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Oxidative stress in the cerebellum of the spastic Han-Wistar (sHW) mutant rat, a model of Ataxic Neurodegeneration

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ABSTRACT

Oxidative stress in the cerebellum of the spastic Han-Wistar (sHW) mutant rat, a model of Ataxic Neurodegeneration

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Neurodegeneration is the progressive breakdown of neurons resulting in impairment of cognitive functions and motor abilities, and has been attributed to such neurological diseases as Alzheimer's, Parkinson's, Huntington's, amyotrophic lateral sclerosis (ALS), and important in this study, ataxia. Oxidative and nitrosative stress occurs when the

damaging action of the reactive oxygen species (ROS) and reactive nitrogen species (RNS) targets key cellular molecules such as proteins, DNA, and RNA. This phenomenon is usually kept in check by the body's natural antioxidant defense system. However, under certain biomolecular or environmental factors, this defense system can be overwhelmed and shuts down, leading to a degenerative state. There has been a growing body of evidence indicating that oxidative and nitrosative damage is directly involved in the pathogenesis of neurodegenerative diseases. Recent research shows that mitochondrial dysfunction increases the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Lin and Beal, 2006). Oxidative stress causes formation of oxidative lesions in the DNA. Specifically, oxidation causes transversion mutations of guanine to thymine with 8-hydroxy-deoxyguanosine (8-OHdG) accumulation as a byproduct in affected cells (Ho et al., 1999). Therefore, 8-OHdG is a good marker for neuronal degeneration due to DNA damage (Won et al., 1999). In contrast, a marker of oxidative damage to proteins is nitrotyrosine, which when combined with nitric oxide (NO), results in the generation of peroxynitrite. Peroxynitrite is a strong and relatively, long-lived oxidizing agent, which can lead to nitration of tyrosine residues in tissue proteins. In my study, I am in interested in the region of the brain that is prone to oxidative stress: the cerebellum. In particular, Purkinje cells are very sensitive to ischemia (Sulkowska et al., 2008). Researchers showed that by seven months of age the harlequin mouse exhibited higher 8-OHdG immunoreactivity because of increased Purkinje cell death (Klein et al., 2002). Research has also shown that increased NT in the Purkinje cells implicates oxidative damage during aging that also involves associated motor deficits (Chung et al., 2002). My project utilizes the mutant *spastic* Han-Wistar

(sHW) rat, an acknowledged model of ataxia. The sHW rat carries an autosomal, recessive gene that is manifested at 25 days of age as spastic paresis, fore limb tremor, rigidity of the hind-limbs, and early death (approximately 65 days; Cohen et al., 1991). Morphological examination of mutant animals indicates losses of cerebellar Purkinje cells, alterations in neurofilament proteins in their cytoskeleton and asymmetries in the granular cell layer compared to its normal siblings (Cohen et al., 1991). Further studies in our lab hypothesize that glutamate excitotoxicity leads to mitochondrial dysfunction, resulting in oxidative cellular damage and cell death (Hildebrandt et al., 2003). The goal of this study is to examine 8-OHdG and NT expression to quantify the amount of oxidative damage in affected Purkinje cells in the sHW rat. Ultimately, our lab hopes to discover the underlying mechanism of oxidative stress in this rat model, emphasizing the different effects in the mutant cerebellum.

Introduction

Neurodegeneration is the progressive breakdown of both structure and/ or function of neurons that results in the impairment of cognitive functions and motor abilities leading to neuronal cell death (Przedborski et al., 2003). The progressive loss of related neuronal systems is attributed to such neurodegenerative diseases as Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), amyotrophic lateral sclerosis (ALS), and important in my study, ataxia (Lin and Beal, 2006). In practice, neurodegenerative diseases comprise a large group of disorders with heterogeneous clinical and pathological expressions affecting specific populations of neurons (Przedborski et al., 2003). With a few exceptions, the etiology of these neurodegenerative diseases remains mysterious. Though there is growing debate surrounding the causes of neurodegenerative disorders, researchers are examining the roles that cellular and environmental factors contribute in the initiation of these diseases.

Recent research indicates that excitotoxicity, resulting in mitochondrial dysfunction and various forms of "oxidative stress", plays a pivotal role in the pathogenesis of these neurodegenerative diseases (Nisim et al., 1999; Andersen et al., 2004; Lin and Beal, 2006;). Excitotoxicity is defined as neuronal cell death caused by overactivation of excitatory amino acid receptors. Specifically, it is the ability of glutamate and structurally related amino acids to destroy neurons (Olney et al., 1969, 1986). The toxic effects of glutamate was first observed by Lucas and Newhouse (1957), who described degeneration of the inner layers of the retina following subcutaneous

injections of glutamate in infant mice. Later, in a landmark paper, Olney (1969) coined the term "glutamate excitotoxicity" describing intracranial brain lesions in response to subcutaneous injections of glutamate in infant and adult mice.

The amino acid L- glutamate is the major excitatory neurotransmitter found in the mammalian central nervous system (CNS). Glutamate receptors are categorized as either ligand-gated, ion channel receptors (ionotropic) or G-protein-coupled receptors (metabotropic) (Bradford et al., 1995). The binding of glutamate to ionotropic receptors activates ion channels allowing cations to flux through the cell membrane, resulting in the formation of excitatory currents. The metabotropic receptors (mGluRs) are linked to intracellular signal transduction systems which are crucial in regulating the size of many currents. The ionotropic receptors can be further divided into NMDA and non-NMDA subtypes (AMPA and kainate) named after the specific pharmacological agents that stimulate them. These receptors are intrinsic to three types of receptors defined pharmacologically by their agonists as *N*-methyl-D-aspartate (NMDA), alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate. NMDA receptors are voltage and ligand gated channels which control the influx of cations, significantly calcium (Ca^{2+}). Non-NMDA receptors (AMPA and kainate receptors) are required for the activation of the NMDA receptors, since non-NMDA receptor activation requires removal of the magnesium (Mg^{2+}) ion blockade for normal NMDA channel function (Choi et al., 1988).

Transmission of glutamate is crucial for normal neurophysiological functions such as cell-cell communication, playing an important role in the establishment and maintenance of synaptic plasticity (Choi et al., 1987, 1988; Collingridge et al., 1990).

However, abnormally high synaptic glutamate levels or dysfunctional receptor systems may result in neurodegeneration (Choi et al., 1987, 1988). Neuronal damage or death occurs because of swelling resulting by the stimulation by Ca^{2+} of intracellular enzymes that degrade proteins, lipids, and nucleic acids. The phenomenon of glutamate excitotoxicity, has been linked to several neurodegenerative disorders including epilepsy, ischemia, ALS, Huntington's disease, Parkinson's disease, Alzheimer's disease and ataxia (Choi et al., 1988; Bradford et al., 1995).

The underlying biochemical mechanism with glutamate excitotoxicity occurs in two phases: the first, cell swelling stemming from increased glutamate receptor activation, resulting in an influx of sodium (Na^{+}) coupled with that of water (Rothman et al., 1985; Olney et al., 1986). The second is delayed Ca^{2+} dependent cell degeneration. It is well established that a strong relationship exists between excessive Ca^{2+} influx and glutamate triggered neuronal injury (Choi et al., 1988). Altered physiological levels of Ca^{2+} may result in activation of active Ca^{2+}-dependent proteases such as calpain I (Siman et al., 1989), altered transcription of various neuronal genes (Mayer et al., 1987), or induction of phospholipase A_2 (Feldman et al., 1997). These events may lead to activation of apoptosis or other calcium-linked biochemical events leading to cell death (Nicotera et al., 1990; Pollard et al., 1994).

A study by Choi (1988) shared that calcium influx was essential to glutamate excitotoxicity. The study examined how glutamate excitotoxicity in neuronal cultures was potentiated in a calcium-rich extracellular solution, whereas a calcium-free extracellular solution markedly reduced neurodegeneration. Based on this initial study by Choi, researchers have found that the removal of extracellular Na^{+} eliminates the acute neuronal

swelling in cortical cell cultures exposed to glutamate (Mayer et al., 1987). However, the absence of extracellular Na^+ failed to prevent long-term (delayed) neuronal degeneration. Furthermore, neuronal degeneration could only be fully attenuated following the removal of extracellular Ca^{2+}. While NMDA receptors play a central role in glutamate-induced excitotoxicity, it is important to examine the potential roles of non-NMDA receptors as well.

One source that can be attributed to the pathogenesis of these neurodegenerative disorders is mitochondrial dysfunction. Mitochondria are considered to be the "power house" of the cell by converting metabolites into ATP through oxidative phosphorylation (Lin and Beal, 2006). Initially mitochondrial dysfunction leads to decreased ATP production resulting in partial neuronal depolarization followed by activation of excitatory amino acid receptors by ambient concentrations of glutamate (Beal, 1996). It also causes detrimental effects, such as impaired intracellular calcium buffering and generation of reactive oxygen species (ROS) (Lin and Beal, 2006). Finally, other possible sources that could contribute to mitochondrial dysfunction via specific forms of "stress" such as oxidative and nitrosative stress. Harmful by-products of oxygen metabolism may produce reactive oxygen species (ROS). ROS are defined as chemical entities that react with cellular components, resulting in detrimental effects on their function (Andersen et al., 2004). Reactive oxygen species include both free radicals (containing highly reactive unpaired electrons), such as superoxide (O_2), nitric oxide (NO), hydroxyl radical (OH), hydrogen peroxide (H_2O_2) and peroxynitrite ($ONOO^-$). ROS are produced during normal metabolic activities catalyzed by mitochondrial electron transport and other redox reactions that take place in microsomes and the inner plasma membrane surface (Kamsler

et. al., 2000). However, most cellular ROS are generated during incomplete metabolic reduction of oxygen to water (Andersen et. al., 2004). All of these species are redox active and can associate with nearby cellular components such as proteins, lipids, and DNA. ROS exerts a regulatory role through interactions with required transcription pathways and signaling pathways, that if left untreated or unchecked results in cell death (Merrill and Murphy, 1997). Though, under certain biomolecular or environmental factors, this defense system can be overwhelmed and shuts down, leading to a degenerative state (Ames et. al., 1993).

ROS are very reactive and will react with a multitude of different molecules to initiate neuronal cell death and hence neurodegeneration through an array of different pathways. Oxidative stress (oxidation of lipids, proteins and DNA) results in impaired cellular functions and the formation of toxic species, such as peroxides, alcohols, aldehydes, and ketones (Ferrari, 2000). Modifications to proteins result in the impairment of enzymes, whereas ROS interactions with DNA lead to mutations (Barnham et al., 2004). One of the downstream events that occurs in response to an ROS-induced calcium influx is an excitotoxic response, in which activation of glutamate receptors triggers a cascade of events leading to cell death (Mattson and Chan, 2003; Yamamoto et al., 1998).

One of the most widely studied markers for cellular oxidative stress is 8-hydroxydeoxyguanosine (8-OHdG) (Won et al., 1999). 8-OHdG is the most abundant, oxidative stress marker resulting in mutations through formation of GC-to-TA transversions (Kasai et al., 1984). Though the presence of 8-OHdG is normal and does not necessarily indicate that of oxidative stress linked mutations. However, high accumulation of 8-OHdG is a good indicator for the mechanisms underlying the

occurrence of "free-radical diseases" (Floyd et al., 1990; Ames et al., 1993). For example, one study showed a statistically significant 108-fold increase in the ratio of 8-OHdG in intact DNA to free 8-OHdG in patients with advanced AD (Lovell et. al., 2001).

8-OHdG formation indicates the presence of oxidative stress in the nuclei, which can induce epigenetic changes (Anisimov et al., 1998). This accumulation of 8-OHdG may alter the expression of specific genes, largely influencing a variety of signal transduction pathways responsible for the maintenance and regulation of cellular functions (Anisimov et al., 1998; Balaban, 2005). In the brain, the cerebellum appears to be susceptible to oxidative damage, for example Purkinje cells are sensitive to oxidative stress linked ischemia (Sulkowska et. al., 2008). Research has shown that 6-OHDA-lesioned hemiparkinsonian rats displayed significantly elevated 8-OHdG levels in urine, serum, and substantia nigra compared to sham controls (Yasuhara et al., 2007). Researchers showed that by seven months of age the harlequin mouse, which has a progressive degeneration of terminally differentiated cerebellar neurons, exhibited higher 8-OHdG immunoreactivity due to increased Purkinje cell death in the cerebellum (Klein et al., 2003).

Nitrosylation damage can also occur in proteins affecting a variety of cellular functions involving proteins such as receptors, transport systems, and enzymes. This can result in increased levels of protein carbonyls (PCO), due to attack from reactive oxygen and nitrogen species (ROS/RNS) (Butterfield et al., 1998). Free radicals are produced primarily as byproducts of metabolism, but may also be obtained from environmental sources. Two major sources of free radicals are superoxide, produced in mitochondria (Chung et al., 2002) and nitric oxide (NO) generated by Ca^{2+}-activated calmodulin-

dependent NO synthase (NOS) in the conversion of arginine to citrulline (Cheng et al., 2002). Nitric oxide (NO) combines rapidly with superoxide anion to form the powerful oxidizing agent peroxynitrite ($ONOO^-$), since it is diffusion limited (Czapski et al., 1995). This occurs especially under conditions of oxidative stress when the production of NO and oxygen radicals is increased. Peroxynitrite is able to oxidize proteins, lipids, thiols, and DNA through a hydroxyl radical-like intermediate and a number of other small organic molecules (Chung et al., 2002). Since peroxynitrite is a strong and relatively long-lived oxidizing agent, it can lead to nitration of tyrosine residues in many tissue proteins. Nitration of proteins can disrupt cell function and results in pathological processes, including neuronal degeneration and DNA damage (Crow et al., 1995).

Recent research has shown that nitrotyrosine (NT) is an early marker for certain neurodegenerative processes involving oxidative stress (Teunissen et al., 2002). Given the fact that NT is an end product of peroxynitrite oxidation, research has shown that increased nitrotyrosine in the Purkinje cells serves as a marker for functionally significant nitrosylative oxidative damage (Chung et al., 2002). Research has shown increased NT formation in animal models for Huntington's disease and Parkinson's disease (Schulz et al., 1997; Klirenyi et al., 2000). Also, NT formation has also been associated with the development of characteristic neurofilament tangles in Alzheimer's disease (Good et al., 1998). Chung and associates concluded that the increase in NT with age is not likely due to the increase in NO production, but rather is due to increased degradation of nitrated proteins that have accumulated during aging. They also showed that NT increases with age in the cerebellum and are linked with the associated behavioral deficits observed during aging and age-associated neurodegenerative diseases involving the cerebellum

like ataxia (Chung et al., 2002).

The cerebellum, located behind the cerebrum and above the pons and fourth ventricle, is involved in coordination of movement and walking, maintenance of body equilibrium and muscular tonus. One of the principal disorders observed in cerebellar dysfunction is ataxia. Ataxia is a progressive, genetically-inherited disorder that affects the central nervous system. Derived from the Greek word meaning "lack of order", consists of a gross lack of coordination of muscle movements. In cerebellar ataxia, there is a loss of Purkinje cells resulting in gait instability and difficulties in motor coordination. There are many types of ataxias, ranging from hereditary ataxias to autosomal dominant ataxias such as spinocerebellar ataxias and episodic ataxia. Friedreich Ataxia (FRDA), the most common recessively inherited ataxia, is caused by decreased expression of the gene for the mitochondrial protein frataxin. Although the function of frataxin is uncertain, its deficit increases mitochondrial oxidative stress and iron accumulation, which are considered to be the major causes of the progressive pathophysiology of FRDA (Jauslin et al., 2003). FRDA is an autosomal recessive degenerative disorder characterized by progressive gait and limb ataxia, loss of limb deep tendon reflexes, spasticity and extensor plantar responses (Durr et al., 1996; Harding, 1981). Ataxia-telangiectasia (A-T) is a rare autosomal recessive genetic disorder characterized by progressive neurodegeneration, a high risk of cancer and immunodeficiency (Lavin et. al., 2007). This is caused by mutational inactivation of the *ATM* (ataxia-telangiectasia mutated) gene. ATM gene normally recognizes DNA damage and signal to the DNA repair machinery and the cell cycle checkpoints to minimize the risk of genetic damage (Lavin et. al., 2007). The most debilitating feature of A-T is the

progressive neurodegeneration due to loss of Purkinje cells in the cerebellum and malfunction of other neuronal cells (Lavin et. al., 2007). Though there is no cure, treatment has focused on slowing the progress of the neurodegeneration.

My project utilized mutant *spastic* Han-Wistar (sHW) rats, an acknowledged animal model of ataxia. This mutant rat, first identified in 1976 by Wolfgang Pittermann, carries an autosomal, recessive gene that is manifested at 25 days of age. It produces spastic paresis (muscular weakness), characterized by fore limb tremor and rigidity of the hind limbs. The earliest behavioral symptoms occur at about 25-30 days of postnatal age when mutants display fore limb tremor. Mutants are also identified at this age by reduced body weight gain compared to their normal littermates and hyperactivity. As mutants reach 40 days of age, their gait becomes unsteady, their hind limbs splay out and the fore limb tremor becomes progressively worse. After 55 days, their hind limbs become extended and rigid resulting in a reduced ability to walk. After 60 days of age, the mutant animals are unable to feed, due to the ataxia in the hind limbs, which leads to eventual death at approximately 10 weeks of age (Cohen et al., 1991). Histological evaluations of the mutant's cerebellum showed the general layering was normal, granule cells and Golgi cells appeared preserved, whereas Purkinje cells revealed signs of late onset neurodegeneration (Wagemann et al., 1991). Further morphological examination of mutant animals indicates losses of cerebellar Purkinje cells, alterations in neurofilament proteins in their cytoskeleton and asymmetries in the granular cell layer (Cohen et al., 1991; Levine et al., 1992). The degeneration of cerebellar Purkinje cells is observed in the sHW mutant as early as 40 days postnatally (Cohen et. al., 1991).

Furthermore, evidence of a dysfunctional glutamatergic system in the cerebellum

and the susceptibility of cultured Purkinje cells to death via glutamate excitotoxicity suggest that glutamate receptors are involved in the observed condition (Cohen et al., 1991). Molecular analysis of altered GluR2 receptor subunit mRNA expression in the mutant offers further evidence of the involvement of the non-NMDA receptors in the progressive neurodegenerative disorder observed in the cerebellum of the sHW mutant (Margulies et al., 1993). At 30 days postnatally, mutants express significantly reduced GluR2 mRNA in both Purkinje and granule cells. However, at this age, there is no histological evidence of neurodegeneration in the mutant cerebellum. An increase in GluR2 mRNA at 60 days (Cohen et al., 1991). This maybe due to an alteration in glutamate receptor assembly in affected areas. During the second and third postnatal weeks, when symptoms first arise, it is an important developmental period when glutamate receptors undergoes changes in abundance and distribution from developmental to adult state (Ben-Ari et. al., 1997). Taking into account the various biochemical mechanisms exhibited in the sHW strain, it makes the sHW mutant an invaluable tool for studying excitotoxicity linked ataxia.

The goal of this study was to examine the role of 8-hydroxydeoxyguanosine (8-OHdG) and nitrotyrosine (NT) expression in order to quantify the amount of oxidative damage in affected neurons in the sHW rat. Ultimately, this will help contribute to discovering the underlying mechanisms of oxidative and nitrosative stress in the *spastic* Han-Wistar (sHW) rat model, with an emphasis on the different effects in the mutant cerebellum and further investigate the cause of pathogenesis in neurodegenerative diseases.

Materials and Methods

Animals: All *spastic* Han-Wistar (sHW) rats used in the following experiments were acquired from the Biology Department's vivarium breeding colony at California State University, Northridge. This colony was obtained from the University of California in Los Angeles in 1994. The original colony was initially derived in 1986 from breeding pairs obtained from Zentralinstitut fur Versuchstierzucht (Hanover, Germany). The animals were housed in groups of 2-4 and were maintained under normal conditions of 12h light/12h dark cycle and room temperature of 23-25ºC. They were provided Purina rat chow and water *ad libitum.* Subsequent to being weaned from their dam, mutant rats and their normal same sex siblings were selected and processed together in the following experiments. The pairing of histology groups consisted of two rats, a mutant and a same sex normal sibling. All protocols mentioned in my thesis have been approved by CSUN's Animal Care and Use Committee.

Tissue Fixation and Preparation: Same-sex, normal and mutant 50-55 day old pairs (n=10 pairs) were utilized for immunohistochemical analysis. Rats were anesthetized with 400mg/kg of chloral hydrate (Sigma Chemical), and then perfused via the following transcardial perfusion procedure. The animal was determined unresponsive using the foot-pad squeeze and eye blink-response methods determining depth of anesthesia. The heart was exposed through a midline ventral chest incision and using hemostats, the descending aorta and inferior vena cava were clamped to prevent whole body perfusion. A 26-gauge catheter needle was inserted into the apex of the left ventricle and the right atrium was immediately incised. Using a varistaltic pump, 150ml of perfusion saline solution [0.1M phosphate-buffered saline (PBS) containing 0.2% heparin] was performed in order to clear most of the blood from the head and neck region. Clearance of blood was

visually confirmed by the paleness/whiteness of the lungs, eyes, and mouth. Afterwards, approximately 250ml of 4% paraformaldehyde in 0.1M PBS (pH 7.4) was perfused until animal tissues appeared fixed (confirmed by physical inspection of stiffness of the neck and forelimbs). The brains were removed using Rangier pliers and placed in 4% paraformaldehyde/PBS for at least 24 hours at 4ºC to ensure thorough fixation. The tissue was finally cryoprotected in 20% sucrose in 4% paraformaldehyde/PBS solution for an additional 48 hours prior to sectioning to avoid potential freeze fracturing of the tissue upon exposure to low (-20ºC) sectioning temperatures.

Tissue Sectioning: Histological processing of mutant and normal brains occurred simultaneously to avoid processing artifacts. Harvested tissue was sectioned using a Reichert-Jung Cryocut 1800. The cerebellum was mounted onto a cryostat mounting platform using Tissue Freezing Medium (Triangle Biomedical Sciences) on dry ice. Brains were sectioned at -20ºC into 30µm sagittal slices of the cerebellum and stored in 24 well tissue culture plates containing 0.1M PBS at 4°C until immunohistochemistry was performed within 96 hours.

Immunohistochemistry: Immunohistochemical staining was utilized to investigate the expression of 8-hydroxydeoxyguanosine (8-OHdG) and nitrotyrosine (NT) in cerebellar sections of 50-55 day old mutant and normal sHW rats (n=10 pairs for each antibody). The premise for this powerful technique lies in the fact that monoclonal antibodies have highly selective binding affinities for specific proteins. In order to expose antigens that have been masked during the tissue fixation process, antigen retrieval using heat induced epitope retrieval was utilized after the blocking step but prior to incubation with primary antibody during immunohistochemical staining. The 30µM cerebellar sections were

incubated in a 10mM citrate buffer for 30 minutes at 37ºC. Tissue sections were washed in a series of three changes of 0.1M phosphate buffer saline (PBS) buffer. Samples were then placed in 10% H_2O_2 treatment for 10 minutes to remove endogenous peroxidase activity, preventing non-specific binding. Excess hydrogen peroxide was removed with three 5 minute washes of 0.1M PBS. Samples were then incubated in 5% normal goat serum (NGS) for 1 hour to block non-specific binding. Affinity purified anti-8-hydroxydeoxyguanosine (mouse monoclonal IgG; Northwest Life Science Specialties, Vancouver, WA; Catalog number NWA-MO020, 10µg/ml) and anti-nitrotyrosine, clone 2A8.2 (mouse monoclonal IgG; Fisher Scientific, Houston, TX; Catalog number MAB5404, 1mg/ml) primary antibody was added in PBS and was incubated for 48 hours. During this incubation period of primary antibodies, the tissue was held at 4°C on an orbital shaker (Lab Line). The tissue slices were then washed with five repetitions of 0.1M PBS at 5 minutes each and a 1 hour application of biotinylated anti-mouse secondary antibody (ABC Elite kit; Vector Laboratories, Inc.). This was followed by three 5 minute rinses in 0.1M PBS. The tissue was then immediately placed in an Avidin-Biotin solution (Vector Laboratories, Inc.,) for another hour. This complex serves as a substrate for the chromagen diaminobenzidine (DAB; Sigma). DAB was applied to tissue sections for 2 minutes, and resulted in reddish brown precipitate at the primary antibody/receptor sites. Excess DAB was removed with three 5 minute rinses of 0.1M PBS. Finally, tissue sections were mounted on glass slides, dehydrated in alcohol, cleared with xylene and cover slipped with Permount (Fisher Scientific). Control sections were treated as above, with the exception of primary antibodies application, to determine non-specific staining. Combining the selectivity of monoclonal antibodies with a color

producing chromogen, staining intensity was used to determine qualitatively the distribution, localization and density of 8-OHdG and NT within each section.

Quantitative Analysis of Immunohistochemistry: Cerebellar sections from 50-55 day old mutants and littermate normals were examined at a high magnification (X20 and X40 objective) on a Nikon Optiphot photomicroscope using transmitted epi-illumination. Computer images of individual Purkinje cells and of the granular cell and molecular cell layers were generated using a Sony analog camera and were analyzed using ImageJ Analysis Software. All TIF images were then imported using image sequence and converted to an 8-bit grayscale for contrast, reducing color information to black and white for thresholding. Next, an analysis box was established at a standard width of 300µm and a height of 100µm, producing an area of 30,000 pixels (1pixel=1µm). Image was then adjusted to a threshold of 100 for 8-OHdG and 120 for NT (depending on the staining intensity), to remove background staining. Finally, the area fraction and display label were added to the set measurements and percent area staining was measured. Afterwards, the data were normalized and then analyzed using t-tests with equal variance.

Results

Immunohistochemistry Study:

The immunohistochemistry study was performed to quantify oxidative stress damage in the brains of 55 day old mutant sHW rats compared to their normal siblings. Based on previous studies, it was expected that there would be higher oxidative stress observed in the mutant sHW rats compared to their normal siblings. To accomplish this, I utilized two markers, 8-hydroxydeoxyguanosine and 3-nitrotyrosine as indicators of two distinctly different forms of neuronal stress.

8-Hydroxydeoxyguanosine Staining:

The compound 8-hydroxydeoxyguanosine (8-OHdG) is one of the most common oxidative stress marker resulting from formation of GC-to-TA transversions (Kasai et al., 1984; Cheng et al., 1992). Previous studies in the Harlequin mouse, which has a progressive degeneration of terminally differentiated cerebellar neurons, researchers observed that by seven months of age these mice exhibited higher 8-OHdG immunoreactivity due to increased Purkinje cell deaths in the caudal lobules of the cerebellum (Klein et al., 2002). Therefore, 8-OHdG was expected to be present in significantly higher amounts in the cerebellums of mutant sHW rats due to increased degeneration of cerebellar Purkinje cells (Cohen et al., 1991). In the cerebellum of mutant animals, cerebellar Purkinje cells degenerate, alterations in neurofilament proteins underlying the Purkinje cell cytoskeletal structure occur, and there are asymmetries in the granule cell layer (Levine et al., 1992). Also, the frequency of gaps and the distance are

increased between adjacent Purkinje cells with age, gaps suggesting regions of cell death (Levine et al., 1992).

Cerebellar sagittal sections of 50-55 day old mutant and normal sHW rats were stained with 8-OHdG antibody. The sections showed uniform distribution of 8-OHdG in both normal and mutant cerebellums but with the mutant sHW rat displaying significantly higher staining compared to normal sHW rats (Figure 1). Specifically, mutant sHW rats showed much greater staining intensity within the Purkinje cells and within the deep cerebellar nuclei. There were also significant gaps between adjacent Purkinje cells in the mutants, exhibiting the neurodegeneration seen at this age. However, both the molecular and granular cell layers showed little or no staining. Normal sibling sHW rats showed very little staining in the Purkinje cells. In fact, there was uniformly light expression of 8-OHdG among the molecular, Purkinje, and granular cell layers in normal rats at this age.

Photomicrograph images were converted to an 8-bit grayscale using ImageJ software for quantification of 8-OHdG staining, reducing the color information to black and white for enhanced contrast thresholding. Images were adjusted to a threshold of 100 for staining intensity to remove background staining using ImageJ software and eventually allowing quantification of 8-OhdG expression. By converting the images to false color photomicrographs, contrasting images showed different immunohistochemistry staining intensities within normal and mutant sHW 55 day old siblings (Figure 2). The false red color, indicative of antibody staining, again showed significantly higher 8-OHdG expression in the Purkinje cells of the mutants compared to their normal siblings that had no staining in the Purkinje cells. Finally, 8-OHdG

immunohistochemistry staining in the cerebellum of normal and mutant sHW 55 day old siblings was quantified. Data were first normalized by subtracting the control data from the experimental data and then analyzed using t-tests with equal variance. Control data were tissue that was not treated with any antibody and experimental data were tissue that had been treated with antibody. Mutant sHW rats showed significantly higher amounts of 8-OHdG staining than normal animals ($t=3.52$; $p<0.05$) (Figure 3).

Nitrotyrosine Staining:

Nitrotyrosine (NT) is an early marker for neurodegenerative processes involving oxidative stress (Teunissen et al., 2002). Nitration of proteins can disturb cell function and result in pathological processes, including neuronal degeneration and DNA damage (Crow et al., 1995). Since NT is an end product of peroxynitrite oxidation, research has shown that increased nitrotyrosine in the Purkinje cells serves a marker for functionally significant nitrosylative oxidative damage (Chung et al., 2002). Increased NT formation has been linked to animal models for Huntington's disease and Parkinson's disease (Schulz et al., 1997; Klirenyi et al., 2000) as well as with neurofilament tangles in Alzheimer's disease (Good et al., 1998). Therefore, it was expected that NT immunoreactivity would be higher in the mutant sHW rat compared to their normal siblings.

Cerebellar sagittal sections were stained with NT antibody from 50-55 day old mutant and normal sHW rats. The immunohistochemistry staining of NT in the cerebellum showed very light, yet similar staining intensities in both the normal and mutant sHW 55 day old siblings (Figure 4). Examining both the normal and mutant sHW

rat, Purkinje cells are visible, however none were stained with NT. There were no differences in NT expression among the molecular, Purkinje and granular cell layers of both genotypes. In comparison to control sections that were not treated with NT antibody, there were no differences between these sections and sections treated with anti-NT in both types of cerebellums. These results suggest that there is no abnormal protein nitration occurring in the cerebellum of the sHW mutant or normal rats.

Like I did with 8-OHdG staining, photomicrographs were converted to an 8-bit grayscale in ImageJ, reducing the color information to black and white for quantitative thresholding. Next, a false color photomicrograph of NT immunohistochemical staining in sagittal cerebellar sections of 55 day old normal and mutant sHW rats were developed (Figure 5). Images were adjusted to a threshold of 120 for staining intensity to remove background staining. The false red color again is indicative of antibody staining, and these results showed no NT expression in the cerebellums of either group. Data were first normalized by subtracting the control data from the experimental data and then analyzed using t-tests with equal variance. Control data were tissue that was not treated with any antibody and experimental data were tissue that had been treated with antibody. There were no significant staining differences seen between the normal and mutant sHW rats ($t=0.06$; $p > 0.05$) (Figure 6).

Discussion

Neurodegeneration consists of a progressive neurological decline and is associated with neuronal loss (Palop et al., 2006). Data from experimental animal models and post-mortem human brain studies suggest that oxidative stress may be directly linked to the observed neuronal degeneration in diseases such as Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis (ALS) and ataxia (Simonian and Coyle, 1996). Normally, oxygen is essential for the survival of aerobic organisms and due to its high redox potential, it serves as the terminal electron acceptor in the process of metabolic energy generation. Unfortunately, this high redox potential can also damage vulnerable cells if it is not completely reduced. Partial reduction of molecular oxygen is an unintended consequence during aerobic metabolism and may lead to an imbalance between the generation and detoxification of reactive oxygen and nitrogen species (ROS/RNS) (Wang and Michaelis, 2010). While ROS/RNS serve as signaling molecules at physiological levels, an excessive amount of these molecules leads to oxidative modification and therefore, dysfunction of proteins, nucleic acids, and lipids. In the CNS, excessive production of ROS and RNS has been proved to be a mechanism for neurogeneration associated with various insults to neurons as seen in Alzheimer's disease, Parkinson's disease, ALS and important in my study, ataxia (Zhu et al., 2004b; Jenner, 2003; Carri et al., 2003).

Ataxia is a progressive neurological disease characterized by incoordination, postural abnormalities, difficulties with gait, and problems with speech (Lavin et al.,

2007). Studies have showed that oxidative stress may be an important cause of the degeneration of cerebellar neurons in human forms of ataxia (Kamsler et al., 2001). Cells derived from ataxic patients and mice models exhibit genomic instability and show hypersensitivity to ionizing radiation and other treatments that generate ROS easily (Rotman and Shiloh, 1999; Lavin, 1998). The *Tottering* mouse is an ataxic mutant characterized by a mild ataxia, generalized absence-like seizures, and paroxysmal dyskinesia (Green and Sidman, 1962). This mutant mouse carries a recessive autosomal allele of *tottering* locus on chromosome 8 that encodes a gene for the alpha 1A subunit of the P/Q-type Ca^{2+} channel ($Ca_v2.1$) (Fletcher et al., 1996). The dysfunction of $Ca_v2.1$ channel in Purkinje cells is involved in highly sustained intracellular Ca^{2+} concentrations. Purkinje cell death apparently occurs through mitochondrial dysfunction related to oxidative stress (Dove et al., 2000). Research has shown a parasaggital pattern of Purkinje cell loss in the cerebellum of the *tottering* mouse (Sawada et al., 2009). Ataxia-telangiectasia (A-T) is an inherited disease causing progressive neurological dysfunction, especially in cerebellar Purkinje cells (Barlow et al., 1996). A hallmark of A-T is a markedly increased sensitivity to oxidative stress particularly Purkinje cells (Barlow et al., 1996; Rotman and Shiloh, 1998; Brown et al., 1999).

Our animal model of ataxia, the spastic Han-Wistar (sHW) rat, is a mutant strain first identified in 1967 (Pittermann et al., 1976). Behaviorally, the earliest indications of degeneration are detectable as early as 15 days postnatally with mutants displaying forelimb tremor and hyperactivity (Pittermann et al., 1976; Cohen et al., 1991; Levine et al., 1992). By 50 days, there is a marked worsening of the forelimb tremor, increased rigidity of the hindlimbs and an onset of ataxia becomes apparent (Cohen et al., 1991). At

approximately 60 days of age, the mutants are unable to feed or drink and die shortly after (Cohen et al., 1991). Histological evaluations of the mutant's cerebellum indicate this structure is significantly affected by this condition (Cohen et al., 1991, 1997; Wagemann et al., 1991). Specifically, degeneration of cerebellar Purkinje cells is observed in the sHW mutant as early as 40 days postnatally (Cohen et al., 1991; Wagemann et al., 1991; Levine et al., 1992). A previous study has shown that in the cerebellum of most mutant sHW animals the general layering was normal, granule cells and Golgi cells appeared preserved, whereas several Purkinje cells revealed signs of degeneration (Wagemann et al., 1991). In another study, it was shown that the frequency of gaps and the distance are increased between adjacent Purkinje cells and is correlated with mutant age, suggesting regions of progressive cell death (Levine et al., 1992). Looking at Figure 1, there were also significant gaps between adjacent Purkinje cells in the mutants, exhibiting the neurodegeneration seen at this age. The sHW mutant rat is an excellent model of ataxia due to the loss of Purkinje cells and an undetermined autosomal recessive mutation (Cohen et al., 1991,1997; Levine et al., 1992).

In ataxia, motor abilities are impaired; thus, one can examine the Purkinje cell loss in the *spastic* Han-Wistar rat model for possible oxidative stress. The mechanism of Purkinje cell degeneration induced by ROS should include the formation of oxidative lesions in DNA accompanied by subsequent 8-hydroxydeoxyguanosine (8-OHdG) accumulation (Kasai and Nishimura, 1986). Thus, the detection of 8-OHdG by immunohistochemistry would be a useful method for estimating the oxidative DNA damage in cells and regions of the brain (Won et al., 1999).

As seen in Figure 1, there was uniform expression of 8-OHdG antibody in both

the normal and mutant cerebellum sections, with the mutant sHW rat displaying significantly higher staining compared to normal sHW rats. Mutant sHW rats also showed much increased staining intensity within the deep cerebellar nuclei as well. Since 8-OHdG is a common oxidative stress marker, the results support the hypothesis that oxidative stress may be occurring in the mutant sHW cerebellum. Using t-tests with equal variance to analyze the data, Figure 3 showed that mutant sHW rats had significantly higher amounts of 8-OHdG staining than normal animals ($t=3.52$; $p<0.05$). In another study performed by our lab, it was revealed that there was also significantly higher 8-OHdG staining also occurring in the hippocampus of mutant sHW rats (N. Alfulaij pers. comm.).

Another early marker for neurodegenerative processes involving oxidative stress is nitrotyrosine (NT) (Teunissen et al., 2002). Nitration of proteins can result in neuronal degeneration (Crow et al., 1995). However, unlike increased 8OHdG expression in the mutant, Figure 4 showed little to no staining of NT in the cerebellum of both mutant and normal sHW rats. One can see the visible Purkinje cells; however, neither the mutant nor the normal cerebellums were stained with NT. Statistically, there were no significant staining differences seen between the normal and mutant sHW rats (Figure 6; $t=0.06$; $p> 0.05$). Therefore, these results suggest that there is no abnormal protein nitration occurring in the cerebellum of the sHW mutant or normal rats. The NT results taken together with the observed 8OhdG expression suggest that the form of oxidative stress occurring in the cerebellums of mutant spastic-Han Wistar rat is strictly DNA based damage.

The response of stressful neurodegenerative conditions within different neuronal

populations displays selective neuronal vulnerability (SNV). SNV refers to the differential sensitivity of neuronal populations in the CNS to similar stresses that cause cells to respond differently, leading to neurodegeneration in some susceptible populations (Wang and Michaelis, 2010). Currently, the known molecular and cellular factors that contribute to oxidative stress and subsequent SNV can range from high demand for ROS/RNS- based signaling, low ATP production, mitochondrial dysfunction, high inflammatory response in vulnerable neurons, to glutamate excitotoxicity (Wang and Michaelis, 2010).

Within the sHW rat cerebellum, the vulnerability of Purkinje cells to glutamate excitotoxicity triggers possible oxidative stress. My research shows that the neurodegenerative pathways take the DNA oxidation route, leading to the observed ataxic condition. Is this cell death pathway unique to the sHW rat? Interestingly, researchers have seen similar results. In an autopsy study, cerebellar degeneration in human siblings with ataxia-telangiectasia-like disorder was examined. The siblings had increased expression 8-OHdG in the nuclei of granule cells and Purkinje cells (Oba et al., 2010). Do vulnerable Purkinje cells normally die via the oxidation of DNA in cerebellar ataxic diseases?

Other diseases show different oxidative stress pathways leading to neurodegeneration. ALS is a progressive, fatal neurodegenerative disease characterized by gradual degeneration of motor neurons in the cortex, brainstem and spinal cord (Mariani et al., 2005). Results showed plasma and urine 8-OHdG levels increased significantly with time in ALS patients and increased rate of urine 8-OHdG levels with time significantly correlated with disease severity (Bogdanov et al., 2000). Yet, there is

evidence for increased protein nitration in ALS, in which there was increased immunocytochemical staining for 3-nitrotyrosine in spinal cord motor neurons of both sporadic and FALS patients (Beal et al., 1997). Thus, depending on neuron type, cell death may take distinct forms of oxidative stress.

Research from my study and previous research in our lab has shown that both the cerebellum and hippocampus are susceptible to oxidative DNA damage as detected by 8-OHdG marker. However, neither brain regions showed any nitrosative protein damage as detected by nitrotyrosine. Therefore, our sHW mutant rat is a good example of SNV and that oxidative stress may be occurring and contributes to our animal model of ataxic neurodegeneration. Future research might look at different time points, maybe starting from 20 days until 55 days and examine at what time point is there an increase in 8-OHdG DNA oxidative damage. Research into other oxidative stress markers in conjunction with 8-OHdG might also help support the idea that the oxidative stress occurring in the sHW mutant rat is nuclear based. Finally, based upon my research, future researchers may wish to examine treatment options with antioxidants to help suppress DNA damage.

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Appendix

Figure 1. 8-OHdG Staining

(a) Normal

(b) Mutant

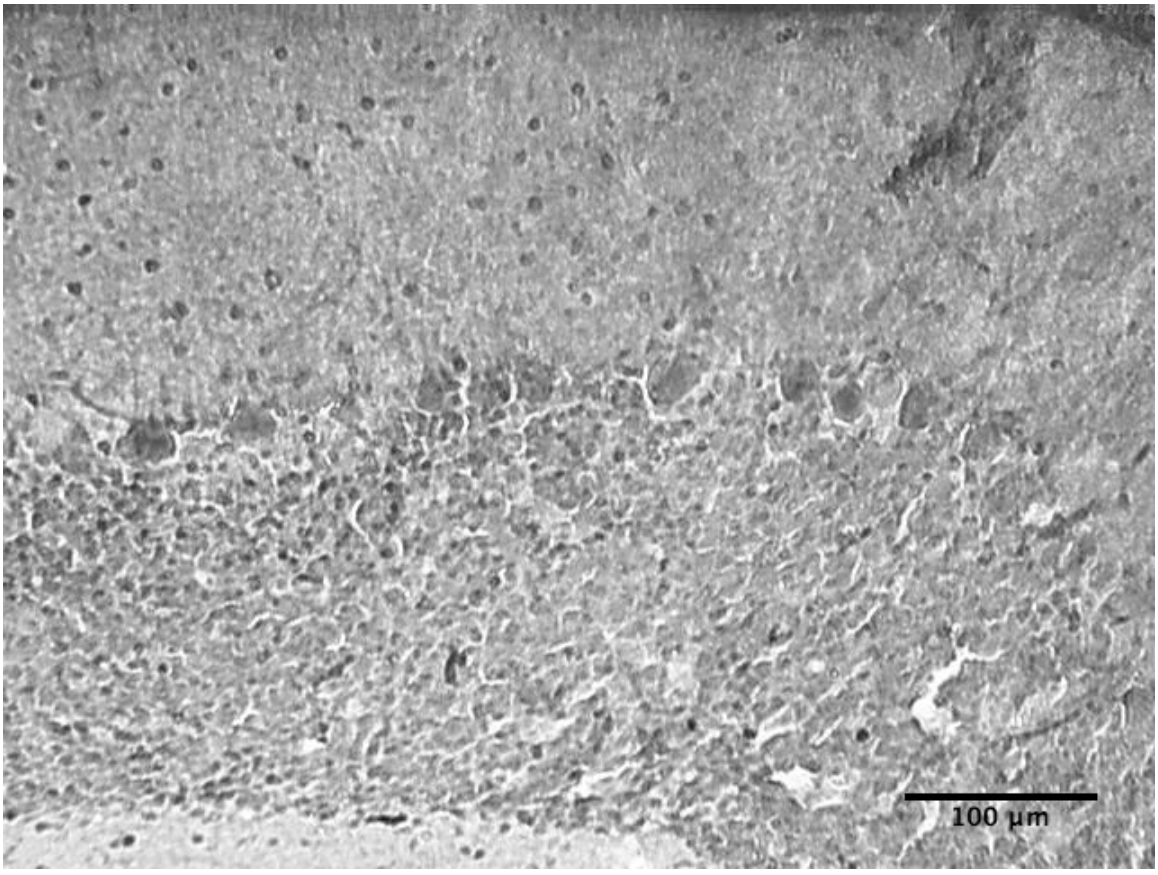
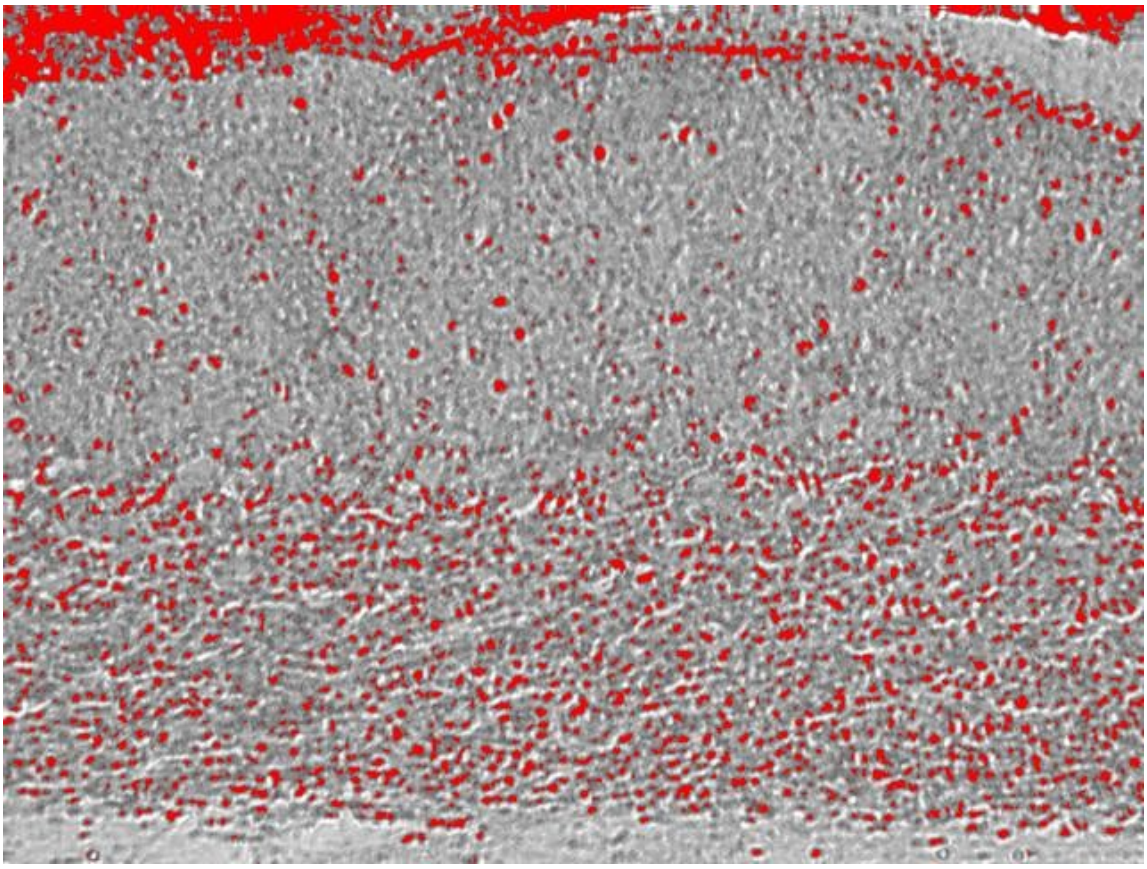


Figure 1: Photomicrograph of 8-OHdG immunohistochemical staining in sagittal cerebellar sections of 55 day old Normal (a) and Mutant (b) sHW rats magnified to 20X. Images were converted to an 8-bit grayscale for contrast, reducing the color information to black and white for thresholding. Results showed significantly higher staining in mutants compared to normal sHW rats, especially in the Purkinje cells. Calibration bar in

mutant panel refers to normal panel.

Figure 2. False color photomicrograph of 8-OHdG staining

(a) Normal



(b)Mutant

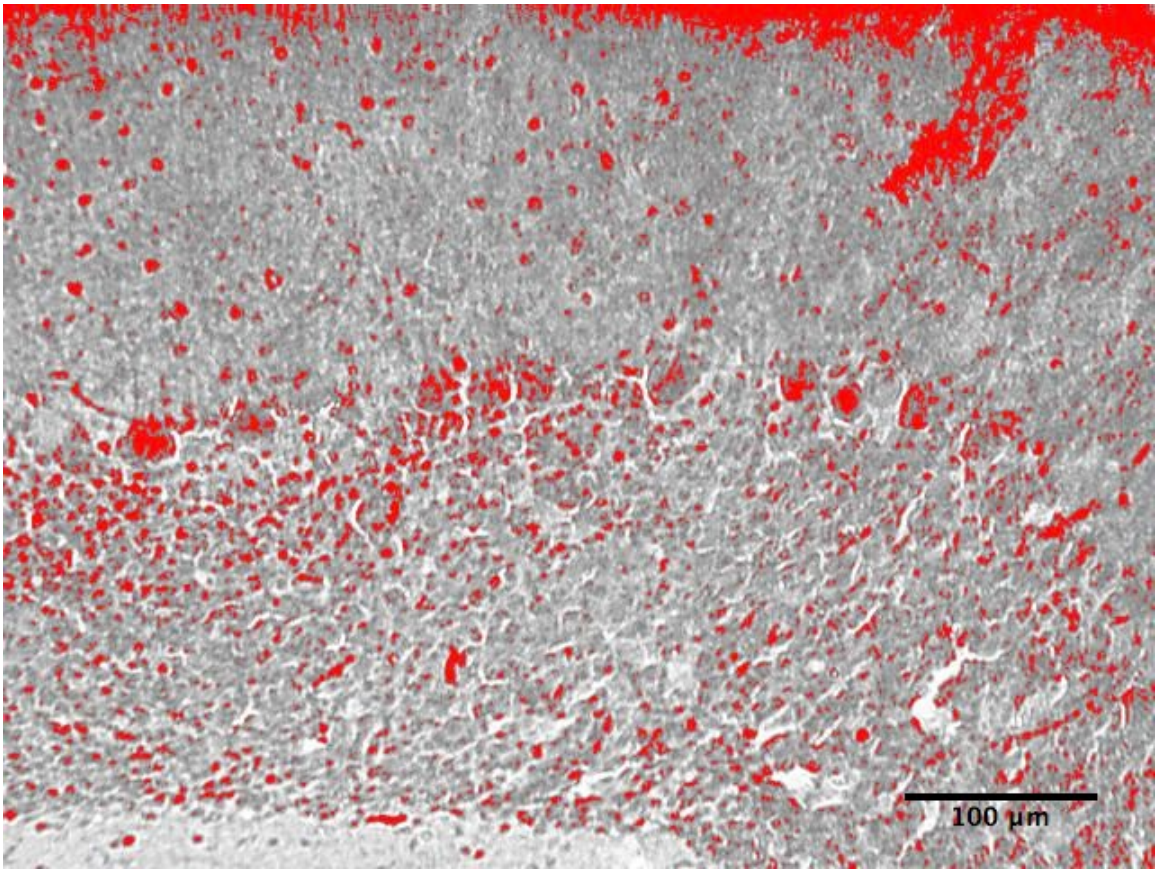


Figure 2: False color photomicrograph of 8-OHdG immunohistochemical staining in sagittal cerebellar sections of 55 day old Normal (a) and Mutant (b) sHW rats magnified to 20X. Images were adjusted to a threshold of 100 for staining intensity to remove background staining. Red color indicates antibody staining. Results showed significantly higher staining in mutants compared to normal sHW rats, especially in the Purkinje cells.

Calibration bar in mutant panel refers to normal panel.

Figure 3. Quantification of 8-OHdG staining

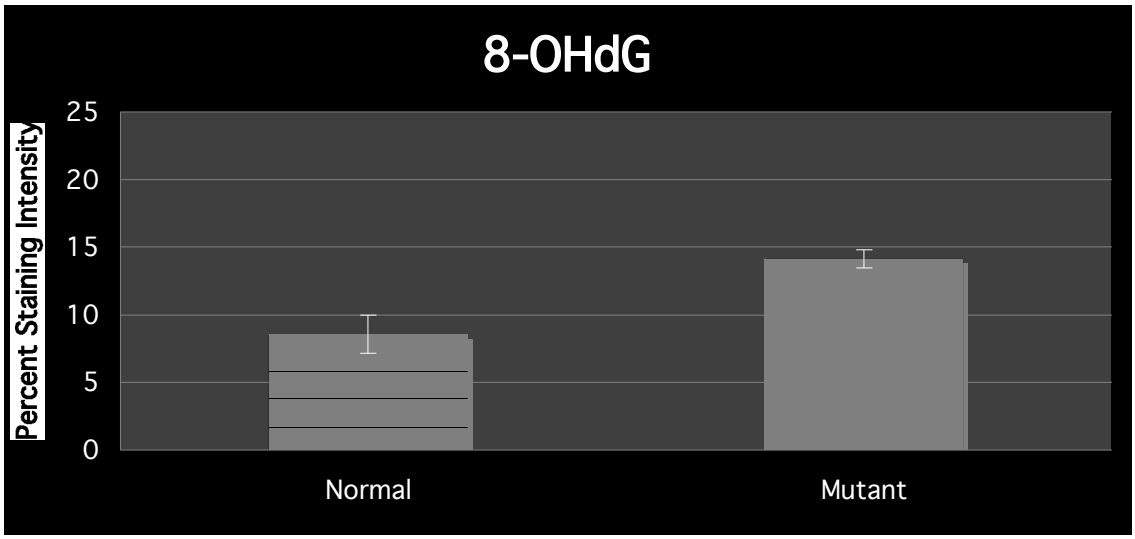


Figure 3: Quantification of 8-OHdG immunohistochemistry performed on normal and mutant sHW rats at 55 days old. Mutant sHW rats showed significantly higher amounts of 8-OHdG staining than normal animals ($t= 3.52$; $p<0.05$). Error bars represent the standard error.

Figure 4. NT staining

(a) Normal

(b) Mutant

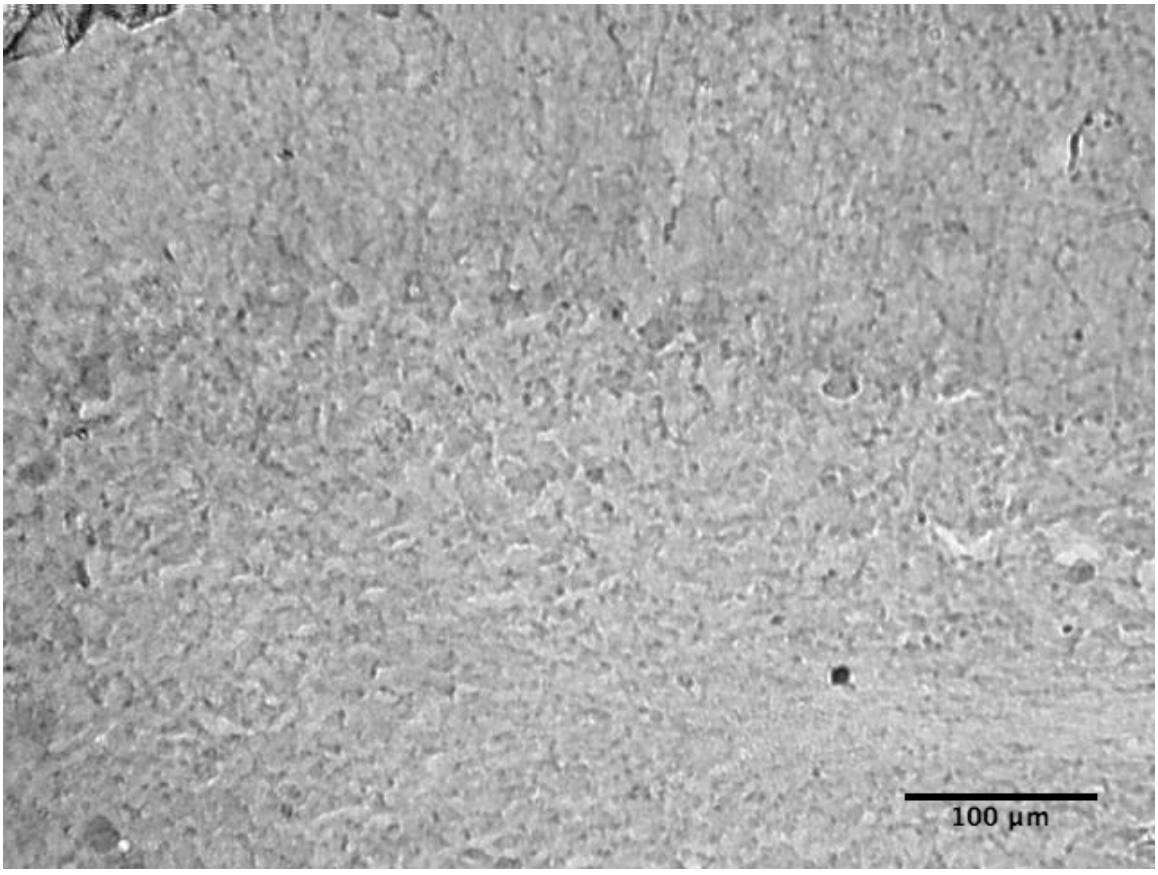
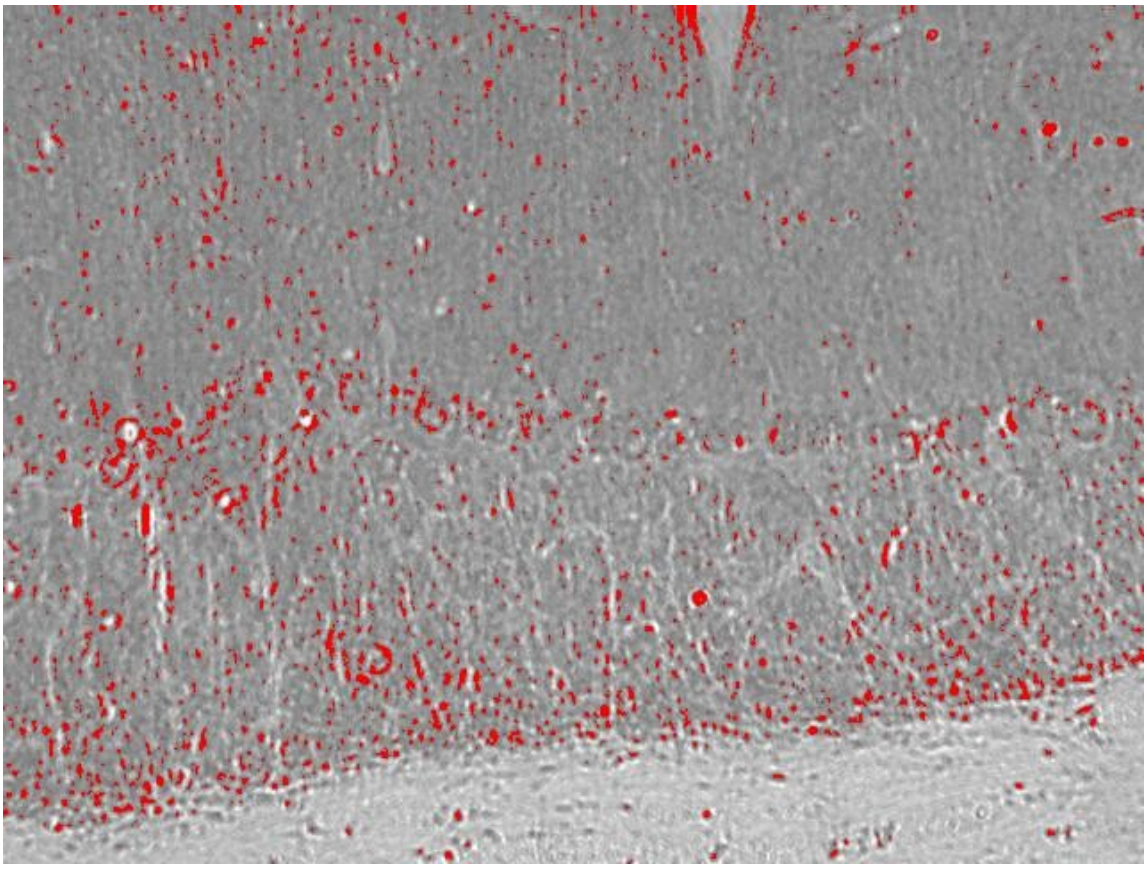


Figure 4: Photomicrograph of NT immunohistochemical staining in sagittal cerebellar sections of 55 day old Normal (a) and Mutant (b) sHW rats magnified to 20X. Images were converted to an 8-bit grayscale for contrast, reducing the color information to black and white for thresholding. Results showed no significant staining differences in either group. Calibration bar in mutant panel refers to normal panel.

Figure 5. False color photomicrograph of NT staining

(a) Normal NT



(b) Mutant NT

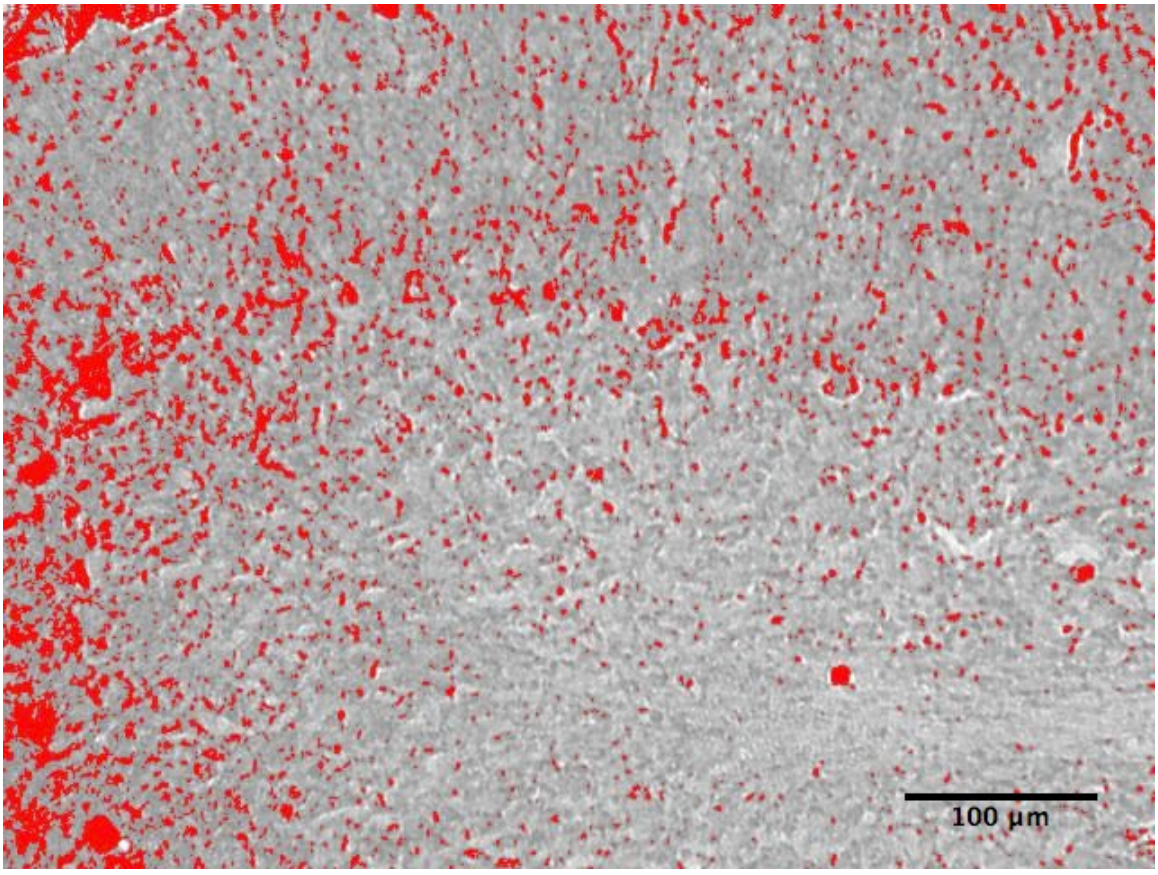


Figure 5: False color photomicrograph of NT immunohistochemical staining in sagittal cerebellar sections of 55 day old Normal (a) and Mutant (b) sHW rats magnified to 20X. Images were adjusted to a threshold of 120 for staining intensity to remove background staining. Red color indicates antibody staining. Results showed no significant staining differences in either group. Calibration bar in mutant panel refers to normal panel.

Figure 6. Quantification of NT staining

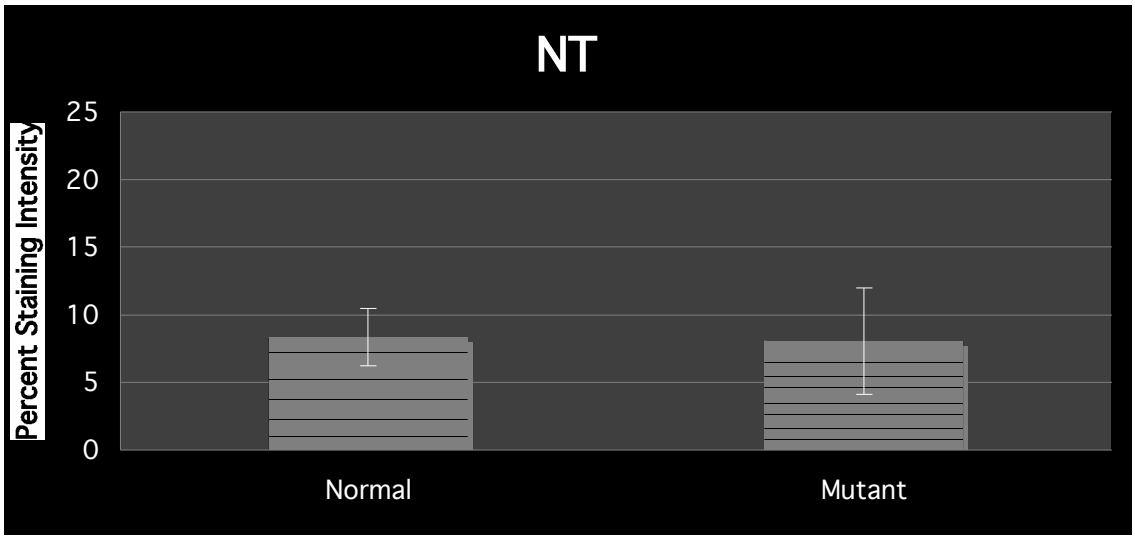


Figure 6: Quantification of NT immunohistochemistry performed on normal and mutant sHW rats at 55 days old. Results showed no significant differences ($t= 0.06$; $p>0.05$). Error bars represent the standard error.