Glucose-6-phosphate dehydrogenase deficiency: A possible risk factor for neurodegeneration associated with aging and amphetamine use

by

Margaret M. Loniewska

A thesis submitted in conformity with the requirements for the Degree of Doctor of Philosophy
Graduate Department of Pharmaceutical Sciences
University of Toronto

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2013

ABSTRACT

Glucose-6-phosphate dehydrogenase (G6PD) regenerates the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH), which is important for reactive oxygen species (ROS) detoxification. ROS have been implicated in the development of neurodegenerative diseases in aging as well as the neurotoxic effects of amphetamine analogs. Using a mutant G6PD-deficient mouse model, we investigated the potential neuroprotective role of G6PD in aging-related and amphetamine-initiated neurotoxicity.

Aging G6PD-deficient mice exhibited increased DNA damage along with neurodegenerative changes including a marked loss of Purkinje cells. Locomotor
deficits linked to cerebellar function were observed in aging G6PD-deficient mice, but no cognitive deficits were detected. Electrophysiological measurements in hippocampal slices showed substantial amplifications of synaptic function in aging G6PD-deficient animals. Conversely, lifespan was increased in aging homozygous G6PD-deficient mice compared to heterozygous G6PD-deficient mice and wild-type G6PD-normal mice.

The potential protective role of G6PD against ROS-initiating neurotoxins was examined using 3,4-methylenedioxyamphetamine (MDA), the major active metabolite of 3,4-methylenedioxymethamphetamine (MDMA, Ecstasy). Although MDA caused deficits in motor function linked to the substantia nigra, and in cognition measured by taste aversion learning, these deficits were not exacerbated in G6PD-deficient mice, and homozygous G6PD-deficient females were actually protected from one gender-specific MDA-initiated learning deficit observed in wild-type animals.

My results show a complex role for G6PD. On one hand, G6PD protected against the neurodegenerative effects of oxidative stress in aging mice at the macromolecular, cellular and behavioural levels while the substantial electrophysiological alterations in G6PD-deficient mice suggest that cognitive deficits remain to be discovered. In contrast, the enhanced lifespan of G6PD-deficient mice suggests that a reduction of products from the pentose phosphate pathway including NADPH may have benefits, which mechanisms also remain to be examined. The absence of a modulatory role for G6PD in some learning deficits caused by MDA in adult mice suggests limitations in the protective ability of G6PD, while the reduction in
another MDA-initiated behavioural deficit in G6PD-deficient females reveals the same paradoxical potential seen in aging mice. While G6PD appears to exert a predominantly neuroprotective role, particularly in aging, the beneficial outcomes from G6PD deficiency may be relevant to the high prevalence of it in humans.
ACKNOWLEDGEMENTS

I would like to thank all the wonderful people in my life that have supported me in this long process of finishing this thesis. First and foremost I would like to thank my parents, Waleria Loniewska and Zbigniew Loniewski, who have encourage me all my life to be curious about the world and to ask questions. As well my parents have provided me with a life in Canada while giving up their own lives in Poland. I could never repay them for their sacrifices. I would like to also give a special thanks to my partner RJ Pauloski for joining me on this long journey. Without him this would never have been accomplished. Thank you for the endless support and believing in me and keeping me on my toes. I would also like to thank all my friends. They filled my life with plenty of love, support, fun times and swap goodies. Without plenty of friends life is empty.

I would also like to give my endless gratitude to Dr. Peter Wells for being a very understanding, encouraging and persistent supervisor. It has been a pleasure working for him. As well, I would like to send my gratitude to members of my advisory committee who have guided me throughout this research project. Also, thanks to the defense committee who have taken the time to review this thesis.

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it!’. This motto has stuck with me throughout this degree as well as in my life. Julia Abramov was my partner in crime and my long-term bench and desk buddy. I don’t think I could have picked a better person to share space, long conversations and many laughs with. I bet I will never find a better bench mate since I am the messy one and she never complained. The new students that joined the lab later, Kyla and Stephanie, who worked so hard to help us move, and have become great and successful members of the lab and friends. Kyla became my great source of food and new restaurants and of course city politics discussions. As well, Kyla became my partner in behavioural testing with the development of the passive avoidance apparatus. Maybe one day we will get to use fancy equipment that we did not have to build or put together from scraps. Stephanie is a bright scientist that will definitely do something great in the future. Michelle and Nicole arrived together and also became not just co-workers but friends. They completed very interesting projects on methanol toxicity. Michelle was at first a great friend, and became a motivational work-out buddy with our Phit Phriday workouts which turned into very fun gatherings (thanks team! I hope we stay phit always). Nicole is a great inspiration with her love of her new family and such amazing knitting skills. Aaron and Lutfiya were the last additions and I greatly enjoyed all our outside the lab outings and Aaron’s wonderful long stories. Lutfiya’s dedication can be seen not only in her research but her own healthy living. I would also like to thank all of the current and former students in the Department of Pharmaceutical Sciences. They definitely made coming to school a wonderful and experience. I wish everyone great success and hope to stay in touch in the future.
# CONTENTS

Abstract .............................................................................................................. ii
Acknowledgements .......................................................................................... v
List of Tables ..................................................................................................... ix
List of Figures ..................................................................................................... x
List of Abbreviations ......................................................................................... xii
List of Publications and Abstracts Arising from This Thesis ......................... xxi

1. Introduction .................................................................................................... 1
  1.1. Rationale and Research Objectives ......................................................... 2
    Hypothesis ....................................................................................................... 4
    Objectives and Justification of Approach ...................................................... 5
  1.2. Introduction to Oxidative Stress in Disease ............................................. 7
    1.2.1 Reactive Oxygen Species ....................................................................... 7
    1.2.1.2 Sources of ROS in the brain .............................................................. 8
      1.2.1.2.1 Mitochondrial respiration .......................................................... 11
      1.2.1.2.2 Excitotoxicity ........................................................................... 13
      1.2.1.2.3 Transition metals and autooxidation .......................................... 16
      1.2.1.2.4 Microglia and oxidative burst .................................................... 16
      1.2.1.2.5 Enzymatic oxidation .................................................................. 20
    1.2.1.3 Antioxidative and protective systems of the brain ......................... 22
      1.2.1.3.1 Blood-brain barrier .................................................................... 22
      1.2.1.3.2 Dietary and non-enzymatic antioxidants .................................... 26
      1.2.1.3.3 Antioxidative Enzymes ............................................................... 31
        1.2.1.3.3.1 Superoxide Dismutase .......................................................... 31
        1.2.1.3.3.2 Catalase ............................................................................ 32
        1.2.1.3.3.3 Glutathione Peroxidase ......................................................... 33
        1.2.1.3.3.4 NAD(P)H:Quinone Oxidoreductase .................................. 34
        1.2.1.3.3.5 Peroxiredoxins and Thioredoxins ....................................... 34
        1.2.1.3.3.6 Glucose-6-phosphate Dehydrogenase (G6PD) .................... 35
          1.2.1.3.3.6.1 Genetics and Regulation of G6PD ................................... 35
          1.2.1.3.3.6.2 G6PD Protein Structure ............................................... 37
          1.2.1.3.3.6.3 G6PD Enzymology ....................................................... 38
          1.2.1.3.3.6.4 G6PD Deficiency and Classification ............................... 41
          1.2.1.3.3.6.5 Protective Role of G6PD in Blood Cells ....................... 42
          1.2.1.3.3.6.6 G6PD Protection in Development .................................. 44
          1.2.1.3.3.6.7 Protective Role of G6PD in the Brain ............................. 45
    1.2.1.4 ROS in Signal Transduction .............................................................. 47
      1.2.1.4.1 Mitogen-activated Protein Kinases (MAPKs) ............................ 49
      1.2.1.4.2 Protein Kinases C (PKCs) ......................................................... 51
| 1.2.1.4.3 | Nuclear Factor-kappa B (NF-κB) | 51 |
| 1.2.1.4.4 | Activator Protein-1 (AP-1) | 52 |
| 1.2.1.4.5 | p45-nuclear factor erythroid-derived 2 related factors (Nrfs – Nrf2) | 54 |
| 1.2.1.5 | Free Radical Theory of Aging | 56 |
| 1.2.1.6 | Amphetamines as sources of ROS | 61 |

2. **Studies** .................................................................................................................. 66

2.2. **Study 1: BRAIN GLUCOSE-6-PHOSPHATE DEHYDROGENASE PROTECTS AGAINST ENDOGENOUS OXIDATIVE DNA DAMAGE AND NEURODEGENERATION IN AGED MICE** .................................................... 67

Abstract ......................................................................................................................... 67
Introduction ....................................................................................................................... 68
Results ............................................................................................................................... 69
Discussion ......................................................................................................................... 73
Conclusion ......................................................................................................................... 76
Materials and Methods ................................................................................................. 80
Acknowledgements .......................................................................................................... 83

2.2. **Study 2: GLUCOSE-6-PHOSPHATE DEHYDROGENASE DEFICIENCY IN AGING: DNA DAMAGE, ELECTROPHYSIOLOGICAL AND BEHAVIOURAL CONSEQUENCES AND SURVIVAL** ................................................. 101

Abstract ......................................................................................................................... 101
Introduction ....................................................................................................................... 102
Methods ............................................................................................................................ 106
Results ............................................................................................................................... 110
Discussion ......................................................................................................................... 114

2.3. **Study 3: FUNCTIONAL ASSESSMENT OF NEURODEGENERATION IN AGING AND AMPHETAMINE-TREATED GLUCOSE-6-PHOSPHATE DEHYDROGENASE-DEFICIENT MICE** ................................................................. 137

Abstract ......................................................................................................................... 137
Introduction ....................................................................................................................... 138
Results ............................................................................................................................... 142
Discussion ......................................................................................................................... 146

3. **Summary, Conclusions, & Future Studies** ................................................................. 159

3.1 **Summary and Conclusions** .................................................................................. 160

3.2 **Future Proposed Studies** ..................................................................................... 167

References ....................................................................................................................... 170

4. **Appendix** ................................................................................................................ 196
LIST OF TABLES

Table 1. Enzymes which generate ROS in the brain ..................................................20
Table 2. Enzymes requiring NADPH .................................................................41
Table 3. Classes of G6PD-deficiency .................................................................42
LIST OF FIGURES

Fig. 1. Role of reactive oxygen species (ROS) in disease................................................................. 9
Fig. 2. Sources of reactive oxygen species (ROS) in the brain..................................................... 10
Fig. 3. Sites of superoxide formation in the respiratory chain....................................................... 12
Fig. 4. Increase in calcium leading to toxicity.................................................................................. 14
Fig. 5. Excitotoxic factors for the genesis of neurodegenerative disorders....................................... 15
Fig. 6. Reactive microgliosis.............................................................................................................. 18
Fig. 7. Activation of the phagocytic NADPH oxidase complex.......................................................... 19
Fig. 8. Schematic diagram of the BBB.............................................................................................. 23
Fig. 9. Transport across the BBB...................................................................................................... 25
Fig. 10. Ascorbate uptake and metabolism in the CNS.................................................................... 27
Fig. 11. Production of glutathione (GSH) in astrocytes and neurons.............................................. 30
Fig. 12. Pentose Phosphate Pathway............................................................................................... 39
Fig. 13. Cysteine biochemistry allows for redox-dependent signaling............................................ 48
Fig. 14. Model of MAPK-mediated detection of and response to cellular redox state...50
Fig. 15 Crosstalk of ROS with NF-kB signaling pathways.............................................................. 53
Fig. 16. Redox regulation of the KEAP1-Nrf2 system in response to ROS................................. 55
Fig. 17. Involvement of situins in ROS signaling........................................................................... 60
Fig. 18. Scheme of pathways involved in METH-induced DA terminal degeneration and ROS involvement................................................................................................................. 65

Fig. 1.1. Postulated mechanism of neuroprotection against endogenous reactive oxygen species-mediated oxidative stress and damage by glucose-6-phosphate dehydrogenase................................................................................................................................. 72
Fig. 1.2. Increased levels of DNA oxidation in aged G6PD-deficient mice.................................... 74
Fig. 1.3. Increased localized oxidatively damaged DNA in aged G6PD-deficient mice. ............... 75
Fig. 1.4. Increased CNS cell death and morphological changes in aged G6PD-deficient mice........ 80
Fig. 1.5. Quantitative analysis of Purkinje cells of the cerebellum in aged G6PD-normal and heterozygous G6PD-deficient mice.......................................................... 81
Fig. 1.6. G6PD activity in whole brains of aged G6PD-normal and G6PD-deficient mice. ......... 83
Fig. 1.7. G6PD activity in specific brain regions of aged G6PD-normal and G6PD-deficient mice... 84
Fig. 1.8. Relation of regional DNA oxidation and G6PD activity in G6PD+/+ and G6PDdef/def mice. ................................................................................................................................. 85
Fig. 2.1. Increase in DNA damage in the cerebellum and hippocampus of aging G6PD-deficient mice measured by the comet assay................................................................. 85
Fig. 2.2. Decrease in Purkinje cell (PC) numbers and calbindin-D-28K expression in aging G6PD-deficient mice................................................................................................. 117
Fig. 2.3. Positive correlation of G6PD activity in erythrocytes with cerebellar tissues, and no change in G6PD activity with aging.................................................................................... 119
Fig. 2.4. Motor coordination during aging in G6PD-deficient mice measured by rotarod performance. ................................................................. 120
Fig. 2.5. G6PD activity was not correlated with rotarod motor coordination performance during aging. ................................................................. 121
Fig. 2.6. Effect of G6PD-deficiency on the ledge balance test but not the hindlimb clasp test during aging. ......................................................... 122
Fig. 2.7. No effect of G6PD deficiency on cognitive function in aging mice. ............ 123
Fig. 2.8. Hippocampal electrophysiological function is altered in aging G6PD-deficient mice. ................................................................. 127
Fig. 2.9. Enhanced survival in aged G6PD-deficient mice ................................................................. 128
Fig. 3.1. Motor coordination in adult G6PD-normal and -deficient mice measured by rotarod performance. ................................................................. 147
Fig. 3.2. Rotarod, ledge balance test and hindlimb clasp test in aging MDA-treated G6PD-normal and G6PD-deficient mice. ................................................................. 148
Fig. 3.3. No effect of MDA treatment or G6PD-deficiency on passive avoidance. ..... 150
Fig. 3.4. No effect of METH treatment or G6PD-deficiency on passive avoidance. .... 151
Fig. 3.5. Effects of scopolamine on passive avoidance ................................................. 152
Fig. 3.6. Taste Aversion in MDA-treated G6PD-normal and G6PD-deficient young female mice. ................................................................. 153
Fig. S1. Physiological role of glucose-6-phosphate dehydrogenase (G6PD). ............ 163
Fig. S2. The antioxidative and pro-oxidative duality of G6PD. ...................................... 166
Fig. A1. Comet Assay ................................................................. 197
Fig. A2. Olfactory Discrimination ............................................................................. 198
Fig. A3. Detection of threshold of olfactory sensitivity.............................................. 199
Fig. A4. Rectal temperature after MDA or saline treatment in G6PD-normal and G6PD-deficient mice. ................................................................. 200
Fig. A5. Blood G6PD and catalase activity. ................................................................. 201
Fig. A6. Weights throughout life in G6PD-normal and deficient animals .................. 202
Fig. A7. G6PD activity assay principles ...................................................................... 203
Fig. A8. G6PD activity assay set up .......................................................................... 204
Fig. A9. Maternal and child G6PD activity ................................................................. 206
Fig. A10. Child G6PD did not correlate to maternal G6PD. .......................................... 207
Fig. A11. Child G6PD activity did not affect oxidative stress birth outcomes. ........... 208
Fig. A12. Maternal G6PD activity did not affect oxidative stress birth outcomes. ....... 209
Fig. A13. G6PD activity and neurodevelopmental measures ........................................ 210
Fig. A14. G6PD activity and IQ ................................................................................. 211
Fig. A15. Maternal or child G6PD activity did and IQ – drugs combined. ................. 212
**LIST OF ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>Aβ</td>
<td>beta-amyloid</td>
</tr>
<tr>
<td>ABC</td>
<td>ATP-binding cassette</td>
</tr>
<tr>
<td>ACSF</td>
<td>artificial cerebrospinal fluid</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimer's disease</td>
</tr>
<tr>
<td>AFR</td>
<td>ascorbate free radical</td>
</tr>
<tr>
<td>Akt1</td>
<td>v-Akt murine thymoma viral oncogene homolog 1</td>
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<tr>
<td>ALS</td>
<td>Amyotrophic lateral sclerosis (Lou Gehrig's disease)</td>
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<td>AMPA</td>
<td>α-amino-3-hydroxy-5-methyl-4-isoxasole-proprioninc acid</td>
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<td>AMT</td>
<td>adsorptive-mediated transcytosis</td>
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<td>ANOVA</td>
<td>analysis of variance</td>
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<tr>
<td>AP-1</td>
<td>activator protein 1</td>
</tr>
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<td>AP-2</td>
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<td>antioxidant response element/s</td>
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<td>ascorbate</td>
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<td>ASK-1</td>
<td>apoptosis signal-regulating kinase 1</td>
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<tr>
<td>ATM</td>
<td>ataxia telangiectasia mutated</td>
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<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BBB</td>
<td>blood-brain barrier</td>
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<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>BMK</td>
<td>big MAP kinase</td>
</tr>
<tr>
<td>Ca$^{2+}$</td>
<td>calcium cation</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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</tr>
<tr>
<td>CCD</td>
<td>charge coupled device</td>
</tr>
<tr>
<td>CSF</td>
<td>cerebrospinal fluid</td>
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<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CNSHA</td>
<td>chronic non-spherocytic hemolytic anemia</td>
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<tr>
<td>COX</td>
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<td>caloric restriction</td>
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<td>CSB</td>
<td>Cockayne syndrome B</td>
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<td>CSF</td>
<td>cerebrospinal fluid</td>
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<td>CuZnSOD</td>
<td>CuZn superoxide dismutase</td>
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<td>DA</td>
<td>dopamine</td>
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<td>DHA</td>
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<td>DNA</td>
<td>deoxyribose nucleic acid</td>
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<td>DMSO</td>
<td>dimethyl sulfoxide</td>
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<tr>
<td>EAAC1</td>
<td>excitatory amino acid carrier 1</td>
</tr>
<tr>
<td>ECL</td>
<td>enhanced chemiluminescence</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
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<tr>
<td>eNOS</td>
<td>endothelial nitric oxide synthase</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<tr>
<td>EPSPs</td>
<td>excitatory postsynaptic potentials</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>ERKs</td>
<td>extracellular signal-regulated kinases, aka MAPK</td>
</tr>
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<td>Erk2</td>
<td>extracellular signal-regulated kinase 2</td>
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ETC  electron transport chain
fEPSPs  field excitatory postsynaptic potentials
Fe  iron
Fe$^{2+}$  ferrous ion
Fe$^{3+}$  ferric ion
FFAs  free fatty acids
FOXO3  forkhead homeobox type O factor 3
FR  free radical
G6P  glucose-6-phosphate
G6PD  glucose-6-phosphate dehydrogenase
G6PD$^{+/+}$  G6PD normal female mice
G6PD$^{+/def}$  G6PD heterozygous deficient female mice
G6PD$^{def/def}$  G6PD homozygous deficient female mice
G6PD$^{+/y}$  G6PD normal male mice
G6PD$^{def/y}$  G6PD hemizygous deficient male mice
γ-GCS (GCS)  γ-glutamylcysteine synthetase
GDI  guanine nucleotide dissociation inhibitor;
GDP  guanosine diphosphate
GGT  gamma (γ)-glutamyl transpeptidase
GLUT  glucose transporter
GPx  glutathione peroxidase
GRx  glutathione reductase
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Definition</th>
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<tr>
<td>GS</td>
<td>glutathione synthetase</td>
</tr>
<tr>
<td>GS'</td>
<td>glutathione radical, oxidized glutathione</td>
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<td>GSH</td>
<td>glutathione</td>
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<tr>
<td>GSSG</td>
<td>oxidized glutathione, glutathione disulphide</td>
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<td>immunoglobulin G</td>
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<td>IkB</td>
<td>nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor</td>
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<td>IkB kinase</td>
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<tr>
<td>IL-1</td>
<td>interleukin-1</td>
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<tr>
<td>iNOS</td>
<td>inducible nitric oxide synthase</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>interferon-gamma</td>
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<tr>
<td>ip</td>
<td>intraperitoneally</td>
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<td>JNK/s</td>
<td>c-Jun N-terminal kinase</td>
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<tr>
<td>kb</td>
<td>kilobase</td>
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<td>Keap1</td>
<td>Kelch-like erythroid cell-derived protein with CNC homolog (ECH)-associated protein 1</td>
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<td>L-DOPA</td>
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<td>lipopolysaccharide</td>
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<tr>
<td>LTP</td>
<td>long term potentiation</td>
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<td>MΩ</td>
<td>megaohm</td>
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<td>MAO</td>
<td>monoamine oxidase</td>
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<td>MAP3K5</td>
<td>mitogen-activated protein kinase kinase kinase 5</td>
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<td>MAPK/s</td>
<td>mitogen-activated protein kinase</td>
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<td>MDA</td>
<td>3,4-methylenedioxyamphetamine</td>
</tr>
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<td>MDMA</td>
<td>3,4-methylenedioxymethamphetamine</td>
</tr>
<tr>
<td>MEK1/2</td>
<td>mitogen-activated protein kinase kinase, aka MAP2K1/2</td>
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<td>METH</td>
<td>methamphetamine</td>
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<td>MnSOD</td>
<td>manganese superoxide dismutase</td>
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<tr>
<td>MPTP</td>
<td>1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine</td>
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<td>mRNA</td>
<td>messenger RNA</td>
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<td>Abbreviation/Definition</td>
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<tr>
<td>MRPs</td>
<td>multidrug resistance proteins</td>
</tr>
<tr>
<td>MS</td>
<td>multiple sclerosis</td>
</tr>
<tr>
<td>mtNOS</td>
<td>mitochondrial nitric oxide synthase</td>
</tr>
<tr>
<td>Na⁺</td>
<td>sodium cation</td>
</tr>
<tr>
<td>NADPH</td>
<td>nicotinamide adenine dinucleotide phosphate (reduced)</td>
</tr>
<tr>
<td>NADP⁺</td>
<td>nicotinamide adenine dinucleotide phosphate (oxidized)</td>
</tr>
<tr>
<td>NEMO</td>
<td>NF-kappa-B essential modulator</td>
</tr>
<tr>
<td>NF-E2</td>
<td>p45-nuclear factor erythroid-derived 2</td>
</tr>
<tr>
<td>NF-κB</td>
<td>nuclear factor κB</td>
</tr>
<tr>
<td>NGF</td>
<td>nerve growth factor</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>nNOS</td>
<td>neuronal nitric oxide synthase</td>
</tr>
<tr>
<td>NO⁻</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>nitric oxide synthase</td>
</tr>
<tr>
<td>NOX/s</td>
<td>NADPH oxidase/s</td>
</tr>
<tr>
<td>NP-40</td>
<td>nonyl phenoxypolyethoxylethanol</td>
</tr>
<tr>
<td>NQO1</td>
<td>NAD(P)H:quinone oxidoreductase</td>
</tr>
<tr>
<td>Nrf/s</td>
<td>NF-E2-related factors</td>
</tr>
<tr>
<td>NSAIDs</td>
<td>non-steroidal antiinflammatory drugs</td>
</tr>
<tr>
<td>O₂⁻</td>
<td>superoxide anion</td>
</tr>
<tr>
<td>‘OH, HO’</td>
<td>hydroxyl radical</td>
</tr>
<tr>
<td>6-OHDA</td>
<td>6-hydroxydopamine</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>OGG1</td>
<td>oxoguanine glycosylase 1</td>
</tr>
<tr>
<td>ONOO-</td>
<td>Peroxynitrite</td>
</tr>
<tr>
<td>8-oxo-dG</td>
<td>8-oxo-2'-deoxyguanosine</td>
</tr>
<tr>
<td>P450s</td>
<td>cytochromes P450</td>
</tr>
<tr>
<td>PBN</td>
<td>alpha-phenyl-N-t-butylnitrone</td>
</tr>
<tr>
<td>PC</td>
<td>Purkinje cells</td>
</tr>
<tr>
<td>PD</td>
<td>Parkinson's disease</td>
</tr>
<tr>
<td>p-gp</td>
<td>p-glycoprotein</td>
</tr>
<tr>
<td>PG</td>
<td>prostaglandin</td>
</tr>
<tr>
<td>PGC-1</td>
<td>Peroxisome proliferator-activated receptor gamma coactivator 1- α</td>
</tr>
<tr>
<td>PGE&lt;sub&gt;2&lt;/sub&gt;</td>
<td>prostaglandin E2</td>
</tr>
<tr>
<td>PGG&lt;sub&gt;2&lt;/sub&gt;</td>
<td>hydroperoxy endoperoxide</td>
</tr>
<tr>
<td>PGH&lt;sub&gt;2&lt;/sub&gt;</td>
<td>hydroxy endoperoxide</td>
</tr>
<tr>
<td>PGK</td>
<td>phosphoglycerol kinase</td>
</tr>
<tr>
<td>PHS/s</td>
<td>prostaglandin H synthase/s</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PLA&lt;sub&gt;2&lt;/sub&gt;</td>
<td>phospholipase A&lt;sub&gt;2&lt;/sub&gt;</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethanesulfonylfluoride</td>
</tr>
<tr>
<td>PPF</td>
<td>pair-pulse facilitation</td>
</tr>
<tr>
<td>PPP</td>
<td>petose phosphate pathway</td>
</tr>
<tr>
<td>Prx</td>
<td>peroxiredoxin</td>
</tr>
</tbody>
</table>
PTK  protein tyrosine kinase
PUFA  polyunsaturated fatty acid element
PVDF  polyvinylidene difluoride
R5P  ribose-5-phosphate
Rac  ras-associated C3 botulinum toxin substrate
RMT  receptor-mediated transcytosis
RNA  ribose nucleic acid
RNS  reactive nitrogen species
ROS  reactive oxygen species
SCGE  single cell gel electrophoresis
SDS-PAGE  sodium dodecyl sulfate-polyacrylamide gel electrophoresis
Sir2  silent information regulator 2, sirtuins
SIRT/s1-7  sirtuins 1-7
SLC  solute carriers
SOD  superoxide dismutase
SOD1  CuZnSOD
SOD2  MnSOD
SOD3  extracellular CuZnSOD
SOH  sulfinic acid
SO2H  sulfenic acid
SO3H  sulfonic acid
Sp1  stimulatory protein 1
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>SVCT2</td>
<td>sodium-dependent vitamin C transporter type 2</td>
</tr>
<tr>
<td>TAT</td>
<td>trans-activator of transcription</td>
</tr>
<tr>
<td>TBP</td>
<td>TATA binding protein</td>
</tr>
<tr>
<td>TBS</td>
<td>theta burst stimulation</td>
</tr>
<tr>
<td>TNFα</td>
<td>tumor necrosis factor alpha</td>
</tr>
<tr>
<td>Trx</td>
<td>thioredoxin</td>
</tr>
<tr>
<td>Trx1</td>
<td>thioredoxin 1</td>
</tr>
<tr>
<td>UGT</td>
<td>UDP-glucuronosyltransferase</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>VMAT2</td>
<td>Vesicular monoamine transporter 2</td>
</tr>
<tr>
<td>X*</td>
<td>free radical species (general)</td>
</tr>
</tbody>
</table>
LIST OF PUBLICATIONS AND ABSTRACTS ARISING FROM THIS THESIS

Publications


Manuscripts


Chapters


Abstracts (Posters and Presentations)


1. INTRODUCTION
1.1. RATIONALE AND RESEARCH OBJECTIVES

In recent years, reactive oxygen species (ROS) have been implicated in neurodegenerative diseases such as Alzheimer’s disease (AD) and Parkinson’s disease (PD) (Jenner and Olanow 1996; Lyras et al. 1997; Markesbery 1997). Our laboratory has in the past focused on ROS-mediated damage to developing embryos using several mutant, transgenic and knockout rat and mouse models (Nicol et al. 1995; Kim and Wells 1996; Parman et al. 1998; Nicol et al. 2000; Parman and Wells 2002; Laposa et al. 2003). More recently, we have used several of these models to elucidate the role of ROS-mediated damage to the central nervous system (CNS) associated with aging, and with exposure to ROS-initiating drugs (Jeng et al. 2005; Jeng et al. 2006; Wong et al. 2008; Wells et al. 2009; Jeng and Wells 2010; Jeng et al. 2011; McCallum et al. 2011).

Glucose-6-phosphate dehydrogenase (G6PD) is the first and rate-limiting enzyme in the hexose monophosphate pathway (HMP), important for its role in the regeneration of the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) and the production of ribose-5-phosphate (Buehler 1993; Luzzatto et al. 2001). During cellular oxidative stress, NADPH is critical for maintaining glutathione in its reduced form (GSH), which is essential for the detoxification of reactive free radicals and lipid hydroperoxides (Halliwell and Gutteridge 2007). Another important role of NADPH is the maintenance of the catalytic activity of catalase, which is required for the detoxification of hydrogen peroxide (Kirkman et al. 1987; Kirkman et al. 1999).
It is generally believed that G6PD deficiencies constitute a problem only for mature red blood cells (Luzzatto et al. 2001; Cappellini and Fiorelli 2008); however, our laboratory has shown that G6PD also plays a role in protecting embryos from oxidative stress and chemical teratogenesis (Nicol et al. 2000). G6PD-deficient mice have been shown to be more sensitive to menadione-induced oxidative stress, with increases in DNA oxidation, lipid peroxide and protein carbonyl levels in hepatic tissue (Nichols and Kirby 2008). Also, preliminary studies with the brains of untreated aged G6PD-deficient mice suggest a neuroprotective role. Histological examination of these mice revealed enhanced degenerative changes compared to wild type mice, which displayed typical age-related changes. The changes associated with G6PD deficiency included cell loss in the frontal cortex, cerebellum and hippocampus, along with increased DNA oxidation in the same regions, among others (Jeng et al. 2013). The risk of ROS-mediated neuronal damage in G6PD-deficient mice may have important clinical implications, as this is the most common human enzymopathy, affecting over 400 million people and up to 60% of some populations (Sodeinde 1992). The widespread prevalence of malaria is believed to be the reason why G6PD deficiency is common throughout the world, as G6PD-deficient blood cells are inhospitable to the Plasmodium falciparum parasite responsible for malaria (Cappellini and Fiorelli 2008).

Previous work from our laboratory and elsewhere has implicated ROS in the neurotoxic mechanism of several amphetamine derivatives including 3,4-methylenedioxymethamphetamine (MDMA, Ecstasy), its demethylated active metabolite 3,4-methylenedioxyamphetamine (MDA), and methamphetamine (METH)
(Cadet et al. 1994a; Cadet et al. 1994b; Colado et al. 1997; Cadet et al. 2001; Jeng et al. 2006; Jeng and Wells 2010). Although the molecular mechanism of neurodegeneration remains unclear, studies from our laboratory have implicated prostaglandin H synthases (PHSs) in the bioactivation of amphetamines to neurotoxic free radical intermediates (Jeng et al. 2006; Jeng and Wells 2010), and a similar mechanism may contribute to neurodegeneration associated with aging, via PHS-catalyzed bioactivation of endogenous neurotransmitters, their precursors and metabolites (Goncalves et al. 2009; Ramkissoon and Wells 2011a). Since MDMA and related amphetamines have gained popularity as recreational drugs of choice in young adults, these drugs along with untreated aging mice are clinically relevant models for elucidating the ROS-mediated mechanisms of neurodegeneration and determinants of risk.

Hypothesis

Some neurodegenerative effects associated with aging, and those caused by MDMA and structurally related drugs, are mediated by ROS. Bioactivation of endogenous substrates with aging, and of MDMA and related amphetamines, forms free radical intermediates that initiate ROS formation. Mutant glucose-6-phosphate dehydrogenase (G6PD)-deficient mice will be more susceptible to ROS-mediated CNS damage caused by endogenous (untreated, aging) and exogenous (amphetamines, adults) substrates, revealing the neuroprotective role of G6PD.
Objectives and Justification of Approach

1. To develop and standardize several behavioural methods of testing motor coordination and cognition in mice, and to use these tests in assessing neurodegeneration. Previously, our laboratory has used the rotarod test to measure motor coordination. We required a broader array of behavioural tests of motor function as well as cognitive function to study ROS-initiated neurodegenerative changes caused by normal aging or xenobiotic-induced toxicity. For motor coordination, I have utilized the ledge balance test and the hindlimb clasping reflex. I have found that these tests are complementary to the rotarod test, and provide more detailed information regarding target brain regions. I also have developed and used the taste aversion learning test, which provided the first evidence that amphetamine-induced toxicity may affect this type of learning. Additionally, I employed the passive avoidance paradigm to study another form of cognitive function.

2. To develop a measure of DNA damage that was sensitive and useful in small samples like individual brain regions. Our laboratory utilizes a measure of oxidatively damaged DNA, namely the 8-hydroxy-2’-deoxyguanine (8-oxo-dG) lesion, measured by high-performance liquid chromatography with electrochemical detection (HPLC-EC) (Jeng et al. 2013) or with detection and characterization by tandem mass spectrometry (HPLC-MS/MS) (Miller et al. 2013a; Miller et al. 2013b). However, this method is difficult to use with small samples and may suffer from high background noise, which makes it difficult to determine small differences. As a
complementary approach, I instituted the use of the comet assay to measure a functional outcome of oxidatively damaged DNA.

3. **To evaluate the neuroprotective potential of G6PD against ROS-mediated molecular and functional neurotoxicity generated by endogenous processes in aging.** G6PD-deficient mice had increased levels of oxidatively damaged DNA and various pathological changes including the loss of Purkinje cells. Using the comet assay, DNA damage was determined in several brain regions. As well, motor and cognitive function were determined using several of the tests described in Objective 1. Tests like the ledge balance test are more indicative of cerebellar decline when compared to the rotarod or other tests of motor function. As well, hippocampal function was studied electrophysiologically via measures of long-term potentiation (LTP), basal synaptic strength and paired-pulse facilitation in G6PD-normal and G6PD-deficient animals. To further gauge the effects of G6PD deficiency, lifespan was compared among genotypes.

4. **To evaluate the neuroprotective potential of G6PD against ROS-initiating xenobiotics.** Acute doses of 3,4-methylenedioxyamphetamine (MDA), the major active metabolite of 3,4-methylenedioxymethamphetamine (MDMA), were used to evaluate the neuroprotective role of G6PD. I tested the effects of amphetamine treatment on measures of cognition using the passive avoidance test and the taste aversion test. The effects of an acute dose of MDA on motor function were also assessed, as amphetamines have deleterious effects in the striatum and substantia nigra.
1.2. **INTRODUCTION TO OXIDATIVE STRESS IN DISEASE**

1.2.1 **Reactive Oxygen Species**

Every organism is exposed, on a daily basis, to potentially damaging agents. The origins of these may be endogenous (by-products of cellular respiration and other basic cellular functions) as well as exogenous, from exposure to a variety of chemicals including air particulates, pesticides, insecticides and other environmental pollutants, as well as metals, radiation and drugs of abuse. Exposure to these agents may be toxic and lead to various diseases. This extensive list of toxicants can lead to the formation of free radical intermediates via bioactivation by various endogenous pathways including cytochromes P450 (P450s) and prostaglandin H synthases (PHSs) (Fig. 1).

Free radicals are chemical species capable of independent existence that contain one or more unpaired valence electrons (Halliwell and Gutteridge 2007), which makes them highly reactive. Free radicals tend to react quickly in a non-specific manner with nearby molecules by abstracting or “stealing” an electron leaving the “attacked” molecule a free radical itself (oxidation). This process can go on indefinitely in a chain reaction and the cascading effect can result in the disruption of normal cellular processes. Free radicals can also act as reducing agents by donating a single electron to a non-radical converting the recipient into a radical. And finally, a radical can join another molecule creating an adduct that contains an unpaired electron (Halliwell and Gutteridge 2007).

Free radical intermediates can react directly or indirectly with molecular oxygen (O\textsubscript{2}) leading to the formation of reactive oxygen species (ROS). ROS can also be created during cellular respiration in the electron transport chain. These include
superoxide anion \((O_2^-)\), hydrogen peroxide \((H_2O_2)\), hydroxyl radical \((\cdot OH)\) and nitric oxide \((NO^-)\). Superoxide anion is the product of a one-electron reduction of oxygen and is the precursor of most ROS. Dismutation of \(O_2^-\) produces hydrogen peroxide and this may fully be reduced to water or partially reduced to hydroxyl radicals. As mentioned above, ROS are highly reactive and can oxidize molecular targets such as proteins, lipids and DNA in a process known as oxidative stress (Halliwell and Gutteridge 2007). ROS can also alter signal transduction and affect gene transcription. There are several antioxidant and repair pathways that will be discussed later. Such damage, if not repaired, can accumulate over time and can lead to loss of cellular function and even cell death and disease (Fig. 1). The brain is especially susceptible to oxidative stress due to the high rate of oxygen consumption, presence of excitotoxic amino acids, modest antioxidant levels and several ROS-generating cellular functions and enzymatic reactions (Halliwell 2006). ROS have been implicated in many neurodegenerative diseases such as Alzheimer’s disease (AD) (Markesbery 1999), Parkinson’s disease (PD) (Shimura-Miura et al. 1999) and multiple sclerosis (MS) (Vladimirova et al. 1998), among others.

1.2.1.2 Sources of ROS in the brain

ROS in the brain are formed via many physiological and pathophysiological reactions. These include mitochondrial respiration, enzymatic oxidation, excitotoxicity, and microglia. As well, endogenous and exogenous substrates can be bioactivated into free radical intermediates. These pathways are summarized in Fig. 2.
Fig. 1. Role of reactive oxygen species (ROS) in disease.

Xenobiotics can be bioactivated by drug metabolizing enzymes into free radical intermediates which react directly or indirectly with oxygen to form reactive oxygen species (ROS). Excessive ROS cause oxidative stress in the forms of macromolecular damage and changes to signal transduction pathways, which eventually lead to disease including neurodegeneration. Several cytoprotective enzymes normally maintain low ROS levels in the cell.
Fig. 2. Sources of reactive oxygen species (ROS) in the brain.

When pro-oxidants exceed the antioxidative and repair mechanisms, oxidative damage can occur leading to neurotoxicity. Bolded enzymes involve NADPH in their activity. Abbreviations: ATM, ataxia telangiectasia mutated; CSB, Cockayne syndrome B; Fe, iron; G-6-P, glucose-6-phosphate; GSH, glutathione; GSSG, glutathione disulfide; H$_2$O$_2$, hydrogen peroxide; HO$^\cdot$, hydroxyl radical; LPO, lipoxygenase; NADP$^+$, nicotinamide adenine dinucleotide phosphate; O$_2^\cdot$, superoxide anion, OGG1, oxoguanine glycosylase 1; P450, cytochromes P450; PHS, prostaglandin H synthase; SOD, superoxide dismutase. Modified with permission from (Wells et al. 2009) and (Ramkissoon et al. 2013)
1.2.1.2.1 **Mitochondrial respiration**

In any cell, a major source of ROS is the mitochondrial respiratory chain. The main role of the mitochondria is to generate adenosine-5'-triphosphate (ATP), which is the major source of energy for the entire cell. The brain uses approximately 20% of the body’s O\(_2\) because of the vast amounts of ATP required to maintain neuronal intracellular ion homoeostasis (Halliwell 2006). The mitochondrial electron transport chain may leak electrons to oxygen, constituting the primary source of O\(_2^•^-\) and H\(_2\)O\(_2\) in most tissues (Turrens 2003). **Fig. 3** shows an overview of the mitochondria as a source of ROS. Interestingly, the relative contribution of each component of the mitochondrial electron transport chain to ROS production varies from organ to organ. In the brain, under normal conditions, Complex I appears to be the main source of O\(_2^•^-\) (Barja and Herrero 1998; Barja 1999). In Fig. 3, it is shown that the O\(_2^•^-\) produced in the mitochondria can be removed by superoxide dismutases (MnSOD, CuZnSOD). These enzymes are partly responsible for keeping the mitochondria from overwhelming the cells with oxidative stress. Several neurodegenerative diseases have been associated with failure of these antioxidative systems including PD (Shimoda-Matsubayashi *et al.* 1996; Grasbon-Frodl *et al.* 1999), and amyotrophic lateral sclerosis (ALS) (Valentine and Hart 2003).
Fig. 3. Sites of superoxide formation in the respiratory chain.

Various respiratory complexes leak electrons to oxygen producing primarily superoxide anion (O$_2^-$). This species may reduce cytochrome c (in the intermembrane space), or may be converted to hydrogen peroxide (H$_2$O$_2$) and oxygen (in both the matrix and the intermembrane space). Increased steady state concentrations of O$_2^-$ may reduce transition metals (which in turn react with H$_2$O$_2$ producing hydroxyl radicals ('OH)) or may react with nitric oxide to form peroxynitrite. Both 'OH and peroxynitrite are strong oxidants which indiscriminately react with nucleic acids, lipids, and proteins. With permission from (Turrens 2003).
1.2.1.2.2  *Excitotoxicity*

Excitotoxicity is the prolonged activation of excitatory amino acid receptors potentially leading to cell death (Lucas and Newhouse 1957; Olney and Sharpe 1969), and glutamic acid (glutamate) is the main excitatory transmitter within the mammalian brain (Watkins and Evans 1981; Doble 1999). During normal rapid excitatory synaptic transmission, glutamate is released from glutamatergic nerve terminals in response to depolarisation. It crosses the synaptic cleft, and acts on postsynaptic receptors. These receptors are membrane ion channels, and their activation leads to the entry of cations (Ca$^{2+}$, Na$^+$) into the postsynaptic neuron, causing depolarisation. When depolarisation of the postsynaptic neuronal membrane reaches a certain threshold, the action potential is generated (Doble 1999). There are two types of glutamate receptors, ionotropic, which are ligand-gated ion channels, and metabotropic receptors that are linked to G-proteins (Dong et al. 2009). The ionotropic receptors are characterized by their selective affinity for the specific agonists N-methyl-d-aspartate (NMDA), α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) and kainic acid (kainite) (Mehta et al. 2013). Prolonged high glutamate concentrations result in excessive intracellular calcium accumulation, which leads to ROS production (Fig. 4). AD, ALS, PD and Huntington’s disease (HD) and have all been linked to excitotoxicity, as summarized in Fig. 5 (reviewed in - Mehta et al. 2013).
Fig. 4. Increase in calcium leading to toxicity.

A schematic and descriptive presentation of proposed mechanisms by which intracellular Ca\(^{2+}\) elevation may trigger secondary Ca\(^{2+}\)-dependent phenomena, which result in neurotoxicity. ATP, adenosine triphosphate; DNA, desoxyribonucleic acid; NO\(^\cdot\), nitric oxide; ONOO\(^-\), peroxynitrite; ROS, reactive oxygen species. Modified with permission from (Sattler and Tymianski 2000).
Fig. 5. Excitotoxic factors for the genesis of neurodegenerative disorders.

The number of factors such as alteration in neurotransmitters level in the brain, energy impairment, development of Lewy bodies along with age progression and various genetic factors results in the overactivation of glutamate, the excitatory amino acid which when present in excess leads to excitotoxic responses causing neuronal cell death. With permission from (Mehta et al. 2013).
1.2.1.2.3 Transition metals and autooxidation

Neural tissue contains high concentration of non-heme iron (Schenck and Zimmerman 2004). Ferrous ion ($\text{Fe}^{2+}$) can become oxidized by $\text{O}_2$ to produce $\text{O}_2^{-}$ and ferric ion ($\text{Fe}^{3+}$), in the process of autooxidation (Halliwell and Gutteridge 2007). In addition to autooxidation, transition metal ions can play a major role in the generation of free radicals by participating in the Fenton reaction that generates 'OH from $\text{O}_2^{-}$ and $\text{H}_2\text{O}_2$ (Fig. 2). Endogenous substances that can be autooxidized include hemoglobin, myoglobin, reduced cytochrome C and thiols. In the brain several other endogenous substrates including the neurotransmitters dopamine, its precursor levodopa (L-DOPA), serotonin, and norepinephrine can react with $\text{O}_2$ to generate $\text{O}_2^{-}$ (Halliwell 2006) as well as quinones/semiquinones (Graham 1978; Spencer et al. 1998). The CNS contains high concentrations of antioxidant, ascorbic acid (vitamin C). Antioxidants like Vitamin C and Vitamin E in the presence of transition metals can become pro-oxidant by reducing the transition metals and generate metal ions that can produce ROS (Kadiiska et al. 1995; Burkitt and Milne 1996).

1.2.1.2.4 Microglia and oxidative burst

Microglia are referred to as immune cells of the brain and are found in many regions, although their local distribution can vary highly (Mittelbronn et al. 2001), with highest concentrations found in the grey matter mostly in the hippocampus, olfactory telencephalon, basal ganglia and substantia nigra (Lawson et al. 1990; Mittelbronn et al. 2001). Neurodegenerative disorders, as well as physiological aging in humans and animals, are associated with signs of CNS inflammation. These cells provide immune
surveillance and are mobilized in response to neuronal damage, which in a healthy brain is beneficial (Aguzzi et al. 2013; Hellwig et al. 2013). Microglial cells are activated from their resting state by detecting lipopolysaccharide (LPS), beta-amyloid (Aβ), thrombin, interferon-gamma (IFN-γ), other proinflammatory cytokines, and CNS injury (Nimmerjahn et al. 2005; Dheen et al. 2007). Reactive microglia can produce and release a variety of cytotoxic or pro-inflammatory factors, including interleukin-1 (IL-1), tumor necrosis factor-alpha (TNF-α), prostanoids, glutamate, ROS and reactive nitrogen species (RNS), including NO⁺ (Koutsilieri et al. 1999; Halliwell 2006), and become phagocytic when neurons are damaged (Colton et al. 2000). This is summarized in Fig. 6. Oxidative burst refers to a rapid cellular release of ROS, which are produced by membrane-bound NADPH oxidase in the microglia. When the microglia are activated, the phagocytic NADPH oxidase complex becomes active and generates large amounts of O₂⁻ (Wilkinson and Landreth 2006) (Fig. 7).
Fig. 6. Reactive microgliosis.

Microglia are activated by several pro-inflammatory triggers. In response they produce neurotoxic proinflammatory factors. Aβ, amyloid-β; H$_2$O$_2$, hydrogen peroxide; IL-1, interleukin 1; LPS, lipopolysaccharide; NO$^-$, nitric oxide; NOX, NADPH oxidase; ONOO$^-$, peroxynitrite; O$_2^{•-}$, superoxide; PGE$_2$, prostaglandin E2; TNFα, tumour necrosis factor-alpha. Modified with creative commons license from (Block 2008).
Fig. 7. Activation of the phagocytic NADPH oxidase complex.

When NADPH oxidase is inactive, gp91$^{phox}$ and p22$^{phox}$ are membrane-bound while the four remaining subunits, p67$^{phox}$, p47$^{phox}$, p40$^{phox}$, and Rac are cytosolic. Upon stimulation, the cytosolic subunits translocate to the membrane to form a complex with the membrane-bound components. The assembly of all subunits yields an active enzyme that catalyzes the oxidation of NADPH into NADP$^+$, releasing an electron in the process. The electron is coupled to oxygen to generate superoxide. Although gp91$^{phox}$ is the catalytic core of the enzyme, responsible for the transmission of an electron to oxygen, the presence and correct positioning of all subunits is required for full function of the enzyme. GDI, guanine nucleotide dissociation inhibitor; GDP, guanosine diphosphate; Rac, ras-associated C3 botulinum toxin substrate. With permission from (Massaad and Klann 2011).
1.2.1.2.5 **Enzymatic oxidation**

There are numerous enzyme systems in the brain capable of generating significant amounts of ROS and free radicals as products or by-products of their reactions. These are described in Table 1.

**Table 1. Enzymes which generate ROS in the brain**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Location in the Brain</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monoamine oxidase</td>
<td>Neurons, mitochondrial membranes</td>
<td>Oxidizes catecholamines. Produces H$_2$O$_2$ and ‘OH.</td>
</tr>
<tr>
<td>- MAO-A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- MAO-B</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Xanthine oxidase</td>
<td>Vascular endothelium</td>
<td>Catalyzes the oxidation of hypoxanthine to xanthine, and of xanthine to uric acid. Increased calcium maintains activity via Ca-dependant proteases.</td>
</tr>
<tr>
<td>NADPH oxidase</td>
<td>Microglia, astrocytes, neurons</td>
<td>Transmembrane proteins that transfer electrons across membranes to reduce oxygen to superoxide</td>
</tr>
<tr>
<td>Superoxide dismutases</td>
<td>All cells</td>
<td>Dismutation of O$_2^-$ to H$_2$O$_2$ which leads to ‘OH.</td>
</tr>
<tr>
<td>Nitric oxide synthase</td>
<td>Microglia, astrocytes, neurons (nNOS)</td>
<td>Catalyzes conversion of L-arginine to NO’. Release of O$_2^-$ when arginine is low. NO’ can react with O$_2^-$ to yield ONOO- which decomposes to ‘OH.</td>
</tr>
<tr>
<td>- iNOS (inducible)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- nNOS (neuronal)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- eNOS (endothelial)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- mtNOS (mitochondrial)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phospholipase A2</td>
<td>Astrocytes, neurons</td>
<td>Releases arachidonic acid from lipid membrane which subsequently yields superoxide through LPO and PHS. Regulated by calcium, ROS and neurotransmitters.</td>
</tr>
<tr>
<td>Cytochromes P450</td>
<td>All cells</td>
<td>Responsible for bioactivation, metabolism and bioactivation of various compounds. Can reduce O$_2$ to produce ROS</td>
</tr>
<tr>
<td>Prostaglandin H synthases</td>
<td>Various brain regions</td>
<td>Co-oxidation of endogenous and exogenous substrates can occur during the catalytic process of leukotriene synthesis.</td>
</tr>
<tr>
<td>- PHS-1 constitutive</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- PHS-2 inducible</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lipoygenases</td>
<td>Co-oxidation of endogenous and exogenous substrates can occur during the catalytic process of prostaglandin synthesis.</td>
<td></td>
</tr>
</tbody>
</table>

Adapted from (Coyle and Puttfarcken 1993; Wells et al. 2009; Ramkissoon et al. 2013)
1.2.1.3 **Antioxidative and protective systems of the brain**

The balance between ROS formation and subsequent elimination is maintained by antioxidative and other protective systems in the brain. As mentioned previously, the brain may be particularly susceptible to oxidative damage compared to other organs due to cell-specific localization and lower levels of antioxidative enzymes. Due to the complexity of the neural networks, unrepaired damage can lead to structural and functional deficits.

The brain is not defenseless against oxidative damage. These defenses include the blood brain barrier, non-enzymatic and dietary antioxidants, as well as several enzyme systems. This section describes some of the protective systems and enzymes found in the brain. The focus is on enzymatic defenses particularly the glucose-6-phosphate dehydrogenase enzyme and its potential to defend the brain.

### 1.2.1.3.1 Blood-brain barrier

The central nervous system is separated from the bloodstream by the blood-brain barrier (**BBB**). This term is used to describe a series of properties possessed by blood vessels of the CNS. These unique properties tightly regulate the movement of molecules, ions and even cells between the blood and neural tissue. The BBB protects the brain from injury by limiting the entry of many toxins, pathogens and effects of the body's immune system (Daneman 2012). This unique barrier is supported by the endothelial cells of the brain capillaries, which form tight junctions unique in the CNS (**Fig. 8**). Surrounding the endothelial cells is the basement membrane composed of
different extra cellular matrix structural proteins (Zlokovic 2008). The capillary is discontinuously surrounded by pericytes, which are related to vascular smooth muscles cells. They are responsible for controlling blood flow (Peppiatt et al. 2006) and providing vessel stability by depositing matrix structure. These cells also release a number of growth factors that regulate remodeling and angiogenesis (Zlokovic 2008). Astrocytes ensheath blood vessels with their endfoot processes, allowing their communication between the blood and neurons (Abbott and Ronnback 2006).

**Fig. 8. Schematic diagram of the BBB.**

Schematic drawing of the neurovascular unit showing the close spatial relationship and the complex physiologic interactions between the various cells at the blood-brain barrier. In the brain, endothelial cells form tight junctions at their margins, sealing off paracellular pathways between cells. Pericytes distributed discontinuously along the cerebral capillaries serve as first line of defense when brain–endothelial cell integrity is compromised. Cerebral endothelial cells and pericytes are both surrounded by basement membrane, and also contribute to its formation. Foot processes from astrocytes form a network surrounding the capillaries, and microglial cells represent the resident immunocompetent cells of the brain. Modified with permission from (Abbott and Friedman 2012).
Together these components regulate the transport of nutrients, ions and hormones that are required to maintain optimal conditions for brain functions (LeFevre and Peters 1966; Reese and Karnovsky 1967; Pardridge et al. 1990; Abbott et al. 2010). A wide range of lipid-soluble molecules can diffuse though the BBB and enter the brain passively. In addition, a number of ATP-binding cassette (ABC) energy-dependent efflux transporters (ATP-binding cassette transporters; e.g. P-glycoprotein (p-gp), multidrug resistance proteins (MRPs)) actively pump many of these agents out of the brain (Begley 2004; Dauchy et al. 2008). The transport systems in the BBB are described in Fig. 9. These unique features of the BBB function as protectors against endogenous and exogenous toxins. When dysfunction occurs, either through structural changes, alteration of transporters or oxidative stress (Pun et al. 2009; Freeman and Keller 2012), the BBB may contribute to neurotoxicity associated with several neurodegenerative diseases like MS, PD, AD and epilepsy (Bowman et al. 2007; Zlokovic 2008; Jeynes and Provias 2011; Daneman 2012). The BBB creates an interesting and difficult obstacle to drug development for brain disease because this barrier is such a good blockade for many substrates (Chen and Liu 2012; Pardridge 2012). In terms of protecting the brain from oxidative stress, the endothelial cells have comparatively high levels of antioxidative systems compared to the rest of the brain (Tayarani et al. 1987) and GSH appears to be important in maintaining barrier integrity (Agarwal and Shukla 1999).
Fig. 9. Transport across the BBB.

(a) Solutes may passively diffuse through the cell membrane and cross the endothelium. (b) Active efflux carriers (ABC transporters) may intercept some of these passively penetrating solutes and pump them out of the endothelial cell either as they diffuse through the cell membrane or from the cytoplasm. (c) Carrier-mediated influx via solute carriers (SLCs) may be passive or primarily or secondarily active and can transport many essential polar molecules such as glucose, amino acids and nucleosides into the CNS. The solute carriers may be bidirectional, the direction of net transport being determined by the substrate concentration gradient (1), unidirectional either into or out of the cell (2/3), or involve an exchange of one substrate for another or be driven by an ion gradient (4). (d) Receptor-mediated transcytosis (RMT) requires receptor binding of ligand and can transport a variety of macromolecules such as peptides and proteins across the cerebral endothelium. Adsorptive-mediated transcytosis (AMT) appears to be induced in a non-specific manner by positively charged macromolecules and can also transport across the endothelium. (e) Leukocytes cross the BBB either by a process of diapedesis through the endothelial cells (penetrating close to the tight junctional regions), or via modified tight junctions. With permission from (Abbott et al. 2010)
1.2.1.3.2 Dietary and non-enzymatic antioxidants

Once ROS are produced in the brain, there are several enzymatic and non-enzymatic systems that can eliminate and halt the progression of oxidative stress. The function of vitamin C (ascorbate, ascorbic acid) in the brain is a double-edged sword with respect to free radical damage (Halliwell and Gutteridge 2007). The brain and spinal cord as well as the adrenal glands have the highest concentration of Vitamin C in the body (Hornig 1975). To achieve these very high concentrations (millimolar concentrations in neuron-rich areas), a multi-level system of transport has been elucidated (Fig.10). Vitamin C enters the CNS primarily via active transport at the choroid plexus (site of production of the cerebral spinal fluid). From there it diffuses into the brain cerebrospinal fluid (CSF). From the CSF, ascorbate is taken up into brain cells via the sodium-dependent vitamin C transporter type 2 (SVCT2) ascorbate transporter, where it accumulates at high concentrations (Rice 2000; Harrison and May 2009). Neurons are reported to have concentrations of up to 10 mM and glia about 1 mM (Rice and Russo-Menna 1998). Aside from acting as a broad ROS scavenger, ascorbate is involved in dopamine hydroxylation, neurotransmission, collagen and neuropeptide synthesis and formation of the myelin sheath (Rice 2000; Passage et al. 2004; Halliwell and Gutteridge 2007). However, in the presence of iron or copper vitamin C can act as a pro-oxidant (Reiter 1995; Carr and Frei 1999). The proposed mechanism involves ascorbate maintaining metal ions in their reduced state and therefore leading to reaction of the reduced metal ions with hydrogen peroxide, generating hydroxyl radicals (Carr and Frei 1999).
Fig. 10. Ascorbate uptake and metabolism in the CNS.

Ascorbate enters the CSF either directly through the choroid plexus via the SVCT2 or possibly as DHA via GLUTs across the blood–brain barrier. Similarly, ascorbate enters the neuron through the SVCT2 or as DHA via the GLUTs. AFR generated by X· dismutates to form DHA and ascorbate. Both the AFR and the DHA are recycled back to ascorbate by cellular metabolism. Glial cells obtain ascorbate from the recycling of DHA that enters via GLUTs, whereas neurons also have the SVCT2 for direct acquisition of ascorbate. ASC, ascorbate; AFR, ascorbate free radical; DHA, dehydroascorbic acid; CSF, cerebrospinal fluid; glucose transporters, GLUT; sodium-dependent vitamin C transporter type 2, SVCT2; X·, oxidizing free radical species. With permission from (Harrison and May 2009).
Vitamin E, also known as α-tocopherol, is a lipid soluble antioxidant concentrated in the hydrophobic layer of cell membranes. It contributes an electron to the peroxyl radical that is formed during the chain reaction of lipid peroxidation, and therefore is classified as a chain-breaking antioxidant (Reiter 1995; Halliwell and Gutteridge 2007). The vitamin E radical produced when the parent compound donates an electron is unreactive, and it eventually degrades or is recycled to vitamin E by ascorbate. α-Tocopherol is important for normal brain physiology, as patients with a prolonged deficiency of this vitamin suffer from neurological deficits, and the concentration of α-tocopherol is highly regulated as supplementation takes several weeks to affect brain concentrations (Muller and Goss-Sampson 1990).

The third major non-enzymatic antioxidant is the tripeptide γ-glutamyl-cysteinyl-glycine (γ-GluCysGly), commonly called glutathione (GSH). The GSH system is considered as one of the most important antioxidant systems in the brain. GSH is synthesized from L-glutamate and cysteine via the enzyme γ-glutamylcysteine synthetase (γ-GCS) forming γ-glutamylcysteine (γ-GluCys). This reaction is the rate-limiting step in glutathione synthesis. Glycine is added to the C-terminal of γ-GluCys via the enzyme GSH synthetase (GS) to form GSH (Dringen 2000). GSH helps to eliminate ROS in three different ways. Firstly, it can directly non-enzymatically react with several radicals (Halliwell and Gutteridge 2007). It conjugates xenobiotics, catalyzed by glutathione S-transferase (GST), before they are able to generate ROS. And finally, GSH can reduce H₂O₂ into water, catalyzed by the glutathione peroxidase (GPx). During this reduction of peroxides, GSH is oxidized by GPx to GS•, two molecules of
which combine to form glutathione disulfide (GSSG). Within cells, GSH is regenerated from GSSG in a reaction catalyzed by GSH reductase (GRx) (Fig. 1). This enzyme transfers electrons from NADPH to GSSG, thereby regenerating GSH. In the brain, GSH is predominately found in astrocytes with reduced concentrations in neurons (Dringen 2000). Furthermore, astroglial cells appear to supply cysteine and γ-GluCys to neurons for GSH synthesis (Fig. 11) (Aoyama et al. 2008; Johnson et al. 2012). The expression of the enzymes responsible for GSH synthesis and transport are controlled by the transcription factor nuclear factor-erythroid 2-related factor 2 (Nrf2). When ROS are increased in the cell, Nrf2 is translocated to the nucleus, where it forms a complex with other nuclear proteins that binds to the antioxidant response element (ARE) to induce antioxidant and phase II detoxification enzymes (Fernandez-Fernandez et al. 2012).

There are several other antioxidant compounds that could potentially provide protection. Coenzyme Q (ubiquinone) is associated with the mitochondrial oxidative phosphorylation enzyme complexes where it serves an antioxidant function. Several studies including protection against 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) toxicity have pointed to a protective potential of ubiquinone supplementation (Beal et al. 1998; Mattson et al. 2002). Carotenoids and flavonoids and particularly the supplement ginkgo biloba (containing the flavonoids myricetin and quercetin) and have also been studied as potential protective supplements (Halliwell 2007). The results of these studies are mixed and antioxidant supplementation is difficult to evaluate, as many oral antioxidants do not cross the BBB (Moosmann and Behl 2002).
Fig. 11. Production of glutathione (GSH) in astrocytes and neurons.

The basic routes of synthesis of GSH in astrocytes and neurons within the brain are depicted diagrammatically. In the rate-limiting step of GSH synthesis, the amino acids glutamate and cysteine are ligated to form the dipeptide γ-GluCys by the γ-glutamylcysteine synthetase (GCS) enzyme at the expense of ATP. γ-GluCys is then converted to GSH (γ-GluCysGly) by glutathione synthetase (GS). This reaction occurs in both astrocytes and neurons. Astrocytes, however, can secrete GSH into the intracellular space where it is cleaved into Glu and CysGly by γ-glutamyltranspeptidase (GGT). The CysGly dipeptide is cleaved into single amino acids (the cysteine of which can be taken up by neurons) by aminopeptidase N. The rate limiting amino acid cysteine is imported directly into neurons via the EAAC1, and cystine is imported into astrocytes via the Xc− transporter. The CysGly dipeptide is taken up much more effectively than GSH by both the neurons and astrocytes, but especially by the neurons. Direct uptake of GSH across the blood-brain barrier and into brain cells has been suggested, but it is unlikely that such direct uptake contributes in a quantitatively important way to the GSH concentration in brain cells. With open access permission from (Johnson et al. 2012)
1.2.1.3.3 **Antioxidative Enzymes**

1.2.1.3.3.1 **Superoxide Dismutase**

Superoxide dismutases (SODs) are metal-containing enzymes that catalyze the dismutation of $\text{O}_2^-$ to $\text{H}_2\text{O}_2$ and molecular oxygen. Mammalian tissues contain three forms of constitutively expressed SOD. They include CuZnSOD (SOD1) found in the peroxisomes and cytoplasm, MnSOD (SOD2) found solely in the mitochondrial matrix and an extracellular CuZnSOD (SOD3) (Johnson and Giulivi 2005).

SODs have been extensively investigated and several transgenic animal models have been developed. Animal models of SOD1 knockout mice are more sensitive to neurotoxicity caused by MPTP (Zhang *et al.* 2000), and they show increased cell death after axonal injury to motor neurons (Reaume *et al.* 1996). As well, animals that overexpress human SOD1 are protected from several neurotoxins including METH, MDA, MDMA, 6-hydroxydopamine (6-OHDA) and MPTP (Przedborski *et al.* 1992; Cadet *et al.* 1994a; Cadet *et al.* 1994b; Cadet *et al.* 1994c; Jayanthi *et al.* 1999; Halliwell and Gutteridge 2007), and are resistant to ischemia-reperfusion damage (Chan *et al.* 1991). These same animals also exhibited mild pathologic changes in the innervation of muscle that are suggestive of premature aging (Epstein *et al.* 1987; Ceballos-Picot *et al.* 1992). In humans, SOD1 mutations (many of which have no effect on activity) have been linked to ALS (Deng *et al.* 1993; Valentine and Hart 2003), and mice that were engineered to harbor several of these mutations also displayed a phenotype similar to ALS (Gurney *et al.* 1994). SOD2 knockout mice suffer from postnatal neurodegeneration highlighted by cell death in the cortex and brainstem.
regions and severe motor disturbances (Melov et al. 1998). In the case of SOD3, overexpression has been found to be detrimental to neurons, impairing long-term potentiation (Thiels et al. 2000; Thiels and Klann 2002); however, others have found mice overexpressing SOD3 may be protected from neurobehavioural deficits during aging (Hu et al. 2006).

SOD is generally considered as a protective enzyme; however, when increased SOD activity is not accompanied by a corresponding increase in catalase or glutathione peroxidase, this can lead to the accumulation of H$_2$O$_2$. For example, enhanced phenytoin-initiated embryotoxicity has been observed with a high maternal dose of PEG-SOD, unlike with PEG-catalase, which was embryoprotective (Winn and Wells 1999). Overexpression of CuZnSOD, producing an imbalance between H$_2$O$_2$ generation and its removal, has been suggested to contribute to the pathology of trisomy 21 (Down’s syndrome) (Kedziora and Bartosz 1988; Groner et al. 1994). Evidence from these animal studies demonstrate that not only too little but also too much SOD can be harmful to the nervous system (Halliwell and Gutteridge 2007).

1.2.1.3.3.2 Catalase

Catalase is an intracellular heme-containing metalloenzyme that protects against H$_2$O$_2$ cytotoxicity by converting hydrogen peroxide into water and molecular oxygen (Halliwell and Gutteridge 2007). It is located in the peroxisomes and to a lesser extent in the cytosol and mitochondria (Gaunt and de Duve 1976; Brannan et al. 1981; Vitorica et al. 1984). Catalase is ubiquitously expressed in a wide variety of brain cells;
however, its expression is relatively low in brain compared to other tissues, and has been considered inconsequential (Reiter 1995; Halliwell 2006). However, more recently it was shown that even these low brain activities of catalase are essential in protecting the developing fetal brain against ROS-initiated postnatal neurodevelopmental effects. Mice with deficient catalase activity (acatalasemic) were more susceptible to phenytoin-induced neurodevelopmental deficits, and aged acatalasemic mice showed greater motor coordination deficits, indicating that the relatively lower levels of catalase in the brain are not inconsequential (Abramov et al. 2012).

1.2.1.3.3.3 Glutathione Peroxidase

Due to the low levels of catalase in the brain, selenium-dependent glutathione peroxidase (GPx) may play the major role in detoxifying H₂O₂ in the brain (Reiter 1995; Brigelius-Flohe 1999). GPx detoxification of H₂O₂ and other peroxides is coupled with the oxidation of GSH to GSSG in the cytosol and mitochondria of glia cells (Vitorica et al. 1984; Savaskan et al. 2007). GPx-knockout mice exhibited increased striatal DNA oxidation, dopamine depletion and striatal damage upon administration of a ROS-initiating exogenous toxin (Klivenyi et al. 2000). Under normal conditions, a balance may exist between the rate of H₂O₂ formation via dismutation of superoxide by SOD and its elimination by GPx, as well as catalase, to prevent oxidative stress (Halliwell and Gutteridge 2007).
1.2.1.3.3.4 NAD(P)H:Quinone Oxidoreductase

NAD(P)H:quinone oxidoreductase (NQO1) is a cytosolic enzyme that catalyzes the two-electron reduction of quinones to hydroquinones, using either NADPH or NADH as electron donors (Halliwell and Gutteridge 2007). In addition, the hydroquinone products of the NQO1 reaction can be further metabolized to glucuronide and sulfate conjugates, which can then be excreted. NQO1 is mainly expressed in astrocytes and brain endothelial cells and prevents the generation of ROS (Siegel and Ross 2000; van Muiswinkel et al. 2004; van Horssen et al. 2006).

1.2.1.3.3.5 Peroxiredoxins and Thioredoxins

The family of peroxiredoxin (Prx) enzymes is involved in the degradation of H$_2$O$_2$, organic hydroperoxides and peroxynitrite, and are dependent on a reactive cysteine at the active site (Rhee et al. 2005). Prx proteins reduce peroxides by employing electron donating and redox-sensitive cysteines that undergo oxidation/reduction cycles (Sarafian et al. 1999). Different brain regions and cell type demonstrate unique basal expression profiles of Prx. For example, Prx proteins 1 and 6 are expressed in glial cells, whereas Prx proteins 2–5 are localized in neurons (Sarafian et al. 1999). Prx can be inactivated by H$_2$O$_2$. At physiological concentrations, Prx are reported to dispose of much of the H$_2$O$_2$, but at higher concentrations (during injury or inflammation) the Prxs are inactivated, allowing the cell to react to the ROS signal (Rhee et al. 2005; Halliwell 2006).
Thioredoxins (Trxs) are another family of proteins acting in conjunction with Prx to repair oxidatively damaged proteins. They act like antioxidants by facilitating the reduction of other proteins by cysteine thiol-disulfide exchange becoming oxidized themselves. Oxidized Trx proteins can be reduced through the action of Trx reductase using NADPH as a cofactor (Patenaude et al. 2005).

1.2.1.3.3.6 Glucose-6-phosphate Dehydrogenase (G6PD)

Note: The following section on G6PD appears as a component of a book chapter on ROS and neurodegeneration and is reproduced with permission from publisher:


This section will discuss the highly polymorphic enzyme glucose-6-phosphate dehydrogenase (G6PD), which is important in ROS detoxification.

1.2.1.3.3.6.1 Genetics and Regulation of G6PD

Glucose-6-phosphate dehydrogenase (D-glucose 6-phosphate: NADP oxidoreductase, EC 1.1.1.49) gene has been mapped to the telomeric region of the long arm of the X chromosome (band Xq28) (Pai et al. 1980; Mason et al. 1990). G6PD is a typical X-linked gene that has helped in the development of the X-chromosome inactivation hypothesis (Lyon Law), where one of the two X chromosomes in the female
cell is randomly inactivated (Lyon 1961; Beutler et al. 1962). The gene consists of 13 exons and 12 introns and covers about 18.5 kb (Martini et al. 1986). Exon 1 contains no coding sequence and the ATG start site is found in exon 2. The first intron is highly conserved (Toniolo et al. 1991), while the rest of the intron sequences vary among species. The second intron is unusually long, measuring about 11 kb (Chen et al. 1991). The promoter is embedded in a CpG island spanning from nt -1200 to intron 1, and X chromosome inactivation is associated with the methylation of this entire island region (Toniolo et al. 1988). The promoter region contains several stimulatory protein 1 (Sp1) and activator protein 2 (AP-2) binding sites (Philippe et al. 1994) and an atypical TATA box (ATTAAAT) at -30 to -25 bp (Toniolo et al. 1991). Deletion experiments have shown that the core promoter resides between nt -147 and +45, inhibitory sequences are located between nt -358 and -147, and upstream stimulatory sequences are present between nt -613 and -358 (Philippe et al. 1994). Although the gene is typically regarded as a “housekeeping” gene, expression has been shown to be tissue- and species-dependent. The genetic elements that determine the rate of transcription of the gene in different cell types and in response to cellular changes are not yet fully known (Luzzatto et al. 2001). The mRNA product of the G6PD contains a relatively short 5’ untranslated region of 69 bp, which corresponds to all of exon 1 and part of exon 2, and a longer 3’ untranslated region of 655 bp. The gene product is about 2.4 kb in size (including the poly (A) tail) (Luzzatto et al. 2001). Kletzien and coworkers (1994) partially reviews regulation of G6PD expression by hormones, diet and oxidative stress.
Mutations in the G6PD gene are well studied and reviewed, and the most current database describes 186 mutations (Minucci et al. 2012). Historically, the number of distinct variants has fluctuated, reaching as high as 442 (Beutler 1994), but current sequencing advances have shown that many of the reported variants were caused by the same mutation. Most of the mutations (85%) are single nucleotide substitutions throughout the entire coding region except in Exon 1, and 2 mutations have even been found in the intronic regions. Some of the highest prevalence rates are found in tropical Africa, the Middle East, tropical and subtropical Asia, areas of the Mediterranean and Papua New Guinea, where the incidence of G6PD polymorphisms can approach 60% of the population (Sodeinde 1992).

G6PD is constitutively but not uniformly expressed in all cells, with basal activity varying up to about 10-fold among different organs and tissues (Kletzien et al. 1994; Corcoran et al. 1996). In various animal models and humans, the levels of mRNA and protein expression in the brain are constitutive in neurons and glial cells (Cammer and Zimmerman 1982; Philbert et al. 1991; Biagiotti et al. 2001).

1.2.1.3.3.6.2 **G6PD Protein Structure**

The mature G6PD protein contains 514 amino acids and has an approximate molecular weight of 59 kDa. The gene codes for 515 amino acids; the N-terminal amino acid of the mature protein present in human erythrocytes is an acetylated alanine; the initiating methionine is cleaved and the following alanine is acetylated during post-translational processing (Camardella et al. 1995). The active enzyme exists in an
equilibrium of dimers and tetramers determined by ion concentrations and pH (Cohen and Rosemeyer 1969). Each mammalian G6PD monomer contains a catalytic site and a second structural NADP$^+$ binding site (Au et al. 2000), the latter of which is not found in the bacterial enzyme. The role of the second structural NADP$^+$ binding site has been postulated to provide long-term stability, and many of the mutations that affect activity are found in this region (Wang et al. 2008). Multiple sequence alignments reveal a conserved nine-residue peptide (198-RIDHYLGKE-206 in the human) (Kotaka et al. 2005) where the aspartate, histidine and lysine residues are important in glucose-6-phosphate binding (Cosgrove et al. 2000). The consensus nucleotide-binding fingerprint, 38-GASGDLA-44, has been associated with coenzyme NADP$^+$ binding (Levy et al. 1996).

1.2.1.3.3.6.3 G6PD Enzymology

Glucose-6-phosphate dehydrogenase is the first and rate-limiting enzyme in the hexose monophosphate pathway (HMP) also known as the pentose phosphate pathway (PPP). In this pathway glucose-6-phosphate (G6P) is ultimately converted to ribose-5-phosphate (R5P). R5P is required for glycolysis and for DNA and RNA synthesis. During the conversion of G6P to 6-phosphogluconolactone, G6PD generates nicotinamide adenine dinucleotide phosphate (NADPH), which is essential for many reductive biosynthetic pathways including cholesterol and fatty acid synthesis (Fig. 12).
Fig. 12. Pentose Phosphate Pathway.

The oxidative phase of the pentose phosphate pathway (PPP) converts NADP$^+$ to NADPH. The non-oxidative phase forms nucleic acids and amino acids.
During cellular oxidative stress, NADPH is critical for the regeneration of reduced glutathione (GSH), catalyzed by NADPH-dependent glutathione reductase. GSH is essential for the detoxification of reactive free radicals and lipid hydroperoxides by GSH peroxidase. Another important role of NADPH is the maintenance of the catalytic activity of catalase, which is required for the detoxification of hydrogen peroxide (Kirkman et al. 1987; Kirkman et al. 1999). NADPH binds to catalase and prevents the formation of inactive catalase (compound II), as well as mediating the rapid reduction of catalase compound II back to its active form. Impaired catalase activity was found to contribute largely to a H_2O_2-mediated enhancement of oxidant sensitivity in G6PD-deficient erythrocytes. However, some catalase activity did remain in the G6PD-deficient cells since catalase activity did not drop below 50% of its initial level (Scott et al. 1993). Furthermore, the amount of NADPH required for the prevention of catalase inactivation is very low (below 0.1 µM) *in vivo*, and the reduction of catalase compound II to the active form (compound I) is known to occur in the absence of NADPH, albeit at much slower rates (Kirkman et al. 1987). Other enzymes and systems requiring NADPH are summarized in Table 2.
Table 2. Enzymes requiring NADPH

<table>
<thead>
<tr>
<th>Enzyme/System</th>
<th>Reference</th>
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<tbody>
<tr>
<td>Antioxidative systems (GSH reductase, catalase) (Halliwell and Gutteridge 2007)</td>
<td></td>
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<tr>
<td>Fatty acid synthesis (β-ketoacyl-ACP reductase, enoyl-ACP reductase) (Wakil et al. 1983)</td>
<td></td>
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<tr>
<td>Cholesterol synthesis (3-hydroxy-3-methyl-glutaryl-CoA reductase, squalene synthase) (Berg et al. 2002)</td>
<td></td>
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<tr>
<td>NADPH oxidases (Bedard and Krause 2007)</td>
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<tr>
<td>Nitric Oxide synthase (Stuehr 1999)</td>
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</tr>
<tr>
<td>Cytochrome p450 oxidoreductase (Iyanagi 2007)</td>
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1.2.1.3.3.6.4 G6PD Deficiency and Classification

G6PD deficiency refers to the condition of reduced activity of the enzyme. It has frequently been referred to as the most common human enzymopathy with a prevalence worldwide of 4.9% (Nkhoma et al. 2009). As mentioned previously, the G6PD gene is highly mutated, and the World Health Organization has created a 5-class system to classify G6PD variants according to the level of enzyme activity in the red blood cell, and the clinical manifestations. Class I refers to severely deficient variants with less than 10% of activity that are associated with chronic non-spherocytic hemolytic anemia (CNSHA). Class II variants are severely deficient resulting in less
than 10% of residual enzyme activity but are not associated with CNSHA. Class III variants are moderately deficient (10-60% of activity). Class IV are the variants resulting in normal enzyme activity (60%-150%) and the Class V produce G6PD activity higher than normal (>150%) (WHO 1989).

Table 3. Classes of G6PD-deficiency

<table>
<thead>
<tr>
<th>CLASS</th>
<th>PERCENT ACTIVITY</th>
<th>SYMPTOMS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Severe Deficiency, I</td>
<td>&lt;10%</td>
<td>chronic nonspherocytic hemolytic anemia</td>
</tr>
<tr>
<td>Severe Deficiency, II</td>
<td>&lt;10%</td>
<td>Intermittent hemolysis</td>
</tr>
<tr>
<td>Moderate Deficiency, III</td>
<td>10-60%</td>
<td>Drug induced hemolysis</td>
</tr>
<tr>
<td>Non-deficient, IV</td>
<td>60-150%</td>
<td>----</td>
</tr>
<tr>
<td>Increased Activity, V</td>
<td>&gt;150%</td>
<td>----</td>
</tr>
</tbody>
</table>

1.2.1.3.6.5  **Protective Role of G6PD in Blood Cells**

Most individuals with a G6PD deficiency are normally asymptomatic, but can exhibit a clinical syndrome in response to an enhanced oxidative insult or exogenous factors. Although some deficiencies belong to the Class I of G6PD variants associated with CNSHA, there is no link to date between a specific genetic G6PD variant and a
single clinical syndrome. It is generally believed that G6PD deficiencies constitute a problem only for mature red blood cells (Cappellini and Fiorelli 2008), which lack a nucleus and the ability to increase G6PD expression in response to oxidative stress.

G6PD deficiency generally manifests as acute red blood cell hemolysis and anemia caused by oxidative stress initiated by drugs, infection or exposure to fava beans (favism, see below). Since G6PD is the only source of NADPH in the red blood cell, it is an essential part of the defense against oxidative stress (Pandolfi et al. 1995). G6PD deficiency was discovered when certain patients receiving the anti-malarial drug primaquine developed acute hemolysis (Beutler 1959). Since that discovery, several drugs have been linked to this manifestation (Luzzatto et al. 2001), making G6PD a highly important pharmacogene (McDonagh et al. 2012). The other and probably most common causes of oxidative stress leading to hemolysis in G6PD-deficient patients are various types of infections including hepatitis viruses A and B, cyclomegalovirus, pneumonia and typhoid fever (Cappellini and Fiorelli 2008). Favism is a unique type of hemolysis caused by the ingestion of the fava bean. It is believed that divicine, isouramil and convicine are the compounds found in the beans that increase the activity of the HMP and promote hemolysis in G6PD-deficient individuals (Arese and De Flora 1990). In the case of another manifestation, neonatal jaundice, it is still unclear why G6PD-deficient neonates are more susceptible to this syndrome, but these neonates may have an impaired ability to conjugate and clear bilirubin in the liver but the mechanism is yet unknown (Mason et al. 2007). Neonatal jaundice, if not treated
timely, leads to chronic bilirubin encephalopathy (kernicterus) and can leave the child mentally impaired (Mason et al. 2007).

The widespread prevalence of malaria is believed to be the reason why G6PD deficiency is common throughout the world (especially in areas affect by outbreaks of the host parasite). It is commonly believed that G6PD-deficient red blood cells are inhospitable to the survival and reproduction of the Plasmodium falciparum parasite responsible for malaria (Hedrick 2011). G6PD deficiency may also protect against Plasmodium vivax (Carter and Mendis 2002; Leslie et al. 2010), which could provide further selective pressure for the high frequency of this enzymopathy. This benefit makes G6PD mutations “balanced” polymorphisms, since they provide a selective advantage even though they have the potential for facilitating a disease state (Beutler 1996).

1.2.1.3.3.6.6 G6PD Protection in Development

Using a mutant G6PD-deficient mouse model, G6PD was discovered to be a developmentally critical antioxidative enzyme that protects the embryo from the pathological effects of both endogenous and xenobiotic-enhanced oxidative stress and DNA damage (Nicol et al. 2000). Similarly, G6PD-inactivated embryonic stem cells were found to be viable but highly sensitive to oxidative stress (Pandolfi et al. 1995). Attempts were made to create G6PD null mice, but the resulting embryos died in utero by E10.5 (Longo et al. 2002), confirming the fundamental importance of this enzyme in development.
1.2.1.3.3.6.7 **Protective Role of G6PD in the Brain**

G6PD is coordinately regulated with the expression of other antioxidative enzymes, including GSH peroxidase and glutathione reductase, in the developing and adult rat brain (Ninfali *et al.* 1996; Ninfali *et al.* 1998). Moreover, NADPH generated via G6PD is necessary for the maintenance of reduced glutathione by glutathione reductase. Hence, in addition to G6PD deficiencies, other antioxidative defense mechanisms may be compromised, which would increase susceptibility of the brain to ROS-mediated oxidative stress and subsequent neuronal damage. Only a few studies have examined the protective role of G6PD in the brain. One of these studied the carcinogenic implications of G6PD deficiency in brain employing young mice that contained the X-ray-induced low efficiency allele of G6PD along with a transgenic shuttle vector for measuring mutagenesis in vivo (Felix *et al.* 2002). The brains of these G6PD-deficient male mice exhibited a decrease in the ratio of reduced glutathione to oxidized glutathione, an accumulation of promutagenic etheno DNA adducts secondary to lipid peroxidation, and an increased somatic mutation rate suggesting an enhanced risk for brain cancer.

Selective overexpression of G6PD activity in the dopaminergic nigrostriatal system of mice was protective against the toxic effects of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Mejias *et al.* 2006). These findings suggest that G6PD plays a protective role in the brain. Further gene expression studies in these G6PD overexpressing mice displayed changes mainly in the expression of proteins related to
antioxidant defense (eg. peroxiredoxin, GST), detoxification (eg. aconitase 2 – iron regulation) and synaptic function (eg. dynamin-1) (Romero-Ruiz et al. 2010).

Histochemical and immunohistochemical analyses revealed that the highest expression of cerebellar G6PD activity and protein was in the Purkinje cells (Biagiotti et al. 2001; Biagiotti et al. 2003), and later was found to be co-localized with NADPH-dependent enzymes including NADPH-cytochrome P450 reductase and glutathione reductase (Ferri et al. 2005). G6PD activity may be particularly important in the hippocampus, where increased neuronal G6PD and reactive sulfhydryl levels were found in patients with Alzheimer’s disease, presumably in compensation for increased oxidative stress in that region (Russell et al. 1999). The levels of CuZnSOD mRNA and protein are particularly high in hippocampal pyramidal neurons and granular cells (Ceballos-Picot et al. 1992), which in the absence of adequate G6PD-mediated detoxification may result in \( \text{H}_2\text{O}_2 \) overproduction and peroxidative damage within these cells. In addition to the cytoprotective role of G6PD against ROS-mediated oxidative damage, G6PD may be required for normal cell growth by providing NADPH for various cell processes including reductive biosynthesis (Pandolfi et al. 1995; Ninfali et al. 1996; Tian et al. 1998).

Note: End of book section.
1.2.1.4 **ROS in Signal Transduction**

Signal transduction describes the mechanism by which cells sense and respond to their environment. The signaling cascade can occur inter- and intracellularly. ROS can influence this mechanism by interacting with many proteins along the signal pathway leading to changes in gene expression, cell growth and cell death (Allen and Tresini 2000; Halliwell and Gutteridge 2007). The research in this field is expanding quickly and the effects of ROS have turned from being a solely toxic byproduct of cellular processes to being necessary for cell survival, growth and differentiation, participating in tightly controlled redox pathways (Droge 2002; Maher 2006; Rhee 2006; Valko et al. 2007; Forman et al. 2010; Klomsiri et al. 2011; Veal and Day 2011; Herrmann and Dick 2012; Corcoran and Cotter 2013). ROS signaling a major mechanism of communication between the mitochondria and nucleus of the cell (Antico Arciuch et al. 2012). It is proposed that oxidation and reduction of protein cysteine sulfhydryl (SH) groups may work as a molecular switch to start or to stop the signaling (Klomsiri et al. 2011). The biochemistry allowing for these reactions to occur is diagramed in **Fig. 13**. Several signaling pathways associated with ROS will be briefly discussed below.
Fig. 13. Cysteine biochemistry allows for redox-dependent signaling.

Specific reactive cysteine (Cys) residues within target proteins can be covalently modified by oxidative stress. Much like phosphorylation on serine or threonine residues, alteration of the thiol group can in turn modify enzymatic activity. Although the sulfenic form (SOH) is readily reversible, higher states of oxidation generally, but not always, lead to irreversible modification. With creative commons license from (Finkel 2011)
1.2.1.4.1 Mitogen-activated Protein Kinases (MAPKs)

The mitogen-activated protein kinases (MAPKs) are a large family of enzymes that use ATP to phosphorylate their protein targets on serine and threonine residues (Halliwell and Gutteridge 2007). Growth factors and cytokines can activate the MAP kinase signaling pathway, which transmit signals to the nucleus where gene expression is regulated (Karin and Hunter 1995). The MAPK subfamilies include: (1) extracellular-regulated kinases (ERKs), (2) c-jun-N-terminal kinases (JNKs), (3) p38 MAPK and (4) big MAP kinase (BMK) (Valko et al. 2007). All of these proteins contain redox-sensitive sites (Allen and Tresini 2000). H₂O₂ is able to activate these kinases and increase phosphorylation at several areas in the pathway. This activation of each of the signaling pathways is type-, stimulus- and concentration-specific (Halliwell and Gutteridge 2007; Valko et al. 2007). Fig. 14 illustrates a potential model of ROS activation of MAPKs at high and low concentrations of ROS.

These kinase pathways can also be activated upstream by H₂O₂ inducing guanine nucleotide exchange on Ras by reacting with cysteine 118 (Lander et al. 1995). Apoptosis signal-regulating kinase 1 (ASK1), also known as mitogen-activated protein kinase kinase kinase 5 (MAP3K5), is another upstream protein in the MAPK signaling pathway. ASK1 is subject to a complex mode of both direct and indirect redox-regulation through its association with inhibitor thioredoxin-1 (Trx1) (Saitoh et al. 1998). In the first step of the redox activation of ASK1, the oxidation of Trx1 results in its dissociation from the kinase, rendering ASK1 free to become oxidized itself, which
triggers the formation of a multimeric kinase through the formation of many intermolecular disulfide bonds (Liu and Min 2002; Corcoran and Cotter 2013).

**Fig. 14. Model of MAPK-mediated detection of and response to cellular redox state.**

Proposed by (Galli et al. 2008). MAPKs gauge the levels of intracellular H$_2$O$_2$ and dictate the fate of the cell accordingly. Low levels of H$_2$O$_2$ function in a signalling capacity, pushing the proliferative agenda of Erk2 by oxidizing its cysteine residues (C38 and C214) to sulfenic (SO$_2$H) and sulfonic acid (SO$_3$H), leading to conformational changes in the enzyme that increase its binding to MEK1/2 (MAP2K1/2) and trigger the relocation of Erk2 to the nucleus. When H$_2$O$_2$ levels become elevated to point of toxicity, the same cysteine residues of Erk2 become insensitive to oxidation. At this concentration of H$_2$O$_2$, cysteines of JNK2 (C41) and p38 (C162) become oxidized to sulfonic acid (SO$_3$H), facilitating the activation of these kinases and stimulating a cell-cycle arrest programme. Illustrated in (Corcoran and Cotter 2013), reproduced with permission.
1.2.1.4.2  **Protein Kinases C (PKCs)**

Protein kinases C (PKCs) are another group of serine/threonine kinases responsible for cell growth regulation (Halliwell and Gutteridge 2007). They contain several cysteine-rich regions in the regulatory domain and the catalytic site that can be modified by oxidants (Gopalakrishna and Jaken 2000). Similarly as MAPKs, in the presence of very high levels of ROS, PKCs are inactivated, but at physiologically normal or slightly elevated concentrations selective oxidation of the N-terminal regulatory domain can stimulate and maintain PKC activity (Gopalakrishna and Jaken 2000).

1.2.1.4.3  **Nuclear Factor-kappa B (NF-κB)**

Nuclear factor-kappa b (NF-κB) is a transcription factor inducing the expression of hundreds of genes involved in innate and adaptive immune response, cell growth and survival, differentiation, development, inflammation, stress response and apoptosis (Siomek 2012). This dimeric transcription factor is composed of different members of the Rel family. The mammalian NF-κB protein family contains five members: NF-κB1 (p50/p105), NF-κB2 (p52/p100), RelA (p65), RelB, and c-Rel. These proteins share evolutionarily conserved domains for dimerization, DNA binding, and nuclear localization (Gloire and Piette 2009; Siomek 2012). These dimers bind to the promoters of a diversity of genes at sequences known as κB elements (Bowie and O'Neill 2000). NF-κB is kept in the cytoplasm by binding to the inhibitory subunit, IκB. During activation, IκB becomes phosphorylated and NF-κB is released and translocates to the
nucleus (Halliwell and Gutteridge 2007). As already mentioned, NF-κB is involved in the transcriptional control of many genes, which include antioxidative enzymes like SOD, catalase, and NQO1 as well as pro-oxidant enzymes like NOX and iNOS (Morgan and Liu 2011). However, not only is NF-κB responsible for controlling the amount of ROS in the cell, but also ROS are postulated to activate NF-κB. Fig. 15 demonstrates the sites in the NF-κB pathway that have been linked to ROS signalling (Morgan and Liu 2011).

1.2.1.4.4 **Activator Protein-1 (AP-1)**

Another transcription factor involved in the sensing of redox state and regulating expression of genes involved in stress response, growth and differentiation is the activator protein-1 (AP-1) factor (Halliwell and Gutteridge 2007; Valko *et al.* 2007). AP-1 is a dimer of proteins from the Fos and Jun families, and ROS can alter its activity. Most direct control involves the cysteine residues in the DNA-binding domain and decreased DNA binding (Halliwell and Gutteridge 2007).
ROS interact with NF-κB at various places within the signaling pathway. Many of these interactions occur in a cell type-specific manner. ROS have been proposed to both activate and inactivate the IKK complex leading to an effect on the downstream targets. Often ROS have been shown to activate NF-κB through alternative IκBα phosphorylation, which may or may not result in the degradation of IκBα. Lastly, ROS may influence the DNA binding properties of the NF-κB proteins themselves. Oxidation of p50 on its DNA binding domain has been shown to prevent its DNA binding, and must be reversed in the nucleus by a Trx1-dependent process involving Ref-1. On the other hand, the phosphorylation of RelA that is influenced by ROS-dependent processes leads to greater NF-κB activation. From (Morgan and Liu 2011)
1.2.1.4.5  **p45-nuclear factor erythroid-derived 2 related factors (Nrf – Nrf2)**

The p45-nuclear factor erythroid-derived 2 (NF-E2) and the NF-E2-related factors Nrf1, Nrf2 and Nrf3 are another set of transcription factors that respond to ROS by ultimately increasing the transcription of genes that contain antioxidant response elements (AREs) (Kobayashi et al. 2002; Halliwell and Gutteridge 2007). Nrf2, in particular, is part of a vital network in protective cellular responses in many tissues. In normal cellular conditions, Nrf2 binds to Keap1 (Kelch-like erythroid cell-derived protein with CNC homolog (ECH)-associated protein 1), which keeps Nrf2 in the cytosol and hence inactive (Itoh et al. 1999). The Nrf2-Keap1 dimer is also bound to an ubiquitin ligase complex which promotes proteasomal degradation of Nrf2. When the cell is exposed to large amounts of ROS, the cysteine-rich Keap1 is oxidized and releases Nrf2, which is translocated into the nucleus. The proposed mechanisms of ROS effects on Keap1 are shown in Fig. 16. Once in the nucleus, Nrf2 can dimerize with other proteins such as Maf and Jun and modulate transcription by binding to AREs (Itoh et al. 2004; Antelmann and Helmann 2011).
Fig. 16. Redox regulation of the KEAP1-Nfr2 system in response to ROS.

The Keap1 sensor protein contains more than 25 Cys residues, including the redox-sensitive C151 and the C273 and C288 residues that coordinate Zn. Under nonstress conditions, Keap1 binds to the DLG and ETGE sites in the Nrf2 transcription factor, positioning the lysine residues of Nrf2 optimal for ubiquitination by the E3 ligase and subsequent degradation of Nrf2. On exposure to ROS, the C151 residues form an intersubunit disulfide bond in Keap1, and intramolecular disulfide bonds between C236-C613 have also been detected. Oxidation of C273 and C288 leads to Zn release. S* indicates unknown oxoforms. Disulfide bonds or S-alkylation of Keap1 decreases binding to the DLG site of Nrf2. Oxidation of Keap1 also masks the NES, leading to nuclear accumulation of Nrf2, which is further stabilized by binding to p21. This leads to transcriptional activation of Nrf2 that binds to antioxidant response elements (AREs) in the promoters of phase-2 genes encoding heme oxygenase (HO-1), quinone oxidoreductase (NQQ1), gamma-glutamylcysteine synthetase (g-GCS), glutathione S-transferase (GST), catalase (Cat), superoxide dismutase (MnSOD), or metallothioneins (MT-1,2) and other antioxidant-function genes. With permission from (Antelmann and Helmann 2011) and (Paulsen and Carroll 2010).
1.2.1.5 Free Radical Theory of Aging

The free radical theory of aging originated in 1956 when Denham Harman first proposed it based on observations studying ionizing radiation (Harman 1956; Harman 2009). The theory states that the generation and accumulation of free radicals during normal cellular processes over time (aging) results in oxidative damage to cellular macromolecules leading to the eventual disease and failure of the organism. Focusing on the brain in this introduction, the various sources of ROS and ways of removing these compounds have been discussed. Several neurodegenerative diseases associated with aging, including AD, PD, ALS, and HD, have been linked to oxidative stress (Markesbery 1997; Jenner 1998; Mariani et al. 2005; Valko et al. 2007; Melo et al. 2011).

The theory is straightforward and researchers have since tried to prove it, including the proposition that stopping oxidative stress should stop or slow the aging process. In several mouse models, treatment with various antioxidants was able to slow aging-related cognitive decline (Behl and Moosmann 2002; Milgram et al. 2002a; Milgram et al. 2002b; Arzi et al. 2004; Sung et al. 2004). In humans, however, results have been mixed (Moosmann and Behl 2002; Blacker 2005). There have been several large studies of antioxidant treatment as well as research with transgenic animals, which have not yielded definitive proof for the theory, leading to some doubt being cast on the free radical theory of aging (Perez et al. 2009a; Speakman and Selman 2011; Moyer 2013).
For example, a recent study of the potential of NF-κB involvement in aging has been published. NF-κB, which is activated by ROS in different cell types (Gloire et al. 2006; Gloire and Piette 2009), has been shown to be an effective controller of body aging (Zhang et al. 2013). These researchers showed that specifically increasing NF-κB in the hypothalamus caused mice to age faster, while inhibiting NF-κB allowed the mice to live longer. The animals with NF-κB inhibited not only showed better survival, but also better memory, strength and skin condition. The involvement of NF-κB shows a possible mechanism by which ROS affects aging (Zhang et al. 2013). On the other hand, overexpression of antioxidative enzymes has given mixed results. Several studies have shown that overexpression of antioxidant enzymes in mice has a protective effect against oxidative stress, with a diminished accumulation of oxidative damage to macromolecules (Muller et al., 2007). When catalase was targeted to the mitochondria, a 21% extension in the lifespan in this mouse model was reported (Schriner et al. 2005). However, transgenic mice overexpressing CuZnSOD and catalase in the whole cell did not show any increases in lifespan (Huang et al. 2000; Perez et al. 2009b).

In 1935, for the first time, the life-extending effects of caloric restriction (CR) were described (McCay et al. 1989). CR is defined as a decrease of 30% to 60% of food intake without malnutrition. Several model systems have been used to test this lifespan-extending treatment, and it has been shown to be effective in yeast, worms, flies, spiders, mice and most recently rhesus monkeys (Colman et al. 2009; Libert and Guarente 2013). There are some human epidemiological studies supporting the role of
CR in lifespan extension in humans. One comes from a population in Okinawa, Japan where there is 4-5 times the average number of centenarians. A low caloric intake was confirmed in this population, with a 20% CR in adults residing on Okinawa compared to mainland Japan (Suzuki et al. 2001), attributed to laborious occupations and daily activities as farmers and a diet that was rich in nutrients yet low in energy density (Willcox et al. 2007). The second study is of Spanish nursing home residents who underwent long-term alternate day feeding resulting in a 35% CR compared to a control group (Vallejo 1957; Redman and Ravussin 2011). Results indicated that death rate tended to be lower in the CR group (but not significant because of the low numbers) and hospital admissions were significantly reduced in these individuals (Stunkard 1976; Johnson et al. 2006; Redman and Ravussin 2011).

The mechanism by which CR expands lifespan is still largely unknown, but ROS have been implicated. It is proposed that CR creates a shift in energy metabolism (Anderson et al. 2009), thereby reducing overall ROS production in the organism (Barja 2002). Long-term CR decreased the rate of mitochondrial ROS generation by 45% at complex I of the electron transport chain in the heart of rats and significantly lowered the level of oxidative damage to mtDNA by 30% (Gredilla et al. 2001b). Similar results were subsequently observed in rat liver mitochondria even only after 6 weeks of caloric restriction (Gredilla et al. 2001a). In mice, DNA oxidation was reduced in all organs with 60% caloric intake (Sohal et al. 1994).

Sirtuins (silent information regulator 2 (Sir2) 1 proteins, SIRTs 1 through 7) belong to an ancient family of evolutionarily conserved NAD⁺-dependent enzymes with
deacetylase and/or mono-ADP-ribosyltransferase activity, and are implicated in diverse cellular processes. Sirtuin-catalyzed deacetylation of key transcription factors results in altered gene expression of key antioxidant enzymes or those producing ROS. Mammalian sirtuins are centrally involved in metabolism (Houtkooper et al. 2012; Kanfi et al. 2012), and CR has been shown to have an effect on these enzymes. SIRT1 is downstream in ROS signaling because of a dependence on the availability of NAD+, but it can be important upstream in cellular regulators, including forkhead homeobox type O factor 3 (FOXO3), muscle-specific RING finger protein1, and the v-Akt murine thymoma viral oncogene homolog1 (Akt1) (Radak et al. 2013). The proposed mechanism of SIRT1 in redox signaling is illustrated in Fig. 18. When 12-month-old rats were subjected to CR, as 60% of the daily food allotment, the result was a downregulation of the insulin growth factor 1 (IGF-1) pathway and SIRT1-mediated regulation of p53, Ku70, and FOXO3 (Cohen et al. 2004).
Fig. 17. Involvement of sirtuins in ROS signaling.

Sirtuins are involved in a complex fashion in redox regulation. By the deacytlation of transcription factors by SIRT1, such as p53, NF-kB, and PGC-1α, they decrease the transcription of pro-oxidant and apoptotic pathways. While on the other hand deacytlation of FOXO-3a enhances its activity and increasing the transcription of the antioxidant enzymes such as MnSOD and catalase. These modulations allows a sensitive way of controlling the transcription of antioxidant genes, mitochondrial biogenesis, activity of oxidative damage-repairing enzymes, and chromatin structure via the reduced activity of histone acetyltransferases (HAT) maintaining uncondensed DNA. With permission from (Radak et al. 2013)
1.2.1.6  **Amphetamines as sources of ROS**

ROS have been also been associated with the neurotoxic mechanisms of several amphetamine derivatives including 3,4-methylenedioxymethamphetamine (MDMA, Ecstasy, Molly), its demethylated active metabolite 3,4-methylenedioxyamphetamine (MDA) and methamphetamine (METH) (Cadet et al. 1994b; Hirata et al. 1995; Colado et al. 1997; Cadet et al. 2001; Jeng et al. 2006; Jeng and Wells 2010). For example, studies with transgenic mice overexpressing Cu/Zn superoxide dismutase (Cu/Zn SOD) showed resistance to the lethal effects of both MDA and MDMA (Cadet et al. 1994b), as well as the acute and chronic effects of METH on dopaminergic systems (Cadet et al. 1994a; Hirata et al. 1995). In addition, nNOS knockout mice were protected from METH-initiated striatal damage, implicating involvement of reactive nitrogen species (RNS) in the neurotoxic mechanism (Itzhak et al. 1998; Imam et al. 2001). The free radical trapping agent α-phenyl-N-t-butylnitrone (PBN) prevented MDMA-initiated neurodegeneration of serotonergic neurons and the associated loss of 5-HT in rat cortex and hippocampus (Colado and Green 1995). As well, MDMA and METH increase lipid peroxidation in the brain (Sprague and Nichols 1995; Jayanthi et al. 1998; 1999). Although the molecular mechanism of neurodegeneration remains unclear, several studies have implicated oxidative reactions in the neurotoxic mechanism. METH and its analogs can increase the levels of neurotransmitters dopamine (DA) and serotonin at the synapse and more importantly in the cytosol, rendering them more sensitive to oxidative reactions. DA can: (1) be metabolized by MAO-B generating H₂O₂, (2) auto-oxidize generating O₂⁻.
Halliwell 2006); and, (3) be metabolized to quinone/semiquinone metabolites that redox cycle (Graham 1978; Hiramatsu et al. 1990). Secondly, METH has been shown to increase glutamate release and potentially lead to excitotoxicity via influx of Ca+ into the neurons (Battaglia et al. 2002; Yamamoto and Bankson 2005). Thirdly, METH, MDMA and MDA can be co-oxidized by PHSs, bioactivating these amphetamines to neurotoxic free radical intermediates (Jeng et al. 2006; Jeng and Wells 2010; Ramkissoon and Wells 2011b). A similar PHS-catalyzed bioactivation to a free radical intermediate can also occur with neurotransmitters like DA, as well as their precursors and metabolites (Goncalves et al. 2009; Ramkissoon and Wells 2011a). **Fig. 18** combines some of the ROS-mediated pathways recently implicated in neuronal degeneration.

MDMA and MDA are absorbed in the intestinal tract and reach their peak concentration in the plasma of humans about 2 hr after oral administration (de la Torre et al. 2000). METH is also easily absorbed, but peak concentrations are reached between 3-6 hours (Cook et al. 1992; Cook et al. 1993; Schepers et al. 2003; Huestis and Cone 2007). The half-life of MDMA in humans ranges between 9 and 12 hr (Cook et al. 1992; Cook et al. 1993; Shappell et al. 1996; Kalant 2001; Schepers et al. 2003). In rodents the half-life is only 70 min to 3 hr, which is substantially less than that in humans (Cho et al. 2001; de la Torre and Farre 2004). To mimic human exposure, mice typically are administered 4 doses of METH, each 5-20 mg/kg ip, with a 2-6-hr interval between each dose (Cho et al. 2001; de la Torre and Farre 2004). Plasma concentrations after these doses in mice are not typically measured but several
measures of neurotoxicity from this dosing regimen have been observed (Krasnova and Cadet 2009). After a single administration, the distribution of METH is relatively uniform throughout the brain of experimental animals, with concentrations in the range of 45-65 nmol/g in various brain regions (Jonsson and Nwanze 1982; Shiue et al. 1995; O'Neil et al. 2006).

The potential of METH and MDMA to lead to cognitive deficits have recently been reviewed. A large meta-analysis of studies of current and former recreational MDMA users (not administered at the time of testing) found that ecstasy users consistently performed worse than controls across a wide range of cognitive tests and psychopathological instruments, although the authors cautioned that this field of research is comprised of relatively weak studies (Rogers et al. 2009). The effects were most consistent in measures of memory, particularly verbal and working memory, but are also seen particularly strongly in self-rated measures of depression, memory, anxiety and impulsivity (Rogers et al. 2009). Most recently a study that garnered media attention showed that new MDMA users after a year of use showed only cognitive impairment in visual paired associates learning, with no impairment in other tests studied. These researchers conclude that the results point to a specific serotonergic dysfunction in hippocampal regions (Wagner et al. 2013).

In a review of METH and its potential to cause cognitive decline, the results were mixed (Dean et al. 2013). There was some support for a causal relationship between METH abuse and cognitive decline (Hoffman et al. 2006; Kim et al. 2006; Salo et al. 2007; Salo et al. 2009), while other findings suggested that there is no relationship
(Dawidzik et al. 2003; Chang et al. 2005; King et al. 2010; Simon et al. 2010). The preponderance of the data, however, supports the possibility that METH abuse causes cognitive decline, of unknown duration, in at least some users of the drug. When averaged across individuals, this decline is likely to be mild in early-to-middle adulthood. Confounding variables like age, length of abuse, other drug use, education, social standing and disease status are likely to contribute to the presence and/or severity of cognitive decline exhibited by a given individual (Dean et al. 2013). The animal data was more definitive with doses reflecting high-dose, “binge” use of METH in humans causing deficits in object recognition memory, odor recognition memory, spatial learning, sequential learning, path integration learning and working memory (reviewed in Dean et al. 2013).
METH enters dopaminergic neurons via DAT and passive diffusion. There it enters synaptic vesicles through VMAT-2 and causes the release of DA into the cytoplasm by reversing the DA transporter and changing of pH. Once there, DA auto-oxidizes to form toxic quinones with generation of ROS via quinone cycling. Subsequent formation of hydroxyl radicals through interactions of $\text{O}_2^-$ and hydrogen peroxide with transition metals leads to oxidative stress, mitochondrial dysfunction and peroxidative damage to pre-synaptic membranes. The involvement of endogenous DA in METH neurotoxicity is supported by findings that the tyrosine hydroxylase inhibitor, $\alpha$-methyl-p-tyrosine, which blocks DA synthesis, protects against METH toxicity. The role of DA is also supported by observations that pretreatment with the MAO inhibitor, clorgyline, and with the irreversible inhibitor of vesicular transport, reserpine, which increases cytoplasmic DA levels, both exacerbate METH-induced toxicity. Together, these events are thought to be partly responsible for the loss of DA terminals. DA release from the terminals is also involved because the dopamine transporter inhibitor, amphonelic acid, which blocks METH-induced DA release from DA terminals, also prevents damage to DA axons. The toxic effects of released DA may occur through activation of DA receptors, because DA receptor antagonists block degeneration of DA terminals. Interactions of DA with D1 receptors on the post-synaptic membrane cause activation of various transcription factors and subsequent upregulation of death cascades in post-synaptic neurons. These death cascades can be inhibited, in part, by the DA D1 antagonist, SCH23390. METH, DA, L-DOPA and DOPAC can be bioactivated into a free radical by PHSs. Modified with permission from (Krasnova and Cadet 2009)
2. **STUDIES**
2.2. Study 1: BRAIN GLUCOSE-6-PHOSPHATE DEHYDROGENASE PROTECTS AGAINST ENDOGENOUS OXIDATIVE DNA DAMAGE AND NEURODEGENERATION IN AGED MICE

Winnie Jeng¹, Margaret M. Loniewska², and Peter G. Wells

a. Preliminary reports of this research were presented at the 2000 annual meeting of the Society of Toxicology (U.S.A.) [Toxicological Sciences (Supplement: The Toxicologist), 54:178 (No. 838), 2000], and the 2005 annual meeting of the Society of Toxicology of Canada [STC Proceedings, abstract no. 26]. These studies were supported by a grant from the Canadian Institutes of Health Research (CIHR). WJ was supported by a doctoral award from the CIHR/Rx&D Health Research Foundation, and the Covance doctoral fellowship from the Society of Toxicology. Current address for WJ: Drug Safety Evaluation Projects, Sanofi-Aventis US Inc., Bridgewater, NJ 08807.

b. This research has been published in a peer reviewed journal and reprinted with permission from:


1. Winnie Jeng preformed the DNA oxidation analysis, immunohistochemical staining for 8-oxo-dG, and a large section of the text
2. Margaret Loniewska preformed all the G6PD activity analysis, and Purkinje Cells analysis, several H&E pictures, and several rounds of revisions for reviewers
3. Colin McKerlie provided some of the H&E pictures and pathological analysis
Abstract

Glucose-6-phosphate dehydrogenase (G6PD) protects the embryo from endogenous and xenobiotic-enhanced oxidative DNA damage and embryopathies. Here we show in aged mice that G6PD similarly protects against endogenous reactive oxygen species (ROS)-mediated neurodegeneration. In G6PD-normal (G6PD+/+), and heterozygous (G6PD+/def) and homozygous (G6PDdef/def) G6PD-deficient male and female mice at about 2 years of age, oxidative DNA damage in various brain regions was assessed by 8-oxo-2′-deoxyguanosine formation using high-performance liquid chromatography and immunohistochemistry. Morphological changes in brain sections...
were assessed by H&E staining. DNA oxidation was increased in G6PD^{def/def} mice in the cortex (p<0.02), hippocampus (p<0.01) and cerebellum (p<0.006) compared to G6PD^{+/+} mice, and was localized to distinct cell types. Histologically, in G6PD^{+/def} mice, enhanced regionally and cellularly specific neurodegenerative changes were observed in those brain regions exhibiting elevated DNA oxidation, with a 53% reduction in the Purkinje cell count. These results show G6PD is important in protecting against the neurodegenerative effects of endogenous ROS in aging, and suggest that common hereditary G6PD deficiencies may constitute a risk factor for some neurodegenerative diseases.

**Introduction**

Glucose-6-phosphate dehydrogenase (G6PD) is a cytoplasmic enzyme responsible for catalyzing the first and rate-limiting step in the hexose monophosphate pathway. The end product of this pathway, ribose-5-phosphate, is required for nucleic acid synthesis and, hence, essential for cell growth. During the conversion of glucose-6-phosphate to 6-phosphogluconolactone, G6PD generates nicotinamide adenine dinucleotide phosphate (NADPH) which, in addition to being essential for many reductive biosynthetic pathways including cholesterol and fatty acid synthesis, also is required for catalase stability and the regeneration of reduced glutathione (GSH) (Luzzatto et al. 2001). Since catalase and GSH peroxidase are essential for the detoxification of H_{2}O_{2}, G6PD provides critical protection against reactive oxygen species (ROS) and oxidative stress (Fig. 1.1).
Commonly recognized clinical manifestations of G6PD deficiency include neonatal jaundice, acute hemolytic anemia and life-threatening neonatal kernicterus (Luzzatto et al. 2001; Cappellini and Fiorelli 2008). The inheritance of G6PD shows a characteristic X-linked pattern, with deficiency more likely to affect males than females. G6PD deficiency is the most common human enzymopathy, affecting over 400 million people worldwide with a global prevalence of 4.9% (Nkhoma et al. 2009). Over 400 variant G6PD alleles exist in the human population, and to date 186 mutants have been reported (Luzzatto et al. 2001; Minucci et al. 2012).

Previously, using a mutant G6PD-deficient mouse model, G6PD was discovered to be a developmentally critical antioxidative enzyme that protects the embryo from the pathological effects of both endogenous and xenobiotic-enhanced oxidative stress and DNA damage (Nicol et al. 2000), raising the possibility that G6PD might be similarly important at other times of biochemical susceptibility, such as in aging.

G6PD is constitutively but not uniformly expressed in all cells, with basal activity varying up to about 10-fold among different organs and tissues (Kletzien et al. 1994; Corcoran et al. 1996), including in the mutant mouse strain used in the study herein (Nicol et al. 2000). G6PD activities in the fetus, and in all tissues including brain of young adults of the strain used here, are about 30% and 80% lower in G6PD+/def and G6PDdef/def mice respectively (Nicol et al. 2000). In various animal models and humans, the levels of mRNA and protein expression in the brain are constitutive in neurons and glial cells (Cammer and Zimmerman 1982; Philbert et al. 1991; Biagiotti et
al. 2001). With increased oxidative stress, G6PD expression and/or activity in vitro (Preville et al. 1999; Ho et al. 2000) and in vivo is increased (Palmer 1999; Russell et al. 1999). Increased neuronal G6PD expression has been observed in the hippocampus of Alzheimer’s disease patients compared to age-matched controls (Russell et al. 1999).

In this study, we used mutant G6PD-deficient mice to investigate the role of G6PD in protecting against endogenous ROS-mediated oxidative DNA damage and neurodegeneration in the brains of aged mice. This relatively G6PD-deficient strain was used because the knockout is embryolethal (Longo et al. 2002). The deficiency in this strain results from a decrease in total G6PD protein expression causing an overall reduction in activity (Pretsch et al. 1988). The results suggest that G6PD may be an essential protective enzyme preventing ROS-initiated neurodegenerative oxidative damage associated with aging. Since G6PD deficiencies are the most common human enzymopathy (Luzzatto et al. 2001), the mutant G6PD-deficient mouse may provide important insights into previously unappreciated risk factors for some neurodegenerative diseases.
Glucose-6-phosphate dehydrogenase (G6PD), a cytoplasmic enzyme, is responsible for the catalyzing the first step in the hexose monophosphate pathway. The end product of this pathway, ribose-5-phosphate, is required for nucleic acid synthesis, and hence is essential for cell growth. During the conversion of glucose-6-phosphate to 6-phosphogluconolactone, G6PD generates NADPH essential for many reductive biosynthetic pathways including cholesterol and fatty acid synthesis, catalase stability and the regeneration of glutathione (GSH). Catalase and GSH (via GSH peroxidase) are essential for the elimination of \( \text{H}_2\text{O}_2 \), which, if not detoxified, can cause oxidative stress and/or oxidatively damage cellular macromolecules (DNA, protein, lipid), thereby initiating diseases such as teratogenesis or neurodegeneration.

Abbreviations: NADP\(^+\), nicotinamide adenine dinucleotide phosphate; NADPH, nicotinamide adenine nucleotide phosphate, reduced; GSH, glutathione; GSSG, oxidized GSH; \( \text{O}_2^- \), superoxide; \( \text{H}_2\text{O}_2 \), hydrogen peroxide; CuZnSOD, copper/zinc superoxide dismutase.
**Results**

*Increased oxidatively damaged DNA in the brains of aged G6PD-deficient mice*

HPLC-EC was used to quantify the level of endogenous 8-oxo-2'-deoxyguanosine (8-oxo-dG), one form of oxidative DNA damage, in the cortex, hippocampus, striatum, mesencephalon/diencephalon, brainstem and cerebellum of aged G6PD\(^{+/+}\) and G6PD\(^{def/def}\) female mice. Aged G6PD\(^{def/def}\) mice had regionally dependent increases in endogenous brain DNA oxidation compared to their wild-type counterparts ([Fig. 1.2](#)). The G6PD\(^{def/def}\) mice showed a 23% increase in oxidative DNA damage in the cortex (p<0.02), a 45% increase in the hippocampus (p<0.01), and a 26% increase in the cerebellum (p<0.006) compared to their age-matched G6PD\(^{+/+}\) mice. No significant differences were observed in the striatum, mesencephalon/diencephalon and brainstem of the deficient mice compared to G6PD-normal controls.

Immunohistochemical staining confirmed the HPLC analysis of increased 8-oxo-dG formation in cortex, hippocampus and cerebellum in the G6PD\(^{+/def}\) mice compared to the G6PD\(^{+/+}\) mice ([Fig. 1.3](#)). Moreover, oxidative DNA damage was localized to specific regions and cell types. In the cerebellum, the Purkinje cells of G6PD\(^{+/def}\) mice had increased oxidized DNA ([Fig. 1.3b](#)), evidenced by the increased number and intensity of staining, compared to the G6PD\(^{+/+}\) mice ([Fig. 1.3a](#)). Increased localized DNA damage in G6PD\(^{+/def}\) mice was similarly observed in the cortex and corpus callosum ([Fig. 1.3c,d](#)) and the dentate gyrus of the hippocampus ([Fig. 1.3e,f](#)), but not in the brainstem ([Fig. 1.3g,h](#)).
Fig. 1.2. Increased levels of DNA oxidation in aged G6PD-deficient mice.

Brain regions from G6PD^{+/+} and G6PD^{def/def} mice were isolated and analyzed by high-performance liquid chromatography with electrochemical detection (HPLC-EC) for oxidatively damaged DNA, quantified by the formation of 8-oxo-2'-deoxyguanosine (8-oxo-dG). “p<0.02, ”p<0.01 and ”p<0.006 compared to aged-matched G6PD^{+/+} controls. The number of mice in each group is given in parentheses.
Fig. 1.3. Increased localized oxidatively damaged DNA in aged G6PD-deficient mice.

G6PD⁺/⁺ and G6PD⁺/def mice were sacrificed and perfused with 10% formalin. Brain sections were analyzed for oxidatively damaged DNA by immunohistochemistry using the N45.1 anti-8-oxo-dG antibody. Immunohistochemical staining is representative of a minimum of n=5/genotype. Comparisons of specific brain areas between paired G6PD⁺/⁺ and G6PD⁺/def mice are enclosed within a black border. **A,C,E,G**, G6PD⁺/⁺ mice; **B,D,F,H**, G6PD⁺/def mice. **A**, cerebellum from G6PD⁺/⁺ mice: black arrowheads indicate Purkinje cells. **B**, cerebellum from G6PD⁺/def mice: black arrowheads indicate atrophic Purkinje cells with increased oxidatively damaged DNA. **C,D**, cortex (C), corpus callosum (CC); black arrows indicate examples of oxidatively damaged DNA. **E,F**, dentate gyrus of the hippocampus; black arrow indicates increased immunoreactivity to oxidatively damaged DNA. **G,H**, brainstem.
Increased oxidative DNA damage in aged G6PD-deficient mice is associated with increased CNS cell death and morphological changes

The localization of enhanced endogenous ROS-mediated oxidative DNA damage in particular brain regions and cell types of aged G6PD-deficient mice was associated with a similar regional and cellular specificity in enhanced morphological changes in the brains of aged G6PD+/def mice assessed semi-quantitatively by H&E staining (Fig. 1.4). Some changes characteristic of aging were common to all animals, including reduced Nissl substance and increased neuronal nuclear diameter in the brainstem nuclei (Fig. 1.4q), scattered apoptotic or pyknotic neuronal bodies in the frontal cortex and hippocampus (Fig. 1.4g), scattered vacuolation in the white matter tracts of the neuropil (Fig. 1.4n), and the accumulation of cytoplasmic lipofuscin (Fig. 1.4r). However, G6PD+/def mice exhibited further degenerative changes compared to the age-matched G6PD+/+ controls. In the cerebellum of the deficient mice, there was increased pathological loss of Purkinje cells at the junction of the molecular and granule cell layers (Fig. 1.4b,d,e) compared to G6PD+/+ mice (Fig. 1.4a,c). The remaining neurons of the Purkinje layer are characterized by moderate anisokaryosis with a variety of bizarre nuclear shapes (Fig. 1.4e). G6PD-deficient mice also had more vacuolation in the deep cerebellar white matter (Fig. 1.4f).

In the aged G6PD+/+ mice, there were scattered examples of apoptotic or necrotic bodies, presumably dead or dying neurons, particularly in the outer layers of the parietal cortex. In many cases, the apoptotic remnants have an adjacent astrocyte (Fig. 1.4g). However, in the frontal cortex of the G6PD+/def mice, significant changes included an increase in the glia-to-neuron ratio, particularly in the mid- to outer regions,
and an increased number of chromatolytic or pyknotic neurons in the G6PD+/def mice compared to G6PD+/+ controls (Fig. 1.4h).

The densely packed spherical or oval neurons of the pyramidal layer were normal in the G6PD+/+ mice with very occasional shrunken neurons in the Ammon’s horn. In the polymorph layer, there was patchy condensation of neurons with cell shrinkage and pyknosis. In addition, there were numerous condensed and pyknotic centrally chromatolytic neurons at the lateral border of the hippocampus, as well as in the pyramidal layer (Fig. 1.4j). However, in the G6PD+/def mice, there were increased numbers of chromatolytic neurons within deeper layers of the pyramidal layer (Fig. 1.4k). The pyramidal layer and granule cell layer of the Ammon’s horn of the hippocampus and the dentate gyrus, respectively, are characterized by greater vacuolation and spongiosis in the G6PD-deficient mice (Fig. 1.4m) compared to the G6PD+/+ controls (Fig. 1.4l). In addition, numerous condensed and pyknotic centrally chromatolytic neurons at the lateral border of the hippocampus were observed (Fig. 1.4k), along with scattered examples of large vacuolated mononuclear cells adjacent to blood vessels.

The pathological findings in the brainstem were increased in the aged G6PD+/def mice compared to the G6PD+/+ controls. Although there was increased neuronal nuclear diameter in the brainstem nuclei in the G6PD+/+ mice, in the G6PD+/def mice, the brainstem nuclei had moderate to marked regional accumulation of lipofuscin in neuronal cell bodies, particularly in the hypoglossal nuclei (Fig. 1.4p). There is also more prominent vacuolation of the white matter tracts in the G6PD+/def mice (Fig. 1.4o)
than the G6PD+/+ mice (Fig. 1.4n). Thickening and neoplastic proliferation of cells in the brainstem was seen in one G6PD+/def case with mitotic figures scattered throughout the pleomorphic cell population (Fig. 1.4s). The proliferative tissue was characterized by myxomatous tissue composed of stromal fibroblastic cells with a variable degree of differentiation. There were also examples of marked dysplasia/anaplasia in the cell population with up to 3x anisokaryosis. The G6PD+/def mice also showed more examples of foamy and vacuolated mononuclear cells compared to G6PD+/+ mice (Fig. 1.4i).

Increased lipofuscin accumulation was seen in the frontal cortex, hippocampus and cerebellum in the G6PD+/def mice.

The neuronal population of the striatum and hypothalamus was interpreted to be relatively normal, with no apparent difference between G6PD-normal and G6PD-deficient mice. Histological findings in the cerebellum, cortex, hippocampus and brainstem of homozygous G6PD-deficient females and hemizygous-deficient males were similar to those observed in the heterozygous G6PD-deficient females.

The Purkinje cell total count in the cerebellum for 10 fields was 55.0 ± 3.6 in G6PD+/+ mice, and 25.7 ± 7.6 in G6PD+/def mice, indicating a 53% reduction in G6PD+/def mice compared to G6PD+/+ controls (p< 0.001) (Fig. 1.5). No visible ataxia or other motor deficits were apparent.
Fig. 1.4. Increased CNS cell death and morphological changes in aged G6PD-deficient mice.

Brain sections from G6PD\(^{+/+}\) and G6PD\(^{+/def}\) mice were stained with H&E and analyzed for morphological changes, with panels representative of a minimum of \(n=5\)/genotype. The brain area is show by row. Comparisons of specific brain areas between paired G6PD\(^{+/+}\) and G6PD\(^{+/def}\) mice are enclosed within a black border. \(A,C,G,J,L,N\): G6PD\(^{+/+}\) mice. \(B,D,E,F,H,I,K,M,O,P,S\): G6PD\(^{+/def}\) mice. \(Q,R\): Common to both G6PD\(^{+/+}\) and G6PD\(^{+/def}\) mice. \(A,C\), Purkinje cells (black arrow heads), and granular (G) and molecular (M) layers of the cerebellum. \(B,D\), loss of Purkinje cells. \(E\), moderate anisokaryosis and bizarre nuclear Purkinje cells. \(F\), vacuolation in the deep cerebellar white matter. \(G\), chromatolytic or pyknotic neurons in the frontal cortex, which are increased in \(H\), where they are often associated with astrocytes. \(I\), single, foamy and vacuolated mononuclear cell in lumen of dorsal third ventricle. \(J\), lateral boundary of hippocampal formation (black arrow head); \(K\), condensed neurons in both pyramidal layer (P) of hippocampus and granular layer of dentate gyrus (DG). \(L\), vacuolation and spongiosis (black arrow head) of hippocampus, which is increased in \(M\). \(N\), white matter vacuolation of brainstem, which is increased in \(O\). \(P\), Lipofuscin in neuronal cell bodies of the brainstem. \(Q,R\), pathological changes common to both G6PD\(^{+/+}\) and G6PD\(^{+/def}\) mice: \(Q\), increased nuclear diameter of brainstem nuclei; \(R\), silver staining of cytoplasmic lipofuscin. \(S\), Thickening and neoplastic proliferation of the brainstem meninges. All sections are x 400 magnification, unless otherwise noted.
Fig. 1.5. Quantitative analysis of Purkinje cells of the cerebellum in aged G6PD-normal and heterozygous G6PD-deficient mice.

Purkinje cells were counted in 10 distinct fields at 40X to give a total of cells for each sample. There was a 54% decrease in cell number in the G6PD-deficient mice compared to G6PD-normal controls (asterisk indicates p<0.0009), consistent with the semi-quantitative histological assessment.
Brain G6PD Activity

Whole brain G6PD activity was decreased respectively to 40.7% and 16.1% of normal in heterozygous and homozygous G6PD-deficient mice compared to G6PD-normal mice (Fig. 1.6). Regional differences in G6PD activity were evident in G6PD-normal mice, with activity varying 3.0-fold increasing from hippocampus, cortex, striatum, olfactory bulb, substantia nigra, brain stem and cerebellum (Fig. 1.7). A similar 2.8-fold regional variation was observed in heterozygous G6PD-deficient mice, increasing from hippocampus, striatum, cortex, substantia nigra, olfactory bulb, cerebellum and brain stem. A greater 4.3-fold variation was observed in homozygous G6PD deficient-mice, increasing from hippocampus, striatum, substantia nigra, cortex, brain stem, olfactory bulb and cerebellum.

In comparing regional brain DNA oxidation and G6PD activity in the same mice, in both G6PD-normal and homozygous G6PD-deficient mice, brain regions with the lowest (hippocampus) and highest (cerebellum) G6PD activities showed respectively higher and lower levels of DNA oxidation, suggesting that extremes of G6PD activity may be an important determinant of regional ROS-mediated macromolecular damage (Fig. 1.8). This association was not evident in tissues with intermediary G6PD activities.

In summary, the striatum of G6PD-deficient mice exhibited no increase in oxidative DNA damage or histological changes. Similarly, no significant pathological changes were reported in the hypothalamus as well as in the areas associated with the mesencephalon and diencephalon. In contrast, the cortex, hippocampus and
The cerebellum exhibited both enhanced DNA oxidation and degenerative histological changes in G6PD-deficient mice. The brainstem, which showed histological changes, exhibited a small but non-significant increase in DNA oxidation determined by HPLC, and no increase by immunohistochemistry.

Our studies used both males and females together as we did not find any differences between sexes in our observations.

**Fig. 1.6.** G6PD activity in whole brains of aged G6PD-normal and G6PD-deficient mice. 

α indicates a decrease from G6PD+/+ mice (p<0.001), and δ indicates a decrease from G6PD+/def (p<0.001). N = 3, 6, and 4 respectively for G6PD+/+, G6PD+/def and G6PD+/def/def groups.
Fig. 1.7. G6PD activity in specific brain regions of aged G6PD-normal and G6PD-deficient mice.

Note the scale differences in the X axes. α indicates a difference between G6PD+/+ and G6PD+/def groups, or G6PD+/def and G6PDdef/def groups, in G6PD activity for the same brain region (p<0.001). N = 3, 6, and 4 for G6PD+/+, G6PD+/def, and G6PDdef/def.
Fig. 1.8. Relation of regional DNA oxidation and G6PD activity in G6PD+/+ and G6PD^def/def mice.

Oxidatively damaged DNA was assessed by 8-oxo-dG formation using HPLC-EC. G6PD activity was measured by a kinetic assay measuring the formation of NADPH. (DNA oxidation: G6PD+/+, n=4; G6PD^def/def, n=6. G6PD activity: G6PD+/+, n=3; G6PD^def/def, n=4). * indicates an increase in DNA oxidation compared to G6PD-normal mice (p<0.05). α indicates a decrease in activity from G6PD+/+ mice for each of the brain regions matched to the same brain region in the G6PD^def/def mice (p<0.001).
Discussion

The increased endogenous oxidative DNA damage in regions of the brain associated with enhanced neuropathological damage in aged G6PD-deficient mice suggests that G6PD is important in protecting the brain from ROS-mediated neurodegeneration associated with aging. Remarkably, oxidative damage and neurodegeneration were enhanced in aged heterozygous as well as homozygous G6PD-deficient mice. Given that G6PD activity in this strain is maximally decreased by only about 30% in heterozygous G6PD-deficient mice, depending upon the tissue (Nicol et al. 2000), the enhanced susceptibility observed with even a heterozygous deficiency indicates that even the modest decrease in G6PD activity associated with the loss of one copy of the gene carries an increased lifetime risk of neurodegeneration. In comparison with other organs, the CNS may for a number of biochemical, physiological and anatomical reasons are especially vulnerable to ROS-mediated injury. Endogenous ROS may be generated from numerous sources, including: (1) the relatively high level of CNS oxidative metabolic activity (mitochondrial respiration); (2) high concentrations of non-heme iron and ascorbic acid; (3) glutamate-mediated excitotoxicity and a disruption in Ca^{2+} homeostasis; (4) Ah receptor-mediated oxidative stress; (5) auto-oxidation of neurotransmitters; (6) redox cycling of endobiotics and xenobiotics with catechol structures; (7) as well as various neuronal enzyme-catalyzed reactions, including ROS formation via the metabolism of endobiotics and xenobiotics by prostaglandin H synthase in the endoplasmic reticulum (ER) and nuclear membrane, and cytochromes P450 in the ER (Coyle and Puttfarcken
1993; Hassoun et al. 1998; Jeng et al. 2006; Goncalves et al. 2009; Jeng and Wells 2010). The lifetime assault of relatively high levels of oxidative stress and accumulation of macromolecular damage, coupled with the relatively low CNS levels of antioxidative enzymes, such as catalase and glutathione peroxidase, compared to the liver or kidney (Sohal et al. 1990), may render the neural anatomical network vulnerable to disruption. Given the non-replicating nature of neuronal cells, such ROS-mediated effects cause permanent and cumulative damage to the CNS.

The neurodegenerative mechanism in G6PD-deficient mice could involve increased ROS-dependent signal transduction and/or ROS-initiated oxidative damage to cellular macromolecules, including DNA, RNA, proteins and lipids. We focused on oxidative DNA damage as a potential macromolecular target involved in neurodegeneration since that molecular lesion appears to contribute to the embryopathic mechanism of some ROS-initiating teratogens (Wells et al. 2009), including neurodevelopmental deficits (Wong et al. 2008), as well contributing to neurodegeneration initiated by amphetamines (Jeng et al. 2006; Jeng and Wells 2010), and by some endogenous neurotransmitters and their precursors and metabolites (Goncalves et al. 2009). DNA oxidation also can be considered as a biomarker for oxidative stress and the concomitant involvement of other potential ROS-dependent mechanisms of toxicity. Analogous to the theory of carcinogenesis (Klaunig and Kamendulis 2007), ROS might be involved via macromolecular damage in the initiation of neurodegeneration, and/or via signal transduction in the “promotion/progression” of a degenerative process initiated by other mechanisms.
In the aged G6PD-deficient mice, there was a remarkable association of increased oxidatively damaged DNA (8-oxo-dG, a marker for increased HO•), determined by HPLC and/or immunohistochemistry, and neurodegenerative changes in the frontal cortex, hippocampus and cerebellum, as well as a converse absence of oxidative DNA damage and neurodegeneration in the striatum, consistent with a causative role for oxidative stress, and possibly oxidative DNA damage in particular, in the mechanism of neurodegeneration. The one exception to this pattern was the brainstem, wherein increased DNA oxidation was not detected by HPLC or immunohistochemistry, but increased pathological changes were evident in G6PD-deficient mice. The enhanced neurodegenerative effects in this particular brain region may result from a lower level of ROS-dependent signal transduction rather than macromolecular damage, and/or may be co-dependent upon regionally specific expression of other antioxidative enzymes as discussed below. In addition, we observed a similar decrease on G6PD activity in all brain areas measured but DNA oxidation and morphological changes were only seen in selected brain areas. Pathways regulating the endogenous formation of ROS, levels of antioxidants and other antioxidative enzymes, and levels of enzymes involved in DNA repair all likely differ not only among tissues, but among cell types. If these assumptions are correct, one would expect that the neurodegenerative impact of a G6PD-deficiency would vary among tissues and cell types, as observed in our study. There were no apparent gender differences in the semi-quantitative histological observations, although further
detailed morphometrics are needed to address this topic and also to definitively quantify the pathological findings observed in different brain sections.

Hereditary G6PD deficiencies, which are the most common human enzymopathies, may accordingly constitute an important risk factor for some neurodegenerative diseases. In addition to the neuroprotective relevance of G6PD itself in the adult brain, the expression of G6PD has been shown to be coordinately modulated with that of other antioxidative enzymes, including glutathione peroxidase and glutathione reductase, in the developing and adult rat brain (Ninfali et al. 1996; Ninfali et al. 1998). The mechanism for this coordinated expression is unknown; however, it is thought that nerve growth factor (NGF) and insulin-like growth factor (IGF), which respond to changes in oxidant levels, may act as transcriptional regulators of these genes (Harris 1992; Yu 1994). Hence, in addition to G6PD deficiencies, other antioxidative defense mechanisms may be compromised, which would increase susceptibility of the brain to ROS-mediated oxidative stress and subsequent neuronal damage.

Our studies showing increased endogenous ROS-mediated oxidative damage and neuronal cell death in specific brain regions and cell types particularly in G6PD-deficient mice are associated with areas of high G6PD expression. Histochemical and immunohistochemical analyses revealed that the highest expression of cerebellar G6PD activity and protein was found in the Purkinje cells (Biagiotti et al. 2001; Biagiotti et al. 2003) and later was found to be co-localized with NADPH-dependent enzymes including NADPH-cytochrome P450 reductase and glutathione reductase (Ferri et al.
We found that the absence of even one copy of the G6PD gene resulted in increased DNA oxidation in Purkinje cells, with a pathological loss of these cells and atrophy of the survivors. This suggests that G6PD expression is required for Purkinje cell viability and possibly for normal functioning of these cells. Previously, we reported the brain activities of G6PD in this strain were approximately 50, 35 and 10 U/g protein in wild-type, heterozygous and homozygous-deficient mice, respectively (Nicol et al. 2000), and the current results suggest that activity higher than 35 U/g protein is required at least in mice to protect against ROS-initiated neurodegeneration. G6PD activity may be particularly important in the hippocampus, where increased neuronal G6PD and sulfhydryl levels were found in patients with Alzheimer’s disease, presumably in compensation for increased oxidative stress in that region (Russell et al. 1999). The levels of CuZnSOD mRNA and protein are particularly high in hippocampal pyramidal neurons and granular cells (Ceballos-Picot et al. 1992), which in the absence of adequate G6PD-mediated detoxification may result in $H_2O_2$ overproduction and peroxidative damage within these cells. In addition to the cytoprotective role of G6PD against ROS-mediated oxidative damage, G6PD may be required for normal cell growth by providing NADPH for redox regulation (Pandolfi et al. 1995; Ninfali et al. 1996; Tian et al. 1998).

The study herein is the first to show enhanced endogenous DNA oxidation in specific brain regions as a consequence of decreased G6PD activity in aged mice, and the associated regionally andcellularly specific neurodegenerative changes. The carcinogenic implications of G6PD deficiency in brain have been investigated in
another study employing young mice that contained the X-ray-induced low efficiency allele of G6PD (Felix et al. 2002). The brains of these G6PD-deficient males exhibited a decrease in the ratio of reduced glutathione to oxidized glutathione, an accumulation of promutagenic etheno DNA adducts secondary to lipid peroxidation, and an increased somatic mutation rate suggesting an enhanced risk for brain cancer. If such molecular events occur in the strain of aged G6PD-deficient mice used herein, they also could contribute to neurodegeneration. However, no enhanced risk for brain cancer has been reported, and we found no evidence for enhanced carcinogenesis in any tissue of the aged G6PD-deficient mice herein (Loniewska and Wells, unpublished). It may be that the promutagenic etheno DNA adducts in young G6PD-deficient mice observed by Felix and coworkers have less functional consequences than the 8-oxo-dG lesions measured herein in aged G6PD-deficient mice. The 8-oxo-dG lesion has also been implicated in the molecular mechanism of embryopathies and neurodevelopmental deficits caused by ROS-initiating teratogens, likely via non-mutagenic mechanisms involving alterations in gene transcription (Wells et al. 2009). A similar mechanism has been implicated in the potential neurodegenerative effects of endogenous (Goncalves et al. 2009) and amphetamine-enhanced (Jeng et al. 2006; Jeng and Wells 2010) oxidative stress. Further studies of learning and memory, and sensorimotor coordination, will be necessary to determine the functional consequences of the molecular lesions and neurodegenerative cellular changes observed herein.

When G6PD activity was selectively overexpressed in the dopaminergic nigrostriatal system of mice, it was protective against the toxic effects of the wells
studied neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Mejias et al. 2006). As with our results, these findings provided evidence that G6PD function has protective potential in the brain. Further gene expression studies in these G6PD-overexpressing mice displayed changes mainly in the expression of proteins related to antioxidant defense, detoxification and synaptic function (Romero-Ruiz et al. 2010).

G6PD deficiency impairs the ability to generate NADPH, which is required for the regeneration of GSH by NADPH-dependent glutathione reductase (Fig. 1). The loss of GSH in turn prevents the detoxification of ROS by GSH peroxidases, thus leading to increased ROS levels. However, we cannot rule out a contributing role for catalase in protecting against oxidative stress in the brain. Similar to glutathione reductase, catalase also requires NADPH for normal function, although in this case NADPH binds to catalase and prevents the formation of inactive catalase (compound II), as well as mediating the rapid reduction of catalase compound II back to its active form (Kirkman et al. 1987; Kirkman et al. 1999). Impaired catalase activity was found to contribute largely to H₂O₂-mediated enhancement of oxidant sensitivity of G6PD-deficient erythrocytes. However, it should be noted that some catalase activity did remain in the G6PD-deficient cells since catalase activity did not drop below 50% of its initial level (Scott et al. 1993). Furthermore, the amount of NADPH required for the prevention of catalase inactivation is very low (below 0.1 µM) in vivo, and the reduction of catalase compound II to the active form (compound I) is known to occur in the absence of NADPH, albeit at much slower rates (Kirkman et al. 1987). Therefore, a contribution from catalase inactivation may be more likely with xenobiotic-enhanced
oxidative stress than with endogenous oxidative stress in the face of a G6PD deficiency.

Most individuals with a G6PD deficiency are normally asymptomatic, but can exhibit a clinical syndrome in response to an enhanced oxidative insult or exogenous factors (e.g. xenobiotics, ingestion of fava beans). Although some deficiencies belonging to the Class I of G6PD variants, which exhibit the most severe deficiency, are associated with chronic non-spherocytic hemolytic anemia (Luzzatto et al. 2001), there is no link to date between a specific genetic G6PD variant and a single clinical syndrome. Some of the highest prevalence rates reside in tropical Africa, the Middle East, tropical and subtropical Asia, areas of the Mediterranean and Papua New Guinea, with the incidence of G6PD deficiency approaching 60% in some populations (Sodeinde 1992). In such populations, based on the results of our mouse studies, neurodegeneration associated with aging should be considered as a potential outcome in molecular epidemiological studies of G6PD deficiencies, particularly among more severely deficient variants.

**Conclusion**

In summary, our studies provide the first direct evidence that G6PD deficiency can result in the accumulation of ROS-mediated oxidative damage to DNA in specific areas of the brain with aging. The increased levels of oxidative DNA damage in different brain regions and cell types were associated with pathological changes, including the loss of Purkinje cells, increased neuronal nuclear diameter, increased vacuolation and increased prevalence of chromatolytic neurons. These results suggest
a novel role for G6PD in neuroprotection in addition to its known developmental (Nicol
et al. 2000) and hematological (Luzzatto et al. 2001) importance. In light of the
susceptibility of even heterozygous deficient mice observed herein, and the high
prevalence of human G6PD deficiencies, this factor may also prove to be an important
risk determinant for neurodegenerative diseases.

Materials and Methods

Chemicals

8-Hydroxy-2’-deoxyguanosine was obtained from Cayman Chemical Co. Nuclease P1 and Escherichia coli alkaline phosphatase were obtained from Sigma-Aldrich, chloroform:isoamyl alcohol:phenol (CIP, 24:1:25) and DdeI restriction enzyme from Life Technologies, Inc. and proteinase K from Roche Diagnostics. PCR primers were purchased from ACGT Corporation. Taq polymerase and dNTPs were purchased from Perkin-Elmer. All other reagents used were of analytical or HPLC grade.

Animals

Female and male mutant G6PD-deficient mice (Pretsch et al. 1988) (Medical Research Council, UK), 20-30 g, 1.5-2.0 years of age, were housed four per plastic cage with ground corn cob bedding (Beta Chip, Northeastern Products Corp.). The basis of the mutation is an ethylnitrosourea-induced A-T transversion mutation in the exon 1 of the G6PD gene, which is believed to cause a splicing error during post-translational processing. Animals were kept in a temperature-controlled room with a 12 h light/dark cycle automatically maintained. Food (Laboratory Rodent Chow 5001;
Ralston Purina) and tap water were provided ad libitum. All animals were monitored regularly by a veterinarian, and found to be in overtly normal health, with no premature deaths in the colony. All G6PD-deficient genotypes were confirmed as described below. These studies were approved by the University of Toronto Animal Care Committee in accordance with the standards of the Canadian Council on Animal Care.

**Genotyping**

Mouse tail snips were obtained from the mice and DNA was subsequently isolated from each sample using a standard DNA extraction kit (QIAGEN, Inc.). Purified DNA (100 to 300 ng) was added to a final concentration of 1X PCR buffer, 0.2 mM dNTPs, 1.5 mM MgCl₂, 0.5 μM forward and reverse primers, ddH₂O and 0.04 units of Taq polymerase. The G6PD mouse PCR primers (sense: 5’-GGAAACTGGCTGTGCGCTAC-3’; antisense: 5’-TCAGCTCCGGCTCTTTCTTG-3’) were made between exon 1 and intron 1, which are designed to generate a 269 bp product targeted around the reported mutation site (Sanders et al. 1997). The final reaction volume was 30 μL. Samples were placed in a thermal cycler (Eppendorf Mastercycler® Gradient, Eppendorf Scientific, Inc.) and run under the following conditions: 94°C, 2 min; 94°C, 20 sec; 58°C, 20 sec; 72°C, 30 sec for 35 cycles and a final extension step of 72°C, 5 min and kept at 4°C until ready for digestion. PCR products were digested using Ddel restriction enzyme at 37°C for at least 1 hr. Digested PCR samples were combined with 6X gel loading buffer (0.25% bromophenol blue; 0.25% xylene cyanol and 15% Ficoll type 400 in ddH₂O) and loaded onto a 3%
agarose gel containing ethidium bromide. The agarose gel was viewed under UV light and photographed.

Upon Ddel digestion, fragments of 214 bp and 55 bp were produced for G6PD^{+/+} or +/y mice, no change in the molecular size (269 bp) in G6PD^{def/def} or def/y mice, and all three fragments in G6PD^{+/def} mice (Nicol et al. 2000).

**Animal treatment & dissection**

The mice were anesthetized by isofluorane and killed by cervical dislocation. For DNA oxidation to be quantified by HPLC, the brains of untreated 1.5 to 2 year old female G6PD-normal and -deficient mice were rinsed with ice-cold 1.15% (w/v) KCl solution and subsequently microdissected on ice to obtain the cortex, hippocampus, striatum, mesencephalon/diencephalons, brainstem and cerebellum, which were immediately snap frozen in liquid nitrogen and stored at –80°C until the day of the assay.

For H&E staining and immunohistochemistry, the brains of the female and male G6PD-deficient mice were perfused with PBS, followed by 10% neutral buffered formalin, isolated and further fixed overnight in formalin before being embedded in paraffin. Paraffin-embedded brains were sectioned into 5 μm-thick sections and mounted onto glass microscope slides for further H&E staining or immunohistochemical analysis. H&E stained slides were assessed semi-quantitatively for normal signs of aging and pathological indices of neurodegeneration by a veterinary pathologist, Dr. Colin McKerlie (Toronto Centre for Phenogenomics and the Samuel Lunenfeld Research Institute, Toronto), who was blinded to the genotype of the animals. For
quantitative assessment, Purkinje cells of the cerebellum were counted on the H&E-stained slides from 4 G6PD+/+ mice and 3 G6PD+/def mice. For each slide, the mean number of Purkinje cells was determined from 10 randomly selected fields.

**DNA extraction and digestion**

Brain regions dissected from female G6PD-normal or deficient mice were homogenized in 500 µl DNA digestion buffer (100 mM Tris-HCl, pH 8.0, 5 mM EDTA, pH 8.0, 0.2% (w/v) SDS, 200 mM NaCl) and allowed to digest overnight with proteinase K (50 µg mL-1) at 55°C. DNA was extracted as described elsewhere (Liu and Wells 1995). Isolated DNA (RNA free) was then digested with nuclease P1 (67 µg mL-1) at 37°C for 30 min, followed by a 60 min incubation with Escherichia coli alkaline phosphatase (0.37 units mL-1) at 37°C. The mixture was syringe tip-filtered (0.22 µm) and analyzed using a high-performance liquid chromatography (HPLC) with an electrochemical (EC) detector.

**Detection of 8-oxo-2’-deoxyguanosine (8-oxo-dG)**

Oxidation of 2’-dG to 8-oxo-dG was quantified using an isocratic HPLC (Series 200, PerkinElmer Instruments LLC) equipped with a 5-µm Exsil 80A-ODS C-18 column (5 cm x 4.6 mm, Jones Chromatography, Ltd.), an electrochemical detector (Coulochem® II), a guard cell (model 5020), an analytical cell (model 5010) (Coulochem, ESA Inc.) and an integrator (PerkinElmer NCI 900 Interface). Samples were filtered (0.22 µm), injected into the HPLC-EC system and eluted using a mobile phase consisting of 50 mM KH₂PO₄ buffer (pH 5.5)-methanol (95:5, v/v) at a flow rate
of 0.8 mL min^{-1} with a detector oxidation potential of +0.4 V (Winn and Wells 1997). Chromatographs were analyzed using a commercial chromatography software program (TotalChrom version 6.2.0, PerkinElmer Instruments LLC).

**Immunohistochemistry**

Paraffin-embedded mouse brain sections were deparaffinized in xylene, hydrated in decreasing dilutions of ethanol and rinsed in ddH₂O. Sections were treated with RNase (100 µg mL^{-1}) for 1 hr at 37°C followed by blocking of endogenous mouse IgG with MOM Mouse IgG blocking reagent (Vector Laboratories) for 3 hr. Tissue sections were then incubated with the N45.1 anti-8-oxo-dG antibody (Wako Chemicals, USA), diluted 1:20 overnight at 4°C, followed by a 1-hr incubation with MOM biotinylated horse anti-mouse IgG secondary antibody. To quench endogenous peroxidase, slides were incubated in 3% (v/v) H₂O₂ in methanol for 30 min at room temperature. Sections were incubated with Vectastain Elite ABC reagent (Vector Laboratories) for 10 min and subsequently detected by DAB reaction. Slides were counterstained, dehydrated with xylene and mounted.

**Glucose-6-phosphate dehydrogenase activity in individual brain areas**

Mice were sacrificed by decapitation, and the brain was removed and rinsed in ice cold 1.15 % (w/v) KCl. The brain areas were dissected in the order of olfactory bulb, hippocampus, cortex, striatum, substantia nigra, cerebellum and brain stem, and snap frozen in liquid nitrogen. The samples were stored in a -80°C freezer until analysis. Each sample was homogenized with a hand homogenizer in 500 µL of
50 mM Tris-HCl pH 7.4, and the homogenate was sonicated with a hand sonicator (Fisher Scientific 60 Sonic Dismembrator) for 10 sec. The homogenates were centrifuged for 30 min at 15,000 x g in a refrigerated microcentrifuge. The pellet was discarded and the protein content was determined in the supernatant (Bradford 1976), as was G6PD activity (Glock and McLean 1953) using a modified protocol (Ninfali et al. 1997). Reaction rates for G6PD were determined by UV spectrophotometry (Beckman model DU 640) at 340 nm at 15 sec intervals over 5 min. The final activity of G6PD was determined using the following equation: Net O.D. x 40/6200 = µmol/min/mL of clear homogenate (IU mL⁻¹).

*Statistical Analysis*

Statistical significance of differences between paired data was determined by the two-tailed Student’s t-test, while multiple comparisons among groups were analyzed by one-way analysis of variance (ANOVA) followed by a Bonferroni post test using GraphPad InStat® 3.05 (GraphPad Software, Inc., GraphPad Software, San Rafael, CA). The level of significance was determined to be at p < 0.05.
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The authors declare no actual or potential conflicts of interest.
2.2 Study 2: GLUCOSE-6-PHOSPHATE DEHYDROGENASE DEFICIENCY IN AGING: DNA DAMAGE, ELECTROPHYSIOLOGICAL AND BEHAVIOURAL CONSEQUENCES AND SURVIVAL

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1. Margaret Loniewska preformed all the G6PD activity analysis, behavioural tests, comet assay, and manuscript preparation.
2. Zheng Ping Jia performed and analyzed the hippocampal function sections.
Abstract

Glucose-6-phosphate dehydrogenase (G6PD) regenerates the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH), which is important for reactive oxygen species (ROS) detoxification, and may be neuroprotective in aging. Brains from aging (about 1.5 years) mutant G6PD-deficient mice showed increased in DNA damage in the comet assay. Activity of G6PD in cerebellar tissue was accurately reflected by that in red blood cells, which did not change significantly with age. Motor function in aging G6PD-deficient mice declined at a faster rate as observed in the ledge balance test, but not in the accelerating rotarod or hindlimb clasp tests. G6PD deficiency did not affect cognitive decline in the taste aversion learning and passive avoidance learning tests. Functional properties of synaptic transmission and plasticity were determined by electrophysiological recordings in the hippocampus. Aging G6PD-deficient mice exhibited significant alterations in post- and presynaptic function, determined respectively by evoked field excitatory postsynaptic potentials (fEPSPs) (increased stimulus intensity-response curve and maximal response), and increased paired-pulse facilitation (PPF), a measure of short-term synaptic plasticity. Long-term potentiation (LTP) was not affected. Conversely, deficient mice with all G6PD alleles mutated exhibited an increase in survival compared to heterozygous G6PD-deficient mice and wild-type G6PD-normal mice. The substantial electrophysiological abnormalities and selective functional consequences in G6PD-deficient mice combined with increased DNA damage indicate an important protective role for G6PD in ROS-mediated neurodegeneration in aging. However, the conversely protective effects of
G6PD deficiency suggest a complex impact on aging.

**Key Words:** Aging, glucose-6-phosphate dehydrogenase (G6PD); motor coordination; cognition; electrophysiology; reactive oxygen species (ROS); DNA damage; survival

**Introduction**

Glucose-6-phosphate dehydrogenase (G6PD) is the first and rate-limiting enzyme in the hexose monophosphate pathway, important for its role in the regeneration of the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) and the production of ribose-5-phosphate (Buehler 1993; Luzzatto et al. 2001). During cellular oxidative stress, NADPH is critical for maintaining glutathione in its reduced form (GSH), which is essential for the detoxification of reactive free radicals and lipid hydroperoxides (Halliwell and Gutteridge 1999). Another important role of NADPH is the maintenance of the catalytic activity of catalase, which is required for the detoxification of hydrogen peroxide (Kirkman et al. 1987; Kirkman et al. 1999).

It is generally believed that G6PD deficiencies constitute a problem only for mature red blood cells (Cappellini and Fiorelli 2008); however, G6PD also plays a role in protecting embryos from oxidative stress and chemical teratogenesis (Nicol et al. 2000). In hepatic tissue, G6PD-deficient mice exhibit enhanced sensitivity to menadione-induced oxidative stress with increases in DNA oxidation, lipid peroxide and protein carbonyl levels (Nichols and Kirby 2008). In another G6PD-deficient mouse model engineered to express a transgenic shuttle vector for measuring mutagenesis in vivo, the brains of deficient mice exhibited an increase in oxidative mutagenesis with...
accumulation of promutagenic etheno DNA adducts and increased somatic mutation rates suggesting an increased risk of cancer (Felix et al. 2002). Recently, we observed that the brains of untreated aged G6PD-deficient mice have increased levels of oxidatively damaged DNA, measured by 8-oxo-2'-deoxyguanine (8-oxo-dG), and histological examination revealed enhanced degenerative changes (Jeng et al. 2013). The changes associated with G6PD deficiency included qualitative or quantitative cell loss in the frontal cortex, cerebellum and hippocampus, and increased DNA oxidation in the same regions.

The risk of ROS-mediated damage to brain cells in G6PD-deficient mice may have important clinical implications, since this is the most common human enzymopathy, affecting over 400 million people and up to 60% of some populations (Luzzatto et al. 2001). The high incidence of G6PD deficiencies is believed to be reflect an evolutionary adaptation to the widespread prevalence of malaria, as G6PD-deficient host red blood cells are inhospitable to the parasite Plasmodium falciparum (Cappellini and Fiorelli 2008).

In light of our previous observations of oxidatively damaged DNA and related cellular degeneration in the brains of aging G6PD-deficient mice (Jeng et al. 2013), we sought to confirm the quantitative nature of cerebellar Purkinje cell (PC) loss using a specific cellular marker (calbindin D-28K), and to determine if DNA oxidation was associated with a functional measure of DNA damage in the form of single-strand breaks, and with electrophysiological and behavioural consequences. Motor coordination was of particular interest in light of deficits observed in other mouse
models of PC loss (Vogel et al. 2007; Dougherty et al. 2013). Our results show that G6PD is neuroprotective in aging at both the macromolecular and functional levels, although the full extent of its neuroprotective importance remains to be elucidated. However, the biological role of G6PD in aging is complex, as lifespan was enhanced in G6PD-deficient mice with the lowest activity, suggesting alternative hypotheses for the high incidence of this deficiency in humans.

**Methods**

**Animals**

Female and male mutant G6PD-deficient mice (Pretsch et al. 1988) (Medical Research Council, UK) were housed three or four per plastic cage with ground corn cob bedding. The G6PD deficiency mutation is an ethylnitrosourea-induced A-T transversion mutation in exon 1 of the G6PD gene, which causes a splicing error during post-translational processing resulting in decreased protein expression. Animals were kept in a temperature-controlled room with a 12 h light/dark cycle automatically maintained. Food and water were provided ad libitum except during the taste aversion testing. All animals were monitored regularly by a veterinarian, and the studies were approved by the University of Toronto Animal Care Committee in accordance with the standards of the Canadian Council on Animal Care. The G6PD gene is X-linked, and heterozygous (+/def) females were bred with wild-type (+/y) or hemizygous (def/y) males to yield progeny of varied genotypes for females (+/+ , +/def, def/def) and males (+/y, def/y), where “def” indicates a mutated gene resulting in deficient G6PD activity.
G6PD genotypes were confirmed by the TaqMan assay (Holland et al. 1991) preformed by The Centre for Applied Genomics (TCAG) at the Toronto Hospital for Sick Children.

**Comet Assay**

DNA damage was measured by the alkaline single-cell gel electrophoresis (SCGE) or comet assay, from Singh, McCoy et al. (1988) with minor modifications. The general protocol includes dissociation of tissue into individual cells, mounting the cells in agarose onto microscopes slides, lysis of the cells with detergent and high salt to release the DNA, unwinding of the DNA, separating the damaged DNA fragments using electrophoresis, and finally staining and visualizing/scoring the resulting DNA “comet tails”. The technique is based on the principle that any strand break in the DNA will cause the supercoiling to relax, allowing negatively charged loops of DNA to freely extend and migrate, in the electric field, toward the anode creating a “tail”. For the comet assay, animals were anesthetized by isofluorane and killed by cervical dislocation. Separate brain tissue specimens from the two brain areas of interest (hippocampus and cerebellum) were washed in cold mincing solution (Hank’s Balanced Salt Solution, Ca++ and Mg++ free, 20 mM Na$_2$EDTA, 10% DMSO, pH 7.5) and placed in 1 ml of chilled mincing solution (1.5 ml for cerebellum). The tissue was cut into smaller pieces and the mixture was pushed through a 26 ¾ gauge needle to obtain dissociated cells. The sample was flash frozen until embedding. For embedding, the sample was mixed (1:1 v/v) with 0.5% low melting point agarose (prepared in Dulbecco’s phosphate-buffered saline, Ca++, Mg++, and phenol free, pH 7.4) and layered on conventional slides that were pre-dipped in 1% normal melting point
agarose. Slides were immersed in ice-cold freshly prepared lysis solution (2.5 M NaCl, 100 mM Na$_2$EDTA, 10 mM Tris-HCl, 1% Triton X-100 and DMSO 10%, pH 10) for 3 days at 4°C to lyse the cells and allow DNA unfolding.

After lysis, the slides were rinsed with and placed into alkaline electrophoresis buffer (0.3 M NaOH, 1 mM EDTA, pH>13) for 1 hr for DNA unwinding. The slides were positioned in a 20-slide COMET assay tank (Cleaver Scientific Ltd., Rugby, UK) and covered with fresh electrophoresis buffer. Electrophoresis was performed at 0.6 mV/cm and 300 mA for 24 min. Slides were then neutralized with 0.4 M Tris for 5 min, immersed in 100% cold ethanol for 5 min and air dried. Slides were then stained with SYBR Gold (Invitogen, Molecular Probes, Eugene, OR) and visualized with a fluorescence microscope (Zeiss Axioplan 2 Imaging upright fluorescence) and a CCD camera. For each sample a total of at least 100 comets were scored using the TriTek CometScore™ Freeware v1.5. The olive tail moment is the length of the tail multiplied by the fraction of DNA in the tail.

**Cell Analysis and Immunoblotting**

For H&E staining, the brains were perfused via cardiac puncture with PBS, followed by 10% neutral buffered formalin, isolated and further fixed overnight in 10% neutral buffered formalin before being embedded in paraffin. Paraffin-embedded brains were sliced into 5 μm-thick sections, which were mounted onto glass microscope slides for H&E staining. Embedding and staining were carried out at the Centre for Modeling Human Disease (CMHD) Pathology Core at the University of Toronto. H&E-stained slides were used for the assessment of PC numbers. Each G6PD genotype and gender
contained samples from 3 animals. For each slide, the mean number of PCs was determined from 10 randomly selected fields. For immunoblotting, a homogenate was prepared with half of a mouse cerebellum mixed with 500 μL of homogenization buffer (NP-40, 1 mM PMSF, 6 mM KF) and homogenized using a hand homogenizer. The homogenate was centrifuged at 16,000 x g and the supernatant was analyzed for protein concentration using the Bradford assay (Bradford 1976). A 10 μg sample of cellular protein was separated using a 10% SDS-PAGE gel under reducing and denaturing conditions and transferred onto a PVDF membrane (Amersham Hybond-P, GE Healthcare Life Sciences, Little Chalfont, UK). The PC marker calbindin-D-28K was detected with a mouse monoclonal anti-Calbindin-D-28K primary antibody (1:3000, Sigma-Aldrich, Oakville, ON) and a donkey anti-mouse IgG (H+L) HRP-conjugated secondary antibody (1:25000, Jackson ImmunoResearch Laboratories Inc., West Grove, PA). Each sample was standardized by probing the same membrane for β-actin. Peroxidase activity was visualized by an enhanced chemiluminescence (ECL) detection system (Amersham ECL Plus, GE Healthcare Life Sciences, Little Chalfont, UK).

**G6PD Activity**

For cerebellar G6PD activity, mice were anesthetized with isoflurane, sacrificed by decapitation, and the brain was removed and rinsed in ice-cold 1.15 % KCl. The cerebellum was dissected out and snap-frozen in liquid nitrogen. The samples were stored in a -80°C freezer until analysis. Each sample was homogenized with a hand homogenizer in 500 μL of 50 mM Tris-HCl pH 7.4, and the homogenate was sonicated
with a hand sonicator (Fisher Scientific 60 Sonic Dismembrator) for 10 sec. The homogenates were centrifuged for 30 min at 15,000 x g in a refrigerated microcentrifuge. The pellet was discarded and the protein content was determined in the supernatant using the Bradford assay, as was G6PD activity (Glock and McLean 1953) using a modified protocol (Ninfali et al. 1997). Reaction rates for G6PD were determined by UV spectrophotometry using a plate reader (SpectraMax M2, Molecular Devices, Sunnyvale, CA) at 340 nm at 15 sec intervals over 5 min. The final activity of G6PD was determined using the following equation: Net optical density x 40/6200 = µmol/min/ml of clear homogenate (IU/ml).

For analysis of erythrocyte G6PD activity, blood samples were taken from the tail vein without sacrificing the animals using EDTA-coated capillary tubes to avoid blood clotting (Microvette 100, Sarstedt, Montreal, PQ). The red blood cells were separated by centrifugation at 600 x g and washed twice with PBS, each time followed by centrifugation at 600 x g. A 500 µL aliquot of PBS was added after the final wash. The red blood cell count was obtained using a hemocytometer (Brightline, Sigma-Aldrich, Oakville, ON). The blood sample was mixed with cold ddH2O in a 1:4 (blood:water) ratio to burst the cells and release the cytoplasmic enzymes, and G6PD activity was determined using the protocol above.

Rotarod Performance

Motor coordination was measured using an accelerating rotarod apparatus. After a 1 hr acclimatization time, mice were required to perch on a stationary rod for 60 sec, after which the rod began rotating at 5 rpm for 90 sec. This was repeated once and
these trials were used to accustom the animals to the test. The actual test performed an hour after the trials were completed consisted of the same 60 sec stationary period, the 90 sec 5 rpm period and followed by an accelerating period of gradually increasing speed to 25 rpm for up to a total of 5.5 min. The latency to fall during the test was recorded. The test was repeated once and the longest time from the two tests was used as the animal’s measure of motor coordination.

**Ledge Balance Test**

The ledge balance test involved placing a mouse in the middle of a narrow (2 mm) cardboard edge 31 cm in length and allowing it to move towards either side to find a platform. The mice were left on the edge for a total of 2 min. If the mouse reached the platform in under 2 min, it was placed in the middle of the ledge again and the test was repeated. A repeated test was also allowed for mice that fell off the ledge. The test was repeated for each mouse at least 2 times unless the mouse did not move from the middle and could not walk towards the platform in 2 min. Each test was video-recorded, and the videos were scored in a blinded fashion on a scale of 1 to 4. The scores were defined as: 1 – made the platform with no trouble within the time allowed, 2 – reached the platform with some difficulty, 3 – did not reach the platform but remained on the ledge for the test, 4 – mouse fell off the ledge and could not balance. The final results were grouped according to age divided into 200-day intervals.

**Hindlimb Clasp Test**

The hindlimb clasp test was performed by holding a mouse up in the air by the
tail and monitoring the reflex of the hindlimbs. An abnormal test is observed when the animals holds its hindlimbs close to the body and/or clasps the hindlimbs together. This test was scored on a scale of 1 – 4. 1 – normal hindlimb splay and movement, 2 – hindlimbs slightly held to the body for a part of the test, 3 – hindlimbs held close to the body with no movement, 4 – hindlimbs close to the body with clasping in front. The final results were grouped according to ages divided into 200 day intervals.

**Passive Avoidance Test**

Passive avoidance represents a form of single-pass learning in which mice experience a brief unpleasant stimulus (mild foot shock) upon exposure to a one set of environmental cues (dark versus light chamber) (Crawley 2000). Mice were placed in a two-chamber cage with one dark (safe) chamber and one light (unsafe) chamber. The mice were allowed to explore the light chamber for 20 sec with a closed door to the dark chamber. The door was opened and the time to enter the dark chamber was recorded and the animal received a mild shock (1 mV for 4 sec) upon entering the dark area. The following day the animal was placed back into the light chamber and the latency to enter the dark chamber was recorded after the door to the dark chamber was opened. The maximum time allowed was 5 min. If the animal entered the dark chamber in under 5 min, another shock was administered. The test was repeated for one more day in cases where the shock was not administered upon entry, and again 1 week later.
**Taste Aversion Test**

Taste aversion is a cognitive behavioural test that measures the ability of the animal to learn and later recall an association of a specific taste (sweetness) with feelings of malaise (Welzl *et al.* 2001). This test has been used in a model of aging disease (Janus *et al.* 2004). Conditioned taste aversion was measured in aged G6PD-deficient and wild-type control mice up to 25 days after conditioning. Aging mice, 1 mouse per cage, were acclimatized for 7 days to a 7-hr drinking cycle (the animals received water for only 7 hr during a 24 hr period), which trained the mice to drink at least 1 mL of water within the first 30 min of access to water. This was confirmed by measuring how much water has been consumed in the first 30 min. On day 0, the mice were given a 0.5% saccharin solution for the first 30 min of the drinking cycle, and this was paired with a 4% body weight dose of 0.14 M LiCl i.p. to induce a feeling of malaise. At 2, 3, 4, 5, 6, 10, 14 and 25 days later the saccharin was presented again for the first 30 min of the drinking cycle and the amount of saccharin solution consumed was measured.

**Hippocampal Function**

The procedures for electrophysiological recordings were described previously (Meng *et al.* 2002; Meng *et al.* 2003; Zhou *et al.* 2011). Briefly, hippocampal slices (400 μm) were prepared from the wild-type and age-matched G6PD-deficient mice and allowed to recover in a holding chamber for at least 2 hr. A single slice was then transferred to the recording chamber and submerged and superfused with 95% O₂-5% CO₂-saturated artificial cerebrospinal fluid (ACSF, 2 ml/min). The ACSF contained 120
mM NaCl, 2.5 mM KCl, 1.3 mM MgSO$_4$, 1.0 mM NaH$_2$PO$_4$, 26 mM NaHCO$_3$, 2.5 mM CaCl$_2$, and 11 mM D-glucose. The recording pipette (3 megaohm [MΩ]) was filled with ACSF solution. Synaptic responses were evoked by bipolar tungsten electrodes placed 50-100 μm from the cell body layer in the CA1 area. Evoked field excitatory postsynaptic potentials (fEPSPs) were measured by taking the slope of the rising phase between 5% and 60% of the peak response. LTP was induced with the theta burst stimulation (TBS) protocol consisting of 5 bursts of 4 pulses of 100 Hz stimulation at 200 ms intervals. All data acquisition and analysis were done using pCLAMP 8 software (Axon instruments, Molecular Devices, Sunnyvale, CA). In long-term potentiation (LTP) studies, the data were normalized to the average of the baseline responses.

**Statistical Analysis**

Significance between paired data was determined by the two-tailed Student’s t-test (comet assay, PC counts, ledge test, hindlimb clasp). Multiple comparisons among groups were analyzed by one-way ANOVA (immunoblot). For data with two independent variables, two-way ANOVA was performed followed by a Bonferroni post-test (passive avoidance, taste aversion) (GraphPad Prism 5, USA). Linear regression was used to analyze data for the rotarod test and G6PD activity. The minimum level of significance used was p<0.5. Survival was assessed by the log rank Mantel-Cox test and the survival curve for homozygous-deficient female and hemizygous-deficient male mice was significantly different from the other two curves.
Results

DNA Damage and Purkinje Cell Analysis

Compared to age-matched wild-type controls, aging G6PD-deficient mice exhibited increased DNA damage reflected by DNA strand breaks in the cerebellum and hippocampus, with up to about 2-fold higher levels of damage in the latter region (Fig. 2.1). Maximal levels of DNA damage were observed with only a heterozygous mutation. The number of cerebellar PCs was reduced in aging G6PD-deficient males and females compared to wild-type controls (p<0.05), with similar reductions in heterozygous and homozygous mutant females (Fig. 2.2, upper panel). Homozygous G6PD-deficient females appeared to exhibit a greater loss than hemizygous deficient males (63% vs. 75% compared to wild-type), but this difference was not significant. A reduction in PC number with G6PD deficiency was confirmed by western analysis for the PC marker calbindin-D-28K, with a 47% decrease in aging G6PD-deficient mice with either a homozygous (females) or hemizygous (male) G6PD mutation, and a lesser 28% reduction in heterozygous G6PD-deficient females (p<0.05), suggesting a gene dose-dependent trend (Fig. 2.2, lower panel).
Fig. 2.1. Increase in DNA damage in the cerebellum and hippocampus of aging G6PD-deficient mice measured by the comet assay.

Cells were obtained from the hippocampus and cerebellum and prepared and visualized as described in the Methods. Upper pictures depict representative “comet tails” from each brain area and genotype. Lower graphs represent the olive tail moment means from 3 samples in each group. Asterisks indicate a difference from wild-type G6PD-normal mice for the same brain region (p<0.05).
PURKINJE CELL COUNTS

FEMALES

MALES

WESTERN BLOT ANALYSIS

G6PD GENOTYPE
Fig. 2.2. Decrease in Purkinje cell (PC) numbers and calbindin-D-28K expression in aging G6PD-deficient mice.

**Upper panel:** PCs were counted in 10 distinct fields at 40X to give a total of cells for each sample. Asterisks indicate a difference from C6PD-normal controls of the same gender (p<0.05). **Lower panel:** Densitometric analysis of calbindin-D-28K protein, a marker for PCs, was performed for cerebellar tissues from G6PD-normal and G6PD-deficient mice. Gels were loaded with 10 μg of total cellular protein. Asterisks indicate a difference from G6PD-normal wild-type controls (p<0.05).
**G6PD Activity with Age**

The activity of G6PD in red blood cells correlated tightly with that in cerebellar tissue \( r = 0.91, \ p < 0.0001 \) ([Fig. 2.3](#fig2.3), upper panel), allowing repetitive sampling of the same mice to determine activity throughout their lifespan, and provide estimates of G6PD activity in the brains of mice being tested for behavioural performance. G6PD activity did not change significantly with age among any genotype, although there was a non-significant trend among \(+/+, +/\text{def} \) and \(+/y\) mice, but not \text{def}/\text{def} or \text{def}/y mice, for higher activities below 200 days of age and above 200 days of age, approximating a “U”-shaped curve in mice with at least one copy of the G6PD gene ([Fig. 2.3](#fig2.3), lower panel).

**Motor Coordination**

In the rotarod test, the performance of female wild-type, G6PD-normal mice declined slightly but significantly with age ([Fig. 2.4](#fig2.4)). This decline with age was not observed in wild-type males, nor in G6PD-deficient females or males of any genotype. When groups were divided by age (< 7 months, 7-18 months, > 18 months) or analyzed together, there was no correlation between G6PD activity and rotarod performance at any age ([Fig. 2.5](#fig2.5)). In contrast, performance in the ledge balance test decline in performance more quickly with age for G6PD-deficient mice with all alleles mutated compared to heterozygous and hemizygous G6PD-deficient mice and wild-type G6PD-normal animals ([Fig. 2.6](#fig2.6), upper panel). There were no G6PD-dependent differences with age in the hindlimb clasp test ([Fig. 2.6](#fig2.6), lower panel), although all genotypes showed a progressive decline.
Fig. 2.3. Positive correlation of G6PD activity in erythrocytes with cerebellar tissues, and no change in G6PD activity with aging.

**Upper panel:** G6PD activity in red blood cells and cerebellar tissue was measured in the same mouse, and the relationship was assessed by Pearson’s correlation analysis. **Lower panel:** G6PD activity in red blood cells was measured in mice of different ages for all G6PD genotypes.
Fig. 2.4. Motor coordination during aging in G6PD-deficient mice measured by rotarod performance.

Mice of different ages and all G6PD genotypes were tested using the rotarod apparatus, and the latency to fall was recorded. The Pearson r value is shown for significantly correlated groups.
Fig. 2.5. G6PD activity was not correlated with rotarod motor coordination performance during aging.

Mice were tested on the rotarod apparatus and the latency to fall was recorded. Blood G6PD activity was measured in the same mice.
Fig. 2.6. Effect of G6PD-deficiency on the ledge balance test but not the hindlimb clasp test during aging.

**Upper panel:** *Ledge balance test.* The ability of mice to walk across a narrow ledge was scored on a scale of 1-4. The final results were grouped according to ages divided into 200-day intervals. All animals progressively declined with age (p<0.0001, 2-way ANOVA). The decline was different in homozygous G6PD-deficient mice (p<0.05), occurring at an earlier age (200 days). **Lower panel:** *Hindlimb clasp test.* The hindlimb splay reflex was scored on a scale of 1-4. The final results were grouped according to ages divided into 200-day intervals. No differences were found among the genotypes. All of the genotypes progressively declined over age (p<0.0001, 2-way ANOVA)
Fig. 2.7. No effect of G6PD deficiency on cognitive function in aging mice.

**Left panel: Passive avoidance.** Aging mice (1.25-1.5 years) were placed in a two chamber cage with one dark (safe) chamber and one light (unsafe) chamber. The mice were allowed to explore the light chamber for 20 sec with a closed door to the dark chamber. The door was opened, the time to enter the dark chamber was recorded and the animal received a shock when entering the dark area. The following day the subject was placed back into the light chamber and the latency to enter was recorded after the door to the dark chamber was opened. The test was repeated for one more day and again 1 week later. No differences were found for any G6PD genotype. **Right panel: Taste aversion.** Aging mice were acclimatized for 7 days to a 7-hr drinking cycle (animals received water for only 7 hr during a 24 hr period). On Day 0, the mice were given a 0.5% saccharin solution for the first 30 min of the drinking cycle and this was paired with a 4% b/w dose of 0.14 M LiCl i.p. to induce a feeling of malaise. At 2, 3, 4, 5, 6, 10, 14 and 25 days later, the saccharin was presented again for the first 30 min of the drinking cycle and the amount of saccharin solution consumed was measured. No differences were observed.
**Passive Avoidance and Taste Aversion**

No G6PD-dependent differences in cognition were observed with aging as measures by either the passive avoidance or taste aversion tests (**Fig. 2.7**).

**Hippocampal Function**

To determine the effect of G6PD deficiency on the functional properties of synaptic transmission and plasticity, we conducted electrophysiological recordings in the CA1 region of the hippocampus using an established model (Meng *et al.* 2003). First, basal synapse strength was determined by stimulating the Schaffer collateral pathway and recording fEPSPs at the CA1 synapse. Analysis of evoked fEPSPs revealed a substantial and significant enhancement in both the stimulus intensity-response curve and the maximal response in def/y G6PD-deficient mice compared to +/y wild-type G6PD-normal controls (**Fig. 2.8**, upper panel). Secondly, PPF reflecting short-term synaptic plasticity was examined as an indicator of presynaptic function. As shown in Fig. 8 (middle panel), the magnitude of PPF over the range of 25 to 100 ms was also substantially and significantly increased in the G6PD-deficient mice. Finally, we analyzed LTP, an extensively studied form of long-lasting synaptic plasticity widely regarded as a synaptic model for learning and memory (Bliss and Collingridge 1993). We induced LTP by theta burst stimulation (TBS) because it is considered to be a more physiologically relevant protocol to elicit plasticity. There was no difference in the magnitude of LTP between the G6PD-deficient mice and wild-type controls (**Fig. 2.8**, lower panel).
**Survival**

The lifespan of the colony was monitored, and G6PD-deficient females (+/def and def/def) and males (def/y) with all alleles mutated showed a small but significantly improved survival rate compared to heterozygous G6PD-deficient mice and wild-type G6PD-normal animals (Fig. 2.9). The basis for this improved lifespan could not be determined.
Fig. 2.8. Hippocampal electrophysiological function is altered in aging G6PD-deficient mice.

**Upper panel:** Enhanced basal synaptic transmission in aged hemizygous G6PD-deficient male mice. The slope of evoked field excitatory postsynaptic potentials (fEPSPs) was plotted as a function of stimulus intensity, showing significant increases in both the slope and maximal responses in G6PD-deficient mice compared to the age-matched G6PD-normal wild-type control. The distance between the stimulating and recording electrodes was kept constant between slices and mice. **Middle panel:** Increased presynaptic function in aged hemizygous G6PD-deficient male mice. Paired-pulse facilitation ratios (second fEPSP/first fEPSP) were plotted as a function of the interpulse intervals between the two stimulations, showing a significant enhancement in PPF in the aged G6PD-deficient mice compared to G6PD-normal wild-type controls. **Lower panel:** Normal long-term potentiation (LTP) in aged hemizygous G6PD-deficient male mice. LTP induced by theta burst stimulation, showing no differences between genotypes. Traces in the inset above the graph were taken from the recordings immediately before and 40 min after the delivery of TBS given at 20 min time point.
Fig. 2.9. Enhanced survival in aged G6PD-deficient mice.

Untreated mice were monitored throughout their lifetime and times and apparent causes for death were recorded. Survival was assessed by the log rank Mantel-Cox test, and the survival curve for homozygous female and hemizygous male G6PD-deficient mice was significantly different from the curves for both heterozygous G6PD-deficient females and G6PD-normal wild-type males and females.
Discussion

We previously reported an increase in oxidatively damaged DNA in the brains of aging G6PD-deficient mice as determined at the molecular level by the 8-oxo-dG lesion (Jeng et al. 2013). The alkaline comet assay used herein measures single-strand breaks and alkali-labile sites (Tice et al. 2000), providing a complementary functional outcome in single cells, and possibly a more sensitive measure of DNA damage. The increased level of DNA damage in aging G6PD-deficient mice revealed in the comet assay corroborated our previous measures of an oxidative DNA lesion, indicating a consistent neuroprotective role for G6PD in aging at the molecular and macromolecular levels. The approximately 2-fold greater level of DNA damage in hippocampal tissues compared to the cerebellum suggests that the former tissue may be at even greater risk of oxidative stress during aging, due to greater ROS formation or relatively lower levels of pathways for ROS detoxification and/or DNA repair. Maximal DNA damage was observed in aging mice with the loss of only one G6PD allele, indicating that even modest reductions in this enzyme may have important neurodegenerative consequences, as G6PD activity in heterozygous mice is decreased to about 40% of normal (Jeng et al. 2013).

We similarly sought to more definitively confirm the loss of cerebellar PCs previously reported for female heterozygous G6PD-deficient mice determined by cell counting (Jeng et al. 2013). Herein, we definitively identified PC numbers by immunoblotting for calbindin-D-28K, which is a specific marker for PCs (Jande et al. 1981; Baurle and Grusser-Cornehls 1994), and found a decrease in PCs in the
cerebella of aged G6PD-deficient mice similar to that determined by counting, corroborating the neuroprotective importance of G6PD in aging at the cellular level. We also found that both male and female aging G6PD-deficient mice exhibited a loss of PCs, and that a maximal loss was observed with the mutation of only a single G6PD allele, as was similarly noted above for DNA damage. The remarkable maximal cellular loss in aging G6PD-deficient mice indicates that even a modest reduction in the expression of this enzyme has neurodegenerative consequences for cellular lost in aging. Interestingly, G6PD expression is reported to be highest in PCs (Biagiotti et al. 2001), and is associated with increased activity of NADPH-dependent reactions and enzymes in the brain of rats (Biagiotti et al. 2003; Ferri et al. 2005). The congruency of maximal DNA damage and PC loss in heterozygous aging G6PD-deficient mice observed herein is consistent with a causal association of low levels of G6PD leading to enhanced oxidative DNA damage and cellular neurodegeneration.

The high correlation of G6PD activity in red blood cells with that in cerebellar tissues ($r = 0.91$) confirmed that RBC activity provides an accurate estimate of cerebellar activity, which did not change significantly with a given G6PD genotype with age. However, there was an interesting trend for higher G6PD activity in the youngest and oldest age groups, suggesting that these two age groups may be experiencing higher levels of oxidative stress.

A reduction in PCs often leads to motor function deficits in mouse models such as the Lurcher mouse (Vogel et al. 2007) and the Purkinje Cell Degradation (PCD) mouse (Wang and Morgan 2007), as well as in models of Huntington’s Disease.
(Dougherty et al. 2013). We have previously used the rotarod assay to determine the effects of enhanced in utero oxidative stress and oxidatively damaged DNA in fetal brain on postnatal motor coordination caused by maternal treatment with the ROS-initiating teratogen methamphetamine in normal (Jeng et al. 2005) and DNA repair-deficient pregnant mice (Wong et al. 2008). In the aging G6PD-deficient mice herein, we did not observe a decline in rotarod performance, although there was a small decline in performance in the aging G6PD-normal female mice. The basis for the decline only in female G6PD-normal mice is not known, but was unrelated to G6PD activity, which did not change with age. G6PD activity was similarly unchanged with age within each of the G6PD-deficient genotypes, which may or may not have contributed to the absence of any change in rotarod performance. In other models, the Lurcher mouse and the PCD mouse show an almost total degeneration of PCs as well as other cells in the cerebellum, and these mice display major motor deficits in old age (Caddy and Biscoe 1975; Vogel et al. 2007; Wang and Morgan 2007). Interestingly, the Lurcher mice, even with almost complete loss of PCs, were able to learn to match their wild-type littermate controls in a tilted platform test, which is similar to the rotarod test (Lalonde 1994). Other studies found that the degree of PC loss did not correlate with motor coordination deficits in several mouse models including Staggerer mice, Lurcher mice and Hot-foot mutants, as well as some specific null mutations affecting PCs (Caston et al. 1998). These studies are consistent with the absence of a decrease in rotarod performance in aging G6PD-deficient mice herein, despite their substantial loss of PCs.
Additional tests were employed to discern deficits in motor function not detected by the rotarod test. The hindlimb clasp test showed a general aging change in all genotypes, possibly indicative of specific damage in the substantia nigra. Animal models for Parkinson's disease with specific damage in the substantia nigra exhibit deficits in the hindlimb clasping test but not in balance tests (Lieu et al. 2013). The ledge balance test also showed a general decline in performance with aging in all genotypes, but revealed that homozygous and hemizygous G6PD-deficient mice exhibited a maximal decline earlier in life, at 400 days, whereas the heterozygous G6PD-deficient mice and wild-type normal mice did not exhibit the same maximal decline until 600 days. Similar results have been reported for mouse models of Huntington's disease (Heng et al. 2007; Heng et al. 2010), which show damage specifically to PCs in the cerebellum (Dougherty et al. 2012; Dougherty et al. 2013). These behavioural outcomes are consistent with our current and previous observations; herein, we found a reduction of PCs, and previously, we found no increase in DNA oxidation or morphological changes in the substantia nigra or striatum in aged G6PD-deficient mice (Jeng et al. 2013).

Aging G6PD-deficient mice did not exhibit a deficit in cognitive function assessed by passive avoidance and taste aversion compared to age-matched wild-type G6PD-normal mice. In light of our electrophysiological studies discussed below, this was unexpected but perhaps not surprising, given that more severe damage in the relevant brain regions may be required for alterations in the selected tests, similar to the substantial loss of dopaminergic neurons required for behavioural evidence of
Parkinson's disease where at the onset of symptoms in humans striatal dopamine is reduced by 80% and cell numbers are reduced by 50% (Fearnley and Lees 1991). In light of the molecular and cellular changes in the brains of G6PD-deficient mice shown herein and in our previous study (Jeng et al. 2013), we would anticipate that other behavioural tests, perhaps including measures of more demanding tasks like executive function using new testing paradigms (Karlsson et al. 2011), may reveal enhanced deficits in cognition in aging G6PD-deficient mice. Even the current tests may show increased cognitive deficits in aging G6PD-deficient mice concurrently exposed to environmental conditions that enhance brain ROS formation, and/or reduce pathways of antioxidative defense or DNA repair.

Although no behaviour changes reflecting cognitive deficits were observed in aging G6PD-deficient mice, electrophysiological studies showed that excitatory synaptic function is substantially altered in G6PD deficiency. Specifically, hippocampal slices from aging G6PD-deficient mice exhibit increases in both basal synaptic strength and presynaptic short-term plasticity. These findings are somewhat surprising because chronic oxidative stress is generally understood to cause cell death. Indeed, the G6PD-deficient mice show reduced PC number in the cerebellum. However, whether specific cell loss occurs in the hippocampus remains to be investigated. It is possible that chronic oxidative stress-induced neuronal loss may lead to altered synaptic connections that result in enhanced synaptic responses. Further studies on neuronal numbers, neuronal morphology, synapse and synaptic proteins will be important for elucidating the underlying mechanisms. Given the critical role of excitatory synaptic
transmission and plasticity in various aspects of brain function (Collingridge et al. 2010; Luscher and Huber 2010), our results raise the possibilities that genetic abnormalities may have effects on behavioral responses and cognition. Since G6PD deficiencies constitute the most common enzymopathy in humans, our results also have important clinical implications, suggesting that reduced G6PD function has not only metabolic effects, but also serious consequences for brain development and function.

G6PD is primarily studied for its importance in red blood cells, and the high human prevalence of G6PD deficiencies is generally believed to result from the evolutionary pressure of enhanced survival of G6PD-deficient people with malaria. Unexpectedly, we found that homozygous and hemizygous G6PD-deficient animals survived longer than heterozygous G6PD-deficient or wild-type G6PD-normal animals, none of which had malaria. The mechanism underlying this novel observation is unknown, and could include alterations in NADPH-dependent pathways aside from G6PD, and/or other biochemical changes due to a reduction in the pentose phosphate pathway, perhaps analogous to the increased survival observed in animals on a severely restricted diet. G6PD deficiency has recently been reported to have apparently contradictory health benefits, perhaps due at least in part to contrasting roles of this enzyme. For example, G6PD deficiency has been reported to reduce: (1) human retinopathy and mortality from cardiovascular disease in a discreet Mediterranean population (Gupte 2010); (2) cholesterol synthesis, production of superoxide that leads to ROS formation, and reductive stress in mice (Gupte et al. 2006; Hecker et al. 2013a); and, (3) angiotensin II-dependent hypertension (Matsui et al. 2005).
contrast, other studies have reported that G6PD deficiency in mice results in a spectrum of cardiomyopathies including age-associated cardiac hypertrophy (Hecker et al. 2012; Hecker et al. 2013b), which would not be consistent with the enhanced survival observed in our aging G6PD-deficient mice. It has been postulated that G6PD deficiency may decrease the development of cardiovascular disease, but may aggravate already established disease (Hecker et al. 2013a). One study reported no change in survival for G6PD-deficient mice (Hecker et al. 2012) but this study ended at week 35 (day 245) in contrast to our study that followed the mice until their death (around 800 days). A population of humans with increased longevity in Sardinia, Italy, has been found to highly conserve a mutant variant of G6PD resulting in an enzymatic deficiency. This population exhibited a decrease in deaths from ischemic heart disease, cerebrovascular disease and liver cirrhosis, which is consistent with the extended lifespan we found in aging G6PD-deficient mice (Long et al. 1967; Cocco et al. 1998; Meloni et al. 2008). On the other hand, this same population exhibited an increase in mortality from non-Hodgkin’s lymphoma in individuals with G6PD-deficiency, which we did not observe in aged G6PD-deficient mice. The importance of environmental factors affecting G6PD, and thereby potentially altering health, is virtually unknown. Interestingly, analogs of dehydroepiandrosterone (DHEA), a potent inhibitor of G6PD (Raineri and Levy 1970; Gordon et al. 1995), as well as DHEA itself, are being evaluated in animal models and in human clinical trials as potential therapeutic agents for numerous diseases including cancer and cardiovascular disease (Schwartz and Pashko 2004; Gupte 2010), although these agents likely have confounding effects in
addition of G6PD inhibition. However, our studies (Nicol et al. 2000; Jeng et al. 2013) including the results herein, along with several studies from other laboratories (Gupte et al. 2006; Hecker et al. 2013b), suggest that G6PD inhibition may have adverse consequences in addition to any anticipated therapeutic benefits.

In conclusion, G6PD deficiencies constitute the most common human enzymopathy, affecting over 400 million people and up to 60% of some populations. Although medical interest in deficiencies in this enzyme has been largely confined to red blood cells, more recent evidence suggests broader implications for health, including an expanding list of potential outcomes that includes birth defects, heart disease and cancer. Our results suggest that reduced G6PD activity has not only metabolic effects, but also serious consequences for brain development and function, which may have important clinical implications.
2.3 Study 3: FUNCTIONAL ASSESSMENT OF NEURODEGENERATION IN AGING AND AMPHETAMINE-TREATED GLUCOSE-6-PHOSPHATE DEHYDROGENASE-DEFICIENT MICE

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a. Preliminary reports of this research were presented at the 11th International Congress of Toxicology (ICT Proceedings, Abstract No. PM5.195, 2007) and the 2011 annual meeting of the Society of Toxicology of Canada (STC Proceedings, Abstract No. 24, 2011). These studies were supported by a grant from the Canadian Institutes of Health Research (CIHR).
Abstract

Glucose-6-phosphate dehydrogenase (G6PD) is important for the detoxification of reactive oxygen species (ROS). Using mutant G6PD-deficient mice, we evaluated several behavioural tests of motor coordination and learning and memory to assess the neuroprotective role of G6PD against amphetamines and in aging. Motor coordination, taste aversion learning (TAL) and passive avoidance learning (PAL) were measured in adult (4-6 months old) and aging (1.25-1.5 years old) G6PD-normal and G6PD-deficient mice that were either untreated or treated with a single dose of 3,4-methylenedioxyamphetamine (MDA), the major active metabolite of 3,4-methylenedioxymethamphetamine (MDMA, Ecstasy), or methamphetamine (METH), all of which enhance ROS formation. Motor coordination measured by rotarod and ledge balance performance were not affected in MDA-treated mice in either gender or any G6PD genotype, but hindlimb clasp reflex was impaired by MDA in aging mice. MDA decreased TAL in females but not males, and homozygous but not heterozygous G6PD-deficient animals were paradoxically protected. PAL was not affected by MDA or METH in adult or aging mice. Although the gender-dependent deficit in TAL caused by MDA is consistent with ROS-mediated neurodegeneration, the limited behavioural tests employed herein do not provide evidence of a neuroprotective role for G6PD in amphetamine-enhanced oxidative stress. The remarkable and unexpected protection in G6PD-deficient mice against MDA-initiated TAL deficits, together with the absence of a G6PD effect on PAL, suggest complexity in the sensitivity and specificity of behavioural tests for neurodegenerative effects of amphetamines.
Key Words: Glucose-6-phosphate dehydrogenase; motor coordination; cognition; neurodegeneration; reactive oxygen species; ROS; amphetamines; methamphetamine; 3,4-methylenedioxyamphetamine

Introduction

Glucose-6-phosphate dehydrogenase (G6PD) is the first and rate-limiting enzyme in the hexose monophosphate pathway, important for its role in the regeneration of the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) and the production of ribose-5-phosphate (Buehler 1993). During cellular oxidative stress, NADPH is critical for maintaining glutathione in its reduced form (GSH), which is essential for the detoxification of reactive free radicals and lipid hydroperoxides (Halliwell and Gutteridge 2007). Another important role of NADPH is the maintenance of the catalytic activity of catalase, which is required for the detoxification of hydrogen peroxide (Kirkman et al. 1987; Kirkman et al. 1999).

Over the years, it has been shown that reactive oxygen species (ROS) are implicated in neurodegenerative diseases such as Alzheimer’s disease (AD) and Parkinson’s disease (PD) (Markesbery 1997; Owen et al. 1997; Lyras et al. 1998; Jenner 2003; Nunomura et al. 2006). ROS have been also been associated with the neurotoxic mechanisms of several amphetamine derivatives including 3,4-methylenedioxymethamphetamine (MDMA, Ecstasy), its demethylated active metabolite 3,4-methylenedioxyamphetamine (MDA) and methamphetamine (METH) (Cadet et al. 1994b; Colado et al. 1997; Cadet et al. 2001; Jeng et al. 2006; Jeng and Wells 2010). Although the molecular mechanism of neurodegeneration remains
unclear, studies from our laboratory have linked prostaglandin H synthases (PHSs) in the bioactivation of amphetamines to neurotoxic free radical intermediates (Jeng et al. 2006; Jeng and Wells 2010; Ramkissoon and Wells 2011b), and a similar mechanism may contribute to neurodegeneration associated with aging, via PHS-catalyzed bioactivation of endogenous neurotransmitters, their precursors and metabolites (Goncalves et al. 2009; Jeng et al. 2011; Ramkissoon and Wells 2011a). Since MDMA and related amphetamines have gained popularity as recreational drugs of choice in young adults (Freese et al. 2002), these drugs are clinically relevant models for elucidating the ROS-mediated mechanisms of neurodegeneration and determinants of risk.

G6PD is an important embryoprotective enzyme, and evidence from our laboratory suggests that G6PD similarly protects the brain from DNA oxidation and cellular neurodegeneration caused by oxidative stress associated with aging (Jeng et al. 2013). Herein, we evaluated the neuroprotective role of G6PD in aging and exposure to the amphetamines MDA and METH using: (1) a complementary measure of DNA damage in single cells, reflected by DNA strand breaks in the comet assay; (2) a measure of cerebellar Purkinje cell number; and, (3) behavioural tests assessing motor coordination and cognition.
Methods

Animals

Female and male mutant G6PD-deficient mice (Pretsch et al. 1988) (Medical Research Council, UK) were housed three or four per plastic cage with ground corn cob bedding. The G6PD deficiency mutation is an ethylnitrosourea-induced A-T transversion mutation in exon 1 of the G6PD gene, which causes a splicing error during post-translational processing resulting in decreased protein expression. Animals were kept in a temperature-controlled room with a 12 hr light/dark cycle automatically maintained. Food and water were provided ad libitum except during the taste aversion testing. All animals were monitored regularly by a veterinarian, and the studies were approved by the University of Toronto Animal Care Committee in accordance with the standards of the Canadian Council on Animal Care. The G6PD gene is X-linked, and heterozygous (+/def) females were bred with wild-type (+/y) or hemizygous (def/y) males to yield progeny of varied genotypes for females (+/+ , +/def, def/def) and males (+/y, +/def), where “def” indicates a mutated gene resulting in deficient G6PD activity. G6PD genotypes were confirmed by the TaqMan assay (Holland et al. 1991) performed by The Centre for Applied Genomics (TCAG) at the Toronto Hospital for Sick Children.

Drugs and Treatment

Pure racemic (dl)-MDA and d/-METH were provided by the Research Technology Branch of the National Institute on Drug Abuse (Rockville, MD) and by the Healthy Environments and Consumer Safety Branch of Health Canada (Ottawa, ON, Canada). The pure composition and identity was confirmed by an outside lab by a Bio-
Rad REMEDi HS system and confirmed by liquid chromatography-mass spectrometry-mass spectrometry (Clinical Biochemistry, Hospital for Sick Children, Toronto, ON, Canada). Drugs were dissolved with sterilized 0.9% saline and the drug or its vehicle were injected intraperitoneally (ip) in a fixed volume of 0.1 ml/10 g body weight at an ambient temperature of 21-24°C. Mice were administered 4 doses of MDA (20 mg/kg), METH (5 or 10 mg/kg) or 0.9% saline (vehicle) in a single day, with each dose given at 2 hr intervals. Scopolamine (Sigma-Aldrich, Oakville, ON) was administered as a single dose of 0.3 mg/kg ip, either 30 min before initial passive avoidance association or immediately after the association.

**Rotarod**

Motor coordination was measured using an accelerating rotarod apparatus. After a 1 hr acclimatization time, mice were required to perch on a stationary rod for 60 sec, after which the rod began rotating at 5 rpm for 90 sec. This was repeated once and these trials were used to accustom the animals to the test. The actual test consisted of the same 60 sec stationary period, the 90 sec 5 rpm period and followed by an accelerating period of gradually increasing speed to 25 rpm for up to a total of 5.5 min. The latency to fall during the test was recorded. The test was repeated once and the longest time from the two tests was used as the animal’s measure of motor coordination. For the adult animals, the test was repeated every week for 4 weeks starting 5 days after MDA dosing and once more at 8 and 12 weeks. For the aging treated animals the test was done 1 day before MDA treatment and 2 weeks after treatment.
Ledge Balance Test

The ledge balance test involved placing a mouse in the middle of a narrow (2 mm) cardboard edge 31 cm in length and allowing it to move towards either side to find a platform. The mice were left on the edge for a total of 2 min. If the mouse reached the platform in less than 2 min, it was placed in the middle of the ledge again and the test was repeated. A repeated test was also allowed for mice that fell off the ledge. The test was repeated for each mouse at least 2 times unless the mouse did not move from the middle and could not walk towards the platform within 2 min. Each test was video-recorded, and the videos were scored in a blinded fashion on a scale of 1 to 4. The scores were defined as: 1 – made the platform with no trouble within the time allowed, 2 – reached the platform with some difficulty, 3 – did not reach the platform but remained on the ledge for the test, 4 – mouse fell off the ledge and could not balance. For the aging treated animals the test was done 1 day before MDA treatment and 2 weeks after treatment.

Hindlimb Clasp Reflex

The hindlimb clasp test was performed by holding a mouse up in the air by the tail and monitoring the reflex of the hindlimbs. An abnormal test is observed when the animals holds its hindlimbs close to the body and/or clasps the hindlimbs together. This test was scored on a scale of 1 – 4. 1 – normal hindlimb splay and movement, 2 – hindlimbs slightly held to the body for a part of the test, 3 – hindlimbs held close to the body with no movement, 4 – hindlimbs close to the body with clasping in front. For the
aging treated animals the test was done 1 day before MDA treatment and 2 weeks after treatment.

**Passive Avoidance**

Passive avoidance represents a form of single-pass learning in which mice experience a brief unpleasant stimulus (mild foot shock) upon exposure to a set of environmental cues (dark versus light chamber) (Crawley 2000). Mice were placed in a two-chamber cage with one dark (safe) chamber and one light (unsafe) chamber. The mice were allowed to explore the light chamber for 20 sec with a closed door to the dark chamber. The door was opened and the time to enter the dark chamber was recorded and the animal received a mild shock (1 mV for 4 sec) upon entering the dark area. The following day the animal was placed back into the light chamber and the latency to enter the dark chamber was recorded after the door to the dark chamber was opened. The maximum time allowed was 5 min. If the animal entered the dark chamber in less than 5 min, another shock was administered. The first day of testing was 5 days after drug or vehicle treatment was administered to the animals.

**Taste Aversion**

Taste aversion is a cognitive behavioural test that measures the ability of the animal to learn and later recall an association of a specific taste (sweetness) with feelings of malaise (Welzl et al. 2001). This test has been used in a model of aging disease (Janus et al. 2004). Conditioned taste aversion was measured in aging G6PD-deficient and wild-type control mice up to 25 days after conditioning. Aging mice, 1 mouse per cage, were acclimatized for 7 days to a 7-hr drinking cycle (the animals
received water for only 7 hr during a 24 hr period), which trained the mice to drink at least 1 mL of water within the first 30 min of access to water. This was confirmed by measuring how much water has been consumed in the first 30 min. On day 0, the mice were given a 0.5% saccharin solution for the first 30 min of the drinking cycle, and this was paired with a 4% body weight dose of 0.14 M LiCl ip to induce a feeling of malaise. At 2, 3, 4, 5, 6, 10, 14 and 25 days later the saccharin was presented again for the first 30 min of the drinking cycle and the amount of saccharin solution consumed was measured. The animals were treated with MDA or vehicle 5 days before Day 0.

**Statistical Analysis**

Significance between paired data was determined by the two-tailed Student’s t-test (ledge test, hindlimb clasp, taste aversion). For data with two independent variables, two-way ANOVA was performed followed by a Bonferroni post-test (passive avoidance, taste aversion) (GraphPad Prism 5, USA). The minimum level of significance used was p<0.5.
Results

Motor coordination and hindlimb clasping reflex

Performance in the rotarod test was not affected in adult mice by MDA treatment or G6PD genotype (Fig. 3.1), nor was there a gender difference (male data are not shown). The animals did not improve in their performance over the weeks of the test.

In aging mice, rotarod performance was similarly unaffected by MDA treatment or G6PD genotype (Fig. 3.2, upper panel), nor were there any differences in the ledge balance test (Fig. 3.2, middle panel). However, there was an effect of MDA treatment in the hindlimb clasping reflex in aging mice (Fig. 3.2, lower panel). Aging mice treated with MDA were significantly impaired when tested 2 weeks after dosing, compared to both vehicle controls and the same mice before drug treatment. There was no effect of G6PD genotype in the hindlimb clasp test (data not shown).
Fig. 3.1. Motor coordination in adult G6PD-normal and -deficient mice measured by rotarod performance.

Adult mice were tested 5 days after MDA or vehicle treatment using the rotarod apparatus, and the latency to fall was recorded. The left panels compare treatment in different genotypes. The right panels compare different genotypes in treatment groups. The lower right graph compares saline and MDA treatment with the genotypes combined.
Fig. 3.2. Rotarod, ledge balance test and hindlimb clasp test in aging MDA-treated G6PD-normal and G6PD-deficient mice.

Each test was administered 1 day BEFORE treatment and 2 weeks AFTER treatment. Upper Panel Rotarod test. The latency to fall was used as the measure of performance. Middle panel: Ledge balance test. The ability of mice to walk across a narrow ledge was scored on a scale of 1-4. Results for all G6PD genotypes are combined. Lower panel: Hindlimb clasp test. The hindlimb splay reflex was scored on a scale of 1-4. Results did not differ among G6PD genotypes, and data for all G6PD genotypes are combined. γ indicates a difference compared to the same mice before treatment, as well as to vehicle-treated matched controls (p<0.05 by t-test analysis, p=0.056 with repeated measures ANOVA).
**Passive Avoidance**

Passive avoidance learning was not altered by MDA treatment in either adult (Fig. 3.3, left panel) or aging animals (Fig. 3.3, right panel) for any G6PD genotype. Similarly, METH treatment did not cause deficits in passive avoidance learning (Fig. 3.4). To ensure the validity of our test, scopolamine was used as a positive control. Scopolamine is a competitive muscarinic receptor antagonist that prevents the formation of novel memories during spatial memory tasks (Bammer 1982) (Fig. 3.5). When the animals received a dose of scopolamine 30 min before the initial association of a shock when entering the dark chamber, the association was entirely abolished when the animals were tested for recall the following day, showing that the test in our hands was working.

**Taste Aversion**

Adult female G6PD-normal and heterozygous G6PD-deficient mice treated once with MDA exhibited a greater loss of association with the aversive stimulus when compared to vehicle controls (p<0.05) (Fig. 3.6, upper and middle panels). This effect was not observed in the homozygous G6PD-deficient mice, where the MDA-treated mice were not different from vehicle controls, although the trend was similar to that for +/+ and +/-def mice (Fig. 3.6, lower panel).
Fig. 3.3. No effect of MDA treatment or G6PD-deficiency on passive avoidance.

**Passive avoidance.** Mice were placed in a two-chamber cage with one dark (safe) chamber and one light (unsafe) chamber. The mice were allowed to explore the light chamber for 20 sec with a closed door to the dark chamber. The door was opened, the time to enter the dark chamber was recorded and the animal received a shock when entering the dark area. The following day the subject was placed back into the light chamber and the latency to enter was recorded after the door to the dark chamber was opened. The test was repeated for one more day and again 1 week later. **Left panel:** Adult treated G6PD-normal and G6PD-deficient mice. No differences were found with treatment or genotype. **Right panel:** Aging treated G6PD-normal and G6PD-deficient mice. No differences were found with treatment or genotype.
Fig. 3.4. No effect of METH treatment or G6PD-deficiency on passive avoidance.

Passive avoidance. **Upper two graphs:** Adult treated G6PD-normal and heterozygous G6PD-deficient mice treated with high dose of METH (10 mg/kg x 4) or saline. No differences were found with treatment or genotype. **Lower panel:** Adult METH treated homozygous G6PD-deficient mice. These mice were treated with either saline, low-dose METH (5 mg/kg x 4) or high-dose METH (10 mg/kg x 4). No differences were found with treatment or genotype.
Fig. 3.5. Effects of scopolamine on passive avoidance.

Mice were treated with either saline or 0.3 mg/kg ip of scopolamine immediately after or 30 min before first entering the passive avoidance chamber. Saline animals learned not to enter the dark chamber as did the mice that received the scopolamine immediately after the first day test. The animals that received scopolamine 30 min before did not establish the association.
Fig. 3.6. Taste Aversion in MDA-treated G6PD-normal and G6PD-deficient young female mice.

Adult mice (4-6 months) were kept on a water-limited regimen. Mice were treated with either saline or 20 mg/kg MDA x 4 every 2 hr. 5 days after treatment, during the first 30 min of receiving drink, the animals were given a sweet saccharin solution along with an injection of LiCl to induce a feeling of malaise. The association of the sweet taste to the feeling of malaise was established. The following days the mice were given the saccharin solution in the first 30 min of receiving drink and the amount of solution consumed was recorded. MDA caused an extinction of the aversive learning and G6PD deficiency protected against this extinction. αp<0.05, βp<0.01 compared to the same time point.
Discussion

We have previously studied the protective potential of the antioxidative role of G6PD in aging (Jeng et al. 2013; Loniewska et al. 2013). The increased endogenous oxidative DNA damage in regions of the brain associated with enhanced neuropathological damage in aged G6PD-deficient mice suggested that G6PD is important in protecting the brain from ROS-mediated neurodegeneration associated with aging. In the brain, endogenous ROS may be generated by many pathways, including: (1) high levels of metabolic activity (mitochondrial respiration); (2) enzymatic sources like NADPH oxidases, NO synthases, monoamine oxidases, cytochromes P450 and xanthine oxidoreductase; (3) glutamate excitotoxicity and a disruption in Ca$^{2+}$ homeostasis; (4) redox cycling of endogenous substrates; and, (7) bioactivation of endogenous substrates to free radical intermediates by enzymes like prostaglandin H synthase (Goncalves et al. 2009). In comparison with other organs, the CNS may be more vulnerable to ROS-mediated damage. High levels of oxidative stress and accumulation of macromolecular damage, combined with the relatively lower levels of antioxidative enzymes (Sohal et al. 1990), may render the CNS susceptible to disruption.

We used amphetamine derivatives as ROS-initiating neurotoxins to test the potential role of G6PD in protecting against functional deficits caused by xenobiotic-enhanced oxidative stress in the brain. Evidence for MDMA and METH, as well as structurally related drugs like MDA, has implicated ROS in mediating neuronal damage. Amphetamine-initiated ROS generation has been demonstrated by increased levels of
oxidatively damaged DNA, protein carbonyls and lipid peroxidation in various regions of the brain including the striatum, hippocampus and cortex (Jayanthi et al. 1999; Gluck et al. 2001; Camarero et al. 2002; Fornai et al. 2004; Jeng et al. 2006; Jeng and Wells 2010). Moreover, studies with transgenic mice overexpressing Cu/Zn superoxide dismutase (Cu/ZnSOD) showed resistance to the lethal effects of both MDA and MDMA (Cadet et al. 1994b), as well as the acute and chronic effects of METH on dopaminergic systems (Cadet et al. 1994c; Hirata et al. 1995).

In untreated aged G6PD-deficient mice we previously found an increase of oxidatively damaged DNA (Jeng et al. 2013) and a functional consequence of DNA strand breaks from that oxidation (Loniewska et al. 2013). Herein, when we treated adult animals with MDA, we did not observe any effects on motor coordination measured by the rotarod test. We also did not observe any changes with MDA treatment in aging animals on the rotarod test. We have previously used the rotarod test to determine the effects of enhanced in utero oxidative stress and oxidatively damaged DNA in fetal brain on postnatal motor coordination caused by maternal treatment with methamphetamine in normal (Jeng et al. 2005) and DNA repair-deficient pregnant OGG1 knockout mice (Wong et al. 2008). G6PD deficiency also had no effect on rotarod performance. Previously, in aging animals, we did not observe a deficiency in rotarod performance even with substantial Purkinje cell loss (Loniewska et al. 2013). Our observations suggest that the neuronal systems involved in rotarod performance are not a target for CNS damage in aging mice or in adults treated with MDA.

Additional tests in aging mice were employed to discern deficits in motor function
not detected by the rotarod test. The ledge balance test, like the rotarod test, did not show a drug treatment or genotype effect, despite the loss of cerebellar Purkinje cells, suggesting that these tests may not reflect, or at least may be relatively insensitive in detecting, MDA-initiated neurodegeneration, and hence may not reflect the neuroprotective potential of G6PD in aging. On the other hand, aging mice treated with MDA did exhibit a worsening in the hindlimb clasp reflex, which is possibly indicative of specific damage in the substantia nigra. Animal models for PD with specific damage in the substantia nigra exhibit deficits in the hindlimb clasp test but not in balance tests (Lieu et al. 2013). MDMA-initiated DNA damage was shown specifically in the substantia nigra and striatum (Fornai et al. 2004). The absence of protection by G6PD in the hindlimb clasp test is consistent with our previous studies, in which we found no increase in DNA oxidation or morphological changes in the substantia nigra or striatum in aging G6PD-deficient mice (Jeng et al. 2013). This could mean that the oxidative stress from MDA treatment is not affected by G6PD activity in those particular brain regions. It may be that G6PD is protective against neurotoxins like alcohol or methylmercury that specifically target the cerebellum (Fonnum and Lock 2000).

We previously found that untreated aging G6PD-deficient mice did not exhibit a deficit in cognitive function assessed by passive avoidance or taste aversion compared to age-matched wild-type G6PD-normal mice (Loniewska et al. 2013). Herein we similarly found no effect of MDA on the passive avoidance test in adult or aging animals. We confirmed these results using METH, since other investigators reported differential effects of MDMA and METH on the passive avoidance test. These
investigators also found no effect with MDMA, but they did observe a slight deficit after METH treatment (Murnane et al. 2012). We did not see the same effects in our mice. To ensure that our testing procedure was working correctly, using scopolamine as a positive control, we validated the passive avoidance test for detecting memory deficits, in that animals treated with scopolamine 30 min before entering the passive avoidance chamber did not form an association.

Taste aversion was affected with MDA treatment. One acute dose of MDA 5 days before LiCl association leads to a quicker extinction of conditioned taste aversion. Amphetamines have been previously used to induce taste aversion (Lin et al. 1993; Verendeev and Riley 2012), but here we show that MDA actually can cause a learning deficit in this test. This is the first evidence that MDA neurotoxicity includes deficits in the conditioned taste aversion task. Interestingly, the MDA-initiated toxicity only occurred in G6PD-normal and heterozygous G6PD-deficient mice, although a similar but non-significant trend was observed in homozygous G6PD-deficient mice. Recently we examined hippocampal function in tissue slices and found that G6PD-deficient mice had substantial amplification of both basal synaptic strength and presynaptic short-term plasticity (Loniewska et al. 2013). These enhancements in the hippocampus could lead to a protective effect of G6PD-deficiency in this type of learning deficits.

In these studies, we did not observe a protective role of G6PD in several functional consequences of neurodegeneration associated with aging or amphetamines in adults. In other models of neurotoxicity G6PD was shown to be protective. When G6PD activity was selectively overexpressed in the dopaminergic nigrostriatal system
of mice, it was protective against the toxic effects of the well studied neurotoxin, MPTP (Mejias et al. 2006). As with our earlier results (Jeng et al. 2013; Loniewska et al. 2013), the data for MPTP provided evidence that G6PD has protective potential in the brain. Further gene expression studies in these G6PD-overexpressing mice displayed changes mainly in the expression of proteins related to antioxidant defense, detoxification and synaptic function (Romero-Ruiz et al. 2010).

In conclusion, G6PD deficiencies constitute the most common human enzymopathy, affecting over 400 million people and up to 60% of some populations. Most individuals with a G6PD deficiency are normally asymptomatic, but can exhibit a clinical syndrome in response to an enhanced oxidative insult or exogenous factors (e.g. xenobiotics, ingestion of fava beans). The behavioural tests employed herein failed to show a protective effect of G6PD in aging or in MDA-treated mice. In contrast, we found a paradoxically protective role for G6PD-deficiency in reducing MDA-initiated learning deficits reflected in the passive avoidance test. In light of the substantial electrophysiological effects of G6PD deficiency in aging, it would not be surprising if a broader array of functional tests, perhaps including measures of executive function, were to reveal a neuroprotective role for the functional consequences of oxidative stress enhanced with aging or with exposure to amphetamines. Our results herein suggest that role of G6PD in the behavioural consequences of enhanced CNS oxidative stress is complicated, and that deficiencies in G6PD may be good or bad depending upon the brain region and associated functions, among other factors.
3. SUMMARY, CONCLUSIONS, & FUTURE STUDIES
3.1 SUMMARY AND CONCLUSIONS

The goal of this research was to determine whether G6PD plays a protective role in ROS-mediated neurodegeneration initiated by amphetamines or aging. We tested this hypothesis in aged G6PD-deficient mice, adult MDA-treated G6PD-deficient mice and aged MDA-treated G6PD-deficient mice. We have found that aged G6PD-deficient mice show an increase in oxidatively damaged DNA along with significant neurodegenerative changes in the brain. To corroborate the measurement of oxidatively damaged DNA by HPLC-EC, the comet assay was established, which also reflects a functional consequence of DNA damage. To determine the functional consequences of the molecular and cellular changes in brain with G6PD deficiency, we tested mice in several behavioural tests concentrating on: (1) motor coordination deficits relevant to the loss of Purkinje cells in the cerebellum of aged G6PD-deficient mice; and, (2) cognitive deficits potentially relevant to the cellular and electrophysiological changes found in the hippocampus. The rotarod test previously utilized in our lab, along with the ledge balance test and hind limb clasp test, were used to detect motor deficits. The rotarod test did not show deficits in aged G6PD-deficient mice, but their performance in the ledge balance test declined at an earlier age. The ledge balance test appeared to be a more sensitive test for measuring cerebellar functional deficits resulting from the Purkinje cell loss in G6PD-deficient mice. Overall, the objectives of this research were reached. We have shown that G6PD is important and protective in certain brain areas. As well several functional and cognitive tests were developed and used to test the potential of G6PD deficiency as a risk factor in
neurodegeneration. There was an important factor limiting the types of behavioural tests that could be employed. The G6PD-deficient mice are on a C3H/HeJ background, which are believed to be blind by weaning age due to a genetic mutation causing macular degeneration early in life (Chang et al. 2013). We also showed that amphetamines can cause cognitive and motor deficits.

To detect cognitive deficits, a taste aversion test was developed and the passive avoidance test was used. These tests did not find any measurable functional deficits in aging G6PD-deficient mice. This suggests a number of yet to be determined possibilities including: (1) failure to reach a threshold of macromolecular damage and cellular changes necessary for functional neurodegenerative consequences; (2) the particular tests selected were not sufficiently sensitive to detect cognitive functional changes; (3) the observed macromolecular damage and cellular changes do not have functional consequences in aging; and (4) the presence of compensatory systems to mitigate the biochemical and/or neurodegenerative consequences of G6PD deficiency. Interestingly, we found that G6PD-deficient mice with all alleles mutated survived longer than heterozygous G6PD-deficient mice and G6PD-normal mice. As well, G6PD-deficient mice exhibited increases in both basal synaptic strength and presynaptic short-term plasticity. These results suggest that reduced G6PD activity in aging has not only metabolic effects, but also serious consequences for brain development and function, which may have important clinical implications.

In adult MDA-treated mice a single dose of MDA 5 days prior to the taste aversion learning test was sufficient to cause a learning deficit in adult G6PD-normal
and heterozygous G6PD-deficient mice. The homozygous G6PD-deficient mice did not experience this learning deficit, suggesting another protective role of G6PD-deficiency. Since enhanced hippocampal function was observed in G6PD-deficient mice as described above, the G6PD-deficient mice may be in fact protected from the deleterious effects of MDA in the taste aversion learning paradigm. MDA did not have effects in the rotarod test or the ledge balance tests in adult animals or aging animals. The hindlimb clasping reflex, on the other hand, was affected after an acute dose of MDA, which is possibly indicative of specific damage in the substantia nigra. G6PD was not protective in the hindlimb clasp test since G6PD-deficient animals did not perform any worse than G6PD-normal mice. These behavioural outcomes are consistent with our previous observations. We found no increase in DNA oxidation or morphological changes in the substantia nigra or striatum in aged G6PD-deficient mice which could mean that the oxidative stress from MDA treatment is not mitigated by G6PD activity in those particular cells.

Since G6PD is involved upstream of several pathways (Fig. S1) it is probable that different organs and types of cells are affected by G6PD deficiency in a diverse manner. In the cerebellum, Purkinje cells appeared to be the most sensitive, possibly due a higher level of ROS-mediated oxidative stress and/or a greater dependence upon G6PD for the detoxification of excess ROS. MDA is a neurotoxin in dopaminergic neurons in mice, whereas Purkinje cells are GABAergic neurons, which may have contributed to their resistance to the effects of MDA. In the striatum and substantia nigra, G6PD levels were low, and we also did not observe an increase in DNA
oxidation in these regions, suggesting that G6PD may be less important in these regions. There is a mouse model that selectively overexpresses G6PD activity in the dopaminergic nigrostriatal system of mice, and these animals were protected against the toxic effects of MPTP, which is a selective toxin in this brain region (Mejias et al. 2006).

**Fig. S1. Physiological role of glucose-6-phosphate dehydrogenase (G6PD).**

Glucose-6-phosphate dehydrogenase (G6PD) is the rate-limiting enzyme of the pentose phosphate pathway, which leads to the production of ribose-5-phosphate, a basic structural molecule used in the building of many important cell components. G6PD also maintains NADPH in its reduced form, which is important for reductive biosynthesis and detoxification as well as ROS formation.
As mentioned previously we observed some differences in electrophysiological recordings of measures of several aspects of synaptic strength including LTP. Specifically, hippocampal slices from aging G6PD-deficient mice exhibit amplifications in both basal synaptic strength and presynaptic short-term plasticity but overall normal LTP. It has been proposed that the activation of protein kinase C (PKC) is achieved via a reaction with superoxide both in the presynaptic neuron and the post synaptic neuron (Massaad and Klann 2011). Activated PKC in the presynaptic nerve terminal prolongs the release of neurotransmitters, therefore increased ROS in G6PD-deficient mice could be modulating presynaptic function. As well, activation of PKC in the postsynaptic neuron could be causing an amplified synaptic strength. The lack of an effect on LTP could arise from a selective loss of inhibitory neurons in the hippocampus, which would cause an increase in synaptic transmission in the remaining excitatory neurons, thereby nullifying an effect on LTP.

We also found that homozygous and hemizygous G6PD-deficient animals survived longer than heterozygous G6PD-deficient or wild-type G6PD-normal animals. The mechanism underlying this novel observation is unknown, and could include alterations in NADPH-dependent pathways aside from G6PD, and/or other biochemical changes due to a reduction in the pentose phosphate pathway, perhaps analogous to the increased survival observed in animals on a severely restricted diet. G6PD deficiency has been reported to have apparently contradictory health benefits, perhaps due at least in part to contrasting roles of this enzyme. For example, G6PD deficiency has been reported to reduce: (1) human retinopathy and mortality from cardiovascular
disease in a discreet Mediterranean population (Gupte 2010); (2) cholesterol synthesis, production of superoxide that leads to ROS formation, and reductive stress in mice (Gupte et al. 2006; Hecker et al. 2013a); and, (3) angiotensin II-dependent hypertension (Matsui et al. 2005). In contrast, other studies have reported that G6PD deficiency in mice results in a spectrum of cardiomyopathies including age-associated cardiac hypertrophy (Hecker et al. 2012; Hecker et al. 2013b), which would not be consistent with the enhanced survival observed in our aging G6PD-deficient mice. It has been postulated that G6PD deficiency may decrease the development of cardiovascular disease, but may aggravate already established disease (Hecker et al. 2013a). A population of humans with increased longevity in Sardinia, Italy, has been found to highly conserve a mutant variant of G6PD resulting in an enzymatic deficiency (Long et al. 1967; Cocco et al. 1998; Meloni et al. 2008). On the other hand, this same population exhibited an increase in mortality from non-Hodgkin’s lymphoma in individuals with G6PD-deficiency.

A scheme of proposed detoxifying and pro-oxidant pathways, associated with G6PD and its deficiency, are diagramed in Fig. S2. The potential of a dual role of G6PD is presented. Some effects of G6PD deficiency may be deleterious, like the loss of Purkinje cells, and other effects of the same deficiency may be beneficial, such as an increase in survival. As well, there are some observations that were more difficult to categorize, such as the amplification of synaptic signals. Although G6PD is an enzyme generally studied only with respect to its effects in red blood cells, the research presented in this thesis, as well as previous mouse studies in the developing embryo
and fetus, suggest that G6PD deficiencies merit further studies in humans to determine the clinical implications for development and the aging brain.

Fig. S2. The antioxidative and pro-oxidative duality of G6PD.
3.2 Future Proposed Studies

Our findings indicate that G6PD has a role in the CNS. We also found that the effect of G6PD deficiency varied with the brain region. In the cerebellum, we found a marked reduction in the number of Purkinje cells and a complementary decline in balance in aging. The mechanism underlying this apparently selective loss of Purkinje cells warrants further investigation. We propose that ROS reduces the formation of Purkinje cells and/or enhances their death, which could be determined in primary Purkinje cell lines derived from these animals, and compared to other types of cells resistant to such loss. These cells can be similarly studied following exposure to ROS-initiating toxins. We did not find a protective role of G6PD with amphetamine treatment but using neurotoxins like alcohol or methylmercury that target the cerebellum (Fonnum and Lock 2000) could reveal a more robust protective role for G6PD in that particular brain region.

We also did not find cognitive functional changes in aging G6PD-deficient mice using the tests employed. It is possible that different tests like novel recognition or tests of higher executive function could reveal a further role of G6PD. The taste aversion test proved to be a good measure of learning and memory and can be used in the laboratory with different models of neurotoxicity as a measure of cognition. We observed increased basal synaptic transmission, the cause of which merits further investigation. Electrophysiological measurements can be performed in different areas of the brain, including other areas of the hippocampus as well as recordings from Purkinje cells. The cause of these amplified signals also should be elucidated. It is
possible that a selective loss of inhibitory pathways in the hippocampus could be occurring. Further immunohistochemical analysis of the hippocampus could elucidate which cells are being affected by the deficiency in G6PD.

To better understand the altered biochemical milieu in which G6PD-deficiency leads to ROS-mediated neurodegeneration, a large battery of enzymes and systems can be studied. I did not find a change in catalase activity in the blood of these animals (Appendix – Fig A5) but brain cells may show a more measurable difference in catalase activity. The multitude of other detoxifying enzymes, which often differ in their intracellular localization, can be similarly measured for their contribution to the consequences of G6PD deficiency. Conversely, with regard to ROS formation, the potential of NADPH oxidase activity or NO synthase activity to be affected by G6PD status should also be investigated.

Certain aspects of G6PD-deficiency proved to be interesting in the whole animal model system. G6PD-deficient animals with all alleles mutated survived longer than heterozygous G6PD-deficient and G6PD-normal mice. The mechanism of this is not known, and its elucidation might provide useful therapeutic insights. Clinical studies with G6PD inhibitors are being currently completed (Schwartz and Pashko 2004; Gupte 2010) but because of the effects we found in the aging brain it could be difficult to separate the beneficial and deleterious effects in global enzyme inhibition. The use of a G6PD overexpressing mouse model could prove to be useful in further study of these mechanisms. Survival studies with these mice could show us if over expression of G6PD could have an effect on survival rate. As well, since G6PD is a common human
enzymopathy, the potential of studying the human population is vast. We managed to get some human data with G6PD and cognition from the Neurodevelopmental Effects of Antiepileptic Drugs (NEAD) Study (Meador et al. 2009; Cohen et al. 2011; Meador et al. 2013). Unfortunately the numbers were small and we were not able to discern an effect but the potential of G6PD status can be studied in the human populations particularly in areas of high prevalence like the Mediterranean or Sub-Saharan Africa (Nkhoma et al. 2009).

The comet assay was used as a measure of DNA damage but could be further developed to study specific lesions like 8-oxo-dG with the use of specific enzyme treatments. Including specific DNA repair enzymes has been utilized to modify the Comet assay to measure specific DNA lesions. For example, after lysis, treating the slides with Ogg1 or fpg enzymes creates single strand breaks and therefore increasing the size of the comets in cells that have increased 8-oxo-dG lesions. This test could be used in small samples like brain regions and fetal tissue to study oxidatively damaged DNA in the future.


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4. APPENDIX
1. **Positive Control of Comet Assay – ethyl methanesulfonate treatment**

Comet Assay validated with ethyl methanesulfonate, an alkylating agent.

![Fig. A1. Comet Assay.](image)

DNA damage is shown by representative electrophoresis migration of DNA fragments, or “comet tails” (arrows), in single cells from liver (A-D) and cerebellum (E-H) from C3H/HeJ mice treated with either saline (A,B,E,F) or the DNA alkylating agent ethyl methanesulfonate [EMS] (C,D,G,H). Liver and cerebellar cells were obtained from fresh tissue, embedded onto microscope slides in low melting agarose, and lysed over night in lysis buffer (100 mM EDTA, 2.5 M sodium chloride, and 10 mM TRIS, pH 10, 1% (v/v) triton-X100 and 10% DMSO). Electrophoresis was performed at 0.6 mV/cm and 300 mA in 0.3 M NaOH, 1 mM EDTA buffer (pH>13) for 35 minutes. DNA was stained with SYBR Gold and slides were visualized and photographed with a fluorescence microscope (Zeiss “Axioplan 2 Imaging” upright fluorescence) and a CCD camera. A, C, E, and G are viewed at 20X. B, D, F, and H are representative cells from each treatment group magnified.
2. Olfactory discrimination

Mice were initially tested to see if they could discern a foreign odour at increasing concentrations of the odor. Two petri dishes were presented to the animal in a clean cage. Time spent exploring the water petri dish and the odor petri dish. Only at the highest concentrations the females explored the odor for slightly longer. N for females is 12, N for males is 10. The odor was cinnamon.

Fig. A2. Olfactory Discrimination.

Mice were initially tested to see if they could discern a foreign odour at increasing concentrations of the odor. Two petri dishes were presented to the animal in a clean cage. Time spent exploring the water petri dish and the odor petri dish. Only at the highest concentrations the females explored the odor for slightly longer. N for females is 12, N for males is 10. The odor was cinnamon.
Fig. A3. Detection of threshold of olfactory sensitivity.

Mice were initially tested to see if they could discern a foreign odour at increasing concentrations of the odor. Two petri dishes were presented to the animal in a clean cage. Time spent exploring the water petri dish and the odor petri dish. The ratio of odor exploration to total petri dish exploration is shown. Animals are deemed as sensitive to the odor when this ratio is significantly above 50%. None of the concentrations were able to reach this threshold. With this paradigm, the G6PD mouse model was not sensitive to odor discrimination testing. N for females is 12, N for males is 10. The odor was cinnamon.
3. Temperature with MDA-treatment

**Fig. A4.** Rectal temperature after MDA or saline treatment in G6PD-normal and G6PD-deficient mice. No differences in temperature change with MDA treatment between G6PD-normal and G6PD-deficient mice. The arrows across the x-axis indicate time of each dose of 20 mg/kg of MDA ip.
4. Blood Catalase Activity vs G6PD Activity

Fig. A5. Blood G6PD and catalase activity.

No correlation between G6PD blood activity and catalase blood activity.
5. Weights of G6PD-normal and G6PD-deficient animals throughout age

Fig. A6. Weights throughout life in G6PD-normal and deficient animals.
No differences with weight gain in G6PD-normal and G6PD-deficient mice.
6. G6PD Activity

G6PD Activity Assay

**Measurement of NADPH by subtraction.**

The G6PD activity assay measures the rate of production of NADPH which is the product of G6PD but NADPH is also a product of 6-phosphogluconate dehydrogenase (6-PGD) downstream in the pentose phosphate pathway. The second source of NADPH is confounding to the measurement of G6PD activity and therefore has to be subtracted from the total NADPH measure. This is done using the method of Glock and McLean (1) which takes into account the second source of NADPH and corrects for it.

Glucose-6-phosphate + NADP+

G6PD

6-phosphogluconolactone + NADPH

6-PGA + NADP+

6-PGD

Ribulose-5-phosphate + NADPH

**Total rate of production measured at 340 nm**

**Measured separately and subtracted from the total rate of production**

---

Fig. A7. G6PD activity assay principles
# G6PD Activity Assay

We used a modified protocol of Glock and McLean which consists of a 4 cuvette system corrected for a blank reading (cuvette 1) and for the 6-PGD activity (cuvette 3). Reagents were added to cuvettes with a final volume of 1 mL.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Cuvette</th>
<th>1 (µl)</th>
<th>2 (µl)</th>
<th>3 (µl)</th>
<th>4 (µl)</th>
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<tr>
<td>Tris-HCl, 1 M, EDTA, 5mM, pH 8.0</td>
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<tr>
<td>MgCl₂, 0.1 M</td>
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<tr>
<td>NADP, 2 mM</td>
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<td>25</td>
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</tr>
<tr>
<td>Distilled water</td>
<td></td>
<td>750</td>
<td>725</td>
<td>675</td>
<td>625</td>
</tr>
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</table>

*Incubate 10 min. at 37 degrees C*

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<th>2 (µl)</th>
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<tbody>
<tr>
<td>10 mM glucose-6-phosphate</td>
<td></td>
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<td>---</td>
<td>50</td>
</tr>
<tr>
<td>10 mM 6-phosphogluconic acid</td>
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Rates were read with the Beckman DU 640 Spectrophotometer at 340 nm at 15 sec intervals for 5 minutes total.

<table>
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<tr>
<th>Net optical density</th>
<th>Net O.D./6.22 x 1/0.025</th>
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<tr>
<td>= rate of cuv. 4 − (rate of cuv. 1 + cuv. 3)</td>
<td>= µmol/min/ml of homogenate (IU/ml)</td>
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</table>

*Fig. A8. G6PD activity assay set up.*
7. Neurodevelopmental Effects of Antiepileptic Drugs (NEAD) Study

The NEAD study was used to determine the neurodevelopmental effects of in utero exposure of antiepileptic drugs. The four drugs studied were carbamazepine (*CBZ*), phenytoin (*PHT*), valproate (*VPA*) and lamotrigine (*LTG*). Children of mothers who took epileptic drugs were followed from birth until 6 years of age. Adverse birth events were recorded and various measures of motor and cognitive development were measured. In the scope of this thesis, the G6PD activities of some of the mothers and children were measured. Some questions we tried to answer were:

1. Is there a different in G6PD activity in the child compared to the mother and if there is a correlation between the two activities?
2. Can G6PD activity modulate the number of adverse birth events linked to oxidative stress?
3. Can G6PD activity in the child modulate the neurodevelopmental outcomes measured in the children?
Fig. A9. Maternal and child G6PD activity.

Child G6PD was significantly higher than mother. P<0.01.
Fig. A10. Child G6PD did not correlate to maternal G6PD.
Fig. A11. Child G6PD activity did not affect oxidative stress birth outcomes.
Fig. A12. Maternal G6PD activity did not affect oxidative stress birth outcomes.
Fig. A13. G6PD activity and neurodevelopmental measures.

G6PD activity did not have an effect on infant mental and motor development in separated epileptic drug treatments. The Bayley Scales of Infant Development (BSID) measures the mental and motor development and test the behavior of infants from one to 42 months of age.
Fig. A14. G6PD activity and IQ.

G6PD activity did not have an effect on toddler IQ. The Bayley Scales of Infant Development (BSID) measures the mental and motor development and test the behavior of infants from one to 42 months of age. The Differential Ability Scales is an individually administered battery of cognitive and achievement tests for children and adolescents aged 2 years, 6 months through 17 years, 11 months. Because the DAS covers such a wide age range, it is divided into three levels: Lower Preschool (ages 2 years, 6 months through 3 years, 5 months), Upper Preschool (aged 3 years, 6 months through 5 years, 11 months), and School-Age (6 years, 0 months through 17 years, 11 months). In this study the Lower Preschool level was utilized. The DAS was designed to measure specific, definable abilities and to provide interpretable profiles of strengths and weaknesses. The DAS also contains three achievement tests, co-normed with the cognitive battery, which allows direct ability-achievement discrepancy analysis. The DAS is considered suitable for use in any setting in which the cognitive abilities of children and adolescents are to be evaluated, although many of the DAS subtests are not appropriate for students with severe sensory or motor disabilities. The DAS cognitive battery yields a composite score labeled General Conceptual Ability (GCA) that is defined as "the general ability of an individual to perform complex mental processing that involves conceptualization and transformation of information".
Fig. A15. Maternal or child G6PD activity did and IQ – drugs combined.

All treatment groups combined. Mental and motor BSID scores were measure at 2 years and DAS-GDA scores were measured at 3 yr. (Tests described in Figs A13, and A14)
Our observations from NEAD data may be due to:

- G6PD deficiency alone is not sufficient to increase risk of adverse oxidative birth outcomes
- There are insufficient subjects with a severe G6PD deficiency to adequately test the hypothesis
- ROS and G6PD activity do not contribute to the outcomes measured in this study
- More time may be required for the neurodevelopmental differences to become pronounced