induced oxidative stress. When the raw data from all 4 Wt MEF lines and all 5 DJ-1 KO MEF lines were pooled into two groups, it was clear that 24h 100µM PQ exposure decreased cell viability in both groups, but the elimination of DJ-1 did not significantly enhance sensitivity to PQ (Figure 24A). Data expressed as percent of control (Figure 24B) further illustrate that 24h 100µM PQ exposure decreased cell viability in both groups, but DJ-1 KO had no effect on cell viability. Data were analyzed using the Student’s t-test.

Table 1 shows the average cell viability and how it changes after PQ exposure for the data presented in Figure 23A. Based on these data, Wt 5-6 and KO 5-2 were selected as sensitive MEF lines for further experiments. Wt 5-5 was selected as a resistant Wt MEF line for further experimentation and KO 4-4 was selected as its match due to a similar decrease in viability after PQ exposure. Raw data were not pooled into Wt and DJ-1 KO groups for all subsequent experiments because raw absorbance values for MEFs under control conditions differed between experiments that were performed on different days. Therefore, all other MTT reduction assay data were expressed as a percent of each respective MEF control.

Wt and DJ-1 KO MEFs were treated with 50µM PQ for 24h and 48h or 100µM H2O2 for 1h. Cell viability was assessed using the MTT reduction assay. The 2 Wt lines and the 2 DJ-1 KO lines selected, in addition to Wt 4-3, were analyzed for potential differences within and between genotypic groups. After 24h PQ exposure, cell viability in one MEF line (Wt 5-6) was significantly reduced to 80% ± 4% of its own controls that were treated with water vehicle (Figure 25A; p < 0.05, n = 3, 6 samples/experiment). By 48h, cell viability was reduced in all of the MEF lines except Wt 5-5 (p < 0.05, n = 4 experiments for Wt 5-6 and Wt 4-3, n = 5 experiments for all other Wt and DJ-1 KO MEF lines, 6 samples/experiment). When these percent of control data after 48h PQ exposure were pooled into a single Wt group and a single DJ-1 KO group, cell viability was found to be significantly decreased in both groups to the same degree (Figure 25B; 76% ± 5% of control for Wt and 78% ± 5% of control for DJ-1 KO). Data were analyzed using one-way ANOVA with Bonferroni post-hoc analysis. Taken together, these results indicate that KO of DJ-1 in MEFs did not confer increased sensitivity to PQ.
Figure 24: Pooled data: MTT reduction after 24h 100µM PQ showed no difference in cell viability between Wt and DJ-1 KO MEFs. (A) The 4 Wt MEF lines and the 5 DJ-1 KO MEF lines presented in Figure 23 were pooled into their respective genotypic groups. Raw data are presented as Arbitrary Units (A.U.). 24h 100µM PQ decreased cell viability in both Wt and DJ-1 KO MEFs versus their own controls and DJ-1 KO did not affect the viability of MEFs in response to PQ-induced oxidative stress. (B) The data presented in (A) are presented as percent of control. It is clear that 24h 100µM PQ decreased cell viability in both Wt and DJ-1 KO MEFs versus their own controls, but DJ-1 KO had no effect (p > 0.05). * denotes p < 0.05. Error bars shown are ± SEM.
Table 1: MTT reduction in Wt and DJ-1 KO MEFs after 24h 100μM PQ.
Table of the absorbance values shown in Figure 23A. Each sample was repeated 8 times for one experiment and averaged. Means and variances in the 8 readings per sample are shown. MEFs lines selected for further analysis are shown in dark grey (resistant lines) and light grey (sensitive lines).

<table>
<thead>
<tr>
<th>MEF Line</th>
<th>Control</th>
<th>24h 100μM PQ</th>
<th>PQ/Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wt 5-6</td>
<td>0.289 ± 0.043</td>
<td>0.169 ± 0.024</td>
<td>0.58</td>
</tr>
<tr>
<td>Wt 5-3</td>
<td>0.182 ± 0.02</td>
<td>0.125 ± 0.018</td>
<td>0.69</td>
</tr>
<tr>
<td>Wt 4-3</td>
<td>0.265 ± 0.017</td>
<td>0.228 ± 0.013</td>
<td>0.86</td>
</tr>
<tr>
<td>Wt 5-5</td>
<td>0.213 ± 0.041</td>
<td>0.183 ± 0.018</td>
<td>0.86</td>
</tr>
<tr>
<td>KO 5-9</td>
<td>0.239 ± 0.012</td>
<td>0.216 ± 0.014</td>
<td>0.90</td>
</tr>
<tr>
<td>KO 5-2</td>
<td>0.255 ± 0.017</td>
<td>0.17 ± 0.02</td>
<td>0.67</td>
</tr>
<tr>
<td>KO 4-11</td>
<td>0.217 ± 0.035</td>
<td>0.206 ± 0.025</td>
<td>0.95</td>
</tr>
<tr>
<td>KO 3-3</td>
<td>0.222 ± 0.01</td>
<td>0.206 ± 0.019</td>
<td>0.93</td>
</tr>
<tr>
<td>KO 4-4</td>
<td>0.189 ± 0.014</td>
<td>0.164 ± 0.014</td>
<td>0.87</td>
</tr>
</tbody>
</table>
MTT reduction assay: 48h 50μM PQ exposure decreased cell viability in MEFs, but no differential effects were observed between Wt and DJ-1 KO. At 24h, Wt 5-6 displayed decreased cell viability to 80% ± 4% versus water treated controls. By 48h, cell viability was compromised in all cell lines except Wt 5-5, relative to each respective MEF line control (Wt 5-6: 64% ± 6%; Wt 4-3: 82% ± 5%; KO 5-2: 70% ± 6%; KO 4-4: 89% ± 6% versus controls). Values shown are the averages of 4 experiments for Wt 5-6 and Wt 4-3 (n = 4) and 5 experiments (n = 5) for all other MEF lines, with 6 samples per experiment. (B) The 48h data shown in (A) were pooled into a Wt and a DJ-1 KO group. Cell viability after 48h 50μM PQ was significantly decreased to 76% ± 5% of control in Wt MEFs and to 78% ± 5% of control in DJ-1 KO MEFs. * denotes p < 0.05 and ** denotes p < 0.001. Error bars shown are ± SEM.
3.15. \( \text{H}_2\text{O}_2 \)-induced changes in cell viability in MEFs were independent of DJ-1 KO

To determine whether Wt and DJ-1 KO MEFs are differentially sensitive to direct treatment with ROS, cells were exposed to \( 100\mu\text{M} \ \text{H}_2\text{O}_2 \) for 1h and cell viability was assessed by the MTT reduction assay. All MEF lines tested demonstrated reduced cell viability in response to 1h \( 100\mu\text{M} \ \text{H}_2\text{O}_2 \) treatment (Figure 26; Wt 4-3: 34\% \pm 9\%; Wt 5-6: 76\% \pm 5\% of control; Wt 5-5: 66\% \pm 6\% of control; KO 5-2: 71\% \pm 9\% of control; KO 4-4: 72\% \pm 12\% of control, \( p < 0.05 \), \( n = 3 \) experiments for Wt 4-3, \( n = 4 \) experiments for all other MEF lines, 6 samples/experiment). Taken together, the fact that DJ-1 KO MEFs did not show reductions in cell viability to PQ or \( \text{H}_2\text{O}_2 \) relative to Wt MEFs suggests that the elimination of DJ-1 in MEFs does not confer heightened sensitivity to oxidative stress. Data were analyzed using one-way ANOVA with Bonferroni post-hoc analysis.

3.16. ROS levels were increased in MEF lines after 24h PQ with no effect of DJ-1 KO

To confirm that PQ exposure generated oxidative stress in MEFs, ROS levels were measured using CM-H\(_2\)DCFDA and flow cytometry. MEFs were exposed to \( 50\mu\text{M} \ - 1000\mu\text{M} \) PQ for 24h. CM-H\(_2\)DCFDA is cleaved by cellular esterases and subsequently retained in the cell, where it can be oxidized to a highly fluorescent form, 2′,7′-dichlorofluorescein (DCF), in the presence of ROS; consequently, the fluorescent signal reflects levels of ROS in the cell (reviewed in Armstrong & Whiteman, 2007). ROS levels were only assessed in the selected sensitive and resistant MEF lines described in section 3.14. These data are presented as percent of control due to differences in DCF signal on different experimental days. Basal levels of ROS did not significantly differ between the 2 Wt and 2 DJ-1 KO MEF lines tested (Figure 27A). Notably, DJ-1 KO had no effect on ROS production induced by PQ exposure (Figure 27B). 24h \( 400\mu\text{M} \) PQ treatment resulted in significant ROS levels in all Wt and DJ-1 KO MEF lines tested; lower concentrations had no effect (Figure 27C; \( p < 0.05 \), \( n = 2 \) experiments for KO 5-2 and \( n = 3 \) experiments for all other MEF lines, 3 samples/experiment). ROS production tapered off at \( 800\mu\text{M} \) to \( 1000\mu\text{M} \) PQ relative to lower concentrations, but still remained significantly elevated relative to respective controls. Data were analyzed using one-way ANOVA with Bonferroni post-hoc analysis.
Figure 26: MTT reduction assay: 1h H₂O₂ exposure compromised cell viability in MEFs, but no differential effects were observed between Wt and DJ-1 KO MEFs. Cell viability was decreased after 1h 100μM H₂O₂ exposure in all MEF lines versus respective water treated controls (Wt 4-3: 34 ± 9%; Wt 5-6: 76% ± 5%; Wt 5-5: 66% ± 6%; KO 5-2: 71% ± 9%; KO 4-4: 72% ± 12% of control). DJ-1 KO did not affect cell viability at this dose of H₂O₂. Values shown are the averages of 4 experiments (n = 4), with 6 samples per experiment. * denotes p < 0.05 and ** denotes p < 0.001. Error bars shown are ± SEM.
Figure 27: Flow cytometry: ROS levels are increased after 24h PQ in Wt and DJ-1 KO MEFs. For all traces, green: Wt 5-5, dark blue: Wt 5-6, pink: KO 5-2, and orange: KO 4-4. (A) Representative flow cytometry traces illustrating relative DCF fluorescence in Wt 5-6, Wt 5-5, KO 5-2, and KO 4-4 under basal conditions. (B) Representative flow cytometry traces illustrate that Wt and DJ-1 KO MEFs generate similar ROS levels in response to 24h 400μM PQ. (C) ROS levels were increased in all Wt and DJ-1 KO MEF lines after 24h 400μM PQ exposure. Note that ROS levels began to drop at 800μM PQ - 1000μM, but still remained elevated relative to respective controls. Values shown are the averages of 2 experiments (n = 2) for KO 5-2, and 3 experiments (n = 3) for all other MEF lines, with each sample performed in triplicate per experiment. * denotes p < 0.05 and ** denotes p < 0.001. Error bars shown are ± SEM.
3.17. Confocal imaging indicated that ROS levels increased after 1h H$_2$O$_2$ in MEFs

Although sustained PQ exposure increased ROS levels in all MEF lines, it was possible that sustained exposure allowed sufficient time for upregulation of anti-oxidant defences in DJ-1 KO MEFs, thereby masking potential effects of DJ-1 KO. It was also unknown if increased ROS production could be induced after an acute PQ treatment in MEFs; therefore, ROS levels in MEFs were assessed after 1h to 4h of oxidative stress. MEFs were challenged with 400µM PQ for 2h and 100µM H$_2$O$_2$ for 1h and ROS levels were examined by confocal fluorescence microscopy and qualitatively scored. Preliminary data suggest that at 1h of 100µM H$_2$O$_2$ exposure, an increase in DCF fluorescence was observed in all MEF lines tested; however, no effect of DJ-1 KO on H$_2$O$_2$-induced ROS levels was evident (Figure 28I-L). Wt and DJ-1 KO MEFs were also treated with 2h 400µM PQ to assess changes in ROS levels after short term PQ exposure. Preliminary data showed no evident increase in ROS levels in all MEF lines after short term PQ exposure (Figure 28E-H). DJ-1 KO did not have an effect on ROS generation after acute PQ stress. Of note is the fact that DJ-1 KO MEFs had slightly lower basal ROS levels compared to Wt controls (Figure 28A-D); however, this experiment needs to be repeated to evaluate statistical significance. Table 2 and Table 3 show the average measurements of ROS fluorescence in one experiment after PQ and H$_2$O$_2$ exposure, respectively, expressed as arbitrary units (A.U.). Taken together, fluorescence imaging data suggests that DJ-1 KO had no effect on ROS production in MEFs after short term oxidative stress.

3.18. Confocal imaging indicated that 24h PQ exposure did not alter MTP in MEFs

Confocal fluorescence microscopy analysis of Rh123 dye uptake was used to assess MTP in Wt and DJ-1 KO MEFs stressed with PQ for 24h. Rh123 is a membrane permeable cation that accumulates preferentially in mitochondria depending on the magnitude of its negative transmembrane potential; hence, cellular fluorescence of Rh123 is directly proportional to MTP. Increased intracellular ROS levels are known to depress MTP (reviewed in Drechsel & Patel, 2008). Wt and DJ-1 KO MEFs were exposed to 100µM PQ for 24h and confocal imaging was used to visualize Rh123 uptake. All MEF lines displayed basal MTPs that were comparable to each other (Figure 29A-D). At 24h 100µM PQ, no change in MTP was observed in all MEF lines tested, relative to respective controls (Figure 29E-H; n = 1). DJ-1 KO did not
Figure 28: Confocal fluorescence microscopy: ROS levels increased after 1h H$_2$O$_2$, but not after short term PQ, in Wt and DJ-1 KO MEFs. Basal ROS levels were the same in Wt 5-6 (A), Wt 5-5 (B), KO 5-2 (C), and KO 4-4 (D). No increase in ROS production after 2h exposure to 400μM PQ was observed in Wt 5-6 (E), Wt 5-5 (F), KO 5-2 (G), and KO 4-4 (H). ROS generation was increased after 1h 100μM H$_2$O$_2$ exposure in Wt 5-6 (I), Wt 5-5 (J), KO 5-2 (K), and KO 4-4 (L). Images were taken with a 20x fluor objective, 1x zoom.
Table 2: ROS levels in MEFs after 2h 400μM PQ. MEFs were treated with 400μM PQ for 2h and examined by confocal fluorescence microscopy. DCF measurements in 10 cells were taken from one image and averaged. Values shown are means of raw data presented as arbitrary units (A.U.) along with the corresponding standard deviation. These data suggest that 2h 400μM PQ did not change ROS levels in both Wt and DJ-1 KO MEFs.

<table>
<thead>
<tr>
<th>MEF line</th>
<th>Control (A.U.)</th>
<th>PQ (A.U.)</th>
<th>PQ/Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wt 5-6</td>
<td>59 ± 8</td>
<td>62 ± 6</td>
<td>1.06</td>
</tr>
<tr>
<td>Wt 5-5</td>
<td>58 ± 14</td>
<td>57 ± 5</td>
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<td>KO 5-2</td>
<td>50 ± 10</td>
<td>50 ± 7</td>
<td>1.00</td>
</tr>
<tr>
<td>KO 4-4</td>
<td>39 ± 3</td>
<td>43 ± 4</td>
<td>1.10</td>
</tr>
</tbody>
</table>

Table 3: ROS levels in MEFs after 1h 100μM H₂O₂. MEFs were treated with 100μM H₂O₂ for 1h and examined by confocal fluorescence microscopy. DCF measurements in 10 cells were taken from one image and averaged. Values shown are means of raw data presented as arbitrary units (A.U.) along with the corresponding standard deviation. These data suggest that 1h 100μM H₂O₂ increased ROS levels in both Wt and DJ-1 KO MEFs.

<table>
<thead>
<tr>
<th>MEF line</th>
<th>Control (A.U.)</th>
<th>H₂O₂ (A.U.)</th>
<th>H₂O₂/Control</th>
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</thead>
<tbody>
<tr>
<td>Wt 5-6</td>
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<td>KO 4-4</td>
<td>39 ± 3</td>
<td>66 ± 9</td>
<td>1.68</td>
</tr>
</tbody>
</table>
Figure 29: Confocal fluorescence microscopy: Rh123 uptake showed that MTP did not change after 24h 100μM PQ exposure in MEFs. MTP did not differ between Wt 5-6 (A), Wt 5-5 (B), KO 5-2 (C), and KO 4-4 (D) under basal conditions. No changes in MTP after exposure to 24h 100μM PQ were detected in Wt 5-6 (E), Wt 5-5 (F), KO 5-2 (G), and KO 4-4 (H). Images were taken with 60x fluor objective, 1x zoom.
have any effect on MTP after PQ exposure. Raw values from the fluorescence quantification of the images in Figure 29 are shown in Table 4 as raw arbitrary units (A.U.). Wt 5-6 appeared to have increased MTP after 24h 100µM PQ exposure; however, additional experiments to increase sample size are necessary for statistical analysis. As 24h 100µM PQ did decrease cell viability, but did not significantly increase ROS in Wt 5-5, KO 5-2 or KO 4-4, this suggests that this dose may have been insufficient to alter MTP. Ongoing experiments are examining the effects of higher doses of PQ on MTP.

3.19. Basal CnA levels were the same between Wt and DJ-1 KO MEFs

Cn is a cytoplasmic serine-threonine phosphatase that is involved in cell survival/cell death pathways and is especially abundant in brain regions that are susceptible to neurodegeneration (Polli et al., 1991). Some literature indicates that Cn is redox sensitive, although reports on the effects on Cn after exposure to oxidative stress are mixed (Lee et al., 2007; See & Loeffler, 2001). A recent study reported that overexpression of CnB, the regulatory subunit of Cn, resulted in upregulation of DJ-1 (Wang et al., 2008). We sought to investigate a potential relationship between the expression of CnA, the catalytic subunit of Cn, and DJ-1 KO in MEF by Western blot of WCL. 4 Wt MEF lines (Wt 5-6, Wt 5-5, Wt 4-2, and Wt 4-3) and 5 DJ-1 KO MEF lines (KO 4-11, KO 5-2, KO 4-4, KO 3-3, KO 5-9) were used in the analysis of basal CnA expression. No differences in basal CnA expression were observed among Wt MEF lines and among DJ-1 KO MEF lines (Figure 30A); consequently, data from all lines were pooled into the two genotypic groups to give a sample size of 4 for Wt (n = 4) and a sample size of 5 for DJ-1 KO (n = 5; Figure 30B). Western blotting revealed no difference in CnA expression between Wt and DJ-1 KO MEFs under unstressed basal conditions (n = 4 for Wt, n = 5 for DJ-1 KO, 1 sample/experiment, p > 0.05). Data were analyzed using the Student’s t-test.

3.20. Sub-lethal PQ did not alter CnA levels in Wt and DJ-1 KO MEFs

Both CnA and DJ-1 are redox-sensitive proteins and DJ-1 is known to have anti-oxidant properties (Lee et al., 2007; Lev et al., 2008). To investigate whether PQ-induced oxidative
Table 4: MTP in MEFs after 2h 400μM PQ. MEFs were treated with 100μM PQ for 24h and examined by confocal fluorescence microscopy. Ten Rh123 measurements were taken from 10 different cells in one image and averaged. Values shown are means of raw data presented as arbitrary units (A.U.) along with the corresponding standard deviation. These data suggest that 24h 100μM PQ did not change ROS levels in both Wt and DJ-1 KO MEFs.

<table>
<thead>
<tr>
<th>MEF line</th>
<th>Control (A.U.)</th>
<th>PQ (A.U.)</th>
<th>PQ/Control</th>
</tr>
</thead>
<tbody>
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<td>Wt 5-6</td>
<td>65 ± 12</td>
<td>84 ± 14</td>
<td>1.30</td>
</tr>
<tr>
<td>Wt 5-5</td>
<td>72 ± 15</td>
<td>75 ± 14</td>
<td>1.04</td>
</tr>
<tr>
<td>KO 5-2</td>
<td>77 ± 13</td>
<td>81 ± 15</td>
<td>1.05</td>
</tr>
<tr>
<td>KO 4-4</td>
<td>58 ± 10</td>
<td>58 ± 12</td>
<td>1.00</td>
</tr>
</tbody>
</table>
Figure 30: Western blot: CnA expression is the same between Wt and DJ-1 KO MEFs. (A) Representative Western blot of CnA expression levels in WCL of Wt 4-2 and KO 4-11 under basal conditions. GAPDH was used as a loading control. (B) CnA expression was the same in Wt and DJ-1 KO MEFs under unstressed conditions. Wt 5-6, Wt 5-5, Wt 4-2, Wt 4-3, KO 4-4, KO 3-3, KO 5-2, KO 5-9, and KO 4-11 were used and results were pooled into Wt and DJ-1 KO groups. Values shown are the averages of 4 experiments for Wt (n = 4) and 5 experiments for DJ-1 KO (n = 5), p > 0.05.
stress affects CnA levels in Wt and DJ-1 KO MEFs, Wt and DJ-1 KO MEFs were exposed to 50μM PQ for 1h - 24h and CnA levels were measured by Western blot of WCL. CnA levels remained unchanged relative to controls in both Wt and DJ-1 KO MEFs after 50 μM PQ exposure at all time points (1h - 24h) examined (Figure 31A-B). Due to the small sample sizes for some MEF lines, not all lines were analyzed separately. When Wt lines and DJ-1 KO lines were pooled into two genotypic groups for the purpose of data analysis, no difference was observed. Furthermore, analyses where experiment (n) numbers were matched between individual MEF lines also did not show any significant differences in CnA levels between the two genotypic groups after PQ exposure. The MEF lines used and the number of times each line was used for a time point is indicated at the bottom of Figure 31B. Experiments assessing ROS levels in MEFs using 50μM PQ suggested that this dose may not have generated sufficient oxidative stress (see section 3.16). To assess if a higher sub-lethal dose of PQ had effects on CnA levels, Wt and DJ-1 KO MEFs were exposed to 100μM PQ. A preliminary experiment showed that at 24h, CnA levels were unchanged in Wt samples, but marginally decreased in DJ-1 KO samples (data not shown; n = 1, 1 sample/experiment). Increased sample size is required to validate this finding. At this time, my data indicates that DJ-1 KO had no effect on CnA levels after exposure to low dose sub-lethal PQ. Ongoing experiments are addressing the effects of higher sub-lethal doses of PQ (200μM-400μM) in Wt and DJ-1 KO MEFs, but my data indicates that at least at the doses tested, the elimination of DJ-1 had no effect on sensitivity. Data were analyzed using one-way ANOVA with Bonferroni post-hoc analysis.

3.21. Sub-lethal 1h 100μM H₂O₂ induced CnA cleavage and had differential effects on Wt and DJ-1 KO MEFs

My data in PC12 cells and rat cortical neurons indicated that PQ and H₂O₂ have differential effects on CnA. To investigate if PQ versus H₂O₂ has different effects on Wt versus DJ-1 KO MEFs, H₂O₂ was used to generate oxidative stress directly in Wt and DJ-1 KO MEFs. Western blot was used to analyze CnA levels in WCL obtained from cells that had been exposed to 100μM H₂O₂ for 1h. Due to the small sample sizes for the MEF lines analyzed, it was not possible to analyze all lines separately. Therefore, Wt lines and DJ-1 KO lines were pooled into two genotypic groups for the purpose of data analysis, although experiments were not
**Figure 31:** Western blot: CnA expression did not change after sub-lethal PQ exposure in MEFs. (A) Representative Western blot of CnA expression in WCL of Wt 4-2 and KO 4-2 after exposure to 50µM PQ for 1h-24h. GAPDH was used as a loading control. (B) CnA expression did not change in both Wt and DJ-1 KO MEFs after sub-lethal 50µM PQ for 1h-24h. Values shown are the averages of 3 experiments for 1h and 2h (n = 3), 5 experiments for 4h (n = 5), and 4 experiments for Wt (n = 4) and 6 experiments for DJ-1 KO (n = 6) at 24h. p > 0.05 for all comparisons. The MEF lines used for each time point are indicated below the graph, with the number of experiments each line was used for in brackets. Error bars shown are ± SEM.
performed in all MEF lines for the same number of times. After 1h 100µM H$_2$O$_2$ treatment, a CnA cleavage fragment of approximately 32kDa was detected in both Wt and DJ-1 KO samples (Figure 32A). The 32kDa cleavage fragment has been previously reported by Lee et al. (2007) to be inactive. Significantly greater CnA cleavage was detected in DJ-1 KO samples after H$_2$O$_2$ treatment than in Wt samples (Figure 32B). Both Wt and DJ-1 KO controls had negligible cleaved CnA levels. In addition, whole CnA levels significantly decreased in DJ-1 KO MEFs after H$_2$O$_2$ exposure, compared to whole CnA levels in Wt MEFs which did not decrease (Figure 32C; p < 0.05, n = 3 experiments in Wt and n = 4 experiments in DJ-1 KO, 1 sample/experiment). Where experiment (n) numbers were matched between the Wt and DJ-1 KO MEFs analyzed, DJ-1 KO MEFs still had decreased whole CnA levels and increased CnA cleavage relative to Wt MEFs. Data were analyzed using Student's t-test. These results suggest that DJ-1 KO enhanced sensitivity of MEFs to H$_2$O$_2$-induced CnA cleavage.

3.22. Preliminary data: NFATc4 subcellular localization differed between Wt and DJ-1 KO MEFs under basal conditions

NFATc4 is a substrate of Cn that is involved in the upregulation of apoptotic pathways through the activation of Fas ligand transcription. NFATc4 exists in an inactive, phosphorylated state in the cytoplasm under basal conditions, with some localization to the nucleus (Loh et al., 1996). While examining the downstream effects of H$_2$O$_2$-induced CnA cleavage by Western blot of subcellular fractions, it was unexpectedly discovered that subcellular localization of NFATc4 in Wt and DJ-1 KO MEFs differed under basal conditions (Figure 33A). Preliminary data obtained using KO 5-2 and KO 4-4 suggest that basal NFATc4 nuclear localization is less than Wt 5-5, with levels in KO 5-2 at 29% ± 9% of Wt 5-5 (p < 0.05, n = 3 experiments, 1 sample/experiment) and levels in KO 4-4 at 48% ± 20% of Wt 5-5 (p < 0.05, n = 4 experiments, 1 sample/experiment). My preliminary experiments also showed that cytoplasmic NFATc4 localization also differed between Wt 5-5 and KO 5-2 or KO 4-4, but with cell line specific effects. Cytoplasmic NFATc4 localization in KO 5-2 was 73% ± 7% of Wt 5-5 under basal conditions (p < 0.05, n = 3 experiments, 1 sample/experiment), while cytoplasmic NFATc4 localization in KO 4-4 was 191% ± 23% of Wt 5-5 under basal conditions (p < 0.05, n = 4 experiments, 1 sample/experiment). Data were analyzed using Student's t-test. Preliminary experiments were also performed using Wt 5-6, which displayed similar cytoplasmic and
Figure 32: Western blot: 1h 100μM H₂O₂ cleaved CnA in both Wt and DJ-1 KO MEFs and decreased CnA levels in DJ-1 KO MEFs. (A) Representative Western blot of CnA levels in WCL of Wt 5-6 and KO 5-2 after exposure to 100μM H₂O₂ for 1h. Cleaved CnA is 32kDa and are outlined in black ovals. Blots were probed for DJ-1 to confirm KO. GAPDH was used as a loading control. (B) CnA cleavage was greater in DJ-1 KO MEFs than in Wt. (C) Whole CnA levels were decreased in DJ-1 KO MEFs after 1h 100μM H₂O₂. Values shown are the averages of 2 experiments in Wt 5-6 and 1 experiment in Wt 5-5 to give n = 3 for Wt. 2 experiments in both KO 5-2 and KO 4-4 were averaged to give n = 4 for DJ-1 KO. * denotes p < 0.05. Error bars shown are ± SEM.
Figure 33: Preliminary data showed that NFATc4 subcellular localization differed between Wt and DJ-1 KO MEFs under basal conditions. (A) Representative raw data from a single experiment that illustrates the densitometry of cytoplasmic NFATc4 normalized to the densitometry of GAPDH in cytoplasmic fractions of Wt and DJ-1 KO MEFs. (B) Representative raw data from a single experiment that illustrates the densitometry of nuclear NFATc4 in Wt and DJ-1 KO MEFs. (C) Cytoplasmic NFATc4 levels in KO 5-2 were 73% ± 7% of Wt 5-5, while cytoplasmic levels in KO 4-4 were 191% ± 23% of Wt 5-5 under basal conditions. Nuclear localization of NFATc4 was lower in both DJ-1 KO MEFs lines and were 29% ± 9% of Wt 5-5 in KO 5-2 and 48% ± 20% of Wt 5-5 in KO 4-4. Values shown are the averages 3 and 4 experiments in KO 5-2 and KO 4-4, respectively). * denotes p < 0.05. Error bars shown are ± SEM.
nuclear NFATc4 levels as Wt 5-5 (Figure 33B-C). Only Wt 5-5 was used for comparison purposes. These preliminary data suggest that there are MEF line specific differences in Wt and DJ-1 KO MEFs; therefore, experiments using more Wt and DJ-1 KO MEF lines are required in order to validate these differences in cytoplasmic and nuclear NFATc4 levels under basal conditions.

3.23. Preliminary data: sub-lethal 2h 100μM H₂O₂ induced an increase in cytoplasmic NFATc4 in Wt MEFs, but not in DJ-1 KO MEFs

Upon dephosphorylation by Cn, NFATc4 is activated and translocates to the nucleus, where it mediates changes in gene transcription (Jayanthi et al., 2005). Under conditions of Cn inactivation, less cytoplasmic NFATc4 is dephosphorylated and activated, while nuclear NFATc4 is expected to be dephosphorylated by nuclear phosphatases and translocated from the nucleus to the cytoplasm. To assess potential downstream effects of CnA cleavage, Western blot of subcellular fractions was used to examine the activation and localization of NFATc4 after exposure to 2h H₂O₂. This longer exposure was selected to allow time for downstream effects of Cn inactivation to occur and corresponds to the time point selected by Lee et al. (2007) that was best able to show alterations in NFATc4 activity after Cn inactivation. As shown in the preliminary data illustrated in Figure 34A-C, Western blotting of cytoplasmic and nuclear fractions of Wt and DJ-1 KO MEFs showed that after 2h 100μM H₂O₂, cytoplasmic NFATc4 was significantly increased in Wt MEFs to 147% ± 17% versus control, but nuclear NFATc4 showed no significant change (Figure 34C; p < 0.05, n = 4 experiments [3 experiments with Wt 5-5 and 1 experiment with Wt 5-6], 1 sample/experiment). In preliminary experiments, fractions from DJ-1 KO MEFs showed no changes in cytoplasmic or nuclear localization after H₂O₂ treatment (n = 7 experiments [4 experiments with KO 4-4 and 3 experiments with KO 5-2], 1 sample/experiment), suggesting that DJ-1 KO affected CnA cleavage after H₂O₂ exposure without inducing downstream effects in subcellular NFATc4 localization. Data were analyzed using Student's t-test. It was mentioned in section 3.22 that there are cell line specific differences in basal cytoplasmic and nuclear NFATc4 levels. Therefore, experiments using more Wt and DJ-1 KO cell lines are required in order to validate these preliminary data.
Figure 34: Western blot: Preliminary data indicated that 2h 100μM H₂O₂ changed NFATc4 levels in cytoplasmic fractions of Wt MEFs only. (A) Representative Western blot of NFATc4 expression in cytoplasmic fractions of Wt and DJ-1 KO MEFs after exposure to 100μM H₂O₂ for 2h. GAPDH was used as a cytoplasmic loading control. (B) Representative Western blot of NFATc4 expression in nuclear fractions of Wt and DJ-1 KO MEFs after exposure to 100μM H₂O₂ for 2h. Histone H3 was used as a nuclear loading control. (C) 2h 100μM H₂O₂ increased cytoplasmic NFATc4 in Wt MEFs only. No changes in NFATc4 were observed in the cytoplasmic or the nuclear fractions in DJ-1 KO MEFs. Values shown are the averages 4 and 7 experiments in Wt and DJ-1 KO MEFs, respectively (n = at least 3). * denotes p < 0.05. Error bars shown are ± SEM.
Similar to assessment of NFATc4 localization in rat cortical neurons, histone H3 was used as a nuclear loading control. Problems with extraction of histone H3 into the cytoplasmic fraction was also observed in MEF fractions (see Figure 34A). The same amount of protein was loaded into each well; therefore, experimental bands were compared against respective controls without normalization to a nuclear loading control. It should be noted that although we do not know the purity of cytoplasmic factions, nuclear fractions are relatively free from cytoplasmic contamination due to minimal GAPDH detected in nuclear fractions (see Figure 34B). We can be relatively certain that NFATc4 in nuclear fractions is specifically localized to the nucleus, although results were not normalized against a nuclear loading control.

3.24. DJ-1 is localized to the cytoplasm, mitochondria, and nucleus under basal conditions in PC12 cells

A literature review shows that there is no consensus about the subcellular localization of DJ-1 under basal conditions. To address this issue, I used immunofluorescence and Western blotting to visualize the subcellular localization of DJ-1 in PC12 cells. Immunofluorescence revealed DJ-1 fluorescence throughout the cell in PC12 cells. The diffuse nature of this staining and its relatively even distribution within the cell is not consistent with a predominantly mitochondrial localization of DJ-1, although some mitochondrial localization cannot be ruled out (Figure 35A). Optical sectioning of cells by confocal microscopy confirmed that DJ-1 was also present in the nucleus (see red arrow in Figure 35A) and that this staining is greater than the non-specific staining seen in the nuclei of DJ-1 KO MEFs (see Figure 2). Western blotting of cytoplasmic, mitochondrial, and nuclear subcellular fractions revealed the presence of DJ-1 in these three subcellular compartments under unstressed conditions, with the majority of cellular DJ-1 in the cytoplasm (Figure 35B-C). Collectively, both immunofluorescence and Western blot data support DJ-1 localization in the cytoplasm, mitochondria, and nucleus under basal conditions.
Figure 35: DJ-1 is localized to the cytoplasm, mitochondria and nucleus under basal conditions in PC12 cells. (A) Immunofluorescence showed that there is diffuse DJ-1 staining in the cytoplasm and nucleus (red arrow). Images were taken with a 60x fluor objective, 1x zoom. (B) Western blot of cytoplasmic (cp) and mitochondrial (mt) fractions showed that DJ-1 is present in the cytoplasm and mitochondria under basal conditions. (C) Western blot of nuclear (nc) fractions of PC12 cells showed that DJ-1 is present in the nucleus under basal conditions. β-actin or GAPDH, TOM20, and histone H3 (H3) were used as cytoplasmic, mitochondrial, and nuclear loading controls, respectively.
3.25. **PQ did not induce translocation of DJ-1 to mitochondria in PC12 cells**

Oxidative stress is reported to induce DJ-1 translocation to the mitochondria and also to the nucleus (Junn et al., 2009). To address this issue in our PC12 cell model, I used two approaches. In the first approach, PC12 cells were exposed to PQ and processed for immunofluorescence at various times post-PQ. The second approach involved subcellular fractionation of PC12 cells exposed to PQ and Western blotting to probe for DJ-1. At 24h 40μM PQ exposure, a small but significant increase in cytoplasmic DJ-1 (116% ± 4% versus controls) was detected (Figure 36A-B; p < 0.05, n = 9 experiments, 1 sample/experiment); however, there was no increase in DJ-1 levels in mitochondrial fractions. Data were analyzed using Student's t-test. In PC12 cells labelled with DJ-1 antibodies and fixed after 3h, 6h, or 24h of 40μM or 80μM PQ, immunofluorescence was scored qualitatively. Examination of these images showed there was no increased mitochondrial translocation after PQ exposure (Figure 37A-I; n = 3, 1 sample/experiment). There was no enhanced mitochondrial localization observed at all time points and concentrations tested (n = 3, 1 sample/experiment). These data argue that DJ-1 does not translocate to the mitochondria after PQ-induced oxidative stress.

3.26. **Immunofluorescence revealed upregulation of DJ-1 after H₂O₂ exposure in PC12 cells**

As PQ produces ROS through redox cycling mechanisms, the effects of direct ROS addition, namely H₂O₂, on DJ-1 translocation were investigated. Immunofluorescence of PC12 cells exposed to 3h 50μM or 100μM H₂O₂ revealed no obvious mitochondrial or nuclear translocation of DJ-1 (Figure 37J and M), while general cytoplasmic upregulation of DJ-1 was evident (Figure 38J and M). As cell viability in response to H₂O₂ is density-dependent (see section 3.2) and imaging of immunofluorescence in cells required cultures of lower density, these lower doses of H₂O₂ were selected so that cellular toxicity could be limited. By 24h, general upregulation of DJ-1 still persisted (Figure 38L and O), and there was still no evident translocation to the mitochondria or nucleus (Figure 37L and O). DJ-1 upregulation was most pronounced in the cell body and processes. This contrasts with data obtained after PQ exposure, which did not cause upregulation of DJ-1 (Figure 38D-I), suggesting that DJ-1 may be more sensitive to oxidative stress from H₂O₂.
Figure 36: DJ-1 did not translocate to the mitochondria after oxidative stress with PQ in PC12 cells. (A) Representative Western blot showing no change in mitochondrial DJ-1 localization after 24h 40μM PQ in PC12 cells. (C) By 24h 40μM PQ, DJ-1 was upregulated in the cytoplasm to 116% ± 4% versus controls, while there was no change in mitochondrial DJ-1 localization. Values shown are the averages of 9 experiments (n = 9). * denotes p < 0.05. Error bars shown are ± SEM.
Figure 37: Immunofluorescence showed no increased translocation of DJ-1 to mitochondria after PQ or H₂O₂ exposure in PC12 cells. Control PC12 cells at 3h (A), 6h (B), and 24h (C). PC12 cells exposed to 40μM PQ for 3h (D), 6h (E), and 24h (F). PC12 cells exposed to 80μM PQ for 3h (G), 6h (H), and 24h (I). PC12 cells exposed to 50μM H₂O₂ for 3h (J), 6h (K), and 24h (L). PC12 cells exposed to 100μM H₂O₂ for 3h (M), 6h (N), and 24h (O). Images were taken with a 60x fluor objective, 1x zoom, with the exception of (E), (F), and (I), which are 1.5x zoom.
Figure 38: Immunofluorescence showed that DJ-1 is upregulated after H$_2$O$_2$, but not PQ, exposure in PC12 cells. White represents areas of saturated signal, while black represents areas of low signal. Note that DJ-1 is upregulated after H$_2$O$_2$ exposure, but not after PQ exposure.
3.27. \( \text{H}_2\text{O}_2 \) did not increase translocation of DJ-1 to mitochondria, but did increase DJ-1 levels in the nucleus of PC12 cells

Junn et al. (2009) reported that mitochondrial and nuclear translocation of DJ-1 may be time-dependent, with mitochondrial translocation occurring within hours after \( \text{H}_2\text{O}_2 \) exposure and nuclear translocation occurring between 12h - 24h of oxidative stress after mitochondrial DJ-1 levels have declined. Their subcellular fractionation protocol utilized a digitonin-free buffer to disrupt the plasma membrane in order to facilitate mitochondrial isolation, while our fractionation protocol included digitonin. The high molecular weight of digitonin facilitates its intercalation into the cholesterol-rich plasma membranes of vertebrates, producing high yield mitochondrial fractions that are more pure than those isolated in the absence of digitonin. However, the OMM is also rich in cholesterol and digitonin may disturb the OMM and lead to the release of associated proteins, thereby making digitonin-based protocols unfavourable when mitochondrial proteins are to be examined (Cremel et al., 1990; Dorbani et al., 1987).

Junn et al. (2009) reported an increase in mitochondrial DJ-1 localization as early as 3h \( \text{H}_2\text{O}_2 \) exposure. We followed their subcellular fractionation protocol using a digitonin-free buffer from Active Motif to isolate cytoplasmic, mitochondrial and nuclear fractions from PC12 cells exposed to 50\( \mu \text{M} \) \( \text{H}_2\text{O}_2 \) for 3h, which matches the time and concentration that was used for immunofluorescence. At 3h 50\( \mu \text{M} \) \( \text{H}_2\text{O}_2 \), no increase in mitochondrial or cytoplasmic DJ-1 levels was observed by Western blot (Figure 39A). Levels of DJ-1 in the nucleus, however, were increased to 116\% \pm 3\% versus controls (Figure 39B and C; \( p < 0.05 \), \( n = 3 \) experiments, 1 sample/experiment). Data were analyzed using Student's \( t \)-test. Collectively, my immunofluorescence and Western blotting data does not support mitochondrial translocation of DJ-1 after oxidative stress with PQ or \( \text{H}_2\text{O}_2 \), but does argue that DJ-1 levels are increased in the nucleus after exposure to \( \text{H}_2\text{O}_2 \) only. Further, \( \text{H}_2\text{O}_2 \) appeared to upregulate DJ-1 by 24h, but no such effect was observed with PQ.

3.28. \( \text{H}_2\text{O}_2 \) induced upregulation of DJ-1 in rat cortical neurons

We addressed the issue of DJ-1 mitochondrial translocation in rat cortical neurons. Rat cortical neurons cultured for 14 days were exposed to 50\( \mu \text{M} \) \( \text{H}_2\text{O}_2 \) for 3h or 24h, after which DJ-1 levels were assessed qualitatively by immunofluorescence. Immunofluorescence data
Figure 39: DJ-1 did not translocate to the mitochondria, but did increase in the nucleus after oxidative stress with H$_2$O$_2$ in PC12 cells. (A) Representative Western blot showing no change in mitochondrial DJ-1 after 3h 50μM H$_2$O$_2$. (B) Representative Western blot showing increased nuclear DJ-1 after 3h 50μM H$_2$O$_2$. (C) Nuclear localization of DJ-1 was increased by 116% ± 3% versus controls after 3h 50μM H$_2$O$_2$. Values shown are the averages of 3 experiments (n = 3). * denotes p < 0.05. Error bars shown are ± SEM.
confirmed that DJ-1 was present as diffuse staining in the cytoplasm and nucleus, with no evidence of a predominantly mitochondrial localization pattern (see Figure 40A-B). After 3h of 50μM H₂O₂, there was marked upregulation of DJ-1 in rat cortical neurons (Figure 40C). By 24h H₂O₂ exposure, however, DJ-1 levels had returned to baseline and appeared no different from control (Figure 40D). Taken together, this data confirms that DJ-1 is present in the cytoplasm and nucleus under basal conditions and supports my finding of H₂O₂-induced DJ-1 upregulation in PC12 cells.
Figure 40: Immunofluorescence showed that DJ-1 is upregulated after 3h 50μM H₂O₂ in rat cortical neurons. (A) & (B) Control rat cortical neurons show diffuse DJ-1 staining throughout the cytoplasm. (C) DJ-1 was upregulated in rat cortical neurons after 3h 50μM H₂O₂. (D) By 24h of 50μM H₂O₂, DJ-1 expression in rat cortical neurons was not different from control. Images were taken with a 20x fluor objective, 1x zoom.
DISCUSSION
PART I: THE EFFECTS OF DJ-1 KO IN MEFs AFTER OXIDATIVE STRESS

4.1.1. Introduction

Oxidative stress is a key factor in the development of PD (Mitsumoto et al., 2001). Post-mortem analyses have found free radical mediated damage to lipids, proteins and DNA in the brains of PD patients (Schapira et al., 1989). The pathogenesis of PD is not well understood, but the loss of DJ-1, a redox sensitive protein, is thought to be a causative factor in one familial form of PD (Bonifati et al, 2003a). This study was undertaken to investigate the neuroprotective potential of DJ-1 in models of oxidative stress. In addition, I examined potential links between DJ-1 and Cn, a cytoplasmic serine/threonine phosphatase. Cn is activated by Ca$^{2+}$ and is involved in multiple cellular activities, including but not limited to, cell survival and apoptosis (Asai et al., 1999), learning and memory (Miyata et al., 2001), and regulation of gene transcription (Loh et al., 1996).

My study investigated changes in DJ-1 localization and expression and changes in Cn levels in response to oxidative stress. Oxidative stress did not induce translocation of DJ-1 to the mitochondria or nucleus in PC12 cells and rat cortical neurons. Assessment of multiple parameters after oxidative stress did not reveal differential responses between Wt MEFs and DJ-1 KO MEFs. CnA was cleaved after H$_2$O$_2$ exposure in both Wt and DJ-1 KO MEFs. Cleavage of CnA increased in DJ-1 KO MEFs after H$_2$O$_2$ exposure, suggesting that DJ-1 and Cn pathways may converge, although the exact links has yet to be elucidated.

4.1.2. DJ-1 KO does not decrease cell viability after oxidative stress

Missense mutations in DJ-1 lead to early onset PD, whereas complete loss of function in the protein is a rare cause for familial PD (Lockhart et al., 2004). Studies investigating the effects of DJ-1 KO or knockdown using siRNA have been useful in developing an understanding of the mechanisms underlying PD pathology. My data showed no difference in cell viability between Wt and DJ-1 KO cells under basal conditions, which is consistent with a previous report that DJ-1 downregulation in Neuro2A cells and 293T cells has no effect on basal cell viability (Yokota et al., 2003). There was also no difference in cell viability between 3 different Wt MEF lines and 2 different DJ-1 KO MEF lines after oxidative stress with H$_2$O$_2$ for
1h or with 50μM PQ for 24h and 48h. Cell viability between 4 different WT MEF lines and 5 different DJ-1 KO MEF lines treated with 100μM PQ for 24h also showed no differences in cell viability between Wt and DJ-1 KO MEFs. My PQ data on MEFs conflicts with the results of previous studies on neuronal cells lines where the reduction of endogenous DJ-1 increased cell vulnerability to oxidative stress (see below).

Previous studies showed that downregulation of DJ-1 by siRNA in mouse Neuro2a (Yokota et al., 2003), human 293T cells (Yokota et al., 2003), neuroblastoma cells (Taira et al., 2004), SK-N-BE cells (Batelli et al., 2008), and murine primary midbrain cultures (Martinat et al., 2004) resulted in increased sensitivity to H$_2$O$_2$. In addition, mouse cortical neurons (Kim et al., 2005) and embryonic stem cells (Martinat et al., 2004) from DJ-1 KO mice showed increased sensitivity to H$_2$O$_2$. I selected PQ for my studies because environmental exposure to PQ is linked to the development of PD (Hertzman et al., 1990; Liou et al., 1997). Redox cycling of PQ generates superoxide, which does not easily cross biological membranes. Superoxide is rapidly dismutated to H$_2$O$_2$, which is the primary ROS through which PQ exerts its effects (reviewed in Armstrong & Whiteman, 2007). While it is possible that some of the discrepancy between my data and these other studies can be explained by my use of PQ, Wt and DJ-1 KO MEFs exposed to H$_2$O$_2$ also showed no differential sensitivity. It seems likely that the differences are due to cell type and that, specifically, the elimination of DJ-1 does not result in decreased cell viability in the MEF model. It has been reported the DJ-1 binds to and stabilizes Nrf2. This interaction facilitates the translocation of Nrf2 into the nucleus where it activates the transcription of a number of anti-oxidant genes, thereby providing one means of DJ-1-mediated cellular protection after oxidative stress (Clements et al., 2006). Since the DJ-1 KO MEFs used in this study were not conditional KOs, it may be that MEFs can more readily upregulate alternative anti-oxidant pathways/genes that are not regulated by the interaction between DJ-1 and Nrf2.

4.1.3. DJ-1 KO does not affect ROS levels in MEFs under basal conditions

Basal ROS levels did not differ between Wt and DJ-1 KO MEFs, which suggests that DJ-1 does not play a role in basal anti-oxidant defences. Andres-Mateos et al. (2007) found that isolated brain mitochondria from DJ-1 KO mice have increased H$_2$O$_2$, which suggests that DJ-1 plays a role in basal anti-oxidant defenses. In SH-SY5Y cells, overexpression of DJ-1 reduced
H$_2$O$_2$ under basal conditions (Junn et al., 2005). Since these reports used neuronal models and MEF are not a neuronal model, this may partially explain why they are not consistent with my results. Neuronal cells have lower anti-oxidant capability than MEFs (Sinet et al., 1980). Therefore, when DJ-1 is downregulated, MEFs may be better able to maintain low levels of ROS under basal levels than neuronal cells.

**4.1.4. DJ-1 KO increases ROS generation in MEFs after sustained PQ**

DJ-1 stabilizes Nrf2, a master regulator of the transcription of ARE genes in the oxidative stress response (Clements et al., 2006). In the absence of DJ-1, the inhibitor of Nrf2, Keap1, targets Nrf2 for degradation by ubiquitination (Furukawa & Xiong, 2005). This leads to decreased expression of ARE genes and a decreased antioxidant response, consequently I hypothesized that DJ-1 KO in MEFs would result in enhanced ROS levels after oxidative stress. Flow cytometry using DCF in Wt and DJ-1 KO MEFs after 24h PQ exposure indicated that ROS generation increased with increasing concentrations of PQ, but no effect of DJ-1 KO was observed. Trypsinization induces oxidative stress during collection of adherent cells (Solaini et al., 2007). However, it is unlikely that this procedure stressed Wt cells more than DJ-1 KO cells, masking any increased sensitivity of the DJ-1 KO MEFs. Multiple anti-oxidant defences exist in the cell, many of which do not involve DJ-1 (see section 1.6). Consequently, the effects of eliminating DJ-1 may have been masked by upregulation of other anti-oxidant mechanisms/genes that are not regulated by DJ-1. Interestingly, ROS levels at higher doses of PQ were reduced compared to lower doses of PQ. One explanation for this decline could be inactivation of esterases at higher doses. As a result, less DCFDA would accumulate inside the cell, leading to lower DCF fluorescence.

Fluorescence microscopy was used to assess ROS levels after acute exposure to oxidative stress. No differential effects were observed between Wt and DJ-1 KO MEFs after 1h H$_2$O$_2$, but increased DCF was observed in all cell lines. Treatment of MEFs with 400µM PQ for 2h failed to increase DCF fluorescence in all cell lines. Since superoxide is the primary ROS produced immediately after PQ exposure, MitoSOX, which measures mitochondrial superoxide production, may have been a better ROS indicator. However, a study that exposed rat brain fractions to 250µM PQ found that DCF signal was increased almost immediately, indicating that there was a detectable rise in H$_2$O$_2$ by DCF instantaneously after PQ exposure (Castello et al.,
In another study, DCF signal was shown to increase in SH-SY5Y cells after 4h 400μM PQ exposure (Maracchioni et al., 2007). These reports support the use of DCF as a ROS indicator after PQ exposure. The results of my study indicate that PQ does not induce immediate increases in ROS generation in MEFs, rather, sustained PQ exposure is required for increased ROS production. While it cannot be precluded that anti-oxidant responses were upregulated in DJ-1 KO MEFs at 24h, this possibility is unlikely because 1h H₂O₂ generated an increase in ROS in Wt 5-6, Wt 5-5, KO 5-2, and KO 4-4 without unveiling differential responses between Wt and DJ-1 KO MEFs. Although assessment of ROS levels after 1h H₂O₂ has only been performed once in MEFs, the similar observations between 2 different Wt MEF lines and between 2 different DJ-1 KO MEF lines are adequate to suggest that ROS levels are not different between Wt and DJ-1 KO MEFs after acute H₂O₂ exposure. Collectively, in the 4 MEF lines tested, DJ-1 KO had no effect on ROS generation evoked by short term or long term oxidative stress.

4.1.5. Direct assessments of ROS after oxidative challenges in DJ-1 KO

Multiple studies have investigated the effects of DJ-1 downregulation in combination with oxidative stress. The majority assessed cell viability, however few actually examine ROS generation directly under basal conditions and the oxidatively stressed state. Notably, PQ exposure was reported to decrease cell viability in DJ-1 KO cells, but no direct correlation between cell viability and increased ROS was made (Lavara-Culebras & Paricio, 2007; Meulener et al., 2005). To date, no study in DJ-1 KO cells has assessed ROS levels after PQ exposure. My data showing that ROS production is not increased in DJ-1 KO cells exposed to PQ is the first such report.

4.1.6. The pitfalls of H₂DCFDA

Dichlorodihydrofluorescein diacetate (H₂DCFDA) is frequently used to monitor ROS production in live cells. H₂DCFDA is non-fluorescent and passively enters cells, where it becomes trapped after its acetate groups are cleaved by intracellular esterases to generate 2’, 7’-dichlorodihydrofluorescein (H₂DCF) (LeBel et al., 1992). H₂DCF is non-fluorescent, but can be oxidized by H₂O₂ to highly fluorescent 2’, 7’-dichlorofluorescein (DCF) (Bass et al., 1983). Because DCF primarily measures H₂O₂, measurements with DCF may underestimate the total
ROS burden. However, it was recently reported that H$_2$DCF can be oxidized by peroxynitrite, other peroxides, and hypochlorous acid as well (Whiteman et al., 2004; Whiteman et al., 2005). An inherent drawback of H$_2$DCF is its high degree of photosensitivity, given that H$_2$DCF can be auto-oxidized by excitation light or ambient illumination alone to produce large background fluorescence in the absence of ROS (Marchesi et al., 1999). Another contributor to background fluorescence is ROS formation in the incubation media (Grzelak et al., 2000). Extracellular fluorescence is further increased by DCF extrusion from cells (Hempel et al., 1999).

4.1.7. Rh123 as a fluorescence indicator for MTP

Rh123, a membrane permeable cation, was used as an indicator of MTP. In the mitochondria of living cells, a proton gradient is directed inwards towards the mitochondrial matrix, resulting in a MTP of -150mV to -180mV in comparison to the cytosol. Since this negative potential depends upon an active respiratory chain, use of Rh123 as a mitochondrial marker is restricted to living cells. Rh123 distributes across all membranes but accumulates preferentially in mitochondria because mitochondria are the most electrically negative compartment in the cell. Mitochondrial fluorescence of Rh123 can be linearly related to MTP, thereby allowing the MTP to be estimated by the Nernst equation (Foster et al., 2006; Solaini et al., 2007). Rh123 can be used in quenched and non-quenched modes and it is important to determine which mode is appropriate.

4.1.8. MTP is unchanged after sustained PQ exposure

ROS levels and MTP are intimately linked. Mitochondria generate the majority of basal cellular ROS as a byproduct of oxidative phosphorylation and oxidative phosphorylation is dependent upon maintenance of MTP by a functional ETC. When there is an imbalance between ROS production and ROS removal, excess ROS inhibit the ETC. This inhibition permits free electrons to pass from respiratory complexes to O$_2$ instead of to the next electron carrier, resulting in increased mitochondrial superoxide production, which further exacerbates ETC inhibition. Maintenance of MTP requires the coupling of energy released by electrons moving down the ETC and consequent proton extrusion to the pumping of protons into the IMS. Therefore, one consequence of ETC inhibition is decreased MTP (reviewed in Cadenas & Davies, 2000).
We assessed Rh123 by fluorescence microscopy. Fluorescence microscopy revealed no change in MTP after 24h 100μM PQ. 24h PQ exposure has been reported to decrease MTP in SH-SY5Y cells (Yang & Tiffany-Castiglioni, 2008), PC12 cells (Kang et al., 2007), and isolated rat liver mitochondria (Palmeira et al., 1995). In MEFs, 100μM PQ (24h) did not increase ROS levels or cell death, which suggests that this dose was too low to produce significant oxidative stress. This is in marked contrast to PC12 cells and rat cortical neurons where 100μM PQ is associated with increased cell toxicity. Further experiments using higher concentrations of PQ and/or other ROS indicators could address this issue.

4.1.9. Microscopy versus flow cytometry for fluorescence measurements

Fluorescent probes can be visualized using fluorescence microscopy or flow cytometry, the first of which yields qualitative data while the latter is able to quantitatively estimate intracellular fluorescence. Fluorescence microscopy permits visualization of only a subset of cells at any given time, making evaluation of culture heterogeneity difficult (Mukhopadhyay et al., 2007). Photoactivation of fluorescent probes is also an issue with fluorescence microscopy. Flow cytometry, on the other hand, can evaluate entire cell populations and detect heterogeneity in fluorescence signal with limited photoactivation (Solaini et al., 2007). Although the utilization of lasers or UV light to excite fluorescent probes in either technique can increase ROS generation, the effect is much less in flow cytometry due to the lower intensity of excitation light (Mukhopadhyay et al., 2007). Conversely, the requirement of suspended cells for flow cytometry necessitates scraping, or trypsinization and this stress may alter MTP and increase ROS levels (Solaini et al., 2007).

4.1.10. MEFs

MEFs are useful cellular models where KO lethality is an issue because MEFs are harvested from embryos. MEFs can be grown and readily passaged in culture so that the effects of gene KO can be studied, even when KO lethality would render in vivo studies, or harvesting cells from post-natal animals, impossible. One issue of using MEFs is culture heterogeneity. Although heterogeneity is reduced by culture conditions, it is never completely eliminated. Introduction of MEFs to culture conditions causes a 2-3 fold increase in mutations that further
accumulate with each successive passage (Busuttil et al., 2003; Matsumura et al., 1989). Subsequent passaging may lead to selective propagation of sub-populations of cells, leading to enhancement of distinct clonal characteristics (Tolstonog et al., 2005). For example, MEFs deficient in SOD2 were able to increasingly overcome their initial growth deficits with each passage and eventually immortalize (Samper et al., 2003).

As MEFs accumulate mutations, they undergo a progressive decline in growth potential until around passage 15-20, at which time they either spontaneously immortalize and their growth increases until it equals or exceeds that of the original culture, or they senesce and die due to telomere shortening that occurs with increased passaging (Todaro & Green, 1963). MEFs are also less sensitive to oxidative stress than neurons due to greater expression of anti-oxidant enzymes (Sinet et al., 1980). Thus, while useful, studies using MEFs to study neurodegenerative processes should be complemented with neuronal studies.

PART II: EXPRESSION AND SUBCELLULAR TRANSLOCATION OF DJ-1 AFTER OXIDATIVE STRESS

4.2.1. DJ-1 and subcellular translocation

DJ-1 is a redox sensitive protein that undergoes a shift in predominant pI isoform upon exposure to oxidative stress (Canet-Aviles et al., 2004; Mitsumoto et al., 2001). Studies have shown that DJ-1 is protective against oxidative stress induced by H₂O₂ (Junn et al., 2009; Kim et al., 2005; Yokota et al., 2003; Zhou & Freed, 2005), RT (Lev et al., 2008), 6-OHDA (Batelli et al., 2008; Zhou & Freed, 2005), and PQ (Canet-Aviles et al., 2004; Meulener et al., 2005). It is thought that oxidative stress-induced alterations in DJ-1 are cytoprotective (Canet-Aviles et al., 2004; Fan et al., 2008; Junn et al., 2009; Lev et al., 2008); however, the mechanism through which DJ-1 mediates protective effects after exposure to oxidative stress is unknown. Still hotly debated is the idea that the protective capabilities of DJ-1 involve translocation of DJ-1 to different subcellular compartments. Specifically it is hypothesized that DJ-1 mediates cellular protection through translocation to mitochondria after oxidative stress. Some studies also suggest that nuclear translocation of DJ-1 may be important. To understand how DJ-1 mediates neuroprotection, it is imperative that the localization and role of DJ-1 under basal conditions is established.
Although the majority of studies have focused on a role of mitochondrial translocation of DJ-1 in protection against oxidative stress, it is still not clear how DJ-1 mediates cytoprotection, and where its primary site of action is in the cell. Mitochondria are attractive as a translocation target given their role in ROS generation and cell survival/apoptotic pathways. Most oxidative stressors used in the study of PD are mitochondrial toxins that specifically target the ETC to increase ROS production (Castello et al., 2007; Fallon et al., 1997; Panov et al., 2005; Sachs & Jonsson, 1975). It has been proposed that DJ-1 acts directly as an anti-oxidant and this, combined with its increased translocation to mitochondria, could help combat lethal rises in ROS levels. Studies have also proposed that oxidative stress induces a direct DJ-1 association with the OMM, a key regulator in apoptosis (Betarbet et al., 2006; Canet-Aviles et al., 2004; Junn et al., 2009; Ramsey & Giasson, 2008). Permeabilization of the OMM leads to release of apoptotic factors and is considered a point of no return in apoptosis (Chipuk et al., 2006). In addition, apoptosis regulators of the Bcl-2 family preside on the OMM. Mitochondrial translocation of DJ-1 after oxidative stress could prevent apoptosis by blocking permeabilization of the OMM or by interacting with pro-apoptotic Bcl-2 family proteins. However, no study to date has directly addressed such interactions. Further, how DJ-1 is targeted to the mitochondria is an open question since it lacks a mitochondrial localization sequence (Junn et al., 2009).

4.2.2. DJ-1 is localized to the cytoplasm, mitochondria, and nucleus

In my studies, immunofluorescence and Western blotting were used to determine the constitutive localization of endogenous DJ-1 in PC12 cells, rat cortical neurons and Wt MEFs. My immunofluorescence results from PC12 cells and rat cortical neurons are consistent with previous reports of a predominantly cytoplasmic localization. Mitochondria labelled with MitoTracker, or antibodies to TOM20, appear as spheroid to snake-like structures throughout the cell. Using DJ-1 antibodies, I observed only a diffuse distribution of DJ-1 throughout the cell cytoplasm. These results indicate that DJ-1 is not primarily localized to mitochondria under basal conditions. Studies using different anti-DJ-1 antibodies also reported no DJ-1 co-localization with mitochondria under basal conditions (Blackinton et al., 2005; Canet-Aviles et al., 2004; Miller et al., 2003). My experiments using two different primary anti-DJ-1 antibodies did show some nuclear localization, suggesting that DJ-1 is present in the nucleus under basal conditions.
Immunofluorescence studies in SH-SY5Y cells (Lev et al., 2008), SK-N-BE(2)C cells (Junn et al., 2009), and rat cortical and nigral neurons (Betarbet et al., 2006) showed that DJ-1 has a similar diffuse pattern to the one I observed in PC12 cells and rat cortical neurons. Exogenous DJ-1 in transfected M17 neuroblastoma cells (Blackinton et al., 2005; Canet-Aviles et al., 2004), COS7 cells (Miller et al., 2003), Neuro2A cells (Fan et al., 2008; Ramsey & Giasson, 2008), SH-SY5Y neuroblastoma cells (Fan et al., 2008), COS1 cells (Park et al., 2005), and H1299 cells (Shinbo et al., 2006) also showed that DJ-1 is predominantly localized to the cytoplasm under basal conditions. These studies reported some overlap with MitoTracker in some mitochondria in some cells. However, it is important to note that when two markers are used that label different proteins within cytoplasm, some degree of overlap is almost inevitable. Consequently, observations of DJ-1 overlap with MitoTracker under basal conditions must be regarded with caution. Finally, two other studies reported a primarily nuclear localization of DJ-1 in NIH3T3 (Li et al., 2005), SH-SY5Y cells and Neuro2A cells (Fan et al., 2008) which conflicts with the generally agreed upon predominant cytoplasmic localization.

Western blots showed that DJ-1 is present in the cytoplasm, mitochondria and nucleus under basal conditions in PC12 cells. These results are consistent with those of Miller et al. (2003), who observed no mitochondrial localization of DJ-1 by immunofluorescence, although Western blotting revealed mitochondrial DJ-1. Junn et al. (2009) also reported similar results in SK-N-BE(2) cells by Western blot. Immunogold electron microscopy and subcellular fractionation also support mitochondrial localization of DJ-1 under basal conditions (Zhang et al., 2005). However, DJ-1 does not contain a known mitochondrial localization sequence (Junn et al., 2009), so it is not clear how DJ-1 is able to translocate to the mitochondria under any conditions. My results are in agreement with previous reports of nuclear localization of DJ-1 in M17 cells (Canet-Aviles et al., 2004; Miller et al., 2003) and SK-N-BE(2) cells (Junn et al., 2009) under basal conditions as assessed by Western blotting. In summary, my results are consistent with a cytoplasmic, mitochondrial and nuclear localization of DJ-1 under basal conditions.

4.2.3. H$_2$O$_2$ does not increase mitochondrial translocation of DJ-1

DJ-1 translocation to mitochondria has been reported to occur in response to H$_2$O$_2$-induced oxidative stress. However, my studies indicate that such translocation does not occur in
PC12 cells and rat cortical neurons. Junn et al. (2009) reported that mitochondrial localization of DJ-1 in SK-N-BE(2)C cells peaked after 3h of H2O2 exposure. Using their subcellular fractionation protocol on PC12 cells exposed to a comparable dose of H2O2 for 3h, I failed to see any mitochondrial translocation by Western blot. Immunostaining of PC12 cells also showed no mitochondrial translocation of DJ-1 after 3h - 24h of H2O2 exposure. The literature investigating DJ-1 translocation is inconsistent. Several studies use the DJ-1 L166P mutant to study mitochondrial translocation of DJ-1 and this form of DJ-1 may not behave like endogenous DJ-1. Results also vary depending on the type of oxidative stress applied, the duration of oxidative challenge, and the type of cell line used, which suggests that DJ-1 is not universally sensitive to all types of oxidative stress and that DJ-1 responses follow different timelines in different cell types. Studies using H2O2 as an acute (1h-3h) oxidative stress in SK-N-BE(2)C cells (Junn et al., 2009) and Neuro2A cells (Ramsey & Giasson, 2008) found increased mitochondrial translocation of endogenous DJ-1; specifically, limited trypsin digestion and subcellular fractionations suggested that DJ-1 was associated with the cytoplasmic side of the OMM or located in close proximity to mitochondria, but did not enter mitochondria. This is consistent with the fact that DJ-1 does not have a mitochondrial targeting sequence (Junn et al., 2009). In contrast, despite our use of two different cell types, different concentrations of H2O2, and different exposure times we did not observe mitochondrial translocation of DJ-1. While we cannot rule out the possibility that DJ-1 translocation to mitochondria occurred in a few cells, my results suggest that H2O2 does not alter the distribution of DJ-1 in primary neurons or neuronal-like PC12 cells.

4.2.4. DJ-1 is upregulated after H2O2

H2O2 exposure for 3h-24h increased DJ-1 immunofluorescence in PC12 cells, consistent with the results of Taira et al. (2004), who reported upregulation of DJ-1 expression at 12h of H2O2 exposure in SH-SY5Y cells by Western blotting. In my studies in rat cortical neurons, H2O2 induced a marked upregulation of DJ-1. In contrast to PC12 cells, this increase was transient and DJ-1 levels fell to basal levels by 24h. Upregulation of DJ-1 after H2O2 exposure has also been demonstrated in zebrafish brains by Western blotting (Baulac et al., 2009). Overall, my data indicates that H2O2 induces upregulation of DJ-1 but not in mitochondria.
4.2.5. PQ does not increase translocation of DJ-1 to mitochondria

Western blotting of mitochondrial fractions showed no increase in mitochondrial DJ-1 in response to low and high doses of PQ added for 3h or 24h. Parallel immunofluorescence studies also showed no increase in DJ-1 in mitochondria. Taken together, these data demonstrate that PQ-induced oxidative stress does not result in translocation of DJ-1 to mitochondria. The literature regarding PQ-induced mitochondrial translocation of DJ-1 is inconsistent. Canet-Aviles et al. (2004) reported that 24h PQ exposure induced mitochondrial translocation of DJ-1 to the cytoplasmic side of the OMM in M17 neuroblastoma cells. These findings were confirmed by Blackinton et al. (2005), although they did not establish a preferential site of DJ-1 localization in mitochondria. Conversely, Zhang et al. (2005) treated SH-SY5Y cells with PQ for 24h and found no increased DJ-1 localization to mitochondria by Western blot. To date, no studies have investigated total DJ-1 expression after PQ exposure, although a number of studies have indicated that PQ treatment leads to accumulation of the acidic isoform of DJ-1 (Canet-Aviles et al., 2004; Mitsumoto et al., 2001). There are also no reports of the effects of acute PQ exposure on potential DJ-1 translocation. Overall despite the relationship between PD and environmental exposure to PQ, a link between PQ and DJ-1 is only weakly supported by current literature. This is consistent with my results showing no DJ-1 translocation in response to PQ.

4.2.6. Mitochondria and DJ-1

Junn et al. (2009) targeted DJ-1 to mitochondria through cloning of the mitochondrial targeting signal of Bcl-XL upstream of the DJ-1 gene and reported that mitochondrial targeted DJ-1 was more effective at protecting cells against oxidative stress when compared to cytoplasmic and nuclear targeted DJ-1. The physiological relevance of this study is not clear, as DJ-1 does not have a mitochondrial targeting sequence and it is still unknown how DJ-1 translocates to mitochondria. Blackinton et al. (2005) casted doubt on mitochondrial translocation of DJ-1 being neuroprotective, reporting that while PQ induced translocation of Wt, L166P, and M26I mutant DJ-1 to mitochondria, only Wt DJ-1 was neuroprotective for M17 neuroblastoma cells against PQ. Other studies on SH-SY5Y cells reported that Wt and mutant DJ-1 did not translocate to mitochondria in response to PQ, but that Wt DJ-1 cells showed increased viability compared to DJ-1 mutants (Zhang et al., 2005). This suggests that the
neuroprotective effects of DJ-1 do not involve mitochondrial translocation, which is consistent with my results showing no translocation of DJ-1 to the mitochondria after PQ.

4.2.7. The nucleus as a target of DJ-1 translocation

My Western blotting data suggests that nuclear translocation of DJ-1 may occur after oxidative stress. To date, little is known about the physiological relevance of nuclear DJ-1 localization under basal conditions, and even less about DJ-1 translocation to the nucleus after oxidative stress. DJ-1 does not have a nuclear targeting sequence, but sumoylation is also important for nuclear localization and DJ-1 can be sumoylated on a number of its amino acids, the most important of which being lysine 130 (Fan et al., 2008). Junn et al. (2009) was the first to report time-dependent changes in DJ-1 translocation after oxidative stress induced by H₂O₂ in SK-N-BE(2)C cells, demonstrating that mitochondrial translocation of DJ-1 after oxidative stress is an early event that is subsequently followed by nuclear translocation, at which time mitochondrial DJ-1 levels are not different from controls. In a UV-induced oxidative stress model, DJ-1 translocated to the nucleus after 1h and gradually returned to the cytoplasm between 1h and 24h post-UV exposure in ME180 cells (Shinbo et al., 2006). Another study reported a primarily nuclear localization of DJ-1 under basal conditions and DJ-1 translocation to the cytoplasm after H₂O₂ exposure in NIH3T3 cells (Li et al., 2005a).

Three studies have investigated the nucleus as a site of DJ-1 neuroprotective action thus far. Work by Junn et al. (2005) found that DJ-1 interacts with Daxx in the nucleus. Daxx is a nuclear protein that is capable of cytoplasmic translocation and subsequent interaction with the intracellular domain of the Fas receptor to trigger apoptotic cell death. Daxx can also interact with and activate ASK1 to induce caspase-independent cell death. By translocating to the nucleus and sequestering Daxx, DJ-1 is able to prevent Daxx-mediated cell death (Junn et al., 2005).

DJ-1 also prevents apoptosis by inhibiting transcriptional silencing induced by PSF (pyrimidine tract binding protein associated splicing factor) in cooperation with p54nrb in the nucleus (Xu et al., 2005). p54nrb is normally repressed by PSF, resulting in decreased transcription. When DJ-1 binds p54nrb, survival gene transcription is increased. Similarly, the association of DJ-1 with PSF increases the transcription of tyrosine hydroxylase, which is a key enzyme in the synthesis of DA. Under conditions of oxidative stress, PSF becomes sumoylated
and dissociates from DJ-1. Sumoylated PSF is capable of PGC-1α activation, leading to subsequent increased transcription of MnSOD and an increased anti-oxidant response (Xu et al., 2005).

Most recently it was shown that DJ-1 binds to and represses p53 transcriptional activity in the nucleus, leading to decreased expression of the proapoptotic protein, Bax (Bretaud et al., 2007; Fan et al., 2008). In DJ-1 KO zebrafish embryos, p53 expression is elevated under basal conditions (Bretaud et al., 2007). Furthermore, Fan et al. (2008) showed that mutation of lysine 130 resulted in decreased sumoylation of DJ-1 and subsequent decreased nuclear localization and increased cell death, providing both a mechanism for DJ-1 nuclear localization, and additional evidence that nuclear DJ-1 localization is neuroprotective. However, no studies to date have examined whether oxidative stress affects any of these cell death pathways that DJ-1 is purported to play a role in. DJ-1 stabilizes Nrf2 in the cytoplasm after oxidative stress. Subsequently Nrf2 translocates to the nucleus to mediate transcription of anti-oxidant genes (Clements et al., 2006), but it is not known whether DJ-1 and Nrf2 translocate to the nucleus together to mediate anti-oxidant effects. Further experiments should focus on identifying if any of the reported nuclear associations with DJ-1 hold true in cells exposed to oxidative stress.

4.2.8. Summary of DJ-1 translocation data

DJ-1 is present in the mitochondria under basal conditions, but the function of DJ-1 in the mitochondria is not known. Furthermore, DJ-1 does not have a mitochondrial targeting sequence, which raises questions about how it is able to localize to mitochondria under basal condition and how translocation after oxidative stress is mediated. **Cytoplasmic and mitochondrial DJ-1 localization patterns did not significantly change after oxidative stress in this study.** It remains possible that mitochondrial translocation occurred in a few cells but if so, it is not a widespread phenomenon. To detect DJ-1 mitochondrial translocation after oxidative stress, I used two neuronal models, different doses of two oxidative stressors, and both short term and long term exposure. **Overall, my studies indicate that mitochondrial translocation of DJ-1 does not occur after oxidative stress in neurons but that DJ-1 translocation to the nucleus may occur after oxidative stress in PC12 cells.**
PART III: THE EFFECTS OF OXIDATIVE STRESS ON Cn ACTIVITY AND CnA EXPRESSION

4.3.1. DJ-1 and Cn are potentially linked

DJ-1 is purported to be a redox-sensitive protein whose upregulation leads to cancer and downregulation leads to neurodegenerative disease (Bonifati et al., 2003; Hod, 2004). Another redox-sensitive protein, Cn, follows the same pattern and is also upregulated in tumorigenesis and downregulated in neurodegeneration diseases (Agbas et al., 2005; Madoz-Gurpide et al., 2007). To date, only Wang et al. (2008) has suggested a link between these two proteins, reporting that overexpression of the regulatory subunit of Cn (CnB) in HEK293 cells led to increased oncogenic potential as well as a concomitant increase in DJ-1. It is unknown whether any interactions occur between DJ-1 and Cn and if there are, which cellular compartment it occurs in. I aimed to study whether the reverse relationship is true, that is, whether downregulation of DJ-1 leads to downregulation of CnA and how oxidative stress affects this relationship. The genes coding for CnA and CnB are located on different chromosomes and can be differentially expressed (Wang et al., 1996a). CnA was selected for expression studies because CnA contains the catalytic subunit of Cn; therefore, Cn activity can only be as great as the expression of CnA. Prior to the investigation of effects of DJ-1 KO on CnA expression and Cn activity in response to oxidative stress, it was essential to first elucidate how Cn is altered after oxidative stress only.

4.3.2. PQ, H$_2$O$_2$ and menadione affect CnA levels via distinct mechanisms

4.3.2.1. H$_2$O$_2$ decreases CnA levels via cleavage of CnA

H$_2$O$_2$ exposure resulted in the cleavage of CnA into a 32kDa fragment in rat cortical neurons, which is consistent with a previous report of CnA cleavage after exposure to H$_2$O$_2$ (Lee et al., 2007). To date, there are only two known CnA cleavage products: 1) a 45kDa fragment that is constitutively active (Mukerjee et al., 2000; Wu et al., 2004), and 2) a 32kDa fragment that is permanently inactive (Lee et al., 2007). The 32kDa fragment I observed is likely an inactive form of CnA. In mouse primary cortical neurons, chloroquine, a lysosome protease inhibitor, prevents CnA cleavage, suggesting that CnA cleavage can be mediated by
lysosomal proteases that have leaked into the cytoplasm after H$_2$O$_2$ exposure (Brunk et al., 1995; Lee et al., 2007).

Whole CnA levels after H$_2$O$_2$ exposure were not different from controls suggesting that CnA protein is continuously synthesized as CnA is cleaved, and that the rates of the two processes are coupled. Lee et al. (2007) reported that cleaved CnA is degraded by the proteasome; therefore, the elevated levels of cleaved CnA at 24h may reflect a delay in degradation by the proteasomal degradation pathway. However, it is not possible for us to conclude whether the progressively declining levels of CnA cleavage fragment from 1h to 24h are the result of gradually declining de novo cleavage, or delayed degradation of a static CnA fragment pool, or the combination of both.

### 4.3.2.2. PQ decreases CnA levels without cleavage of CnA

In PC12 cells and rat cortical neurons, PQ did not cause CnA cleavage, but whole CnA levels were reduced at 24h. This PQ data differs from my H$_2$O$_2$ data in 2 ways: 1) CnA cleavage was detected after a 1h H$_2$O$_2$ exposure, in contrast PQ caused no cleavage at any time examined, 2) whole CnA levels did not decline after H$_2$O$_2$ treatment, in contrast PQ did decrease whole CnA levels. My data support time-dependent effects of H$_2$O$_2$ and PQ on CnA, and suggest that H$_2$O$_2$ and PQ mediate changes in CnA levels by different mechanisms. Moreover, my data represent the first report of decreased CnA levels in neuronal models after PQ exposure.

H$_2$O$_2$ and PQ have distinct oxidative stress-generating mechanisms. The half-life of H$_2$O$_2$ is short, so cells exposed to H$_2$O$_2$ may recover as H$_2$O$_2$ levels decline. Conversely, PQ continuously produces ROS at a low rate, resulting in constant stress that may not allow recovery from oxidative damage. As a result, short-term PQ may have generated only very low levels of ROS that were insufficient to cause oxidative stress while longer PQ exposure may cause degradation of CnA as a consequence of cumulative ROS production and oxidative damage. My data do not indicate whether the decline in CnA levels is due to increased degradation, or decreased expression of CnA. Toxic doses of PQ at 48h also resulted in reduced whole CnA levels in PC12 cells, suggesting that decreased CnA is either a response that predisposes cells to death, or a protective response in surviving cells to prevent cell death. My results argue that H$_2$O$_2$ and PQ affect CnA via distinct mechanisms, with the former...
cleaving CnA without altering whole CnA levels and the latter causing a decrease in whole CnA levels in the absence of lysosomal proteolytic cleavage.

4.3.2.3. CnA cleavage is also induced by menadione

Menadione is a redox-cycling compound that is lipophilic, and crosses membranes easily. Redox cycling of menadione is initiated rapidly due to increased contact with suitable electron donors (reviewed in Comporti, 1989 and Lamson & Plaza, 2003), which may explain why a prominent cleavage fragment of CnA was observed early after menadione exposure. Conversely, redox cycling of PQ may be retarded due to its hydrophilic nature and lack of available electron donors (Clejan & Cederbaum, 1989), resulting in slower generation of ROS and lack of CnA cleavage. Menadione can also cause release of cathepsins from lysosomes into the cytoplasm (Baumgartner et al., 2007). Although Lee et al. (2007) reported that cathepsins are not involved in the cleavage of CnA after H₂O₂ exposure, it is conceivable that H₂O₂ and menadione disrupt lysosomal membranes, thereby resulting in release of lysosomal proteases. Information on how menadione affects the integrity of lysosomal membrane are lacking. My results suggest that, although PQ and menadione are both redox cycling compounds, H₂O₂ and menadione may be more similar in their mechanisms of actions on CnA due to their ease in crossing cellular membranes, and their potential for causing an early burst of ROS.

4.3.3. Oxidative stress decreases cell viability

Oxidative stress is defined as the imbalance between cellular ROS production and ROS removal. Anti-oxidant defences exist in the cell to combat ROS; however, exogenous addition of ROS sets off a feed forward mechanism that results in the generation of more ROS, leading to mitochondrial dysfunction and damage to proteins, lipids and DNA, which ultimately culminates in cell death (reviewed in Cadenas & Davies, 2000). As a result, oxidative stress is expected to decrease cell viability, as was observed in PC12 cells and rat cortical neurons. Previous studies have also shown that oxidative stress decreases viability in embryonic stem cells (Martinat et al., 2004), mouse primary cortical neurons (Kim et al., 2005) and SK-N-BE cells (Batelli et al., 2008).
The results in rat cortical neurons most clearly showed the differential toxicities of H$_2$O$_2$, PQ and menadione. H$_2$O$_2$ was expected to significantly reduce cell viability soon after exposure as it is permeable to biological membranes, which is consistent with my observation of decreased cell viability in rat cortical neurons after 1h of H$_2$O$_2$ exposure. A comparison of cell viabilities after exposure to H$_2$O$_2$, PQ, or menadione for 24h showed that H$_2$O$_2$ was the least effective at decreasing cell viability. This result was expected, given that H$_2$O$_2$ has a short half-life and is rapidly removed by catalase (reviewed in Cadenas & Davies, 2000). As PQ and menadione are redox cycling compounds that produce ROS continuously, I anticipated that they would reduce cell viability more than H$_2$O$_2$. Menadione is a highly cytotoxic anti-tumour agent in rodents, even at micromolar concentrations (Kuriyama et al., 2005; Osada et al., 2001; Sasaki et al., 2008), which explains its high toxicity. One reason for the differential toxicity of PQ and menadione may lie in their varying abilities to cross biological membranes and generate oxidative stress. Menadione is fat soluble and readily crosses membranes, while PQ is charged and requires Na$^+$-dependent uptake (Fukushima et al., 1995; reviewed in Lamson & Plaza, 2003). Their rates of electron capture and donation to produce superoxide may also differ significantly, resulting in differential effects on cell viability depending on the rates of ROS production.

4.3.4. Low dose menadione increases cell viability

Menadione is not a naturally occurring analog of vitamin K. It can function as a synthetic provitamin that can be alkylated to form biologically active isoprenylated menaquinone, otherwise known as vitamin K$_2$, in bacteria. Bacteria can use vitamin K$_2$ in anaerobic respiration to generate ATP (reviewed in Lamson & Plaza, 2003). Although high concentrations of menadione or vitamin K$_2$ are cytotoxic due to the generation of oxidative stress, pharmacological doses of vitamin K$_2$ protect against fractures and bone loss and are vital in blood coagulation (Merli & Fink, 2008; Shea & Booth, 2008). Low dose menadione may therefore have as yet unidentified neuroprotective effects, which could translate into the increased cell viability seen in cortical neurons exposed to low dose menadione for 4h.
4.3.5. Cn activity does not change after oxidative stress

4.3.5.1. Cn is not inactivated after PQ exposure in PC12 cells and rat cortical neurons or after H₂O₂ exposure in rat cortical neurons

CnA levels and Cn activity are not strictly correlated, as Cn inactivation may occur via oxidation of Fe²⁺ in the active site, which does not impact CnA levels. My data show that Cn activity was unchanged after 1h-24h 40µM PQ exposure in PC12 cells, and after 4h 10µM or 20µM PQ exposure in rat cortical neurons, consistent with my findings that CnA levels were unchanged in PC12 cells and rat cortical neurons at these doses and time points. Although CnA was cleaved into an inactive 32kDa fragment after 1h H₂O₂, Cn activity did not change in rat cortical neurons exposed to H₂O₂. This is also consistent with my findings that 1h to 24h of 50µM H₂O₂ exposure did not change whole CnA levels. My results suggest that whole CnA levels, rather than cleaved CnA levels, correlate more closely with Cn activity.

A previous study reported that superoxide, which is generated by PQ through redox cycling, inactivates Cn in Jurkat T lymphocytes (Namgaladze et al., 2002). Inactivation of Cn after PQ treatment has also been reported in SK-N-SH neuroblastoma cells (Sommer et al., 2002) and SH-SY5Y neuroblastoma cells (Ferri et al., 2000). Studies in neutrophils (Carballo et al., 1999) and NK cell lyastes (Furuke et al., 1999) found that H₂O₂ treatment decreased Cn activity. Chronic oxidative stress in neuroblastoma cells is reported to inactivate Cn (Ferri et al., 2000; Sommer et al., 2002). Other reports indicate that Cn is activated by oxidative stress induced by β-amyloid and prion protein in rat cortical neurons (Agostinho et al., 2008), by H₂O₂ in mouse cerebellar granule neurons, and by β-amyloid in human SH-SY5Y cells (Reese et al., 2008). Another study reported no change in Cn activity after rat cortical neurons, rat brain cytosol, and purified Cn were treated with PQ or the xanthine/xanthine oxidase superoxide-generating system (Agbas et al., 2007), which is consistent with my results. It is clear that there is no consensus in the literature regarding how Cn activity changes in response to oxidative stress in any cell type.

Despite attempts to optimize the assay, our results showed high variability between experiments. A positive control was not done so it is possible that the sensitivity of the Cn activity assay was not sufficient, although it would appear that the assay can measure changes in activity. Cn activity assays are also fraught with inherent problems (see section 4.3.6 below). Ongoing experiments are addressing these issues.
4.3.5.2. *Cn activity and CnA levels do not correlate after 24h PQ exposure in rat cortical neurons*

Cn activity was unchanged at 24h of PQ exposure in rat cortical neurons, whereas CnA levels had significantly declined at this time point. This suggests that the remaining pool of Cn must have been maximally activated in order for Cn activity to be unchanged versus controls. While sustained PQ exposure in astrocytes is reported to upregulate gene transcription of anti-oxidative enzymes (Olesen et al., 2008), other studies show that SOD is upregulated in rat brain by acute oxidative stress only (Filipovic & Radojcic, 2005). Further experiments are required to determine if the upregulation of anti-oxidant defences prevents the inactivation of Cn during long term PQ stress (increased ROS), in cortical neurons, leading to sustained activation of Cn. Since Cn activation is involved in the upregulation of apoptotic pathways, Cn downregulation may provide neuroprotection against oxidative stress in neurons, while Cn activation may promote cell death. In fact, a ‘tug-of-war’ phenomenon may be occurring after sustained PQ challenge, which provides a possible explanation for the simultaneous observations of decreased CnA levels and maintained Cn activity seen in this study. This idea needs to be further investigated.

4.3.6. *Pitfalls of the Cn activity assay*

Problems exist with all methods of Cn activity assessment. Measurement of released radioactive phosphate was the preferred method prior to the development of the colorimetric assay, in which free phosphate is detected through complexing with malachite green. Contamination of samples with phosphate is common, as phosphate is ubiquitous in the environment. It is difficult to measure Cn activity in cellular extracts because Ca$^{2+}$ and CaM in the extract and assay buffer can reactivate Cn that was inactivated during experimental treatments, thereby causing an overestimation of actual Cn activity (Shibasaki et al., 1997). All current methods measure the amount of phosphate released when Cn activity is inhibited, and subtract that from the amount of total phosphate released due to total cellular phosphatase activity, and are thus an indirect measure of Cn activity. One way to circumvent this is to measure the activity of purified Cn; yet, the purification process has been shown to oxidize Cn, resulting in underestimation of Cn activity (Klee et al., 1979; Wang et al., 1996b). To summarize, measurements of Cn activity must be interpreted with care.
4.3.7. Cn downregulation mediates neuroprotection

The brain regions that are most vulnerable to stroke, epilepsy and neurodegenerative diseases are also the areas that have the greatest Cn expression (Polli et al., 1991). Overexpression of constitutively active Cn in PC12 cells and neurons increases susceptibility to apoptosis under nutrient- and growth factor-stressed conditions and cell death can be rescued through the administration of the Cn inhibitors FK506 or CsA (Asai et al., 1999). CsA and FK506 are neuroprotective against ischemic injury in animal models, further suggesting a neuroprotective effect of Cn downregulation (Sharkey & Butcher, 1994; Uchino et al., 2008; Wang et al., 1999). Cn activity has been linked to apoptosis via NFAT, where NFAT dephosphorylation and activation by Cn leads to nuclear translocation of NFAT and activation of the Fas ligand gene. Increased Fas ligand production results in increased binding to the Fas death receptor on the cell membrane, leading to caspase-dependent apoptosis (Jayanthi et al., 2005; Shibasaki et al., 1997; Shioda et al., 2006; Shioda et al., 2007). Cn has a number of substrates, including CREB, NF-κB, NFAT, and Bad. Lee et al. (2007) showed that H$_2$O$_2$-mediated cleavage of CnA resulted in inactivation that led to downstream NFATc4 inactivation and extrusion from the nucleus, which may have neuroprotective effects. Since I observed CnA cleavage after H$_2$O$_2$ as well, and NFATc4 has been reported to be involved in cell death pathways in the brain (Jayanthi et al., 2005), I elected to study NFATc4 localization after H$_2$O$_2$ stress as a potential downstream neuroprotective effect of Cn inactivation.

4.3.8. NFATc4 translocation in rat cortical neurons

As H$_2$O$_2$ exposure led to CnA cleavage in rat cortical neurons, I hypothesized that this might have downstream effects on NFATc4, as previously reported in mouse cortical neurons (Lee et al., 2007). In two separate preliminary experiments, I observed increased cytoplasmic localization along with decreased nuclear localization, although the data could not be analyzed for statistical significance due to small sample size. The phosphorylation status of NFATc4 in the cytoplasm is maintained by constitutively active kinases, which was not expected to be disturbed significantly by the dose and duration of H$_2$O$_2$ applied (Bradley et al., 2005; Jayanthi et al., 2005). Therefore, under conditions of oxidative stress where Cn is inactivated, I would expect to see increased cytoplasmic accumulation of NFATc4 together with decreased nuclear localization of NFATc4, which is consistent with my preliminary data.
4.3.9. Pure nuclear fractions are difficult to obtain

Complete cell lysis and absolute nuclear isolation could not be assured, although all samples were checked qualitatively under the microscope for isolated nuclei. Therefore, the nuclear pellet may contain unlysed cells, which would contaminate the nuclear fraction with cytoplasmic components. This explains why GAPDH, a cytoplasmic marker, was present in my nuclear fractions in some cases. Cytoplasmic contamination of my nuclear fractions was especially problematic as I was assessing cytoplasmic/nuclear translocation; thus, nuclear localization of NFATc4 could have been overestimated. Another concern is the use of histone H3 as a nuclear marker; this proved to be ineffective as it was later revealed by Active Motif that there was a risk of histone extraction into cytoplasmic fractions using their kits. For future experiments, Sp1, a transcription factor, may be a more reliable alternative nuclear loading control than histone H3. Based on these technical issues, I analyzed NFATc4 localization data with the assumptions that total protein loading was equal in all nuclear samples and that cytoplasmic contamination, if present, was present to the same degree in all nuclear samples. Furthermore, there are only a limited number of weak commercially available antibodies for NFATc4, and these produce non-specific banding. Ongoing experiments will determine if alternate techniques are more suitable for NFAT translocation studies.

PART IV: THE EFFECTS OF DJ-1 KO ON Cn AFTER OXIDATIVE STRESS

4.4.1. DJ-1 KO does not affect basal CnA

The majority of literature supports the inactivation of Cn by oxidative stress. I had anticipated finding reduced CnA levels in DJ-1 KO MEFs under basal conditions because DJ-1 deficiency has been reported to increase ROS under basal conditions (Andres-Mateos et al., 2007). My data showed that basal ROS levels did not differ between Wt and DJ-1 KO MEFs, which is consistent with my finding that basal CnA expression was the same between Wt and DJ-1 KO MEFs. Levels of CnA expression were comparable among 4 Wt lines and among 5 DJ-1 KO lines. Since Cn activity was not assessed, Cn activity may have differed between Wt and DJ-1 KO MEFs, although CnA expression was the same.
Wang et al. (2008) reported that overexpression of CnB in HEK293 cells led to upregulation of DJ-1, which suggests that DJ-1 is downstream of CnB. I opted to study CnA because it contains the active site of the Cn heterodimer and Cn activity can only be as great as the level of CnA present. From Wang et al. (2008)’s study, it is not known whether downregulation of CnB results in downregulation of DJ-1, and most importantly, whether the reverse is true (i.e.: whether downregulation of DJ-1 leads to downregulation of CnB). CnA and CnB are encoded by different genes that reside on different chromosomes (Wang et al., 1996a). Therefore, it is possible that they do not undergo tandem regulation and DJ-1 overexpression or downregulation does not affect CnA at all.

### 4.4.2 CnA levels in DJ-1 KO MEFs after PQ

My results in PC12 cells and rat cortical neurons indicate that sustained PQ led to decreased whole CnA levels. The downregulation of DJ-1 has been previously reported to render cells more susceptible to oxidative stress (Canet-Aviles et al., 2004; Taira et al., 2004; Yokota et al., 2003; Zhou & Freed, 2005). This suggests that DJ-1 KO should result in lower CnA levels in DJ-1 KO MEFs than Wt. However, my results indicate that CnA levels are the same between Wt and DJ-1 KO MEFs after PQ exposure and that there was no change in CnA levels in both genotypes at all time points tested. Given that I did not observe increased ROS production in DJ-1 KO MEFs relative to Wt MEFs after 50μM PQ exposure, the lack of differential response between Wt and DJ-1 KO MEFs is consistent with my ROS data. Cell viability was not significantly reduced nor were ROS levels significantly increased after 24h 50μM PQ exposure, which suggests that the dose of PQ I selected could have been too low. However, my results in PC12 cells indicate that a sub-lethal dose of PQ need not decrease cell viability to have an effect on CnA, as 24h 40μM -80μM PQ decreased CnA levels without reducing cell viability. Further experiments are required to address this issue.

### 4.4.3. DJ-1 KO MEFs are more susceptible to Cn cleavage induced by H2O2

1h H2O2 exposure resulted in CnA cleavage into a 32 kDa fragment that was more pronounced in DJ-1 KO MEFs than Wt controls. CnA cleavage in MEFs after H2O2 mirrored my data in rat cortical neurons and is also consistent with a published report (Lee et al., 2007).
Whole CnA levels were also decreased in DJ-1 KO MEFs, but not in Wt MEFs. The results in Wt MEFs are consistent with my finding of no reduction in whole CnA levels in rat cortical neurons after H$_2$O$_2$. The rate of CnA synthesis may have been sufficient to replenish whole CnA in Wt MEFs, but not in DJ-1 KO MEFs. Confocal fluorescence microscopy of ROS levels in MEFs exposed to 1h H$_2$O$_2$ showed increased ROS in Wt and DJ-1 KO MEFs, which suggests that CnA cleavage may be due to increased ROS. However, after a 1h H$_2$O$_2$ exposure, ROS levels increased to the same degree in Wt and DJ-1 KO cells. This makes the increased CnA cleavage in DJ-1 KO cells puzzling if CnA cleavage is solely due to lysosomal protease leakage, as was suggested by Lee et al. (2007). Plausibly, other mechanisms of CnA cleavage exist.

DJ-1 is proposed to mediate neuroprotective effects after oxidative stress, but the precise mechanism through which it occurs is still a subject of debate. In SH-SY5Y cells, DJ-1 upregulation is neuroprotective (Lev et al., 2008). DJ-1 downregulation increases vulnerability to oxidative stress (Canet-Aviles et al., 2004; Taira et al., 2004; Yokota et al., 2003; Zhou & Freed, 2005). When DJ-1 is downregulated, other protective pathways, including Cn, may be activated. With respect to Cn, downregulation is thought to be protective (Asai et al., 1999; Lee et al., 2007; Sharkey & Butcher, 1994; Uchino et al., 2008). The consequences of DJ-1 downregulation, which may include increased ROS and changes in Ca$^{2+}$, could lead to downregulation of the Cn pathway, and consequent increased protection. Future experiments are needed to investigate potential links between DJ-1- and Cn-mediated neuroprotective mechanisms.

4.4.4. NFATc4 and DJ-1 KO

Cn mediates apoptosis through multiple pathways and the downregulation of any one is neuroprotective. I opted to study NFATc4 as a downstream effector of Cn after H$_2$O$_2$-induced oxidative stress in MEFs, as NFATc4-mediated apoptosis has been previously studied in neurons and is linked to deafferentation during normal development (Jayanthi et al., 2005; Luoma & Zirpel, 2008). My own data in rat cortical neurons, albeit preliminary, is also suggestive of cytoplasmic accumulation and decreased nuclear localization of NFATc4 after H$_2$O$_2$ exposure. Therefore, I hypothesized that DJ-1 KO MEFs would have increased cytoplasmic accumulation and decreased nuclear localization of NFATc4 after H$_2$O$_2$ stress when compared to Wt MEFs.
4.4.5. DJ-1 KO MEFs and NFATc4 under basal conditions

Preliminary data suggested that KO 4-4 consistently had increased cytoplasmic accumulation of NFATc4 relative to Wt MEFs under basal conditions, whereas KO 5-2 consistently had lower cytoplasmic accumulation of NFATc4 relative to Wt controls. Both DJ-1 KO MEF lines displayed significantly decreased NFATc4 nuclear localization relative to Wt under basal conditions. This suggests that basal compartmentalization and baseline levels of NFATc4 differ between Wt and DJ-1 KO MEFs and must be taken into account when interpreting how NFATc4 localization changes after oxidative stress.

It is important to note that nuclear fractions were not normalized because histone H3 was extracted into cytoplasmic factions (see section 4.3.9). However, given the negligible cytoplasmic GAPDH in nuclear fractions, along with the fact that NFATc4 is only localized to the cytoplasm and nucleus, it is likely that the NFATc4 seen in the nuclear fractions was specifically localized to the nucleus. Therefore, within the limitations of my data, I observed consistently lower nuclear NFATc4 localization in DJ-1 KO MEFs under basal conditions.

4.4.6. Wt MEFs and NFATc4 after H$_2$O$_2$ exposure

Preliminary experiments showed that Wt MEFs had significant cytoplasmic accumulation of NFATc4 after 2h H$_2$O$_2$ exposure. Since I did not observe a corresponding decrease in NFATc4 localization in the nucleus, cytoplasmic accumulation of NFATc4 may have been due to increased synthesis, or decreased degradation, or both. Cn activity is required to maintain NFATc4 activation and nuclear localization. Therefore, the maintenance of NFATc4 nuclear localization after H$_2$O$_2$ exposure suggests that Cn activity was not altered from baseline, which is consistent with the observation that whole CnA levels did not change after H$_2$O$_2$ treatment in Wt MEFs. Furthermore, this suggests that the rates of NFATc4 entry into the nucleus and NFATc4 extrusion from the nucleus are coupled, such that nuclear localization of NFATc4 remains unchanged after oxidative stress. It is unclear whether cytoplasmic accumulation of NFATc4 is a protective response of the cell towards oxidative stress, or an indirect consequence of other processes affected by oxidative stress. Moreover, the technical difficulties in subcellular fractionation for nuclear fractions (see section 4.3.9 and 4.4.6) also make this data difficult to interpret. Studies of downstream effects of NFATc4 cytoplasmic
accumulation in this model are needed to see if cytoplasmic accumulation of NFATc4 has any protective effects.

4.4.7. DJ-1 KO MEFs do not display changes in subcellular NFATc4 localization after H$_2$O$_2$ exposure

In preliminary experiments, DJ-1 KO MEFs did not display cytoplasmic or nuclear levels of NFATc4 that were significantly different from controls after 2h H$_2$O$_2$ exposure. NFATc4 levels were evaluated after 2h of H$_2$O$_2$ exposure as opposed to 1h because it was previously reported that 1h H$_2$O$_2$ leads to CnA cleavage, with extrusion of NFATc4 evident by 2h of H$_2$O$_2$ exposure (Lee et al., 2007). As previously discussed, preliminary data suggested that both DJ-1 KO MEF lines displayed significantly less nuclear NFATc4 localization when compared to Wt MEFs under basal conditions. Since nuclear NFATc4 was already low under basal conditions, any further declines may not have been detectable. This suggests that there was no new synthesis of NFATc4 or that the rates of synthesis and degradation were coupled.

My preliminary data suggest that DJ-1 KO MEFs already suppress NFATc4-activated apoptotic pathways under basal conditions, and that no changes to this pathway occur in response to H$_2$O$_2$. CnA is clearly cleaved after short term H$_2$O$_2$, but since I did not assess whole CnA levels and CnA cleavage after 2h, it is possible that whole Cn levels rebounded by this point. However, H$_2$O$_2$ for 2h is reportedly sufficient to inactivate Cn (Carballo et al., 1999; Ferri et al., 2000; Lee et al., 2007; Sommer et al., 2000; Sommer et al., 2002). Consequently, it is highly likely that Cn activity was still decreased after 2h of H$_2$O$_2$, although whether CnA cleavage was greater in DJ-1 KO MEFs at this time is unknown. Although H$_2$O$_2$ induced greater CnA cleavage in DJ-1 KO MEFs than controls, the cytoplasmic and nuclear localization of NFATc4 was unchanged. This suggests that increased CnA cleavage does not affect the NFAT pathway in DJ-1 KO MEFs. NFAT is not the only substrate of Cn and the downstream substrate of Cn in DJ-1 KO cells under H$_2$O$_2$-stressed conditions remains to be identified. Further, it is unknown what mechanisms maintain low levels of NFATc4 in the nucleus of DJ-1 KO MEFs under basal conditions.
4.4.8. Summary and Working Model

DJ-1 has been reported by many studies to be protective against the toxins linked to the development of PD, most of which generate oxidative stress. However, the mechanism(s) by which DJ-1 mediates its neuroprotective effects are largely unknown. Since DJ-1 KO mice weren’t readily available during the course of my work I used Wt and DJ-1 KO MEFs. Rat cortical neurons and PC12 cells were used to validate some of my results on oxidative stress and Cn. My results show that: 1) DJ-1 does not translocate to mitochondria after oxidative stress, but it is upregulated after H$_2$O$_2$ exposure in PC12 cells and rat cortical neurons; 2) oxidative stress induces CnA cleavage and/or decreased whole CnA levels in PC12 cells, rat cortical neurons, and MEFs; 3) DJ-1 KO MEFs show increased CnA cleavage after H$_2$O$_2$ exposure compared to Wt controls; 4) preliminary data indicate that nuclear NFATc4 localization is significantly lower in DJ-1 KO MEFs, and; 5) preliminary experiments show that cytoplasmic NFATc4 is increased and nuclear NFATc4 unchanged after H$_2$O$_2$ exposure in Wt MEFs, but not in DJ-1 KO MEFs. Figure 41 is a modified version of my initial model (see Figure 1), revised according to my results. Figure 42 illustrates some of the key findings of this study, with the modifications in Figure 41 taken into consideration. The results of my study suggest that DJ-1 and Cn cytoprotective pathways run in parallel in the cell under normal conditions, appearing to interact only under the conditions where DJ-1 KO cells are subjected to oxidative stress.

Based on my results, I propose the following working model. DJ-1 and Cn-mediated neuroprotective pathways against oxidative stress are only two of multiple neuroprotective pathways running in parallel (Figure 43). Under basal conditions, DJ-1 and Cn mediated pathways contribute towards neuroprotection against oxidative stress, but do not converge. Specifically, DJ-1 is upregulated, while Cn is downregulated. I propose that when DJ-1 is eliminated, other neuroprotective pathways are activated (Figure 44). In the case of Cn, this means increased downregulation to compensate for the loss of protection via DJ-1 pathways. Since NFATc4-mediated cell death mechanisms are not altered in DJ-1 KOs after oxidative stress, while Cn is downregulated, downregulation of other Cn-mediated apoptotic pathways (e.g.: decreased Bad dephosphorylation by Cn) may compensate for the loss of DJ-1. Furthermore, oxidative stress and decreased Cn levels may alter other pro-/anti-apoptotic pathways. Ultimately, the net effect of eliminating DJ-1 may be a progressive replacement of DJ-1-mediated cytoprotection by other pathways. This may explain why our DJ-1 KO MEFs did not display enhanced susceptibility to oxidative stress when compared to Wt controls.
Figure 41: Initial model of the responses of DJ-1 and Cn after oxidative stress. In my study, it was observed that there was no increased translocation of DJ-1 to the mitochondria after oxidative stress. It was also found that the changes in CnA cleavage and CnA levels were not the same for different types of oxidative stress and/or exposure times (outlined in red oval). A direct link between DJ-1 and Cn was not found under basal conditions.
Figure 42: Revised model of the responses of DJ-1 and Cn after oxidative stress. No increased translocation of DJ-1 to the mitochondria was observed after oxidative stress, but DJ-1 translocation to the nucleus was observed. Increased DJ-1 in the cytoplasm was also observed. Acute H₂O₂ or menadione exposure cleaved CnA, whereas chronic PQ did not, but decreased CnA levels. Such changes could alter interactions with Bcl-2 family proteins, resulting in protection. Both increased translocation of DJ-1 to the nucleus and changes in CnA cleavage/levels could alter transcription factor activation, which could also enhance protection. KO of DJ-1 resulted in increased CnA cleavage after H₂O₂ exposure, suggesting an interaction between DJ-1 and Cn-mediated protective pathways.
Figure 43: Working model of DJ-1 and Cn protective pathways. Oxidative stress leads to changes in DJ-1 subcellular localization and expression, resulting in increased cellular survival. CnA levels are decreased and CnA is cleaved after oxidative stress, which may lead to altered transcription factor activation (e.g.: NFATc4) and subsequent increased survival. Alternatively, changes in Cn may alter alternative survival/apoptotic pathways, such as Bad phosphorylation, which also culminates in decreased cell death. Under conditions of oxidative stress, DJ-1 and Cn protective pathways run in parallel to enhance cellular survival.
**Figure 44: Working model of protective pathways in DJ-1 KO cells.** When DJ-1 is absent, CnA cleavage increases in response to oxidative stress. No change in nuclear translocation of NFATc4 is observed, although CnA levels are decreased. Decreased CnA may downregulate other Cn associated apoptotic pathways (e.g.: decreased Bad dephosphorylation). Oxidative stress and decreased CnA levels may also affect pro-/anti-apoptotic pathways. Collectively, these events enhance cellular survival, which may explain why cell viability in DJ-1 KO cells was not decreased versus Wt cells.
Ongoing Studies and Future Directions

1. NFATc4 localization experiments after oxidative stress need to be repeated using a luciferase reporter assay, because existing commercial NFATc4 antibodies are poor and loading controls used to confirm the purity of nuclear fractions are difficult to obtain.

2. Many of my experiments were performed using 2 Wt MEF lines and 2 DJ-1 KO MEF lines and my results revealed a high degree of variability between the lines in the Wt and KO groups. While such variability could have masked some of the differences between Wt and DJ-1 KO cells, my results indicate that in the lines tested, the elimination of DJ-1 does result in increased susceptibility to oxidative stress. Although repeating the ROS and MTP experiments using more lines could reveal some effects of DJ-1, this seems unlikely given that my MTT results show no differences when data from all 4 Wt MEF lines and all 5 DJ-1 KO MEF lines are pooled.

3. Since changes in CnA expression and Cn activity do not correlate, and only CnA levels were assessed in Wt and DJ-1 KO MEFs, Cn activity should be determined in MEFs under basal and oxidative conditions before making any conclusions regarding Cn and DJ-1 in KOs.

4. In DJ-1 KO CnA cleavage increased, but NFATc4-mediated cell death was unchanged; consequently other apoptotic pathways (e.g.: Bad dephosphorylation) should be assessed.

Conclusions and Implications

Cancer and neurodegeneration are two extremes of a fine balance between cell survival and cell death. Both DJ-1 and Cn are involved in these processes. Wang et al. (2008) first demonstrated a link between DJ-1 and Cn pathways, showing that CnB overexpression induced upregulation of DJ-1. My study indicates that DJ-1 downregulation does not affect CnA expression. Taken together, this suggests that Cn expression is involved in the regulation of DJ-1, but DJ-1 does not reciprocally regulate Cn. However, I also demonstrated that oxidative stress reveals a link between CnA and DJ-1, where the lack of DJ-1 induces increased cleavage of CnA, which may impact both pro- and anti-apoptotic pathways. Overall, my results suggest that simultaneous downregulation of both DJ-1 and CnA are important in neurodegenerative models involving oxidative stress, although the precise relationships between these pathways
are still unclear. Improving our understanding of how both DJ-1 and Cn are involved in neuroprotection could potentially provide new strategies for treating PD that prevent/reduce the symptoms by targeting DJ-1, while at same time increasing neuroprotection by targeting Cn.
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