Cerebellar degeneration in harlequin mice is associated with inflammation unaltered by low-dose phenobarbital treatment

Anita Prtenjaca
The University of Western Ontario

Supervisor
Dr. Kathleen Hill
The University of Western Ontario

Graduate Program in Biology
A thesis submitted in partial fulfillment of the requirements for the degree in Doctor of Philosophy
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Cerebellar degeneration in *harlequin* mice is associated with inflammation
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By:

Anita Prtenjaca

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The thesis by

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ABSTRACT

Canadian population demographics are shifting to an increase in aged individuals and an increase in the prevalence of neurodegenerative diseases. The post-mitotic nature of most neurons highlights the need to understand the etiology and early mechanisms in neurodegenerative diseases and design targeted therapies. Currently, the etiologies of neurodegeneration are poorly understood but oxidative stress, mitochondrial dysfunction and inflammation are early mechanisms in Alzheimer’s disease, Parkinson’s disease and Amyotrophic Lateral Sclerosis. An anti-aging strategy that can be adapted for use in neurodegeneration is hormesis, where repeated low-level exposures to stressors are beneficial to the cell. Hormesis has demonstrated efficacy in inhibiting hepatocarcinoma in the rat through administration of dietary phenobarbital, which decreases DNA damage, increases DNA repair, and decreases cell proliferation. Given that phenobarbital decreases DNA damage and increases DNA repair, phenobarbital was tested as a hormetic agent in murine neurodegeneration. The harlequin (hq) mouse is a model of mitochondrial dysfunction and oxidative stress with cerebellar degeneration. One-month-old wild type (WT) and hq mice were administered phenobarbital in drinking water at 0, 2 or 4 ppm provided ad libitum, until euthanized at 3, 7, or 10 months of age. Eleven parameters of nocturnal behaviour were examined in WT and hq mice (n=9 to 14). In situ, post-mortem cerebellar tissue sections were examined for neuron loss, cell damage, and reactive oxygen species (ROS) (n=3). In vivo cerebellar mutations were detected using the Big Blue® cII mutation assay (n=5 or 6). The transcriptome was analyzed to survey global markers in hq cerebella and response to phenobarbital exposure (n=3). The hq phenotype had no behavioural changes, but had increased neuron loss, limited cell
damage, a mutation signature of ROS, and elevated ROS with age. Phenobarbital administration did not prevent cerebellar degeneration in *hq* mice. Transcriptome data revealed inflammation as an early disease mechanism in *hq* mice. In *hq* mice, a down-regulation in the GABA_A receptor was found which potentially limits the efficacy of phenobarbital. The *hq* mouse could benefit from a strategy addressing mitochondrial dysfunction through supplementation with riboflavin, thus increasing activity of complex I of the electron transport chain and increasing ATP production.

**Key words:** *harlequin* mouse, cerebellar degeneration, *Apoptosis-inducing factor*, phenobarbital, hormesis, inflammation, mitochondrial dysfunction, aging, murine behaviour, Big Blue® *cII* mutation detection assay, oxidative stress, microarray.
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<table>
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<tr>
<td>3xTg-AD</td>
<td>Triple Transgenic Alzheimer’s Disease mouse</td>
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<td>8-OHdG</td>
<td>8-hydroxy-2’-deoxyguanosine</td>
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<tr>
<td>Acta2</td>
<td>Actin Alpha-2 Smooth Muscle Aorta</td>
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<td>AD</td>
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<td>Aldh3b2</td>
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<td>AIF</td>
<td>Apoptosis-Inducing Factor</td>
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<td>ALS</td>
<td>Amyotrophic Lateral Sclerosis</td>
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<tr>
<td>AMP</td>
<td>Adenosine Monophosphate</td>
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<td>AMPT</td>
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CHAPTER ONE - INTRODUCTION

1.1 Aging-associated neurodegenerative diseases

Neurodegenerative diseases are associated with substantial economic impact on society. In 1998, the cost of neurodegenerative diseases totaled over $400 million dollars, which included hospital stays, treatments, and money lost from long-term disability and premature death [1]. As of the last Canadian census, individuals aged 65 and over made up a record 13.7% of the total population [2]. Last year, 2011, was momentous as it marked the year the first cohort of the baby-boomer generation reached the age of 65. The proportion of individuals over the age of 65 is expected to continue increasing until 2056, at which point seniors will constitute 25% to 30% of the total population, more than double the current proportion [2]. As the proportion of Canadians over the age of 65 increases, so will the frequency of neurodegenerative disease, as it is highly correlated with age [3]. Current estimates are that over 800,000 Canadians suffer from either Alzheimer’s disease (AD), Parkinson’s disease (PD), or Amyotrophic Lateral Sclerosis (ALS) [2]. Biomarkers for neurodegeneration include testing cerebral spinal fluid for amyloid β and/or tau in AD [4], or tumor necrosis factor-α (TNF-α), and 8-hydroxy-2’-deoxyguanosine (8-OHdG) in PD [5], and glutamate in ALS [6], but lumbar punctures required to obtain cerebral spinal fluid are painful and invasive procedures. Positron emission tomography imaging can be used to visualize the location of neurofibrillary tangles and amyloid plaques in AD [7], the decrease of dopamine transporters with disease progression in PD [8], or to measure glucose hypermetabolism in ALS [9], all which require the injection of radioactive tracers. Even with the development of early biomarkers, by the time symptoms appear, a significant number of
neurons have already been lost [10, 11]. Currently we are unable to prevent neurodegenerative diseases and the treatments that are available are limited to alleviating symptoms and are not able to slow disease progression or cure neurodegeneration. A limiting step in drug development is determining the etiology and mechanisms of neurodegeneration.

1.2 Genetic basis behind early-onset neurodegenerative diseases

Families with early-onset neurodegenerative disorders due to single-gene mutations have been identified and display simple Mendelian inheritance patterns [12-14]. Single-gene mutations in *Amyloid precursor protein*, *Presenilin-1*, *Presenilin-2*, *α-Synuclein*, *Parkin*, and *Copper/zinc superoxide dismutase* genes have been identified in familial AD [14-17], PD [12, 18], or ALS [13]. Single-gene mutations cause 5% of the cases of neurodegeneration [19-21], although most individuals who develop late-onset neurodegeneration have no family history of neurodegenerative disorders. Thus early-onset neurodegenerative disorders are a limited model when studying late-onset neurodegenerative diseases.

1.3 Late-onset neurodegenerative disorders

The etiology of late-onset neurodegeneration is poorly understood, has no clear genetic basis and combines complex interactions between genes and the environment. Several susceptibility genes have been identified in sporadic cases of neurodegeneration, but brain injuries and environmental exposure to pesticides as well as heavy metals all increase the risk of developing neurodegeneration. Individuals who experience a
traumatic brain injury have an increased risk of AD [22] that is further exacerbated when they also have the epsilon 4 allele of the Apolipoprotein E gene [23]. Individuals chronically exposed to pesticides have a 70% higher incidence of PD compared to individuals not chronically exposed to pesticides [24]. It is thought that the link between pesticides and PD lies in the activity of the enzymes that are involved with the detoxification, metabolism, and transport of xenobiotics. The cytochrome P450 enzyme 2D6 (CYP2D6) is involved in the metabolism of pesticides. Individuals with a polymorphism in the CYP2D6 enzyme causing lower CYP2D6 activity and poor pesticide metabolism have a higher incidence of PD [25, 26]. In ALS patients, elevated iron levels are detected [27] that can be due to alterations in a number of genes involved in iron transport [28]. Iron enters neurons though a complex composed of transferrin and its receptor [29, 30] and the gene Ferroportin-1 is responsible for neuronal iron export [31]. Dysregulation of genes involved in iron transport could lead to increased intracellular levels of iron, causing oxidative stress, inflammation, and microglial activation [28]. This highlights the importance of gene and environmental interactions and the complexity of disease triggers and genetic basis of disease mechanisms.

1.4 Early mechanisms underlying aging-associated neurodegeneration

1.4.1 Oxidative stress as a common denominator in aging-associated neurodegeneration

One suggested theory of neurodegeneration is that aged neurons have increased levels of oxidative stress with negative impact on lipids, proteins, RNA, and DNA. Oxidative stress is caused by reactive oxygen species (ROS), metabolites of molecular oxygen that include lipid peroxide, hydrogen peroxide, and the superoxide anion [32].
The free radical theory of aging suggests that ROS are generated as a byproduct of metabolism and cause damage to cellular components that accumulate over time, resulting in aging and death [33]. The brain is particularly susceptible to oxidative stress because of its high levels of oxygen consumption [34], metabolic activity [35], and concentration of polyunsaturated fatty acids (PUFA) [36, 37]. Oxidative stress-induced damage to proteins, lipid membranes, and nucleic acids are observed in neurodegenerative diseases [38–40]. Protein oxidation leads to structural alterations and subsequent loss of enzymatic activity critical for neuronal function [41]. Membrane integrity is disrupted given the vulnerability of PUFAs to free radical attack since double bonds allow easy removal of hydrogen atoms by ROS [42]. Unrepaired DNA damage can result in an accumulation of DNA adducts in both nuclear and mitochondrial genomes and can inhibit binding of transcriptional and regulatory factors. Lipofuscin is a fluorescent pigment composed of approximately 60-70% proteins, 16-27% oxidized lipids, and 1.0 – 1.7% metals [43] that accumulates with age in lysosomes of post-mitotic cells such as cardiac myocytes [44], retinal cells [45], and other neurons [44, 46]. The presence of lipofuscin is detected with age in neurons in numerous organisms including rodents [46], monkeys [47], and humans [48]. Lipofuscin cannot be degraded by lysosomal enzymes due to the presence of peptides cross-linked by aldehydes into plastic-like structures [49] that is of particular importance since lipofuscin can negatively impact cellular processes including protein degradation [45]. Collectively, oxidative damage can lead to abnormal functioning of neurons and apoptosis or necrosis.
1.4.2 Mitochondrial dysfunction in aging-associated neurodegeneration

The mitochondria are responsible for producing energy in the form of adenosine triphosphate (ATP) during oxidative phosphorylation but they are also the major source of ROS. NADH is oxidized by complex I of the electron transport chain and the electrons are transferred to coenzyme Q while pumping protons across the mitochondrial membrane, creating a proton gradient [50]. Approximately 4-5% of the time, oxygen is capable of stealing electrons from the electron transport chain components to generate the superoxide radical [51]. The main sources of the superoxide radical are complexes I and III of the electron transport chain [52, 53]. Mitochondria are particularly vulnerable to ROS damage as the mitochondrial DNA is in close proximity to ROS production and does not contain histones or other DNA-associated proteins and thus is exposed directly to attack by ROS. Mitochondrial DNA lacks introns [54] and has a low repair rate [55], resulting in a high probability of ROS damaging or modifying a coding region. As a result of damage to mitochondrial components, there is a decrease in the amount of energy production and an increase in ROS production which can lead to apoptosis [56]. Both decreased energy production and mitochondrial dysfunction have been detected in AD [57, 58], PD [59, 60], and ALS [61].

1.4.3 Inflammation in aging-associated neurodegeneration

Inflammation, which is due to the activation of the immune response, is generally protective in that it is an attempt by the organism to remove invading microorganisms as well as damaged and dying cells to initiate the healing process. Inflammation in the central nervous system occurs through the activation of microglia. Microglia are the
resident macrophages of the central nervous system [62], capable of scavenging infected [63], damaged [64], or apoptotic neurons [65]. Once microglia become activated, they express surface receptors including the major histocompatibility complex [66] and complement receptors [67]. These cells also undergo changes in morphology from a ramified shape to a mobile amoeboid shape [68]. Activated microglia can secrete complement proteins [69], cytokines [70, 71], chemokines [72], ROS [73], proteases [74, 75], excitatory amino acids [76], and nitric oxide [77]. If the agent causing inflammation is not removed, then this can then lead to constant microglial activation and a chronic inflammatory state. Activated microglia can be found in close proximity to amyloid β deposits in AD [78] and amyloid β is known to stimulate the release of interleukin-1 (IL-1) [72], interleukin-6 (IL-6) [79], tumour necrosis factor-α (TNF-α) [72, 80], macrophage inflammatory protein-1 α (MIP-1α) [72], monocyte chemoattractant protein-1 (MCP-1) [79], nitric oxide [77], and superoxide radicals [81] from activated microglia. Activated microglia are detected in the substantia nigra of PD patients [82]. Similar to AD, activated microglia in PD release TNF-α [83], IL-1β, interferon-γ (IFN-γ) [84], inducible nitric oxide synthase (iNOS) [83], and cyclooxygenase-2 (COX-2) [85]. The release of inflammatory cytokines as well as factors released from dying neurons can amplify the inflammation and lead to further neuronal injury and death. Microglial activation is found in the prefrontal cortex, motor cortex, thalamus, and the pons in patients with ALS [86]. Targeting the inflammatory response may be of therapeutic benefit since nonsteroidal anti-inflammatory drugs decrease the risk of AD [3], inhibition of iNOS provides neuroprotection in PD [87], and minocycline improves motor performance and survival in a mouse model of ALS [88].
1.4.4 Gene expression in aging-associated neurodegeneration

The neurodegenerative disease process is complex and involves the interaction of many different pathways and genes. Changes in the gene expression profile with neurodegenerative diseases can provide information about the onset and progression of the diseased state and lead to understanding of the disease pathology. In AD, an up-regulated immune response involving complement genes is detected in the entorhinal cortex, hippocampus, and midtemporal gyrus in the brain [89]. Elevated expression of chemokines and cytokines in AD [90] are capable of inducing iNOS activity [91]. Cytokines are also able to activate microglia [92] and astrocytes [93], further propagating inflammation. There is reduced expression of genes involved in energy metabolism leading to decreased mitochondrial function and ATP production in AD [94]. Also, there is a down-regulation in genes that code for cytoskeleton proteins [95] that can lead to dysfunction in cellular transport [96].

Gene expression changes in PD include up-regulated genes involved in the immune and inflammatory response [97], indicating a role in the activation of glial cells in PD pathology. In PD, there is an up-regulation of genes involved in apoptosis leading to increased cell death [98]. Genes involved in cytoskeleton maintenance are down-regulated [98] that can affect cellular trafficking, cell-to-cell contact, and stability. Mitochondrial genes involved in proper function and energy synthesis are down-regulated [97, 98] that could lead to impaired energy production. There is a down-regulation in an antioxidant enzyme [98], leading to decreased protection in the cell from ROS [99]. Genes involved in proteolytic machinery to degrade misfolded and damaged proteins are down-regulated [97, 98] that can lead to accumulation of aggregated proteins
and inhibition of proteasomal function [100]. Genes involved in synaptic function (γ-aminobutyric acid (GABA) transporter and receptors), are down-regulated [98] that disrupts signal transmission and communication between neurons. Ion channels are also down-regulated [97, 98], leading to altered maintenance of the electrochemical gradients. Dysregulation of ion channels has been implicated in PD pathogenesis [101].

Gene expression changes in ALS diseased brains include an up-regulation of glutamate receptors [102, 103] that can lead to excitotoxicity [104]. There is down-regulation of genes involved in zinc and iron homeostasis [102], suggesting a role of metal imbalance in ALS [105]. Down-regulation in proteasomal function is also detected in ALS [102, 103] and can lead to an increase in misfolded proteins [106]. Genes involved in the organization of the cytoskeleton are also down-regulated [102, 103] and can lead to disruption in axonal transport [107]. There is also a down-regulation of genes that inhibit apoptosis [102], thus promoting apoptosis [108, 109]. Genes involved in mitochondrial metabolism and energy production are down-regulated [102, 103] and can lead to impaired energy production. Genes associated with neuroprotection, such as heat shock proteins and antioxidants are down-regulated [102, 110], indicating a lack of ability to deal with increased oxidative stress and inflammation. Up-regulation of genes involved in inflammation cascades including the Interleukin-1 receptor accessory protein (IL-1RAP), the Chemokine (C-C motif) ligand 5 (CCL5), and Glial fibrilary acidic protein (GFAP) have been identified [111]. IL-1RAP is a transmembrane receptor complex that binds to IL-1 [112], which can lead to the downstream transduction of inflammatory effects of IL-1 [113]. The cytokine CCL5 is a proinflammatory
chemoattractant for glial cells [114] and GFAP, a marker of astrogliosis [115], indicates that glial cell activation is a pathological event in ALS.

1.5 The use of mice in studying neurodegeneration

Studying neurodegeneration in humans is difficult to do, as scientists cannot control for diet, environment, or the genetics of human subjects, not to mention that acquiring access to neurodegenerative human brain tissue could be a lengthy process due to limited supplies from the declining rate of autopsies [116]. Also, precautions need to be taken when handling human tissue, as it may contain infectious agents such as the hepatitis virus, human immunodeficiency virus, and prions, all of which can be transmitted to other humans [117]. Model organisms of neurodegeneration are valuable tools allowing the study of the pathological mechanisms as well as new treatments in complex diseases. *Drosophila melanogaster, Caenorhabditis elegans* and *Danio rerio* have been used to study neurodegenerative disease mechanisms [118-120], but their simpler nervous systems limit the translation to human-specific neurodegenerative diseases. Mice are commonly used in neurodegenerative research as a mammalian model of human disease due to similar genetic composition to humans with 99% of human genes having a mouse homolog [121]. Mice also have similar physiology to humans [122], are small in size, easy to handle and house, and have large litter sizes and short generation times. Despite the obvious difference in size, all the major brain structures that are present in humans are also present in rodents. However, one major difference is that in humans, the cortex contains a large area devoted to vision, while in rodents the visual area is smaller since they rely more on auditory and olfactory cues. Despite these differences, gene expression
in the brain is similar between the two species [123]. Thus mice can be useful tools in studying neurodegeneration.

1.6 Mice having aging-associated neurodegeneration

Mice show limited neuron loss with aging [124-126], but transgenic animal models have been created in order to mimic human neurodegenerative diseases. Although no transgenic model is able to replicate exactly all features of a particular neurodegenerative disease, mouse models can nonetheless display the different disease pathologies. The triple transgenic mouse (3xTg) for AD, (3xTg-AD), is the only AD mouse that displays both amyloid β plaques and neurofibrillary tangles [127]. At 6 months of age, the 3xTg-AD mouse has a 14.8 fold up-regulation of the pro-inflammatory cytokine, TNF-α, and a 10.8 fold up-regulation of the pro-inflammatory chemokine, MCP-1, as well as activated microglia in the entorhinal cortex [128]. The 3xTg-AD mouse also shows elevated oxidative stress, a 20% reduction in hippocampal mitochondrial DNA, and decreased complex IV and V of the electron transport chain [129]. Another 3xTg-AD mouse model shows decreased activity of complex I and IV in the mitochondrial electron transport chain, decreased membrane potential, and increased ROS [130].

A PD mouse model that overexpresses α-synuclein leads to microglial activation, increased TNF-α, IL-1β, COX2, NOX2, iNOS, and increased ROS production [131]. α-Synuclein localizes to complex I in neuronal cells [132] and it has been documented in vitro and in vivo that overexpression of α-synuclein inhibits complex I and increases ROS production [133, 134].

A mouse model of ALS that contains a mutated Superoxide dismutase-1 (mSOD1)
gene with a glycine to alanine substitution at codon 93 (G93A), has swollen mitochondria, decreases in activity of complex IV of the electron transport chain, and elevated levels of ROS [99]. In cell cultures, extracellular mSOD1-G93A is capable of activating microglia [135] but activated microglia containing the mSOD1-G93A are more neurotoxic than activated wild type (WT) microglia [136], likely through the elevated production of superoxide anions, nitric oxide, and peroxynitrite radicals [99]. In fact, replacement of microglia in mSOD1-G93A transgenic mice with microglia expressing WT SOD1 slows disease progression and increases survival [137], indicating microglial activation as a source of neuroinflammation in mSOD1-G93A mice.

1.7 Altered mouse behaviour with neurodegeneration

In humans, neurodegenerative diseases are characterized by cognitive decline and motor deficits including slower gait and decreased stride length that can be detected in individuals prior to confirmation with post-mortem analysis [138-141]. Behavioural differences can also be detected in mouse models of neurodegeneration. The 3xTg-AD mouse has similar locomotor activity as non-transgenic WT controls prior to disease onset and has decreased activity at 15 months of age, but with no impairment in balance [142]. A different mouse model of AD, expressing human P301S tau protein, demonstrates elevated locomotor activity but decreased motor co-ordination compared to non-transgenic WT controls [143]. Mouse models of PD caused by administration of the neurotoxin 1-methyl 4-phenyl 1,2,3, 6-tetrahydropyridine (MPTP), depleting the brain of dopamine, display motor deficits [144, 145]. The major changes include a decrease in stride length and duration that leads to an increase in frequency of steps, indicating that
MPTP-treated mice are taking more steps in a shorter period of time when compared to controls [145]. A different mouse model of PD, which overexpresses a mutated version of the human \(\alpha\)-Synuclein gene, displays decreased locomotion, with impaired motor coordination and balance [146]. The ALS mouse model containing a G93A substitution in the \textit{SOD1} gene shows gait disturbances with an increase of time spent in the propulsion portion of the stride, an increase in stride time, a decrease in stride frequency, and a decrease in walking velocity [147]. Thus, mouse models of neurodegeneration exhibit phenotypes consistent with affected humans suggesting that they are useful tools in the study of neurodegenerative diseases through the monitoring of early biological markers of altered animal behaviour. Early markers widen the window of opportunity for treatment to delay or prevent the occurrence of severe disease symptoms.

**1.8 Human cerebellar ataxias are a model of neurodegeneration**

Human cerebellar ataxias have a strong behavioural component and the genetics behind the most common cerebellar ataxias in humans are well known. The most common autosomal recessive cerebellar ataxia is Friedreich’s ataxia due to a GAA triplet repeat expansion in the \textit{Frataxin} gene [148]. Ataxia telangiectasia is the second most common autosomal recessive cerebellar ataxia due to a mutation in the \textit{Ataxia telangiectasia mutated} gene [149]. There are also autosomal dominant cerebellar ataxias that are caused by the trinucleotide CAG repeat expansion leading to spinocerebellar ataxia [150]. Cerebellar ataxias have early onset of disease [151, 152] and known genetic etiologies making these diseases very specific examples of neurodegeneration. To understand general mechanisms of aging-associated neurodegenerative disorders, genetic
models of cerebellar degeneration with predominant neurodegenerative phenotypes are needed.

1.9 The cerebellum as a model for studying neurodegenerative diseases

The brain is a complex tissue to study as it consists of different anatomically and histologically distinct areas with different neuronal and non-neuronal cell types [153]. Also, neurodegeneration tends to occur in distinct regions of the brain such as the cerebral cortex and hippocampus in AD, substantia nigra in PD, and in upper and lower motor neurons in the motor cortex, brain stem, and spinal cord in ALS. Thus, in order to study pathological changes in the brain, it would be better to focus on a specific area with a certain cell type rather than a whole brain region.

The cerebellum is an ideal brain area of study since the majority of neurons are located in the cerebellum [154]. Also, the cerebellum shows degenerative changes in humans with neurodegenerative disorders such as AD, PD, and ALS. Atrophy of the neuronal layers, decreased neuronal density, decreased synapses, and amyloid β accumulations are observed in the cerebella of patients with AD [155-159]. Atrophy of the posterior cerebellum is also associated with poorer cognitive performance in individuals with AD [160]. In PD, there is a reduction in the amount of cerebellar inhibition leading to the generation of postural tremor [161]. Changes in the cerebella have been reported in ALS, including loss of Purkinje cells and basophilic inclusions, which are a pathologic feature in ALS [162]. Also, uncoordinated movement is a common feature in neurodegenerative diseases [163-165] and the cerebellum plays a role in motor control [166].
1.10 The function and organization of the cerebellum

The cerebellum is involved in motor control but specifically co-ordination and fine-tuning of movements. Electrical stimulation of the cerebellum does not cause sensation or produce motor movements, yet the cerebellum is vital for movements that require co-ordination of different muscle groups like running and playing the piano. The role of the cerebellum in movement was first discovered after World War I, where individuals with lesions in the cerebellum lost the ability to move properly [167]. Their movements were ataxic and they had problems with muscle weakness, posture, and balance without signs of paralysis [167, 168]. The cerebellum receives sensory information from voluntary muscle movements, compares those movements with what was intended by the motor cortex, and is able to make corrective adjustments during execution [169-171].

In order to understand the function of the cerebellum, knowledge of the structure and organization of the cerebellar circuitry is needed. The cerebellum is located at the base of the brainstem and can be divided medio-laterally and anterior-posteriorly (Figure 1.1a). Medio-laterally the cerebellum can be divided into the vermis and two lateral hemispheres [172]. In the anterior-posterior direction, two fissures divide the vermis cerebellum into three major lobes. The primary fissure separates the anterior and posterior lobes, and the posterolateral fissure separates the flocculonodular lobe from the
Figure 1.1 – A dorsal view of the mouse brain. A) The location of the cerebellum in relation to other structures of the mouse brain. The cerebellum is divided medio-laterally into the vermis and the hemispheres. The image of the whole mouse brain was taken by Eric Diehl. B) A schematic diagram of the unrolled cerebellum from anterior to posterior. The fissures separate the cerebellum into different lobes.
posterior lobe [172] (Figure 1.1b). Due to the fact that the cerebellum contains the majority of neurons in the brain in such a small space [173-175], the cerebellum is intricately folded and the lobes can be further divided into ten lobules numbered with Roman numerals I–X [172] (Figure 1.2a). Gene expression patterns however, suggest that there are four different zones including the anterior zone (AZ: lobules, I-V), the central zone (CZ: lobules VI, VII), the posterior zone (PZ: lobules VIII, IX), and the nodular zone (NZ: lobule X) [176].

The cerebellum consists of four distinct layers (Figure 1.2b). The molecular layer contains scattered stellate and basket neurons that are inhibitory in nature. The next layer is the single cell layer of Purkinje cells. Underneath the Purkinje cell layer is the granule layer, which consists of densely packed granule cells and scattered Golgi cells with a granule cell to Golgi cell ratio of 414:1 [177]. And lastly, the white matter layer contains myelinated afferent (input) and efferent (output) fibers as well as the deep cerebellar nuclei. The afferents to the cerebellum are climbing fibers and mossy fibers (Figure 1.3). The climbing fibers originate from the inferior olive in the medulla oblongata in the brainstem and synapse on the deep cerebellar nuclei as well as on Purkinje cells in the cerebellar cortex. Mossy fibers on the other hand originate from various regions including the spinal cord and the precerebellar nuclei in the hindbrain [178], and synapse on the deep cerebellar nuclei [179] and granule cells [180]. Both climbing and mossy fibers are excitatory. Climbing fibers release aspartate [181] and glutamate [182], and mossy fibers release glutamate [183] and acetylcholine [184, 185]. The axons of granule cells, also called parallel fibers, synapse onto the dendrites of Purkinje, basket, stellate, and Golgi cells and are able to induce excitation.
Figure 1.2 – The structure and organization of the mouse cerebellum.  

A) An image of a sagittally sectioned mouse cerebellum stained with hematoxylin and eosin showing the organization of the lobules labeled with Roman numerals I-X [172].  

B) Histology of the four different layers in the mouse cerebellum.  The cryopreserved cerebellum was sagittally sectioned to a thickness of 10 µm and stained with hematoxylin and eosin.  Arrows are pointing to the different layers of the cerebellum.  The molecular layer contains stellate and basket neurons.  The Purkinje cell layer is a single cell layer comprised of Purkinje cells.  The granule layer contains granule cells and Golgi cells.  The layer of white matter contains both afferent (input) and efferent (output) fibers of the cerebellum.  Scale bars: A = 1 mm, B = 100 µm
Figure 1.3 - A schematic diagram of the circuitry of the cerebellum. Input into the cerebellum in the form of mossy and climbing fibers originates in the precerebellar nuclei and the inferior olive respectively. Mossy fibers synapse onto granule cells and the deep cerebellar nuclei and climbing fibers synapse onto Purkinje cells and the deep cerebellar nuclei. Purkinje cells are the point of convergence of multiple signals from climbing fibers, granule cells, basket cells, and stellate cells. Purkinje cell output can be inhibited by basket and stellate cells and excited by granule cells. Purkinje cells then send inhibitory signals to the deep cerebellar nuclei in the white matter. Golgi cells can inhibit granule cell output. Mossy fibers, climbing fibers, and granule cells induce excitation, all other neurons in the cerebellum induce inhibition.
Basket cells and stellate cells inhibit Purkinje cells, and Golgi cells inhibit the mossy fiber-granule cell synapse [180]. Purkinje cells are the point of convergence of separate incoming signals and are the sole efferents of the cerebellar cortex, which is inhibitory in nature. The axons of Purkinje cells synapse onto the deep cerebellar nuclei in the white matter of the cerebellum [186], as well as the vestibular nuclei located in the medulla [187]. The efferent fibers of the deep cerebellar nuclei send excitatory signals to the thalamus and various regions in the brainstem including red nucleus, the vestibular nuclei, and the inferior olive [188, 189] (Figure 1.4). The thalamus receives input from the cerebellum and the basal ganglia, and projects the input to the motor cortex [190, 191]. The brainstem sends output to muscles [192] and the motor cortex [193, 194] and the motor cortex relays the information to the brainstem. Mossy and climbing fibers of the precerebellar nuclei and inferior olive synapse on granule cells and Purkinje cells respectively, but also on deep cerebellar nuclei in the cerebellum, thus forming the cerebrocerebellar communication loop.

1.11 Age-associated changes in the cerebellum

The human cerebellum shows a number of age-associated changes including the reduction of cerebellar mass, loss of white matter volume, reduction of the volume of the perikaryon, and loss of Purkinje and granule cells [195]. A previous study determined that there is a 27% reduction of myelinated fibers with age in the cerebrum [196], which could potentially be the origin of the decrease in white matter in the cerebellum. Gene expression analysis of the aging cerebellum in mice shows alterations in lipid biosynthesis [197], possibly leading to defective myelin synthesis and signal transduction. Aged mice also have a reduced number of Purkinje cells [124, 198, 199],
Figure 1.4 – A schematic diagram of the involvement of the cerebellum in coordinated movement. To initiate movement, the motor cortex sends signals to the muscles and the cerebellum. The cerebellum receives input from the motor cortex and the muscles, compares the two signals and modifies the signal back to the motor cortex and muscles via the thalamus, brainstem, and spinal cord.
basket cells, and stellate cells [124]. Decreased Purkinje cell numbers are detected at 18 months of age in C57Bl/6J [198] and CBA [199] inbred mouse strains. In addition, genes involved in cellular immune and inflammatory responses are up-regulated in the cerebellum with age in seven different inbred mouse strains (129sv, BALB/c, CBA, DBA, C57BL, C3H and B6C3F1) [200]. Other studies have shown increases in genes associated with apoptosis and mitochondrial dysfunction and decreases in neurotrophic factors [201, 202]. All of these responses have been implicated in normal human brain aging [203].

1.12 Similarities and differences in the human and mouse cerebellum

The most obvious difference between the human and mouse cerebellum is size. The average surface area of the human cerebellum is 1,128 cm$^2$ compared to the mouse cerebellum at 190 mm$^2$ [204]. The larger surface area in the human cerebellum increases the complexity of the convolutions in the cerebellum, but the major cerebellar lobules can be identified in humans and mice. Due to evolution from a common ancestor, the cerebellum is present in all vertebrate brains [205]. Despite the similar structures and organization of the cerebellum, the functional topography of the cerebellum, on the other hand, is complex and assumptions cannot be made regarding the functionality of the same lobule in different species as determined through electrophysiological mapping [206]. Humans have four deep cerebellar nuclei (dentate, emboliform, globose, fastigial), and mice have three (dentate, interpositus, fastigial), but the neurons in the cerebellum and their connections are the same in humans and mice. Overall, the similarities in the cerebellar structure, organization, function, and aging in the human and mouse
cerebellum make mouse models an acceptable choice for the examination of cerebellar disease mechanisms.

1.13 Mouse models of cerebellar degeneration

Several mouse models of cerebellar degeneration have been characterized. *Leaner* mice are the result of a recessive mutation for a component of the voltage-gated calcium channel, mimicking spinocerebellar ataxia type 6 [207]. Granule cell death is detected at postnatal day 10 with the most dramatic loss occurring between postnatal day 15 and 40 in *leaner* mice [208]. Purkinje cell loss is minor until postnatal day 26 then degeneration accelerates for the next 4 to 6 weeks in *leaner* mice [208]. The heterozygous *lurcher* mouse contains a mutation in the *Glutamate receptor delta-2 subunit* gene [209], causing large scale degeneration of the cerebellar Purkinje and granule cells starting at postnatal day 8 [210], with almost no Purkinje cells and few granule cells remaining at postnatal day 90 [211]. The *staggerer* mutant mouse has a deletion of the *Rora* gene which codes for a retinoid-like nuclear receptor involved in neuronal differentiation and maturation and is expressed in high levels in the cerebellar Purkinje cells [212]. In *staggerer* mice, Purkinje cells are reduced in number prior to postnatal day 5 and only 25% of Purkinje cells are left at the end of the first postnatal month [213]. Granule cells are nearly all degenerated by the end of the first postnatal month [214], but this is secondary to the Purkinje cell degeneration [215]. *Reeler* mice have a mutation in the *Reln* gene, which encodes the protein REELIN, an extracellular matrix protein involved in neural adhesion and migration during development [216]. The mutation in *Reln* leads to disorganization of cells in the cerebellum [217], inferior olive
[218], cochlear nucleus [219], dopaminergic neurons [220], and hippocampus [221]. Approximately 50% of Purkinje cells degenerate in reeler mice [222]. The weaver mouse contains a mutation in the G-protein-gated potassium channel 2 (Grik2) gene that codes for a rectifying potassium channel [223] resulting in loss of almost all granule cells [224] and 28% of Purkinje cells are lost by 3 months of age [225]. The loss of granule cells occurs just shortly after birth, prior to migration into the internal granule cell layer [224]. In addition, these mouse models of cerebellar degeneration leaner, lurcher, reeler, staggerer, and weaver display ataxia or disturbances in gait [226-231]. However, it is important to note that these mouse models of cerebellar degeneration all display loss of Purkinje and granule cells with early disease onset in the postnatal period, which does not make them ideal mouse models in testing anti-aging strategies. An ideal mouse model would be symptom-free at young ages, and have a late onset of cerebellar degeneration that allows for a wide treatment window.

### 1.14 The harlequin mouse as a model of oxidative stress and cerebellar neurodegeneration

The harlequin (hq, X<sup>hq</sup>Y) mouse is a model of mitochondrial dysfunction leading to elevated oxidative stress and retinal and cerebellar neurodegeneration [232-234]. The hq mouse contains a proviral insertion in the first intron of the X-linked Apoptosis-inducing factor (Aif) gene, also known as Programmed cell death 8 (Pdcd8) [232]. The proviral insertion results in aberrant splicing and a reduction in Aif expression in all tissues examined including the cerebrum, cerebellum, retina, heart, kidney, muscle, lung, spinal cord, stomach and liver [232, 233, 235]. Under normal conditions, AIF is located
in the inner [236] and outer [237] mitochondrial membrane, but AIF induces apoptosis when translocated to the nucleus inducing chromatin condensation and large scale DNA fragmentation [238].

Despite the fact that hq mice arose due to a single gene mutation, the phenotype of the hq mouse is quite complex and can be used to model changes in behaviour, oxidative stress, and mitochondrial dysfunction. Aif has sequence similarity to bacterial oxidoreductases [238] and thus may be able to convert hydrogen peroxide to water, with AIF deficiency leading to an increase in ROS production and oxidative stress [232]. Neurons and cardiomyocytes from hq mice are sensitive to hydrogen peroxide induced cell death [232, 235], and heart tissue when examined for increased oxidative stress has elevated levels of the superoxide radical [239]. Elevated levels of superoxide radicals are also detected in the skin of hq mice, which are also accompanied by a 1.7-fold increase in mutation frequency [240]. Using the human placental alkaline phosphatase (PLAP) assay, capable of detecting a single guanine deletion, the mutant frequency in the brain of hq mice is 3-fold higher when compared to WT mice [241]. This elevation in brain mutant frequency does not change with age and is not seen in the kidney, heart, and liver in hq mice [241]. This elevated mutant and mutation frequency in hq mice contrasts to previous studies where spontaneous mutation frequency in C57Bl/6J mice is similar regardless of tissue type from 3 to 10 months of age [242, 243]. Increases in mutation frequency from 10 to 25 months of age are reported in liver and adipose tissue, while the mutation frequencies in brain and male germ-line remain low throughout development and into old age in C57Bl/6J mice [242-244]. In hq mice, the olfactory epithelium has increased lipofuscin autofluorescence and increased oxidative damage to RNA and
mitochondrial DNA as well as increased apoptotic cells [245]. The sensitivity displayed by neurons, cardiomyocytes, skin, and olfactory epithelium to oxidative stress and increased markers of oxidative stress in tissues from hq mice and AIF-knockout mice suggests a role of AIF as an antioxidant enzyme [232, 239-241, 246, 247].

AIF plays an undefined role in oxidative phosphorylation (OXPHOS), perhaps related to correct assembly and/or maintenance of the respiratory chain complex I [248]. Decreased activity of complex I and III of the electron transport chain are detected in the brain and retinal tissue but not in the muscle or kidney from hq mice [248]. AIF-knockout embryonic stem cells or AIF knockdown cell lines are associated with decreased complex I proteins [248-250]. A targeted deletion of the Aif gene in striated muscle leads to increased mitochondrial dysfunction, dilated cardiomyopathy, atrophy of skeletal muscle, and death at 4 months of age [246]. Complete Aif-knockout is lethal in mouse embryos with death occurring at embryonic day 11 [251]. Human patients with mitochondrial disorders also have similar defects in oxidative phosphorylation in a tissue-specific manner [252, 253]. Two related individuals, with a trinucleotide deletion in exon 5 of the Aif gene, were identified recently [254]. Both individuals presented with developmental delay, hypotonia, an absence of reflexes, muscle atrophy, and reduced activity of the electron transport chain complexes III and IV [254]. Altogether these results suggest a role of AIF in mitochondrial electron transport chain assembly or maintenance.

In the olfactory epithelial tissue, global analysis of gene expression in hq mice has only been examined at 6 months of age. Altered genes were classified into categories of cell signaling/apoptosis, oxidative stress/aging, cytoskeleton/extracellular matrix
transport, and neurogenesis [245]. The *Programmed cell death 6 (Pdcd6)* gene is up-regulated in *hq* mice, and is induced by endoplasmic reticulum stress, and mediates a cell death pathway [255]. *Pdcd6* also accelerates apoptosis mediated by caspase-3 [256]. Another gene induced by endoplasmic reticulum stress, *Dnaj homolog, subfamily C, member 10 (Dnajc10)*, is up-regulated in *hq* mice. *Dnajc10* is required for the correct folding of proteins in the endoplasmic reticulum [257]. Up-regulated genes involved in the endoplasmic reticulum stress response could be due to misfolded proteins that can be induced by oxidative stress [258]. Genes involved in DNA repair, are also up-regulated in the olfactory epithelial tissue in *hq* mice [245]. The expression of the *Damage-specific DNA binding protein-1* gene, a component of nucleotide excision repair [259], and the *Werner syndrome* gene, a DNA repair gene [260], are elevated consistent with oxidative DNA damage. The *Chemokine (C-X-C motif) ligand-12 (Cxcl12)* gene is up-regulated in the olfactory epithelium and attracts immune cells to a site of injury or infection [261] and could lead to immune system activation and inflammation. In fact, a previous study shows an increase in macrophage recruitment in the olfactory epithelium following large-scale apoptosis [262]. Down-regulated genes in the olfactory epithelium in *hq* mice are involved in cytoskeleton organization, which includes *Protein tyrosine kinase-2* [245]. *Protein tyrosine kinase-2* is involved in the disassembly of focal adhesions [263] and may lead to increased cell migration or limit the proliferation of progenitor cells. The *Cyclin D2* gene is down-regulated in the olfactory epithelium in *hq* mice and is required for adult neurogenesis [264] that occurs in the olfactory epithelium [265, 266]. The *Bone morphogenetic protein receptor type-I A* gene is also down-regulated and promotes neurogenesis in the olfactory epithelium [267]. The transcriptome of the olfactory
epithelium is consistent with increased oxidative stress, apoptosis, and decreased neurogenesis.

1.15 Disease progression timeline in hq mice

As a result of Aif down-regulation, hq mice can be distinguished from WT mice at birth due to decreased body mass that persists into adulthood, as well as patchy or no hair growth, and minimal accumulation of intraperitoneal fat stores [233]. Deficits in retinal function and loss of cells from multiple retinal layers are evident by four months of age [232]. Ataxia onset, an unsteady gait, and lateral tremor are observed at five months of age [268]. At six months of age, hq mice have increased levels of oxidative stress and apoptosis in the olfactory epithelium [245]. By seven months of age, necrotic Purkinje cells are evident and there is a significant decrease in the size of the cerebellum [232]. Complete vision loss is reported for 10-month-old hq mice [232, 269]. The hq phenotype demonstrates the effects of mitochondrial dysfunction and neurodegeneration.

1.16 The hq mouse is not specifically a model of Alzheimer’s Disease, Parkinson’s Disease, or Amyotrophic Lateral Sclerosis

The most obvious difference between neurodegeneration in hq mice and neurodegeneration in AD, PD, and ALS is the location of neurodegeneration. In hq mice neuron loss is limited to the retina and the cerebellum whereas in AD, PD, and ALS neuron loss occurs in the neocortex, limbic system, basal ganglia, or spinal cord. Individuals with AD lose neurons in the entorhinal cortex [10], hippocampus [270], motor cortex, and sensory cortex [271]. Individuals with PD lose dopaminergic neurons
in the substantia nigra [272] and those with ALS lose upper and lower motor neurons in the frontal cortex and spinal cord [273, 274]. The pathological features in AD, PD, and ALS are also different compared to those seen in hq mice. In individuals with AD, the brain contains amyloid β plaques and neurofibrillary tangles comprised of hyperphosphorylated tau protein [275]. In PD, the brain contains Lewy bodies [276], and in ALS diseased brains, the motor neurons contain Lewy body-like inclusions [277], skein-like inclusions [278], and Bunina bodies [279]. Hyperphosphorylated tau protein is not elevated in hq mice compared to WT mice [280], but levels of amyloid β plaques, Lewy bodies, Lewy body-like inclusions, skein-like inclusions, and Bunina bodies have not been studied in hq mice. Although cerebellar degeneration of hq mice does not strictly resemble neurodegeneration in AD, PD, and ALS, neurodegeneration in hq mice does share common mechanisms with AD, PD, and ALS. These mechanisms include disturbances in movement, increased oxidative stress, mitochondrial dysfunction, inflammation, and a progressive loss of neurons with age. Thus, hq mice share features that are in common with AD, PD, and ALS and can permit the examination of the neurodegenerative processes that are common to all.

1.17 Hormesis as an anti-aging strategy

Hormesis is a potential anti-aging strategy with a long history in lifespan extension and cancer prevention, but very little has been studied in the context of neurodegeneration. In hormesis, low doses of a stressor stimulates resistance to that stressor and are beneficial to an organism [281]. Hormesis has been studied in a variety of different organisms with different stressors. Seedlings of Vicia faba (fava beans)
exposed to low doses of lead in the soil grew taller [282]. The low doses of lead induced expression of heat shock proteins, as well as antioxidant enzymes to produce an enhanced defense against oxidative stress [282]. In *Drosophila*, low doses of ethanol increases the longevity in three different strains [283]. This increase in *Drosophila* lifespan could be due to elevated levels of sirtuin mRNA which is increased after ethanol feeding [284]. Exposure of *C. elegans* to a low dose of juglone, a chemical that stimulates oxidative stress, increases survival by 23% when exposed to lethal doses of juglone and juglone produces cross-tolerance to a different oxidative stressor [285]. In another study using an oxidative stressor in *C. elegans*, pretreatment with high concentrations of oxygen increases survival from X-irradiation through increases in the expression of heat shock proteins [286], suggesting that a single stressor can induce resistance to different stressors. Mallards (*Anas platyrhynchos*) that consume a diet with low concentrations of mercury exhibit enhanced hatching success and increased mean weights of six-day-old ducklings [287]. Caloric restriction increases the lifespan of animals by preventing diseases associated with age including cancer [288], diabetes [289], obesity [288, 289], and cardiovascular disease [289]. Gene expression in calorically restricted mice reveals decreased expression of genes involved in immunity, defence, and inflammation [200, 202, 290, 291]. In a mouse model of retinitis pigmentosa, low-dose radiation delays retinal loss due to up-regulation of the *peroxdoxin-2* gene, which is an antioxidant enzyme [292]. Voluntary exercise on the running wheel is not able to reduce amyloid β and tau accumulation in 3xTg-AD mice but is able to reduce lipid peroxidation, as well as increase glutathione peroxidase to levels seen in control mice [293]. Long-term endurance exercise improves movement, balance, mitochondrial function, and prevents
loss of dopaminergic neurons in the substantia nigra in a mouse model of PD [294]. Two ppm of phenobarbital (PB) decreases the amount of liver cancer in rats treated with N-diethylnitrosamine, a potent inducer of hepatocarcinoma [295]. Aside from studies of caloric restriction and exercise and their effects on lifespan extension and neurodegeneration, other hormetic agents have not yet been studied in a mouse model of neurodegeneration.

1.18 Phenobarbital as a hormetic agent

Phenobarbital is a barbiturate commonly used to treat epilepsy in humans. Phenobarbital binds the \( \gamma \)-aminobutyric acid (GABA\( \text{A} \)) receptor, allowing chloride ions to enter neurons causing hyperpolarization and suppression of neuronal activity [296-298] and locomotor activity [299]. Cases of increased physical activity are also observed with PB administration [299, 300]. Phenobarbital is known to target neurons in the cortex, hypothalamus, limbic system, caudate nucleus, cervical spinal cord, trigeminal ganglion, and the cerebellum [301]. Treatment of pregnant mice with high doses of PB leads to structural abnormalities in the cerebellum of the offspring, in particular to the mitochondria, myelin sheaths, and lamellar bodies of the granule cell layer [302]. Phenobarbital is a known tumour promoter in rodents [303-306] but is considered to be a weak mutagen, since in the absence of liver microsomal enzymes, PB induces only a two-fold increase in mutations in Salmonella typhimurium strain TA 1535 [307, 308] and is negative in strains TA98, TA100, TA1537, and TA1538 [309, 310]. However, in vivo studies using the Big Blue\textsuperscript{®} mouse transgenic mutation detection assay, PB is not able to increase the frequency of mutations at a dose of 2500 ppm but is able to change the
mutation spectra of mutation types [305, 306]. Phenobarbital would be of use as a hormetic agent since at high doses, PB inhibits locomotor activity, causes structural damage to the cerebellum, changes mutation spectra, and is a known promoter of liver tumours in rodents [303-306], but at low doses PB increases locomotor activity [300] and prevents liver cancer in a rat model of hepatocarcinoma [295]. Low doses of PB in the range of 1 to 7.5 ppm reduced the number of preneoplastic lesions in rats injected with diethylnitrosamine [311]. The molecular mechanisms underlying the hormetic effects of different stressors have not been elucidated, but in the case of the rat model of hepatocarcinoma, 2 ppm of PB decreases the amount of 8-hydroxy-2'-deoxyguanosine (8-OHdG), a marker of oxidative DNA damage, through the up-regulation of 8-OHdG repair enzyme Oxoguanine glycosylase-1 [295]. Phenobarbital also suppresses cellular proliferation and apoptosis in the liver through decreased expression of Mitogen activated protein kinase p38 as well as other kinases [295].

Hormesis has thus demonstrated success as an anti-aging and anti-carcinogenesis strategy and PB has demonstrated success in vivo with anti-carcinogenesis. If the mechanisms by which PB is able to reduce hepatocarcinoma are similar to the mechanisms demonstrated in exercise and caloric restriction in anti-aging, then PB should be successful as an anti-aging strategy in the hq mouse, a model of neurodegeneration. We know that 2 ppm of PB reduces ROS-induced DNA damage and increases DNA repair [295], while the hq mouse has elevated ROS-induced mutations [241], and neurodegenerative diseases have elevated DNA damage in nuclear and mitochondrial DNA [40]. Neuronal cells have reduced base-excision repair [203] and DNA repair was decreased in fibroblast and lymphoblastoid cell lines from individuals
with neurodegenerative diseases [312, 313]. Since both cancer and neurodegeneration display increased ROS, DNA damage, and decreased DNA repair, then it could be hypothesized that PB would prevent neurodegeneration even though PB hormesis has not been demonstrated in an anti-aging capacity.

**1.19 Phenobarbital metabolism in mice**

Phenobarbital is metabolized and inactivated in the liver. The metabolism of PB occurs in two phases. In the first phase, PB undergoes hydroxylation by the cytochrome P450 monooxygenases, producing p-hydroxyphenobarbital [314, 315]. In the second phase, PB and p-hydroxyphenobarbital can be conjugated with glucuronic acid (N-glycosylation) by the mircosomal enzyme glucuronyl transferase to produce PB N-glucuronides and p-hydroxyphenobarbital O-glucuronides respectively [316-318]. In addition, PB can be conjugated with glucose (N-glucosylation) by the enzyme glucosyl transferase to produce PB N-glucosides [319-321]. Once PB is hydroxylated or conjugated it is considered inactivated [319, 322]. Phenobarbital enters the brain through passive diffusion [323] and is eliminated from the kidney through urine, from the liver through bile, and from the gastrointestinal tract through feces [314]. Urine analysis in mice shows that most of the PB is eliminated as p-hydroxyphenobarbital followed by unmetabolized PB, and finally PB N-glucuronides and PB N-glucosides [317]. The half-life of PB in mice is 11.5 hours [324]. PB eliminated via the bile can be reabsorbed in the intestine or eliminated in feces [314, 325, 326].
1.20 Biomarkers for the assessment of an anti-aging strategy

Behavioural assays can be used as markers to assess cerebellar dysfunction. Using rotarod, a rotating cylinder suspended above the ground, *hq* mice display deficits in balance and coordination [280]. Locomotor assays have been used previously in mouse models prior to the onset of neurodegeneration [327-329]. However, a global assessment of nocturnal behaviour would provide insight into different parameters involved in movement and an automated home cage decreases the amount of time spent on manual observations while also decreasing observer bias [330]. Mouse models of cerebellar degeneration, despite having ataxia, display either higher or lower activity levels when compared to control mice. The *Lurcher* mutant mouse has higher levels of activity compared to control mice [227, 331] and spends more time walking and covers more distance compared to WT mice [331]. The *reeler* mutant mouse has higher activity levels but a lower number of rearing events compared to WT control mice [332]. However both *weaver* [333] and *staggerer* [334] mutant mice are hypoactive and rear less compared to control mice. *Leaner* mutant mice also have decreased motor activity [335]. An assessment of nocturnal behaviour would also determine whether low-dose administration of PB is able to target the brain and induce the expected effect of hyperactivity in WT and *hq* mice.

Another neurodegenerative assessment marker is tissue pathology. Tissue pathology can be identified using hematoxylin and eosin staining of tissue sections to examine tissue structure, architecture, thinning of the granule cell layer, and cell loss of the Purkinje cell layer. Hematoxylin is a basic dye that binds acidic compounds of the nuclei a blue/purple, while eosin stains basic compounds in the cytoplasm a red/pink
Neuropathology has been assessed previously using hematoxylin and eosin [232, 277], and will give a qualitative assessment of neuron loss. A quantitative measurement of the number of degenerating neurons can be determined through the use of Fluoro-Jade® B staining, a fluorochrome capable of staining apoptotic neurons resulting from exposure to a variety of different neurotoxic insults [337].

Another marker employed to assess neurodegeneration is the level of ROS in the cerebellum. The effect of elevated ROS can be examined by measuring the damage to cellular components including lipids, proteins, and DNA. Damage to lipids and proteins can be assayed by lipofuscin accumulation. Lipofuscin is a fluorescent pigment composed of oxidized lipids, proteins, and metals [43, 338] that accumulate with age in post-mitotic neurons [46-48]. Lipofuscin accumulation is detected in mouse models of neurodegeneration [339, 340]. Mutation frequency and pattern can be assessed to see if ROS-associated DNA damage is replicated in the DNA as mutations leading to elevated mutation load in hq mice. Mutations in the DNA can be determined through the use of transgenic mutation detection assays. Using the human placental alkaline phosphatase (PLAP) assay, hq mice have a 3-fold elevation in mutant frequency in the brain and this is not seen in other tissues in hq or WT mice [241]. However, the PLAP assay is restricted to the detection of single base pair deletions, which is only one signature of oxidative stress. Oxidative stress is associated with chromosome loss [341], DNA strand breaks [342], chromosome rearrangements [343], mitotic recombination [344], large [345] and small deletions [346], and the induction of point mutations with the most common being G:C to T:A transversions [347], although other base pair changes are implicated as well [345, 347]. In contrast to the PLAP assay, a forward mutation
detection assay such as the $cII$ assay in Big Blue® mice [348] offers analysis of mutation load using multiple susceptible DNA sequence contexts.

The production of ROS in the form of superoxide anions may be detected using dihydroethidium (DHE), a dye that fluoresces when oxidized by superoxide anions into ethidium [349]. Ethidium is a DNA intercalating agent that fluoresces red when excited by ultraviolet light and is capable of detection with fluorescence microscopy [350]. Levels of superoxide anions in the brain have been assessed by DHE previously [351].

Lastly, microarray technology is a powerful tool that provides researchers with the opportunity to analyze the level of mRNA of thousands of genes simultaneously [352]. Gene expression analyses in mouse models of neurodegeneration have implicated oxidative stress, mitochondrial dysfunction, and inflammatory response [353-357], as well as mitochondrial dysfunction leading to decreased energy metabolism [358]. The use of high-density microarrays can identify genome-wide changes in the levels of mRNA in the mouse cerebellum and can identify altered pathways early in the neurodegeneration process.

1.21 Central hypothesis and Experimental Aims

Given that cancer and neurodegenerative diseases are associated with elevated DNA damage and decreased DNA repair and given that hormesis has demonstrated efficacy as an anti-carcinogenesis strategy in a mouse model of hepatocarcinoma, I hypothesize that chronic low doses of PB treatment in $hq$ mice will delay cerebellar neurodegeneration.
Given that cerebellar function is required for coordinated movement, I hypothesize that *hq* mice will have decreased nocturnal behaviours compared to WT mice. In addition, given that PB administration increases activity in rodents, I hypothesize that PB administration will limit age-related decreases in nocturnal behaviours in *hq* mice through mechanisms that will reduce cerebellar degeneration. Three age groups will be tested, representing three stages in the progression of cerebellar degeneration in *hq* mice. Three months of age represents early disease onset, 7 months of age represents mid-disease onset, and 10 months of age represents late-stage disease. Mice at 3 and 7 months of age will be administered 0, 2, or 4 ppm of PB in the drinking water *ad libitum*. Nocturnal behaviours will be measured overnight. I predict that *hq* mice will have cerebellar deficits leading to decreased nocturnal activity compared to WT mice and the nocturnal activity will decrease with age. I also predict that PB will reverse age-related declines in nocturnal behaviours and that it will increase nocturnal activity in WT mice, given the evidence reported to date.

Given that *hq* mice are undergoing cerebellar neurodegeneration, I hypothesize that there will be an increase in cell loss, apoptosis, lipofuscin accumulation, and superoxide anion levels compared to WT mice. In addition, given that PB administration affects different anti-aging pathways, I hypothesize that hormetic PB administration will limit cell loss, apoptosis, lipofuscin accumulation, and superoxide anion levels. Hematoxylin and eosin histological assessments will be used to look at cerebellum size, structure, and evidence of cell loss in the granule and Purkinje cell layers. *In situ* Fluoro-Jade® B staining will be used to assess the number and location of neurons undergoing apoptosis. The accumulation of damaged cellular components in the form of lipofuscin
will be assessed. The amount of superoxide anions will be measured to determine whether increased ROS is associated with cerebellar neurodegeneration in hq mice. I predict that hq mice will have smaller cerebella and increased cell loss of the granule and Purkinje cell layers over time. In addition, I predict that biomarkers of neurodegeneration will be elevated in hq mice and will increase with age compared to WT mice. Phenobarbital treatment is predicted to decrease the biomarkers of neurodegeneration.

Given that hq mice had an elevated number of single nucleotide deletions in the whole brain compared to WT mice, I hypothesize there will be an increase in the spontaneous mutant and mutation frequency of the cII gene and a change in the mutation pattern in the cerebellum of hq mice when compared to WT mice. The hq mice will be bred with Big Blue® transgenic mice to allow for mutation detection. Spontaneous mutant and mutation frequency and pattern will be studied in 3- and 7-month-old WT and hq mice in the absence of PB administration. I predict that hq mice will have an elevated mutant and mutation frequency when compared to WT mice. In addition, the mutation pattern will be altered and contain signatures of oxidative stress in hq mice.

Given that pathways involving oxidative stress, mitochondrial dysfunction, and inflammation have been detected in neurodegenerative disorders, I hypothesize that hq mice will have altered pathways involved in oxidative stress, mitochondrial dysfunction, and inflammation when compared to WT mice. Also, given that PB administration may affect anti-cancer pathways, I hypothesize that PB administration will counteract oxidative stress, mitochondrial dysfunction, and inflammation in hq mice. Gene expression in hq and WT mice will be assayed prior to onset of cerebellar degeneration at
3 months of age. I predict that *hq* mice will have down-regulated gene expression in pathways involved in cellular repair and up-regulated genes involved antioxidant response, mitochondrial dysfunction, and inflammation when compared to WT mice and that PB administration will up-regulate genes involved in cellular repair or down-regulate genes involved in mitochondrial dysfunction and inflammation.
CHAPTER TWO – MATERIALS AND METHODS

2.1 Animals

2.1.1 General care

Animal protocols were approved by The Animal Use Subcommittee of the University Council on Animal Care of The University of Western Ontario (Appendix A). All mice were housed two or three in a cage on a 12 h light/dark cycle at a constant temperature of 21 ± 1°C and relative humidity of 44 to 60%. Mice were fed a standard diet (PMI Foods, St. Louis, MO) ad libitum. All experimental mice were male and either wild type (WT) for the Aif gene or harlequin (hq; B6CBACa A<sup>wt</sup>/A-Pdcd8<sup>hq</sup>/J; Bar Harbor, Maine).

2.1.2 Hormetic treatment

Mice of both genotypes were given phenobarbital (PB) (Sigma-Aldrich, St. Louis, MO) in concentrations of 2 or 4 ppm administered in the drinking water provided ad libitum from 1 month of age until euthanized at 3 or 7 months of age. Drinking water intake was measured everyday for a three-week period for a subset of mice at 7 months of age. This was done to determine the amount of water consumed (mL/g/day) for each genotype and to confirm no PB taste aversion. A two-factor Analysis of Variance (ANOVA) for genotype and treatment was performed on the amount of water consumed in WT and hq mice with and without PB treatment.
2.1.3 Breeding hq/Big Blue\textsuperscript{®} transgenic mice for transgene mutation detection and increased inbred mouse background

Four-month-old heterozygous female hq (X\textsuperscript{hq}X) mice (B6CBACa A\textsuperscript{w-j}/A-Pdcd8\textsuperscript{hq}/J; The Jackson Laboratory, Bar Harbor, ME), were bred to 3-month-old male Big Blue\textsuperscript{®} mice (C57Bl/6) homozygous for the lambda phage cII transgene (Taconic Farms, Germantown, NY) in order to incorporate the cII mutation detection target in hq mice (Figure 2.1). The first generation progeny were either WT (XY or XX) or had the hq mutation (X\textsuperscript{hq}Y or X\textsuperscript{hq}X) and all mice were hemizygous for the cII transgene (cII/0) and were brother and sister mated to generate additional mice with both the hq mutation and the cII transgene. This mating strategy produced an increased number of mice having hydrocephaly and microphthalmia, due to the background of C57Bl/6J mice [359, 360]. As a result, both male and female progeny from the second generation were bred with inbred CBA/CaJ mice (The Jackson Laboratory, Bar Harbor, ME). The inbreeding of hq mice to the CBA/CaJ background continued for a total of four generations. Additional brother to sister matings were performed for three more generations to increase the number of male mice with the hq mutation and the (hemizygous or homozygous) presence of the cII transgene. The zygosity of Big Blue\textsuperscript{®} mice for the cII gene does not affect the spontaneous mutant or mutation frequencies [361]. Mice used in this study were male WT mice (XY) or hemizygous hq mice (X\textsuperscript{hq}Y) from generations four through nine (G4 – G9) with 75 to 94% CBA/CaJ inbred mouse background.
Heterozygous $hq$ female mice ($X^{hq}X$: B6CBACa) were initially bred with Big Blue® transgenic mice (C57Bl/6J) homozygous for the $cII$ transgene. The offspring were brother and sister mated (G1) to increase the number of $hq$ mice with the $cII$ transgene. Mice from this generation were mated with CBA/CaJ inbred mice and this breeding strategy continued for four generations (G2 - G5) in order to decrease the genetic variability in the mice on a primarily CBA/CaJ background. Additional brother and sister matings were performed to increase the number of $hq$ male mice with the $cII$ mutation target (G6 - G8).
$X^{hq}X$ (B6CBACa) x C57Bl/6J $cll/0$ Big Blue® transgenic mouse

G1

$X^{hq}X^{cll/0} \times X^{hq}Y^{cll/0}$ (sibling mating)

G2

$X^{hq}X^{cll/0} \times$ CBA/CaJ

G3

$X^{hq}X^{cll/0} \times$ CBA/CaJ

G4

$X^{hq}X^{cll/0} \times$ CBA/CaJ

G5

$X^{hq}X^{cll/0} \times$ CBA/CaJ

G6

$X^{hq}X^{cll/0} \times XY^{cll/0}$ (sibling mating)

G7

$X^{hq}X^{cll/0} \times X^{hq}Y^{cll/0}$ (sibling mating)

G8

$X^{hq}X^{hq}^{cll/0} \times X^{hq}Y^{cll/0}$ (sibling mating)
2.1.4 Genotyping of WT and hq mice

Animals were ear notched and tail clipped at 10 to 12 days of age and DNA was extracted using a small-scale genomic DNA extraction protocol. Tail clippings were digested overnight at 55°C in TENS buffer and Proteinase K (Invitrogen, Burlington, ON). After digestion, the suspension was transferred to a 1.5 mL Phase Lock Gel™ tube (Qiagen, Mississauga, ON) with an equal volume of a 50:50 phenol:chloroform mix. The suspension was centrifuged for 5 min at 14,000 rpm. The aqueous layer was transferred to a clean 1.5 mL Eppendorf tube. DNA was precipitated with 100% ethanol and centrifuged at 14,000 rpm for 10 min. The supernatant was decanted and the pellet was allowed to air dry. The pellet was dissolved in 30 µl of TE buffer. DNA was stored at 4°C until used for genotyping the cII and Aif gene.

The presence of the cII sequence in Big Blue® mice was detected using polymerase chain reaction (PCR) amplification. A 476 bp sequence containing the cII gene was amplified using the primer set ASPCIIF 38248: 5’ CCG CTC TTA CAC ATT CCA GC 3’ and JAKCIIR 38723: 5’ CCT CTG CCG AAG TTG AGT AT 3’ [362]. PCR was performed with the following conditions: 1.2 µg of sample DNA, 0.32 mM dNTP, 1X PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl) 2 mM MgCl₂, 0.08 µM of each primer, and 0.02 U/µl Taq DNA polymerase in a 30 µl reaction volume. The reaction consisted of an initial denaturation at 94°C for 5 min, followed by 35 cycles of template denaturation at 94°C for 20 sec, primer annealing at 53°C for 80 sec, primer extension at 72°C for 2 min, with a final primer elongation at 72°C for 10 min (GeneAmp PCR System 9600, Applied Biosystems, Foster City, CA).
The *Aif* genotype was determined by PCR amplification using a tri-primer protocol [232]. The forward, *Aif* 3468 5' AGT GTC CAG TCA AAG TAC CGG G 3' and reverse, *Aif* 4000 5' CTA TGC CCT TCT CCA TGT AGT T 3' primers were designed for exons one and two respectively of the *Aif* gene. Another forward primer was designed from the long terminal repeat of the murine C-type ecotropic virus *hq*LTR2 5' GAA CAA GGA AGT ACA GAG AGG C 3' located in intron one of the *Aif* gene in *hq* and carrier mice. PCR was performed with the following conditions 1.2 µg of sample DNA, 0.32 mM dNTP, 1X PCR buffer, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 3.5 mM MgCl₂, 0.08 µM of each primer, and 0.02 U/µl *Taq* DNA polymerase in a 30 µl reaction volume. Cycling conditions included an initial denaturation at 94°C for 3 min, followed by 35 cycles of 94°C for 30 sec, 62°C for 1 min, 72°C for 1 min, followed by 72°C for 2 min (GeneAmp PCR System 9600, Applied Biosystems, Foster City, CA).

### 2.2 The experimental cohorts

The experimental design consisted of 152 mice from G4 – G9, with 70 WT (XY) and 82 *hq* (X*hq*Y) mice (Figure 2.2). The mice were further divided into three groups according to age; 3 months of age (WT n=29, *hq* n=35), 7 months of age (WT n=32, *hq* n=38) and 10 months of age (WT n=9, *hq* n=9). Each genotype was administered 0, 2, and 4 ppm of PB in the drinking water provided *ad libitum* from 1 month of age until euthanized at 3 or 7 months of age. The control cohorts of mice received untreated drinking water *ad libitum*. Mice were euthanized according to the standard operating procedure (protocol# 320-02) at 3, 7, or 10 months of age by CO₂ inhalation and mouse body mass was recorded.
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Figure 2.2 – Experimental design used to assess cerebellar degeneration and a potential intervention strategy using phenobarbital (PB). The experimental design included 152 male mice, 70 wild type (WT) and 82 harlequin (hq) mice. The mice were further divided into three different age groups representing three different disease time points. Mice at 3 months of age represent the early disease time point, mice at 7 months of age represent the mid-disease time point, and mice at 10 months of age represent the late stage of the disease time point. In the 3 and 7 months of age cohorts, mice were treated with 0, 2, or 4 ppm of PB. The untreated cohorts received drinking water ad libitum and the treated cohorts received 2 or 4 ppm of PB dissolved in the drinking water ad libitum. Cerebellar function and response to PB were assessed by monitoring nocturnal cage behaviour. Postmortem in situ histopathology assays were performed on the cerebellum to determine changes with disease progression. Mutation detection with the cII transgene mutation detection assay was performed on the cerebellum to determine whether mutations were elevated and accumulated with age in the cerebellum of hq mice compared to age-matched WT mice. Gene expression analysis was performed to determine early changes in gene expression associated with the hq genotype.
3 Months

Genotypes

Ages

Treatments

Phenotypic Markers

9 WT 9 hq Mouse Behaviour
5 WT 5 hq cll mutants
3 WT 3 hq Cerebellar histology
3 WT 3 hq Cerebellar Transcriptome

10 WT 12 hq Mouse Behaviour

10 WT 10 hq Mouse Behaviour

13 WT 12 hq Mouse Behaviour

11 WT 13 hq Mouse Behaviour

9 WT 9 hq Mouse Behaviour

7 Months

10 Months
2.3 Behaviour testing of WT and hq mice

Mouse behaviour was recorded using the ActiTrack® apparatus and software (Panlab Harvard Apparatus, Barcelona, ES) in a mouse test cage that simulates the home cage environment. Eleven different parameters of mouse movement were quantified using the ActiTrack® system: activity, locomotion, stereotypes, distance, resting time, time spent moving slowly, time spent moving quickly, maximum velocity, mean velocity, number of rearings, and mean rearing duration, as they best characterized mouse behaviour (Table 2.1). Each mouse was placed individually into a clear plastic test cage measuring 14.7 inches deep x 9.2 inches wide x 5.5 inches high (Innovive, San Diego, CA), which was similar in size to a home cage, with a thin layer of cedar shavings (Nepco, Warrensburg, NY). Five to six food pellets were placed in one corner and water (containing 0, 2, or 4 ppm PB as per the animal treatment cohort study) was made available overhead ad libitum. Mouse access to the water bottle does not register as a rearing event because the upper infrared beam that records movement in the z-axis is higher than the water bottle spout. No nestlet material was provided to avoid interference with the infrared beams used to track mouse movement in the ActiTrack® apparatus. The test cage was assembled and the mouse placed into the testing arena at 1400 hours, 4 hours before testing was started to allow familiarization with the new environment. The threshold values for velocity were set so resting was defined as any movement less than 2 cm/s, moving slow as any movement between 2 cm/s and 5 cm/s, and moving fast was as any movement above 5 cm/s [363]. Rearings were measured when the mouse stood on its hind legs and blocked the infrared beams of the upper grid in the arena. The upper
Table 2.1 Description of the behaviours measured in *harlequin* and wild type mice using the ActiTrack® apparatus

<table>
<thead>
<tr>
<th>Mouse Behaviour (units of measure)</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activity (events)</td>
<td>Measures the mouse’s total activity during the analyzed time interval and is the summation of locomotion and stereotypes</td>
</tr>
<tr>
<td>Locomotion (events)</td>
<td>Measures the mouse’s locomotion during the analyzed time interval where the position of the mouse is different from the position of the previous location</td>
</tr>
<tr>
<td>Stereotypes (events)</td>
<td>Measures the number of times where the position of the mouse is different from the position of the previous location and equal to the position of the second location back in time</td>
</tr>
<tr>
<td>Distance (cm)</td>
<td>Total distance travelled by the mouse during the time interval analyzed</td>
</tr>
<tr>
<td>Time spent resting (s)</td>
<td>Time period during which the speed of the mouse is below the resting threshold (2 cm/s) over the time interval analyzed</td>
</tr>
<tr>
<td>Time spent moving slowly (s)</td>
<td>Time period during which the speed of the mouse is between the resting and fast thresholds (2 cm/s-5 cm/s) over the time interval analyzed</td>
</tr>
<tr>
<td>Time spent moving quickly (s)</td>
<td>Time period during which the speed of the mouse is above the fast threshold (5 cm/s) over the time interval analyzed</td>
</tr>
<tr>
<td>Maximum velocity (cm/s)</td>
<td>The maximum velocity (cm/s) reached by the mouse</td>
</tr>
<tr>
<td>Mean velocity (cm/s)</td>
<td>Mean velocity is calculated as the distance travelled by the mouse, divided by the total interval analyzed</td>
</tr>
<tr>
<td>Number of rearings (events)</td>
<td>Measures the number of times the mouse is standing on its hind legs and moving vertically</td>
</tr>
<tr>
<td>Mean rearing duration (s)</td>
<td>Measures the mean time of the rearing events for the total interval analyzed</td>
</tr>
</tbody>
</table>
beam was adjusted according to the mouse body size, which was empirically determined, to ensure the mouse was able to trigger the upper beam. Daylight and nocturnal behaviour were recorded at hourly intervals beginning at 1800 hours until 2000 hours and 2000 hours to 0300 hours, respectively. Behaviour was studied for two consecutive nights for each mouse and analyses were performed on overnight data for WT and hq mice at 3 months of age (n=9 WT and n=14 hq mice, no drug treatment; n=10 WT and hq mice, 2 ppm PB; n=10 WT and n=11 hq mice, 4 ppm PB), 7 months of age (n=9 WT and n=12 hq disease mice, no drug treatment; n=12, WT and hq mice, 2 ppm PB; n=11 WT and n=14 hq mice, 4 ppm PB), and 10 months of age (n=9, WT and hq mice, no drug treatment).

2.3.1 Statistical analysis of WT and hq mouse behaviour

A principal component analysis (PCA) with varimax rotation and Kaiser normalization was performed to identify interrelationships between nocturnal test cage behavior parameters. Missing values were converted to group means for PCA. Factors with eigenvalues >1 were used for further analysis. A three-factor (genotype, age and treatment) MANOVA (p<0.05) followed by Tukey’s test for post hoc analysis was used to compare the 11 parameters of behaviour measured by the Actitrack in WT and hq mice at 3, 7, and 10 months of age with three different concentrations of phenobarbital 0, 2, and 4 ppm in the drinking water. One-factor ANOVA (p<0.05) was performed to further analyze significant interactions found with the three-factor MANOVA, or three-factor ANOVA. All statistics were performed with Statistical Package for the Social Sciences (SPSS) Version 17.0 (IBM, Somers, NY).
2.4 In situ histological assays for neurodegeneration and testing ROS as an underlying hypothesized mechanism of disease

2.4.1 Mouse tissue collection

Mice were euthanized according to standard operating procedures (protocol# 320-02) at 3, 7, and 10 months of age by CO₂ inhalation and body mass was recorded. The whole cerebellum was harvested and either flash frozen in liquid nitrogen for some mice for use in the cII assay or half of the cerebellum was placed into a cryomold containing optimal cutting temperature media (Somagen Diagnostics, Edmonton, Alberta) for cryosectioning and the other half of the cerebellum was flash frozen in liquid nitrogen for RNA isolation and stored at -80°C until further use. All animals were healthy at the time of tissue harvest, with a healthy shiny fur coat with no visible wounds and were able to move and obtain food and water. No gross tissue pathology was observed for any major organ.

2.4.2 Cryosectioning of the mouse cerebellum

Half of the cerebellum of WT and hq mice was cryosectioned sagittally at a 10 μm thickness, 200-450 μm distal to the midline using a Leica CM350 Cryostat (Leica Microsystems, Houston, TX), with two sections being placed per slide on a positively charged poly-L-lysine microscope slide (VWR, Mississauga, ON), and stored at -20°C.
2.4.3 Hematoxylin and eosin tissue section staining to assay cerebellar histopathology including cell loss

Hematoxylin and eosin staining was performed on frozen unfixed cerebellar tissue. Slides were immersed in 70% ethanol and then stained with hematoxylin for 5 min and rinsed with water until runoff was colourless. Slides were dipped in 500 mL of 70% ethanol containing 5 drops of 1N hydrochloric acid, immersed in tap water, and immersed in 1% lithium carbonate (BDH Laboratory Supplies, Poole, England). Sections were counterstained with Eosin Y (Sigma-Aldrich, Oakville, ON) for 30 sec and dehydrated by rinsing in increasing concentrations of ethanol 50%, 70%, 95%, and 100%, followed by a wash in fresh 100% ethanol. Slides were then submerged in xylene until coverslipped (VWR, Mississauga, ON) with Permount® Mounting Media (Ottawa, ON).

Images of the cerebellum were captured at 20X magnification using the Arcturus Veritas® Microdissection System (Molecular Devices, Sunnyvale, CA) to assess the size and extent of the nature of gross abnormalities. Further imaging was performed at 200X magnification to quantify the nuclear density. Images at the base and apex of lobules I/II, III, VIa, IX and X were captured representing four different zones in the cerebellum [176] (Figure 2.3). Images were converted into grayscale and the threshold was set to 140 to highlight nuclei (Figure 2.4) using ImageJ analysis software (National Institute of Health, Bethesda, MD). ImageJ quantified the percentage area containing granule cell nuclei and are representative of cell numbers. Three nuclear count measurements were taken per image using a 120 x 90 pixel box corresponding to 57.5 μm x 43.1 μm. Two independent scorers, blinded to animal cohort identifiers, determined the percentage of the area containing nuclei to determine granule cell loss.
Figure 2.3 – An illustration depicting where images were taken in the sagittal hematoxylin and eosin stained sections of the mouse cerebellum in order to determine the neuron counts in the granule cell layer of specific lobules. The images were taken at the base (B) and apex (A) of lobules I/II, III, VIa, IX, and X representing four different zones, anterior zone (AZ: lobules, I-V), the central zone (CZ: lobules VI, VII), the posterior zone (PZ: lobules VIII, IX), and the nodular zone (NZ: lobule X), in the mouse cerebellum based on the previously reported unique expression of molecular markers for determining compartmentalization of the cerebellum [176]. Scale bar = 1 mm
Figure 2.4 - Neuron counting in the granule cell layer in wild type (WT) and harlequin (hq) mice. A) A hematoxylin and eosin image of the apex of lobule I/II of the cerebellum in 3-month-old WT mice. B) Images were converted into white nuclei on a black background in ImageJ. White nuclei were calculated and converted into percentage area occupied by nuclei. Three nuclear counts were made in both the base and apex of each lobule for two different cerebellar sections. The nuclear counts in the base and apex were compared with a one-way ANOVA. The nuclear counts in the base and apex were similar and the average was taken for the six nuclear counts. Nuclear counts were taken in three different mice per experimental group by two independent observers. Scale bar = 100 µm.
2.4.4 Purkinje cell counts to assay Purkinje cell loss

Tissue sections were stained with hematoxylin and eosin as in section 2.5.3. Purkinje cells, based on morphology and location, were counted from the base to the apex, and from the apex to the base of lobules I/II, III, VIa, IX, and X for two different sections per mouse (Figure 2.5). The Purkinje cell counts for each section were averaged together for each mouse. Purkinje cell counts were taken in three different mice per experimental group.

2.4.5 Fluoro-Jade® B to assay degenerating neurons with paraquat-treated tissue controls

Frozen and unfixed cryosections were heated on a 45°C heat block to evaporate moisture, stained with hematoxylin and eosin (see section 2.4.3), and subsequently stained with Fluoro-Jade® B as described previously [337]. Hematoxylin and eosin stained sections were washed in deionized water for 1 min and transferred to 0.001% Fluoro-Jade® B (Millipore, Temecula, CA) in deionized water with 0.1% acetic acid for 30 min on a rotating platform. Sections were rinsed in water for 3 min and air dried before immersing in xylene for 5 min and coverslipping using Permount® mounting media (Fisher Scientific, Pittsburgh, PA). Sections were viewed at 200X under the FITC filter of the Arcturus Veritas® Microdissection System (Molecular Devices, Sunnyvale, CA). Fluoro-Jade® B positive cells were counted per whole section with a minimum of 2 sections per animal. Positive and negative controls were created by injecting WT mice with 10 mg/kg of paraquat or a vehicle control [239], since paraquat is known to
Figure 2.5 – A hematoxylin and eosin image of the cerebellum of a wild type (WT) mouse indicating the location were the Purkinje cell counts were made. Purkinje cells were identified by location and morphology and counted in lobules I/II, III, VIa, IX, and X for two separate sections and the average was calculated for each lobule. There were three different mice per experimental group. Scale bar = 1 mm.
degenerate neurons in the substantia nigra [364] and Fluoro-Jade® B is able to stain paraquat-treated cells [365]. These mice were euthanized 15 days after injection with paraquat and the cerebrum was fixed in 4% paraformaldehyde, embedded in paraffin wax (Leica ASP300, Leica Microsystems, Richmond Hill, ON) [239] and sectioned sagittally at a thickness of 5 μm (Leica RM2255 Microtome, Leica Microsystems) and deparaffinized in xylene prior to hematoxylin and eosin staining. Hematoxylin, eosin, and Fluoro-Jade® B staining were performed as described above (Appendix B, Figure B.1).

2.4.6 Autofluorescence of the cerebellar tissue to assay lipofuscin accumulation and ROS damage

Frozen and unfixed cryosections were examined for lipofuscin accumulation through yellow tissue autofluorescence [44] using the FITC filter (Excitation: 455-495 nm; Emission: > 510 nm) of the Arcturus Veritas® Microdissection System (Molecular Devices, Sunnyvale, CA). Images were captured at 200X magnification and one image was taken at the apex of each lobule (I/II, III, VIa, IX, and X) of the cerebellum across two tissue sections for each mouse. All images where set to a threshold of 140 to get rid of the green autofluorescence and six measurements were taken in the Purkinje cell layer using a 100 x 50 pixel box to determine the yellow lipofuscin autofluorescence (Appendix B, Figure B.2). Quantification was performed by two independent scorers, blinded to animal cohort identifiers, using ImageJ analysis software (National Institute of Health, Bethesda, MD).
2.4.7 Dihydroethidium (DHE) staining of cerebellar tissue sections to assess superoxide anion levels

Dihydroethidium staining was performed on frozen sectioned cerebellar tissue that was embedded in optimal cutting temperature media. Frozen slides were heated on a 45°C heat block to evaporate moisture. A negative control was created by pre-treating the slide with 30 µl of 1000 U/ml superoxide dismutase (SOD, Sigma-Aldrich Canada Inc., Oakville, ON) for 10 min at room temperature to remove superoxide anions. A positive control was created by pre-treating the slide with 20 µl of 0.5 M hydrogen peroxide solution for 5 min at room temperature. The SOD and hydrogen peroxide solutions were removed before proceeding with DHE staining in a dark room.

Cryosectioned cerebella were treated with 50 µl of 50 µM DHE in dimethyl-sulphoxide (DMSO) and incubated in a humidified chamber at 37°C for 30 min [349] (Appendix B, Figure B.3). Superoxide anions were quantified on the tissue sections using the green fluorescence filter (Excitation: 503-548 nm; Emission: > 565 nm) on an inverted Zeiss microscope (Carl Zeiss Incorporated, Toronto, ON) capable of detecting the DHE emission wavelength of 590 nm. The fluorescent lamp was set to a gain of ‘1’ and exposure time of 500 ms for all images and analyses. DHE staining has a very short duration of fluorescence before quenching begins to occur so time to image capture was consistently restricted to 3 min per section. Images were captured at 200 X magnification and one image was taken at the apex of lobules I/II, III, VIa, IX, and X representing four different zones in the cerebellum determined by gene expression for two tissue sections for each DHE treatment and control. Fluorescence intensity was determined in three regions per image by drawing a 120 x 90 pixel box in ImageJ analysis software (National Institute of Health, Bethesda, MD). ImageJ quantifies the fluorescence intensity by
assigning the detected fluorescence a grey scale brightness value, thus it allows one to measure the DHE fluorescence.

2.4.8 Statistical analysis for in situ cerebellar phenotyping assays to characterize hq mice and response to PB treatment

Three-factor MANOVA followed by one-factor ANOVAs (p<0.05) were performed to further analyze statistically significant interactions found with the three-factor MANOVA. Tukey’s test for post hoc comparison was used to determine differences in mean granule cell and Purkinje neuron counts, mean fluorescence intensity of lipofuscin and DHE across genotype, age, and drug treatment, with the level of significance p<0.05. A three-factor ANOVA was used to determine mean differences in the number of Fluoro-Jade® B positive cells across, genotype, age, and drug treatment. All data were analyzed using SPSS version 17.0 (IBM, Somers, NY).

2.5 The Big Blue® cII assay for mutation detection

To analyze mutations in the cII gene, high molecular weight genomic DNA was extracted from 50% of the cerebellum (60 mg) of WT mice and the entire cerebellum (90 mg) from hq mice using the RecoverEase™ DNA Isolation Kit protocol (Stratagene, LaJolla, CA). Tissues were homogenized, digested with Proteinase K and dialyzed in TE (pH 7.5) at room temperature for 48 hours. DNA was transferred into a sterile microcentrifuge tube using a flamed glass capillary rod and stored at 4ºC. The Big Blue® cII mutation detection assay was carried out using the Transpak® and the lambda Select-cII™ protocol [366]. Briefly, lambda phage genomes containing the Big Blue® cII
construct were excised from extracted genomic DNA from 21 mice and packaged in vitro to produce virulent lambda phage, used to infect *E. coli* strain G1250 and plated. Plates were incubated under cII mutant selective (24°C, 40 hours) or nonselective control (37°C, 24 hours) conditions.

Putative cII mutants were cored using sterile pipette tips and stored at 4°C in 500 μl of SM buffer containing 5 μl chloroform and were used to reinfect *E. coli* to confirm the mutant phenotype under cII mutant selective conditions [366]. Mutants with confirmed phenotype were cored and placed in 100 μL of sterile TE (pH 7.5) and stored at -20°C until needed. Mutant frequency was calculated by dividing the number of mutant plaques by the total number of plaque forming units (pfu’s) as estimated from the number of plaques on titer plates incubated under the nonselective temperature condition [366].

The cII gene was PCR amplified using primers ASPCIIF 38248 and JAKCIIR 38723 [366]. To remove excess primer and unincorporated dNTPs for sequencing, the amplified PCR products were incubated with 10 U/μl exonuclease I and 2 U/μl shrimp alkaline phosphatase for 15 min at 37°C followed by heat inactivation at 80°C for 15 min (USB Corporation, Cleveland, Ohio). Purified products were sequenced at the Robarts Research Institute (London, ON) with 2 μM of KOHCIIF 38272 cII primer 5’ AAA AAG GGC ATC AAA TTA AAC C 3’ [366]. Sequences were compared in a multiple sequence alignment to the WT cII Genbank reference sequence (J02459) [362] and mutations were analyzed using Seqscape® v2.0 (ABI) and Sequencher software (Gene Codes Inc. Ann Arbor, MI). Sequence chromatograms were analyzed by two individuals. Mutation frequency was obtained for each cerebellar sample and mouse by correcting the
mutant frequency for recurrent mutations in the same mouse tissue and dividing by the total pfu’s assayed.

At least five mice were used per experimental group at 3 and 7 months of age. Three to six estimates of mutant frequency per mouse were made using the Big Blue® cII assay with an average packaging efficiency of about 130,000 mutation targets assayed. The experimental design for the Big Blue® cII assay exceeded recommended guidelines to achieve the statistical power (66 to 88%) necessary to detect as significant a 50% change in mutant frequency [367-369]. Also, the mutation(s) in each mutant was(were) identified and clones removed from the estimate of mutation frequency, further reducing inter-animal variation in the estimate of mutation frequency [370] and further increasing statistical power. Mutant and mutation frequencies in the cerebellum of WT and hq mice were analyzed using a two-factor ANOVA (Microsoft Excel 2007 and SPSS, Somers, NY). The frequency of Class II deletions [i.e., >1 bp but < 1 kb] [371] in the cerebellum of young hq mice was compared to the other mouse cohorts in this study and to our database of cII mutations in WT and hq mice (Ward, unpublished) [239, 240] using a the Fisher-Freeman-Halton test in StatXact statistical analysis software (CYTEL Software, Cambridge, MA). Differences in mutation frequencies were significant if p<0.05. The mutation pattern, the proportion of eleven different mutations, was analyzed using Fisher-Freeman-Halton test (Monte Carlo simulation) in the StatXact statistical analysis software package (CYTEL Software, Cambridge, MA) and was considered significantly different if p<0.05. The mutation spectrum, a visual representation of all the mutations in the cII gene, was analyzed by plotting all the mutations on the cII sequence to visualize areas of mutation hot spots.
2.6 Gene expression in cerebellar tissues to study early mechanisms of degeneration and response to PB treatment

2.6.1 RNA isolation and purification for gene expression analysis

Total RNA was extracted from half of the cerebellum of WT and hq mice using 1 mL Qiazol lysis reagent and homogenized with 20 strokes in a 7 mL dounce homogenizer with pestle A (Wheaton, Millville, NJ). The remainder of the RNA isolation was processed using the RNeasy® Mini Kit according to the manufacturer’s protocol (Qiagen, Valencia, CA). Total RNA was purified with 0.25 volumes of 3 M sodium acetate and 4 volumes of 100% ethanol and left overnight in a -20°C freezer. RNA was pelleted by centrifugation at 12,000 xg for 30 min, washed twice in 80% ethanol and centrifuged at 4°C for 30 min at 12,000 xg. The pellet was air dried and resuspended in RNase free water.

Total RNA purity was determined with spectrophotometric $A_{260/280}$ and $A_{260/230}$ ratios in the range of 2.0-2.2, and 1.8-2.1, respectively with the NanoVue™ (GE Healthcare Bio-Sciences, Piscataway, NJ). RNA integrity was determined by the presence of prominent 28S and 18S peaks on an electropherogram using the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). RNA was used if the RNA integrity number (RIN) was 8.8 or greater. RNA was stored at -80°C.

2.6.2 Gene expression assayed by microarray hybridization

Total RNA (100 ng) was used to synthesize mRNA into double stranded cDNA with T7-Oligo (dT) Promoter Primer (Affymetrix, Santa Clara, CA) followed by an in vitro transcription with T7 RNA polymerase and biotin-labeled nucleotides to form cRNA
(Affymetrix GeneChip® user manual, 2005). The cRNA was amplified, fragmented, and hybridized. Each sample was hybridized to a single GeneChip® Mouse Gene 1.0 ST Array (Affymetrix, Santa Clara, CA) containing 770,317 probes corresponding to 28,853 genes to obtain expression data. After hybridization, Streptavidin-Phycoerythrin (SAPE) solution was used to stain the chip by binding to the biotin molecules and emitting fluorescence which was detected when scanned. The scanned files containing the raw expression values from the GeneChip® Mouse Gene 1.0 ST Array were imported into Partek Genomics Suite v6.5 (Partek Inc, St Louis, MO), normalized using Robust Multichip Average (RMA) protocols and were log₂ transformed and analyzed in an a two-factor ANOVA to obtain a p-value and a fold change. The GeneChips® were processed at the London Regional Genomics Centre (Robarts Research Institute, London, Ontario, Canada). Initially, genes with a fold change > 2.0 or < -2.0 with a p-value <0.05 were considered to be differentially-expressed, but the conditions were too stringent and only a handful of genes (<10) met the criteria. The filtering parameters were changed to genes with a fold change > 1.5 or < -1.5 [372] with a p-value <0.05 to be considered differentially-expressed.

2.6.3 Pathway analysis of gene expression data

Ingenuity Systems Pathway Analysis® (IPA®) software version 9 (Ingenuity Systems, Redwood City, CA) was used to determine which predefined biological pathways had altered gene expression. A list of the Affymetrix gene identifiers, corresponding fold changes and p-values were uploaded onto the IPA® website for all the filtered genes. Each Affymetrix gene identifier was mapped to its corresponding gene in
the Ingenuity’s database. The canonical pathways analysis identified biological pathways whose genes were altered so that it may have a significant impact on the pathway. Genes from the data set that were associated with the canonical pathway in Ingenuity’s database were considered for further analysis. The significance of the association between the differentially-expressed genes and the canonical pathway was measured in two ways. First, a ratio was generated of the number of differentially-expressed genes from the data set that map to the pathway, divided by the total number of genes in the canonical pathway. Second, a Fisher’s exact test (IPA, Ingenuity Systems, Redwood City, CA) was used to calculate a p-value to determine the probability that the association between the genes in the dataset and the canonical pathway was explained by chance alone.
3.1 *Wild type (WT) and harlequin (hq) mice consumed the same amount of water regardless of phenobarbital (PB) concentration*

A two-factor analysis of variance (ANOVA) determined that untreated WT and hq mice consumed similar amounts of water (WT: 0.14 ± 0.04 ml/g/day; hq: 0.16 ± 0.06 ml/g/day) and that there was no taste aversion due to PB treatment (WT 2 ppm: 0.15 ± 0.04; hq 2 ppm: 0.20 ± 0.01; hq 4 ppm: 0.16 ± 0.03 ml/g/day).

3.2. *Compared to WT mice, hq mice were smaller and gained less mass with age*

A three-factor ANOVA showed that body mass was significantly lower in hq mice compared to WT mice at all ages (Figure 3.1a; p<0.001). At 3, 7, and 10 months of age, hq mice weighed 30%, 36%, and 35% less than age-matched WT mice, respectively. Body mass increased with age in WT and hq mice (p<0.001). However, WT mice had a faster rate of body mass increase with age, gaining 6.6% of WT body mass per month compared to 4.9% of hq body mass per month between 3 and 7 months of age.

3.2.1 *WT, more so than hq mice, gain mass with PB treatment*

Phenobarbital treatment increased body mass in WT and hq mice when compared to untreated control mice (Figure 3.1b; p=0.007). The rate of body mass increase was higher in WT mice with 2 ppm and 4 ppm PB treatment, gaining 7.9% and 8.1% of WT
Figure 3.1 – Mouse body mass increased with age and phenobarbital (PB) treatment for wild type (WT) and harlequin (hq) mice. A) Mean final body mass (g) was determined for WT and hq mice at 3, 7, or 10 months of age. Body mass was lower in hq mice compared to WT mice at all three ages (p<0.001). Body mass increased with age for both genotypes (p<0.001). B) Mean final body mass (g) was determined for WT and hq mice at 3, 7, or 10 months of age with three PB treatments (0, 2, and 4 ppm) at the younger ages. Body mass increased with PB treatment in WT and hq mice (p=0.007). Error bars represent ± S.E.M.
body mass per month compared to 5.6% and 7.0% of hq body mass per month, respectively. The rate of change in body mass when compared to the untreated WT and hq mice was Δ1.3% and Δ1.5% in WT mice at 2 and 4 ppm, while it was Δ0.7% and Δ2.1% in hq mice at 2 and 4 ppm.

3.3. WT and not hq mice were responsive to PB treatment showing increased activity

A three-factor multivariate analysis of variance (MANOVA) indicated a significant three-way interaction of genotype, age, and treatment in the total amount of overnight activity (p=0.006; Figure 3.2). A genotype-treatment interaction (p=0.001) was also detected as well as the main effects of genotype (p<0.001) and treatment (p<0.001). Activity was similar for untreated WT and hq mice and did not change with increased age. Phenobarbital had a different effect on each genotype. In 3-month-old WT mice, activity increased with PB treatment (p<0.001), with both 2 and 4 ppm increasing activity (2 ppm: p=0.006; 4 ppm: p<0.001). In 7-month-old WT mice, activity also increased with PB treatment (p=0.019), but only 2 ppm PB (2 ppm: p=0.017) and not 4 ppm increased activity. In hq mice, PB treatment did not have any effect on activity levels at either age.

3.3.1 Phenobarbital-treated WT mice had greater locomotion

There was a three-factor interaction of genotype, age, and treatment (p=0.010) as well as an interaction of genotype and treatment (p=0.002) on the total number of
Figure 3.2 – Mouse activity increased with phenobarbital (PB) treatment in wild type (WT) but not harlequin (hq) mice. Mean activity (events) over 9 hours was determined for WT and hq mice in three age groups (3, 7, and 10 months) with three PB treatments (0, 2, and 4 ppm). Activity was similar for untreated WT and hq mice and did not change with aging. Phenobarbital treatment increased activity in WT mice at 3 (p<0.001) and 7 (p=0.019) months of age. At 3 months of age, 2 and 4 ppm of PB increased activity levels of WT mice (2 ppm: p=0.006; 4 ppm: p<0.001), but at 7 months of age, only 2 ppm increased the activity levels of WT mice (2 ppm: p=0.017). Error bars represent S.E.M.
locomotion events. Each individual factor had an effect on locomotion as well: genotype (p=0.001), age (p=0.005), and treatment (p<0.001; Figure 3.3). Locomotor activity was similar for WT and hq mice without any treatment. Increased age did not alter locomotor activity in WT or hq mice. In WT mice, PB treatment increased locomotion at 3 months of age (p<0.001). Both 2 and 4 ppm of PB increased locomotion in 3-month-old WT mice (2 ppm: p=0.004; 4 ppm: p<0.001). At 7 months of age, PB treatment increased locomotion (p=0.048), but after post hoc analysis 2 and 4 ppm had p-values > 0.05. In hq mice, locomotion did not change with PB treatment.

3.3.2 Phenobarbital-treated WT mice had more stereotypic movements

An age-treatment interaction was detected for the number of stereotypic movements (p=0.006; Figure 3.4). Stereotypic movements were similar in untreated WT and hq mice and there was no change with increased age. Phenobarbital treatment increased stereotypic movements in 3-month-old WT mice (p=0.026), with 4 ppm of PB showing a significant increase in the number of stereotypic movements (4 ppm: p=0.042). However, in 7-month-old WT mice, PB did not increase stereotypic movements. Phenobarbital treatment had no effect on hq mice at either age.

3.3.3 Phenobarbital-treated WT mice traveled greater distances

An age-treatment interaction was detected for the distance travelled (p=0.018; Figure 3.5). For untreated WT and hq mice, distance travelled was similar and did not change with increased age for either genotype. In WT mice, 4 ppm PB
Figure 3.3 – Mouse locomotion increased with phenobarbital treatment in wild type (WT) but not harlequin (hq) mice. Mean locomotion (events) over 9 hours was determined for WT and harlequin (hq) mice in three age groups (3, 7, and 10 months) with three PB treatments (0, 2, and 4 ppm). Locomotion was similar for untreated WT and hq mice and did not change with aging. Phenobarbital treatment increased locomotion in WT mice at both 3 (p<0.001) and 7 (p=0.048) months of age. At 3 months of age, 2 and 4 ppm of PB increased locomotion levels of WT mice (2 ppm: p=0.004; 4 ppm: p<0.001), but at 7 months of age, after post hoc analysis, 2 and 4 ppm had p-values >0.05. Error bars represent ± S.E.M.
Figure 3.4 — Stereotypic behaviour increased with phenobarbital (PB) treatment in wild type (WT) but not harlequin (hq) mice. Mean stereotypes (events) over 9 hours was determined for WT and hq mice in three age groups (3, 7, and 10 months) with three PB treatments (0, 2, and 4 ppm). Stereotypic behaviour was similar for untreated WT and hq mice and did not change with aging. Phenobarbital treatment increased stereotypic behaviour in WT mice at 3 (p=0.026) and not 7 months of age. Error bars represent ± S.E.M.
Distance travelled increased with phenobarbital (PB) treatment in wild type (WT) but not harlequin (hq) mice. Mean total distance travelled (cm) over 9 hours was determined for WT and hq mice in three age groups (3, 7, and 10 months) with three PB treatments (0, 2, and 4 ppm). Distance travelled was similar for untreated WT and hq mice and did not change with aging. Phenobarbital treatment increased distance travelled in WT mice at 3 (p=0.010) and 7 (p=0.038) months of age. At 3 months of age, only 4 ppm of PB increased distance travelled in WT mice (4 ppm: p=0.009), and at 7 months of age, only 2 ppm of PB increased distance travelled (p=0.037). Error bars ±represent S.E.M.
increased distance travelled at 3 months of age (treatment: p=0.010; 4 ppm: p=0.009),
and at 7 months of age, 2 ppm of PB increased distance travelled (treatment: p=0.038; 2
ppm: p=0.037). Phenobarbital did not affect distance travelled in hq mice at either age.

3.3.4 Phenobarbital-treated WT mice spent less time resting

There was a significant three-way interaction of genotype, age, and treatment for
the total amount of time spent resting (p=0.004; Figure 3.6). A genotype-age interaction
(p=0.032) and a genotype-treatment interaction (p=0.002) were detected. The individual
factors of genotype (p=0.008) and treatment (p<0.001) also had an effect on the amount
of time spent resting. Untreated WT and hq mice spent a similar amount of time resting
and did not change with increased age. Phenobarbital treatment was associated with
decreased resting time in WT mice at 3 months of age (treatment: p<0.001; 2 ppm:
p=0.033; 4 ppm: p<0.001) and 7 months of age (treatment: p=0.009; 2 ppm: p=0.007).
Phenobarbital treatment had no effect on the amount of time spent resting in 3-month-old
hq mice. Phenobarbital treatment decreased the amount of time spent resting in 7-month-
old hq mice (treatment: p=0.038) but after post hoc analysis both 2 and 4 ppm of PB had
p-values > 0.05.

3.3.5 Phenobarbital-treated WT mice spent more time moving

There was a significant three-way interaction in the amount of time spent moving
slowly (p=0.004; Figure 3.7). There was a genotype-age interaction (p=0.006) and a
genotype-treatment interaction (p=0.036) on the amount of time moving slowly. The
Figure 3.6 – Time spent resting decreased with phenobarbital (PB) treatment in wild type (WT) but not in harlequin (hq) mice. Mean time (s) spent resting (moving <2 cm/s) over 9 hours was determined for WT and hq mice in three age groups (3, 7, and 10 months) with three PB treatments (0, 2, and 4 ppm). The amount of time spent resting was similar for untreated WT and hq mice and did not change with aging. Phenobarbital treatment decreased time spent resting in WT mice at 3 (p<0.001) and 7 (p=0.009) months of age. At 3 months of age, 2 and 4 ppm of PB decreased time spent resting in WT mice (2 ppm: p=0.033; 4 ppm: p<0.001), and at 7 months of age, only 2 ppm of PB decreased time spent resting (p=0.007). Phenobarbital did not have any effect on time spent resting in 3-month-old hq mice, but PB treatment decreased time spent resting in hq mice at 7 months of age (p=0.038), but after post hoc analysis 2 and 4 ppm of PB had p-values > 0.05. Error bars represent ± S.E.M.
Figure 3.7 – Time spent moving slowly increased with phenobarbital (PB) treatment in wild type (WT) but not in harlequin (hq) mice. The time (s) spent moving slowly (moving between 2 cm/s - 5 cm/s) over 9 hours for WT and hq mice in three age groups (3, 7, and 10 months) with three PB treatments (0, 2, and 4 ppm). The amount of time spent moving slowly was similar for untreated WT and hq mice and did not change with aging. Phenobarbital treatment increased time spent moving slowly in WT mice at 3 months of age (p<0.001) and 7 months of age (p=0.005). At 3 months of age, 2 and 4 ppm of PB increased the amount of time spent moving slowly in WT mice (2 ppm: p=0.012; 4 ppm: p<0.001), and at 7 months of age, 2 and 4 ppm of PB also increased the amount of time spent moving slowly in WT mice (2 ppm: p=0.005; 4 ppm: p=0.037). Phenobarbital did not have any effect on time spent moving slowly in 3-month-old hq mice, but PB treatment increased the amount of time spent moving slowly in hq mice at 7 months of age (p=0.025). At 7 months of age, 4 ppm of PB increased the amount of time spent moving slowly in hq mice (p=0.020). Error bars represent ± S.E.M.
individual factors of genotype (p=0.018) and treatment (p<0.001) had an effect on the amount of time spent moving slowly. The amount of time spent moving slowly was similar in untreated WT and hq mice and did not change with increased age for either genotype. Phenobarbital increased the amount of time spent moving slowly in WT mice at 3 months of age (treatment: p<0.001; 2 ppm: p=0.012; 4 ppm: p<0.001) and 7 months of age (treatment: p=0.005; 2 ppm: p=0.005; 4 ppm: p=0.037). The amount of time spent moving slowly in hq mice at 3 months of age was unaltered by PB. However, 4 ppm of PB did increase the amount of time spent moving slowly in hq mice at 7 months of age (treatment: p=0.025; 4 ppm: p=0.020).

The time spent moving quickly was influenced by a genotype-treatment (p=0.009) interaction (Figure 3.8). The individual factor of treatment (p=0.028) also had an effect on the time spent moving quickly. The amount of time spent moving quickly was similar in untreated WT and hq mice did not change with increased age in either genotype. Treatment with 4 ppm of PB increased time spent moving quickly in WT mice at 3 months of age (treatment: p=0.003, 4 ppm: p=0.002). At 7 months of age, 2 ppm of PB increased time spent moving quickly in WT mice (treatment: p=0.035; 2 ppm: p=0.027). Treatment with PB had no effect on the amount of time spent moving quickly in hq mice.

### 3.3.6 Phenobarbital-treated WT mice attained greater maximum and mean velocities

A significant three-way interaction was detected with maximum velocity (p=0.047; Figure 3.9). There was also a genotype-treatment interaction (p=0.031) effect on the maximum velocity. Individual factors of genotype (p=0.016) and age (p=0.010) had an effect on maximum velocity. Maximum velocity was similar for untreated WT
Figure 3.8 – Time spent moving quickly increased with phenobarbital (PB) treatment in wild type (WT) but not in harlequin (hq) mice. Mean time (s) spent moving quickly (moving >5 cm/s) over 9 hours was determined for WT and hq mice in three age groups (3, 7, and 10 months) with three PB treatments (0, 2, and 4 ppm). The amount of time spent moving quickly was similar for untreated WT and hq mice and did not change with aging. Phenobarbital treatment increased time spent moving quickly in WT mice at 3 months of age (p=0.003) and at 7 months of age (p=0.035). At 3 months of age, 4 ppm of PB increased the amount of time spent moving quickly in WT mice (p=0.002), and at 7 months of age, 2 ppm of PB increased the amount of time spent moving quickly in WT mice (p=0.027). Error bars represent ± S.E.M.
Figure 3.9 – Maximum velocity increased with phenobarbital (PB) treatment in wild type (WT) mice but not in harlequin (hq) mice. Mean maximum velocity (cm/s) over 9 hours was determined for WT and hq mice in three age groups (3, 7, and 10 months) with three PB treatments (0, 2, and 4 ppm). Maximum velocity was similar for untreated WT and hq mice and did not change with aging. Phenobarbital treatment increased maximum velocity in WT mice at 3 months of age (p=0.006) but not at 7 months of age. Error bars represent ± S.E.M.
and $hq$ mice and there was no change with increased age. In WT mice, PB treatment increased maximum velocity at 3 months of age (treatment: $p=0.006$; 4 ppm: $p=0.005$), but there was no change with maximum velocity with PB treatment at 7 months of age. Phenobarbital did not affect maximum velocity in $hq$ mice at either age.

Mean velocity was influenced by a significant age-treatment interaction ($p=0.016$; Figure 3.10). There was no difference in mean velocity between WT and $hq$ mice and mean velocity did not change with age for either genotype (Figure 3.10). Treatment with 4 ppm of PB increased mean velocity in 3-month-old WT mice (treatment: $p=0.010$; 4 ppm: $p=0.010$). At 7 months of age, 2 ppm of PB increased mean velocity in WT mice (treatment: $p=0.043$; 2 ppm: $p=0.043$). Phenobarbital treatment did not affect mean velocity in $hq$ mice for either age.

### 3.3.7 Phenobarbital-treated WT mice reared more but the duration of the events was unchanged

No interactions were detected in the number of rearings with a three-way MANOVA but two of the main factors had an effect on the number of rearings: genotype ($p=0.009$), and age ($p=0.026$; Figure 3.11). There was no difference in the number of rearing events in untreated WT and $hq$ mice and age did not affect the number of rearing events in either genotype. Both 2 and 4 ppm of PB increased the number of rearing events in WT mice at 3 months of age (treatment: $p=0.006$; 2ppm: $p=0.019$; 4 ppm: $p=0.008$) but PB did not have an effect on the number of rearing events at 7 months of age. Phenobarbital had no effect on the number of rearing events in $hq$ mice at either age. There was no difference in mean duration of rearing events for genotype, age, or PB treatment (Figure 3.12).
Figure 3.10 - Mean velocity increased with phenobarbital (PB) in wild type (WT) mice but not in harlequin (hq) mice. Mean velocity (cm/s) over 9 hours was determined for WT and hq mice in three age groups (3, 7, and 10 months) with three PB treatments (0, 2, and 4 ppm). Mean velocity was similar for untreated WT and hq mice and did not change with aging. Phenobarbital treatment increased mean velocity in WT mice at 3 months of age mice (p=0.010) and at 7 months of age (p=0.043). At 3 months of age, 4 ppm of PB increased mean velocity in WT mice (p=0.010) and at 7 months of age, 2 ppm of PB increased mean velocity in WT mice (p=0.043). Error bars represent ± S.E.M.
Figure 3.11 – Rearing behaviour increased with phenobarbital (PB) treatment in WT but not harlequin (hq) mice. Mean number of rearings (events) over 9 hours was determined for WT and hq mice in three age groups (3, 7, and 10 months) with three PB treatments (0, 2, and 4 ppm). Rearings behaviour was similar for untreated WT and hq mice and did not change with aging. Phenobarbital treatment increased the rearing behaviour in WT mice at 3 months of age (p=0.006) but not at 7 months of age. At 3 months of age, 2 and 4 ppm of PB increased the rearing behaviour (2ppm: p=0.019; 4 ppm: p=0.008). Error bars represent ± S.E.M.
Figure 3.12 – Rearing duration showed no effect of genotype, age, or phenobarbital (PB) treatment. Mean rearing duration (s) over 9 hours was determined for wild type (WT) and harlequin (hq) mice in three age groups (3, 7, and 10 months) with three PB treatments (0, 2, and 4 ppm). Error bars represent ± S.E.M.
3.4. Smaller cerebella were characteristic of PB-treated and untreated hq mice

A three-factor analysis of variance (ANOVA) showed a main effect of genotype (p=0.001) on the mass of the cerebellum, and that the cerebellar mass did not change with age or PB treatment in WT or hq mice. One-way ANOVA also confirmed that the hq cerebellum had a significantly smaller mass at 3, 7, and 10 months of age when compared to age-matched WT mice (p=0.001; Figure 3.13a). A three-way ANOVA for the ratio of the cerebellar mass to body mass showed a main effect of age (p<0.001). The cerebellar mass to body mass ratio was similar in WT and hq mice and decreased with age for each genotype (WT: p<0.001; hq: p=0.009; Figure 3.13b). For both genotypes, the cerebellar mass to body mass ratio decreased at 7 and 10 months of age when compared to mice at 3 months of age (WT 7 months: p=0.001; WT 10 months: p=0.001; hq 7 months: p=0.009; hq: 10 months: p=0.031). The hq cerebellum is visibly smaller at 7 and 10 months of age when compared to age-matched WT controls (Figure 3.14). Phenobarbital did not have an effect on the mass of the cerebellum in WT or hq mice (Figure 3.15; Figure 3.16).

3.4.1 Lobule specific early loss of granule neurons progressed across hq cerebella unaffected by PB treatment

A three-factor MANOVA was performed on the nuclear counts of the granule cell layer in WT and hq mice at three different ages with PB treatments and detected a
Figure 3.13 – Cerebellar mass is lower in harlequin (hq) mice but the cerebellar mass to body mass ratio is similar to wild type (WT) mice at all ages. A) WT mice had a greater cerebellar mass at all three ages (p<0.001) and cerebellar mass did not change with age or phenobarbital (PB) treatment in either genotype. B) The cerebellar mass to body mass ratio was similar in WT and hq mice and decreased with age in both genotypes (p<0.001). Five mice were used per experimental group. Error bars represent ± S.E.M.
Figure 3.14 - Histology of the cerebellum in 3-, 7-, and 10-month-old mice displays visible aging-associated cerebellar degeneration in hq mice with age.  

A) Hematoxylin and eosin staining of a 10 μm sagittal section of the cerebellum in 3-month-old WT with Roman numerals indicating cerebellar lobule numbers.  

B) Hematoxylin and eosin staining of a 10 μm sagittal section of the cerebellum in 3-month-old hq mice with absence of histopathology.  

C) An image of the cerebellum from 7-month-old WT and (D) hq mice with visible loss of the granule cell layer.  

E) The cerebellum of 10-month-old WT and (F) hq mice, where the hq cerebellum remained similar in size to (D) 7-month-old hq mice but is still visibly smaller compared to (E) 10-month-old WT mice.  

Scale bars: A-F = 1 mm
WT mice

3 months

A

hq mice

7 months

C

D

10 months

E

F
Figure 3.15 - Phenobarbital (PB) had no visible effect on the histology of the cerebellum of wild type (WT) mice. The histopathology of the cerebellum in 3-, and 7-month-old WT mice with PB treatment at 0, 2, or 4 ppm was examined. Hematoxylin and eosin staining of a 10 μm sagittal section of the cerebellum in (A) 3-month-old WT mouse with no PB treatment. Roman numerals indicate cerebellar lobule numbers. B) An image of 3-month-old WT mouse treated with 2 ppm of PB treatment. C) An image of 3-month-old WT mouse treated with 4 ppm of PB treatment. D) An image of the cerebellum from 7-month-old WT mouse with no PB treatment. E) An image of the cerebellum from 7-month-old WT mouse treated with 2 ppm of PB. F) An image of the cerebellum from 7-month-old WT mouse treated with 4 ppm of PB. Scale bars: A-F = 1 mm
Figure 3.16 – Phenobarbital (PB) had no visible effect on the histology of the cerebellum of harlequin (hq) mice. The histopathology of the cerebellum in 3-, and 7-month-old hq mice with PB treatment at 0, 2, or 4 ppm. Hematoxylin and eosin staining of a 10 μm sagittal section of the cerebellum in (A) 3-month-old hq mouse with no PB treatment. Roman numerals indicate lobule cerebellar numbers. B) An image of 3-month-old hq mouse treated with 2 ppm of PB. C) An image of 3-month-old hq mouse treated with 4 ppm of PB. D) An image of the cerebellum from 7-month-old hq mouse with no PB treatment. E) An image of the cerebellum from 7-month-old hq mouse treated with 2 ppm of PB. F) An image of the cerebellum from 7-month-old hq mouse treated with 4 ppm of PB. Scale bars: A-F = 1 mm
significant interaction between genotype and age for lobules IX and X (IX: \( p=0.019 \); X: \( p=0.037 \)). Main effects for genotype and age were also detected in all lobules (genotype, all lobules: \( p<0.001 \); age, I/II: \( p=0.002 \); III: \( p=0.002 \); VIa: \( p=0.001 \); IX: \( p=0.001 \); X: \( p=0.006 \); Figure 3.17). At 3 months of age, \( hq \) mice had similar nuclear counts for all lobules studied when compared to WT mice. At 7 months of age, \( hq \) mice had significantly decreased nuclear counts in lobules IX and X when compared to WT mice (IX: \( p=0.003 \); X: \( p=0.009 \)). At 10 months of age, all lobules examined had a decreased nuclear count when compared to WT mice (I/II: \( p=0.004 \); III: \( p=0.004 \); VIa: \( p=0.009 \); IX: \( p=0.002 \); X: \( p=0.001 \)).

The nuclear count did not change with age in WT mice for all lobules studied. In contrast, the nuclear count decreased with age in \( hq \) mice (I/II: \( p=0.036 \); III: \( p=0.012 \); VIa: \( p=0.007 \); IX: \( p=0.016 \); X: \( p=0.012 \); Figure 3.17). At 7 months of age, degeneration was visible in the granule cell layer as \( hq \) mice had decreased nuclear counts for lobules IX and X when compared to 3-month-old \( hq \) mice (IX: \( p=0.045 \); X: \( p=0.045 \)). At 10 months of age, all of the lobules studied had decreased nuclear counts (I/II: \( p=0.042 \); III: \( p=0.028 \); VIa: \( p=0.014 \); IX: \( p=0.017 \); X: \( p=0.013 \)) when compared to \( hq \) mice at 3 months of age. Phenobarbital treatment did not have any effect on the nuclear count in any of the cerebellar lobules in WT or \( hq \) mice (Figure 3.17).

### 3.4.2 Loss of Purkinje cells in \( hq \) mice was lobule specific, progressed with age, and was unaffected by PB treatment

A three-factor MANOVA was performed on the number of Purkinje cells in WT and \( hq \) mice at three different ages with PB treatments detected a significant
Figure 3.17 – Granule neuron cell counts decreased with age in all lobules examined in harlequin (hq) mice. For all lobules, the granule neuron count did not change with age in wild type (WT) mice and PB treatment had no effect on the granule neuron count in either WT or hq mice. A) The nuclear count in lobule I/II of the cerebellum. The nuclear count was similar for WT and hq mice at 3 and 7 months of age. At 10 months of age, hq mice had a decreased nuclear count when compared to WT mice (p=0.004). The nuclear count of lobule I/II decreased with age in hq mice (p=0.036) with decreased nuclear count in 10-month-old hq mice when compared to 3-month-old hq mice (p=0.042). B) The nuclear count in lobule III of the cerebellum. The nuclear count was similar for WT and hq mice at 3 and 7 months of age. At 10 months of age, hq mice had a decreased nuclear count when compared to WT mice (p=0.004). The nuclear count decreased with age in hq mice (p=0.012) with decreased nuclear count in 10-month-old hq mice when compared to 3-month-old hq mice (p=0.028). C) The nuclear count in lobule VIa of the cerebellum. The nuclear count was similar for WT and hq mice at 3 and 7 months of age. At 10 months of age, hq mice had a decreased nuclear count when compared to WT mice (p=0.009). The nuclear count decreased with age in hq mice (p=0.007) with decreased nuclear count in 10-month-old hq mice when compared to 3-month-old hq mice (p=0.014). D) The nuclear count in lobule IX of the cerebellum. The nuclear count was similar for WT and hq mice at 3 months of age. At 7 and 10 months of age, hq mice had a decreased nuclear count when compared to WT mice (7: p=0.003; 10: p=0.002). The nuclear count decreased with age in hq mice (p=0.016). In 7- and 10-month-old hq mice, the nuclear count was decreased when compared to 3-month-old hq mice (7 months: p=0.045; 10 months: p=0.017). E) The nuclear count in lobule X of the cerebellum. The nuclear count was similar for WT and hq mice at 3 months of age. At 7 and 10 months of age, hq mice had a decreased nuclear count when compared to WT mice (7: p=0.009; 10: p=0.001). The nuclear count decreased with age in hq mice (p=0.002). In 7- and 10-month-old hq mice, the nuclear count was decreased when compared to 3-month-old hq mice (7 months: p=0.045; 10 months: p=0.013). Error bars represent ± S.E.M.
C

Lobule Vla

Granule neuron count (% area) mean ± SEM

Mouse Age
- △ 10 months
- □ 7 months
- ○ 3 months

0 2 4 0 2 4
Phenobarbital Treatment (ppm)
WT hq
Mouse Genotype

D

Lobule IX

Granule neuron count (% area) mean ± SEM

Mouse Age
- △ 10 months
- □ 7 months
- ○ 3 months

0 2 4 0 2 4
Phenobarbital Treatment (ppm)
WT hq
Mouse Genotype
E

Granule neuron count (% area) mean ± SEM

Lobule X

Mouse Age

△ 10 months

□ 7 months

○ 3 months

Phenobarbital Treatment (ppm)

WT

*hq*

Mouse Genotype
interaction between genotype and age for lobules I/II, III, VIa, IX, and X (I/II: \(p=0.006\); III: \(p=0.002\); VIa: \(p<0.001\); IX: \(p<0.001\); X: \(p=0.002\); Figure 3.18). The main effect of genotype was detected in all lobules, and the main effect of age was detected in lobules III, VIa, IX, and X (genotype, all lobules: \(p<0.001\); age, III: \(p<0.001\); VIa: \(p<0.001\); IX: \(p<0.001\); X: \(p=0.022\); Figure 3.18). At 3 months of age, WT and \(hq\) mice had a similar number of Purkinje cells for all lobules studied. At 7 months of age, \(hq\) mice had significantly decreased number of Purkinje cells in lobules III, VIa, IX, and X when compared to WT mice (III: \(p=0.001\); VIa: \(p=0.036\); IX: \(p=0.024\); X: \(p=0.025\)). At 10 months of age, \(hq\) mice had a decreased number of Purkinje cells in lobules III, VIa, and IX when compared to WT mice (III: \(p=0.017\); VIa: \(p=0.007\); IX: \(p=0.017\)).

The number of Purkinje cells did not change with age in WT mice for any of the lobules studied. In contrast, the number of Purkinje cells decreased with age in lobules I/II, III, VIa, and IX in \(hq\) mice (I/II: \(p=0.010\), III: \(p<0.001\); VIa: \(p=0.006\); IX: \(p=0.014\); Figure 3.18). At 7 months of age, Purkinje cells were reduced in lobules I/II, III, VIa, and IX when compared to \(hq\) mice at 3 months of age (I/II: \(p=0.021\); III: \(p<0.001\); VIa: \(p=0.046\); IX: \(p=0.030\)). At 10 months of age, Purkinje cells were reduced in lobules I/II, III, VIa, and IX when compared to \(hq\) mice at 3 months of age (I/II: \(p=0.013\); III: \(p<0.001\); VIa: \(p=0.005\); IX: \(p=0.017\)). In addition, at 10 months of age, lobule VIa had a reduced number of Purkinje cells when compared to \(hq\) mice at 7 months of age (\(p=0.001\)). Phenobarbital treatment did not have any effect on the number of Purkinje cells in any of the cerebellar lobules in WT or \(hq\) mice (Figure 3.18).
Figure 3.18 – Purkinje cell numbers were decreased in *harlequin (hq)* mice. For all lobules the number of Purkinje cells did not change with age in wild type (WT) mice and PB treatment had no effect on the number of Purkinje cells in WT or *hq* mice.  

A) The Purkinje cell numbers in lobule I/II of the cerebellum. The Purkinje cell number was similar for WT and *hq* mice at 3, 7, and 10 months of age. The number of Purkinje cells decreased with age in lobule I/II in *hq* mice (*p*=0.010). At 7 and 10 months of age, Purkinje cells were significantly decreased when compared to *hq* mice at 3 months of age (7 months: *p*=0.021; 10 months: *p*=0.013).  

B) The Purkinje cell numbers in lobule III of the cerebellum. The Purkinje cell number was similar for WT and *hq* mice at 3 months of age. At 7 and 10 months of age, *hq* mice had decreased numbers of Purkinje cells in lobule III when compared to WT mice (7 months: *p*=0.001; 10 months: *p*=0.017). The number of Purkinje cells decreased with age in lobule III in *hq* mice (*p*<0.001). At 7 and 10 months of age, Purkinje cells were significantly decreased when compared to *hq* mice at 3 months of age (7 months: *p*<0.001; 10 months: *p*<0.001).  

C) The Purkinje cell numbers in lobule VIa of the cerebellum. The Purkinje cell number was similar for WT and *hq* mice at 3 months of age. At 7 and 10 months of age, *hq* mice had decreased numbers of Purkinje cells in lobule VIa when compared to WT mice (7 months: *p*=0.06; 10 months: *p*=0.007). The number of Purkinje cells decreased with age in lobule VIa in *hq* mice (*p*<0.001). At 7 and 10 months of age, Purkinje cells were significantly decreased when compared to *hq* mice at 3 months of age (7 months: *p*=0.046; 10 months: *p*=0.005). At 10 months of age, lobule VIa had a decreased number of Purkinje cells when compared to *hq* mice at 7 months of age (*p*=0.001).  

D) The Purkinje cell numbers in lobule IX of the cerebellum. The Purkinje cell number was similar for WT and *hq* mice at 3 months of age. At 7 and 10 months of age, *hq* mice had decreased numbers of Purkinje cells in lobule IX when compared to WT mice (7 months: *p*=0.024; 10 months: *p*=0.007). The number of Purkinje cells decreased with age in lobule IX in *hq* mice (*p*=0.014). At 7 and 10 months of age, Purkinje cells were significantly decreased when compared to *hq* mice at 3 months of age (7 months: *p*=0.030; 10 months: *p*=0.017).  

E) The Purkinje cell numbers in lobule X of the cerebellum. The Purkinje cell number was similar for WT and *hq* mice at 3 months of age. At 7 months of age, *hq* mice had decreased numbers of Purkinje cells in lobule X when compared to WT mice (7 months: *p*=0.025). At 10 months of age, the number of Purkinje cells in WT and *hq* mice were similar. Error bars represent ± S.E.M.
A  

Lobule I/II

Mouse Age

△ 10 months  
- 7 months  
○ 3 months

Phenobarbital Treatment (ppm)

WT hq

Mouse Genotype

Purkinje cell count (number) mean ± SEM

B  

Lobule III

Mouse Age

△ 10 months  
- 7 months  
○ 3 months

Phenobarbital Treatment (ppm)

WT hq

Mouse Genotype

Purkinje cell count (number) mean ± SEM
3.4.3 Neurodegeneration was evident in 7-month-old hq mice and unaffected by PB treatment

A three-factor ANOVA for the number of Fluoro-Jade® B positive cells indicated that there was a significant interaction between genotype and age (p<0.001). At 3 months of age, WT and hq mice had a similar number of Fluoro-Jade® B positive cells (Figure 3.19). At 7 months of age, hq mice had an elevated number of Fluoro-Jade® B positive cells compared to WT mice at 7 months of age (p=0.014). At 10 months of age, WT and hq mice had a similar number of Fluoro-Jade® B positive cells. In WT mice, the number of Fluoro-Jade® B positive cells did not change with age. Fluoro-Jade® B positive cells were more numerous at 7 months of age in hq mice when compared to hq mice at 3 and 10 months of age (3 months: p=0.003, 10 months: p=0.002). Phenobarbital treatment had no effect on the number of Fluoro-Jade® B positive cells in WT or hq mice.

3.4.4 Lipofuscin accumulation with age was lobule specific in both WT and hq mice and unaffected by PB treatment

A three-factor MANOVA on lipofuscin accumulation indicated that there was a significant genotype and age interaction in lobule III (p=0.049), as well as a genotype effect in lobules IX and X (IX: p<0.001; X: p=0.012) and age effect in lobules I/II, VIa, and IX (I/II: p=0.002; VIa: p<0.001; IX: p=0.012). WT and hq mice had a similar amount of lipofuscin in 3-, 7-, and 10-month-old mice for all lobules despite the reported genotype effect for lobules IX and X (Figure 3.20).
Figure 3.19 – There were elevated numbers of dying cells in 7-month-old harlequin (hq) mice. Fluoro-Jade® B positive cells were similar in wild type (WT) and hq mice at 3 and 10 months of age. The number of Fluoro-Jade® B positive cells did not change with age in WT mice. Fluoro-Jade® B positive cells were elevated at 7 months of age in hq mice when compared to age-matched WT mice (p=0.014). Fluoro-Jade® B positive cells were elevated at 7 months of age in hq mice when compared to hq mice at 3 and 10 months of age (3 months: p=0.003, 10 months: p=0.002). Phenobarbital had no effect on the number of Fluoro-Jade® positive cells in WT or hq mice. Error bars represent ± S.E.M.
Figure 3.20 – Lipofuscin accumulation was similar in wild type (WT) and harlequin (hq) mice and increased with age in certain lobules. For all lobules PB treatment had no effect on lipofuscin accumulation. A) The lipofuscin autofluorescence profile in lobule I/II of the cerebellum. Lipofuscin autofluorescence was similar in WT and hq mice for all ages studied and PB treatment. Lipofuscin autofluorescence did not change with age in WT mice. Within hq mice, lipofuscin autofluorescence increased with age in lobule I/II (p=0.016). Lipofuscin autofluorescence in lobule I/II was elevated in 7- and 10-month-old hq mice when compared to 3-month-old hq mice (7 months: p=0.026; 10 months: p=0.023). B) The lipofuscin autofluorescence profile in lobule III of the cerebellum. The lipofuscin autofluorescence was similar in WT and hq mice for all ages studied. C) The lipofuscin autofluorescence profile in lobule VIa of the cerebellum. The lipofuscin autofluorescence was similar in WT and hq mice for all ages studied. In WT mice, lipofuscin autofluorescence in lobule VIa was elevated with age (p=0.037). At 10 months of age, WT mice had elevated lipofuscin autofluorescence in lobule VIa when compared to WT mice at 3 months of age (p=0.040). In hq mice, lipofuscin autofluorescence in lobule VIa was elevated with age (p=0.037), with increased lipofuscin autofluorescence at 10 months of age when compared to 3 months of age (p=0.040). D) The lipofuscin autofluorescence profile in lobule IX of the cerebellum. The lipofuscin autofluorescence was similar in WT and hq mice and did not change with age. E) The lipofuscin autofluorescence profile in lobule X of the cerebellum. The lipofuscin autofluorescence was similar in WT and hq mice and did not change with age. Error bars represent ± S.E.M.
Lipofuscin accumulated with age in lobule VIa (p=0.037) in WT mice (Figure 3.20). In lobule VIa, lipofuscin was elevated at 10 months (p=0.040) when compared to WT mice at 3 months of age. In hq mice, lipofuscin also accumulated with age in lobule I/II and VIa (I/II: p=0.016; VIa: p=0.037). Lipofuscin in lobule I/II was elevated at 7 and 10 months of age when compared to hq mice at 3 months of age (7 months: p=0.026; 10 months: p=0.023). In lobule VIa, lipofuscin was elevated at 10 months (p=0.040) when compared to hq mice at 3 months of age. Despite the genotype and age interaction reported for lobule III, and the age effect for lobule I/II and IX, further analysis with one way-ANOVA testing both showed no change with genotype or age. Both 2 and 4 ppm PB had no effect on lipofuscin accumulation in WT and hq mice (Figure 3.20).

3.4.5 ROS increased with age in lobules I/II, III, VIa in both PB-treated and untreated hq mice

A three-factor MANOVA for DHE fluorescence detected a significant effect of age in lobules I/II, III, and VIa (I/II: p=0.050; III: p=0.017; VIa: p=0.002). There was no difference in DHE fluorescence between WT and hq mice at any age in lobules I/II, III, VIa, XI, and X of the cerebellum (Figure 3.21). In WT mice, the DHE fluorescence did not increase with age in any lobule. In hq mice, DHE fluorescence was increased at 10 months of age when compared to 3 months of age for lobules I/II, III, and VIa (I/II: p=0.011; III: p=0.003; VIa: p=0.009). Phenobarbital had no effect on DHE fluorescence in WT or hq mice.
Figure 3.21 - The ROS profiles in wild type (WT) and harlequin (hq) mice were similar at all three ages but ROS did increase with age in hq mice. A) The ROS profile in lobule I/II of the cerebellum. The DHE intensity was similar in WT and hq mice for all ages studied and PB treatment. The DHE intensity increased at 10 months of age within hq mice (p=0.011), however it was still not different from WT mice. B) The ROS profile in lobule III of the cerebellum. The DHE intensity was similar in WT and hq mice for all ages studied and PB treatment. The DHE intensity increased at 10 months of age within hq mice (p=0.003), however it was still not different from WT mice. C) The ROS profile in lobule VIa of the cerebellum. The DHE intensity was similar in WT and hq mice both genotypes for all ages studied and PB treatment. The DHE intensity increased at 10 months of age within hq mice (p=0.009), however it was still not different from WT mice. D) The ROS profile in lobule IX of the cerebellum. The DHE intensity was similar in WT and hq mice for all ages studied and PB treatment. E) The ROS profile in lobule X of the cerebellum. The DHE intensity was similar in WT and hq mice for all ages studied and PB treatment. Error bars represent ± S.E.M.
3.5. Mutation frequencies were similar in WT and hq mice but a ROS mutation signature was more frequent in young hq mice

In total, 12.5 million plaque forming units (pfu’s) were assayed, 414 mutants were collected and 273 independent cII mutations were identified (Table 3.1). The average packaging efficiency was 130,000 pfu’s with a minimum of three and a maximum of six packaging reactions per mouse. A linear regression showed that mutation frequency in hq mice was not correlated with body mass. Spontaneous mutant and mutation frequency showed no significant differences with mouse genotype or age. Mutation patterns were similar between mouse genotypes and ages (Table 3.2). The majority of mutations were G:C to A:T transitions at CpG dinucleotides followed by small deletions less than 50 base pairs in size. Hotspots for mutation in the cII sequence were at CpG dinucleotides and mononucleotide repeats (Figure 3.2). With one exception, single nucleotide insertions and deletions extended or shortened mononucleotide runs. Single base pair deletions and insertions at monobasic runs were not elevated significantly in the cerebellum of hq mice compared to age-matched WT mice. One GC to CT tandem-base mutation was found in a young WT mouse. This same mouse also had one mutant with seven proximal point mutations spanning only 21 base pairs (Figure 3.2).

Four deletions, ranging in length from 10 to 21 base pairs, were found in four of the five young hq mice. Sequences both upstream and downstream the deletion junctions were analyzed for direct sequence repeats but none were found. These deletions were found exclusively in the cerebellum of hq mice in this study (p=0.004) and not in our larger database of 657 cII mutations in kidney, heart, and skin of hq and WT mice (p=0.0001).
Table 3.1  Mutant and mutation frequency in the cerebellum of \( hq \) (\( X^{hq} Y \)) and wild (\( Y \)) (\( XY \)) mice at three and seven months of age

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Age (months)</th>
<th>Mouse Number</th>
<th>Final Body Mass (g)</th>
<th>SEM* Body Mass</th>
<th>Pfu’s* Assayed x 10^6</th>
<th>Mutants with ( cII ) Mutations</th>
<th>Mutant Frequency(^{\circ} ) x 10^6</th>
<th>SEM (Mutant Frequency)</th>
<th>Independent Mutations(^{\circ} )</th>
<th>% Recurrent Mutations(^{\circ} )</th>
<th>Mutation Frequency(^{\circ} ) x 10^6</th>
<th>SEM (Mutation Frequency)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( X^{hq} Y )</td>
<td>3</td>
<td>78</td>
<td>16.6</td>
<td>6.4</td>
<td>4.1</td>
<td>2.1</td>
<td>5.2</td>
<td>11</td>
<td>48</td>
<td>2.7</td>
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<tr>
<td>Group total (mean)</td>
<td>(19.0)</td>
<td>0.63</td>
<td>37.9</td>
<td>109</td>
<td>(2.9)</td>
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<tr>
<td>( X^{hq} Y )</td>
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<tr>
<td>Group total (mean)</td>
<td>(35.8)</td>
<td>1.98</td>
<td>38.1</td>
<td>108</td>
<td>(2.8)</td>
<td>0.64</td>
<td>72</td>
<td>(33)</td>
<td>(1.9)</td>
<td>0.45</td>
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</tbody>
</table>

*SEM is standard error of the mean.

*Pfu forming units assayed.

The frequency of confirmed \( cII \) mutants in the total.

The number of nonrecurrent mutations identified in a mouse tissue.

The percentage of recurrent mutations in a single mouse sample.

The frequency of nonrecurrent mutations in the total number of plaque forming units assayed.

One mutant contained seven point mutations over 21 base pairs.

One mutant contained two mutations (i.e., a tandem-base substitution).
Table 3.2. Pattern of independent mutations in the cerebellum of hq (X\(^{hq}\)Y) and wild type (XY) mice at three and seven months of age

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Age (months)</th>
<th>No. of independent mutations</th>
<th>TS (\rightarrow) CpG</th>
<th>TS (\rightarrow) non CpG</th>
<th>TV (\rightarrow) CpG</th>
<th>TV (\rightarrow) non CpG</th>
<th>Classes of Mutation (%)</th>
<th>D/I</th>
</tr>
</thead>
<tbody>
<tr>
<td>X(^{hq})Y</td>
<td>3</td>
<td>69</td>
<td>22</td>
<td>7</td>
<td>9</td>
<td>1</td>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td>X(^{hq})Y</td>
<td>7</td>
<td>61</td>
<td>25</td>
<td>11</td>
<td>15</td>
<td>0</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>XY(^{cd})</td>
<td>3</td>
<td>78</td>
<td>22</td>
<td>17</td>
<td>15</td>
<td>0</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>XY</td>
<td>7</td>
<td>72</td>
<td>14</td>
<td>17</td>
<td>17</td>
<td>1</td>
<td>3</td>
<td>3</td>
</tr>
</tbody>
</table>

\(^a\) TS = transversions; TV = transversions; CpG = 5 C followed by G 3; D/I = deletions and insertions.
\(^b\) the number of nonrecurrent mutations in a mouse tissue.
\(^c\) one mutant contained seven point mutations over 21 base pairs.
\(^d\) one mutant contained two base substitutions in tandem.
3.22 - Spontaneous mutations in the cII transgene harvested from the cerebellum of 3- and 7-month-old wild type (WT) and harlequin (hq) Big Blue® mice. The numbering convention used for the cII nucleotide positions is that reported previously [362]. Single base substitutions are written above the base position affected. Each independent mutation type has a superscript to designate the experimental cohort (Y = 3-month-old Young wild type, W = 7-month-old middle adult Wild type, H = 3-month-old young adulthood Harlequin, Q = 7-month-old middle adulthood harleQuin). The boxed base pairs below the wild type sequence are nucleotides inserted and lines below the sequence underline the nucleotides deleted. The tandem-base mutation is circled and the seven mutations observed in a single mutant are marked with triangles.
3.6. The transcriptomes of the cerebellum of hq mice displayed immune and inflammatory response markers

A total of 55 genes were differentially expressed with 48 up-regulated and seven down-regulated in the cerebellum of 3-month-old hq mice compared to WT mice with fold changes greater than 1.5 (p<0.05, Table 3.3). Significantly altered pathways included the classical complement system, crosstalk between dendritic cells and natural killer cells, the neuroprotective role of thimet oligopeptidase-1 (THOP1) in AD, and communication between innate and adaptive immune cells (Table 3.4).

3.6.1 Phenobarbital treatment had a minimal effect on the transcriptomes of WT and hq mice

Phenobarbital treatment (4 ppm) in WT mice resulted in five differentially expressed genes. Four genes were up-regulated and one gene was down-regulated in WT mice treated with 4 ppm of PB when compared to untreated WT mice (Table 3.5). Phenobarbital treatment (4 ppm) in hq mice resulted in eight differentially expressed genes when compared to untreated hq mice. Seven genes were up-regulated and one gene was down-regulated in hq mice treated with 4 ppm of PB when compared to untreated hq mice (Table 3.6). For WT and hq mice treated with PB, too few genes were differentially-expressed to perform pathway analysis.
### Table 3.3 List of differentially-expressed genes in the cerebellum of 3-month-old *hq* mice compared to age-matched wild type mice

<table>
<thead>
<tr>
<th>Rank Order</th>
<th>Gene Symbol</th>
<th>Gene Name</th>
<th>Biological Relevance</th>
<th>p-value</th>
<th>Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Up-regulated genes</td>
<td>Mela</td>
<td>Melanoma antigen</td>
<td>Melanoma antigen; envelope glycoprotein</td>
<td>7.4E-08</td>
<td>14.52</td>
</tr>
<tr>
<td>1</td>
<td><em>Ifitm2</em></td>
<td>Interferon-induced transmembrane protein 2</td>
<td>Inflammatory response; induced by type I and II interferons, induces apoptosis independent of p53 but dependent on caspases, up-regulated in schizophrenia</td>
<td>8.2E-06</td>
<td>1.66</td>
</tr>
<tr>
<td>2</td>
<td><em>Mthfd2</em></td>
<td>Methylene tetrahydrofolate dehydrogenase (NADP+ dependent) 2, methenyltetrahydrofolate cyclohydrolase</td>
<td>Bifunctional mitochondrial enzyme; involved in the folate pathway, up-regulation leads to hyperhomocysteinemia which is associated with increased risk for neurological disorders</td>
<td>4.7E-05</td>
<td>1.61</td>
</tr>
<tr>
<td>3</td>
<td><em>C1qb</em></td>
<td>Complement component 1, q subcomponent, B chain</td>
<td>B chain of the complement subcomponent C1Q; associates with C1R and C1S to yield C1, link between innate and acquired immunity, clears apoptotic cells</td>
<td>8.5E-05</td>
<td>1.92</td>
</tr>
<tr>
<td>4</td>
<td><em>Tm4sf1</em></td>
<td>Transmembrane 4 L six family member 1</td>
<td>Transmembrane protein; mediates signal transduction events, role in the regulation of cell development, activation, growth, adhesion and motility, up-regulation leads to increased cell mobility</td>
<td>1.3E-04</td>
<td>1.51</td>
</tr>
<tr>
<td>5</td>
<td><em>H2-K1</em></td>
<td>Histocompatibility 2, K1, K region</td>
<td>Major histocompatibility complex I component; antigen processing and presentation</td>
<td>1.5E-04</td>
<td>1.54</td>
</tr>
<tr>
<td>6</td>
<td><em>Tcnip</em></td>
<td>Thioredoxin interacting protein</td>
<td>Inhibitor of thioredoxin activity; induced by stress, over expression inhibits cell proliferation and promotes apoptosis</td>
<td>1.6E-04</td>
<td>2.63</td>
</tr>
<tr>
<td>7</td>
<td><em>Reep6</em></td>
<td>Receptor accessory protein 6</td>
<td>Membrane protein; proapoptotic function, involved in intracellular membrane trafficking</td>
<td>1.8E-04</td>
<td>1.70</td>
</tr>
<tr>
<td>8</td>
<td><em>Tmem176a</em></td>
<td>Transmembrane protein 176A</td>
<td>Transmembrane protein; up-regulation in dendritic cells maintains their immature state</td>
<td>2.1E-04</td>
<td>1.71</td>
</tr>
<tr>
<td>9</td>
<td><em>Ifitm3</em></td>
<td>Interferon-induced transmembrane protein 3</td>
<td>Receptor; involved in inflammatory response and potential role in cell adhesion, up-regulated in schizophrenia, autism and cancers</td>
<td>2.4E-04</td>
<td>2.32</td>
</tr>
<tr>
<td></td>
<td>Gene Symbol</td>
<td>Function and Description</td>
<td>Score</td>
<td>Log2 Fold Change</td>
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<tr>
<td>10</td>
<td>Ifitm3</td>
<td>Receptor; involved in inflammatory response and potential role in cell adhesion, up-regulated in schizophrenia, autism and cancers</td>
<td>2.4E-04</td>
<td>2.32</td>
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<tr>
<td>11</td>
<td>Laptm5</td>
<td>Transmembrane receptor in lysosomes; mediates programmed cell death, up-regulated in degenerating neuroblastoma cells through caspase-independent lysosomal cell death due to lysosomal destabilization with lysosomal-membrane permeabilization, with impaired autophagy, rat homologue is up-regulated by cell death in granule neurons</td>
<td>2.7E-04</td>
<td>1.59</td>
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</tr>
<tr>
<td>12</td>
<td>CtsS</td>
<td>Lysosomal protease; involved in the degradation of apoptotic cells, up-regulated by microglia during inflammation</td>
<td>2.9E-04</td>
<td>1.52</td>
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<tr>
<td>13</td>
<td>Cdl4</td>
<td>Hematopoietic stem cell (HSC) antigen; involved in adhesion, migration, vascular permeability and integrity; HSCs are able to migrate into the brain and differentiate into neural cell types, HSCs are increased after brain injury and CD34 is up-regulated in the blood of patients with Alzheimer's Disease with a possible role in brain regeneration</td>
<td>4.0E-04</td>
<td>1.62</td>
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<tr>
<td>14</td>
<td>Tmem176b</td>
<td>Transmembrane protein; maintains immature state of dendritic cells by limiting proinflammatory responses</td>
<td>4.7E-04</td>
<td>1.69</td>
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<tr>
<td>15</td>
<td>Edn1</td>
<td>Peptide hormone; involved in neurite elongation, stress response and apoptosis, up-regulation causes increased blood pressure and decreased blood flow which can cause cerebral ischemia</td>
<td>4.8E-04</td>
<td>2.19</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>Sloc43a3</td>
<td>Transporter; endothelial cell specific and has a microvascular function</td>
<td>4.8E-04</td>
<td>1.77</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>Igfbp7</td>
<td>Cell adhesive glycoprotein; involved in the regulation of cell growth and negative regulation of cell proliferation</td>
<td>5.0E-04</td>
<td>1.56</td>
<td></td>
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<tr>
<td></td>
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</tr>
<tr>
<td>18</td>
<td><strong>Ly86</strong></td>
<td><strong>Lymphocyte antigen86</strong></td>
<td>Lymphocyte antigen; innate immune response by signaling T cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>19</td>
<td><strong>Sle7a5</strong></td>
<td><strong>Solute carrier family 7 member 5</strong></td>
<td>Transporter; transports large neutral amino acids, up-regulated in tumour cells to supply more amino acids to support proliferation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td><strong>Ly6a</strong></td>
<td><strong>Lymphocyte antigen 6 complex locus A</strong></td>
<td>Hematopoietic stem cell antigen, able to migrate into the brain and differentiate into neural cell types</td>
<td></td>
<td></td>
</tr>
<tr>
<td>21</td>
<td><strong>Tyrobp</strong></td>
<td><strong>TYRO protein kinase binding protein</strong></td>
<td>Transmembrane signaling protein; controls the production of microglial superoxide ions, up-regulation can lead to apoptosis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>22</td>
<td><strong>Gm11428</strong></td>
<td><strong>Predicted gene</strong></td>
<td>Unknown</td>
<td></td>
<td></td>
</tr>
<tr>
<td>23</td>
<td><strong>Gfap</strong></td>
<td><strong>Glial fibrillary acidic protein</strong></td>
<td>Astrocyte activation marker; increased Gfap leads to increased cytoplasmic protein aggregates with recruitment of stress proteins, cytoplasmic inclusions, cytoskeleton disruption, decreased proliferation, increased cell death in response to H$_2$O$_2$, reduced proteasomal function and compromised astrocyte resistance to stress</td>
<td></td>
<td></td>
</tr>
<tr>
<td>24</td>
<td><strong>C1qc</strong></td>
<td><strong>Complement component 1, q subcomponent C chain</strong></td>
<td>C chain of the complement subcomponent C1Q; involved in immune response through clearance of apoptotic cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>25</td>
<td><strong>C4b</strong></td>
<td><strong>Complement component 4B</strong></td>
<td>Complement component; classical pathway in inflammatory response, up-regulated in a mouse model of traumatic brain injury</td>
<td></td>
<td></td>
</tr>
<tr>
<td>26</td>
<td><strong>C3ar1</strong></td>
<td><strong>Complement component 3a receptor 1</strong></td>
<td>Chemokine receptor; involved in the inflammatory response, up-regulated in response to meningitis, encephalomyelitis and multiple sclerosis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>27</td>
<td><strong>Emcn</strong></td>
<td><strong>Endomucin</strong></td>
<td>Mucin-like sialoglycoprotein; interferes with focal adhesion complexes and inhibits interaction between cells and the extracellular matrix</td>
<td></td>
<td></td>
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<td></td>
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<td>-----------------------------------------------------------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>28</td>
<td><strong>Cd68</strong></td>
<td><strong>Cluster of differentiation 68</strong></td>
<td>Macrophage/microglial cell surface antigen; expressed in monocytes and macrophages, lysosomal or plasma membrane shuffling protein postulated to participate in cell adhesion and antigen presentation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>29</td>
<td><strong>Ccl3</strong></td>
<td><strong>Chemokine (C-C motif) ligand 3</strong></td>
<td>Cytokine; involved in the accumulation of activated glial cells and inflammation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>30</td>
<td><strong>Lgals3bp</strong></td>
<td><strong>Lectin, galactoside-binding, soluble, 3 binding protein</strong></td>
<td>Beta-galactoside-binding protein; promotes intergrin-mediated cell adhesion, may stimulate host defense against viruses and tumour cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>31</td>
<td><strong>Lyz2</strong></td>
<td><strong>Lysozyme 2</strong></td>
<td>Lysozyme; immune response, breaks down bacterial cell walls as a host defense against bacterial infection, microglia over-express lysozyme when activated as part of the inflammation response</td>
<td></td>
<td></td>
</tr>
<tr>
<td>32</td>
<td><strong>Casp12</strong></td>
<td><strong>Caspase 12</strong></td>
<td>Cysteine protease; involved in neuronal death induced by ischemia/reperfusion</td>
<td></td>
<td></td>
</tr>
<tr>
<td>33</td>
<td><strong>Serpina3n</strong></td>
<td><strong>Serine protease inhibitor, clade A, member 3N</strong></td>
<td>Serine protease inhibitor; component of the inflammatory response and up-regulated in amyloid plaques</td>
<td></td>
<td></td>
</tr>
<tr>
<td>34</td>
<td><strong>Adh1</strong></td>
<td><strong>Alcohol dehydrogenase 1</strong></td>
<td>Enzyme that converts ethanol to acetaldehyde; also has retinol dehydrogenase activity suggesting participation in the retinoic acid synthesis pathway</td>
<td></td>
<td></td>
</tr>
<tr>
<td>35</td>
<td><strong>Slc14a1</strong></td>
<td><strong>Solute carrier family 14 member 1</strong></td>
<td>Transporter; transports urea out of the cell</td>
<td></td>
<td></td>
</tr>
<tr>
<td>36</td>
<td><strong>Ehhd1</strong></td>
<td><strong>EGF, latrophilin and seven transmembrane domain containing 1</strong></td>
<td>Membrane protein; neuropeptide signaling and signal transduction</td>
<td></td>
<td></td>
</tr>
<tr>
<td>37</td>
<td><strong>Itm2a</strong></td>
<td><strong>Integral membrane protein 2A</strong></td>
<td>Membrane protein; involved in chondrogenic and skeletal muscle differentiation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>38</td>
<td>39</td>
<td>40</td>
<td>41</td>
<td>42</td>
<td>43</td>
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<td>----</td>
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<td>----</td>
</tr>
<tr>
<td>38</td>
<td><strong>Eng</strong></td>
<td><strong>Endoglin</strong></td>
<td>Transmembrane glycoprotein; expressed in endothelial cells, vascular smooth muscle cells, macrophages, role in the generation of sporadic brain arteriovenous malformations</td>
<td>5.7E-03</td>
<td>1.52</td>
</tr>
<tr>
<td>39</td>
<td><strong>Agxt2I</strong></td>
<td><strong>Alanine glyoxylate aminotransferase 2 like 1</strong></td>
<td>Enzyme; amino acid catabolism of arginine, glutamate, glutamine and proline, up-regulated in individuals with schizophrenia or bipolar disorder</td>
<td>7.1E-03</td>
<td>1.71</td>
</tr>
<tr>
<td>40</td>
<td><strong>Chrnb4</strong></td>
<td><strong>Cholinergic receptor, nicotinic, beta 4</strong></td>
<td>Nicotine, acetylcholine, and cation ligand channel; involved in physiological processes such as memory, anxiety, sleep control, nociception, and autonomic nervous system function</td>
<td>7.9E-03</td>
<td>1.53</td>
</tr>
<tr>
<td>41</td>
<td><strong>Taf1d</strong></td>
<td><strong>TATA box binding protein (TBP)-associated factor</strong></td>
<td>Transcription factor component; binds to TATA binding proteins to help regulate transcription initiation from RNA polymerase I</td>
<td>8.3E-03</td>
<td>1.64</td>
</tr>
<tr>
<td>42</td>
<td><strong>Gm129</strong></td>
<td><strong>Predicted gene</strong></td>
<td>Unknown</td>
<td>9.7E-03</td>
<td>1.59</td>
</tr>
<tr>
<td>43</td>
<td><strong>Cd93</strong></td>
<td><strong>Cluster of differentiation 93</strong></td>
<td>Transmembrane glycoprotein; required for the engulfment of apoptotic cells</td>
<td>1.0E-02</td>
<td>1.78</td>
</tr>
<tr>
<td>44</td>
<td><strong>Phyhd1</strong></td>
<td><strong>Phytanoyl-CoA dioxygenase domain containing 1</strong></td>
<td>Unknown</td>
<td>1.2E-02</td>
<td>1.59</td>
</tr>
<tr>
<td>45</td>
<td><strong>Snord82</strong></td>
<td><strong>Small nucleolar RNA, C/D box 8</strong></td>
<td>Guide RNA; methylation of pre-rRNA</td>
<td>1.7E-02</td>
<td>1.51</td>
</tr>
<tr>
<td>46</td>
<td><strong>Gpx5</strong></td>
<td><strong>Glutathione peroxidase 3</strong></td>
<td>Peroxidase; protects cells from oxidative damage by reducing hydrogen and lipid peroxides</td>
<td>2.1E-02</td>
<td>1.53</td>
</tr>
<tr>
<td>47</td>
<td><strong>Acta2</strong></td>
<td><strong>Actin alpha 2 smooth muscle aorta</strong></td>
<td>Actin family of proteins; integrity of the vascular contractility, up-regulated in glioma invasion, increased Acta2 could lead to increased proliferation and stroke</td>
<td>2.6E-02</td>
<td>1.57</td>
</tr>
<tr>
<td>48</td>
<td><strong>Plscr2</strong></td>
<td><strong>Phospholipid scramblase 2</strong></td>
<td>Plasma membrane protein; binds to Ca^{2+} which is crucial for reorganization of plasma membrane phospholipids during cell activation, phagocytosis and apoptosis</td>
<td>4.3E-02</td>
<td>1.74</td>
</tr>
<tr>
<td>Rank</td>
<td>Gene</td>
<td>Description</td>
<td>Down-regulated genes</td>
<td></td>
<td></td>
</tr>
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<td>------</td>
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<td>-----------------------------------------------------------------------------</td>
<td>--------------------------------------------------------------------------------------</td>
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<td></td>
</tr>
<tr>
<td>1</td>
<td>Tnc</td>
<td>Tenascin C</td>
<td>Extracellular matrix protein; inhibits olfactory sensory neuron neurite outgrowth;</td>
<td></td>
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</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>the olfactory epithelium, Tnc-null mice have a delayed onset of odor detection</td>
<td></td>
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</tr>
<tr>
<td>2</td>
<td>Aifm1</td>
<td>Apoptosis inducing factor mitochondrial associated 1</td>
<td>Mitochondrial protein; involved in the assembly/maintenance of complex I and</td>
<td></td>
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</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>induces apoptosis when translocated into the nucleus, decreased Aifm1 leads to</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>mitochondrial dysfunction and premature aging</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Gabra6</td>
<td>Gamma-aminobutyric acid (GABA) A receptor, alpha 6</td>
<td>Neurotransmitter receptor; binds the inhibitory neurotransmitter GABA, down-</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>regulation leads to decreased inhibition</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Car4</td>
<td>Carbonic anhydrase 4</td>
<td>Membrane-anchored zinc metalloenzyme; converts carbon dioxide and water to the</td>
<td></td>
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</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>bicarbonate ion and hydrogen ion, located in the endothelial cells of blood vessels</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>in the plasma membrane, it buffers brain extracellular pH, decreased Car4 results</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>in decreased buffering capacity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Bclp2</td>
<td>Chitinase like protein 2</td>
<td>Unknown</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Stap2</td>
<td>Signal transducing adaptor family member 2</td>
<td>Adaptor protein; substrate of a non-receptor tyrosine kinase, negatively-regulates</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>immune and inflammatory responses by strengthening host defense</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Cerk1</td>
<td>Ceramide kinase-like</td>
<td>Lipid kinase; negative regulator of apoptosis, mutations in Cerk1 lead to retinitis</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>pigmentosa in humans</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Differentially-expressed genes were identified as having a minimum fold change of 1.5 and a p-value less than 0.05.

*Gene order was determined according to increasing p-value.
Table 3.4 Pathways affected significantly by differentially-expressed genes in the cerebellum of 3-month-old *hq* mice when compared to wild type mice.

<table>
<thead>
<tr>
<th>Pathway</th>
<th>Effect</th>
<th>p-value</th>
<th>Ratio</th>
<th>Gene symbol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complement System</td>
<td>Up-regulated</td>
<td>1.4E-04</td>
<td>4/32</td>
<td><em>C1qb, C1qc, C4b, C3ar1</em></td>
</tr>
<tr>
<td>Crosstalk between Dendritic Cells and Natural Killer Cells</td>
<td>Up-regulated</td>
<td>1.9E-03</td>
<td>3/75</td>
<td><em>Tyrobp, H2-K1, Acta2</em></td>
</tr>
<tr>
<td>Neuroprotective Role of THOPI in Alzheimer's Disease</td>
<td>Down-regulated</td>
<td>6.6E-03</td>
<td>2/39</td>
<td><em>Sarpina3n, H2-K1</em></td>
</tr>
<tr>
<td>Communication between Innate and Adaptive Immune Cells</td>
<td>Up-regulated</td>
<td>1.9E-02</td>
<td>2/81</td>
<td><em>Ccl3, H2-K1</em></td>
</tr>
</tbody>
</table>

* Analysis was performed using Ingenuity Systems Pathway Analysis (Redwood City, CA).

* Pathway order was determined by increasing p-value.

* Ratio is the number of genes with altered regulation divided by the total number of genes in the given pathway.

* Gene descriptions are given in Table 3.3.
Table 3.5  List of differentially-expressed genes in the cerebellum of 3-month-old wild type mice treated with 4 ppm phenobarbital$^a$ compared to age-matched untreated wild type mice$^b$

<table>
<thead>
<tr>
<th>Rank Order</th>
<th>Gene Symbol</th>
<th>Gene Name</th>
<th>Biological Relevance</th>
<th>p-value</th>
<th>Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Up-regulated</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Sh3pxd2a</td>
<td>SH3 and PX domains 2A</td>
<td>Adaptor protein; stimulates metalloprotease activity and increases proteolytic activity of peptidase proteins as an intermediate step in Amyloidβ neurotoxicity signaling pathway.</td>
<td>1.8E-03</td>
<td>1.52</td>
</tr>
<tr>
<td>2</td>
<td>Aldh3b2</td>
<td>Aldehyde dehydrogenase 3 family, member B2</td>
<td>Unknown</td>
<td>4.0E-03</td>
<td>1.51</td>
</tr>
<tr>
<td>3</td>
<td>Phf11</td>
<td>PHD finger protein 11</td>
<td>Regulator of Th1 type cytokine gene expression; increased Phf11 increases expression of Th1 type genes, up-regulates NF-κB pathway that is involved in cell survival, proliferation and inflammation</td>
<td>1.9E-02</td>
<td>1.54</td>
</tr>
<tr>
<td>4</td>
<td>Hoxa5</td>
<td>Homeo box A5</td>
<td>Transcription factor; increased HOXA5 inhibits angiogenesis in brain hemangioem, stabilizes adherens junctions, decreases permeability of endothelial cells thus suggests a role for a stable vascular phenotype</td>
<td>3.6E-02</td>
<td>1.61</td>
</tr>
<tr>
<td><strong>Down-regulated</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Gpatch8</td>
<td>G patch domain containing 8</td>
<td>Unknown; potentially involved in RNA processing and modification</td>
<td>9.6E-04</td>
<td>-1.64</td>
</tr>
</tbody>
</table>

$^a$Phenobarbital treatment was delivered in the drinking water *ad libitum* and was initiated at 1 month of age until euthanization.

$^b$Differentially expressed genes were identified as having a minimum fold change of 1.5 and a p-value less than 0.05.

$^c$Gene order was determined according to increasing p-value.
Table 3.6 List of differentially-expressed genes in the cerebellum of 3-month-old *hq* mice treated with 4 ppm phenobarbital\(^a\) compared to untreated *hq* mice\(^b\)

<table>
<thead>
<tr>
<th>Rank Order(^a)</th>
<th>Gene Symbol</th>
<th>Gene Name</th>
<th>Biological Relevance</th>
<th>p-value</th>
<th>Fold-Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Up-regulated</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Afaf</td>
<td>Acrosome formation-associated factor</td>
<td>Membrane protein; present in acrosomes and early endosomes, possible role in membrane trafficking, fusion and secretory processes</td>
<td>1.3E-04</td>
<td>1.72</td>
</tr>
<tr>
<td>2</td>
<td>Trdn</td>
<td>Triadin</td>
<td>Membrane protein; calcium release from the sarcoplasmic reticulum, overexpression reduces calcium release</td>
<td>7.1E-04</td>
<td>2.59</td>
</tr>
<tr>
<td>3</td>
<td>Gm7957</td>
<td>Predicted gene</td>
<td>Unknown</td>
<td>1.4E-03</td>
<td>1.59</td>
</tr>
<tr>
<td>4</td>
<td>Igca</td>
<td>IQ motif containing with AAA domain</td>
<td>Unknown</td>
<td>3.8E-03</td>
<td>1.51</td>
</tr>
<tr>
<td>5</td>
<td>Trh</td>
<td>Thyrotropin releasing hormone</td>
<td>Hormone; regulates the release of thyroid-stimulating hormone, inhibits glutamate, neuroprotective activity in human spinal chord injury and animal trauma models</td>
<td>1.0E-02</td>
<td>2.14</td>
</tr>
<tr>
<td>6</td>
<td>Htr5b</td>
<td>5-hydroxytryptamine (serotonin) receptor 5B</td>
<td>Receptor; up-regulated in response to social isolation stress</td>
<td>1.0E-02</td>
<td>1.74</td>
</tr>
<tr>
<td>7</td>
<td>Ctnnap3</td>
<td>Contactin associated protein-like 3</td>
<td>Cell recognition protein; mediates neuron-glial interactions</td>
<td>3.2E-02</td>
<td>1.57</td>
</tr>
<tr>
<td>Down-regulated</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Gm5064</td>
<td>Predicted gene</td>
<td>Unknown</td>
<td>2.5E-02</td>
<td>-1.52</td>
</tr>
</tbody>
</table>

\(^a\)Phenobarbital treatment was delivered in the drinking water *ad libitum* and was initiated at 1 month of age until euthanization.

\(^b\)Differentially-expressed genes were identified as having a minimum fold change of 1.5 and a p-value less than 0.05.

\(^c\)Gene order was determined according to increasing p-value.
CHAPTER FOUR – DISCUSSION

Given that individuals with cancer and neurodegenerative diseases have elevated DNA damage and decreased DNA repair and given that hormesis has demonstrated efficacy as an anti-carcinogenesis strategy in a mouse model of hepatocarcinoma, I hypothesized that chronic low doses of PB treatment would delay cerebellar neurodegeneration. The hq mouse had no detectable change in nocturnal behaviour compared to WT mice and this did not change with increased age. Losses of granule and Purkinje cells were observed in hq mice, but increased ROS and ROS-associated damage were not the initiating factors and likely a consequence of mitochondrial dysfunction. The transcriptome analysis implicated inflammation as an early mechanism of cerebellar degeneration in hq mice. Unfortunately chronic low doses of PB did not delay the onset of the symptoms associated with neurodegeneration in hq mice.

My objective was to study the effect of cerebellar degeneration on 11 different parameters of nocturnal behaviour as measured by the ActiTrack® apparatus with and without PB treatment in WT and hq mice. Given that cerebellar function is required for coordinated movement, I hypothesized that hq mice would have decreased nocturnal behaviours compared to WT mice. Given that phenobarbital administration increases activity in rodents, I hypothesized that phenobarbital administration would ameliorate age-related decreases in nocturnal behaviours in hq mice through mechanisms that would reduce cerebellar degeneration.

My second objective was to measure different markers of cerebellar degeneration with and without PB treatment in WT and hq mice. Given that hq mice undergo cerebellar neurodegeneration, I hypothesized that there would be an increase in cell loss,
apoptosis, lipofuscin accumulation, and superoxide anion levels. Given that phenobarbital administration affects different anti-aging pathways, then hormetic phenobarbital administration would limit cell loss, apoptosis, lipofuscin accumulation and superoxide anion levels.

My third objective was to determine the mutant and mutation frequency and mutation pattern in WT and hq mice. Given that hq mice had an elevated number of single nucleotide deletions in the brain compared to WT mice using the PLAP assay, I hypothesized that there would be an increase in the spontaneous mutant and mutation frequency of the cII gene and a change in the mutation pattern in the cerebellum of hq mice when compared to WT mice.

My fourth objective was to determine what genes and pathways were altered in hq mice with and without PB treatment early in the cerebellar degenerative process. Given that pathways of ROS, mitochondrial dysfunction, and inflammation have been detected in neurodegenerative disorders, I hypothesized that hq mice would have altered pathways involved in ROS, mitochondrial dysfunction, and inflammation. Given that PB administration may affect different anti-cancer pathways, I hypothesized that phenobarbital administration would counter act pathways involved in ROS, mitochondrial dysfunction, and inflammation in hq mice.

4.1 Chronic low doses of PB did not delay or ameliorate cerebellar degeneration in hq mice

Cell loss in the cerebellum of hq mice was evident at 7 months of age and progressed further at 10 months of age. Phenobarbital administration did not delay
granule or Purkinje cell loss. Low doses of PB did not reduce lipofuscin accumulation or superoxide anion levels and did not reverse the inflammatory and immune response in *hq* mice. The transcriptome analysis suggests that *hq* mice have lower expression of a component of the GABA<sub>A</sub> receptor, γ-aminobutyric acid (GABA) <sub>A</sub> receptor α<sub>6</sub>, potentially reducing the effect of PB compared to WT mice. Although low-dose PB treatment in *hq* mice was not effective at delaying or limiting cell loss, there are other treatments available for testing in *hq* mice. Possible hormetic and targeted treatments are discussed herein with the new knowledge of the mechanisms of cerebellar cell loss in *hq* mice.

### 4.1.1 The cerebella of *hq* disease mice display normal early development

Cytologically and histologically there were no differences in the 3-month-old cerebellum of *hq* mice compared to WT mice, suggesting that the cerebellum undergoes normal development despite the down-regulation of *Aif*. *Aif* is required for development as complete knockout of *Aif* leads to embryonic lethality at day 11.5 [251] and targeted knockout of *Aif* in the cerebellum and midbrain at embryonic day 8.75 leads to lethality after birth with both granule and Purkinje cells having aberrant cell cycles [373]. Another targeted knockout of *Aif* further on in development leads to normal granule and Purkinje cell development at postnatal day 1 but lethality occurs between postnatal days 5 and 8 [373]. The two targeted knockout experiments suggest that *Aif* is required for neurogenesis but the embryos are still able to develop normally for a short period of time [373]. Thus, despite the 80% down-regulation of *Aif* in *hq* mice [232], there is enough *Aif* for the cerebellum to develop normally.
4.1.2 Unlike WT mice, hq mice show early cerebellar degeneration

With all of the biomarkers that were used to evaluate neurodegeneration in hq mice, it appeared that in WT mice, the biomarkers did not change across the three age cohorts tested. The number of nuclei in the granule and Purkinje cell layers did not change with increasing age in WT mice. Previous research found that granule cell number is constant in mice ranging from 10 to 31 months of age [124, 374]. Furthermore, granule cell numbers do not vary in the different lobules or change medio-laterally [375]. Decreased numbers of Purkinje cells are found in mice at 18, 28, or 31 months of age [124, 198, 374], which is much older than the cohorts of WT mice in the experiment herein. Out of the 35 degeneration markers examined, the only marker that did change with age was lipofuscin autofluorescence in lobule VIa. Since most of the neurodegenerative markers that were used did not change with increasing age, the mixed CBA/CaJ and C57BL/6 background is a suitable control for studying early neurodegeneration in hq mice.

At 7 months of age, Fluoro-Jade® B positive degenerating neurons were elevated in the cerebellum of hq mice (Figure 3.19). Very few granule cells were positive for apoptosis, but rather it was the white matter layer containing axons, cell bodies, and neurites in the deep cerebellar nuclei where motor outputs leave the cerebellum toward the thalamus that were degenerating at 7 months of age. Cerebellar cell loss was evident affecting both granule and Purkinje cells. Cell loss of the granule layer was confined to lobules IX and X, and at 10 months of age, all lobules studied had fewer granule cells (Figure 3.14 and Figure 3.17). Additionally at 7 months of age, Purkinje cell numbers
were reduced in all lobules assayed but lobule X (Figure 3.18). The regional patterning of granule cell loss observed herein was similar to previous findings [232, 376] suggesting that the down-regulation of *Aif* leads to an initial regional and temporal pattern of neuron loss followed by widespread degeneration in all lobules.

Regional patterning of cell loss is not unique to the granule cell layer but was also detected in the Purkinje cell layer in *hq* mice. The pattern of Purkinje cell loss in the anterior cerebellum herein is different to a previous finding where Purkinje cell loss in 7-month-old *hq* mice is worse in the posterior lobules in the cerebellum [232] although the exact number of Purkinje cells are not quantified. In a different study with 8-month-old *hq* mice, Purkinje cell loss is seen in the hemisphere region of the anterior cerebellum lobules (I-V) and in the central and posterior cerebellar lobules VI-VIII [376]. In 11-month-old *hq* mice, widespread Purkinje cell loss is extended into the vermis cerebellum [376]. By 14 months of age, most Purkinje cells are lost, with surviving Purkinje cells located mostly in lobule X [376]. In *hq* mice, Purkinje cells resistant to degeneration express heat shock protein 25 [376]. Heat shock protein 25 is expressed in a small number of Purkinje cells in lobule VI and VII and is expressed constitutively in lobule X [377]. Even though heat shock protein 25 is also expressed in lobules VI and VII, Purkinje cells in these regions are not protected from degeneration as demonstrated herein and previous reports [376], so something in addition to heat shock protein 25 is protective in Purkinje cells in lobule X. The cerebellum has an intricate pattern of gene expression that is not yet fully understood. As there is a spatial and temporal pattern to cerebellar degeneration in *hq* mice, knowing whether *Aif* or other genes are expressed in
the different lobules at different time points would help shed light on what genes are contributing to the spatial resistance of degeneration in granule or Purkinje cells.

4.1.3 The cerebella of hq mice do not show features of premature aging and are different from other models of cerebellar degeneration

The anterior-posterior patterning of granule and Purkinje cell loss is not restricted to hq mice but has also been seen in other mouse models of cerebellar degeneration. Leaner mutant mice display massive granule cell loss in the anterior lobules of the cerebellum [208]. Lurcher mutant mice, similar to hq mice, lose Purkinje cells in all lobules, however, Purkinje cells in lobule X of lurcher mice that are positive for heat shock protein 25 are more resistant to cell death [378]. In weaver mutant mice, Purkinje cell loss occurs in all lobules but is the worst in the central lobules (VI-VII) followed by the anterior lobules (I-V) [379]. Together, these examples demonstrate the asymmetric molecular organization of the cerebellum, and further analysis needs to be performed to determine whether there are temporal and spatial differences in Aif levels in the lobules of the cerebellum resulting in the pattern of granule and Purkinje cell loss or whether the up-regulation of other stress response genes, like heat shock factors in Purkinje cells are also responsible for granule cell survival.

4.1.4 The hq mouse is a model of mitochondrial dysfunction instead of increased oxidative stress

The hq mouse most resembles a model of mitochondrial dysfunction. Two cases of mutated AIF have been documented in humans [254]. A trinucleotide deletion was detected in the AIF gene at position 601-603 in exon 5, leading to the deletion of an
arginine residue in two related individuals [254]. Both individuals had muscle weakness and atrophy and magnetic resonance imaging of the brain revealed abnormal signals in the neostriatum. Fibroblast cells show a reduction in the activity of complex III and IV of the electron transport chain. Mitochondria are necessary for producing cellular energy for the cell in the form of ATP, and defects in the components of electron transport chain can lead to a multitude of disorders. Mitochondrial dysfunction can affect the retina [252], brain [252, 380, 381], heart [380], skeletal muscle [252, 381], skin [382], and hair [382]. Mitochondrial dysfunction has a frequency of 1 in 7,600 births with complex I dysfunction being the most common [383]. Unfortunately there are no cures for mitochondrial dysfunction. Although hq mice have been studied in different contexts relating to neurodegeneration, the hq mouse would make a useful tool in studying the multisystem disorder that occurs with mitochondrial dysfunction as well as testing possible treatments.

4.2. Cerebellar ataxia did not affect nocturnal cage behaviour in hq mice

To examine the hypothesis that hq mice will have decreased nocturnal behaviours compared to WT mice, 3-, 7-, and 10-month-old WT and hq mice were placed individually into the ActiTrack® home cage and 11 nocturnal behaviours were monitored.

4.2.1 Nocturnal cage behaviour in WT mice is unchanged with age

The observation that WT behaviour did not change with age was not unexpected. Since the mean lifespan of CBA mice is 25 months of age and the lifespan of C57BL/6J mice is 25-28 months of age [384], 7- or 10-month-old mice are still considered to be in
middle adulthood and expected to have minimal behavioural changes. In fact, a recent behavioural study in adult and aged C57BL/6 mice shows that 7-month-old and 29-month-old WT mice have similar locomotor activity levels [385]. However, it is important to keep in mind that there are strain-specific behavioural aging patterns. For example, locomotor activity is elevated in C57BL/6 when compared to BALB/C and DBA/2 mice [386]. Furthermore, C57BL/6 locomotor activity levels do not change between 5 and 27 months of age whereas locomotor activity at the same ages decreases in BALB/C mice and locomotor activity levels remain low and do not change in DBA/2 mice [386]. The mixed CBA/CaJ and C57BL/6J background was suitable as a baseline comparison for early cerebellar degeneration.

4.2.2 *hq* mice have unaltered nocturnal cage behaviour compared to WT mice

Contrary to the central hypothesis, *hq* mice did not show evidence of decreased nocturnal behaviours compared to WT mice and this did not change with age, which was unexpected. Although the cerebellum plays an important role in the coordination of movement, multiple regions of the brain are required for locomotion including the motor cortex, thalamus, basal ganglia, brain stem, and spinal cord. The *hq* mice have some apoptotic neurons in the motor cortex, thalamus, and basal ganglia at 4 months, but neuron loss is not progressive and is limited [234]. Since the parameters of nocturnal behaviour were similar in WT and *hq* mice this suggests that these other regions of the brain involved in locomotion are resistant to neuron loss as a result of Aif deficiency. The cerebellum is the primary site of neurodegenerative disease in *hq* mice and there is limited impact on mouse nocturnal locomotor behaviour up to 10 months of age.
4.2.3 Other models of cerebellar degeneration have behavioural changes

Other mouse models of cerebellar degeneration have either increased or decreased activity levels when compared to control mice [227, 331-335]. Hyperactivity is seen in both *lurcher* [227, 331] and *reeler* [332] mutant mice. Most granule cells and nearly all Purkinje cells degenerate in *lurcher* mice [211]. Since Purkinje cells are inhibitory and the only output of the cerebellar cortex, a decrease in Purkinje cells leads to a decrease in cerebellar inhibition and can lead to an increase in excitatory messages to the motor cortex and subsequent hyperactivity [387]. Increased cerebellar disinhibition is not likely in *reeler* mice as Purkinje cell degeneration is mild with a 50% loss of Purkinje cells [222]. Since the ratio of Purkinje cells to deep cerebellar nuclei is 10:1 in mice [210], severe Purkinje cell degeneration is needed for a 1:1 ratio or less of Purkinje cells to deep cerebellar nuclei and cerebellar disinhibition [226]. The hyperactivity seen in *reeler* mice could be due to increased glutamate dehydrogenase activity in the forebrain [388], possibly due to elevated glutamate, an excitatory neurotransmitter involved in locomotor activity [389]. *Weaver* mutant mice, in addition to cerebellar degeneration also have degeneration in the basal ganglia, specifically of the dopaminergic neurons in substantia nigra pars compacta [390] that can lead to hypoactivity. On the other hand both *staggerer* and *leaner* mutant mice have degeneration in the inferior olive [391], which can also lead to hypoactivity [392]. These mouse models of cerebellar degeneration all show alterations to regions of the brain other than the cerebellum and combined this leads to altered nocturnal cage behaviour, unlike *hq* mice.
4.2.4 *Qualitative changes in behaviour are not tracked with an actimeter*

The ActiTrack® actimeter is able to quantify behaviours in mice involved in simple movements like locomotion, stereotypes, and rearing. However, mouse behaviour is complex and mice with neurodegeneration can have behavioural changes that may not affect parameters of activity. A decrease in stride length and duration is detected in a mouse model of PD [145] and an increase in stride duration and a decrease in coordination are detected in a mouse model of ALS [147, 393]. Although behaviour did not change with age, the location of the most severe degeneration of granule cells occurred in lobules IX and X in *hq* mice, which is the location of afferent and efferent connections with the vestibular nuclei [394-396]. Ablation of lobules IX and X in cats leads to an unsteady gate [397], and lesions in lobules VIII-X in rats enhances tremors induced by harmaline, a reversible inhibitor of monoamine oxidase [398]. It has been well documented that *hq* mice have ataxia characterized by an unsteady gait and lateral tremors [232, 268], but it appears that the ataxia doesn’t alter nocturnal behaviours that were measured in *hq* mice. One caveat of the ActiTrack® system is that it is able to measure quantitative parameters of activity but not able to assess the quality of movement. To better study the behaviour of *hq* mice with progressive neurodegeneration, studies using rotarod, gait analysis, and video surveillance in conjunction with nocturnal behaviour would better indicate the quality of motor coordination and behaviour with neurodegeneration.
4.3 Phenobarbital was delivered in a hormetic dose producing expected behavioural effects in WT mice

4.3.1 Low-dose PB had a stimulatory effect on nocturnal behaviour in WT mice

Phenobarbital administration increases activity in rodents [299, 300], and indeed that is the case in the WT mice herein. All the parameters that were measured showed a significant shift towards increased nocturnal activity with the exception of the duration of rearing events, which was not altered with PB treatment. Phenobarbital treatment of WT mice could be described as having a biphasic or hormetic effect on behaviour. High-dose PB treatment in mice causes inhibition of the central nervous system by binding GABA$_A$ receptors leading to an increase in chloride ion conductivity and subsequent hyperpolarization [296]. Low-dose PB treatment in WT mice had an overall stimulatory affect. This excitatory action of PB has been documented before in humans and in animals [300, 399-401]. Evidence suggests that the excitatory mechanism of PB occurs through elevated levels of dopamine. Rats treated with PB show a two- to five-fold increase in dopamine levels in the caudate nucleus, cortex, midbrain, and the cerebellum and this occurs during the dark phase when rats are at their most active [402]. Another study was able to show that the dopamine receptor blocker, haloperidol, inhibits the PB stimulant effect on locomotor activity in mice [300]. In addition, $\alpha$–methyl-$p$-tyrosine (AMPT), a tyrosine hydroxylase inhibitor, suppresses locomotor activity in mice treated with PB [300]. Tyrosine hydroxylase is required for the conversion of the amino acid L-tyrosine to dihydroxyphenylalanine (DOPA) [403], a dopamine precursor, and suggests that newly synthesized dopamine is involved in the increased locomotor activity of WT mice treated with PB. The exact mechanism of how PB causes an increase in dopamine
levels has not yet been elucidated but low concentrations of PB increases calcium conductance in *Aplysia* [404], and calcium is able to increase tyrosine hydroxylase activity and dopamine production [405]. Even though PB administration resulted in increased nocturnal behaviour, prolonged exposure of WT mice to 4 ppm of PB resulted in desensitization to PB at 7 months of age. This is consistent with physiological tolerance to PB where the organism has increased resistance after repeated exposure to a drug. Chronic exposure to PB in mice leads to an 18% down-regulation in the protein levels of the GABA_A receptor [406]. In rats, chronic exposure to PB leads to a 45% decrease in the protein levels of GABA_A receptor subunit α 1 [407]. This suggests that chronic exposure to 4 ppm of PB could lead to a down-regulation of the GABA_A receptor and should be confirmed. The observed stimulatory effect of PB on WT mice herein, even at low doses, is consistent with an exposure to a dose of PB that is capable of eliciting a response.

4.3.2 Low-dose PB increased body mass in WT and hq mice

A hypothesis that could explain weight gain in both WT and *hq* mice with PB treatment is that PB stimulates feeding systems located in the hypothalamus. There is evidence that GABA and GABA_A receptors are involved in feeding behaviours. GABA agonists, muscimol and flurazepam, when injected into the medial hypothalamus, increases feeding behaviour while GABA antagonists inhibits feeding behaviour [408]. In addition, silencing of the *Huntingtin-associated protein-1* gene, capable of stabilizing GABA_A receptors, reduces the level and activity of hypothalamic GABA_A receptors and leads to a decrease in food intake and body weight [409]. Since *hq* mice have down-
regulated γ-aminobutyric acid A receptor, α6 (Gabra6) which is a GABA<sub>A</sub> receptor subunit, this could decrease feeding behaviour and could explain why hq mice have a smaller body mass and a lower rate of weight gain when compared to WT mice. Since PB binds the GABA<sub>A</sub> receptor, it would also explain why both WT and hq mice gained weight with PB treatment, however, the rate of weight gain was lower in hq mice which would be consistent with a down-regulated GABA<sub>A</sub> receptor gene. The hq mice showed a dose response effect with 4 ppm of PB having a faster rate of weight gain than 2 ppm in hq mice, indicating that more PB is required to increase body mass in hq mice because of the Gabra6 down-regulation. WT mice did not show the same dose response in weight gain possibly because all the GABA<sub>A</sub> receptors were saturated with 2 ppm but also because there may be less capacity for larger WT mice to increase in body mass compared to hq mice. To test the hypothesis that the Gabra6 subunit down-regulation leads to increased feeding behaviour, the daily mass of food consumed could be measured to determine whether PB stimulates appetite in both WT and hq mice.

4.4 Elevated ROS levels do not appear to be a major driver or consequence of hq cerebellar degeneration

To test the hypothesis that hq mice will have elevated levels of ROS, the mutant and mutation frequency and pattern, lipofuscin autofluorescence, and superoxide anion levels were determined for WT and hq mice at 3, 7, and 10 months of age. The transcriptome was also examined for genes involved in the oxidative stress response.
4.4.1 ROS-induced mutations in the nuclear DNA do not appear to be a predominant feature of hq cerebellar degeneration

Unlike the previous observation of elevated frequency of mutation in the brain of hq mice, the cII assay did not reveal significant elevation in spontaneous mutation frequency in the cerebellum of hq mice in young and middle adulthood [410]. This observation of a constant mutation frequency is consistent with previous observations that mutation frequency is constant in the cerebellum over the mouse life span [242, 243]. Even in mouse mimics of premature aging, the frequency of spontaneous mutation would not be expected to be significantly elevated in brain tissues. Elevated mutation frequency in skin [240] but not cerebellum of hq mice may result from replication of skin epithelium and fixation of mutations in comparison to the postmitotic granule neurons of the cerebellum.

There was no change in mutation pattern in the cerebellum of hq mice compared to WT mice at 3 and 7 months of age [410], consistent with previous observations of relatively unchanged mutation pattern over the lifespan of the mouse [242, 243]. A change in mutation pattern would be difficult to detect since 20 different base lesions are associated with oxidative DNA damage [411], thus decreasing the sensitivity of the cII assay to detect individual signature mutations for modest increases above the background level of mutation. The most common mutation signature of oxidative stress, G:T transversions [347], was not over represented in hq mice. Mutagenesis does not appear to be a major driver or consequence of decreased Aif levels.
4.4.2 Contrary to a previous report, mutation frequency in the cerebellum of hq mice was not elevated

The observation that mutation frequency and pattern in hq mice were not different from WT mice conflicted with a previous report where the mutant frequency was elevated three-fold in the brain of hq mice [241]. The cII assay is a forward mutation detection assay capable of detecting all types of base substitutions and small deletions and insertions while the previously used PLAP assay detects the frequency of a −1 base pair deletion at a monobasic run of 11 guanines [241]. The longest monobasic runs in the cII mutation target are runs of six guanines and six adenines. These monobasic runs enable comparison with the PLAP frameshift mutations, but these monobasic runs did not show greater mutant frequency in single nucleotide deletions in the cerebellum of hq mice at either age. Guanines are the most common target of oxidative damage and a run of 11 guanines is hypermutable, consistent with a previous report of increased frequency of mutations in longer repeats [412]. Guanine repeats have an innate stacking nature and an increased susceptibility to adduct formation [413]. Oxidative stress in the brains of hq mice combined with the susceptibility of guanine adducts and inherent instability of guanine stacking could contribute to the previous in situ observation of increased mutation frequency in brains of hq mice. One other possibility is the potential for the PLAP assay to detect transcriptional errors in addition to mutation. Monobasic runs are susceptible to both replication and transcription errors and transcriptional errors are hypothesized to increase with increased oxidative stress associated with aging [414]. In the PLAP assay, clusters of positive cells are consistent with mutation and replication of mutant cells and the reversion mutation has been confirmed [415] but single isolated cells with positive signal could be examined to confirm mutation status and rule out any
contribution by transcriptional error. PLAP over representation could be due to measuring errors in transcription, leading to the inflation in the mutant frequency compared to that measured using the cII transgene.

4.4.3 One ROS mutation signature was observed in young hq mice

There was evidence of oxidative stress-induced mutations in the form of four deletions between 10 and 21 base pairs in length identified in four out of five hq mice in the 3-month-old cohort [410]. No homologous sequences were found in the deletion junctions to implicate sequence context driven mechanisms and as such, these deletions are representative of Class II deletions [371]. Class II deletions have been reported in mice exposed to γ-radiation [371, 416] and dimethylnitrosamine [417] and associated with elevated endogenous oxidative stress [418]. Thus, a ROS mutation signature was detected but overall was not dramatically more frequent and not a major consequence of the down-regulation of Aif.

4.4.4 Mutations in nuclear DNA are not associated with neurodegeneration in hq mice

The hq phenotype is not associated with elevated frequency and significant alterations in the overall pattern of independent mutations in the cerebellum likely for several reasons. Mutation frequency in nuclear DNA is not elevated because nuclear DNA is protected with histones and is distant from the site of ROS production in the mitochondria. A more plausible target of oxidative stress-induced mutation is mitochondrial DNA. Approximately 2% of consumed oxygen is converted to superoxide radicals during respiration [51] that can then go on to cause DNA strand breaks and base
modifications in mitochondrial DNA [419]. Individuals with AD have 10-fold higher levels of oxidized bases in the mitochondrial DNA compared to nuclear DNA [40]. Mitochondrial mutations have been detected in brains of AD [58], PD [420], and ALS patients [421]. Thus in hq mice, mitochondrial DNA may be more susceptible to ROS-induced mutations than nuclear DNA. To test the hypothesis that the mitochondrial DNA in hq mice is more susceptible to ROS-induced mutations, the mitochondrial random mutation capture assay [422] could be used to measure mutations in WT and hq mice.

4.4.5 Lipofuscin levels in hq mice were not elevated compared to WT mice

Lipofuscin accumulation in the cerebellum was detected in hq mice and did increase with age but was similar to cerebellar lipofuscin accumulation in WT mice. Lipofuscin autofluorescence was elevated in lobules I/II and VIa that are located in the anterior and central lobes of the cerebellum. Although only two lobules show an increase of lipofuscin autofluorescence with age, one has to keep in mind that the Purkinje cells, which are demonstrating lipofuscin autofluorescence, are also being lost with age, which would impact the levels detected as Purkinje cells are no longer there. These lipofuscin results in the cerebellum of hq mouse were different when compared to a previous study of lipofuscin in the olfactory epithelium in hq mice, that found elevated lipofuscin autofluorescence in 6-month-old hq mice and not in WT mice [245]. Even though the olfactory epithelium in hq mice has increased apoptotic neurons when compared to age-matched littermate controls, the olfactory epithelium is a site of neurogenesis that is stimulated by neuronal cell death [423-425], thus explaining why the layers of the olfactory epithelium have elevated lipofuscin and do not decrease in thickness or cell number when compared to the retina or the cerebellum [232, 269, 376]. Had the Purkinje
cells in *hq* mice survived, it is reasonable to think that lipofuscin autofluorescence would have been elevated with age in *hq* mice compared to WT mice.

4.4.6 *Superoxide anions were not elevated in *hq* compared to WT mice*

Superoxide anions were elevated with age in *hq* mice in lobules I/II, III, and VIa, but this was not different from WT mice. This pattern of elevated levels of superoxide anions is opposite to the profile of granule cell loss. The lobules with the most initial granule cell loss were lobules IX and X, but the ROS profiles in these lobules did not change with age. Lobules that did not have detectable granule cell loss until late in disease progression had a superoxide anion profile that increased with age. The increased levels of superoxide anions with age are likely a reflection of mitochondrial dysfunction combined with the granule cell density in individual lobules. Since oxidative stress was not elevated initially in the 3-month-old *hq* cerebellum, elevated ROS is not likely responsible for increased cell death in *hq* mice seen with apoptosis staining at 7 months of age, or lipofuscin accumulation in Purkinje cells.

This observation of no initial elevation of superoxide anion levels is similar to a report of superoxide anion levels in the retina of *hq* mice [269] but is in contrast to other reports that ROS is elevated in *hq* heart and skin [239, 240] as well as the retina of female *hq* carrier mice [426]. The reason for this discrepancy is likely due to the lower number of neurons in the cerebellum and retina of *hq* mice, since quantification of DHE intensity depends on the density of nuclei containing ethidium. The heart and skin tissue of *hq* mice shows increased ROS but they do not display any cell loss. The retina of the female *hq* carrier mice does display cell loss, however, the phenotype is not as severe as in *hq* mice [427], indicating that measurement of ROS levels in the cerebellum of *hq* mice were
confounded by granule cell loss. In the future, it is suggested that the ROS levels be normalized to the number of granule cells.

4.4.7 mRNA levels do not indicate a transcriptome response to elevated levels of ROS

Oxidative stress can lead to changes in the expression of many genes including additional antioxidant enzymes like superoxide dismutase, catalase, glutathione S-transferase, glutathione reductase, thioredoxin, peroxiredoxin, as well as genes involved in DNA repair, and heat shock proteins. Only the ROS scavenger, Glutathione peroxidase 3 (Gpx3), was up-regulated in hq cerebellum. Alterations at the mRNA level for antioxidants other than Gpx3 were not detected, nor were there alterations in DNA repair enzymes or heat shock proteins in hq mice. The limited number of genes related to oxidative stress response in the transcriptome data suggests that oxidative stress may not be a major initator of cerebellar cell loss in hq mice.

4.5 The role of AIF in the production or detoxification of ROS remains unknown

The results herein, show that hq mice did not have elevated levels of ROS or lipofuscin at 3 months of age when compared to WT mice, which was contrary to the expectation if one of AIF’s roles was to scavenge ROS. However, complex I deficiency alone can lead to elevated oxidative stress [428], which could explain the increase in ROS in hq mice with age as more mitochondria degenerate. However, AIF’s role in scavenging ROS is based on its amino acid sequence similarity with prokaryotic oxidoreductases [238] but conflicting results have been found about its ability to reduce or produce ROS. In hq mice, AIF-deficiency leads to increased peroxide sensitivity in
granule neurons [232] and cardiomyocytes [235], suggesting a protective effect of AIF as an antioxidant. *In vitro*, AIF has NADH oxidase activity and mediates the reduction of oxygen to superoxide radical [429]. In contrast, there are decreased levels of superoxide anions generated in AIF-deficient tumour cell lines [250]. A different study shows no difference in oxidative damage or ROS levels in WT and AIF-deficient mouse embryonic stems cells [248]. In addition, neither the basal rate of ROS release or hydrogen peroxide removal are different in WT and *hq* mice [430], so AIF’s role in the production or removal of ROS remains unclear.

To further evaluate the ROS production and detoxification function of AIF, the NADH oxidoreductase activity of AIF needs to be further studied. NADH oxidoreductases accept electrons from NADH and in the process convert NADH to NAD$^+$ to be used in various cellular processes [431, 432]. In a cell free system, AIF oxidizes NADH and transfers electrons to molecular oxygen, ferricyanide, and dichlorophenolindophenol (a redox indicator), but does not show any hydrogen peroxidase scavenging activity [429]. However, this superoxide generation of AIF is not detected *in vivo* and suggests that there are other substrates and targets of AIF that have not yet been detected. Until we know what all of the substrates, targets, and proteins that interact with AIF are, we won’t fully understand its function in the mitochondria. If elevated ROS was the mechanism of cerebellar degeneration in *hq* mice, PB administration may have been an effective treatment but more evidence is consistent with mitochondrial dysfunction with AIF deficiency.
4.6 Early hq mechanisms were elucidated from the transcriptome of the cerebellum

To examine the hypothesis that pathways involved in ROS, mitochondrial dysfunction, and inflammation would be up-regulated in hq mice, a gene expression experiment was performed in 3-month-old WT and hq mice.

4.6.1 The up-regulation of Melanoma antigen suggests that the proviral insertion in Aif is transcribed

The Melanoma antigen (Mela) gene was up-regulated 14.5 fold in the cerebella of 3-month-old hq mice compared to WT mice. Mela is expressed during development at embryonic day 14.5 in the central nervous system and the thymus [433]. Mela has a similar nucleotide sequence to the env gene that is located at the 3’ end of the mouse murine leukemia virus. The murine leukemia virus is the proviral insertion located in intron one of the Aif gene [232], suggesting that the microarray is able to detect proviral mRNA from the transcription of Aif. Whether the up-regulation of the env gene leads to ENV production in hq mice is unknown and should be confirmed with a western blot to determine ENV protein expression levels. Whether the proviral insertion in hq mice is able to produce active viral components has not been studied, but ENV is not sufficient on its own to cause infection and requires mature viral components [434]. Infection with ENV induces spongiform neurodegeneration [435], which was not observed in hq mice suggesting that ENV expression is not a cause of cerebellar degeneration in hq mice.

4.6.2 The complement pathway is activated in 3-month-old hq mice

As expected, 3-month-old hq mice had alterations in pathways involving the
immune and inflammatory response. The complement pathway was the most significantly up-regulated pathway in the cerebellum of *hq* mice. The liver is the main source of the complement proteins but *in situ* hybridization has identified complement proteins expressed in the brain [436]. Complement component 1, q subcomponent (C1Q) is the initial target and recognition protein of the complementation pathway and is required for the clearance of pathogens and apoptotic cells [437]. C1Q has a heterotrimeric structure composed of 18 polypeptide chains, 6 each of C1QA, CIQB and C1QC [438]. Both C1qb and C1qc were up-regulated in the cerebellum of 3-month-old *hq* mice, but C1qa is also required to form the C1Q complex. C1qa had a fold increase of 1.3 and did not make the 1.5 fold cut off for the microarray analysis, consistent with a limited sensitivity of the microarray technology to detect small increases in fold change due to the dilution of gene expression by the heterogeneous cell types in the brain [439]. However, small increases in fold change still have a biological impact [440], as evidenced by the fact that full C1Q complex is needed for the activation of the complement pathway, and genes downstream of C1Q were up-regulated in the cerebellum of 3-month-old *hq* mice, including *Complement component 3a receptor 1* (*C3ar1*) and the basic form of *Complement component 4b* (*C4b*). In the cerebellum of 4-month-old *hq* mice, C1qa is up-regulated as well as C1qb and C1qc [427]. With the C1Q complex assembled, it is able to directly bind to apoptotic cells resulting in the activation of the classical complement pathway [437] and facilitates the clearance of apoptotic cells by microglia. The fact that genes in the complement cascade were up-regulated, in particular the early activation products, shows that there was initiation of the phagocytosis response to get rid of apoptotic cells as early as 3 months of age in the
cerebellum of *hq* mice. Also, the limited number of degenerating neurons also supports effective clearance of apoptotic cells. Up-regulation of the complement pathway in *hq* mice compared to WT mice still needs to be validated with real time PCR or protein expression.

Although complement activation is required in defense against pathogens and removal of apoptotic cells, constant up-regulation is detrimental and leads to host tissue damage. Complement activation has been implicated in normal aging [200-202, 290, 291] and the pathogenesis of several diseases including systemic lupus erythematosus/rheumatoid arthritis [441], multiple sclerosis [442], spinal cord injury [443], psoriasis [444], age-related macular degeneration [445], and neurodegenerative diseases [436, 446, 447]. Complement proteins are associated with amyloid deposits in AD [448]. A mouse knockout of *C1q* crossed with APP transgenic mice, shows decreased glial cell activation without reducing amyloid deposition, suggesting a role for complement activation in the pathology of AD [449, 450]. In early stages of AD, the presence of the early complement components is found in plaques including C1Q, C4D and C3D but components C5B-9 are absent [451, 452]. At later stages of AD, in addition to C1Q, C4D, and C3D, C5B-9 are present in plaques and neurofibrillary tangles [453], further suggesting a role for early complement activation in AD. In addition, the prominence of early complement components is in line with a study comparing a transgenic mouse model of AD to WT mice [454], which is similar to what was found here in the cerebellum of 3-month-old *hq* mice suggesting that early complement components play a role in neurodegeneration in *hq* mice.
4.6.3 There was up-regulation of the pathway termed crosstalk between dendritic cells and natural killer cells

There was significant up-regulation in the crosstalk pathway between dendritic cells and natural killer cells. Dendritic cells and natural killer cells are both components of the immune response in the circulatory system. Dendritic cells process antigens and present antigenic material on their surface leading to activation of other immune cells. Natural killer cells are lymphocytes, capable of killing cells that have been infected with viruses or cells that are cancerous. Genes that were up-regulated in this pathway included TYRO protein tyrosine kinase binding protein (Tyrobp), the major histocompatibility complex I (MHC1) component Histocompatibility 2, K1, K region (H2-K1), and Actin alpha 2 smooth muscle aorta (Acta2). TYROBP is an adaptor molecule that binds to activating receptors on the natural killer cells and is required for natural killer cell activation [455]. Since dendritic and natural killer cells are part of the immune system in the circulatory system, it is unlikely that the cells were able to infiltrate the cerebellum through the blood-brain barrier. Since microglial cells are the macrophages of the central nervous system, it is possible that both Tyrobp and H2-K1 were up-regulated due to microglial activation. TYROBP is expressed in microglial cells and up-regulation can lead to an increase in superoxide formation and can lead to increased apoptosis [456]. H2-K1 can also be expressed in microglial cells after engulfment of apoptotic cells [457]. The Acta2 gene codes for α-actin in vascular smooth muscle cells and is important for the contractile function in vasculature [458]. Since down-regulation of Acta2 results in a decrease in the structural integrity of vasculature, and mutations in Acta2 can lead to familial aortic aneurisms [459], up-
regulation of the Acta2 gene could be due to maintenance of the integrity of the blood-brain barrier in hq mice. In hq mice, blood vessels are thicker, longer, and more numerous compared to WT mice [234] indicating maintenance of the blood-brain barrier. Up-regulation of Tyrobp, H2-K1, and Acta2 suggests microglial activation in the cerebellum rather than infiltration from the circulatory system by dendritic and natural killer cells due to a compromised blood-brain barrier in 3-month-old hq mice.

4.6.4 There is maintenance of the extracellular matrix and an active immune response

In hq mice, genes that were up-regulated in the neuroprotective role of THOP1 in Alzheimer’s disease pathway included Serine peptidase inhibitor, clade A, member 3N (Serpina3n) and H2-K1. THOP1 is a metalloprotease that has been associated with APP [460] processing and the indirect degradation of the amyloid β [461] thereby preserving the integrity of axons and dendrites and maintaining connectivity and extracellular matrix functionality of neurons in AD. Despite the neuroprotective role of this pathway, the differentially-expressed genes that were up-regulated have a detrimental role in the THOP1 neuroprotective pathway. SERPINA3N is closely associated with amyloid β [462] and decreased THOP1-dependent amyloid β degradation by 60% [461] either by inhibiting amyloid β degrading proteases or preventing proteases in the brain from clearing APP thus contributing to amyloid deposits. In addition, SERPINA3N is detected in astrocytes in normal human aging [462] suggesting that Serpina3n overexpression is not limited to pathology in AD. Serpina3n co-localizes with astrocytes in the retina after inflammatory stimulation with lipopolysaccharide [463]. The co-localization of Serpina3n and astrocytes could be beneficial as SERPINA3N can inhibit Cathepsin G
and Elastase, which can hydrolyze proteins in the extracellular matrix [464]. Since hq mice don’t have amyloid β pathology, SERPINA3N would have a beneficial effect in limiting breakdown of the extracellular matrix in the cerebellum of hq mice. THOP1 also hydrolyzes oligopeptides ranging in size from 6-17 amino acids in length [465] including antigenic peptides [466] thus limiting MHC I presentation [467], which could eventually lead to suppression of the immune response. However, in the cerebellum of 3-month-old hq mice the MHCI component was up-regulated suggesting an increased immune response. Thus, it is possible that hq mice have down-regulated the neuroprotective role of THOP1 in Alzheimer’s disease pathway leading to a maintenance of the extracellular matrix and increased immune response. Validation is still needed with real time PCR or protein expression.

4.6.5 Immune response activation is consistent with microglial activation

The final pathway up-regulated in hq mice related to immune response was communication between innate and adaptive immune cells. The two genes that were up-regulated in the communication between innate and adaptive immune cells pathway were Chemokine (C-C motif) ligand 3 (Ccl3) and H2-K1. The innate immune system is an immediate defense system used to protect the host from infection from pathogens through the production of chemokines and inflammatory cytokines. The adaptive immune system is activated by the innate immune system and provides an organism with the ability to recognize and remember specific pathogens so that it may trigger stronger attacks each time the same pathogen is encountered. The innate and adaptive immune systems can be activated through microglial activation [468]. Microglial cells are the resident
macrophages of the central nervous system and are activated by infection, injury, or apoptotic cells [62]. CCL3 is a chemokine secreted by activated microglial cells in order to attract additional microglial cells to the point of injury [468]. Microglial cells are antigen presenting cells, capable of expressing MHC I and II. Histocompatibility 2-K1 is one of the components of the MHCI antigen [469] and is presented on the surface of microglial cells after phagocytosis of dead or foreign cells [457]. Since pathways related to immune responses were up-regulated in the cerebella of 3-month-old hq mice without any evidence of invading bacteria, viruses, or a compromised blood-brain barrier, a more plausible hypothesis is that an immune response is occurring in the cerebellum through the activation of microglial cells by damaged or apoptotic neurons. Indeed, manual annotation of the differentially-expressed genes, revealed markers of microglial activation and needs further validation.

4.6.6 Multiple transcripts identified by gene expression indicate microglial activation

Microglial activation is associated with the release of ROS [73], proteases [74], and inflammatory cytokines [70]. Markers of microglial activation in hq mice included Ccl3, which attracts microglia to the site of injury [468]. Cluster of differentiation 68 (Cd68) a cell surface glycoprotein present on activated microglial cells [470]. Cluster of differentiation 93 (Cd93), also known as Complement component 1 subcomponent receptor 1 (C1QR1) that is required for the engulfment of apoptotic cells by microglia [471]. Histocompatibility 2-K1 (H2-K1), is one of the components of MHCI [469] and is presented on the surface of microglial cells after phagocytosis of dead or foreign cells [457]. In addition, Lysozyme 2 (Lyz2) and Cathepsin S (Ctss) are proteases present in
lysosomes of microglial cells. Lyz2 is required for the degradation of apoptotic cells [472] and Ctss is required for MHC II presentation [473] and for degradation of the extracellular matrix [474]. Microglial activation is a necessary component in the removal of cellular debris in the brain, but constant stimulation or unregulated response of microglial cells leads to inflammation. Microglial activation and inflammation is prevalent in neurodegenerative disorders including AD [82], PD [82], and ALS [86]. The up-regulation of microglial markers suggests activated microglia play a role in the early stages of neurodegeneration of the cerebellum of 3-month-old hq mice.

4.6.7 Similar cerebellar transcriptome features exist in 3 and 4-month-old hq mice

Reproducibility of scientific results is a fundamental principle in scientific research. This is of particular importance with microarray technology, which has been known to show a high amount of variability in lists of differentially-expressed genes [475]. Variability can be explained by the use of different platforms and laboratory processing but the reproducibility herein was found in the gene list in the cerebellum of 3- and 4-month-old hq mice. Of the 55 differentially-expressed genes found in the cerebellum of 3-month-old hq compared to WT mice, 48 were also differentially expressed in the cerebellum of 4-month-old hq compared to WT mice (Appendix B, Table B.1) [427]. And in both cases, the fold change was in the same direction for genes that were differentially expressed. Genes that were not reproduced included a predicted gene of unknown function (Gm11428), an enzyme that converts ethanol to acetaldehyde (Adh1), a nicotine, acetylcholine, and cation ligand channel (Chrn6), a guide RNA involved in methylation of pre-rRNA (Snord82), a lipid kinase that is a negative regulator
of apoptosis (Cerkl), a receptor for the neurotransmitter GABA (Gabra6), and a mitochondrial protein involved in the assembly and/or maintenance of complex I in the electron transport chain (Aifm1). However, down-regulation of Aifm1 was confirmed through Taqman® assays in hq mice at 5 weeks of age, 4 months of age and 7 months of age for both the retina and cerebellum (Edwards, unpublished)(Thwaites, unpublished) [476]. Ctss up-regulation was confirmed with Taqman® assays in the cerebella of hq mice at 5 weeks and 4 months of age (Thwaites, unpublished). Thus, most of the genes in the cerebellum of 3-month-old hq mice were replicated in the cerebellum of 4-month-old hq mice.

4.6.8 Similarities and differences exist between cerebellar and retinal transcriptomes in hq mice

Altered pathways in the cerebellum of 3-month-old hq mice included those involved in the immune and inflammatory response. This is similar to a report of gene expression in the retina [427] and cerebellum of 4-month-old hq mice (Appendix B, Table B.1) [427]. At 4 months of age, both the retina and cerebellum show evidence of altered metabolism with a shift to glycolysis [427], as a result of reduced oxidative phosphorylation [248]. When glycolysis is inhibited in AIF-deficient embryonic stems cells, there is increased cell death [248] suggesting an increased dependence on glycolysis for ATP production. Similarly, altered pathways in the retina in 4-month-old hq mice are involved in immune and inflammatory response as well as altered metabolism with a shift to glycolysis [427].

One difference between the cerebellum and retina is that the retina appears to have an earlier onset of degeneration compared to the cerebellum. In the retina,
functional deficits measured by electroretinography, are observed as early as 2 months of age in \textit{hq} mice [269], apoptotic retinal cells are detected at 3 months of age [232], and retinal structural deficits are first seen in the ganglion cell layer and outer nuclear layer at 4 months of age [269]. In the cerebellum, apoptotic cells are first seen at 4 months of age in the \textit{hq} mice leading to a decrease in rotarod performance, a measure of motor coordination, at 6 months of age [233] and structural deficits in the granule and Purkinje cell layer of the cerebellum at 7 months of age [232]. Although, it should be noted that the retina has had a detailed monthly characterization of the degeneration in different layers, whereas, the cerebellum has been studied at a fewer number of time points, so comparisons between the two tissues are difficult. One difference in the disease mechanism between the retina and cerebellum is that the retina of \textit{hq} mice shows evidence of extracellular matrix remodeling. Altered pathways identified in the retina of 4-month-old \textit{hq} mice are related to extracellular matrix receptor interaction, focal adhesions involved with the regulation of the extracellular matrix, and cytoskeletal elements [427]. In addition, hematoxylin and eosin staining shows nuclear displacement in the retina [269, 426], confirming remodeling of extracellular matrix. In contrast, the cerebellum did not show altered gene expression in pathways involved in extracellular matrix remodeling or nuclear displacement of the granule or Purkinje cells.

4.6.9 Transcriptome changes with degeneration of \textit{hq} olfactory epithelium implicated different underlying mechanisms

The reports on gene expression in the retina and cerebellum differ when compared to the olfactory epithelium in \textit{hq} mice. None of the differentially expressed genes in the cerebellum of 3- and 4-month-old \textit{hq} mice were detected in the olfactory epithelium in 6-
month-old *hq* mice [245]. In the olfactory epithelium, altered genes are classified into cell signaling/apoptosis, oxidative stress/aging, cytoskeleton/extracellular matrix, transport, and neurogenesis pathways [245]. Several reasons could explain this discrepancy, the first being that the tissues although they are neural, could have different degeneration timelines. This has been demonstrated for the retina and the cerebellum in *hq* mice. Retinal degeneration is detected as early as 2 or 3 months of age and cerebellar degeneration occurs at 4 months of age [232, 234, 269], but since there is only one study in the olfactory epithelium in *hq* mice [245], a neurodegenerative timeline has not been established for the olfactory epithelium. In addition, different ages of *hq* mice have been studied thus far, and at 6 months of age, disease progression in the olfactory epithelium is well underway, whereas 3 months of age is considered early in the disease onset [232, 234]. Also, the olfactory epithelium is directly exposed to the external environment unlike the cerebellum, and exposure to environmental agents could alter gene expression. Thus, the underlying mechanisms of neurodegeneration in the olfactory epithelium need to be characterized further with an increased number of time points to better compare the differences in the neurodegeneration timeline.

4.7 **Chronic low-dose PB treatment produced few alterations in the transcriptome of the cerebellum**

4.7.1 *The effect of PB on the transcriptome in the cerebellum was limited in WT and hq mice*

Phenobarbital treatment had little effect on the transcriptome in WT and *hq* mice and may not be acting through the induction of gene expression. Administration of 10 mg/kg/day of PB for 28 days leads to very little change in the gene expression in the
liver, as two genes were up-regulated and three genes were down-regulated more than 1.5 fold [477]. Even at a higher dose, 100 mg/kg/day, only seven genes are up-regulated and one gene is down-regulated more than 1.5 fold. The two genes that are up-regulated with 10 mg/kg/day are both cytochrome P450 enzymes. Enzyme activity assays, however, show increased activity for four different cytochrome P450 enzymes even though alteration in the gene expression is not significant [477], suggesting that PB may work through the induction of enzyme activity rather than a change in gene expression in the brain.

4.7.2 Down-regulation of the GABA<sub>A</sub> receptor may impede the effectiveness of PB

The gene expression assay showed that hq mice have a down-regulated Gabra6 gene, potentially leading to down-regulation of functional GABA<sub>A</sub> receptors. A down-regulation in functional GABA<sub>A</sub> receptors could explain why PB treatment had little effect on the behavior of hq mice. Additionally, hq mice require increased levels of ketamine anesthetic for sedation during retinal function testing because hq mice are coming out of sedation earlier than expected. Current literature suggests that ketamine is able to act upon GABA<sub>A</sub> receptors, in particular the alpha<sub>6</sub>beta<sub>2</sub>delta receptor subtype, as well as the N-methyl-D-aspartic acid receptors to produce the anesthetic effect [478]. Both the lack of activity of hq mice with PB administration and the increased ketamine doses required for sedation in hq mice are consistent with the hypothesis that hq mice have decreased Gabra6 levels. To test the hypothesis that hq mice have a decreased amount of the GABRA6 subunit, a western blot could be performed to determine levels
of the subunit in WT and \textit{hq} mice. Decreased \textit{Gabra6} levels potentially make PB an unsuitable hormetric agent in \textit{hq} mice.

4.8 \textit{The hq disease mechanisms and phenotype are consistent with mitochondrial dysfunction}

While PB treatment did not reveal any benefit in WT or \textit{hq} mice, the data are consistent with disease mechanisms associated with mitochondrial function. In cell culture, AIF-deficient cells have compromised oxidative function and rely on ATP generated from glycolysis [248] suggesting that decreased AIF leads to mitochondrial dysfunction. Degenerating mitochondria are seen as early as 2 months of age in \textit{hq} cerebellum and the rate of degenerating mitochondria increases progressively starting at 41\% at 2 months of age to 81\% at 6 months of age in cerebellar granular cells [234]. At 4 months of age, transcriptome analysis in the retina and cerebellum reveals altered metabolism with various genes related to glycolysis [426]. Also, mitochondrial complex I deficiencies are tissue specific. In humans, tissues that can be adversely affected by mitochondrial complex I deficiencies include the retina [252], brain [252, 380, 381], heart [380], skeletal muscle [252, 381], skin [382], and hair [382]. A mouse model of complex I deficiency, displays a decreased growth rate, ataxia, blindness, and encephalomyopathy [479]. In humans and mice, the symptoms of the mitochondrial complex I deficiencies are similar to symptoms in \textit{hq} mice. Since mitochondrial dysfunction leads to elevated ROS production [480], this would explain increased lipofuscin and ROS with age in \textit{hq} mice. Since we know that there is a lobule specific pattern of neuron loss, one caveat of the study regarding mitochondrial dysfunction in \textit{hq} mice [234], was that it did not
specify the mitochondrial dysfunction on a per lobule basis in *hq* mice to see whether lobules IX and X had more dysfunctional mitochondria relative to the other lobules. Elevated ROS are likely a consequence of mitochondrial dysfunction and do not appear to be associated with early cerebellar degeneration in *hq* mice. Thus, the cause of degeneration is likely mitochondrial dysfunction leading to a decrease in ATP production and eventually cell death. In order to prevent cerebellar degeneration in *hq* mice, mitochondrial dysfunction would have to be addressed either through increasing the amount of complex I, or by increasing the efficiency of the electron transport chain. Phenobarbital treatment did not address the mitochondrial dysfunction aspect of the *hq* cerebellar degeneration thus, the targets of PB that made it successful in preventing cancer, did not make it successful in preventing or delaying cerebellar degeneration in *hq* mice. Thus, PB was not a suitable choice as a hormetic agent in *hq* mice.

4.9 Other hormetic therapies can be considered for cerebellar degeneration in *hq* mice

4.9.1 Caloric restriction may reverse inflammation

Although, *hq* mice treated with low doses of PB did not show a hormetic response, this does not mean that other hormetic agents would also prove to be ineffective in *hq* mice. Caloric restriction has been extensively studied for its ability to increase lifespan in species of yeast [481] to mammals [289]. Also, caloric restriction decreases lipofuscin accumulation in the different regions of the brain [482], decreases ROS production [483], decreases damaged lipids and proteins [291, 484], and increases mitochondrial membrane potential in yeast [485]. Mitochondrial energy production relies on the membrane potential so an increase in mitochondrial membrane potential
would lead to an increase in ATP production, however, ATP levels are not altered under caloric restriction and non-caloric restriction conditions [486]. Gene expression analysis in the brain of mice shows that caloric restriction has varied effects depending on the region of the brain studied, but gene expression in regions of the central nervous system are still more similar to each other than they are to other organs [290]. Aging leads to increased expression of complement components as well as antigens (Cd48, Cd52, Cd53) in multiple brain regions, including the cerebellum, and caloric restriction is able to oppose the effects of aging in genes associated with immunity, defense, and inflammation [200, 202, 290, 291]. Generally, caloric restriction can counter some age-associated changes in gene expression, immune response, and inflammation, but is not able to reverse all age-associated changes in gene expression [290]. Thus far, caloric restriction appears to be the most promising anti-aging strategy available. It would be interesting to study caloric restriction in the context of neurodegeneration to see whether the beneficial effects of reduced caloric intake can lead to decreased neurodegeneration inhqmice.

Although, caloric restriction would be a difficult strategy to employ in the human population, understanding of the molecular mechanisms leading to increased longevity is needed. Perhaps in the future there will be developments of drug compounds that can mimic the beneficial effects of caloric restriction without having to decrease caloric intake. Caloric restriction could reduce the inflammatory and immune responses in the cerebellum ofhqmice.
4.9.2 Physical exercise may increase the electron transport chain efficiency

Exercise has shown promise in slowing the decline of neurodegeneration in mouse models of AD, PD, and mitochondrial DNA mutator mice [293, 294, 487]. Exercise is able to ameliorate the decline in mitochondrial DNA and components of the electron transport chain in 3xTg-AD mice [129], and protects against mitochondrial dysfunction in a PD mouse model by increasing mitochondrial respiration, and ATP production [294]. In the mitochondrial DNA mutator mouse, exercise is able to increase mitochondrial copy number while reducing the frequency of mutations in the mitochondrial DNA, increasing the amount of mitochondrial electron transport chain subunits, and increasing mitochondrial activity [487]. Although exercise is beneficial in these mouse models, it might not be as beneficial in hq mice because exercise decreases adipose tissue in the 3xTg-AD mice [129], and hq mice have very little adipose tissue. Nonetheless, exercise shows promise in ameliorating the decline in mitochondrial function in neurodegenerative diseases, and could be a possible strategy to use in hq mice.

4.10 Riboflavin administration is a targeted therapy that may increase the electron transport chain efficiency

Since hq mice have decreased Aif expression leading to decreased complex I assembly [430], activity, and ATP production [248], another possible treatment would be to increase the amount of assembled complex I in the mitochondria to restore complex I activity and ATP production. Riboflavin is a precursor for flavin adenine dinucleotide and flavin mononucleotide that is required for redox enzymes in energy metabolism.
Oral treatment with riboflavin in doses ranging from 9 mg in children to 100 mg in adults, increases complex I activity in the muscle of three out of the five individuals tested, as well as decreasing lactate levels in both the serum and cerebrospinal fluid [488]. The sample size of the previous study is very small but the results in the literature are fairly consistent. Administration of riboflavin, sometimes in conjunction with other components, is able to successfully treat different patients [254, 489-492]. Riboflavin supplementation in *C. elegans* is able to increase the activity and the amount of fully assembled complex I but the exact mechanism is not known [493]. Riboflavin has very low toxicity as demonstrated by administering riboflavin orally at 10 g/kg of body weight in rats with no toxic effects [494]. Riboflavin is also capable of crossing the blood-brain barrier with the human riboflavin transporter *hRFT3*, which is highly expressed in the brain [495]. In humans with manifestation of complex I deficiency in the muscles, rather than manifestation in both muscles and central nervous system, fare better with riboflavin treatment [253, 488, 496], suggesting that riboflavin may not be as effective in *hq* mice as the main effects of complex I deficiency are seen in the cerebellum and retina but defects can also be seen in the cardiac muscle of *hq* mice in conjunction with added stressors [235]. One reason for this could be that riboflavin levels in the brain remain fairly constant despite diets rich or deficient in riboflavin [497]. Despite the drawback, it would still be of interest to test riboflavin administration as a potential treatment in cerebellar degeneration in *hq* mice.
4.11 Caveats

The transcriptome data should be confirmed at the protein level. A change in the mRNA expression of a gene may not reflect a change in the protein expression [498], as there are mechanisms of post-transcriptional control including microRNAs [499], and RNA binding proteins [107]. There are also mechanisms of post-translational control that can regulate protein activity including phosphorylation, acetylation, glycosylation, ubiquitination, and cleavage. Indeed, if there are changes in the protein expression, this may still not result in an up- or down-regulation in a pathway as many genes are involved and a change in the expression of one gene may not lead to a change in the overall pathway. The next step would be to confirm the gene expression data at the protein level with western blotting.

Another caveat regarding the Fluoro-Jade® B stain is that the identity of the compound that Fluoro-Jade® B binds to in degenerating neurons is unknown. Fluoro-Jade® B is a poly-anionic fluorescein and thus is thought to bind to molecules with multiple positive charge groups, but the exact protein has not been determined [500]. Because the mechanism of action is unknown it is still uncertain at what time point during the degeneration process this molecule with affinity to Fluoro-Jade® B is expressed. The use of Fluoro-Jade® B could lead to increased false negatives, neurons that were degenerating but not stained with Fluoro-Jade® B. For an example, a previous study compared the use of terminal deoxynucleotide transferase-mediated, deoxyuridine triphosphate nick-end labeling (TUNEL) and Fluoro-Jade® B in the leaner mutant mouse [501]. A total of 796 granule cells were evaluated using TUNEL, Fluoro-Jade® B and double staining for both TUNEL and Fluoro-Jade® B. Only staining with TUNEL
yielded 29.5% positive granule cells while only staining with Fluoro-Jade® B yielded 17.5% positive granule cells. Double staining for both TUNEL and Fluoro-Jade® B yielded 53% positive granule cells. To improve the accuracy of detecting degenerating neurons, staining with both TUNEL and Fluoro-Jade® B should be incorporated in future studies.

4.12 Summary and Conclusions

This was the first characterization of nocturnal behaviour in hq mice and it was determined that nocturnal activity is not affected by cerebellar degeneration. No new early behavioural markers of cerebellar degeneration were identified. This was the first quantitative characterization of cerebellar degeneration in hq mice with normal aging while previous studies of hq mice focused on qualitative aspects of granule and Purkinje cell loss [232, 376]. Both granule and Purkinje cell numbers decreased in hq mice and cell loss had an anterior-posterior patterning. This pattern of cell loss in the hq cerebellum may provide insight into early mechanisms of degeneration and provide benchmarks for assessing the efficacies of potential treatments. This was the first characterization of mutations in the cerebellum of hq mice using the Big Blue® cII mutation detection assay which determined that nuclear mutations did not play a large role in cerebellar degeneration. A detailed characterization of the transcriptome was achieved in the cerebellum of 3-month-old hq mice. The transcriptome data were consistent with inflammation and microglial activation as early mechanisms in cerebellar degeneration in hq mice. Preventing the inflammatory response early in hq mice may
help to stem cerebellar degeneration. A down-regulation of Gabra6 may explain the lack of response by hq mice to low doses of PB.

Multiple lines of data do not show association of ROS with cerebellar degeneration in hq mice. Superoxide anion levels and ROS-induced damage such as lipofuscin and mutations were not the major contributing factors to cerebellar degeneration as was previously suggested. Lipofuscin accumulation, mutations, and ROS were not elevated initially in the cerebellum of hq mice early in disease progression, suggesting that a decrease in energy production due to mitochondrial dysfunction was the major contributing factor to cell death. Mitochondrial dysfunction also led to an elevation in the mRNA of genes related to the inflammatory response in the cerebellum of hq mice. Thus, mitochondrial dysfunction and inflammation would be potential targets for therapies in preventing cerebellar degeneration in hq mice.

New treatment targets indicated for use in preventing cerebellar degeneration in hq mice were identified. Although, low-dose administration of PB was unsuccessful in treating cerebellar degeneration, this does not mean that hormesis cannot be effective in hq mice. Caloric restriction could be used to reverse inflammation and exercise can increase complex I activity. In addition, one other targeted treatment was documented. Riboflavin administration has shown efficacy in increasing complex I activity of the electron transport chain and has been an effective treatment in humans with mitochondrial dysfunction. Future studies assessing the effectiveness of riboflavin to prevent or delay cerebellar degeneration in hq mice should be done.

The function AIF and its effect on ROS are still unexplained. Even though evidence of ROS-induced damage was not overwhelming in the data presented herein,
superoxide anion levels and lipofuscin increased with age, and a ROS mutation signature was identified. Previous research in *hq* mice has reported evidence of ROS-induced damage [232, 241, 245, 280], as well as sensitivity of neurons and cardiomyocytes to exogenous ROS [232, 235, 502], suggesting an antioxidant role for AIF. However, the rate of ROS production or detoxification was similar in WT and *hq* mice [430] and yet, AIF accepts electrons from NADH and transfers them to molecular oxygen to produce superoxide anions *in vitro* [429]. If AIF has the ability to accept and transfer electrons, it is possible that AIF is involved in the transfer of electrons from NADH or an unidentified associated protein to another associated protein for detoxification. In the absence or down-regulation of AIF, there would not be an increase in ROS but rather a mismanagement of ROS, which would lead to increased oxidative damage but not increased ROS production.

This research has furthered our knowledge of the mechanisms of cerebellar degeneration in *hq* mice. Phenobarbital may be a suitable treatment in preventing hepatocarcinogenesis, but the data do not support its efficacy in preventing neurodegeneration in *hq* mice. Alternatively, potential hormetic and targeted therapies were suggested in treating mitochondrial dysfunction and inflammation in *hq* mice. The key to unlocking the mystery of AIF remains in discovering the function of AIF in the context of ROS and its involvement in the assembly and/or maintenance of complex I. Further understanding of the role of AIF in these two capacities could advance our knowledge of the complex I assembly and the necessary factors required for proper function. Since mitochondrial dysfunction is a common feature of neurodegenerative diseases, insight into the factors required for proper mitochondrial function would be
beneficial in testing treatments for mitochondrial dysfunction or neurodegenerative diseases.
REFERENCES


366. Stratagene, λ Select-cII Mutation Detection System for Big Blue Rodents. La Jolla, California, USA, 2002.


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Appendix A - The University of Western Ontario Ethics Approval for Animal Use in Research

This appendix contains a copy of the ethics approval form for animal use in the Hill laboratory from the University Council on Animal Care and the Animal Use subcommittee.
Dear Dr. Hill:

Your Animal Use Protocol form entitled:

“Hormesis in Mouse Models of Neurodegeneration and Shortened Lifespan: A Novel Anti-aging Strategy”

Funding Agency CIHR - Grant# 134708, BBFP

has been approved by the University Council on Animal Care. This approval is valid from 04.24.09 to 05.01.10.

The protocol number for this project is 2009-031.

1. This number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this number.
3. If no number appears please contact this office when grant approval is received.

If the application for funding is not successful and you wish to proceed with the project, request that an internal scientific peer review be performed by the Animal Use Subcommittee office.

4. Purchases of animals other than through this system must be cleared through the ACVS office. Health certificates will be required.

**ANIMALS APPROVED FOR 4 Years**

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<th>Pain Level</th>
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<td>birth to 3, 4 or 7 months of age; breeding</td>
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**REQUIREMENTS/COMMENTS**

Please ensure that individual(s) performing procedures on live animals, as described in this protocol, are familiar with the contents of this document.

The holder of this Animal Use Protocol is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.

**Hazardous agents have been identified within this Protocol**

---

O.C. J Wasylenko, D Cheshuk

*The University of Western Ontario*

Animal Use Subcommittee / University Council on Animal Care

Health Sciences Centre, ● London, Ontario ● CANADA – N6A 5C1

PH: 519-661-2111 ext. 86770 ● FL 519-661-2028 ● www.uwo.ca / animal
Appendix B – Supplementary Figures and Tables

This appendix contains a supplementary figures and tables used to aid in understanding of research presented within this study.
Figure B.1 – Identification of apoptotic neurons in the mouse cerebellum of wild type (WT) and harlequin (hq) mice.  A) An image of the cerebellum of a WT mouse stained with Fluoro-Jade® B with no visible degenerating neurons.  B) An image of the cerebellum of a hq mouse stained with Fluoro-Jade® B, with the white arrow indicating degenerating neurons.  C) The negative controls were created by injecting WT mice with 10 mg/kg of a vehicle control.  D) The positive controls were created by injecting WT mice with 10 mg/kg of paraquat. Scale bar = 100 µm.
Figure B.2 – Identification of lipofuscin autofluorescence indicative of damaged lipids and proteins in cerebellum of wild type (WT) and harlequin (hq) mice. A) An image of the yellow and green autofluorescence in the apex of lobule I/II of the cerebellum of a WT mouse. The white arrows are indicating lipofuscin autofluorescence. B) An image of the yellow autofluorescence of the apex of lobule I/II of the cerebellum of a WT mouse after setting the 140 threshold to remove the green autofluorescence. The white arrows are indicating lipofuscin autofluorescence. C) A hematoxylin and eosin image of the apex of lobule I/II of the cerebellum of a WT mouse showing the location of the lipofuscin in the Purkinje cell layer. Six yellow autofluorescence measurements were made at the apex of lobules I/II, III, VIa, IX, and X for two different cerebellar sections. The yellow autofluorescence measurements for the two separate sections were averaged together. Yellow autofluorescence measurements were taken in three different mice per experimental group. Scale bars = 100 µm
Figure B.3 – Identification of reactive oxygen species (ROS) in the mouse cerebellum of wild type (WT) and harlequin (hq) mice. A) A hematoxylin and eosin image of lobule I/II of the cerebellum of a 3-month-old WT mouse. B) A fluorescent image of lobule I/II of the cerebellum of a 3-month-old WT mouse stained with dihydroethidium (DHE). C) A fluorescent image of lobule I/II of the cerebellum of a 3-month-old hq mouse stained with DHE. Three DHE fluorescence measurements were made at the apex of lobules I/II, III, VIa, IX, and X for two different cerebellar sections. The DHE fluorescence measurements for the two separate sections were averaged together. DHE fluorescence measurements were taken in three different mice per experimental group. D) An image of a positive control of lobule I/II of the cerebellum of a 3-month-old hq mouse. The positive control was created by exposing sections to 0.5 M hydrogen peroxide for 5 min prior to DHE staining. E) An image of a negative control of lobule I/II of the cerebellum of a 3-month-old hq mouse created by exposing sections to 20 µl of 1000 U/ml superoxide dismutase solution for 10 min prior to DHE staining. F) An image of a negative control of lobule I/II of the cerebellum of a 3-month-old hq mouse created by staining with dimethyl sulfoxide (DMSO) in the absence of DHE staining. Scale bars = 100 µm
Table B.1 Genes that were differentially-expressed at both 3 and 4 months of age in the *hq* cerebellum compared to wild type mice

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\(^{a}\)Differentially expressed genes were identified as having a minimum fold change of 1.5 and a p-value less than 0.05.

\(^{b}\)Gene order was determined according to increasing p-value at 3 months of age.

\(^{c}\)Gene descriptions are given in Table 3.3.

\(^{d}\)The 4-month-old gene expression dataset was obtained from Alex Laliberte's Masters Thesis (unpublished).

\(^{e}\)Atf was not down-regulated in the gene list of 4-month-old hq cerebellum, but Atf down-regulation was later confirmed using Taqman \(^{2}\) probes in the cerebellum of hq mice at 5 weeks, 4 months and 7 months of age.
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Anita Prtenjaca

EDUCATION

*The University of Western Ontario, London, ON* 2005 – Present  
*Ph.D Biology – Molecular Genetics*

*The University of Western Ontario, London, ON* 2001 – 2005  
*B.Sc. Honors Genetics*

AWARDS

**National and Provincial Research Scholarships**  
QEIGSST 2011 - 2012  
NSERC PGS D3 2007 - 2010  
Ontario Graduate Scholarship, declined 2007 - 2008  
NSERC CGS M 2006 - 2007  
Ontario Graduate Scholarship, declined 2006 - 2007  
Ontario Graduate Scholarship 2005 - 2006  
NSERC USRA  
NSERC USRA Summer 2005  
NSERC USRA Summer 2004

*The University of Western Ontario, London, ON*  
Malcolm Ferguson Award 2011  
Graduate Student Travel Award 2008  
Graduate Student Travel Award 2006  
Western Graduate Research Scholarship 2005 - 2010  
Dean’s Honor List 2001 - 2005  
University of Western Ontario Entrance Scholarship 2001

**Research Society Awards**  
Environmental Mutagenesis Society Travel Award 2012

PUBLICATIONS

Prtenjaca A, Tarnowski HE, Marr A, Lacaria M, Sathiamoorthy S, Creamer L, Hill KA. Tissue-specific profiles of spontaneous mutation were observed in pancreas,
submaxillary gland and other epithelial tissues of Big Blue® mice. Environ Mol Mutagen. EMM-12-0152 in review

Prtenjaca A, and Hill KA. Mutation frequency is not elevated in the cerebellum of harlequin/Big Blue® mice but Class II deletions occur preferentially in young harlequin cerebellum. Mutat Res. 2011; 707 (1-2):53-60.


PRESENTATIONS AND PUBLISHED ABSTRACTS

Refereed Publications

Prtenjaca A, and Hill K.A. Inflammation underlies neurodegeneration in harlequin mice but is not a key target of hormetic doses of phenobarbital. International conference for the Annual Meeting of the Environmental Mutagen Society, Seattle, WA; September 8-12 2012 (Poster Presentation).

Prtenjaca A, and Hill KA. Mutation load in the cerebellum of the harlequin mouse, a model of premature aging and aging-associated neurodegeneration. National conference for the Genetics Society of Canada, Hamilton, ON; June 17-20 2010 (Oral Presentation)

Prtenjaca A, and Hill KA. The nature of spontaneous mutations in the developing mouse. National conference for the Genetics Society of Canada, Banff, AB; Feb 28-Mar 2 2008 (Poster Presentation)


Crabbe RA, Prtenjaca A, Tarnowski H and Hill KA. A novel mutation identified in five tissues of five Big Blue® mice is also detected directly in murine tissues using a BI-PASA assay, International conference for the Annual Meeting of the Environmental Mutagen Society, Vancouver, BC; September 16-20 2006 (Oral and Poster Presentation).

Tarnowski H, Prtenjaca A, and Hill KA. Multi-tissue spontaneous mutation load profiles in young adult male Big Blue mice®. International conference for the Annual


Non-refereed Publications


RESEARCH EXPERIENCE

The University of Western Ontario, London, ON
Ph.D Biology – Molecular Genetics 2005 - Present
- Bred and maintained a large mouse colony in both a conventional and barrier facility
- Performed genotyping for detecting transgenic and harlequin disease mice
- Performed the Big Blue® mouse cII mutation detection assay
- Cryosectioned the cerebellum on a Leica Cryostat
- Performed histochemical assays for reactive oxygen species, lipofuscin and apoptotic neurons
- Performed RNA extraction and purification of the cerebellum for an Affymetrix Mouse Array
- Analyzed transcriptome data using Ingenuity Pathway Analysis
- Trained to maintain and operate an Applied Biosystems 3130xl Genetic Analyzer
- Trained to operate an Arcturus Veritas Laser Capture Microdissection System

**Independent Research Project**  
2004 – 2005
- Dissected transgenic mice that were used in mutation research.
- Performed the Big Blue® mouse lacI mutation detection assay
- Made solutions for experimental protocols
- Performed DNA extraction, PCR amplification, gel electrophoresis and disposed of harmful waste
- Designed and optimized primers for a Bi-directional PCR amplification of specific alleles (Bi-PASA) assay

**TEACHING EXPERIENCE**

The University of Western Ontario, London, ON

**Teaching Assistant in Biology**  
2005 - 2012
Molecular Cell Biology of Stress (Biology 3355)  
Sept – Dec 2012
- Organized tutorial sessions
- Created and presented a tutorial on making heatmaps using R software
- Evaluated student assignments and presentations
- Proctored and marked midterms and exams

Introduction to Biology (Biology 1001 and 1201)  
2011 - 2012
- Taught seven two-hour tutorials every two weeks on topics relating to scientific literacy
- Marked weekly assignments for 300 – 400 students
- Proctored and marked midterms and exams

Investigative Techniques in Genetics (Biology 4582)  
2005 - 2010
- Taught basic genetics lab techniques and marked exams and assignments

**Tutor**  
2001 - 2009
- Tutored a second year biology student in Introductory Genetics (Biology 2581)
- Tutored students from 11 to 18 years of age in the topics of math, biology and chemistry
VOLUNTEER ACTIVITIES

The University of Western Ontario, London, ON

Animal Use Subcommittee 2009-2010
- Reviewed and approved animal protocols
- Inspected animal facilities

Biology Graduate Education Committee 2008-2009
- Ranked NSERC, OGS and various other scholarship applications
- Evaluated applications to the Graduate Program in Biology

The Genetics Society of Canada June 2006
- Audiovisual technician for The Genetics Society of Canada

Ontario Biology Day March 2006
- Audiovisual technician for Ontario Biology Day

Biology Outreach Committee Member 2005 - 2007
- Conducted lab tours and talked with high school students about Biology program at Western.

WISDOM Member 2003 - 2005
- Supplied relevant information about opportunities available to young women in science.

Society Participation

Environmental Mutagen Society Newsletter 2006 - 2007