2006

OXIDATIVE STRESS AND REDOX PROTEOMICS STUDIES IN MODELS OF NEURODEGENERATIVE DISORDERS: I. THE CANINE MODEL OF HUMAN AGING; II. INSIGHTS INTO SUCCESSFUL AGING; AND III. TRAUMATIC BRAIN INJURY

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ABSTRACT OF DISSERTATION

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ABSTRACT OF DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Arts and Sciences
at the University of Kentucky

By

Wycliffe Omondi Opii

Lexington, Kentucky

Director: Dr. D. Allan Butterfield, the Alumni Professor of Chemistry

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The studies presented in this dissertation were conducted with the objective of gaining greater understanding into the mechanisms of successful aging, the role of mitochondria dysfunction in traumatic brain injury, and also on the mechanisms of improved learning and cognitive function in the aging.

Aging is usually characterized by impairments in physiological functions increasing its susceptibility to dementia and neurodegenerative disorders. In this dissertation, the mechanisms of dementia-free aging were investigated. The use of an antioxidant fortified diet and a program of behavioral enrichment in the canine model of human aging was shown to result in a significant decrease in the levels of oxidative stress. A proteomic analysis of these brains also demonstrated a significant decrease in the oxidative modification of key brain proteins and an increase in the expression levels of other key brain proteins associated with energy metabolism and antioxidant systems which correlated with improved learning and memory.

We show that following TBI key mitochondrial-related proteins undergo extensive oxidative modification, possibly contributing to the severe loss of mitochondrial energetics and neuronal cell death previously observed in experimental TBI.

Taken together, these findings support the role of oxidative stress in the pathophysiology of aging and age-related neurodegenerative disorders and in CNS injury. These studies also show that antioxidants and a program of behavioral enrichment provide protection against oxidative stress-mediated cognitive impairments.
KEYWORDS: Oxidative stress, Canine, Successful aging, Traumatic brain injury, proteomics

Wycliffe.O.Opii

12/05/06

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Date

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To My Dear Grandmother

The late Nereah Adala Opii

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER ONE

Introduction and Research Aims

The studies presented in this dissertation research were geared towards elucidating biochemical mechanisms associated with successful aging, improved learning and memory following treatment with an antioxidant fortified diet and a program of behavioral enrichment and also in gaining insights into mechanisms of protein oxidation in traumatic brain injury. Aging is defined as the gradual alteration in structure and function that occurs over time, eventually leading to an increased probability of death not associated with disease or trauma (Ashok and Ali, 1999; Gonos, 2000). Aging, and age-related disorders such as Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), and amyotrophic lateral sclerosis (ALS) are usually accompanied by a significant increase in oxidative stress with an eventual decline in memory and cognitive function (Butterfield and Kanski, 2001; Poon et al., 2004a). Though various mechanisms for neurodegeneration have been put forth, the progressive accumulation of oxidative damage has been thought to play a significant role in the development or accumulation of neuropathology typically observed in these neurodegenerative disorders.

The significant increase in oxidative stress and generation of free radicals observed in these neurodegenerative disorders are mediated through a wide range of mechanisms. Mitochondria play a key role through the generation of superoxide anion $O_2^{\cdot -}$, which undergoes various reactions leading to the production of key ROS such as hydroxyl radical (OH·), peroxynitrite ($OONO^-$), and hydrogen peroxide (H_2O_2) among many others (Melov, 2000). For AD, our laboratory has proposed that the significant

increase in the levels of oxidative stress are mediated by amyloid-beta peptide (Aβ 1-42) through methionine 35 (Met-35)-related chemistry (Varadarajan et al., 2000; Varadarajan et al., 2001; Butterfield and Kanski, 2002). These ROS/RNS have been shown to mediate oxidative modification of proteins, lipids and DNA as observed in AD brain when compared to age-matched controls. Oxidative attack to peptides and proteins usually leads to alteration in protein structure (Subramaniam et al., 1997), and a possible loss in function contributing to the pernicious effects on cellular functions (Levine and Stadtman, 2001; Halliwell, 2006).

The mechanisms involved in being able to achieve extended longevity while still maintaining cognitive function are still unknown. However, it has been proposed that the mechanism of successful aging is a product of an interaction between genetic, environmental and lifestyle factors (Petropoulou et al., 2000; Perls et al., 2002a; Perls et al., 2002b; Snowdon, 2003). Significantly, until the present dissertation research, the protein profiles in these groups of individuals have not yet been established. As a result, this dissertation research provides a proteomic expressional profile for the non-demented nonagenarian compared to demented age-match controls, providing possible biochemical pathways towards successful aging.

With an increase in age-related oxidative stress and cognitive dysfunction as reported for neurodegenerative disorders such as AD and models thereof, the use of antioxidants, antioxidant-related compounds and exercise programs have been found to provide beneficial and protective effects against these age-related deficits (Mates et al., 1999; Bickford et al., 2000; Milgram et al., 2002b; Drake et al., 2003b; Farr et al., 2003; Adlard et al., 2005b). These beneficial effects include a reduction in the levels of

oxidative stress, improvements in learning and memory and a delay in the development of age-related cognitive deficits. However, the mechanisms that underlie these effects in the aging brain are still not well established. It has been reported that there is a significant improvement in cognitive function in the canine model of human aging undergoing a program of behavioral enrichment and having a diet fortified with a broad range of antioxidants (Milgram et al., 2004; Milgram et al., 2005). We propose in this dissertation research that this improvement in cognitive function observed is due to a decrease in the level of oxidative stress, up-regulation of key antioxidant enzymes and protection of key brain proteins from oxidative damage. Therefore, this dissertation provides possible mechanism for the improvement in learning and memory following a behavioral enrichment program and an antioxidant fortified diet in a canine model of human aging. This model is highly relevant to AD since Aβ (1-42) in both beagles and humans have the same amino acid sequence.

One of the risk factors associated with developing age-related defects in learning and memory as observed in AD is traumatic brain injury (TBI) (Jellinger, 2004b). TBI is associated with a significant loss in mitochondrial bioenergetics, increased oxidative stress and neuronal cell death mediated by necrosis and apoptosis (Sullivan et al., 2002; Lifshitz et al., 2004a). Despite the well-established notion of increased oxidative stress and decline in mitochondrial function reported in TBI, the identities of specific proteins undergoing oxidative modification following TBI are yet to be established. As a result, one central goal of this dissertation research is to identify specific mitochondria-related proteins that have been oxidatively altered following TBI.

In Summary, this dissertation addresses the following questions:

1. Which proteins are protected following the combined treatment of an antioxidant fortified diet and a program of behavioral enrichment in the brains of the canine (beagle) model of human aging? How do these proteins contribute to the improved learning and memory in the aging canine model?
2. What proteins are differentially expressed in the brains of the canine model of human aging after treatment with an antioxidant-fortified diet and a program behavioral enrichment?
3. Which brain mitochondrial-related proteins are oxidatively modified following experimental traumatic brain injury (TBI)?
4. What brain proteins play a role in the mechanism of successful aging as seen the non-demented nonagenarians?

CHAPTER TWO

Background

2.1 Neuronal structure and function

The central nervous system (CNS) represents the largest part of the nervous system. The CNS is composed of the brain and the spinal cord, which is organized in a network of billions of interconnected components, with the major class of cells being neurons. In addition, this network is composed of glia and astrocytes that provide support to the neurons. Neurons in conjunction with other specialized cells like the astrocytes or microglia mediate the specialized function of the brain which involves the reception, processing and transmission of information as well as neuroprotection.

The anatomy of nerve cells as shown in Fig 2.1 is composed of the cell body also known as the soma or perikaryon, cellular extension process known as dendrites, and a long tube-like projection known as the axon. Just like any other cellular structure, the neuron consists of a cell membrane and organelles that perform specialized functions. These include; the nucleus, mitochondria, Golgi apparatus, lysosomes, endoplasmic reticulum and ribosomes among others. Due to their specialization, neurons can be functionally classified as excitatory, inhibitory and modulatory. As already mentioned, the main function of the neuron is the reception and transmission of information in the form of nerve impulses. Nerve impulses are usually transmitted through electrochemical interactions. Briefly, at rest, the intracellular potassium ions and extracellular sodium ions generate and maintain a neuronal electrical potential of approximately -70 mV. This movement of potassium and sodium ions is facilitated by membrane bound sodium-potassium pumps. At rest, the cell membrane is approximately 100 times more permeable

to potassium ions compared to sodium. In the presence of a stimulus, voltage-gated sodium channels are opened, allowing sodium ions to diffuse into the cell hence making the cell more positive, i.e., depolarizing the cell to about 40+ mV. Once this has stabilized, the sodium channels close and the voltage -dependent potassium channels open, allowing potassium to influx into the cell. This large outward current of potassium ions hyperpolarizes the neuron. The voltage-sensitive potassium channels now close and the continual movement of potassium through potassium leak channels again dominates the membrane potential. Sodium-potassium pumps continue to pump sodium ions out and potassium ions in, preventing any long-term loss of the ion gradients. The resting potential of -70 mV is then re-established and the neuron is said to be repolarized. Fig 2.2 provides a summary of the action potential process.

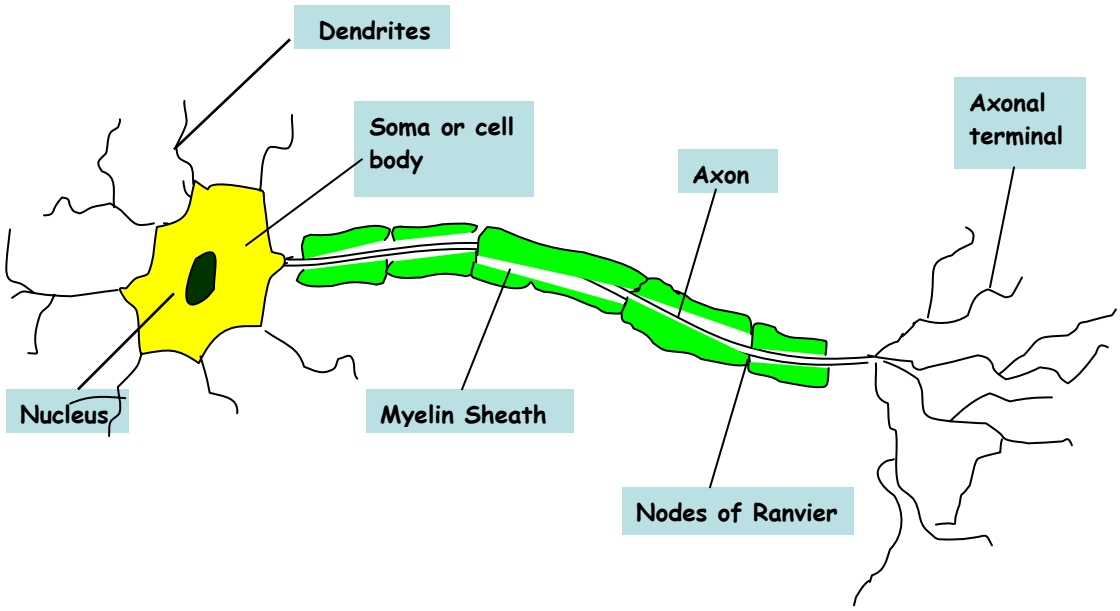


Figure 2.1 Schematic representation of the neuron and its various components.

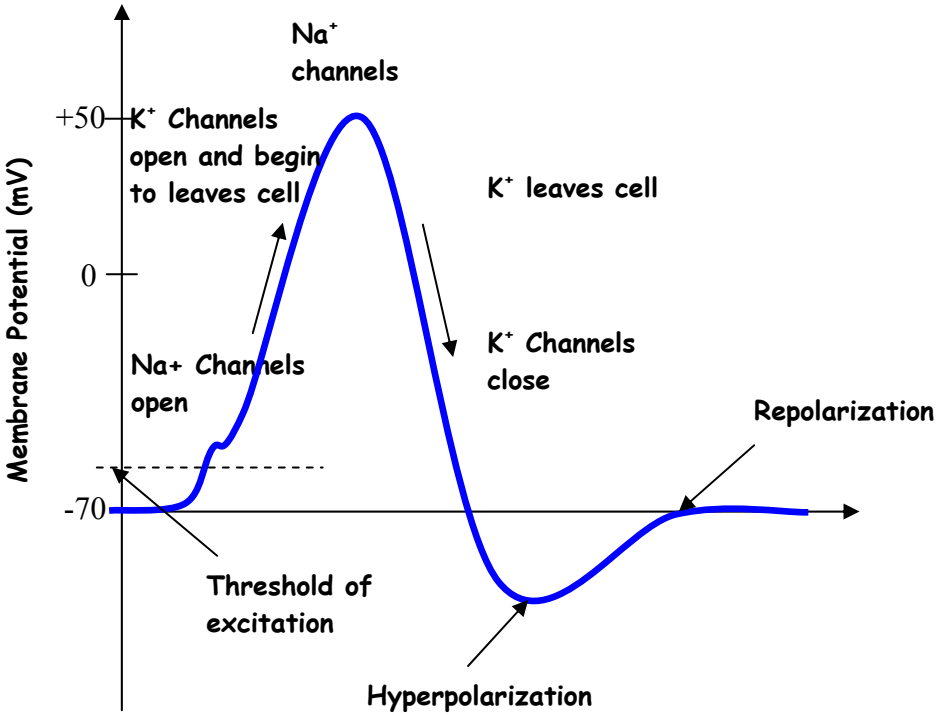


Figure 2.2 Summary of the action potential.

2.2 Oxidative stress

2.2.1 Overview

Under normal physiological conditions, there exists a balance between the amounts of oxidants and antioxidant defenses present in the body. However, as a result of disease, trauma, or environmental factors, there is a shift towards an increase in the production of pro-oxidants or a decline in the levels of antioxidants. Consequently, the rate at which reactive species are generated exceeds the rate at which endogenous antioxidant defenses can scavenge oxidants. This resulting imbalance is what is defined as oxidative stress (Halliwell, 2006) (Fig. 2.3). It has been established that ROS/RNS play a significant role in the normal aging process as well as in various age-related diseases neurodegenerative disorders (Ames et al., 1993a; Markesbery, 1997; Lewen et al., 2000; Poon et al., 2004a; Dalle-Donne et al., 2006). An increase in the levels of oxidative stress has been reported in a number of conditions like: Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), amyotrophic lateral sclerosis (ALS) among many others (Butterfield and Kanski, 2001).

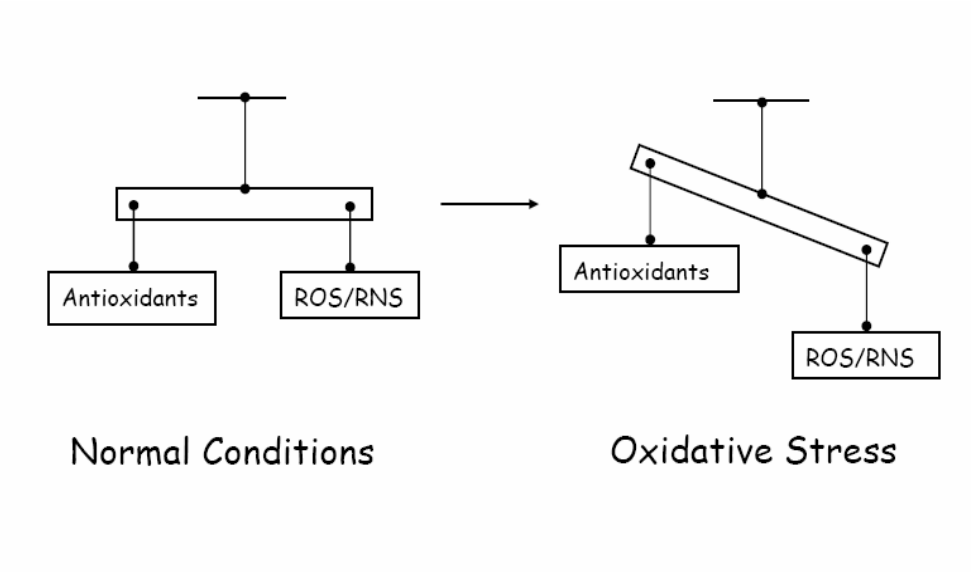


Figure 2.3 A representation of normal vs. oxidative stress conditions.

2.2.2 Free radical production

2.2.2.1 Overview

Free radicals are defined as low molecular weight molecules that contain at least one unpaired electron and have previously been shown to mediate the pathophysiology of most neurodegenerative disorders (Nordberg and Arner, 2001; Droge, 2002). These radical species can be dived into primary radical species of which some examples are OH·, $O_2^{\cdot -}$ CO_2·- NO· and non-radical species are H_2O_2, N_2O_2, O_2, NO_2, HO_2, and HOCl among many others (Halliwell, 2006).

2.2.2.2 Reactive oxygen species (ROS)

ROS are defined as a group of highly reactive molecules usually derived from ground-state oxygen (O_2) (Halliwell, 1996; Fridovich, 1999; Halliwell, 1999; Betteridge, 2000). Some common examples include superoxide anion ($O_2^{\cdot -}$), hydroxyl radical (OH·), and hydrogen peroxide H_2O_2 among many others. This single most important source of ROS is the mitochondria. As a by-product of energy metabolism during normal cellular respiration, there is a 1-2% leakage of electrons from the mitochondria leading to the partial reduction of oxygen (reaction 1) to form a highly toxic free radical known as superoxide (Turrens, 2003). A detailed discussion on mitochondria's role in oxidative stress is provided below (Section 2.4.5). Superoxide dismutase catalyzes the dismutation of superoxide to hydrogen peroxide and water. However, in the presence of transition metal ions in the reduced state, like Fe^{2+} or Cu^{+}, H_2O_2 is further converted to hydroxyl radical OH· (reaction 2). Superoxide can also be produced from the activity of enzymes such as xanthine oxidase and NADPH oxidase. Fig 2.4 below provides a summary of the

pathways to the generation of the ROS mentioned above. A detail discussion of these pathways is provided below in Section (2.4 and 2.7.2).

Reaction 1: $O_2 + e^- \longrightarrow O_2\cdot^-$

Reaction 2: $H_2O_2 + Fe^{2+} \longrightarrow HO\cdot + OH^- + Fe^{3+}$

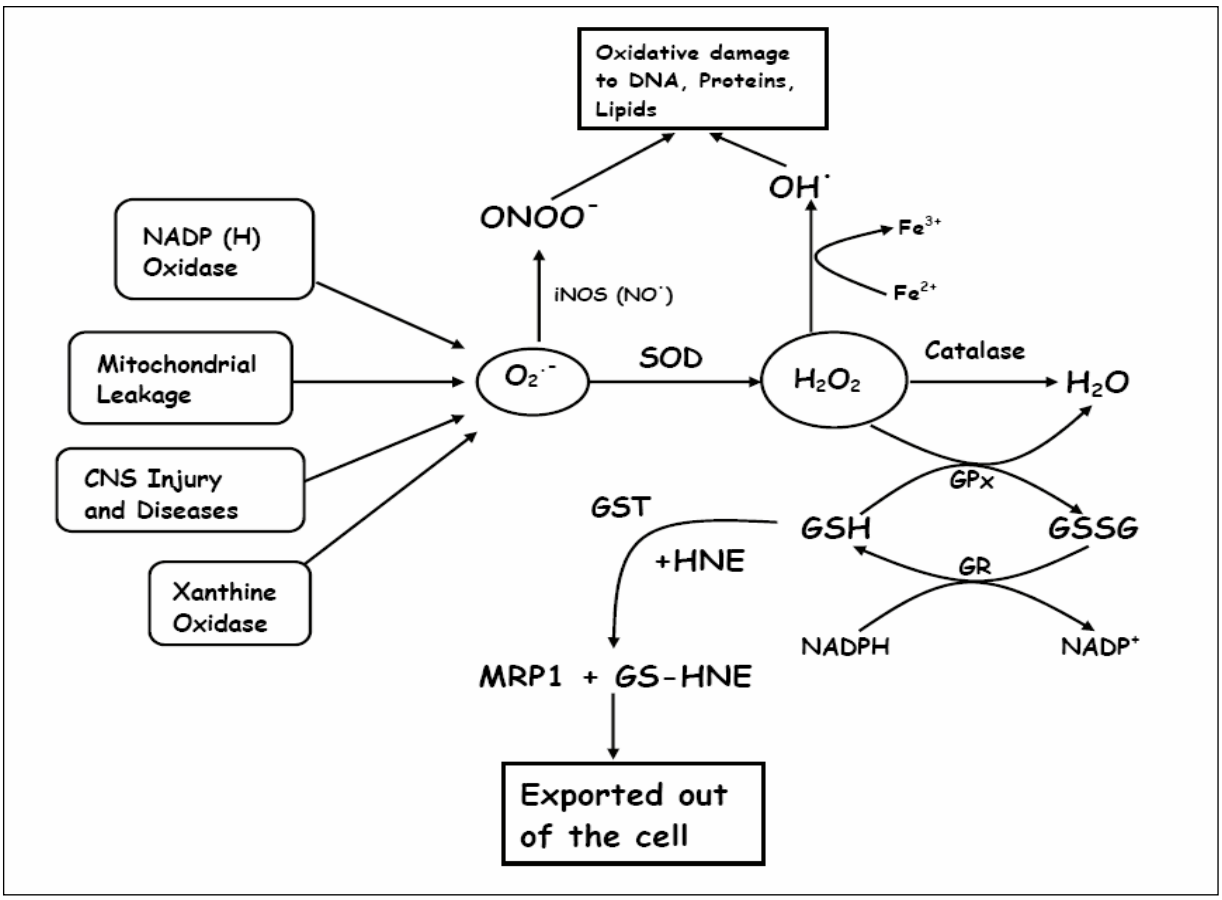


Figure 2.4 Pathways leading to the production of ROS/RNS and antioxidant systems.

2.2.2.3 Reactive nitrogen species (RNS)

RNS represent a group of reactive nitrogen containing compounds with the most common examples being NO, peroxynitrite ($^{-}$OONO), and nitrogen dioxide (NO_2). Nitric oxide radical is produced from the activation of the enzyme nitric oxide synthase (NOS). There are three isoforms of NOS, i.e., inducible NOS (iNOS), endothelial NOS (eNOS), and neuronal NOS (nNOS) (Calabrese et al., 2000). Nitric oxide (NO) in the presence of superoxide reacts to form peroxynitrite reaction 3. Nitric oxide regulates various cellular processes that include vasodilatation, modulation of signaling cascades, and immune responses among many others. On the other hand, it has also been shown that nitric oxide is involved in tissue injury and the progression of various inflammatory pathways in neurodegenerative disorders as reviewed in (Halliwell, 2006). A detailed discussion on the reactions of OONO is provided in Section (2.2.1.3.2).

Reaction 3: $NO + O_2^{\cdot -} \longrightarrow {}^{-}OONO$

2.2.3 Targets of Reactive oxygen species

2.2.3.1 Protein oxidation

Protein oxidation is an important post-translational modification observed in a number of neurodegenerative disorders. Proteins are highly sensitive to oxidative modifications by ROS/RNS. Oxidative damage to proteins can have a wide range of downstream functional consequences. These can include inhibition of enzymatic activity, increased susceptibility to degradation among many others. In AD and other neurodegenerative disorders, there is a significant increase in the levels of protein oxidation (Aksenova et al., 1998; Levine and Stadtman, 2001; Butterfield and Lauderback, 2002; Halliwell, 2006). It should be noted, however, that the progressive accumulation of oxidative damage is a combination of multiple factors. These include; a) rate of formation of ROS/RNS; b) sensitivity of proteins to these ROS/RNS; c) availability of antioxidant defense mechanisms; and d) the effectiveness of the mechanism involve in the repair or elimination of damaged proteins (Stadtman and Berlett, 1997; Stadtman and Levine, 2003). Some examples of oxidative modifications are discussed here.

2.2.3.1.1 Protein carbonyls

The levels of protein carbonyls can index protein oxidation. The levels of protein carbonyls have been shown to be a good marker of oxidative stress (Levine et al., 1990). There is a significant increase in the levels of protein carbonyls in aging and in age-related neurodegenerative disorders (Hensley et al., 1996). Protein carbonyl formation can be introduced into proteins through three major mechanisms (Levine et al., 1990;

Butterfield and Stadtman, 1997; Stadtman and Levine, 2003). These are I) Oxidation of specific amino acids present in protein residues (Fig 2.5) II); β-scission of peptide backbone (Fig 2.6); and III); Covalent modification of amino acids by reactive aldehydes like HNE and acrolein (Fig 2.7). It can therefore be seen that protein carbonyl formation is orders of magnitude greater than other oxidative modifications making it a widely used marker for protein oxidation. The levels of protein carbonyls can be analyzed immunochemically. The details of this procedure will be discussed in Chapter 3.

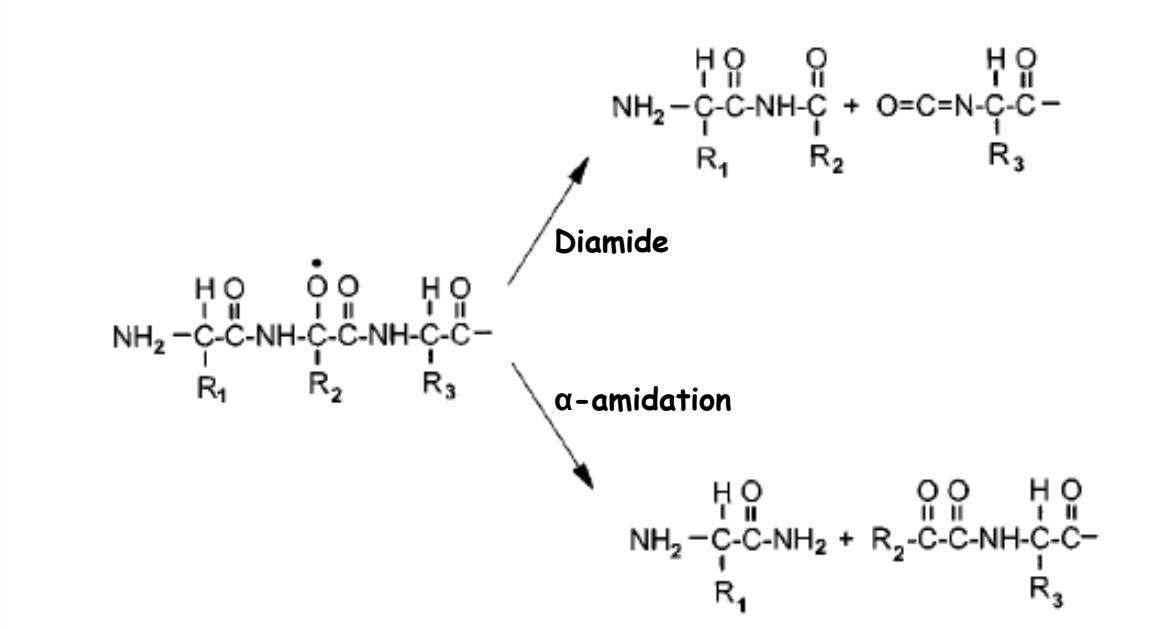


Figure 2.5 Peptide bond cleavage and formation of protein carbonyls through the diamide and α-amidation pathways.

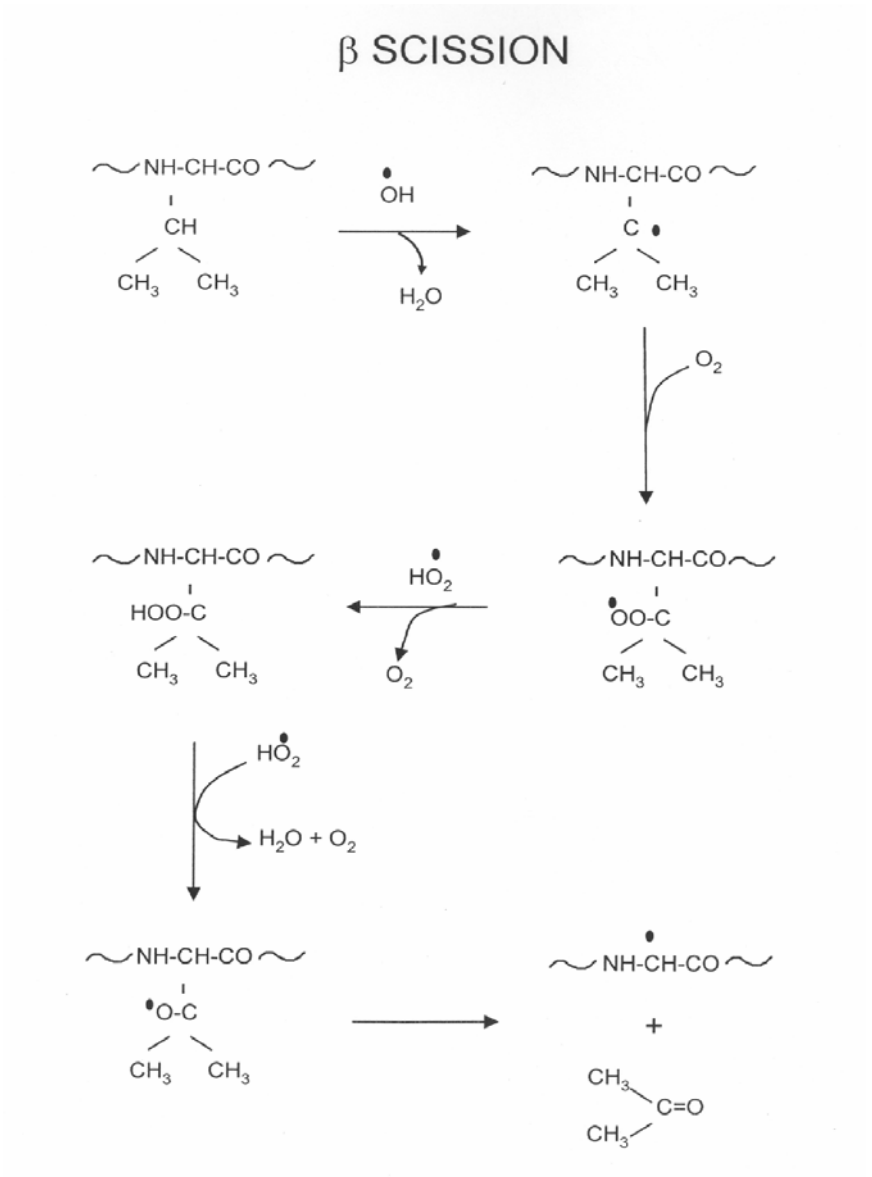


Figure 2.6 Mechanism of protein carbonyl formation from β-scission.

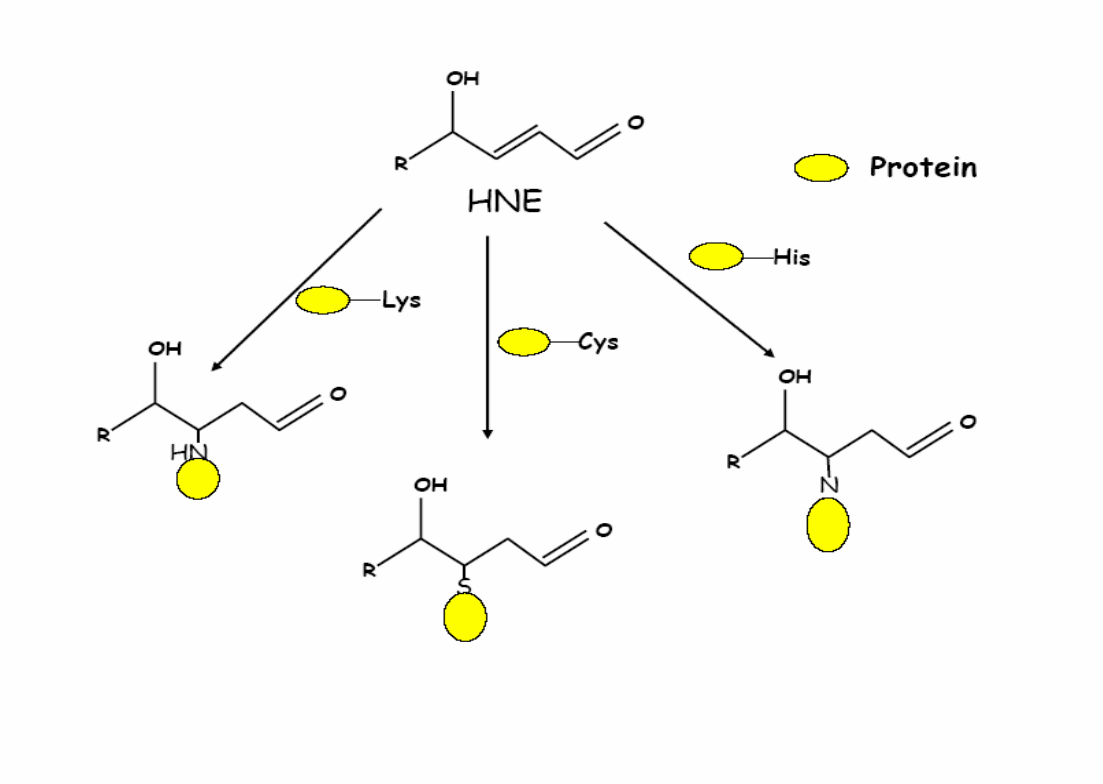


Figure 2.7. Covalent modification of key amino acids by HNE.

2.2.3.1.2 3-Nitrotyrosine (3NT)

Protein oxidation also can be indexed by the levels of 3-nitrotyrosine (3NT). As mentioned previously, the activity of nitric oxide synthase leads to the production of nitric oxide. This is usually through the catalytic conversion of L-arginine to citrulline (Calabrese et al., 2000). Nitric oxide in itself is not a strong oxidant, but the diffusion rate limited reaction with superoxide anion leads to the production of a highly reactive species known as peroxynitrite $ONOO^-$ (Reaction 3). This reaction leading to the formation of peroxynitrite is regulated by the enzymatic activities of NOS and SOD, since they are the two enzymes that produce the needed reactants. Peroxynitrite is highly reactive with a half-life of less than one second and it can exist in two forms; either as the peroxynitrite anion ($ONOO^-$), or as the protonated peroxynitrous acid (ONOOH) (reaction 4). This protonated form can undergo hemolytic cleavage to form the toxic species hydroxyl and nitrite radicals (reaction 5). It should be noted however, that this reaction proceeds via carbon dioxide as shown in Fig 2.8a

Reaction 4: $ONOO^- + H^+ \rightleftharpoons HOONO$

Reaction 5: $HOONO \longrightarrow {}^{\cdot}OH + NO_2{}^{\cdot}$

Under physiological conditions it has been proposed that peroxynitrite reacts with carbon dioxide with the resulting reactive intermediates being responsible for the nitration of proteins (Fig 2.8a) (Denicola et al., 1996). The initial reaction of peroxynitrite and carbon dioxide produces an intermediate known as nitrosoperoxycarbonate (Lymar and Hurst, 1996; Lymar et al., 1996). This species then undergoes rearrangement, forming nitrocarbonate. The homolysis of nitrocarbonate can then generate a carbonate anion and a nitrite radical, which can then react with tyrosine residues on proteins leading

to the formation of 3-nitrotyrosine (3NT) Fig 2.8b). It should be noted however, that in certain cases (like the reaction of peroxynitrite with glutathione) the presence of carbon dioxide inhibits peroxynitrite reaction (Zhang et al., 1997). The levels of 3NT can be detected immunochemically, and it has been shown that in aging and age-related disorders, there is a relative increase in the levels of 3NT, further confirming the notion of increased oxidative stress in these neurodegenerative disorders (Schulz et al., 1997). Though not discussed, it should be noted that there are other possible nitration end products that can be produced from the reactions mentioned above. Some examples are dityrosine and tyrosine peroxide as shown in Fig 2.8b (Ducrocq et al., 1999).

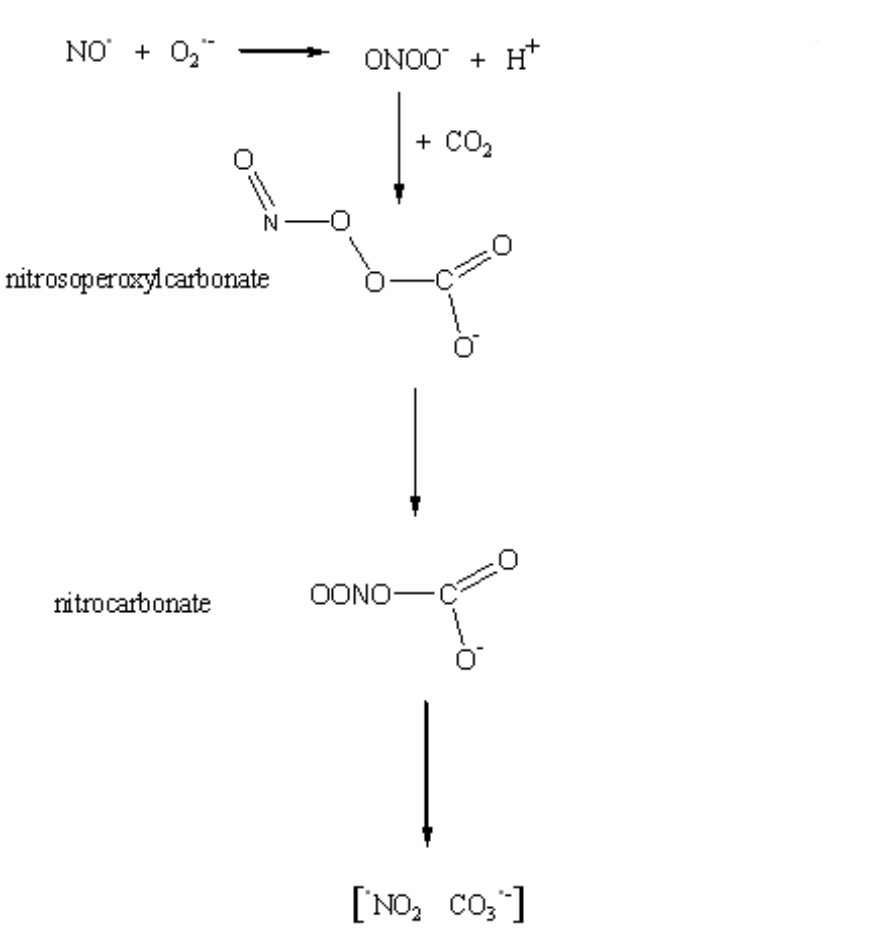


Figure 2.8a: Formation of peroxynitrite and its reaction in the presence of carbon dioxide.

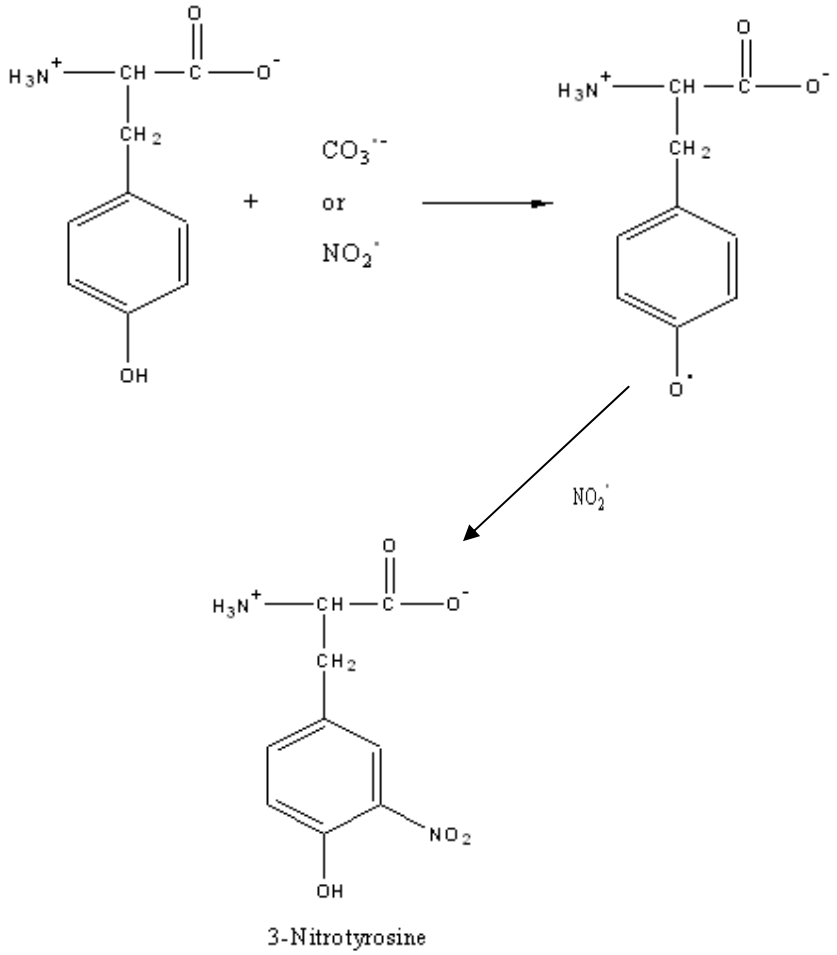


Figure 2.8b: Mechanism of 3NT formation.

2.2.3.2 Lipid peroxidation

There is accumulating evidence implicating lipid peroxidation in the aging process and also in age-related neurodegenerative disorders like AD among other diseases (Butterfield and Lauderback, 2002; Dei et al., 2002). Lipids are particularly vulnerable to oxidative attack due to the availability of unsaturated fatty acid β-chains and the solubility of paramagnetic oxygen in the bilayer. Lipid peroxidation proceeds via a free-radical mediated chain reaction that is initiated by the abstraction of labile hydrogen by the radical forming a carbon centered radical (reaction 6). The lipid radical then reacts rapidly with paramagnetic oxygen forming a peroxyl radical (reaction 7). This peroxyl radical reacts with nearby lipids, abstracting another nearby allylic hydrogen atom, forming lipid hydroperoxides and another carbon-centered radical. If not impeded the chain reaction propagates (reaction 8). Lipid peroxidation is terminated by the quenching reaction of two peroxyl radicals forming oxygen and a nonradical species (Reaction 9). The presence of vitamin E (α- tocopherol), an endogenous lipid soluble antioxidant can also terminate this chain reaction as a chain-breaking antioxidant. The lipid peroxyl radical can abstract a labile hydrogen from α- tocopherol (TOH) forming a tocopheroxyl radical TO·. This radical can be recycled back to TOH by ascorbic acid (vitamin C). Hence, the use of both vitamin E and C as supplements against oxidative stress-mediate disorders is often employed. This will be discussed in detail in Section (2.7.3).

Reaction 6: $LH + X\cdot \longrightarrow L\cdot + XH$

Reaction 7: $L\cdot + O_2 \longrightarrow LOO\cdot$

Reaction 8: $LOO\cdot + LH \longrightarrow LOOH + L\cdot$

Reaction 9: $LOO^{\cdot} + LOO^{\cdot} \longrightarrow$ Nonradical + O_2

2.2.3.2.1 Membrane composition

Biological cellular membranes occur in several locations including the plasma membrane. Biological membranes are selectively permeable membranes composed of lipid bilayers, proteins, cholesterol and carbohydrates (Fig 2.9). The fluid mosaic model of lipid bilayer demonstrates that the cell membrane is composed of two "leaflets". Each leaflet is composed of glycerol-derived phospholipids that contain a polar phosphate head and hydrophobic chains. The leaflets are oriented in such away that one polar phosphate head is towards the cytosol while the other is towards the extracellular milieu. The brain is particularly rich in peroxidizable polyunsaturated fatty acids (PUFA). Some common examples are arachidonic (AA, 20:4n-6) (Fig 2.10), and decosahexaenoic acid (DCH, 22:6n-6) (Fig 2.11) among many others. In neurodegenerative disorders like AD, there is a decline in the levels of PUFA, possibly due to their release from the cell membrane by the actions of phospholipase A_2 (Pratico et al., 1998). These free phospholipids in turn can undergo either enzymatic or non-enzymatic oxidation leading to the generation of reactive aldehydes, prostaglandin isomers and other key biomolecules that play a role in the oxidative stress pathology of neurodegenerative disorders. Figure 2.12 shows the free radical-mediated formation of 4-hydroxy-2-trans-nonenal (HNE) from arachidonic acid.

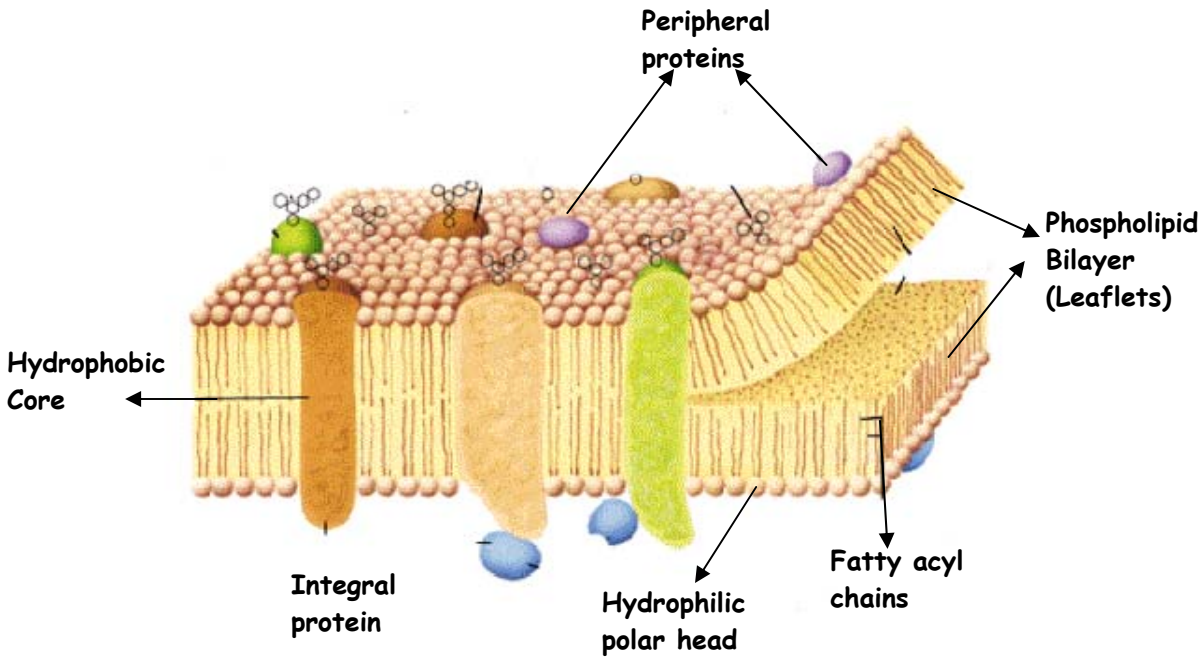


Figure 2.9 Representation of the membrane bilayer.

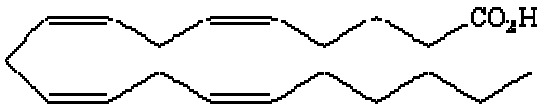


Figure 2.10 Arachidonic acid (AA, 20:4n-6)

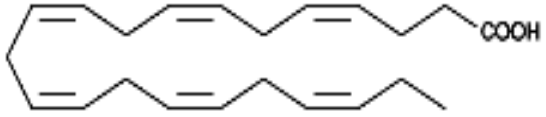


Figure 2.11 Decosahexaenoic acid (DCH, 22:6n-6)

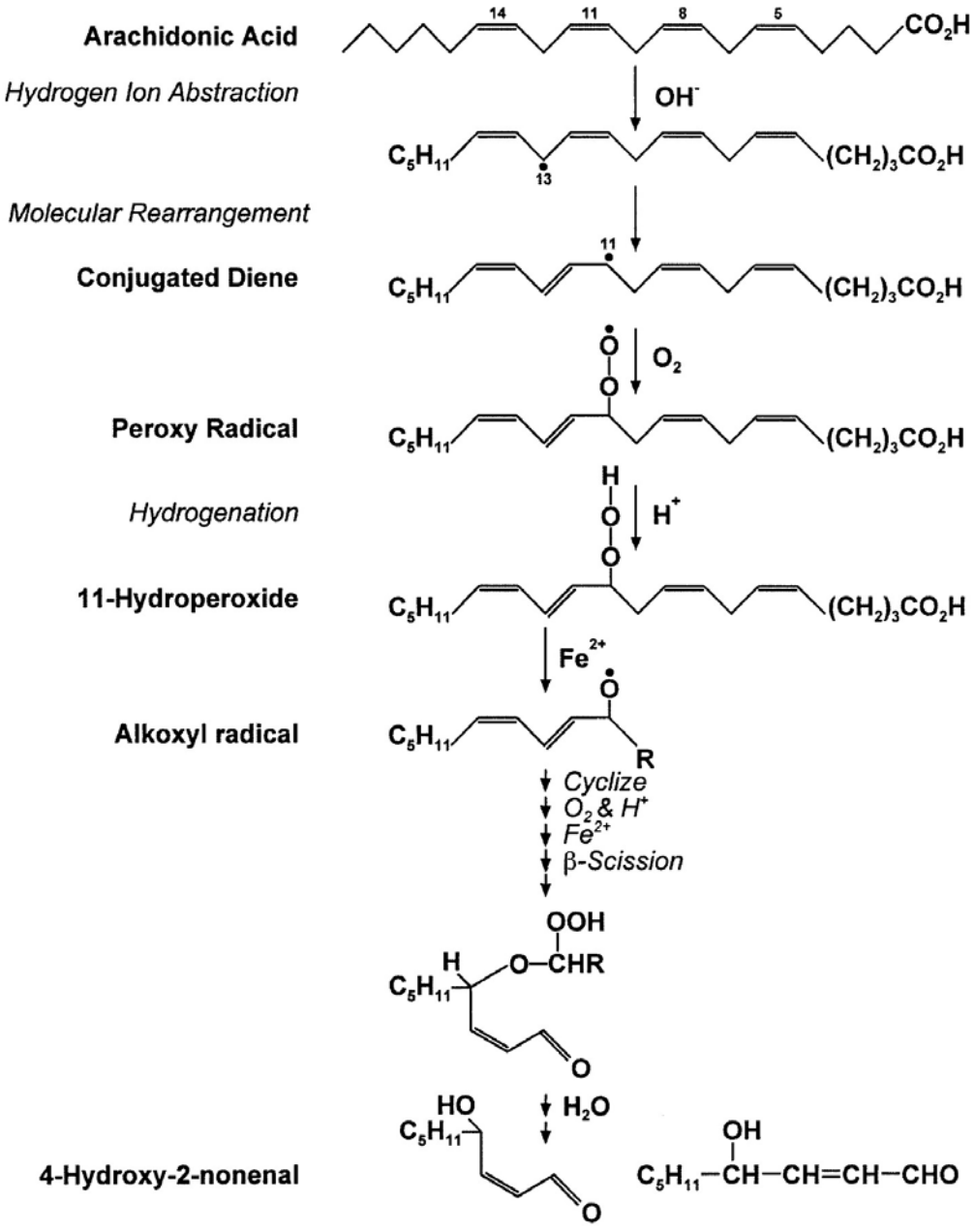


Figure 2.12: Free radical formation of HNE from arachidonic acid.

2.2.3.2.2 Reactive aldehydes

Reactive aldehydes are considered to be the end products of lipid peroxidation. Free radical attack on PUFA leads to the formation of reactive alkenals with varying carbon chain lengths. The most common α, β-unsaturated aldehydes observed in neurodegenerative disorders include: 4-hydroxy-2-nonenal (HNE), acrolein (2-propenal), and malondialdehyde (MDA). Relative to free radicals, these reactive aldehydes have longer half-lives that allow for their diffusion to distant sites from their site of formation. These alkenals can readily react via Michael addition with proteins, leading to the formation of stable covalent protein-bound adducts with cysteine, lysine, and histidine residues (Butterfield and Stadtman, 1997; Subramaniam et al., 1997). These modifications can lead to conformational and structural changes in proteins thereby leading to loss of function and cytopathologic effects observed during oxidative stress. These reactive aldehydes in themselves or as thiobabituric acid species have been used as excellent biomarkers of lipid peroxidation in various neurodegenerative disorders. There is an elevation in the concentrations of free HNE in the ventricular cerebral spinal fluid CSF of AD patient, while the levels of protein-bound HNE are also increased (Lovell et al., 1995; Markesbery and Lovell, 1998; Lauderback et al., 2001). HNE bound to proteins is also elevated in brain of subjects with mild cognitive impairments (Butterfield et al., 2006b) usually the milder form of AD. Acrolein is the most reactive of this group of aldehydes. Acrolein adducts have been observed in the NFT of AD patients compared to control (Lovell et al., 2001). Acrolein has also been shown to bind to NADH-dependent mitochondrial enzymes, pyruvate dehydrogenase and α-ketoglutarate dehydrogenase leading to their inactivation (Pocernich and Butterfield, 2003). On the other hand, MDA

can also undergo similar reactions like HNE. There is an increase in the levels of MDA in the inferior temperal cortexes of aged human brain, (Palinski et al., 1990), and in cytoplasm of neurons and astrocytes (Dei et al., 2002). In the canine model of human aging, it was observed that lipid peroxidation as indexed by MDA was increased as a function of age (Head et al., 2002). As a result, these studies and others continue to provide support for the involvement of oxidative stress and lipid peroxidation in neurodegenerative disorders.

2.2.3.2.3 Isoprostanes and Neuroprostanes

In addition to reactive aldehydes, iso-and neuroprostanes present another index for lipid peroxidation and oxidative stress (Morrow and Roberts, 1999; Milne et al., 2005). The free radical catalyzed non-enzymatic oxidation of the PUFA arachidonic acid and docosahexanoic acid, results in the generation of biologically active prostaglandin derivatives (Morrow, 2006). These prostaglandin isomers are referred to as isoprostanes (IsoPs) and neuroprostanes (NP), respectively, and they represent a reliable index for lipid peroxidation. The levels of isoprostanes and neuroprostanes have been reported to be increased in AD patients, providing additional evidence for lipid peroxidation in AD (Pratico et al., 1998; Montine et al., 2005). In addition, 8-isoprostaglandin F2 alpha has also been used as a marker for lipid peroxidation after experimental brain injury (Hoffman et al., 1996).

2.2.3.3 DNA oxidation

Oxidative modification of DNA has been observed during normal brain aging and also in AD (Mecocci et al., 1993). There is increased damage to both nuclear and mitochondrial DNA. Mitochondrial DNA is particularly vulnerable to oxidative attack when compared to nuclear DNA because of their proximity to the source of production of ROS, the lack of protective histones and also their inability to perform repair mechanism (Wallace, 1992; Ames et al., 1993b). Also, there is recent evidence in AD brains that Aβ (1-42), which produces oxidative stress , resides in mitochondria (Beal, 2004). This damage could probably lead to deleterious effects to the mitochondria and it has been proposed that this is one of the mechanisms resulting to mitochondrial alterations in most neurodegenerative disorders (Beal, 2005; Schapira, 2006). DNA oxidation has been shown to be mediated through the oxidative attack of DNA by ROS particularly hydroxyl radical (OH·). Oxidative attack on DNA by hydroxyl radical yields a large number of possible base-adducts; for example, it could lead to modification of DNA bases, DNA strand breaks, DNA-DNA or DNA-protein crosses links among many others. The most common adduct is 8-hydroxy-2'-deoxyguanosine (8-OHdG), resulting from the hydroxylation of guanine at the C8 position. The quantified levels of 8-OHdG have since been used as the best biomarkers of DNA oxidative damage. During aging and age-related disorders, there is a significant increase in the levels of 8-hydroxy-2'-deoxyguanosine (8-OHdG) in AD patients compared to controls (Mecocci et al., 1993; Lovell et al., 1999).

2.3 Brain aging and oxidative stress

2.3.1 Overview

As noted above, aging is defined as the gradual alteration in structure and function that occurs over time, eventually leading to an increased probability of death not associated with disease or trauma. Aging can be considered to be a product of an interaction between genetic, environmental and lifestyle factors (Ashok and Ali, 1999; Gonos, 2000). Aging is usually characterized by impairments in physiological functions, such as impairments in the brain, mitochondrial dysfunction, increased susceptibility to dementia and neurodegenerative disorders, i.e., Alzheimer's disease (AD) (Calabrese et al., 2001; Lenaz et al., 2006). As was mentioned previously, the aging process usually makes the brain susceptible to oxidative attack leading to neuronal cell death and loss of memory and cognitive functions. The brain is particularly susceptible to oxidative damage due to a number of factors. (a) The brain has one of the highest metabolic rates of any organ and is solely dependent of the supply of oxygen for its function. This characteristic therefore leads to the increased production of ROS through the mitochondria. (b) The brain lacks a relative capacity of antioxidant defense systems. (c) The brain has a high level of polyunsaturated fatty acids (PUFA) that can easily undergo peroxidation leading to the formation of reactive aldehydes among others as discussed previously and (d) There is a high level of redox metal ion (Fe^{2+}), which can participate in lipid peroxidation reaction (Poon et al., 2004a).

2.3.2 Free radical theory of aging

The aging process is an interaction of a wide range of factors leading to the proposition of various theories that try to define and elucidate the aging process. Among these theories is the free radical theory of aging. This was first proposed by Harman in 1956 (Harman, 1956) and it postulates that the production of reactive free radicals leads to the progressive damage of biomolecules such as proteins, lipids or DNA resulting into a loss of cellular function leading to eventual cell death (Beckman and Ames, 1998; Droge, 2002; Harman, 2003, 2006). This theory is supported not only by the involvement of oxidative stress in age-related disorders like AD, but also with the established notion that there is increased oxidative stress and altered antioxidant activity in the aging process (Beckman and Ames, 1998).

2.3.3 Alzheimer's disease

Alzheimer's disease (AD), first reported by Alois Alzheimer in 1907, is one of the most common forms of dementia affecting the elderly population and accounting for 50-60% of all dementia cases. In the western world, the prevalence of AD in people aged 85 year and above is approximately 50% (Blennow et al., 2006). In 2001, it was estimated that approximately 24 million people were affected with AD, and it is believed that due to the increasing life expectancy, 81 million people will be affected by the year 2040 (Ferri et al., 2005). The costs involved with AD in the USA are estimated at least $100 billion annually and it has been suggested that AD has the potential to become an overwhelming public health concern. Consequently, these facts create an urgent need for the development of potential therapeutic interventions. The most common risk factors for developing AD is aging, other risk factors include; a decreased reserve capacity of the

brain, including reduced brain size, low educational and occupational attainment, low mental ability in early life, reduced mental and physical activity during late life, traumatic brain injury, and genetics among many others (Mayeux, 2003; Mortimer et al., 2003; Jellinger, 2004a, b). Clinically, AD is marked by loss in memory and cognitive functions, loss of language function and motor skills, altered behavior and ultimately death. Its pathophysiological hallmarks include senile plaques (SP), neurofibrillary tangles (NFT), and synapse loss starting in the entorhinal cortex and progressing into the hippocampus and cortex (Markesbery, 1997). One underlying mechanism associated with AD is the involvement of oxidative stress as indexed by the increase in the levels of protein oxidation as indexed by protein carbonyls and 3-nitrotyrosine, and also by the levels of lipid peroxidation indexed by levels of free and protein bound HNE among many other indices.

2.3.3.1 Amyloid-beta peptide (Aβ)

Central to the mechanisms of increased oxidative stress in AD is amyloid-β-peptide (Aβ). Senile plaques primarily are composed of Aβ protein, which is a 39-43 amino acid peptide. Aβ is produced by the proteolytic cleavage of amyloid precursor protein (APP) by β-secretase and γ-secretase at the amino terminus by and at the carboxy terminus, respectively (Butterfield and Boyd-Kimball, 2005). The cleavage by γ -secretase can occur at varying positions within the carboxy terminus, hence generating Aβ peptides of varying length. The two main Aβ peptides found in human brain are Aβ (1-40) and Aβ (1-42) with Aβ (1-42) being the main component of senile plaques and the more toxic form (Figure 2.13).

Our laboratory and others have established a role for Aβ-induced oxidative stress in AD. Support for this comes from the fact that Aβ (1-42) has been shown to induce protein oxidation and lipid peroxidation *in vitro* and *in vivo* (Hensley et al., 1995; Markesbery, 1997; Yatin et al., 1999; Butterfield and Lauderback, 2002; Drake et al., 2003a). We have proposed that Aβ1-42 peptide, as a small oligomer, inserts itself in the lipid bilayer in an α-helix conformation. A one-electron oxidation of methionine forms the methionine sulfuranyl radical, which can then abstract a labile hydrogen atom from neighboring unsaturated lipids forming a carbon-centered lipid radical ($L^{\cdot}$), which can react with molecular oxygen to from a peroxyl radical ($LOO^{\cdot}$). This peroxyl radical can abstract hydrogen from a neighboring lipid to form the lipid hydroperoxide LOOH and a carbon centered radical $L^{\cdot}$, which propagates the free radical chain reaction (Varadarajan et al., 2001; Butterfield et al., 2005) (See reactions 6-9 above). It is this mechanism of free radical generation that we believe is responsible for the increased levels of oxidative stress leading to neurodegeneration and a decline in memory and cognitive function in AD patients hence continuing to support the rationale of the free radical theory of aging (Markesbery, 1997; Beckman and Ames, 1998).

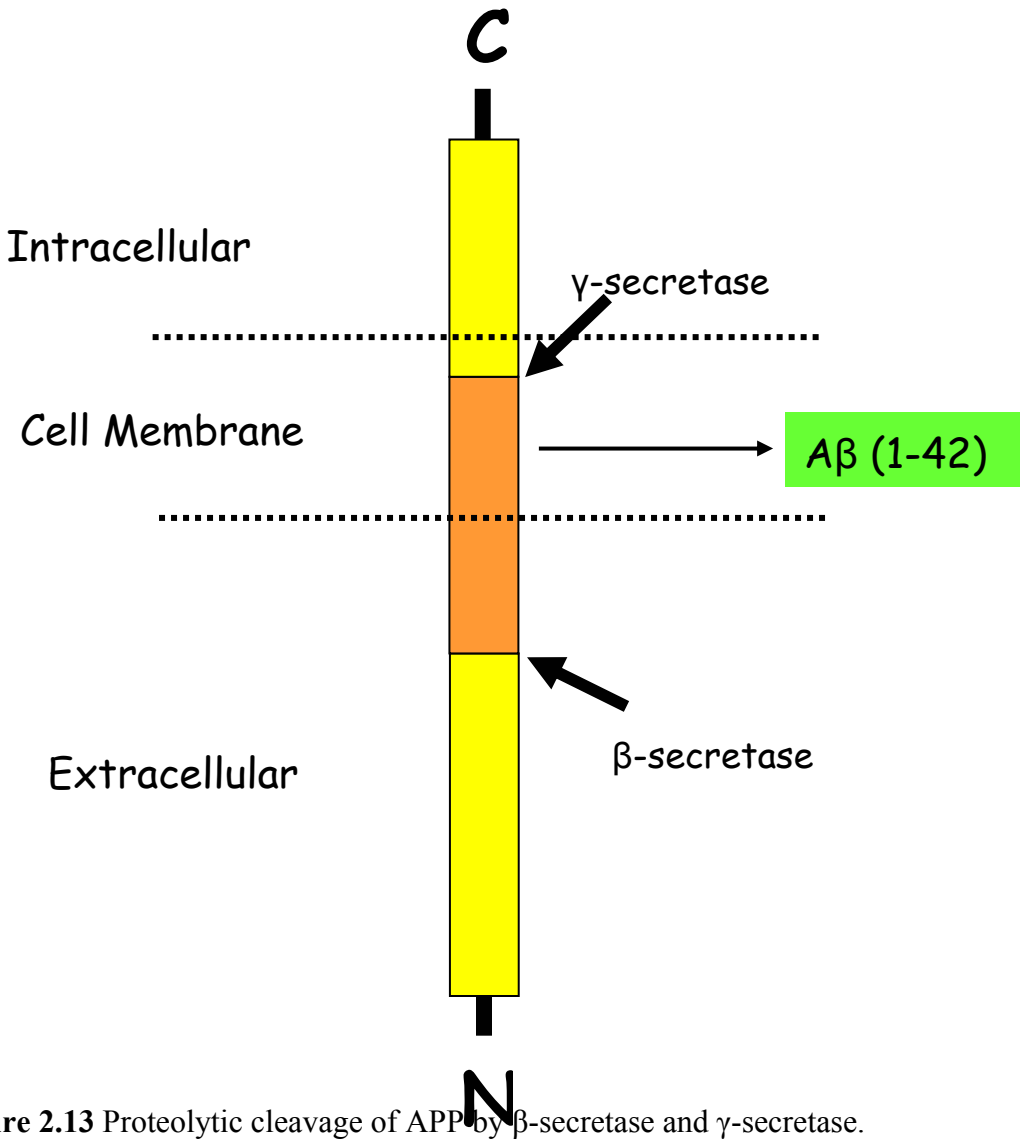


Figure 2.13 Proteolytic cleavage of APP by β-secretase and γ-secretase.

2.3.4 Successful aging

Successful aging or normal aging can be defined as the ability to achieve extreme old age while still maintaining intact cognitive function. Centenarians are the fastest growing age-group in the USA, and it is projected that there will be more that 400,000 persons in the next 30 years over 100 years old (Silver et al., 2002). Studies have shown that there is an increase in the incidence of dementia among people aged 85 years and above (Silver et al., 2001; Perls, 2004b, a), and most people believe that dementia is inevitable to people in this age group (Blansjaar et al., 2000; Snowdon, 2003; Perls, 2004a). However, various studies on nonagenarians and centenarians have shown that approximately 30% of centenarians are cognitively intact and that among those who are demented, 90% show a delay in the time they present signs of cognitive impairments well into their 90's (Hagberg et al., 2001; Silver et al., 2001; Perls, 2004b, a). As a result, the increasing number of cognitively intact nonagenarians and centenarians disputes the notion that the older one becomes that dementia is inevitable (Green et al., 2000; Perls, 2006). The mechanisms involved in the rising number of the dementia-free oldest-old appear to be the result of a complex combination of various factors. These include genetics, environment, and lifestyle among others. These factors, therefore, add to the complexity of understanding the mechanisms involved in the process of successful aging in the human population (Perls, 2005; Perls, 2006). In the current dissertation research, we have sought to gain a better understanding of biochemical mechanisms involved in successful aging.

2.3.5 Animal models of aging

Studies that try to elucidate the mechanisms of human aging and age-related disorders are usually hampered by the lack of a good animal model that can be easily translated to the human population. However, huge strides have been made in developing animal models, e.g. transgenic mice that have led to an increase in the understanding of the mechanisms of human aging. The differences between the genomes of the human and the animal species is one major setback in studying age-related neurodegenerative disorders; moreover, the use of primates which are closer species to humans is not always feasible for age-related studies, due to the length of time required to observe such age-related effects. Recently, the use of the canine model of human aging has gained considerable interest (Cummings et al., 1996b; Cummings et al., 1996a; Cummings et al., 1996c; Head and Torp, 2002). As will be seen in Chapters 4 and 5, the aging canine provides one of the best models for human aging due to a number of reasons. These are: a) Aged canines develop aspects of neuropathology similar to that observed in aged humans; (b) Canines exhibit a clinical syndrome of age-related cognitive dysfunction; (c) Aged canines are deficient on a variety of neuropsychological tests of cognitive function; and (d) Canines develop extensive β-amyloid deposition within neurons and their synaptic fields which appears to give rise to senile plaques. This level of β-amyloid deposition correlates with cognitive dysfunction, and the amino acid sequence of beagle Aβ is the same as that in humans.

2.4 Mitochondria

2.4.1 Overview

There is increasing evidence for mitochondrial involvement in the pathogenesis of neuronal cell death in most neurodegenerative disorders. This evidence applies to aging, age related-disorders like Alzheimer's and Parkinson's disease, amyotrophic lateral sclerosis (ALS) hereditary spastic paraplegia, and cerebellar degenerations, multiple sclerosis (MS) and also in various nervous system injuries like TBI. Oxidative stress and excitotoxicity seem to play a key role in the mechanisms leading towards the observed mitochondrial dysfunction (DiMauro et al., 1993; Melov, 2000; Calabrese et al., 2001; Dalakas et al., 2001; Lenaz et al., 2002; Fiskum et al., 2003; Lifshitz et al., 2004a; Beal, 2005; Zeevalk et al., 2005).

2.4.2 Mitochondria structure

Mitochondria are organelles that evolved from a symbiotic relationship between aerobic bacteria and primordial eukaryotic cells. Mitochondria still carry a functional relic of their original genome (DiDonato et al., 1993; Wallace, 2005). They are intracellular tubular organelles delimited by two membranes. The outer mitochondrial membrane (OMM) is permeable to ions and small proteins of molecular weights < 10 kDa, while the inner mitochondrial membrane houses the multimeric enzyme complexes of the electron transport chain (Schapira, 2002). Human mitochondrial DNA is a circular double stranded molecule of about 16.6 Kb long with the genome containing 37 genes. Of these, 13 genes encode protein subunits of respiratory chain complexes with a significant amount of mitochondrial proteins being encoded by the nuclear genome and

transported into the mitochondria. For example, complex II is solely composed of proteins encoded by nuclear genes (Orth and Schapira, 2001; Schapira, 2006). The varying mitochondrial density in various tissues is usually dictated by the need for oxidative phosphorylation and energy production (Orth and Schapira, 2001) . For example, it is considered that cardiac, skeletal muscles cells, and neurons have the highest density of mitochondria; hence, their sensitivity to energy dependent defects resulting from mitochondrial dysfunctions in aging, age-related disorders or injury are elevated (Schapira, 2006).

2.4.3 Mitochondria and energy metabolism (Bioenergetics)

Central to the role of mitochondria is energy metabolism through the production of ATP. This energy production is usually carried out at the level of the inner mitochondrial membrane through oxidative phosphorylation involving the reduction of oxygen to water by multimeric enzyme complexes (Green and Reed, 1998; Stavrovskaya and Kristal, 2005). The electron transport chain (ETC) is composed of NADH-ubiquinone oxidoreductase (complex I), succinate dehydrogenase-CoQ oxidoreductase (complex II), cytochrome reductase (complex III), cytochrome oxidase (complex IV), and ATP synthase, which is sometimes referred to as complex V (Dudkina et al., 2005; Schapira, 2006). These multimeric complexes are arranged in the inner mitochondrial membrane according to their reduction potentials (Sullivan et al., 2005) (Fig 2.14). During oxidative phosphorylation, ETC complexes are usually involved in reduction and oxidation reactions through reducing equivalents such as NADH or succinate, which are transported into the mitochondria from the cytosol. In the process, protons are pumped

from the matrix into the intermembrane space terminating with the reduction of O_2 to H_2O. The transfer of protons from the matrix to the inner mitochondrial membrane leads to the generation of a mitochondrial membrane potential $\Delta\Psi_m$ of 150-180 mV, which usually determines the energetic status of the mitochondria (Nicholls et al., 1999a). This reserve of potential energy through the electrochemical gradient is then coupled to the generation of ATP, from ADP and inorganic phosphate P_i through the ATP synthase complex (complex V) (Dubinsky et al., 2004). There is a significant compromise in the mitochondrial bioenergetics in aging and neurodegenerative disorders as mentioned above. Since neurons solely depend on the ATP from glycolysis and mitochondria metabolism, this loss in ATP production usually makes the neurons vulnerable to oxidative stress, which eventually results into neuronal cell loss through necrosis or apoptosis.

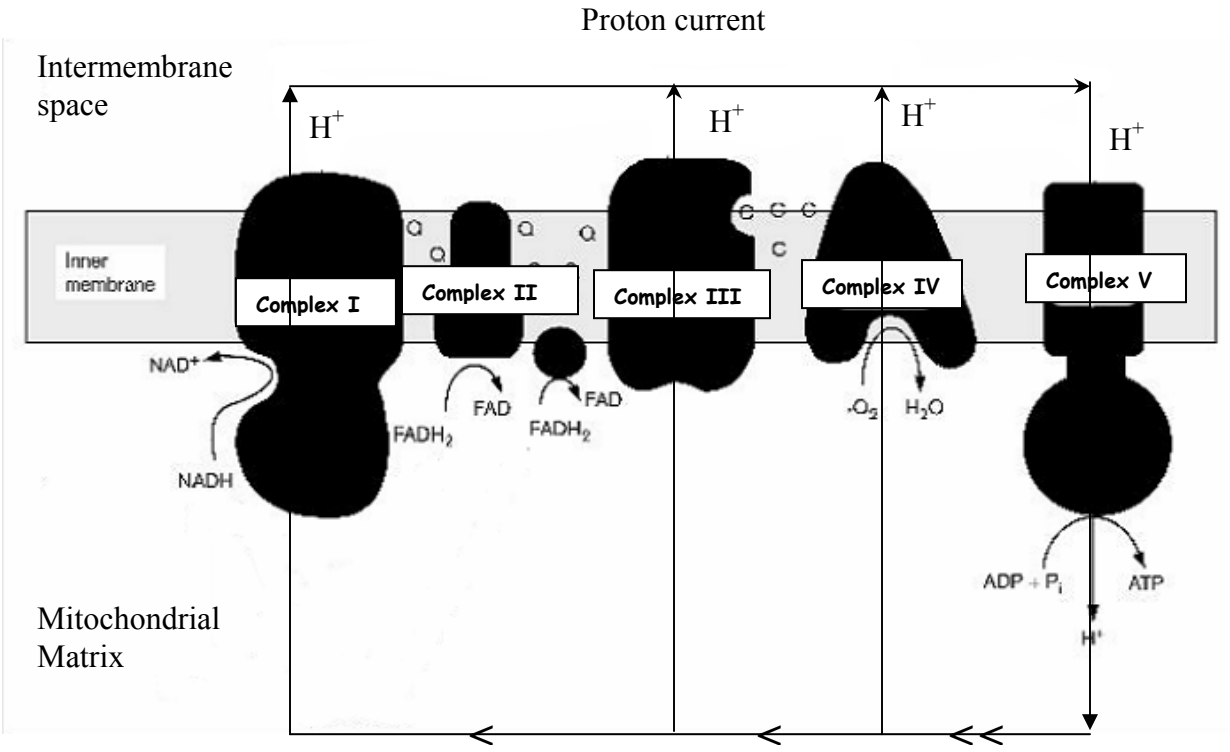


Figure 2.14 Representation of the inner mitochondrial membrane and the ETC complexes.

2.4.4 Excitotoxicity and Ca^{2+} regulation.

Mitochondria under normal conditions usually act as high capacity Ca^{2+} sinks through a highly complex system for the regulation and the transportation of Ca^{2+} (Gunter and Gunter, 1994; Bianchi et al., 2004; Campanella et al., 2004; Szabadkai et al., 2006). Through these processes, mitochondria sense and respond to changes in cytosolic Ca^{2+} loads to maintain cellular Ca^{2+} homeostasis that is required for normal neuronal function (Ichas and Mazat, 1998; Rizzuto et al., 1998; Gunter et al., 2000). Disruption in Ca^{2+} homeostasis is one of the major factors that contribute to the neuropathology following most neurodegenerative disorders like AD and in some CNS injury like traumatic brain injury (Bianchi et al., 2004; Campanella et al., 2004). In neurodegenerative disorders, mitochondrial dysfunction is primarily involved in glutamate neurotoxicity (Globus et al., 1995; Azbill et al., 1997; Montal, 1998). In AD, glutamate excitotoxicity and calcium dysregulation have been shown to be examples of etiologies that follow in addition to oxidative stress. Glutamate excitotoxicity is usually triggered by the excessive influx of calcium following activation and over-stimulation of NMDA subtype of glutamate receptors (Lipton and Rosenberg, 1994; Nicholls and Budd, 1998a; Liu et al., 1999; McAdoo et al., 1999). The resulting increase in intracellular calcium has been shown to lead to impaired function of the mitochondrial ETC, formation of ROS/RNS, and in particular superoxide anion and hydrogen peroxide (White and Reynolds, 1996; Brustovetsky et al., 2002; Brustovetsky et al., 2003; Sullivan et al., 2005). In addition, extreme loads of Ca^{2+}, as will be discussed later, lead to the opening of the mitochondrial permeability transition pore (mPTP) and induction of apoptotic pathways eventually leading to neuronal cell death following most CNS trauma

(Kristal and Dubinsky, 1997; Hirsch et al., 1998; Brustovetsky et al., 2002; Halestrap et al., 2002; Sullivan et al., 2005).

2.4.5 Mitochondria and oxidative stress

As mentioned earlier mitochondria are a key source of ROS. Through the generation of ATP via oxidative phosphorylation by the ETC components, there is always some leakage of electrons (1-2%) from the mitochondria resulting in the partial reduction of O_2 to form superoxide anion $O_2^{\cdot -}$, a majority of which is generated from complex I and complex III of the ETC (Kushnareva et al., 2002; Liu et al., 2002; Zeevalk et al., 2005; Halliwell, 2006). The production of superoxide anion can occur when the electron transport chain is impeded following mitochondrial injury or during neurodegenerative disorders as seen in the case of aging, AD and PD (DiMauro et al., 1993; Lenaz et al., 2002; Lenaz et al., 2006). It should be noted that the production of ROS by mitochondria is tightly linked to the mitochondrial membrane potential, since hyperpolarization or high mitochondrial membrane potential promotes the production of ROS (Liu et al., 2002; Sullivan, 2005). With a high mitochondrial membrane potential, the ETC cannot transfer protons against the electrochemical proton gradient from the matrix. This, in essence, leads to a reduced state of electron carriers and an increase in the half life of semiquinone (Green and Kroemer, 2004). The intermediates thus remain in a prolonged reduced state therefore increasing the chances of electron leakage and partially reduction of O_2 to superoxide anion $O_2^{\cdot -}$ (Liu et al., 2002).

One major pathway predominantly involved in the production of ROS/RNS is the secondary activation of glutamate receptors and excitotoxicity following injury to the

nervous system (White and Reynolds, 1996; Nicholls and Budd, 1998b; Nicholls et al., 1999b). As already mentioned, following elevated cytosolic levels of Ca^{2+} in neurodegenerative disorders or in CNS injury, excess Ca^{2+} is sequestered into the mitochondrial matrix through various transporters (Gunter et al., 1998; Bianchi et al., 2004). However, excessive calcium loads result in the dysregulation of mitochondrial Ca^{2+} homeostasis leading to the activation of nitric oxide synthase (iNOS) and the production of nitric oxide radical (NO·). The presence of nitric oxide NO and superoxide anion $O_2^{\cdot -}$ as earlier discussed leads to the formation of peroxynitrite ONOO-, another significant RNS, which can lead to the oxidative modification of proteins through nitration (Beckman et al., 1990). Elevated intracellular Ca^{2+} levels also activate phospholipase A_2 (PLA_2). This enzyme liberates the unsaturated fatty acid arachidonic acid initiating the formation of free radicals via cyclooxygenase-2 (COX-2) and lipoxygenase pathways. Arachidonic acid can also be oxidized leading to the formation of lipid peroxidation products such as HNE, MDA, acrolein among others (Butterfield and Stadtman, 1997; Lipton, 1999; Mirjany et al., 2002). The activation of inflammatory response following CNS injury also leads to the production of substances such as cytokines, tumor necrosis factor α (TNFα), and interleukin 1 (IL-1β) that can damage neurons. In addition, activation of microglia and astrocytes can also lead to the generation of more ROS. For example, NADPH and xanthine oxidase significantly contribute to $O_2^{\cdot -}$ production upon activation of microglia (Gabryel and Trzeciak, 2001; Zeevalk et al., 2005). If the significant increase in ROS and RNS following CNS injury is not controlled, damage to DNA, proteins or lipids would result, eventually leading to loss of function and possibly neuronal cell death as observed in neurodegenerative disorders.

2.4.6 Mitochondria permeability transition pore (mPTP)

The mitochondrial permeability transition (mPT) is defined as an increase in the permeability of the inner mitochondrial membrane to solutes that have molecular masses lower than 1.5 KDa (Sullivan et al., 2005). The induction of this transition has since been demonstrated to involve the opening of a 2-3mm inner membrane mega channel now referred to as the mitochondria permeability transition pore (mPTP) (Jacotot et al., 1999; Halestrap et al., 2002). This pore is a voltage-dependent channel, which is activated by the elevation of Ca^{2+} levels, oxidative stress and low inner mitochondrial membrane potentials as observed in most nervous system injuries (Hunter and Haworth, 1979). The exact identity of this mega -channel is still under investigation. However, recent progress has been made in trying to provide a logical model for its composition. The minimum configuration of the mPTP requires the adenine nucleotide translocator (ANT), which is located in the inner mitochondrial membrane, in association with outer mitochondrial membrane proteins such as the voltage-dependent anion channel (VDAC) and cyclophilin-D (CyP-D), a peptidyl–prolyl *cis-trans* isomerase whose activity is usually inhibited by immunosuppressant cyclosporine A (CsA) (Bernardi, 1996; Halestrap et al., 2002). In addition, other proteins such as Bcl-2 and Bax have been shown to interact with the ANT, as a result providing a regulatory role for the mPTP (Marzo et al., 1998b; Marzo et al., 1998a; Halestrap et al., 2002). The exact physiological function of the mPTP has not been established, however, there seems to be a clear consensus on its involvement in the induction of permeability transition in the neuropathology of various CNS injuries such as TBI and spinal cord injury (SCI) (Kristal and Dubinsky, 1997; Halestrap et al., 2004; Sullivan et al., 2005). This is particularly

evident, since, it has been established that CNS injury triggers the opening of the mPTP, resulting in a collapse of the electrochemical gradient, the swelling of mitochondria, the inhibition of ATP synthesis, the release of apoptotic factors and the eventual initiation of cell death pathways that are blocked by the use of cyclophilin-D binding immunosuppressant drug cyclosporine A (CsA) (Bernardi, 1996; Sullivan et al., 1999; Brustovetsky et al., 2002; Brustovetsky et al., 2003; Sullivan et al., 2005).

2.4.7 Role of mitochondria in apoptosis and necrosis

Mitochondria also play a central role in the regulation of both programmed cell death (also known as apoptosis) and of uncontrolled cell death, i.e., necrosis (Zamzami et al., 1996; Cai et al., 1998; Green and Reed, 1998; Springer, 2002). Evidence of apoptosis in neurodegenerative disorders like AD and CNS trauma have been well established (DiMauro et al., 1993; Budd and Nicholls, 1998; Hirsch et al., 1998; Lifshitz et al., 2004a). CNS injury usually occur in a biphasic manner, i.e., through a primary necrotic process that results in rapid and significant decline in the levels of ATP and in the ensuing hours, days and weeks, a secondary injury that exacerbates the primary insult occurs through apoptosis (Rabchevsky and Smith, 2001; Springer, 2002; Sullivan et al., 2005). These two mechanisms of neurodegeneration differ in their morphological features of general cell structure. Apoptosis is marked by cell shrinkage and DNA fragmentation, while necrosis is marked by cellular swelling and uncontrolled cell lysis (Hirsch et al., 1997; Green and Reed, 1998; Bras et al., 2005). Due to the duration in the manifestation of pathologies in neurodegenerative disorders, it is believed that apoptosis is the predominant cell death pathway in most neurodegenerative disorders.

As noted above, there is increase in oxidative stress and excitotoxicity leading to Ca^{2+} overload, which in turn triggers the induction of the permeability transition (Kruman and Mattson, 1999; Brustovetsky et al., 2002; Brustovetsky et al., 2003) in most neurodegenerative disorders and CNS injury. This usually leads to excessive mitochondrial swelling and depolarization of the mitochondria and uncoupling of the oxidative phosphorylation eventually leading to a rapid decline in ATP reserves which if not restrained as seen in sever CNS injuries, would more often that not lead to necrotic cell death (Halestrap et al., 2002). However, it has been shown that transient opening of the mPTP is associated with the induction of apoptosis through the release of cytochrome c and other pro-apoptotic proteins (Marchetti et al., 1996; Hirsch et al., 1998; Marzo et al., 1998a; Sullivan et al., 2002). Released cytochrome c usually complexes with apoptosis protease-activating factor 1 (Apaf-1), dATP, and procaspase-9 to form the high molecular weight complex, the apoptosome. The apoptosome then activates down-stream effector caspases, which then initiate the apoptotic cascade (Zamzami et al., 1996; Cai et al., 1998; Green and Reed, 1998; Springer et al., 1999; Eldadah and Faden, 2000). However, apoptotic events still can occur without the opening of the permeability transition pore. Pro-apoptotic proteins, such as Bax and Bad also have been thought to be capable of forming pores in the outer mitochondrial membrane, thereby influencing the release of other pro-apoptotic proteins such as cytochrome *c*, apoptosis inducing factor (AIF), endonuclease G and Smac/Diablo, thereby activating the apoptotic cascade (von Ahsen et al., 2000; Springer, 2002; Bras et al., 2005). These various pathways have been summarized in Fig 2.15.

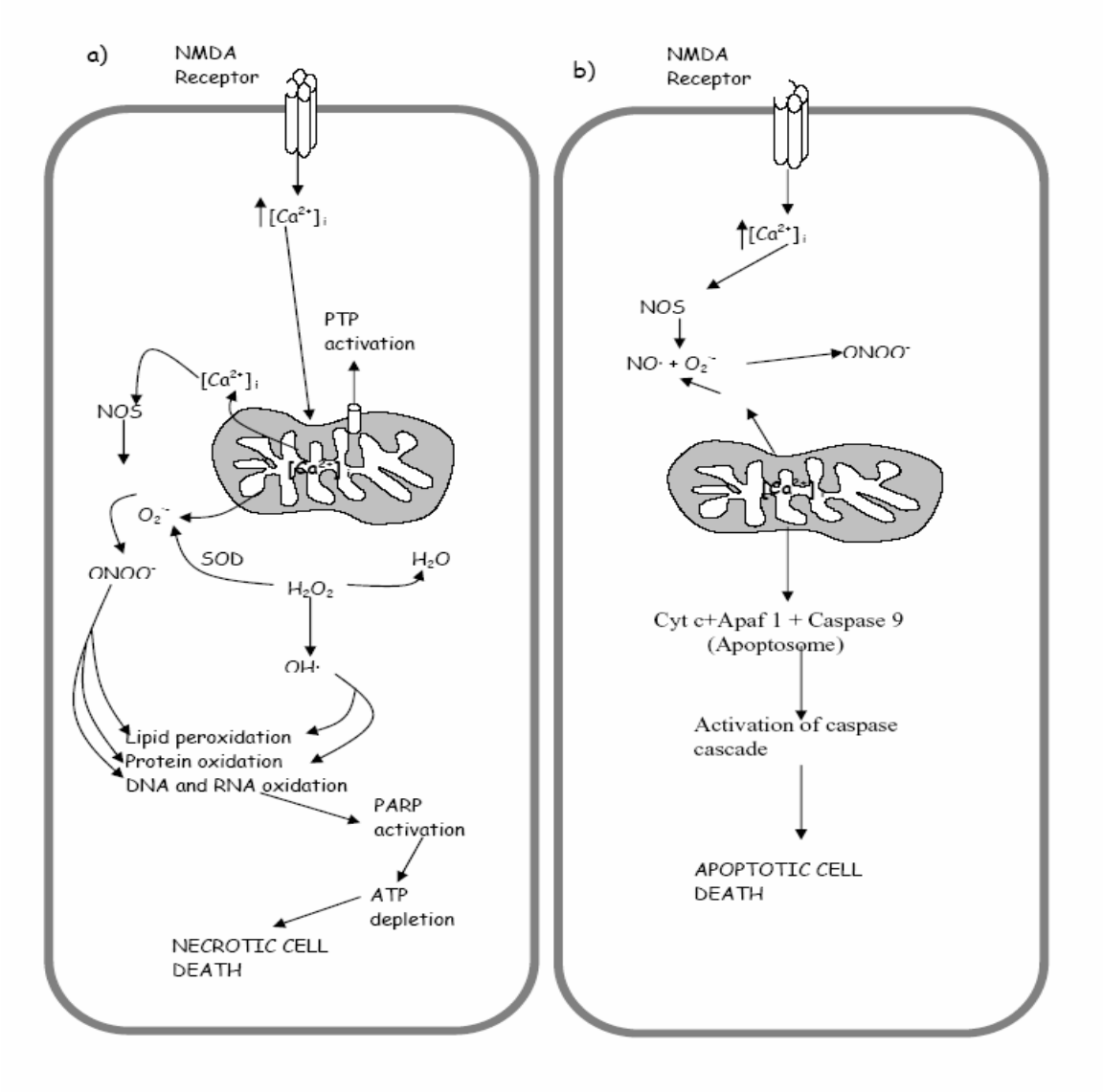


Figure 2.15 Role of mitochondria in glutamate induced excitotoxicity a) represents cell death through necrosis b) depicts cell death through apoptosis. Adapted from Trends Neurosci. (2000) 23, 298–304

2.5 Traumatic brain injury and oxidative stress

Traumatic brain injury (TBI) is a serious health care problem in the United States, with more than 400,000 individuals hospitalized each year and an estimated annual cost of $25 billion (Tibbs et al., 1998; McNair, 1999; Adekoya et al., 2002; Hall and Sullivan, 2004). There is currently no therapeutic intervention for TBI. The pathophysiological events in TBI occur in a biphasic manner. Following TBI the primary insult results in a rapid and significant necrosis of cortical tissue at the site of injury and in the ensuring hours and days, a secondary injury occurs that exacerbates the primary damage (Sullivan et al., 1998b; Robertson, 2004; Sullivan et al., 2005). One underlying feature of this secondary injury in both experimental and clinical TBI is the loss in mitochondrial bioenergetics. This is usually characterized by mitochondrial dysfunction that include exposure of neurons to excitotoxic levels of excitatory neurotransmitters with excessive uptake of Ca^{2+} and eventual overload, generation of reactive oxygen species, induction of the opening of the mitochondrial permeability transition pore (mPTP), release of cytochrome C, inhibition of ATP production and ultimately neuronal cell death (Sullivan et al., 1998b; Nicholls and Budd, 2000; Harris et al., 2001; Xiong et al., 2001; Sullivan et al., 2002; Rego and Oliveira, 2003; Lifshitz et al., 2004b; Robertson, 2004; Sullivan et al., 2005).

Oxidative stress has been shown to be one of the main mechanisms following TBI. Due to the proximity to the source of ROS, the mitochondria are believed to be extremely vulnerable to the effects of these ROS. In this dissertation we have proposed that mitochondrial-related proteins are undergoing significant oxidative modifications leading to their dysfunction hence the decline in mitochondrial related function.

2.6 HIV and AIDS

2.6.1 Overview

Infection with Human Immunodeficiency Virus (HIV) results in the severe compromise of the immune system and increased susceptibility to opportunistic infections (Wang et al., 2006). This leads to the development of acquired immunodeficiency syndrome (AIDS). The risk of developing AIDS after infection with HIV increases over time. It has been established that after HIV infects a cell, it uses the individual's host cell machinery to replicate and produce toxic proteins that contribute to the mechanisms that lead to cell death (Nath and Geiger, 1998; Mattson et al., 2005; Wang et al., 2006). The rise in complications as a result of HIV infection has presented a tremendous challenge to both virologist and immunologists. In recent years, the use of highly active antiretroviral therapies (HAART) has revolutionized the treatment of AIDS, with a suppression of viral load and consequent reduction in complications observed in the late-stages of the disease (Gray et al., 2003). HAART can suppress the replication of the virus in the long term, but this is often accompanied by significant toxicities that can compromise treatment (Egger et al., 1997; Brinkman and Kakuda, 2000). One of the key challenges resulting from HIV infection is the development of neurological disorders resulting in the manifestation of HIV-related dementia (HIVD) (Nath and Geiger, 1998; McArthur et al., 2003; Mattson et al., 2005; Pocernich et al., 2005; Nath and Sacktor, 2006). A detailed discussion of HIVD is provided below.

2.6.2 Toxic HIV proteins and neurodegeneration

It had previously been thought that HIV encephalitis results from the direct infection of the nervous system with the virus (Nath and Geiger, 1998). However, neuronal cell loss occurs without the infection of the neurons (Nath, 2002). It has been established that inflammatory mediators and toxic viral proteins known as "virotoxins" released form activated glial cells following infection with virus mediate this neuronal loss (Pocernich et al., 2005). This is through the release of a number of soluble factors that are either toxic to neurons or cause chemotaxis. Some examples of these virotoxins are gp120, gp41, tat, nef, and vpr among many others. When released, these virotoxins then initiate a cascade of positive feedback loop cascade that cause neuronal cell death (Nath, 2002). Moreover, antioxidants block the effect of tat (Pocernich et al., 2005). A summary of this cascade is provided in (Fig 2.16).

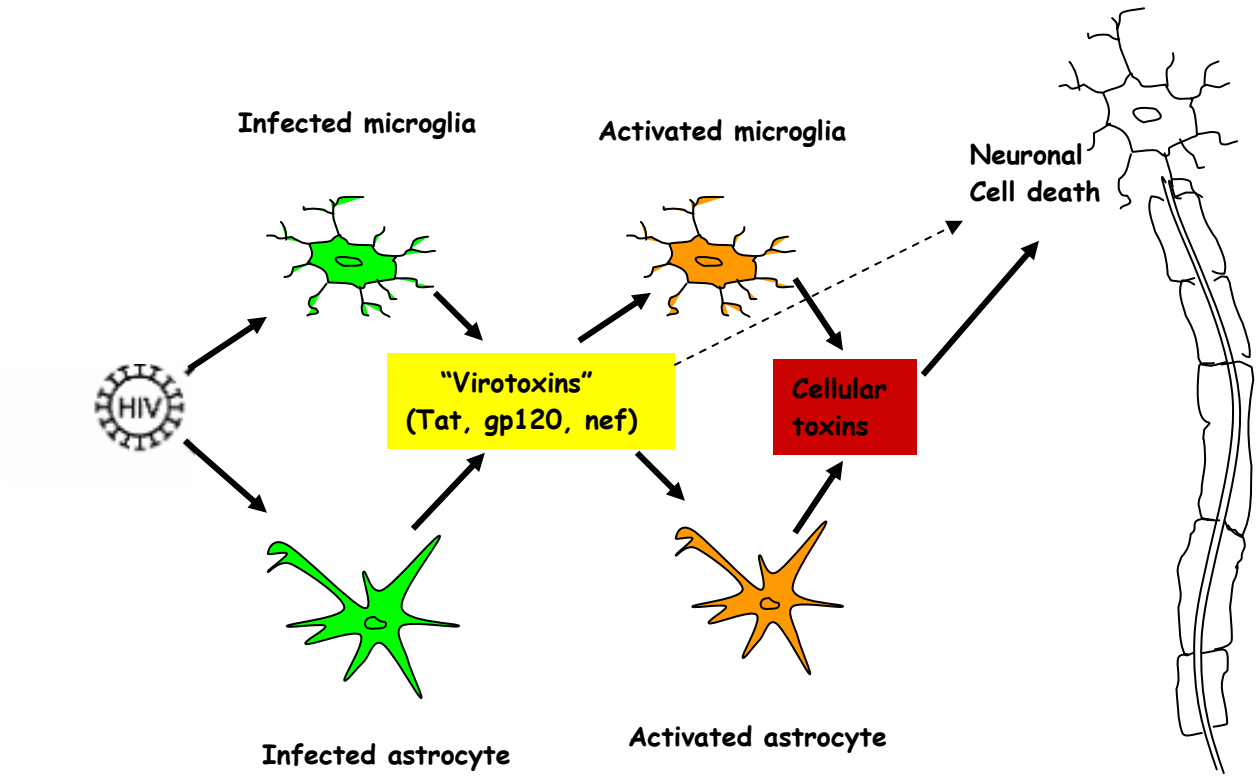


Figure 2.16 Mechanism of neuronal damage following HIV infection.

2.6.3 HIV-Dementia (HIVD) and oxidative stress

Human immunodeficiency virus type-1 (HIV-1) infection is the most common cause of dementia in adults less than 40 years of age. It is estimated that 20-30% of adults infected with (HIV-1) develop HIV dementia (Nath, 2002; Mattson et al., 2005). Currently in the United States, it is estimated that up to 500,000 individuals are affected with HIV-associated brain disease (Wang et al., 2006). The HIV virotoxins-mediated neuronal loss, leads to the development of a condition known as HIV dementia. HIV infection of the brain is characterized by among others, the presence of multinucleated giant cells which are infected macrophages that have fused together, and microglia nodules which are collections of lymphocytes and macrophages (Nath and Geiger, 1998). The cognitive effects are characterized mainly by psychomotor retardation while behavioral manifestations include apathy, social withdrawal, depression, psychosis and emotional incontinence. Motor manifestations include tremors, incoordination, Parkinsonism and impaired balance (Nath et al., 1987). There have been various hypothesis proposed for the entry of HIV into the CNS. One is as a free viral particle or most likely through the migration of infected across the blood-brain barrier, the "Trojan horse" hypothesis (Nath, 2002). The brain is particularly a good reservoir of HIV virus since it as been shown that the incident of HIVD increases with prolonged survival approaching 75% in patients with end-stage AIDS (McArthur et al., 2003; Wang et al., 2006). As already noted, once in the brain, the virus then initiates a cascade of reactions through “virotoxins”. Recent reports have confirmed the clinical significance and involvement of free radicals and oxidative stress in HIV-dementia (Sacktor et al., 2004; Mattson et al., 2005; Pocernich et al., 2005; Wang et al., 2006). This is evident from the

increased oxidative stress as measured by protein oxidation in the brain and cerebrospinal fluid (CSF) of HIVD patients, there is increased expression of iNOS in individuals with server to moderate dementia compared to those with a milder impairments (Adamson et al., 1996). In addition, an increase in the levels of peroxynitrite has been observed in HIVD brains (Boven et al., 1999). It has also been showed that there is a significant decrease in the concentration of the antioxidant glutathione and catalase in HIV-infected patients, thus making them more vulnerable to free radical attack (Castagna et al., 1995; Yano et al., 1998). With the advent of HAART, the incidence of HIVD has fallen, while the cumulative prevalence of HIVD has risen (Neuenburg et al., 2002). Despite the decline of HIVD incidence, neurological complications still remain an important cause of disability and death associated with AIDS (Kandanearatchi et al., 2003; McArthur et al., 2003). For example, in a recent study evaluating HIV-infected outpatients on HAART, it was demonstrated that 9% died with HIVD and, of those, 92% were diagnosed with HIVD within 12 months of death (Welch and Morse, 2002). As a result, from this dissertation and other studies it is proposed that some drugs used in the highly active antiretroviral therapy (HAART) could possibly be involved in the development of HIV dementia through oxidative stress mechanism (Schweinsburg et al., 2005; Nath and Sacktor, 2006).

2.7 Antioxidant defense systems

2.7.1 Overview

As already mentioned increased levels of ROS/RNS can lead to oxidative damage to proteins or enzyme, DNA, and lipids leading to a loss in function and possibly cell death. As a result, cells are equipped with efficient strategies to control and maintain the intracellular levels of ROS/RNS through the use of various endogenous antioxidant systems. These antioxidants and antioxidant defense systems have been reported to be significantly decreased in aging and age-related neurodegenerative disorders (Mates et al., 1999). As a result, various strategies have been developed to modulate these levels of antioxidants and reduce oxidative damage using exogenous antioxidant compounds. The natural antioxidant defense system consists of endogenous antioxidant systems that include glutathione and its related enzymes, superoxide dismutase (SOD), catalase, among others. Other important non-enzymatic low-molecular-weight molecules including vitamin C, vitamin E, lipoic acid, compounds rich in flavonoids, and carotenoids among many others (Sen and Packer, 1996; Mates et al., 1999; Gutteridge and Halliwell, 2000; Martin, 2003; Kahl et al., 2004). These are discussed here in relation to neurodegenerative disorders.

2.7.2 Superoxide dismutase

Superoxide dismutase (SOD) provides the first line of defense against ROS. There are two classes of SOD; manganese SOD (Mn-SOD) which is predominately located in the mitochondrial matrix and copper/zinc SOD (Cu/ZnSOD, SOD1) which is found in the cytosol, though recent studies have shown that Cu/Zn SOD can also be

located in the intermembrane space of the, nucleus, peroxisomes, and mitochondrial intermembrane space of human cells (Liochev and Fridovich, 2005; Selverstone Valentine et al., 2005). SOD acts as an antioxidant enzyme by lowering the steady-state concentration of superoxide. This enzyme catalyzes the disproportionation of $O_2^{\cdot -}$ to H_2O_2 and H_2O thus eliminating the cytotoxic superoxide anion from the cell (Chan, 1996; Gutteridge and Halliwell, 2000; Kahl et al., 2004; Liochev and Fridovich, 2005).

It has been shown that there is a decline in the levels of SOD in neurodegenerative disorders like AD, HIVD and also in normal aging. This thus increases the vulnerability to attack by ROS/RNS. When mutated, SOD can also cause disease as in the case of familial amyotrophic lateral sclerosis (fALS), another neurodegenerative disorder (Selverstone Valentine et al., 2005). The toxic gain of function of mutant SOD (mSOD) leads to the generation of reactive oxygen/nitrogen species (Valentine, 2002; Perluigi et al., 2005a; Poon et al., 2005f). Some researchers believe that the elevated oxidative activity associated with mSOD occurs by enzymes acting as peroxidases (Valentine, 2002) or as superoxide reductases (Liochev and Fridovich, 2000) or by producing $O_2^{\cdot -}$ to form peroxynitrite (Rakhit et al., 2002).

2.7.3 Vitamin E and C

Vitamin E (α-tocopherol) and vitamin C (ascorbic acid) Fig 2.17a/b are key endogenous low molecular weight antioxidants that play a significant role in the protection against neurological disorders associated with oxidative stress. Vitamin E is lipid soluble, thus it predominately provides its antioxidant activity in the lipid-bilayer. On the other hand, vitamin C is predominantly found in the cystosol, where it is involved in the recycling of the tocopheroxyl radical thus maintaining the cellular levels of vitamin

E (Butterfield et al., 1999b; Butterfield et al., 2002c). The levels of vitamin E and C have been reported to be in low concentrations in the CSF of AD patients (Butterfield et al., 2002c). However, there was a significant increase in the levels of both vitamins in the CSF of AD patients who were given both vitamin C and E. The beneficial effects of this supplementation was observed only in the group that had a combination of the vitamins compared to one that had only vitamin E (Morris et al., 1998; Morris et al., 2002b; Martin, 2003). This was thought to be as a result of the need to recycle the tocopheroxyl radical by a reducing agent, such as vitamin C. It has also been observed that dietary intake of antioxidants vitamins E and C, especially vitamin E, is associated with a lower risk of incident AD, though other studies have seen the contrary(Morris et al., 2002a; Petersen et al., 2005). There has also been a significant association found between vitamin E intake and cognitive decline and dementia (Engelhart et al., 2002). Vitamin E has been shown to block *in vitro*, the toxic effects of Aβ-induced toxicity (Koppal et al., 1998; Butterfield et al., 1999b; Yatin et al., 2000). Also, other than its role as an antioxidant, vitamin E has been reported to be involved in the modulation of cellular signaling and transcriptional regulation that play a key role in reducing the risk of developing AD (Morris et al., 1998).

On the other hand, vitamin C another antioxidant has been shown to participate in several enzymatic reactions essential to the synthesis of catecholamines (Launer, 2000). It has been established that medications or vitamins that increase the levels of brain catecholamines and protect against oxidative damage which may reduce the neuronal damage and slow the progression of neurodegenerative disorders like AD (Diliberto et al., 1982). As a result, these studies provided here establish that the use of antioxidants

vitamin C and E could be a potential source of therapeutic intervention against AD and other neurodegenerative disorders. Vitamin E and C were among the antioxidants included in the antioxidant fortified diet given to the aging canine in this dissertation.

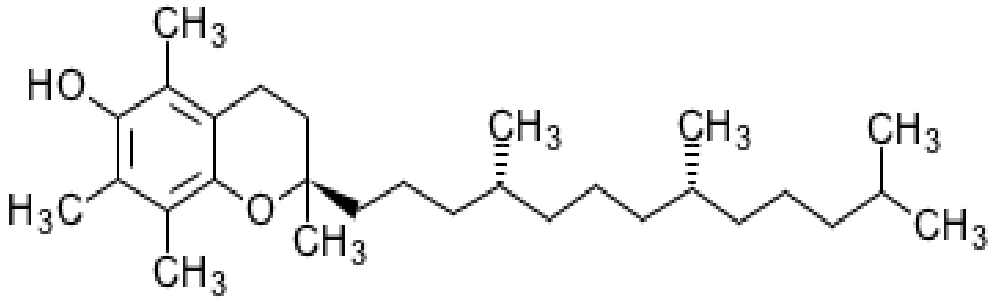


Figure 2.17 a Structure of Vitamin E.

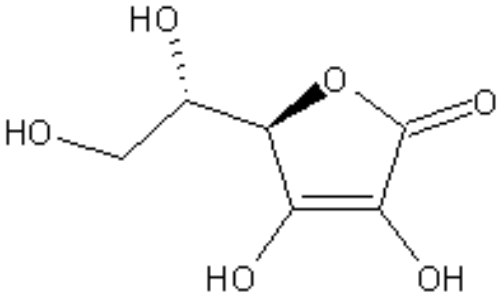


Figure 2.17 b Structure of Vitamin C.

2.7.4 Glutathione system

2.7.4.1 Overview

The glutathione antioxidant system is one of the most important antioxidant defense mechanisms available in living organisms. Glutathione (GSH) is a tripeptide composed of γ-glutamyl-cyteinyl-glycine present in milimolar concentrations in the brain Fig 2.18. It can exist in its oxidized GSSG or reduced for GSH, the ratio of which provides a good indicator of oxidative stress. It has been established that a decrease in GSH concentration is associated with aging and the pathogenesis of most neurodegenerative disorders (Pastore et al., 2003).

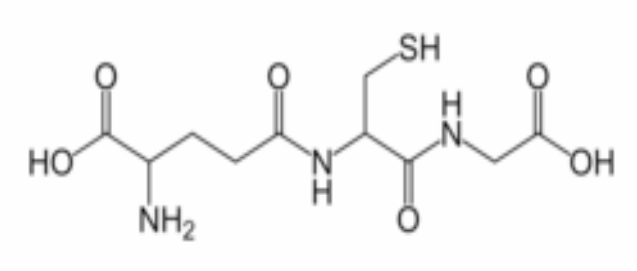


Figure 2.18 Structure of Glutathione.

2.7.4.2 GSH synthesis

GSH is not synthesized in mitochondria. GSH is synthesized in the cytosol from where it is then transported into the mitochondria (Dringen et al., 2000). GSH is synthesized from L-glutamate with L-cysteine forming L-γ-glutamyl-L-cysteine the rate limiting substrate Fig 2.18. This initial reaction is catalyzed by γ-glutamylcysteine synthetase in the presence of Mg^{2+} and ATP. The addition of glycine to L-γ-glutamyl-L-cysteine is catalyzed by GSH synthetase in the presence of Mg^{2+} and ATP to yield GSH (Figure 2.19). It should be noted that the L-cysteine concentration in the brain are in micomolar while the concentration of both L-glutamate and glycine are in millimolar, thus, L-cysteine is the limiting amino acid in the biosynthesis of GSH (Cooper, 1997). Our laboratory and others have successfully modulated the levels of GSH by using compounds that provide cysteine precursors such as N-acetyl-L-cysteine (NAC), glutathione monoethyl ester or through the use of γ-glutamylcysteine ethyl ester (GCEE) that provides the limiting substrate γ-glutamylcysteine of GSH synthesis in various models of neurodegeneration (Pocernich et al., 2001; Drake et al., 2003b; Anderson et al., 2004; Boyd-Kimball et al., 2005a; Boyd-Kimball et al., 2005b).

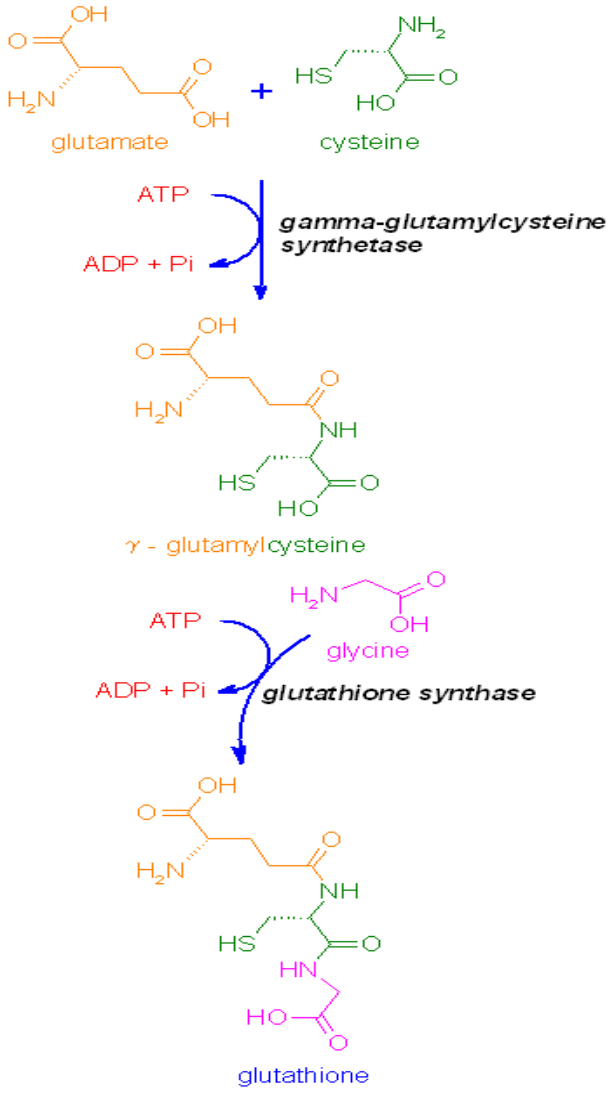


Figure 2.19 Glutathione synthesis.

2.7.4.3 Role of Glutathione

The glutathione system in conjunction with glutathione peroxidase (GPx) and glutathione reductase (GR) are involved in a number of antioxidant defense reactions (Brigelius-Flohe, 1999). As mentioned earlier, the disproportionation of $O_2^{\cdot -}$ generates H_2O_2, another ROS. Though not toxic, H_2O_2 can also mediate oxidative damage through various mechanisms. In the presence of redox transition metal ions such as Fe^{2+} or Cu^{+}, H_2O_2 can undergo a Fenton reaction resulting in the generation of hydroxyl radical OH·, a much more toxic ROS that can exact excessive damage at distant sites from where H_2O_2 was generated (Butterfield and Stadtman, 1997). As a result, there is need for the cell to regulate the intracellular levels of H_2O_2. H_2O_2 can be converted to water by glutathione peroxidase GPx using GSH as the hydrogen donor. The oxidized glutathione (GSSG) is then recycled back to GSH by the enzyme glutathione reductase (GR) that uses NADPH as a cofactor. GSH through the enzyme glutathione-s-transferase (GST) can also form conjugated with reactive aldehydes such as HNE or MDA, which if not eliminated from the cell could lead to additional oxidative damage. With the aid of the multidrug resistant protein-1 (MRP1), the GSH-aldehdye conjugate is exported out of the cell getting rid of the toxic reactive aldehydes (Sultana and Butterfield, 2004). The activities of most of these enzymes have been shown to be decreased in AD. In particular, it has been shown that GST activity is significantly decreased in AD brain and ventricular fluid relative to control (Lovell et al., 1998). Summaries of the glutathione-related reactions described here are given in Fig 2.4 above.

2.7.5 Heme-oxygenase-1 (HO-1)

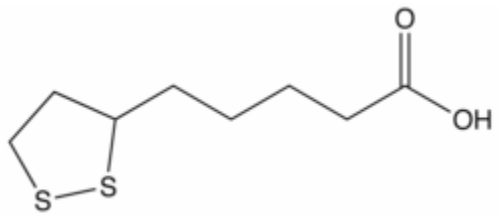
Heme oxygenase or heat shock protein 32 (HSP 32) is a member of the heat shock family of proteins. There are two key isoforms of heme oxygenase, i.e., the inducible form HO-1 and the constitutive form HO-2. Though recent studies have discovered a third isoforms, HO-3, its functions and role are still questionable and it is found only in rat brains. HO catalyzes the degradation of heme to iron (Fe), carbon monoxide, and biliverdin. Biliverdin is further converted to the potent antioxidant bilirubin by biliverdin reductase (Calabrese et al., 2003a; Maines, 2005). The levels of HO-1 are regulated by the activity of the antioxidant response element ARE, which is usually activated in events of increased oxidative stress or a decline in the levels of glutathione as seen in most aging and age-related neurodegenerative disorders. HO-1 levels in particular have been shown to be significantly decreased with age and in age-related neurodegenerative disorders. Interventions with antioxidant compounds that lead to its up-regulation have been found to have cytoprotective effects providing possible leads for the intervention against neurodegenerative disorders in which oxidative stress plays a key role (Calabrese et al., 2003b; Calabrese et al., 2004b; Calabrese et al., 2004a).

2.7.6 Lipoic acid

Lipoic acid (LA) Fig 2.20a is an example of the low molecular antioxidants which can also be found in its reduced form, i.e., dihydrolipoic acid (DHLA) (Fig 2.20b).
LA is a redox-active molecule capable of thiol-disulfide exchange, giving it its antioxidant activity. This antioxidant activity is derived from its ability to a) Scavenge ROS such as H_2O_2, $HO^{\cdot}$, NO and $ONOO^{-}$; b) LA has the ability to chelate ions such as, Cu^{2+} and Fe^{2+} thereby inhibiting further Fenton reactions that could lead to the

generation of additional ROS; c) Lastly, LA antioxidant activity is derived from its ability to recycle endogenous antioxidants. It also protects membranes by interacting with vitamin C and glutathione, which may in turn recycle vitamin E (Packer et al., 1995). Lipoic acid is also known as an essential cofactor of key mitochondrial enzyme complexes particularly pyruvate dehydrogenase (PDH) and alpha ketoglutarate dehydrogenase complex (α-KDH) hence its involvement in energy metabolism (Bogaert et al., 1994; Gibson et al., 2005). The use of lipoic acid by itself or in combination with other antioxidants as a potential therapeutic, is gaining considerable interest. Studies of aging rat have suggested that the use of lipoic acid results in improved memory performance and delayed structural mitochondrial decay. In a study from our laboratory, we have shown that aged SAMP8 mice treated with alpha lipoic acid show improvements in cognitive function as a result of decreased oxidative stress (Farr et al., 2003). This thus suggests that lipoic acid could be a potential therapeutic for those at risk of developing age-related neurodegenerative disorders like AD. Lipoic acid was one of the antioxidants include in the antioxidant fortified diet given to the aging canine.

a)



b)

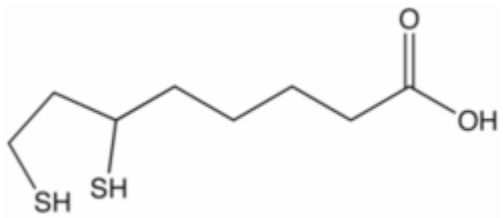


Figure 2.20 Structures of a) lipoic acid and b) dihydrolipoic acid.

2.8 Exercise

It has been established that voluntary physical activity and exercise training can favorably influence brain plasticity by facilitating neurogenerative, neuroadaptive, and neuroprotective processes (Neeper et al., 1995; Cotman and Berchtold, 2002; Cotman and Engesser-Cesar, 2002; Adlard and Cotman, 2004; Adlard et al., 2005a). Physical activity in particular has been shown to be inversely related to cognitive decline as a function of age. The mechanism leading to these beneficial effects of physical activity and exercise on the central nervous system are still unknown, but it is believed that neurotrophic factors like brain derived neurotrophic factor (BDNF) play a key role (Cotman and Berchtold, 2002; Adlard et al., 2005a). BDNF is involved in neuronal survival, enhances learning, and protects against cognitive decline. It has also been established that voluntary exercise decreases amyloid load in a transgenic model of Alzheimer's disease (Adlard et al., 2005b). Also, it has been shown that exercise benefits recovery of neuromuscular function from spinal cord injury (SCI) through the increase in the expression of key protective gene expression in the spinal cord in rats (Perreau et al., 2005). These studies among others continue to provide evidence for the beneficial effects of exercise in delaying the deleterious effects associated with age-related neurodegenerative disorders. The biochemical mechanisms that ensue following exercise have been investigated in the current dissertation.

2.9 Learning and memory

Learning can be defined as the acquisition of an altered behavioral response due to an environmental stimulus. Memory on the other hand, is defined as the process of storing and retrieving what has been learnt resulting from changes in synaptic structure and function (Sweatt, 2003). Memory can further be defined as either short-term or long-term depending on how long it persists. There are various theories that have been put forth to try and define and understand the mechanisms of molecular learning and memory. The most current one being the Hebb's theory that proposes that the simultaneous activation of pre and post synaptic neurons leads to an increase in synaptic efficacy leading to the formation of an associative link (Hebb, 1949).

Age-related neurodegenerative disorders and in particular AD are usually manifested with significant loss in hippocampus-dependent cognitive function, marked by loss in memory and the inability to learn and remember new names, events and spatial information. The mechanisms underlying age-dependent loss in cognitive function are still not well understood. However, using a canine model of human aging among others its emerging that oxidative stress plays a key role since some of this loss in cognitive function can be reversed with the use of antioxidants. The possible mechanisms leading to improved learning and memory in the canine model of human aging will be discussed in detail in Chapters 4 and 5.

CHAPTER THREE

Methods

3.1 Experimental Traumatic Brain injury

3.1.1 Animal and surgical procedures

The University of Kentucky Animal Use and Care Committee approved all procedures using animals. Male Sprague-Dawley rats (250–300 g), (Harlan Laboratories, IN) were used. All animals were housed in group cages on a 12-h light/dark cycle with free access to water and food. Animals were subjected to a moderate unilateral cortical contusion as previously described (Sullivan et al., 1999; Sullivan et al., 2000). This cortical contusion results in severe behavioral deficits, significant loss of cortical tissue, blood–brain barrier disruption and a loss of hippocampal neurons (Sullivan et al., 2002), mimicking some of the events that take place in human closed-head injury. Briefly, the subjects were anesthetized using isoflurane (2%) and placed in a stereotaxic frame (Kopf Instruments, Tujunga, CA). The head was positioned in the horizontal plane with the nose bar set at negative 5. Using sterile procedures, the skin was retracted and a craniotomy made with a hand-held trephine lateral to the sagittal suture and centered between Bregma and lambda. The skullcap was carefully removed without disrupting the underlying dura. The exposed brain was injured using a pneumatically controlled impacting device with a 5-mm beveled tip, which compressed the cortex at 3.5 m/s to a depth of 1.5 mm (Baldwin and Scheff, 1996; Sullivan et al., 1999). After injury, Surgicel (Johnson and Johnson, Arlington, TX) was laid on the dura and the skullcap replaced. A thin coat of dental acrylic was then spread over the craniotomy site and allowed to dry before the wound was stapled closed.

3.2 Mitochondria isolation

3.2.1 Overview

As discussed in Chapter two, mitochondria play a significant role in the pathosphysiology of most neurodegenerative disorders. Despite being the "power house" of the cell through the production of ATP, they are also known to be involved in the production of ROS and regulation of cell death process through either apoptosis or necrosis. As a result they are among the best organelles for the study of mechanism involved in most neurodegenerative disorders.

3.2.2 Mitochondria isolation procedure

Total brain mitochondria were isolated by the method of Sullivan (Sullivan et al., 2003; Sullivan et al., 2004b; Sullivan et al., 2004a). Desired tissues were dissected using a cork punch (d=8 mm). The tissue minced, and then homogenized separately in a glass dounce homogenizer containing approximately five times the volume of isolation buffer with 1 mM EGTA (215 mM mannitol, 75 mM sucrose, 0.1% BSA, 20 mM HEPES, 1 mM EGTA, pH 7.2). The homogenate was spun twice at 1300 × g for 3 min in an eppendorf microcentrifuge at 4◦C. The supernatant were then transferred to new tubes, topped off with isolation buffer with EGTA and spun at 13,000 × g for 10 min. The resulting supernatant was discarded and the pellet resuspended in 500 µL of isolation buffer with EGTA; The resulting pellet was then resuspended in 0.5 ml of mitochondrial isolation buffer (215 mM mannitol, 75 mM sucrose, 0.1% bovine serum albumin, 1 mM EGTA, 20 mM HEPES, pH 7.2), and the plasma membranes were ruptured by nitrogen decompression (Parr Cell Disruption Bomb) at 1000 p.s.i. for 5 min (Brown et al., 2004).

The homogenized tissue and an equal volume of 30 % Percoll in isolation buffer was added (~4 ml). The resultant homogenate was layered on a discontinuous Percoll gradient with the bottom layer containing 40 % Percoll solution in isolation buffer, followed by a 24 % Percoll solution, and finally the sample in a 15 % Percoll solution. The density gradients were spun in a Sorvall RC-5C plus super speed refrigerated centrifuge (Asheville, NC) in a fixed angle SE-12 rotor at 30,400 x g for 10 minutes. Following centrifugation, band 3 (Sims, 1990) were separately removed from the density gradient. The samples were washed by centrifugation at 16,700 x g for 15 minutes. The supernatant was discarded and the loose pellet was resuspended in the 1 ml of isolation buffer. The mitochondrial fractions were then placed in separate 15 ml conical tubes and an equal volume of 30 % Percoll was added to each sample and discontinuous Percoll density gradient centrifugation was performed again as described above. Band 3 was obtained from the gradients and 10 ml of isolation buffer without EGTA (215 mM mannitol, 75 mM sucrose, 0.1 % BSA, 20 mM HEPES, pH is adjusted to 7.2 with KOH) was added. The fractions were centrifuged at 16,700 x g for 15 minutes and subsequently at 11,000 x g for 10 minutes. The resultant pellet was resuspended in 1 ml of isolation buffer without EGTA and centrifuged at 10,000 x g for 10 minutes. The final mitochondrial pellet was resuspended in isolation buffer without EGTA to yield a protein concentration of ~10mg/ml and stored on ice for respiration assays or resuspended in lysis buffer (10 mM HEPES, 137 mM NaCl, 4.6 mM KCl, 1.1 mM KH_2PO_4, 0.6 mM $MgSO_4$ and 0.5 mg/mL leupeptin, 0.7 μg/mL pepstatin, 0.5 μg/mL trypsin inhibitor, and 40 μg/mL PMSF) for subsequent assays.

3.3 Mitochondria respiration

3.3.1 Overview

Isolated mitochondria are a very useful model used for the *in vivo* analysis of oxygen consumption in various paradigms. Isolated mitochondria are always depleted of substrates needed for energy metabolism. As a result, to successfully study respiratory control, mitochondria need to establish and maintain a chemiosmotic gradient unless they are deliberately poisoned. This is usually accomplished by the addition an excess of substrate depending on the state of respiration one is studying. Using an oxygen electrode, the amount of oxygen the isolated mitochondria consumes can then be analyzed. Thus, one can be able to determine how well isolated mitochondria can consume oxygen and hence determine the state of mitochondria in various conditions.

3.3.2 Mitochondria respiration assay

Mitochondrial oxygen consumption was measured by using a Clark-type electrode in a continuously stirred, thermostated sealed chamber (Oxytherm System; Hansatech Instruments Ltd.) at 37°C as previously described (Brown et al., 2004; Jin et al., 2004; Sullivan et al., 2004b). Isolated mitochondrial protein (~30 μg) was suspended in respiration buffer (215 mM mannitol, 75 mM sucrose, 20 mM HEPES, 2 mM $MgCl_2$, 2.5 mM inorganic phosphate, 0.1% BSA, Ph 7.2) in a final volume of 0.25 ml. The respiratory control ratio (RCR) was calculated as the ratio of oxygen consumption in the presence of 10 mM pyruvate and 5 mM malate or 10 mM succinate in the presence of ADP (state III) and in the absence of ADP (State IV).

3.4 Synaptosomes

3.4.1 Overview

Synaptosomes are used as models of functional synapse for the study of neuronal terminal processes. They are usually cleaved from homogenized neurons through the use of ultracentrifugation and a discontinuous sucrose gradient. Synaptosomes are composed of a continuous plasma membrane that have functional pumps and channels capable of ion exchange, and respond to depolarization. They also contain some mitochondria hence are able to carry out respiratory activities. It has also recently been shown that synaptosomes are capable of carrying out protein synthesis; hence they provide a complete model of a functional synapse.

3.4.2 Synaptosomes preparation

The University of Kentucky Animal Care and Use Committee approved animal protocols. Animals were housed in the University of Kentucky Central Animal Facility in 12 hour light/dark conditions and fed standard Purina rodent laboratory chow. During light phase Synaptosomes were isolated from three-month old male Mongolian gerbils (100g) as previously described (Whittaker, 1993). Briefly, the gerbils sacrificed by decapitation and the brain immediately isolated and dissected. The cortex was placed in 0.32 M sucrose isolation buffer containing 4 µg/ml leupeptin, 4 µg /ml pepstatin, 5 µg /ml aprotinin, 2 mM ethylene di-amine tetra-acetic acid (EDTA), 2 mM ethylene glycol-bis-tetraacetic acid (EGTA), 20 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES), 20 µg/ml trypsin inhibitor, and 0.2 mM phenylmethanesulfonyl fluoride (PMSF), pH 7.4. The tissue was homogenized by 20 passes with a Wheaton tissue

homogenizer. The homogenate was centrifuged at 1500 *g* for 10 minutes. The supernatant was retained and centrifuged at 20,000 *g* for 10 minutes. The resulting pellet was resuspended in ~1 ml of 0.32 M sucrose isolation buffer and layered over discontinuous sucrose gradients (0.85 M pH 8.0, 1.0 M pH 8.0, 1.18 M pH 8.5 sucrose solutions each containing 2 mM EDTA, 2 mM EGTA, and 10 mM HEPES) and spun at 82,500 *g* for 1 hr at 4^{o}C. Synaptosomes were collected from the 1.0/1.18M sucrose interfaces, and washed in Locke's buffer (154 mM NaCl, 5.6 mM KCl, 2.3 mM $CaCl_2$, 1.0 mM $MgCl_2$, 3.6 mM $NaHCO_3$, 5 mM glucose, 5 mM HEPES) twice for 10 minutes at 32,000 *g*. The synaptosomal preparation was relatively free of non-synaptic moieties (Whittaker, 1993).

3.5 Protein concentration

The protein concentrations of all samples in this dissertation were performed using the Bicinchoninic acid (BCA) protein assay. This method is based on the protein-mediated reduction of Cu^{2+} to Cu^{+} by the electron-rich peptide bond and amino acids (the biuret reaction). Cu^{+} can then be complex with BCA to from a soluble purple complex exhibits a strong absorbance at 562 nm (Figure 3.1). In this dissertation, bovine serum albumin (BSA) was used as a standard and Beer's law was used to calculate the concentration of protein in sample solution.

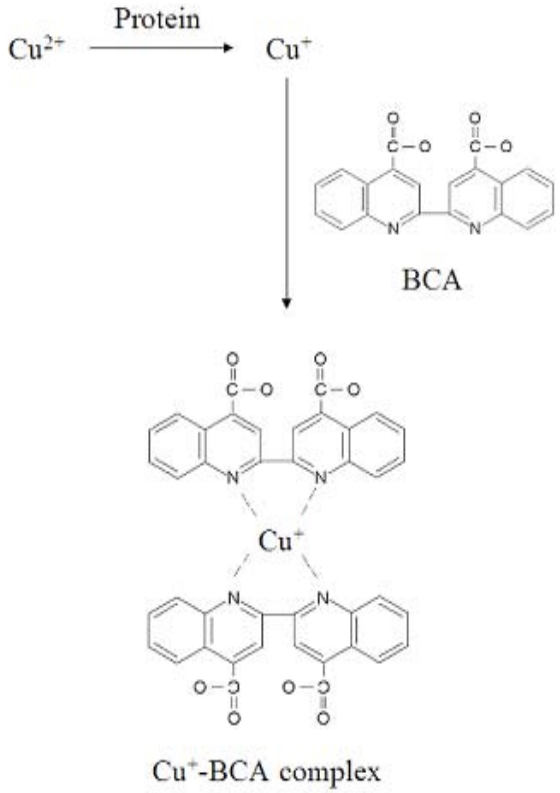


Figure 3.1 Reaction of Cu^{+} with BCA.

3.6 Cytochrome c release

3.6.1 Overview

Cytochrome c usually resides in the inner mitochondrial membrane as a member of the electron transport chain complexes. However, as mentioned in chapter two, its release from the inner mitochondrial membrane to the cytosol marks a key event in the initiation of the apoptotic cascade leading to cell death. As a result, analysis of the levels of Cytochrome c usually serves as a good indicator of cell death processes as seen in neurodegenerative disorders.

3.6.2 Cytochrome c release assay

Cytochrome c release was detected as previously described with slight modifications (Yang et al., 1997). Briefly, after centrifugation of mitochondrial samples, the cytosolic fraction which is the supernatant was used for Western blot analysis for cytochrome c release. The membrane was blocked in blocking buffer (3% bovine serum albumin) in PBS/Tween for 2 h and incubated with a 1:2000 dilution of anti-cytochrome c polyclonal antibody (C-5723; anti-sheep; Sigma) in PBS/Tween for 2 h. The membrane then was washed in PBS/Tween for 5 min three times after incubation. The membrane was incubated for 1 h, after washing, with an anti-sheep IgG alkaline phosphatase secondary antibody diluted in PBS/Tween in a 1:8000 ratio. The membrane then was washed three times in PBS/Tween for 5 min and developed in Sigma Fast tablets. Blots were dried, scanned with Adobe PhotoShop, and quantified using Scion Image software.

3.7 Bcl-2 protein levels

Bcl-2 family of proteins provides a link between apoptosis and mitochondrial physiology. Bcl-2 and Bcl-x_L are anti-apoptotic proteins that suppress release of sequestered matrix Ca^{2+} induced by uncouplers of respiration (Vander Heiden et al., 1999). In isolated mitochondria, Bcl-2 or Bcl-x_L enhances proton extrusion from mitochondria and increases mitochondrial Ca^{2+} buffering capacity. Bcl-2 is also thought to regulate the opening of the mPTP hence regulating the possible induction of apoptosis and eventual cell death. The levels of anti-apoptotic protein Bcl-2 were detected as previously described (Yang et al., 1997) with slight modification as described above, except a 1:2000 dilution of anti-Bcl-2 monoclonal antibody (AAM-072; anti- mouse; stressgen) in PBS/Tween for 2 h was used.

3.8 Oxidative stress parameters

3.8.1 Protein carbonyls

As mentioned in Chapter 2, increased oxidative stress usually results in the oxidative modification of proteins. The introduction of the carbonyl moiety in proteins makes protein carbonyls to be a significant index of protein oxidation. The levels of protein carbonyls in this dissertation were analyzed using the OxyBlot Protein Oxidation Kit (Chemicon International, Temecula, CA, USA). Briefly, samples (5 µg of protein) were derivatized with 10 mM 2, 4-dinitrophenylhydrazine (DNPH) in the presence of 5 µL of 12% sodium dodecyl sulfate for 20 min at room temperature (23°C) forming a Protein-DNP adduct (Fig 3.2). The samples were then neutralized with 7.5 µL of the neutralization solution (2 M Tris in 30% glycerol). Derivatized protein samples were then blotted onto a nitrocellulose membrane with a slot-blot apparatus (250 ng per lane). The

membrane was then washed with wash buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20) and blocked by incubation in the presence of 5% bovine serum albumin, followed by incubation with rabbit polyclonal anti-DNPH antibody (1: 100 dilution) as the primary antibody for 1 h. The membranes were washed with wash buffer and further incubated with alkaline phosphatase-conjugated goat anti-rabbit antibody as the secondary antibody for 1 h. Blots were developed using fast tablet (BCIP/NBT; Sigma-Aldrich) and densitometric levels quantified using Scion Image (PC version of Macintosh-compatible NIH Image) software.

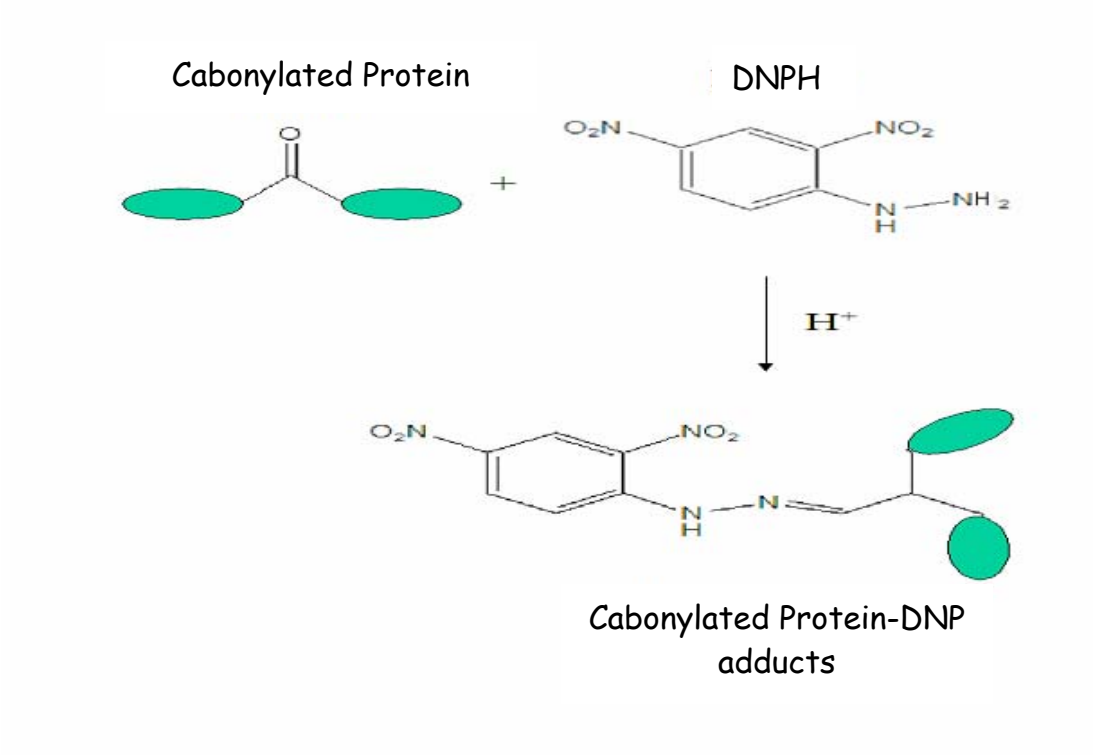


Figure 3.2 Protein carbonyl reactions with DNP forming the protein –DNP adduct.

3.8.2 3-Nitrotyrosine (3NT)

Protein nitration is another form of protein oxidation and can be indexed immunochemically by the levels of 3NT (Castegna et al., 2003). Briefly, samples were incubated with Laemmli sample buffer in a 1:2 ratio (0.125 M Trizma base, pH 6.8, 4% sodium dodecyl sulfate, 20% glycerol) for 20 min. Protein (250 ng) was then blotted onto the nitrocellulose paper using the slot-blot apparatus and immunochemical methods as described above for protein carbonyls. The mouse anti-nitrotyrosine antibody (5: 1000 dilutions) was used as the primary antibody and alkaline phosphatase-conjugated anti-mouse secondary antibody was used for detection. Blots were then scanned using scion imaging and densitometric analysis of bands in images of the blots was used to calculate levels of 3-NT.

3.8.3 4-Hydroxy-2-nonenal (HNE)

The level of lipid peroxidation in this dissertation was determined by the analysis of protein–bound HNE (Figure 2.7). Briefly, 10 µl of sample were incubated with 10 µl of Laemmli buffer containing 0.125 M Tris base pH 6.8, 4 % (v/v) SDS, and 20% (v/v) Glycerol. The resulting sample (250 ng) was loaded per well in the slot blot apparatus containing a nitrocellulose membrane under vacuum pressure. The membrane was blocked with 3% (w/v) bovine serum albumin (BSA) in phosphate buffered saline containing 0.01% (w/v) sodium azide and 0.2% (v/v) Tween 20 (PBST) for 1 h and incubated with a 1:5000 dilution of anti-4-hydroxynonenal (HNE) polyclonal antibody in PBST for 90 min. Following completion of the primary antibody incubation, the membranes were washed three times in PBST. An anti-rabbit IgG alkaline phosphatase secondary antibody was diluted 1:8000 in PBST and added to the membrane. The

membrane was washed in PBST three times and developed using Sigma fast Tablets (BCIP/NBT substrate). Blots were dried, scanned with Adobe Photoshop, and quantified with Scion Image.

3.8.4 Isoprostanes and Neuroprostanes

Another indicator of the levels lipid peroxidation used in this dissertation was determined through the analysis of Isoprostanes and Neuroprostanes. F_2-IsoPs and F_4-NPs were quantified in brain specimens using highly precise and accurate mass spectrometric assays applying stable isotope dilution techniques, as described previously (Morrow and Roberts, 1999; Musiek et al., 2004). Briefly, lipids were extracted from specimens by the method of Folch and colleagues (Folch et al., 1957). F_2-IsoP and F_4-NPs were esterified in tissue and hydrolyzed by chemical saponification, extracted using C_{18} and silica Sep-Pak cartridges (Waters Corporation, Milford, MA), purified by thin-layer chromatography, converted to pentafluorobenzyl ester trimethylsilyl ether derivatives, and quantified using gas chromatography/negative ion chemical ionization/mass spectrometry. The stable isotope dilution techniques used [$^2H_4$]-8-iso-PGF_{2a} as an internal standard for F_2-IsoPs and [$^{18}O_2$]17-F_{4c}-NP as an internal standard for F_4-NPs.

3.9 Enzyme assays

3.9.1Overview

One underlying hypothesis being fronted by our laboratory and others is that oxidative stress leads to the oxidative modification of key proteins and enzymes. In addition, supplementation with antioxidant and antioxidant related compounds has been shown to result in an increase in expression and in some conditions, activity of various protective enzymes. However, it is believed that oxidative modification of critical amino especially those found in the active sites of enzymes it leads to their inactivation and alteration in function. As a result, enzyme activity has particularly been used in this dissertation to try and confirm the hypothesis that oxidative modification of proteins/enzymes leads to their possible inactivation

3.9.2 Glutathione-S-transferase (GST)

The clearance of toxic reactive aldehydes is carried out through their conjugation to GSH catalyzed by GST. In particular, it has been shown that GSTs catalyze the conjugation of GSH to HNE 300 to 600 times faster than the uncatalyzed reaction. In this dissertation, the activity of Glutathione-S-transferase was measured as previously described using 1-chloro-2, 4-dinitrobenzene (CDNB) as substrate (Habig and Jakoby, 1981). Briefly, the standard assay mixture contained CDNB (1 mM), reduced glutathione (1 mM), and potassium phosphate buffer (100 mM; pH = 6.5) in a volume of 100 μL. The thioether formed was determined by reading the absorbance at 340 nm, and quantification was performed by using a molar absorptivity of 9.6 M^{-1}.

3.9.3 Superoxide dismutase (SOD)

As mentioned earlier, the first line of defense against ROS is through the enzyme SOD that catalyzes the disproportionation of superoxide anion to H_2O_2. In this dissertation the activity of total superoxide dismutase (SOD) was measured. Briefly, the reaction mixture of total volume 184 µL contained 160 µL of 50 mmol/l glycine buffers, pH 10.4, and 20.0 µL sample. The reaction was initiated by the addition of 4.0 µL of a 20 mg/ml solution of (–)-epinephrine. Due to its poor solubility, (–)-epinephrine (40 mg) was suspended in 2 ml water and was solubilized by adding 2–3 drops of 2N HCl. The auto-oxidation of (–)-epinephrine was monitored at 480nm and the millimolar absorptivity (4.02 mmol $\cdot$ l^{-1} $\cdot$ cm^{-1}) was used for calculations.

3.9.4 Pyruvate dehydrogenase (PDH)

Pyruvate dehydrogenase (PDH) is a mitochondrial enzyme complex that catalyzes the conversion of pyruvate to acetyl CoA, NADH and CO_2, making this protein the central link between glycolysis and the TCA cycle. In this dissertation, the activity of PDHC was measured using increment in fluorescence measurement of NADH with BioTek Synergy HT plate reader (Winooski, VT, USA). Briefly, mitochondria were freeze thawed and sonicated for 3 cycles. About 8 µg of double ficoll gradient purified mitochondrial protein were added into the buffer containing final concentration of 50mM KCl, 10mM HEPES pH 7.4, 0.3mM thiamine pyrophosphate (TPP), 10µM $CaCl_2$, 0.2mM $MgCl_2$, 5mM pyruvate, 1 µM rotenone and 0.2mM NAD^+. Reactions were started by addition of 0.14mM CoASH and assay was performed at Ex λ 340 nm/ Em λ 460nm and increases in fluorescence were observed at 1 min intervals at 30^0C. The PDHC activity was calculated and expressed as nmol/mg protein

3.9.5 Complex I assay

Complex I activity was measured in isolated mitochondria as the rotenone-sensitive decrease in NADH absorption at 340 nm with ubiquinone-1 as the final acceptor, as previously described (Sriram et al., 1998) with some slight modifications (Sullivan et al., 2004b). Briefly, mitochondria were freeze-thawed and sonicated three times and diluted 1 µg/µl in 10 mM K_3PO_4 buffer. The assay was performed in 25 mM K_3PO_4 buffer (pH 7.2) containing mitochondrial protein (6 µg), 5 mM $MgCl_2$, 1 mM KCN, 1 mg/ml BSA, and 150 µM NADH. The reaction was preincubated for 2 minutes at 30°C, the baseline established, and the reaction initiated by addition of coenzyme Q-1 (50 µM). The activity was measured by monitoring NADH fluorescence (340 nm excitation, >450 nm emission) over time under the same conditions as described above. The assay was also performed in the presence of rotenone (10 µM) to determine the rotenone-insensitive activity and the rotenone-sensitive complex I enzyme activity calculated by subtracting the rotenone-insensitive activity from the total activity. All the assay protocol for a 96-well plate performed with BioTek Synergy HT plate reader (Winooski, VT, USA)

3.9.6 Complex IV activity assay

Cytochrome C Oxidase (Complex IV) of mitochondrial electron transport chain component activity measured with method previously described (Wharton and Tzagoloff, 1967) with slight modification using BioTek Synergy HT plate reader (Winooski, VT, USA). Briefly, mitochondria were freeze thawed and sonicated for 3 cycles. About 8 µg of double ficoll gradient purified mitochondrial protein were added into the 10mM K_3PO_4

buffer pH 8.0, 50µM reduced cytochrome c and the decrease in absorbance was observed with difference with and without addition of Cytochrome c at 1 min interval at 37^0C at 550nm. First the rate constant k was calculated for oxidation reaction of ferricytochrome c calculated and the specific activity A were expressed as (k/mg protein) for cytochrome c oxidase.

3.10 Proteomics

3.10.1 Overview

Proteomics is defined as the study of the entire protein complement of the genome. The proteomic technique encompasses the analysis of complex protein mixtures leading to their identification and detection of possible quantitative changes. This separation of proteins is usually carried out in two dimensions.

In the first dimension, proteins are separated according to their isoelectric point. This is the pH, at which the protein carries no net charge and is therefore immobilized in the electric field (Fig 3.3a). This is carried out through isoelectric focusing (IEF) using immobilized pH gradients (IPG). IPGs are formed by the copolymerization of buffering and titrant groups of acrylamido derivatives into a polyacrylamide gel matrix hence permitting the steady state focusing with high reproducibility of spot positions.

In the second dimension, proteins are separated according to the rate of migration, which in most cases is similar to their molecular weight (Fig 3.3b). Here the low molecular weigh proteins travel faster through the gel and are found on the lower end of the gel. On the other hand, high molecular weight proteins will travel slowly through the polyacrylamide matrix and will be found on the upper end of the gel slab.

With the development of novel mass spectrometry techniques such as MALDI and ESI mass analyzers have been able to better resolve the molecular ion to their mass and charge ratio leading to the proper identification of proteins through various databases. The proteomic techniques in our laboratory and that of others has revolved around the identification of differentially expressed proteins in diseased verse control states and also the identification of the redox status of a protein, specifically oxidatively modified proteins. We have come to refer to the latter as redox proteomics.

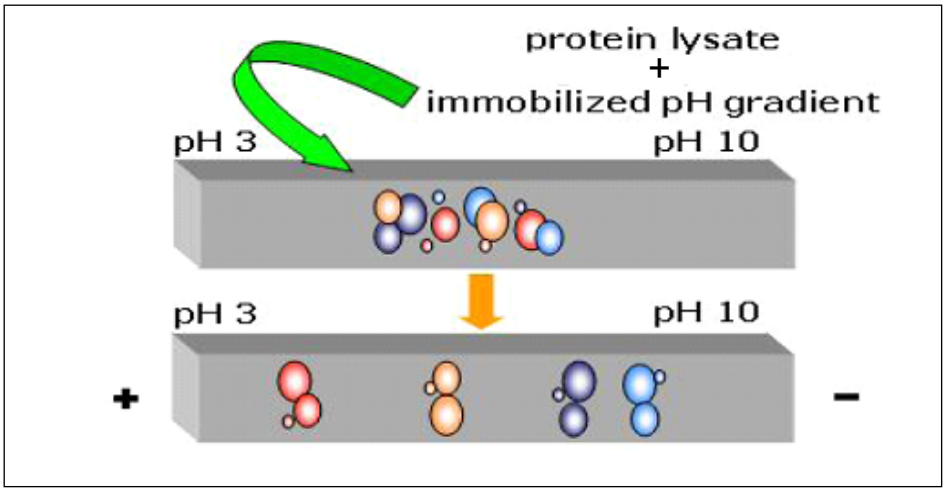


Figure 3.3a First dimension isoelectric focusing (IEF).

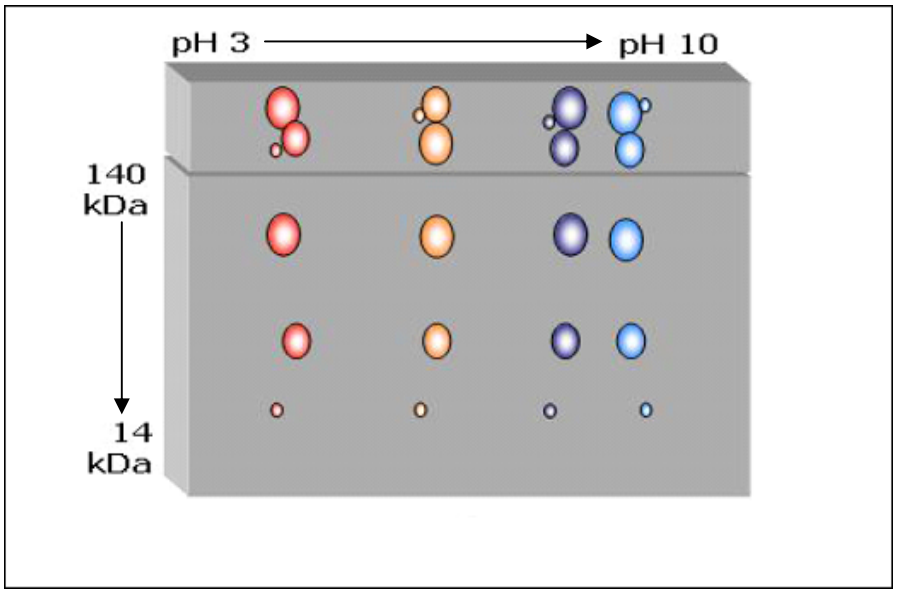


Figure 3.3b Second dimension polyacrylamide gel electrophoresis (PAGE).

3.10.2 Sample preparation

Samples (200 μg) were incubated with 4 volumes of 2N HCl for electrophoresis or 20mM DNPH for western blotting at room temperature for 20 min at room temperature. Proteins were then precipitated by the addition of ice-cold 100% trichloroacetic acid (TCA) to obtain a final concentration of 15% TCA. Samples were then placed on ice for 10 min and precipitate centrifuged at 16,000 g for 3min. The resulting pellet was then washed three times with a 1:1(v/v) ethanol/ethyl acetate solution. The samples were then suspended in 200 μl of rehydration buffer composed of a 1:1 ratio (v/v) of the Zwittergent solubilization buffer (7M urea, 2M thiourea, 2% Chaps, 65 mM DTT, 1% Zwittergent 0.8% 3-10 ampholytes and bromophenol blue) and ASB-14 solubilization buffer (7M urea, 2M thiourea 5Mn TCEP, 1% (w/v) ASB-14, 1% (v/v) Triton X-100, 0.5% Chaps, 0.5% 3-10 ampholytes) for 1 h.

3.10.3 First dimension electrophoresis

For the first-dimension electrophoresis, 200 μL of sample solution was applied to a 110-mm pH 3–10 ReadyStrip™ IPG strips (Bio-Rad, Hercules CA). The strips were then actively rehydrated in the protean IEF cell (Bio-Rad) at 50 V for 18 h. The isoelectric focusing was performed in increasing voltages as follows; 300 V for 1 h, then linear gradient to 8000 V for 5 h and finally 20 000 V/h. Strips were then stored at –80 °C until the 2nd dimension electrophoresis was to be performed.

3.10.4 Second dimension (2D) electrophoresis

For the second dimension, the IPG® Strips, pH 3–10, were equilibrated for 10 min in 50 mM Tris–HCl (pH 6.8) containing 6 M urea, 1% (w/v) sodium dodecyl sulfate (SDS), 30% (v/v) glycerol, and 0.5% dithiothreitol, and then re-equilibrated for 15 min in the same buffer containing 4.5% iodoacetamide instead of dithiothreitol. Linear gradient precast criterion Tris–HCl gels (8–16%) (Bio-Rad) were used to perform second dimension electrophoresis. Precision Protein™ Standards (Bio-Rad, CA) were run along with the sample at 200 V for 65 min.

3.10.5 Western blotting and immunochemical detection

The identification of oxidatively modified proteins was carried out through redox proteomics through the analysis of protein carbonyls levels. Here, the 200 μg proteins incubated with 20mN DNPH described above were used. The strips and gels were run as described above. After the second dimension, the proteins from the gels were transferred onto nitrocellulose papers (Bio-Rad) using the Transblot-Blot® SD semi-Dry Transfer Cell (Bio-Rad), at 15 V for 4 h. The 2,4-dinitrophenyl hydrazone (DNP) adduct of the carbonyls of the proteins was detected on the nitrocellulose paper using a primary rabbit antibody (Chemicon, CA) specific for DNP-protein adducts (1:100), and then a secondary goat anti-rabbit IgG (Sigma, MO) antibody was applied. The resulting stain was developed by application of Sigma-Fast (BCIP/NBT) tablets. In this dissertation these blots have been referred to as 2D-Oxyblots.

3.10.6 Image and statistical analysis

The spots on 2D gels were visualized using SYPRO™ Ruby stain, which is a fluorescent dye with sensitive (1-2ng) detection limits and linear dynamic range over three orders of magnitude (Molloy and Witzmann, 2002). Images from SYPRO Ruby stained gels, were obtained using a UV transilluminator (λ_{ex}=470 nm, λ_{em}=618 nm, Molecular Dynamics, Sunnyvale, CA, USA). On the other hand, 2D Oxyblots were scanned on a Microtek Scanmaker 4900 and saved in TIFF format for further analysis. With the use of PD-Quest (Bio-Rad) imaging software, gel-gel, blot-blot, and gel-blot analysis were performed, generating a large pool of data. After the completion of spot matching, the normalized intensities of each protein spot from individual gels/blots are compared between groups using statistical analysis. Those spots showing significant differential expression or oxidation are then excised from the gel and processed for in-gel digestion.

3.10.7 In-gel trypsin digestion

After the identification of the protein of interest and its excision from the gel, the spot is then subjected to digestion using proteolytic enzymes. In this dissertation, the digestive enzyme trypsin was used in all in gel digestion studies. Trypsin specifically cleaves peptide bonds after positively charged residues amino acids**.** In-gel-digestion reduces the mass of a protein into small peptides and also forms a collection of proteolytically sequence-specific peptides that enable the identification of the protein. These digested proteins are easily eluted from the gel and are ready for mass spectrometry analysis. In this dissertation excised spots were washed with 0.1 M

ammonium bicarbonate (NH_4HCO_3) at room temperature for 15 min. Acetonitrile was then added to the gel pieces and incubated at room temperature for 15 min. This solvent mixture was then removed and gel pieces dried. The protein spots were then incubated with 20 μL of 20 mM DTT in 0.1 M NH_4HCO_3 at 56 °C for 45 min. The DTT solution was removed and replaced with 20 μL of 55 mM iodoacetamide in 0.1 M NH_4HCO_3. The solution was then incubated at room temperature for 30 min. The iodoacetamide was removed and replaced with 0.2 mL of 50 mM NH_4HCO_3 and incubated at room temperature for 15 min. Acetonitrile (200 μL) was added. After 15 min incubation, the solvent was removed, and the gel spots were dried in a flow hood for 30 min. The gel pieces were rehydrated with 20 ng/μL-modified trypsin (Promega, Madison, WI) in 50 mM NH_4HCO_3 with the minimal volume enough to cover the gel pieces. The gel pieces were incubated overnight at 37 °C in a shaking incubator.

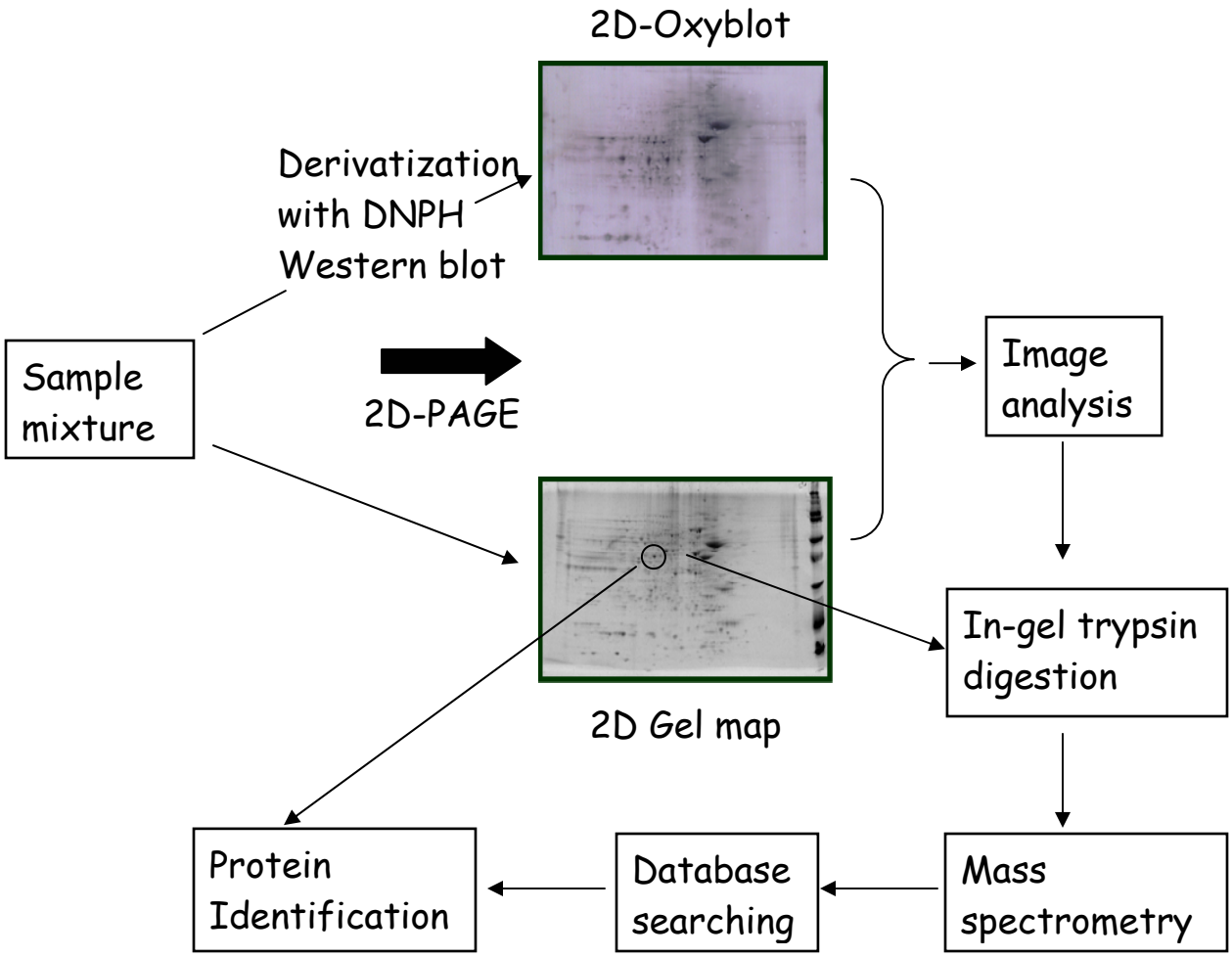


Figure 3.4 Schematic representation of the parallel analysis of 2D-PAGE and oxyblot for the identification of specifically carbonylated or differentially expressed proteins.

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3.11 Mass Spectrometry

3.11.1 Overview

Mass spectrometry technique has recently emerged as very invaluable tool in the rapid identification and characterization of proteins. This is particularly evident from the presentation of the 2005 chemistry Nobel Prize for the development of two novel ionization techniques i.e., matrix assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI). These softer ionization techniques have enabled the identification of large biomolecules with fewer fragmentations.

3.11.2 Matrix Assisted Laser Desorption/Ionization (MALDI)

MALDI is a pulsed ionization technique; generally coupled with TOF (time-of-flight) mass analyzer. In MALDI, the peptide sample is mixed with a matrix (commonly α-cyano-4-hydroxycinnamic acid) and condensed on a plate that is subjected to laser radiation. On application of a high-energy laser to the matrix, the peptides along with the matrix particles are vaporized. This usually occurs in a vacuum to ensure the sublimation of the matrix-peptides solid. Due to the acidic nature of the matrix, the positive ions of the peptides are formed in the gas phase the mechanisms of these are however not well described.

3.11.2.1 The MALDI method

All mass spectrometry data reported in this dissertation were acquired from the Core Proteomic Laboratory, University of Louisville, Louisville, Kentucky headed by Drs Jon. B. Klein and William Pierce. Briefly, MALDI-TOF mass spectrometer in the reflectron mode was used to generate peptide mass fingerprints. Peptides resulting from

in-gel digestion with trypsin were analyzed on a 384 position, 600 μm AnchorChip™ Target (Bruker Daltonics, Bremen, Germany) and prepared according to AnchorChip recommendations (AnchorChip Technology, Rev. 2, Bruker Daltonics, Bremen, Germany). Briefly, 1 μL of digestate was mixed with 1 μL of alpha-cyano-4-hydoxycinnamic acid (0.3 mg/mL in ethanol: acetone, 2:1 ratio) directly on the target and allowed to dry at room temperature. The sample spot was washed with 1 μL of a 1% TFA solution for approximately 60 seconds. The TFA droplet was gently blown off the sample spot with compressed air. The resulting diffuse sample spot was recrystallized (refocused) using 1 μL of a solution of ethanol: acetone: 0. 1 % TFA (6:3:1 ratio). Reported spectra are a summation of 100 laser shots. External calibration of the mass axis was used for acquisition and internal calibration using either trypsin autolysis ions or matrix clusters was applied post acquisition for accurate mass determination.

3.11.3 Electrospray Ionization (ESI)

In ESI the peptide in the solution is sprayed through a narrow capillary tube maintained at ~4000 volts and atmospheric pressure. The potential difference between the outlet and the mass spectrometer leads to the generation of a repulsive columbic force between the like charges on the droplets to extend the solution to form a cone (Taylor cone) from the outlet, causing the solution to disperse into fine droplets (Taylor, 1964). ESI can be coupled to other separation techniques such as HPLC making it more versatile. However the high flow rates and short analysis time presents a drawback when performing multiple analyses.

3.11.4 Data base searching and bioinformatics

Mass spectrometry analysis usually results in a mass spectrum that consists of peaks, which correlate to the mass of peptides obtained following protein digestion. Using various software programs and performing data base searches through different search algorithms, the identity of specific proteins can be obtained. This process is referred to as peptide mass finger printing. The search engines (Table 3.1) provide a theoretical digest of all known proteins, which can be compared with the experimental masses obtained. These comparisons can also be done taking it account several other factors, such as; molecular weight, pI, the probability of a single peptide to occur in the whole database, knowledge of peptide modifications, among many others. For each entry, a probability score is provided based on a mathematical algorithm specific to each search engine. This score, represents the probability that the experimental peptides match those of the theoretical digest. A hit with a probability score corresponding to $p< 0.05$ has a legitimate chance of being the protein cut from a particular spot.

Name	Address
Mass Search	http://cbrg.inf.ethz.ch
Peptide Search	http://www.mann.emblheidelberg.de
Mascot	http://www.matrixscience.com
MOWSE	http://www.hgmp.ac.uk/Bioinformatics/Webapp/mowse
SEQUEST	http://thompson.mbt.washington.edu/sequest

Table 3.1 Table of search engines.

3.11.5 Analysis of peptide sequences

In the current dissertation the MASCOT search engine based on the entire NCBI and SwissProt protein databases. Database searches were conducted allowing for up to one missed trypsin cleavage and using the assumption that the peptides were monoisotopic, oxidized at methionine residues, and carbamidomethylated at cysteine residues. Mass tolerance of 150 ppm, 0.1 Da peptide tolerance and 0.2 Da fragmentation tolerance was the window of error allowed for matching the peptide mass values (Butterfield and Castegna, 2003b). Probability-based MOWSE scores were estimated by comparison of search results against estimated random match population and were reported as -10*log10 (p), where p is the probability that the identification of the protein is a random event. MOWSE scores greater than 63 were considered to be significant ($p < 0.05$).

3.11.6 Protein Interacteome

The proteins identified through proteomics have been found to be involved in a wide range of functions, and are also believed to interact with a plethora of molecules and other proteins. To enable us obtain a consequential possibility that will result from their alterations and how these affect various cellular process, the Interaction Explorer ™ Software Pathway Assist software package (Stratagene, La Jolla, CA) was used in the current dissertation. Pathway Assist is software for functional interaction analysis. It allows for the identification and visualization of pathways, gene regulation networks and protein interaction maps (Donninger et al., 2004). The proteins are first imported as the gene symbols as a set of data. This data set is then searched against ResNet, a database

containing over 500,000 biological interactions built by applying the MedScan text-mining algorithms to all PubMed abstracts. These interactions are then visualized by building interaction networks with shortest-path algorithms. This process can graphically identify all known interaction among the proteins. The information of the function of these proteins and their relevance to diseases are then obtained by using the BIOBASE's Proteome BioKnowledge Library form Incyte Corporation (Incyte, Wilmington, DE) (Hodges et al., 2002).

CHAPTER FOUR

Redox Proteomic Identification of Less Oxidized Brain Proteins Following a Long-Term Treatment with Antioxidants and a Program of Behavioral Enrichment in the Canine Model of Human Aging

4.1 Overview

Oxidative stress may be one of the main mechanisms contributing to neurodegeneration and cognitive decline observed in a number of age-related neurodegenerative disorders such as Alzheimer's disease (AD). The use of antioxidants results in reductions in oxidative damage and in improvements in cognitive function in many animal models. In the present study, the effect of a long-term treatment with an antioxidant fortified diet and a program of behavioral enrichment on oxidative damage was studied in aged canines. The parietal cortex from 21 beagle dogs was obtained from animals placed in one of four treatment groups: i.e., control food- control behavioral enrichment (CC); control food - behavioral enrichment (CE); antioxidant food-control behavioral enrichment (CA); and enriched environment - antioxidant fortified food (EA). We analyzed the levels of the oxidative stress biomarkers, i.e., protein carbonyls, 3-nitrotyroine (3NT), and the lipid peroxidation product, 4-hydroxynonenal (HNE), and observed a decrease in their levels on all treatments paradigms compared to control. The most significant effects were found in the combined treatment, EA. We also used a parallel redox proteomics approach to identify proteins that were less oxidized from the parietal cortex of the beagle dog brain. The specific protein carbonyl levels of glutamate dehydrogenase [NAD (P)], glyceraldehyde-3-phosphate dehydrogenase (GAPDH), α-enolase, neurofilament triplet L protein, glutathione S-transferase (GST) and fascin actin bundling protein were significantly reduced in EA treated

dogs compared to control. In addition, we observed a significant increase in the activities of glutathione-S-transferase (GST) and total superoxide dismutase (SOD), and significant increase in the protein levels of heme oxygenase (HO-1) in EA treated dogs compared to control. In addition, a subset of proteins and antioxidant enzymes were found to correlate with an improvement in cognitive function. These results suggest that the combined treatment reduces the levels of oxidative damage and improves the antioxidant reserve systems in the aging canine brain, and may contribute to improvements in learning and memory, hence providing a possible neurobiological mechanism underlying the effects of the combined treatment. These results support combination treatments as a possible therapeutic approach that could be translated to the aging human population who are at risk for age-related neurodegenerative disorders, including Alzheimer's disease.

4.2 Introduction

Aged dogs naturally develop cognitive deficits and accumulate brain pathology similar to aging humans, providing a useful model for studying the neurobiological mechanisms underlying age- related cognitive dysfunction (Head et al., 2000; Head and Torp, 2002). Aged canine show reduced cerebral volume, cortical atrophy, ventricular widening by in vivo magnetic resonance imaging (Su et al., 1998; Tapp et al., 2004; Tapp et al., 2006). The aging canine also shows impairment on visuospatial working memory and executive function (Chan et al., 2002; Tapp et al., 2003c; Studzinski et al., 2006). Aged beagle brain deposits amyloid-β-peptide (Aβ) that is of the same sequence as humans (Selkoe et al., 1987; Johnstone et al., 1991) and is correlated with decline in cognitive function with age (Cummings et al., 1996b; Head et al., 1998). Beagle dogs are accessible, easy to handle, capable of learning a broad repertoire of cognitive tasks, do not need food deprivation to be motivated and absorb dietary nutrients in similar ways as humans, hence making them a good model for studying human aging (Cummings et al., 1996a). The deposition of Aβ could play a significant role in molecular pathways involving free radical generation and oxidative stress as previously shown in AD-related studies from our laboratory (Butterfield and Lauderback, 2002; Butterfield and Boyd-Kimball, 2004a).

The aging process is associated with a progressive accumulation of oxidative damage that could play a role in the development or accumulation of neuropathology typically observed in age-related neurodegenerative disorders like AD (Hensley et al., 1996; Markesbery, 1997; Lovell et al., 1999; Butterfield and Kanski, 2001). When compared to age-matched controls, the AD brain shows a higher levels of protein and

DNA oxidation, and lipid peroxidation leading to loss of function of key enzymes (Smith et al., 1991; Hensley et al., 1995; Lovell et al., 1999). In various AD studies from our laboratory, we have shown that Aβ 1-42 plays a central role in the oxidative stress observed and that the key to this link is a key amino acid residue methionine 35 (Butterfield and Lauderback, 2002; Butterfield and Boyd-Kimball, 2004a). Similar events may also occur in the canine model of aging as deposits of Aβ1-42 may account for increased oxidative damage, a decline in glutathione content and decreased glutamine synthetase (GS) activity reported previously (Head et al., 2002).

The use of antioxidants and/or related compounds reduces the level of oxidative damage and delays or reduces age-related cognitive decline in both animal models and in humans (Joseph et al., 1998a; Bickford et al., 2000; Milgram et al., 2002c). Previous studies in aged canines show that oxidative damage may be critically involved in the maintenance of cognitive function and long-term treatment with antioxidants and a program of behavioral enrichment reduces cognitive decline (Cotman et al., 2002; Head et al., 2002; Milgram et al., 2004; Milgram et al., 2005). Further, an antioxidant enriched diet alone leads to rapid improvements in complex learning ability in aging dogs (Cotman et al., 2002; Milgram et al., 2002c). However, the neurobiological changes elicited by these two interventions alone or in combination have yet to be established. We show here that there is a significant reduction in the levels of oxidative damage as measured by levels of protein carbonyls, 3NT and HNE, following a combination of the treatments (EA). Using redox proteomics, we identified six proteins that were significantly less oxidized in animal receiving the combined treatment relative to control animals and these included: glutamate dehydrogenase (GDH), glyceraldehyde-3-

phosphate dehydrogenase (GAPDH), α-enolase, neurofilament triplet L protein, glutathione-S-transferase (GST) and fascin actin bundling protein. This improvement was also associated with increased activity of the antioxidant enzymes glutathione-S-transferase (GST) and superoxide dismutase (SOD) and an increase in the protein levels of inducible heme oxygenase (HO-1), a well known neuroprotective chaperone of the family of heat shock proteins (Poon et al., 2004b). A subset of proteins and antioxidant enzymes analyzed were also found to correlate with an improvement in cognitive function. As a result, the use of antioxidants composed of mitochondrial cofactors and cellular antioxidants and a program of behavioral enrichment in the present study could potentially protect proteins from oxidative damage and enhance mitochondrial function leading to the observed improved memory and cognitive function in this model.

4.3 Experimental procedures

The breeding and maintenance of the animals used in the studies described here and in chapter 5 were done by our collaborators at the Lovelace Respiratory Research Institute Albuquerque NM. In addition, behavioral and cognitive tasks and correlational analysis were carried out by Dr Head and group.

4.3.1 Subjects

Twenty-four beagle dogs ranging in from 8.05-12.35 yrs at the start of the study (Mean = 10.69 yrs SE=0.25) were obtained from the colony at the Lovelace Respiratory Research Institute (Table 4.1). These study animals were bred and maintained in the same environment and all had documented dates of birth and comprehensive medical histories. At the time of euthanasia, 23 dogs had received the intervention and ranged in age from 10.72-15.01 yrs (Mean=13.31 yrs, SE=0.26) with one animal not completing the baseline phase of the study. All research was conducted in accordance with approved IACUC protocols.

Table 4.1 Summary of age, treatment groups and cause of death of subjects used

Dog	Date	Animal	Treatment	Age at Start of Study (yrs)	Age at End of Study (yrs)	Duration of Intervention (yrs)	Cause of Death
1494D	10/15/01	1	C/C	12.1	15.0	2.8	Study End
1508U	10/15/01	2	C/C	11.4	13.5	1.9	*Congestive heart failure
1510A	10/15/01	3	C/C	11.3	14.2	2.7	Study End
1521S	10/15/01	4	C/C	10.7	13.6	2.8	Study End
1543S	10/15/01	5	C/C	10.1	13.0	2.8	Study End
B2150	10/15/01	6	C/C	11.6	14.5	2.8	Study End
Mean C/C				11.2	14.0	2.6	
1492B	10/15/01	7	E/C	12.1	12.5	0.3	*Liver degeneration, pancreatitis
1506B	10/15/01	8	E/C	11.5	14.4	2.8	Study End
1518D	10/15/01	9	E/C	10.8	13.7	2.8	Study End
1523U	10/15/01	10	E/C	9.6	12.2	2.5	Anorexia
1529S	10/15/01	11	E/C	10.4	13.3	2.8	Study End
1542S	10/15/01	12	E/C	10.1	13.0	2.8	Study End
Mean E/C				10.7	13.2	2.3	
1491B	10/15/01	13	C/A	12.1	15.0	2.7	Study End
1508A	10/15/01	14	C/A	11.4	14.3	2.8	Study End
1509U	10/15/01	15	C/A	11.3	13.8	2.4	Abscess in left axilla
1523B	10/15/01	16	C/A	9.6	12.5	2.7	Study End
1532S	10/15/01	17	C/A	10.4	13.3	2.8	Study End
1581S	10/15/01	18	C/A	8.1	11.0	2.7	Study End
Mean C/A				10.5	13.3	2.7	
1502S	10/15/01	19	E/A	11.9	14.8	2.8	Study End
1521B	10/15/01	20	E/A	10.7	13.6	2.7	Study End
1541B	10/15/01	21	E/A	10.1	13.0	2.7	Study End
1542T	10/15/01	22	E/A	10.1	13.0	2.8	Study End
1581T	10/15/01	23	E/A	8.1	11.0	2.7	Study End
1585A	10/15/01	24	E/A	7.8	10.7	2.7	Study End
Mean E/A				10.5	13.3	2.7	

C/C - control enrichment/control diet, C/A - control enrichment/antioxidant diet

E/C - behavioral enrichment/control diet, E/A - behavioral enrichment/antioxidant diet

4.3.2 Group Assignments and study timeline

All study dogs underwent extensive baseline cognitive testing as described previously (Milgram et al., 2002a). Animals were subsequently ranked based on cognitive test scores and placed into 4 groups. These four groups were randomly assigned as one of the treatment conditions as follows: C/C – control enrichment/control diet, E/C – behavioral enrichment /control diet, C/A – control enrichment/antioxidant diet, E/A – behavioral enrichment /antioxidant diet.

4.3.3 Behavioral enrichment treatment

The behavioral enrichment protocol consisted of social enrichment, by housing animals in pairs, environmental enrichment, by providing play toys, physical enrichment, by providing two 20-minute outdoor walks per week, and cognitive enrichment, through continuous cognitive testing. The cognitive enrichment consisted of a landmark discrimination task, an oddity discrimination task (Milgram et al., 2002b), and size concept learning (Tapp et al., 2003a). In addition, all animals, regardless of treatment condition were evaluated annually on a test of visuospatial memory (Chan et al., 2002), object recognition memory (Callahan, 2000) and either size discrimination and reversal learning (Tapp et al., 2003b), or black/white discrimination and reversal on consecutive years.

4.3.4 Diet treatment

The two foods were formulated to meet the adult maintenance nutrient profile for the American Association of Feed Control Officials recommendations for adult dogs (AAFCO 1999). Control and test foods were identical in composition, other than

inclusion of a broad-based antioxidant and mitochondrial co-factor supplementation to the test food. The control and enriched foods had the following differences on an as-fed basis, respectively: dl-alpha-tocopherol acetate (vitamin E, approximately 100 vs. 1000 ppm), L-carnitine (<20 ppm vs. approximately 250 ppm), dl-alpha-lipoic acid (<20 ppm vs. approximately 120 ppm), ascorbic acid or vitamin C as Stay-C (<30 ppm vs. approximately 80 ppm), and 1% inclusions of each of the following (1 to 1 exchange for corn): spinach flakes, tomato pomace, grape pomace, carrot granules and citrus pulp. The rationale for these inclusions were as follows: Vitamin E is lipid soluble and acts to protect cell membranes from oxidative damage; vitamin C is essential in maintaining oxidative protection for the soluble phase of cells as well as preventing vitamin E from propagating free radical production (Butterfield et al., 2002c); alpha-lipoic acid is a cofactor for the mitochondrial respiratory chain enzymes, pyruvate and alpha-ketoglutarate dehydrogenase, as well as an antioxidant capable of redox recycling other antioxidants and raising intracellular glutathione levels (Pocernich and Butterfield, 2003); L-carnitine is a precursor to acetyl-L-carnitine and is involved in mitochondrial lipid metabolism and maintaining efficient function (Calabrese et al., 2006). Fruits and vegetables are rich in flavonoids and carotenoids and other antioxidants (Joseph et al., 1998b; Bickford et al., 2000). To define this further, added inclusions were measured for oxygen radical absorbing capacity (ORAC) as well as carotenoid and flavonoid profiles (Cao et al., 1995). Fruit and vegetables selected for inclusion were based on ORAC content and general commercial availability. Results of this analysis revealed that ORAC content of the individual fruit and vegetable inclusions were higher than the corn for which they were substituted. In addition, inclusion of these ingredients, in combination

with the vitamins, resulted in increased ORAC content of the finished product. The food was produced by an extrusion process and a production batch was fed for no more than 6 months before a new lot was manufactured.

4.3.5 Cognitive testing

All animals were given annual tests of cognition to detect changes in response to the different treatments. Within 8 months of euthanasia, animals were given an black/white discrimination and reversal problem that is impaired in aged animals and is significantly improved in both antioxidant treated and/or behaviorally enriched animals (Milgram et al., 2005). Also within a year of the end of the study, spatial memory was tested using a nonmatching to position paradigm described previously to be sensitive to age in dogs (Chan et al., 2002). All of the testing procedures were described in previous publications (Chan et al., 2002; Milgram et al., 2005).

4.3.6 Animal euthanasia

Twenty minutes before induction of general anesthesia, animals were sedated by subcutaneous injection with 0.2 mg/kg acepromazine. General anesthesia was induced by inhalation with 5% isoflurane. While being maintained under anesthesia, dogs were exsanguinated by cardiac puncture and blood samples were collected to obtain plasma and serum for future studies. Within 15 minutes, the brain was removed from the skull and a cerebrospinal fluid sample was obtained from the lateral ventricles. The brain was sectioned midsagitally, with the entire left hemisphere being immediately placed in 4% paraformaldehyde for 48-72 hours at 4°C prior to long term storage in phosphate buffered

saline with 0.05% sodium azide at 4°C. The remaining hemispheres were sectioned coronally and flash frozen at -80°C and the parietal cortex was dissected for use in the current studies. The dissection procedure was completed within 20 minutes. Thus, the post mortem interval for all animals was 35-45 minutes.

4.3.7 Measurement of protein carbonyls

Protein carbonyls are an index of protein oxidation and were determined as described (Butterfield and Stadtman, 1997). Briefly, samples (5 μg of protein) were derivatized with 10 mM 2, 4-dinitrophenylhydrazine (DNPH) in the presence of 5 μL of 12% sodium dodecyl sulfate for 20 min at room temperature (23°C). The samples were then neutralized with 7.5 μL of the neutralization solution (2 M Tris in 30% glycerol). Derivatized protein samples were then blotted onto a nitrocellulose membrane with a slot-blot apparatus (250 ng per lane). The membrane was then washed with wash buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20) and blocked by incubation in the presence of 5% bovine serum albumin, followed by incubation with rabbit polyclonal anti-DNPH antibody (1: 100 dilution) as the primary antibody for 1 h. The membranes were washed with wash buffer and further incubated with alkaline phosphatase-conjugated goat anti-rabbit antibody as the secondary antibody for 1 h. Blots were developed using fast tablet (BCIP/NBT; Sigma-Aldrich) and quantified using Scion Image (PC version of Macintosh-compatible NIH Image) software. No non-specific background binding of the primary or secondary antibodies was found.

4.3.8 Measurement of 3-nitrotyrosine (3-NT)

Nitration of proteins is another form of protein oxidation (Castegna et al., 2003; Sultana et al., 2006b). The nitrotyrosine content was determined immunochemically as previously described (Drake et al., 2003b). Briefly, samples were incubated with Laemmli sample buffer in a 1:2 ratio (0.125 M Trizma base, pH 6.8, 4% sodium dodecyl sulfate, 20% glycerol) for 20 min. Protein (250 ng) was then blotted onto the nitrocellulose paper using the slot-blot apparatus and immunochemical methods as described above for protein carbonyls. The mouse anti-nitrotyrosine antibody (5: 1000 dilutions) was used as the primary antibody and alkaline phosphatase-conjugated anti-mouse secondary antibody was used for detection. Blots were then scanned using scion imaging and densitometric analysis of bands in images of the blots was used to calculate levels of 3-NT. No non-specific binding of the primary or secondary antibodies was found.

4.3.9 Measurement of 4-hydoxynonenal (HNE)

HNE is a marker of lipid oxidation and the assay was performed as previously described (Lauderback et al., 2001). Briefly, 10 µl of sample were incubated with 10 µl of Laemmli buffer containing 0.125 M Tris base pH 6.8, 4 % (v/v) SDS, and 20% (v/v) Glycerol. The resulting sample (250 ng) was loaded per well in the slot blot apparatus containing a nitrocellulose membrane under vacuum pressure. The membrane was blocked with 3% (w/v) bovine serum albumin (BSA) in phosphate buffered saline containing 0.01% (w/v) sodium azide and 0.2% (v/v) Tween 20 (PBST) for 1 h and incubated with a 1:5000 dilution of anti-4-hydroxynonenal (HNE) polyclonal antibody in

PBST for 90 min. Following completion of the primary antibody incubation, the membranes were washed three times in PBST. An anti-rabbit IgG alkaline phosphatase secondary antibody was diluted 1:8000 in PBST and added to the membrane. The membrane was washed in PBST three times and developed using Sigmafast Tablets (BCIP/NBT substrate). Blots were dried, scanned with Adobe Photoshop, and quantified by Scion Image. A small background of the primary antibody binding to the membrane was found, but this was scattered from both control and subject blots.

4.3.10 Two-dimensional electrophoresis

Brain samples (200 μg) were incubated with 4 volumes of 2N HCl at room for electrophoresis or 20mM 2, 4-dinitrophenyl hydrazine (DNPH) for western blotting at room temperature for 20 min. Proteins were then precipitated by the addition of ice-cold 100% trichloroacetic acid (TCA) to obtain a final concentration of 15% TCA. Samples were then placed on ice for 10 min and precipitate centrifuged at 16,000 g for 3min. The resulting pellet was then washed three times with a 1:1(v/v) ethanol/ethyl acetate solution. The samples were then suspended in 200 μl of rehydration buffer composed of a 1:1 ratio (v/v) of the Zwittergent solubilization buffer (7M urea, 2M thiourea, 2% Chaps, 65 mM DTT, 1% Zwittergent 0.8% 3-10 ampholytes and bromophenol blue) for 1 h.

4.3.11 First dimension electrophoresis

For the first-dimension electrophoresis, 200 μL of sample solution was applied to a 110-mm pH 3–10 ReadyStrip™ IPG strips (Bio-Rad, Hercules CA). The strips were then actively rehydrated in the protean IEF cell (Bio-Rad) at 50 V for 18 h. The

isoelectric focusing was performed in increasing voltages as follows; 300 V for 1 h, then linear gradient to 8000 V for 5 h and finally 20 000 V/h. Strips were then stored at –80 °C until the second dimension electrophoresis was to be performed.

4.3.12 Second dimension electrophoresis

For the second dimension, the IPG® Strips, pH 3–10, were equilibrated for 10 min in 50 mM Tris–HCl (pH 6.8) containing 6 M urea, 1% (w/v) sodium dodecyl sulfate (SDS), 30% (v/v) glycerol, and 0.5% dithiothreitol, and then re-equilibrated for 15 min in the same buffer containing 4.5% iodoacetamide instead of dithiothreitol. Linear gradient precast criterion Tris–HCl gels (8–16%) (Bio-Rad) were used to perform second dimension electrophoresis. Precision Protein™ Standards (Bio-Rad, CA) were run along with the sample at 200 V for 65 min.

4.3.13 SYPRO ruby staining

After the second dimension electrophoresis, the gels were incubated in fixing solution (7% acetic acid, 10% methanol) for 20 min and stained overnight at room temperature with 50ml SYPRO Ruby gel stain (Bio-Rad). The SYPRO ruby gel stain was then removed and gels stored in DI water.

4.3.14 Western Blotting

Brain samples (200 μg) incubated with 20mM DNPH were used for western blotting. The strips and gels were run as described above. After the second dimension, the proteins from the gels were transferred onto nitrocellulose papers (Bio-Rad) using the Transblot-Blot® SD semi-Dry Transfer Cell (Bio-Rad), at 15 V for 4 h. The 2,4-

dinitrophenyl hydrazone (DNP) adduct of the carbonyls of the proteins was detected on the nitrocellulose paper using a primary rabbit antibody (Chemicon, CA) specific for DNP-protein adducts (1:100), and then a secondary goat anti-rabbit IgG (Sigma, MO) antibody was applied. The resulting stain was developed by application of Sigma-Fast (BCIP/NBT) tablets.

4.3.15 Image analysis

The nitrocellulose blots (oxyblots) were scanned and saved in TIFF format using Scan jet 3300C (Hewlett Packard, CA). SYPRO ruby-stained gel images were obtained using a STORM phosphoimager (Ex. 470 nm, Em. 618 nm, Molecular Dynamics, Sunnyvale, CA, USA) and also saved in TIFF format. PD-Quest (Bio-Rad) imaging software was then used to match and analyze visualized protein spots among differential 2D gels and 2D oxyblots, with one blot and one gel for each individual sample.

4.3.16 In-gel trypsin digestion

In those brain proteins less oxidized from EA dogs compared to CC dogs as judged by PDQuest analysis, protein spots were digested by trypsin using protocols previously described (Thongboonkerd et al., 2002). Briefly, spots of interest were excised using a clean blade and placed in Eppendorf tubes, which were then washed with 0.1 M ammonium bicarbonate (NH_4HCO_3) at room temperature for 15 min. Acetonitrile was then added to the gel pieces and incubated at room temperature for 15 min. This solvent mixture was then removed and gel pieces dried. The protein spots were then incubated

with 20 μL of 20 mM DTT in 0.1 M NH_4HCO_3 at 56 °C for 45 min. The DTT solution was removed and replaced with 20 μL of 55 mM iodoacetamide in 0.1 M NH_4HCO_3. The solution was then incubated at room temperature for 30 min. The iodoacetamide was removed and replaced with 0.2 mL of 50 mM NH_4HCO_3 and incubated at room temperature for 15 min. Acetonitrile (200 μL) was added. After 15 min incubation, the solvent was removed, and the gel spots were dried in a flow hood for 30 min. The gel pieces were rehydrated with 20 ng/μL-modified trypsin (Promega, Madison, WI) in 50 mM NH_4HCO_3, with the minimal volume enough to cover the gel pieces. The gel pieces were incubated overnight at 37 °C in a shaking incubator.

4.3.17 Mass spectrometry

A MALDI-TOF mass spectrometer in the reflectron mode was used to generate peptide mass fingerprints. Peptides resulting from in-gel digestion with trypsin were analyzed on a 384 position, 600 μm AnchorChip^{TM} Target (Bruker Daltonics, Bremen, Germany) and prepared according to AnchorChip recommendations (AnchorChip Technology, Rev. 2, Bruker Daltonics, Bremen, Germany). Briefly, 1 μL of digestate was mixed with 1 μL of alpha-cyano-4-hydroxycinnamic acid (0.3 mg/mL in ethanol: acetone, 2:1 ratio) directly on the target and allowed to dry at room temperature. The sample spot was washed with 1 μL of a 1% TFA solution for approximately 60 seconds. The TFA droplet was gently blown off the sample spot with compressed air. The resulting diffuse sample spot was recrystallized (refocused) using 1 μL of a solution of ethanol: acetone: 0. 1 % TFA (6:3:1 ratio). Reported spectra are a summation of 100 laser shots. External calibration of the mass axis was used for acquisition and internal

calibration using either trypsin autolysis ions or matrix clusters and was applied post acquisition for accurate mass determination.

4.3.18 Analysis of peptide sequences

Peptide mass fingerprinting was used to identify proteins from tryptic peptide fragments by utilizing the MASCOT search engine based on the entire NCBI and SwissProt protein databases. Database searches were conducted allowing for up to one missed trypsin cleavage and using the assumption that the peptides were monoisotopic, oxidized at methionine residues, and carbamidomethylated at cysteine residues. Mass tolerance of 150 ppm, 0.1 Da peptide tolerances and 0.2 Da fragmentation tolerances was the window of error allowed for matching the peptide mass values. Probability-based MOWSE scores were estimated by comparison of search results against estimated random match population and were reported as -10*log10 (p), where p is the probability that the identification of the protein is a random event. MOWSE scores greater than 63 were considered to be significant ($P < 0.05$). All protein identifications were in the expected size and isoelectric point (pI) range based on the position in the gel.

4.3.19 Immunoprecipitation

Immunoprecipitation of specific proteins was performed as previously described (Sultana and Butterfield, 2004). Brain samples (200 μg) from control or treated animals were incubated overnight with anti-GAPDH antibody. This was followed by three washing steps with buffer B (50Mm Tris HCl (pH-8.0), 150 Mm NaCl, and 1% NP40). The proteins were resolved by SDS-PAGE followed by immunoblotting on a

nitrocellulose membrane (BioRad). The proteins were then detected with alkaline phosphate labeled secondary antibody (Sigma).

4.3.20 Protein Interacteome

The functional protein interacteome was obtained by using Interaction Explorer ™ Software Pathway Assist software package (Stratagene, La Jolla, CA). Pathway Assist is software for functional interaction analysis. It allows for the identification and visualization of pathways, gene regulation networks and protein interaction maps. The proteins are first imported as the gene symbols as a set of data. This data set is then searched against ResNet, a database containing over 500,000 biological interactions built by applying the MedScan text-mining algorithms to all PubMed abstracts. These interactions are then visualized by building interaction networks with shortest-path algorithms. This process can graphically identify all known interaction among the proteins. The information of the function of these proteins and their relevance to diseases are then obtained by using the BIOBASE's Proteome BioKnowledge Library form Incyte Corporation (Incyte, Wilmington, DE) (Hodges et al., 2002).

4.3.21 Determination of glutathione-S-transferase (GST) activity

Glutathione-S-transferase activity was measured as previously described using 1-chloro-2, 4-dinitrobenzene (CDNB) as substrate (Habig and Jakoby, 1981). Briefly, the standard assay mixture contained CDNB (1 mM), reduced glutathione (1 mM), and potassium phosphate buffer (100 mM; pH = 6.5) in a volume of 100 μL. The changes in absorbance were monitored at 340 nm. The thioether formed was determined by reading

the absorbance at 340 nm, and quantification was performed by using a molar absorptivity of 9.6 M^{-1}.

4.3.22 Determination of superoxide dismutase (SOD) activity

Superoxide dismutase (SOD) activity was measured as previously described (Stevens et al., 2000). Briefly, the reaction mixture of total volume 184 µL contained 160 µL of 50 mmol/l glycine buffers, pH 10.4, and 20.0 µL sample. The reaction was initiated by the addition of 4.0 µL of a 20 mg/ml solution of (–)-epinephrine. Due to its poor solubility, (–)-epinephrine (40 mg) was suspended in 2 ml water and was solubilized by adding 2–3 drops of 2N HCl. The auto-oxidation of (–)-epinephrine was monitored at 480nm and the millimolar absorptivity (4.02 mmol $\cdot$ l^{-1} $\cdot$ cm^{-1}) was used for calculations.

4.3.23 Measurement of HO-1 Protein levels

Mixtures of loading buffer and brain samples (50 µg) were denatured and electrophoresed on a 10% SDS-polyacrylamide gel. Proteins were transferred to nitrocellulose at 90 mA/gel for 2 h. The blots were blocked for 1h in fresh wash buffer and incubated with HO-1 primary antibody for 2 h. The membrane was then washed for three times in PBS for 5 min and the incubated with a secondary alkaline phosphatase-conjugated antibody. Proteins were visualized by developing with Sigma fast tablets (BCIP/NBT substrate). Blots were dried, scanned with Adobe Photoshop, and quantified using Scion Image (PC version of Macintosh-compatible NIH Image) software.

4.3.34 Statistics

An analysis of variance was used to compare the 4 treatment groups on measures of oxidative damage (protein carbonyls, 3-NT, HNE). Post hoc comparisons were made using both the Bonferroni correction and Dunnett's post-hoc test. For measures of antioxidant enzymes and HO-1 protein levels, independent t-tests were used. In all of these analyses, raw data were used but percent changes are presented in the plots. Pearson product moment correlations were used to test the linear association between oxidative damage, antioxidant enzymes and cognition. A linear stepwise multiple regression was used to determine which of the measures of oxidative damage best predicted cognition. SPSS for Windows was used and a p value of < 0.05 were used to establish statistical significance. Statistical analysis of specific protein carbonyl levels matched with anti-DNP-positive spots on 2D-oxyblots from brain samples from animals on an enriched environment and antioxidant fortified diet (EA) and age matched control of dogs that were on control food- control environment.(CC) was carried out using Student's *t*-tests. A value of $p < 0.05$ was considered statistically significant. Only proteins that are considered significantly different by Student's *t*-test were subjected to in-gel trypsin digestion and subsequent proteomic analyses. This is the normal procedure for proteomics studies, as sophisticated statistical analysis used for micro array studies are not applicable for proteomics studies (Maurer et al., 2005).

4.4 Results

4.4.1 Combined treatment (EA), decreases in the levels of protein oxidation

As shown in Fig.4.1A and 4.1B, total protein oxidation measured by the accumulation of protein carbonyls ($F(3, 22) = 4.93$ $p=0.011$) and 3-nitrotyrosine (3NT) ($F(3, 22) = 3.82$ $p=0.027$) respectively, were reduced in all treatment conditions. Post hoc comparisons show that the extent of neuroprotection was greater for the combined treatment of the enriched environment and antioxidant-fortified food (EA) ($p=0.013$ and $p=0.031$ for protein carbonyls and 3NT, respectively). The levels of lipid peroxidation, detected as protein-bound 4-hydoxynonenal (HNE), (Fig.4.1C), showed a tendency towards reduction when the groups were compared, but was not significantly different than controls ($F(3, 22) = 1.34$ $p=0.29$).

4.4.2 Combined treatment (EA), results in a decrease in specific protein carbonyl levels

An estimate of the carbonyl levels of specific proteins were obtained by dividing the carbonyl level of a protein spot on the nitrocellulose membrane by the protein level of its corresponding protein spot on the gel. This ratio gives the carbonyl level per unit of protein. We used a parallel approach to quantify the carbonyl protein levels by SYPRO Ruby staining and the extent of DNP-bound proteins by immunoblotting (Fig. 4.2 and Fig 4.3). When we analyzed brain samples from canines that were on the control diet and control environment (CC) and compared them to brain samples from animals that were on an antioxidant fortified diet and in an enriched environment (EA), six proteins were identified that were significantly less oxidized. As shown in (Table 4.2), these proteins were: glutamate dehydrogenase [NAD (P)], glyceraldehyde-3-phosphate dehydrogenase

(GAPDH), α-enolase, neurofilament triplet L protein, glutathione S-transferase (GST), and fascin actin bundling protein. The summary of specific carbonyl levels of the six identified proteins is shown in Table 4.3. The probability of an incorrect identification was established to be minimal (Table 4.2). Nevertheless, to confirm the proteomics identification of GAPDH, we used immunoprecipitation of GAPDH with an anti-GAPDH antibody and Western blot analysis as shown in Fig. 4.4.

4.4.3 Enzyme activities

We next hypothesized that in addition to reduced protein oxidation that antioxidant enzyme activity would be increased in response to treatment. We directly compared the CC animals with the EA animals for these experiments as they showed the largest difference in protein oxidation treatment effects. Thus, the activity and expression of key enzyme systems that contribute to tissue defense against oxidative stress and that are vulnerable to oxidative modification were analyzed following the treatment with an antioxidant fortified diet and a program of behavioral enrichment in the aging canine brain.

The activity of GST in aged canine brain isolated from dogs that had been treated long-term with antioxidants and a program of behavioral enrichment (EA) was found to be significantly ($t(8) = 3.3$ $p = 0.011$) increased by approximately 25% in aged EA animals compared to controls (Fig 4.5). The activity of a second antioxidant enzyme, superoxide dismutase (SOD) was also increased by approximately a 50% (Fig 4.5) in the aging canines after the combined treatment (EA) compared to controls (CC) ($t(8) = 2.29$ $p = 0.05$). This is consistent with the hypothesis that oxidative modification of an enzyme

leads to a loss or decrease in function and the reversal of this oxidative damage can restore the function of an enzyme (Poon et al., 2005e).

4.4.4 Correlation among Cognition, Oxidative Damage and Antioxidant Status

To determine if error scores on individual cognitive tasks were associated with reduced oxidative damage or increased antioxidant enzyme/protein levels, a correlational analysis was used. Table 4.4 shows that generally, higher error scores (i.e. poorer cognition) on tests of black/white discrimination, black/white reversal and spatial memory were associated with higher levels of oxidative damage. Correlations were significant for black/white reversal and 3-NT (Fig 4.7A) and for 3-NT and spatial memory (Fig. 4.7B). Overall, higher levels of antioxidant enzyme activity (SOD, GST) or higher protein levels of HO-1 were generally associated with lower error scores on all the tasks. These were statistically significant for GST and HO-1 (Fig 4.7 C, D) but not SOD, although all showed the same inverse relationship. Because age at death may also be a contributor to both increased error scores and increased oxidative damage, correlations were computed that corrected for age. The correlation between GST activity and black/white discrimination was significant ($r=-0.81$ $p=0.05$) and between black/white reversal learning and HO-1 protein levels was significant ($r=-0.81$ $p=0.05$).

A multiple stepwise regression was used to determine which measures of oxidative damage or antioxidant status best predicted cognitive dysfunction. Age at death was also included in the analysis. The best predictor of error scores on black/white discrimination learning was GST activity ($F(1, 6) = 14.31$ $p=0.013$ $r^2=0.74$), on black/white reversal learning was HO-1 ($F(1, 6) = 11.54$ $p=0.019$ $r^2=0.70$) and on spatial

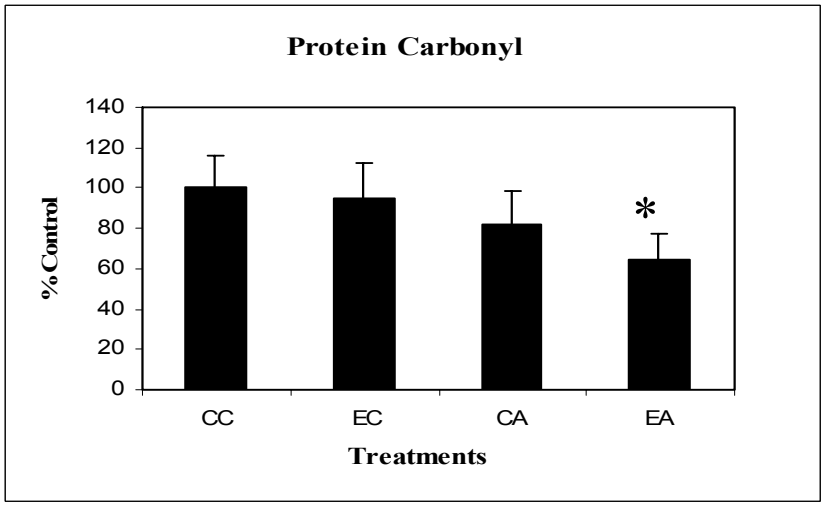
memory was age at death (F (1, 6)-7.22 p=0.044 r^2=0.59). Thus, at least one significant explanatory variable for error scores on tasks administered within 1 year of euthanasia was antioxidant enzyme function.

4.4.5 Protein Interacteome

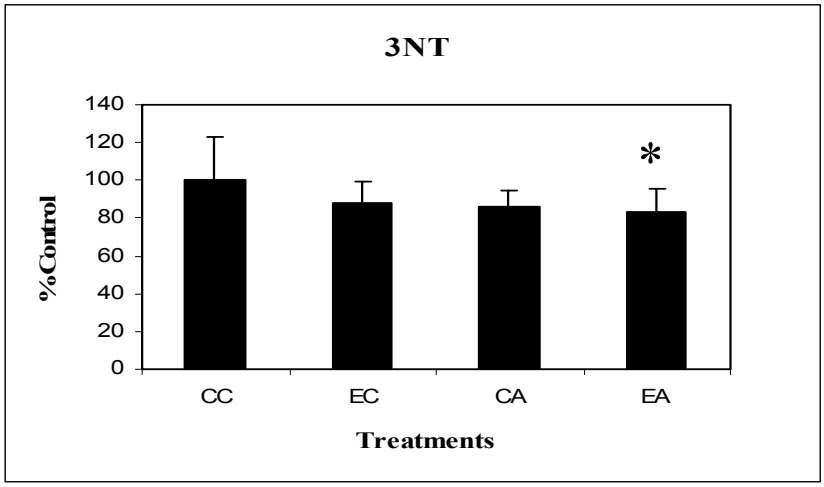
Fig 4.8 shows the protein interacteome of proteomics-identified proteins with decreased oxidation in response to the various intervention paradigms are illustrated by using Interaction Explorer Software Pathway Assist (Stratagene) software. The proteins identified in this study are related to hormone activities, transcription and regulation of signal transduction among others. As a result, the present findings continue to confirm and support previous findings (Poon et al., 2004c; Poon et al., 2005e) that antioxidants and a programme of behavioral enrichment provide beneficial effect of protection and improvement in cognitive functions and memory through the deceased oxidation and increased activity of key proteins

Figure 4.1

(A)



(B)



(C)

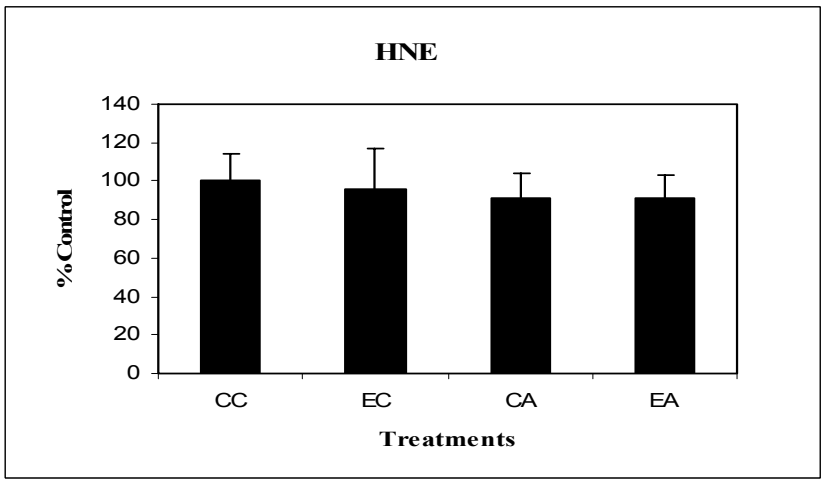


Figure 4.1 Changes in protein carbonyls (A), 3NT (B) and HNE (C) levels in canine brain homogenate samples following treatment. There was a decrease in the levels of protein carbonyls, 3NT and HNE measured from the various treatments i.e. EC, CA and EA compared to the control group CC. Data are represented as % control ± SEM for animals in each treatment group. Measured values are normalized to the CC values (n=6) * $p < 0.05$ for canines on EA treatment.

Figure 4.2

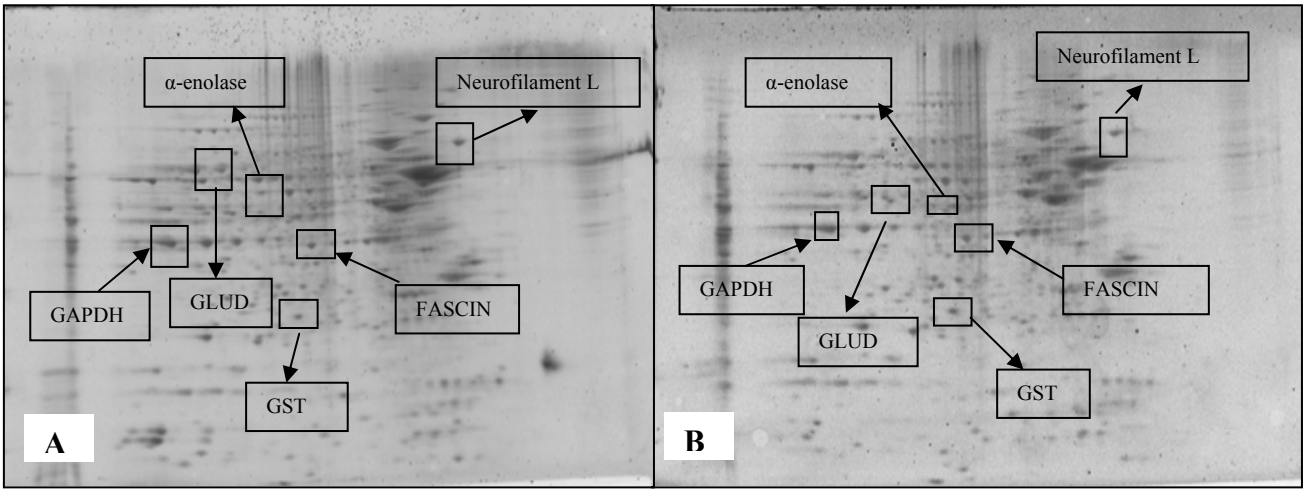


Figure 4.2 Two-dimensional SYPRO Ruby-stained gels from the parietal cortex of canines provided with a combined treatment with an antioxidant fortified diet and behavioral enrichment EA) (B) and compared to control (CC) (A). Positions of the proteins identified by mass spectrometry to be less oxidized are shown as the boxed spots.

Figure 4.3

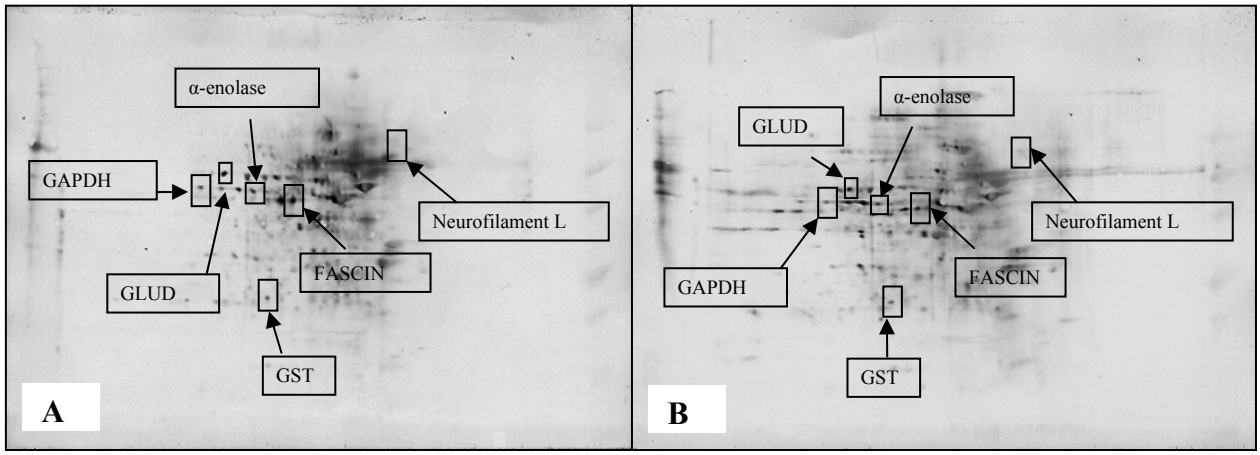


Figure 4.3 Combined treatment of aged dogs with an antioxidant enriched diet and behavioral enrichment leads to reduced protein oxidation. Carbonyl immunoblots showing proteins with less oxidation in the parietal cortex of canines given a combined treatment with an antioxidant fortified diet and an exposure to a behavioral enrichment programme EA) (B) as compared to control (CC) (A)

Figure 4.4

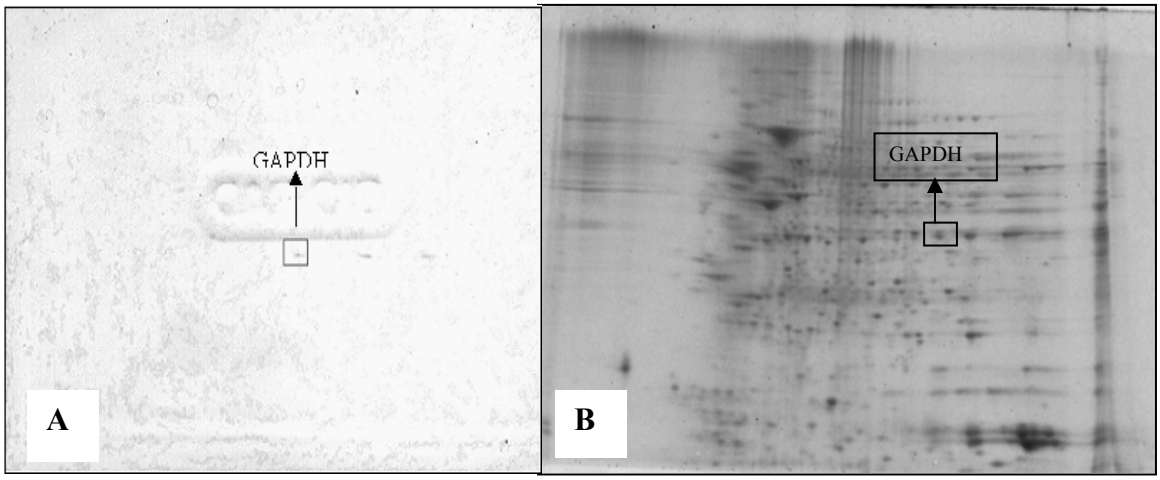


Figure 4.4 Validation of protein identified by proteomics, (A) shows blots probed with anti GADPH antibody and, (B) shows position of GAPDH on the gel.

I

Table 4.2 Summary of the proteins identified showing the MOWSE score, no of peptides matched molecular weight and pI

Identified Protein	GI accession number	Number of peptide matches identified	% Coverage of matched peptides	pI, MrW	Mowse score	Probability of a random identification
Glutamate dehydrogenase [NAD (P)]	gi|81884222	10	22	8.05,61600	123	5.0×10^{-13}
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	gi|62296789	7	25	8.23,35900	69	1.3×10^{-7}
Alpha enolase	gi|13637776	8	21	6.36,47300	94	4.0×10^{-10}
Neurofilament triplet L protein	gi|1709260	13	29	4.63,61200	132	6.3×10^{-14}
Glutathione S-transferase P	gi|73975748	5	30	6.30,23500	71	7.9×10^{-8}
Fascin- actin bundling protein	gi|2498357	14	39	6.81,54900	137	2.0×10^{-14}

Table 4.3 .A summary of the specific carbonyl levels of oxidized proteins		
Identified Protein	Specific protein carbonyl levels of canine on EA % Control ± SEM (n=5)	P value
Glutamate dehydrogenase	27 ± 5	< 0.04
GAPDH	18 ± 8	< 0.05
Alpha enolase	14 ± 3	< 0.05
Neurofilament triplet L protein	16 ± 3	< 0.04
Glutathione S-transferase P	20 ± 6	< 0.02
Fascin actin bundling protein	23 ± 7	< 0.008

Figure 4.5

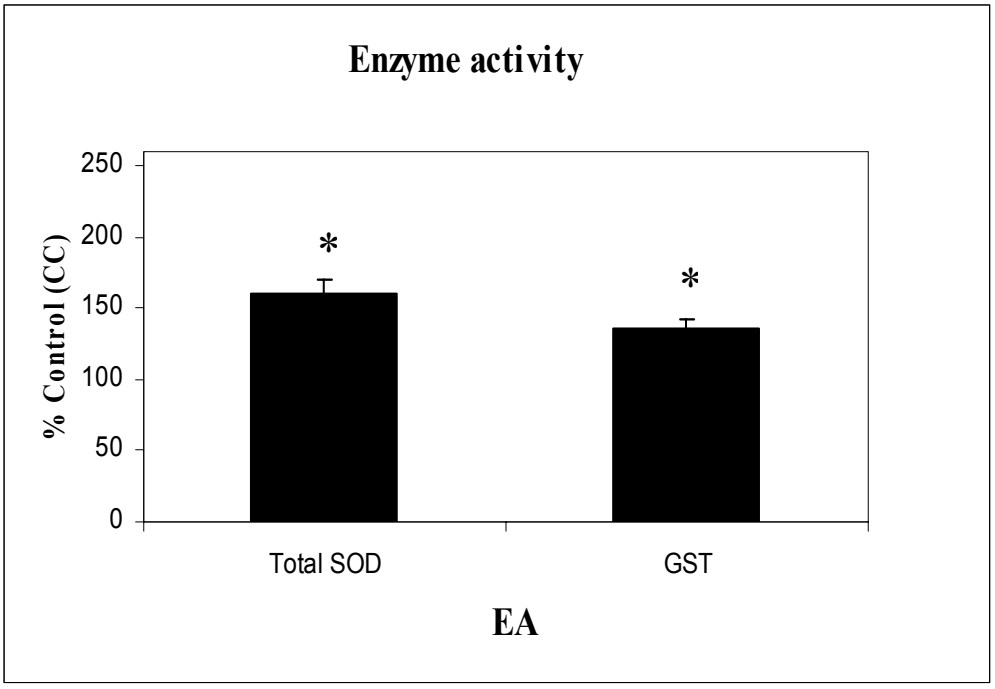


Figure 4.5 SOD and GST activity are significant increased in response to treatment in aged dogs. Dogs provided with the combination of an antioxidant-fortified diet and behavioral enrichment show significantly increased GST and total SOD enzyme activity relative to controls. Activities of GST and SOD are expressed as units per milligram of protein and data are presented as % control ± SEM for animals in each treatment group, (n=5) * p <0.05.

Figure 4.6

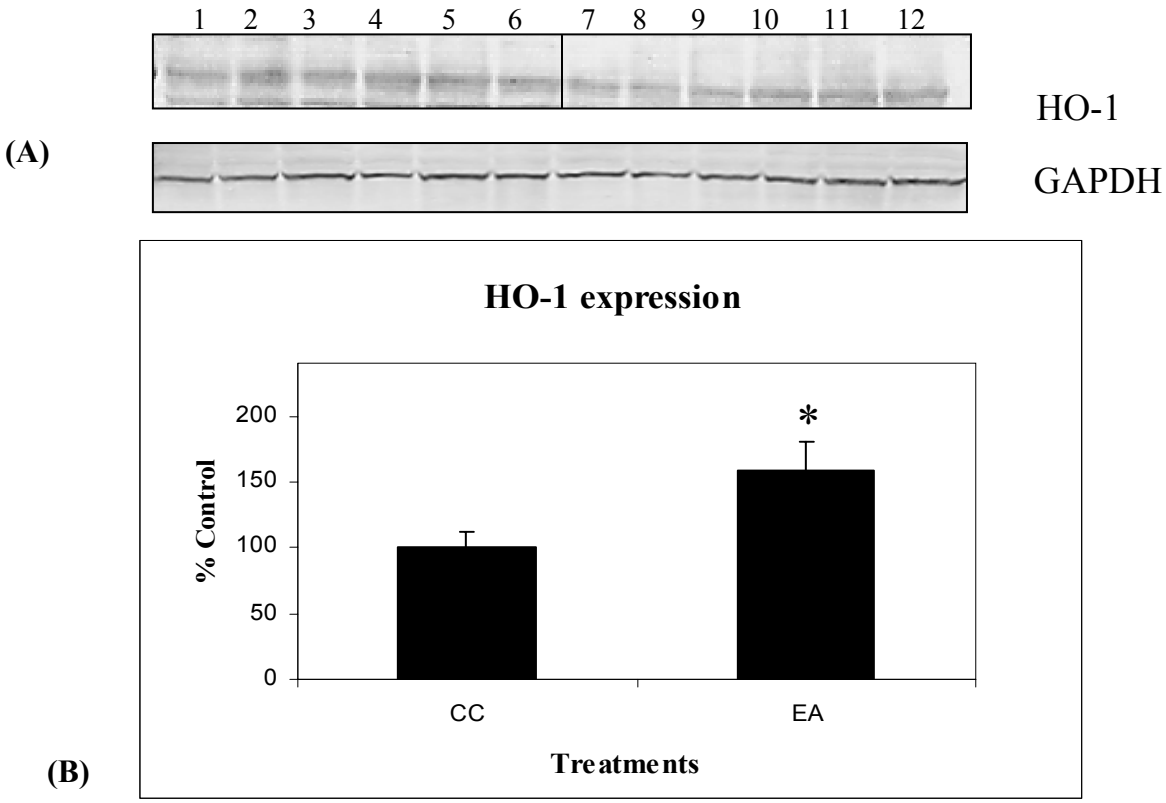


Figure 4.6 HO-1 protein levels increase in response to treatment in aged canines. Western immunoblot analysis and quantification of canine brain homogenates samples containing 50μg of protein loaded onto 10% SDS-PAGE gels were completed using an anti-HO-1 antibody. A representative immunoblot (A) with Lanes (1-6) representing the treatment group EA, and 7-12 represent control group CC is shown and GAPDH was used as a control for equal loading of protein. Densitometric values are plotted as a function of treatment group (B) showing a significant increase in HO-1 expression following the combined treatment of enriched environment and antioxidant-fortified food

(EA) compared to control (CC). GAPDH densitometric data are represented as % control; ± SEM for each group. (n=6), * $p < 0.05$.

Table 4.4 Correlations of Cognition, Oxidative Damage and Antioxidant Status

Cognitive Task	Protein Carbonyls	3-NT	HNE	GR	SOD	GST	HO-1
Black/White Discrimination	r=0.30 p=0.19, n=21	r=0.38 p=0.09 n=21	r=0.08 p=0.73 n=21	r=-0.30 p=0.48 n=8	r=-0.04 p=0.92 n=8	r=-0.67 p=0.07 n=10	r=-0.37 p=0.30 n=10
Black/White Reversal	r=0.40 p=0.18 n=20	r=0.43 p=0.05 n=21	r=0.42 p=0.06 n=21	r=-0.22 p=0.59 n=8	r=-0.34 p=0.40 n=8	r=-0.65 p=0.08 n=8	r=-0.75 p=0.01 n=10
Spatial Memory	r=0.31 p=0.18 n=20	r=0.44 p=0.05 n=20	r=0.32 p=0.17 n=20	r=-0.26 p=0.53 n=8	r=-0.20 p=0.64 n=8	r=-0.16 p=0.71 n=8	r=-0.61 p=0.06 n=10

Figure 4.7

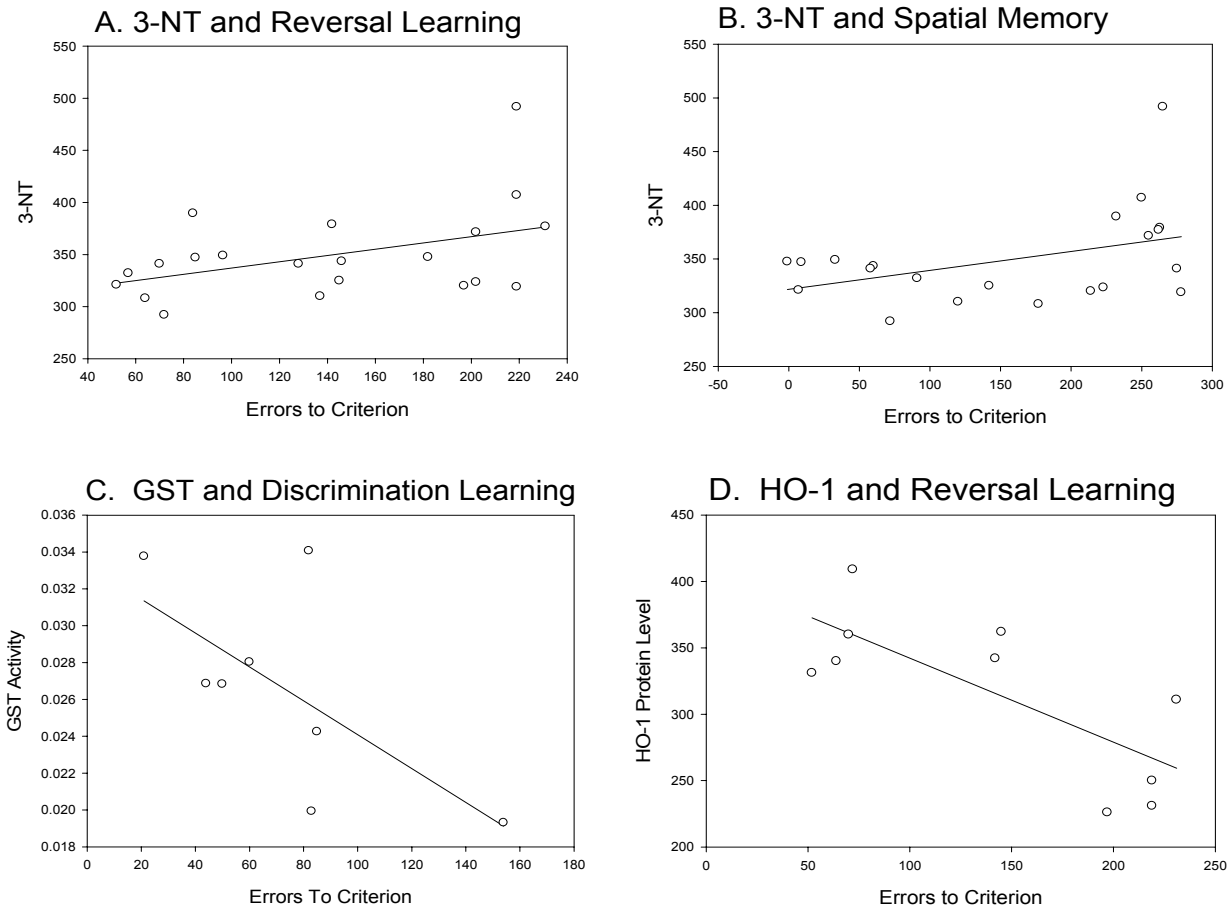


Figure 4.7 Association between cognitive test scores and measures of oxidative damage in treated animals. Shows error scores on individual cognitive tasks associated with reduced oxidative damage or increased antioxidant enzyme/protein levels. Higher error scores on tests of black/white discrimination, black/white reversal and spatial memory were associated with higher levels of oxidative damage. Higher error scores on a reversal learning task (A) and on a visuospatial memory task (B) were correlated with 3-NT. Discrimination learning ability was inversely associated with GST activity (C). Reversal learning error scores were also negatively associated with HO-1, with higher levels of HO-1 associated with better cognition (D).

Figure 4.8

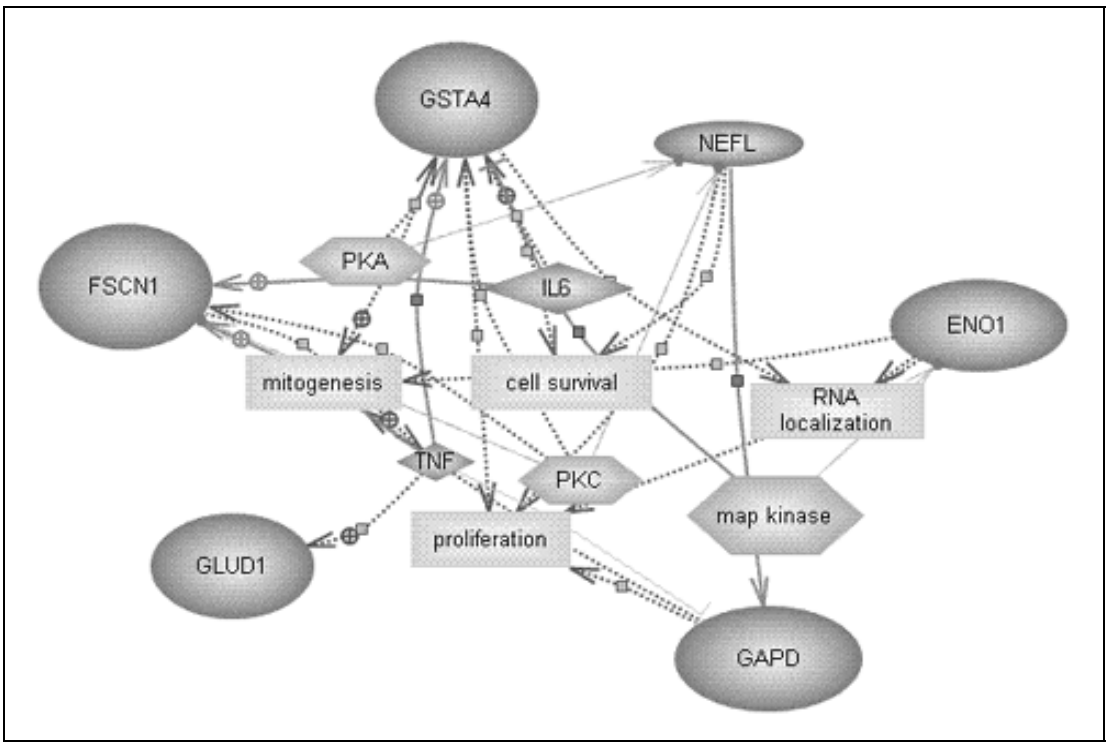


Figure 4.8 Schematic diagram of a functional interacteome of all parietal cortex proteins identified to be significantly less oxidatively modified following the combined treatment of the enriched environment and antioxidant-fortified food (EA). This diagram was generated by the interaction explorer ™ Pathway Module (Stratagene), indicating that all the proteins are directly or indirectly associated with cellular process shown

4.5 Discussion

Oxidative stress may be involved in the development of pathology leading to decline in memory and cognitive functions observed in AD and in other age-related neurodegenerative disorders (Hensley et al., 1995; Butterfield and Kanski, 2001; Butterfield and Lauderback, 2002). However, interventions with antioxidants delays age-related cognitive decline and improves performance in animal models of AD and other age-related neurodegenerative disorders (Joseph et al., 1998b; Bickford et al., 2000; Farr et al., 2003). The present study investigated the effect of a antioxidant-fortified diet and a program of behavioral enrichment on the levels of oxidative damage and in restoring antioxidant reserve systems in the aging canine brain. Four different treatments were compared (CC, CE, CA and EA) in 23 age-matched beagle dogs for a period of 2.8yrs and markers of oxidative stress in the parietal cortex were analyzed. There was a reduction in the levels of brain 3NT and protein carbonyls assayed with all treatments, but only those in the combined treatment EA showed a significant reduction when compared to control. The levels of brain lipid peroxidation as measured by HNE were marginally reduced in all treatments, but none was significantly reduced compared to control. We also used redox proteomics to show that following the combined treatment EA; the aging canine shows less oxidation to key brain proteins involved in energy metabolism, antioxidant systems, and in maintenance and stabilization of cell structure. In addition, there is a significant increase in the activity of antioxidant enzymes GST and SOD in the combined treatment EA when compared to control, and a significant increase in the expression of HO-1 protein, an important defense system in neurons under oxidative stress (Calabrese et al., 2003a). The significant decrease in oxidation of some of

these key brain proteins was also shown to correlate with improved cognitive function in the aged canines undergoing these interventions. These results suggest possible mechanisms for the improved memory and cognitive function previous reported in the (Cotman et al., 2002; Milgram et al., 2004) and are discussed herein with relevance to AD.

In AD, the Aβ peptide plays a central role in the generation of free radicals and oxidative stress (Hensley et al., 1994; Butterfield et al., 2001). In the aging canine, no significant correlation between the levels of Aβ deposition in brain and oxidative damage is observed (Head et al., 2002), however, since the aging canine deposits the more toxic form of Aβ 1-42 as that seen in human aging (Markesbery, 1997; Butterfield et al., 2002b) and since Aβ load and decline in cognitive function events develop in parallel, Aβ could still play a significant role in the mechanism of oxidative stress observed in the aging canine (Cummings et al., 1993; Cummings et al., 1996b; Head et al., 2000). In the peptide sequence of Aβ (1-42), there is a methionine-35 residue that our laboratory has shown to play a critical role in Aβ induced oxidative stress and neurotoxicity observed in AD (Butterfield et al., 2005). We have proposed that the Aβ1-42 peptide, as a small oligomer, inserts itself in the lipid bilayer in an alpha helix conformation. A one-electron oxidation of methionine forms the methionine sulfuranyl radical, which can then abstract a labile hydrogen atom from neighboring unsaturated lipids forming a carbon-centered lipid radical (L$^{\bullet}$), which can react with molecular oxygen to from a peroxyl radical (LOO$^{\bullet}$). This peroxyl radical can abstract hydrogen from a neighboring lipid to form the lipid hydroperoxide LOOH and a carbon centered radical L$^{\bullet}$, which propagates the free radical chain reaction (Varadarajan et al., 2001; Butterfield et al., 2005). It is with this

mechanism of free radical generation that we believe is similarly taking place in the aging canine and is responsible for the increased levels of oxidative stress leading to neurodegeneration and a decline in memory and cognitive function.

The use of dietary intervention with anti-oxidants or free radical quenchers and a regular program of behavioral enrichment (social, cognitive, environmental and physical exercise) is protective against oxidative damage, reduces oxidative stress, protects neurons and consequently improves cognitive function in human aging and in animal models (Joseph et al., 1998b; Bickford et al., 2000; Eckles-Smith et al., 2000; Milgram et al., 2002b; Calabrese et al., 2003a; Adlard et al., 2005b). In the present study, the fortified antioxidant diet included vitamin E and vitamin C, both well-known free radical quenchers. Vitamin E is lipid soluble, hence protects cell membranes from oxidative insults while vitamin C protects the soluble phase of the cell and also regenerates the vitamin E from the vitamin E free radical (Butterfield et al., 2002c), but recent studies in which vitamin C was not included, reported vitamin E did not inhibit the conversion of patients with mild cognitive impairment to AD (Petersen et al., 2005). As a result, the ability of vitamin E in protecting cell membranes provides one possible mechanism through which the fortified diet given to the aging canines provides protection from oxidative damage as seen by the decreased levels of lipid peroxidation assayed by HNE and previously seen to have been elevated as measured by malondialdehyde (Head et al., 2002). In addition, the inclusion of fruits and vegetables rich in flavonoids and carotenoids, could help in quenching the possible free radicals generated by Aβ, which as noted here is deposited in the aging canine brain (Cummings et al., 1996b; Head and Torp, 2002), leading to the low levels of protein oxidation as measured by protein carbonyls and 3NT observed in the present study. Further, there was a significant

correlation between 3NT and spatial memory and black/white reversal learning indicating that there is a correlation between improved cognition and reduction in oxidative damage in the aging canine brain following the combined treatment with a diet fortified with antioxidants and a program of behavioral enrichment.

The behavioral enrichment program used in this study involved a regimen of extra physical exercise, enhanced environmental and social stimulation and cognitive training leading to cognitive improvement (Milgram et al., 2005). Exercise is reported to improve cognitive function, reduce the risk of developing cognitive impairment and reduce neuropathology in humans or in animal models (Hultsch et al., 1999; van Praag et al., 1999; Laurin et al., 2001; Adlard et al., 2005b). In aging dogs, behavioral enrichment leads to significant improvements in visual discrimination learning and frontal-dependent reversal learning (Milgram et al., 2005). The mechanism by which behavioral enrichment provides protection against oxidative damage is still unknown, but the current study provides new insights. Aging usually lowers the expression of antioxidant enzymes and stress protein expression. This loss can be modulated through interventions with diet or exercise (Heydari et al., 1993; Wu et al., 1993; Ji, 2002). One effect of the combined treatment EA was a significant increase in the expression of inducible heme oxygenase (HO-1) also known as HSP32. The heme oxygenase pathway is an important neuronal defense system in conditions of oxidative stress (Chen et al., 2000) and has been reported to be involved in oxidative stress-related neurodegenerative disorders, including AD, (Takahashi et al., 2000). In AD, for example, the expression of HO-1 is significantly altered and is up-regulated during oxidative stress, as well as by GSH depletion (Tyrrell, 1999; Calabrese et al., 2004a). The induction of HO-1 catabolizes heme forming carbon

monoxide (CO) and biliverdin and subsequently bilirubin, a potent antioxidant and anti-inflammatory agent (Calabrese et al., 2004a). In the aging canine increased oxidative stress and depletion of GSH is observed (Head et al., 2002) and with interventions with an antioxidant diet and a program of behavioral enrichment, a perfect environment is created for the induction of HO-1. This in effect could provide an additional antioxidant i.e. bilirubin, contributing to the decreased levels of oxidative damage and improvement in memory and cognitive function in the aging canine. The higher protein levels of HO-1 were also associated with lower error scores on individual cognitive tasks. This correlation was statistically significant even after correction for age at death. As a result HO-1 was one of the best predictors of error scores on black/white reversal learning, i.e., higher HO-1 protein levels were associated with improved cognitive function.

A by-product of mitochondrial respiration is the generation of superoxide, which leaks from the mitochondria inducing more oxidative stress and damage. Supplementation of the diet in the present study with mitochondrial co-factors, could lead to more efficiently functioning mitochondria. We have also shown in the present study that there is increased activity of total SOD, which would then provide protection against an increase in the production of superoxide, leading to a reduction in oxidative damage. On looking at the correlation between increased enzymatic activity and cognition, we found that though high levels of antioxidant activity were associated with lower error scores, though this correlation was not significant for SOD.

We also hypothesized that of the use of mitochondrial co-factors in the canine enriched diet would result in improved mitochondrial function and may increase ATP production, consequently improving cognition (Milgram et al., 2004). Using redox

proteomics in the current study, we were able to identify key brain proteins not only related to energy metabolism but also those related to antioxidant systems, and those involved in the maintenance and stabilization of cell structure to be less oxidized. These proteins may be playing a role in the improved cognitive function observed in these animals.

Energy metabolism

*Alpha enolase (*ENO1) is a glycolytic enzyme that interconverts 2-phosphoglycerate to phosphoenolpyruvate and is one of the proteins recently identified to be significantly oxidatively modified in individuals with mild cognitive impairment (MCI) (Butterfield et al., 2006d), which to some extent, the aged canine models (Cummings et al., 1996a; Cotman et al., 2002). We have also shown that α-enolase is oxidatively modified in AD and in various models of neurodegenerative disorders (Butterfield et al., 2002a; Perluigi et al., 2005a; Perluigi et al., 2005b; Poon et al., 2005c), indicating that this key protein is involved in several age-related neurodegenerative disorders. In addition, we have also shown that following caloric restriction in aging rats (Poon et al., 2005g) and after treatment with lipoic acid in the SAMP8 mice (Poon et al., 2005e), the specific carbonyl levels of α-enolase are significantly decreased leading us to believe that this protein may play a key role in the restoration of cognitive function. Consistent with this idea, our present findings show that following treatment with antioxidants and mitochondrial co-factors (including lipoic acid) and a program of behavioral enrichment in the aging canine, the specific carbonyl levels of α-enolase are significantly reduced.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is another glycolytic enzyme that catalyzes the oxidation of glyceraldehyde-3-phosphate to 1, 3-bisphosphoglycerate and NADH (Chuang et al., 2005). GAPDH can also act as a sensor for nitrosative stress (Hara et al., 2006). Our laboratory has shown that GAPDH undergoes significant nitration, another form of oxidative modification, in the hippocampus of AD patients (Sultana et al., 2006b) and also in rats after intracerebral injection with Aβ (1-42) (Boyd-Kimball et al., 2005c). Interestingly, we have also shown that the use of gamma-glutamylcysteine ethyl ester (GCEE), a compound that leads to increased synthesis of glutathione in neuronal cell culture treated with Aβ (1-42), protects GAPDH against Aβ (1-42)-mediated protein oxidation (Boyd-Kimball et al., 2005b). In the present study we have also identified GAPDH as one of the proteins that is protected from oxidative damage following the combined treatment with antioxidants and behavioral enrichment. As a result, the decreased oxidation of GAPDH and α-enolase could lead to improved glycolytic function and increased ATP production and possible neuronal recovery and improved cognitive function as seen in the canine model of human aging.

Maintenance and stabilization of the integrity of the cell structure

Neurofilament triplet L protein also known as NF68/NF-L is a subunit of neurofilaments (NFs), which give axons their structure and diameter (Hoffman et al., 1987). In addition NFs are involved in cytoskeleton organization, neurogenesis and supports the neuronal architecture in the brain (Poon et al., 2006a). The protein levels of NF-L in brains of AD, Down syndrome, and ALS patients is significantly decreased (Bergeron et al., 1994; Bajo et al., 2001), suggesting that normal NF-L expression could be critical to central nervous system (CNS) function. Oxidation or nitration of

neurofilament (NF) proteins transform the α-helix secondary structure to β-sheet and random coil conformations, destabilizing the interactions between the NF proteins and resulting in axonal damage (Crow et al., 1997) and CNS dysfunction. We have previously shown that NF68 was significantly oxidized in the brain of the gracile axonal dystrophy (gad) mouse (Castegna et al., 2004). NF-66 (α-internexin) another family of the NF's is also significantly oxidized in the brains of old versus young mice (Poon et al., 2005b). In the SAMP 8 mice, following treatment with alpha lipoic acid, we have observed a significant increase in the expression of NF-68 and since alpha lipoic acid treated SAMP8 aged mice have improved learning and memory, this protein could be important for brain function (Poon et al., 2005e). In the present study we established that the levels of protein oxidation for neurofilament triplet L protein were decreased following interventions with antioxidants and a program of behavioral enrichment in aging dogs.

Another cytoskeleton related protein identified to be less oxidized in this study is Fascin. *Fascin,* a 55kD globular protein, is an actin bundling protein responsible for organizing F-actin into well-ordered, tightly packed parallel bundles in vitro and in cells (Adams, 2004b). It is also known to be one of the core actin bundling protein of dendrites among other structures (Adams, 2004a). Fascins function in the organization of two major forms of actin-based structures: dynamic, cortical cell protrusions and cytoplasmic microfilament bundles (Kureishy et al., 2002). Cell protrusions in the plasma membrane sense the cellular environment, provide cell adhesion in the extracellular matrix and act in cellular migration (Adams, 2004b). These cell protrusions usually require a rigid cytoskeleton to support the localized extension of the plasma membrane. Formation of these structures is highly regulated by extracellular and intracellular signals, with a key

point of regulation being the binding of fascin to filamentous actin (F-actin) (Adams, 2004b). Alterations in the expression of fascin are associated with disorders such as cardiovascular diseases and in various carcinomas among others, (Kureishy et al., 2002; Adams, 2004b, a). Fascin was one of the brain proteins identified in the aged canine undergoing treatment with an antioxidant diet and a program of behavioral to be less oxidized. As a result, the identification of NFL and fascin as less oxidized following the combined treatment would possibly lead to a decrease in axonal dystrophy (Poon et al., 2004d), increased cellular migration, cell adhesion and communication, leading to improved neuronal communication and survival and particularly possibly leading to improved memory and cognitive function previously seen in the aging canine. However since the role of fascin in aging or neurodegenerative disorders is not known, the beneficial role of its reduced oxidation and the role it plays in cognitive function remain speculative.

Cellular detoxification

Glutathione-S-transferase (GST) catalyzes the conjugation of a number of exogenous and endogenous compounds such as 4-hydroxynonenal (HNE) or malondialdehyde (MDA) with glutathione inactivating the toxic products of oxygen metabolism (Singh et al., 2002). Hence, GST plays a critical role in cellular protection against oxidative stress. There is a significant decline in the activity of GST in the amygdala, hippocampus and inferior parietal lobule of patients with AD (Lovell et al., 1998), contributing to the accumulation of toxic effects of HNE and related compounds. Our laboratory has previously shown that in the AD brain, GST and multidrug resistant protein MRP1 are oxidatively modified leading to an impairment of detoxification

mechanisms causing increased oxidative stress consistent with elevated HNE in AD (Sultana and Butterfield, 2004). In the aged canine, there is a overall decrease in GSH content and a significant increase in the lipid peroxidation product, MDA (Head et al., 2002). In the present study we show that following the combined treatments of an antioxidant fortified diet and a program of behavioral enrichment, GST was less oxidized. In addition, we also show that the activity of GST is significantly increased. This would potentially enhance the clearance of toxic aldehydes leading to improved memory and cognitive function in aging dogs. The higher activity of GST was also associated with lower error scores on individual cognitive tasks. This correlation was statistically significant even after correction for age at death. Further, increased GST activity was the best predictor of error scores on black/white discrimination learning, thus providing a possible mechanism underlying improved cognitive function following treatment in the aging canine with a diet fortified with antioxidants and a program of behavioral enrichment.

Glutamate dehydrogenase (GDH) is an enzyme located in the mitochondrial matrix that acts in both catabolic and metabolic pathways. GDH can catalyze the reductive amination of α-ketoglutarate with NADPH to yield glutamate in the metabolic pathway and can also catalyze the formation of α-ketoglutarate from glutamate with NAD^+ and ammonium ion in the catabolic pathway (Boyd-Kimball et al., 2005d). The latter pathway is particularly important in eliminating the excitotoxin glutamate. Excess glutamate can stimulate NMDA receptors leading to an increase in Ca^{2+} influx and altered calcium homeostasis, which would lead to alteration in long-term potentiation (LTP) and consequently, learning and memory deficits as seen in AD (Boyd-Kimball et

al., 2005d). We have shown in the present study that following treatment with antioxidants and a program of behavioral enrichment in the canine model of human aging, there is a decrease in the specific carbonyl levels of GDH. This would possibly lead to an increase in its activity, and more importantly its metabolic activity thereby helping to clear excess glutamate in the synaptic cleft. Consequently, this may lead to controlled Ca^{2+} homeostasis, improved LTP, and eventually improvement in cognitive function as observed in the canine model of human aging following interventions with antioxidants and a program of behavioral enrichment.

Using the Interaction Explorer Software PathwayAssist (Stratagene) to analyze our current results as shown in Fig 5, the proteins identified in this study can be divided into three functional categories: those related to energy metabolism, antioxidant systems and maintenance, and stabilization of cell structure. These results therefore continue to support a role for oxidative damage in the development of age-associated cognitive dysfunction and indicate that the use of foods enriched with antioxidants and mitochondrial cofactors can reduce the levels of oxidative damage and result in reducing the effects of aging on memory and cognitive function. In addition, the increased activity of GST continues to confirm our previous notion that oxidation of particular proteins lead to loss of function, and that reduced oxidation of proteins can lead to an improvement in function (Castegna et al., 2004; Perluigi et al., 2005a; Perluigi et al., 2005b; Poon et al., 2005f). This study suggests that cognitive decline can be reversed or cognitive function can be maintained following use of antioxidants and a program of behavioral enrichment and this approach conceivably can be beneficial to the aging human population and in age-related neurodegenerative disorders.

The present study continues to provide additional evidence that oxidative stress may be a key mechanisms contributing to decline in memory and cognitive function with age. We have shown that a diet fortified with antioxidants in combination with a program of behavioral enrichment is capable of reducing the levels of oxidative damage, and increasing the activity and expression of key endogenous antioxidant enzymes in the aging canine brain. As a result of the reduction in the levels of oxidative stress/damage following this intervention, we have also established that key brain proteins associated with energy metabolism, antioxidant systems, and with the maintenance and stabilization of cell structure are protected from oxidative damage. This we believe would lead to improved activity or function consequently leading to the improved memory and cognitive function observed in the aging canine. Further we have also shown that there is a strong correlation between the increased in expression/activity of some of the identified proteins and improved cognitive function. Therefore the present study provides possible mechanisms through which the aging canine, provided with the combined intervention of an antioxidant fortified diet and a program of behavioral enrichment, shows improvements in cognitive function (Milgram et al., 2002b; Milgram et al., 2005) . Further, the increased expression of HO-1, increased activity of GST and SOD together could all have synergistic effects in the reduction of oxidative damage and protection of key proteins from oxidative damage observed in this study. Results from the current study in aging canines may be translatable to humans, providing a possible intervention for Aβ induced cognitive decline observed in AD.

4.6 Acknowledgement

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CHAPTER FIVE

Long-Term Treatment with Antioxidants and a Program of Behavioral Enrichment Leads to Increased Expression of Key Brain Proteins in the Canine Model of Human Aging

5.1 Overview

Aging and age-related disorders such as Alzheimer's disease (AD) are usually accompanied by increased oxidative stress and eventually a decline in memory and cognitive function. Aging canines develop cognitive dysfunction and neuropathology similar to those seen in humans, such as, beta-amyloid deposition, oxidative stress and learning and memory impairments. In the canine model of human aging, interventions with an antioxidant fortified diet and a program of behavioral enrichment improves learning ability of the aging dog. To identify the neurobiological mechanisms underlying these treatment effects, we carried out a comparative proteomics study to identify specific brain proteins that were differentially expressed from the parietal cortex in treated aged dogs. Twenty-one beagle dogs (8.1-12.4 years) were treated for 2.8 years after grouping into 4 treatments: control food with either a control environment (CC) or with behavioral enrichment (CE); antioxidant fortified food with a control environment (CA); and behavioral enrichment and antioxidant fortified food (EA). Analysis of the parietal cortex revealed significant increases in expression of, Cu/Zn superoxide dismutase, fructose-bisphosphate aldolase C, creatine kinase, glutamate dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase. In addition, we also observed a significant increase in the enzymatic activity of superoxide dismutase (SOD) following the combined treatment EA. The increased expression of these proteins in particular Cu/Zn SOD correlated with

improved cognitive function. These findings provide insights into possible mechanisms through which long-term treatment with antioxidants and a program of behavioral enrichment improve and maintain learning ability in aged beagle dogs. In addition it provides possible mechanisms through which this program can be beneficial when translated to the aging human population at risk for age-related neurodegenerative disorders such as AD.

5.2 Introduction

Elucidating mechanisms and developing therapeutic intervention strategies for age-related neurodegenerative disorders such as Alzheimer's disease (AD), Parkinson's disease (PD) and amyotrophic lateral sclerosis (ALS), among others, requires animal models that can be translated to the human population. We have been using a canine model of cognitive and brain aging that develops similar features of normal and pathological aging in humans (Cummings et al., 1996a). Canines show cognitive decline with increased age, (Milgram et al., 1994; Milgram et al., 2002c) , impairments in visuospatial learning and memory (Chan et al., 2002), and age-dependent neuropathology similar to that observed in elderly humans, i.e., accumulation of human-type amyloid β-peptide (Aβ) of the same sequence as humans (Johnstone et al., 1991), which correlates with age-dependent cognitive dysfunction (Cummings et al., 1996b; Head et al., 1998) . In addition, the aging canine brain accumulates oxidative damage and a decline in antioxidant reserves (Kiatipattanasakul et al., 1996; Head et al., 2002) , events similar to those seen in the AD brain (Markesbery, 1997), providing a possible common neurobiological mechanism leading to cognitive dysfunction.

The use of antioxidants and regular exercise has beneficial effects in various models of age-related neurodegenerative disorders, resulting in maintenance, improvement or restoration of memory and cognitive function (Joseph et al., 1998b; Laurin et al., 2001). In the canine model of human aging, short term and long term treatment with a diet rich in a broad spectrum of antioxidants leads to rapid and sustained

learning ability and improved spatial attention; these effects were further enhanced with the addition of behavioral enrichment (Cotman et al., 2002; Milgram et al., 2004).

Improved cognition in response to an antioxidant diet suggests that oxidative stress plays a significant role in the decline of cognitive function observed in this model. In addition, there is a significant decline in antioxidant reserves, making the aging canine more vulnerable to oxidative attack. Consistent with this hypothesis, we previously reported (Chapter 4) that following a program of behavioral enrichment and an antioxidant fortified diet in the aging canine, there is a significant reduction in the levels of protein oxidation as indexed by protein carbonyls and 3-nitrotyrosine (3NT) and also a significant increase in enzymatic activity of key antioxidant proteins. The reduction in oxidative stress and increased activity of antioxidant enzymes provides a possible mechanism for the improvement in memory and cognition following these treatments. Since the aging canine brain is under significant oxidative stress, we believe that this in itself could trigger a stress response leading to the altered transcription of key proteins or enzymes involved in mechanisms for protection against oxidative damage (Calabrese et al., 2003a). Moreover, the use of an antioxidant fortified diet and a program of behavioral enrichment could also trigger this response thereby providing an additive effect. To better understand the mechanisms for protection at the molecular level, we have used proteomics to identify key brain proteins that have been differentially expressed following the treatments provided in this program.

Proteomics is the study of the proteome. Proteome analysis is “the analysis of the entire PROTEin complement expressed by a genOME”. The analysis involves the

systematic separation, identification, and quantification of many proteins simultaneously from a single sample (Butterfield and Castegna, 2003b). Proteomic analysis encompasses the qualitative, quantitative and functional characterization of the entire protein profile of a given cell, tissue and/or organism. Proteomics has become an important and efficient tool in the analyses of protein changes in various disease states (Butterfield, 2004). Our laboratory has been successful in using proteomics to identify key brain proteins that are oxidatively modified and proteins that have shown differential expression in AD and mild cognitive impairment (Butterfield et al., 2002a; Castegna et al., 2002b; Castegna et al., 2003; Butterfield, 2004; Butterfield et al., 2006c; Sultana et al., 2006a), the senescence accelerated prone mouse strain 8 (SAMP8) (Butterfield and Poon, 2005), and mouse models of Huntington's disease (Perluigi et al., 2005b), PD (Poon et al., 2005c) , ALS (Perluigi et al., 2005a; Poon et al., 2005f), among many others.

In the present study, we identified five proteins from the parietal cortex of the aging canine showing a significant increase in expression following treatment with antioxidants, exposure to a program of behavioral enrichment or a combination of both treatments. The proteins identified were, fructose-bisphosphate aldolase C, creatine kinase, glutamate dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase and Cu/Zn superoxide dismutase, whose activity was also shown to be significantly increased following the combined treatment (EA) relative to control. In addition, the increased expression of Cu/Zn SOD correlates with improvements in cognition as shown by an improvement in the black/white reversal and spatial learning. These results suggest that a program of behavioral enrichment and treatment with a diet of antioxidants is capable of improving age-related cognitive function by increasing the activity of a key antioxidant

enzyme and by increasing the expression of key proteins involved in energy metabolism and antioxidant activity.

5.3 Experimental procedures

5.3.1 Subjects

Subjects same as 4.3.1

5.3.2 Group assignments and study timeline

Group assignment and study timeline same as 4.3.

5.3.3 Behavioral enrichment treatment

Behavioral enrichment treatment same as 4.3.3

5.3.4 Diet treatment

Diet treatment same as 4.3.4

5.3.5 Cognitive Testing

Cognitive testing same as 4.3.5

5.3.6 Animal Euthanasia

Animal euthanasia same as 4.3.6

5.3.7 Two-dimensional electrophoresis

Brain samples (200 μg parietal cortex) were incubated with 4 volumes of 2N HCl at room for electrophoresis. Proteins were then precipitated by the addition of ice-cold 100% trichloroacetic acid (TCA) to obtain a final concentration of 15% TCA. Samples were then placed on ice for 10 min and precipitate centrifuged at 16,000 g for 3min. The resulting pellet was then washed three times with a 1:1(v/v) ethanol/ethyl acetate solution. The samples were then suspended in 200 μl of rehydration buffer composed of a

1:1 ratio (v/v) of the Zwittergent solubilization buffer (7M urea, 2M thiourea, 2% Chaps, 65 mM DTT, 1% Zwittergent 0.8% 3-10 ampholytes and bromophenol blue for 1 h.

5.3.8 First dimension electrophoresis

For the first-dimension electrophoresis, 200 µL of sample solution was applied to a 110-mm pH 3–10 ReadyStrip™ IPG strips (Bio-Rad, Hercules CA). The strips were then actively rehydrated in the protean IEF cell (Bio-Rad) at 50 V for 18 h. The isoelectric focusing was performed in increasing voltages as follows; 300 V for 1 h, then linear gradient to 8000 V for 5 h and finally 20 000 V/h. Strips were then stored at –80 °C until the 2nd dimension electrophoresis was to be performed.

5.3.9 Second dimension electrophoresis

For the second dimension, the IPG® Strips, pH 3–10, were equilibrated for 10 min in 50 mM Tris–HCl (pH 6.8) containing 6 M urea, 1% (w/v) sodium dodecyl sulfate (SDS), 30% (v/v) glycerol, and 0.5% dithiothreitol, and then re-equilibrated for 15 min in the same buffer containing 4.5% iodoacetamide instead of dithiothreitol. Linear gradient precast criterion Tris–HCl gels (8–16%) (Bio-Rad) were used to perform second dimension electrophoresis. Precision Protein™ Standards (Bio-Rad, CA) were run along with the sample at 200 V for 65 min.

5.3.10 SYPRO ruby staining

After the second dimension electrophoresis, the gels were incubated in fixing solution (7% acetic acid, 10% methanol) for 20 min and stained overnight at room

temperature with 50ml SYPRO Ruby gel stain (Bio-Rad). The SYPRO ruby gel stain was then removed and gels stored in DI water.

5.3.11 Image analysis

SYPRO ruby-stained gel images were obtained using a STORM phosphoimager (Ex. 470 nm, Em. 618 nm, Molecular Dynamics, Sunnyvale, CA, USA) and also saved in TIFF format. PD-Quest (Bio-Rad) imaging software was then used to match and analyze visualized protein spots among differential 2D gels with one gel for each individual sample.

5.3.12 In-gel trypsin digestion

Protein spots statistically different that controls were digested in-gel by trypsin using protocols previously described and modified by (Thongboonkerd et al., 2002). Briefly, spots of interest were excised using a clean blade and placed in Eppendorf tubes, which were then washed with 0.1 M ammonium bicarbonate (NH_4HCO_3) at room temperature for 15 min. Acetonitrile was then added to the gel pieces and incubated at room temperature for 15 min. This solvent mixture was then removed and gel pieces dried. The protein spots were then incubated with 20 µL of 20 mM DTT in 0.1 M NH_4HCO_3 at 56 °C for 45 min. The DTT solution was removed and replaced with 20 µL of 55 mM iodoacetamide in 0.1 M NH_4HCO_3. The solution was then incubated at room temperature for 30 min. The iodoacetamide was removed and replaced with 0.2 mL of 50 mM NH_4HCO_3 and incubated at room temperature for 15 min. Acetonitrile (200 µL) was added. After 15 min incubation, the solvent was removed, and the gel spots were

dried in a flow hood for 30 min. The gel pieces were rehydrated with 20 ng/μL-modified trypsin (Promega, Madison, WI) in 50 mM NH_4HCO_3 with the minimal volume enough to cover the gel pieces. The gel pieces were incubated overnight at 37 °C in a shaking incubator.

5.3.13 Mass spectrometry

A MALDI-TOF mass spectrometer in the reflectron mode was used to generate peptide mass fingerprints. Peptides resulting from in-gel digestion with trypsin were analyzed on a 384 position, 600 μm AnchorChip^{TM} Target (Bruker Daltonics, Bremen, Germany) and prepared according to AnchorChip recommendations (AnchorChip Technology, Rev. 2, Bruker Daltonics, Bremen, Germany). Briefly, 1 μL of digestate was mixed with 1 μL of alpha-cyano-4-hydroxycinnamic acid (0.3 mg/mL in ethanol: acetone, 2:1 ratio) directly on the target and allowed to dry at room temperature. The sample spot was washed with 1 μL of a 1% TFA solution for approximately 60 seconds. The TFA droplet was gently blown off the sample spot with compressed air. The resulting diffuse sample spot was recrystallized (refocused) using 1 μL of a solution of ethanol: acetone: 0. 1 % TFA (6:3:1 ratio). Reported spectra are a summation of 100 laser shots. External calibration of the mass axis was used for acquisition and internal calibration using either trypsin autolysis ions or matrix clusters and was applied post acquisition for accurate mass determination.

5.3.14 Analysis of peptide sequences

Peptide mass fingerprinting was used to identify proteins from tryptic peptide fragments by utilizing the MASCOT search engine based on the entire NCBI and SwissProt protein databases. Database searches were conducted allowing for up to one missed trypsin cleavage and using the assumption that the peptides were monoisotopic, oxidized at methionine residues, and carbamidomethylated at cysteine residues. Mass tolerance of 150 ppm, 0.1 Da peptide tolerance and 0.2 Da fragmentation tolerance was the window of error allowed for matching the peptide mass values (Butterfield and Castegna, 2003b). Probability-based MOWSE scores were estimated by comparison of search results against estimated random match population and were reported as -10*log10 (p), where p is the probability that the identification of the protein is a random event. MOWSE scores greater than 63 were considered to be significant ($p < 0.05$). All protein identifications were in the expected size and isoelectric point (pI) range based on the position in the gel.

5.3.15 Immunoprecipitation

Immunoprecipitation of specific proteins was performed as previously described (Sultana and Butterfield, 2004). Brain samples (200 μg) from control or treated animals were incubated overnight with anti-GAPDH antibody. This was followed by three washing steps with buffer B (50Mm Tris Hcl (pH-8.0), 150 Mm NaCl, and 1% NP40). The proteins were resolved by SDS-PAGE followed by immunoblotting on a nitrocellulose membrane (BioRad). The proteins were then detected with alkaline phosphate labeled secondary antibody (Sigma).

5.3.16 Protein Interacteome

The functional protein interacteome was obtained by using Interaction Explorer ™ Software Pathway Assist software package (Stratagene, La Jolla, CA). Pathway Assist is software for functional interaction analysis. It allows for the identification and visualization of pathways, gene regulation networks and protein interaction maps (Donninger et al., 2004). The proteins are first imported as the gene symbols as a set of data. This data set is then searched against ResNet, a database containing over 500,000 biological interactions built by applying the MedScan text-mining algorithms to all PubMed abstracts. These interactions are then visualized by building interaction networks with shortest-path algorithms. This process can graphically identify all known interaction among the proteins. The information of the function of these proteins and their relevance to diseases are then obtained by using the BIOBASE's Proteome BioKnowledge Library form Incyte Corporation (Incyte, Wilmington, DE) (Hodges et al., 2002).

5.3.17 Statistical analysis

Statistical analysis of differentially expressed protein levels matched with spots on 2D-gels from parietal cortex brain samples from the various treatment groups control food- control environment (CC), control food - enriched environment (CE), control food- antioxidant fortified food (CA) and enriched environment - antioxidant fortified food (EA) were used and carried out using Student's *t*-tests. Here we report a comparative proteomics analysis of CC vs. CE, CA, and EA .A value of $p < 0.05$ was considered statistically significant. Only proteins that are considered significantly different by Student's *t*-test were subjected to in-gel trypsin digestion and subsequent proteomic analysis. This is the normal procedure for proteomics studies, since sophisticated

statistical analysis used for micro array studies are not applicable for proteomics studies (Maurer et al., 2005). To test for associations between cognitive test scores and differentially expressed proteins identified in the study, we computed correlation co-efficients both with and without age at death as a covariate.

5.4 Results

5.4.1 Antioxidant diet and behavioral enrichment program induces increased protein expression

Two-dimensional electrophoresis offers an excellent tool for the screening of abundant protein changes in various disease states (Butterfield et al., 2003; Butterfield, 2004; Perluigi et al., 2005b; Poon et al., 2005a). In the present study, we investigated the pattern of protein expression in the parietal cortex of aged canines following a 2.8 year treatment with an antioxidant fortified diet and/or a program of behavioral enrichment and compared this with age-matched controls in four different groups. We had a four-way analysis consisting of 23 beagle dogs divided into the following groups: CC, CE, CA and EA. The final comparison was made as follows 1) CC vs. CE; 2) CC vs. CA, and 3) CC vs. EA. Fig 5.1 shows SYPRO ruby stained 2D gels of the groups mentioned above with identified protein boxed and labeled. Compared to control, all treatment groups showed a significant increase in the expression of specific proteins. Some proteins showed an increase in expression in all treatment groups while others were specific for a particular treatment. The proteins identified were: Cu/Zn superoxide dismutase, fructose-bisphosphate aldolase C, creatine kinase, glutamate dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase, showing that the antioxidant and behavioral enrichment programme was successful in increasing the expression of proteins associated with energy metabolism and also a protein associated with antioxidant reserve. These proteins were identified by mass spectrometry and are shown in Table 5.1. Table 5.2 provides the changes in protein levels expressed as % control ± S.E.M across the treatment conditions. As a representative protein to validate these proteomic identifications, we used

immunoprecipitation of GAPDH with anti-GAPDH as shown (Fig 5.2), confirming our previous identification by mass spectrometry.

5.4.2 Combined treatment (EA), increases superoxide dismutase (SOD) activity

We have previously shown that the oxidative modification of specific enzymes generally decreases their activity (Perluigi et al., 2005b; Poon et al., 2006a). Therefore, in the present study we hypothesized that since increased oxidation leads to loss of enzymatic activity, then protection from oxidative damage could restore or maintain the activity of enzymes with up-regulated expression levels. To test this hypothesis, we measured the activities of total SOD, which was significantly increased following the combined treatment of an antioxidant fortified diet and exposure to a behavioral enrichment program in the aging canine. There was a about a 50% increase in the activity of SOD (Fig 5.3) in canine brain after the combined treatment (EA), and this activity was found to be significantly increased when compared to control (CC). These results suggest that oxidative damage plays a key role in the loss of function of key enzymes and that use of antioxidants not only increases their expression but also likely protects them from oxidative damage and significantly increases their enzymatic activities.

5.4.3 Correlation between protein expression levels and cognitive function

To determine if error scores on individual cognitive tasks were associated with increased protein expression of CuZnSOD, FBP, CK, GLUD or GAPDH a correlational analysis was used. CuZnSOD protein level was negatively correlated with error scores on a black/white reversal task and on a spatial memory task with higher SOD levels, i.e.

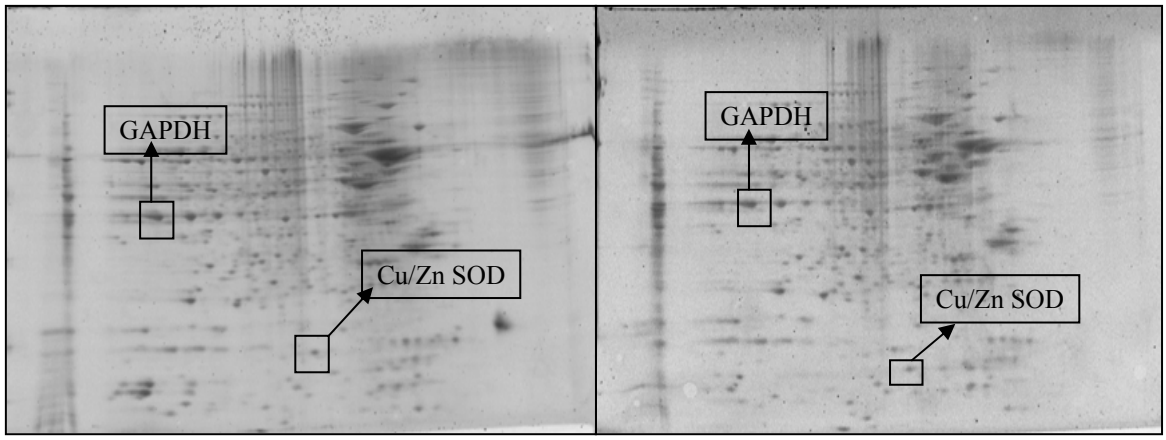
higher antioxidant protein level, being associated with lower error scores (improved cognition). Figure 5.4 shows the linear association between CuZnSOD protein and cognitive ability. Because age at death may also be a contributor to either increased error scores or increased protein expression, correlations were also computed and corrected for age. The significant association between CuZnSOD and cognition remained. Other protein measures (FBP, CK, GLUD, and GAPDH) did not correlate with cognitive scores.

5.4.4 Protein Interacteome

Fig 5.5 shows the protein interacteome of proteomics-identified proteins of increase expression following various intervention paradigms. The Interaction Explorer Software PathwayAssist (Stratagene) software results are shown in (Fig 5). The proteins identified in this study are related to hormone activities, transcription and regulation of signal transduction among others. As a result the present findings continue to confirm and support previous findings (Poon et al., 2004c; Poon et al., 2005e) that antioxidants and a program of regular exercise provide beneficial effect of protection and improvement in cognitive functions and memory through the increase in expression and activity of key proteins.

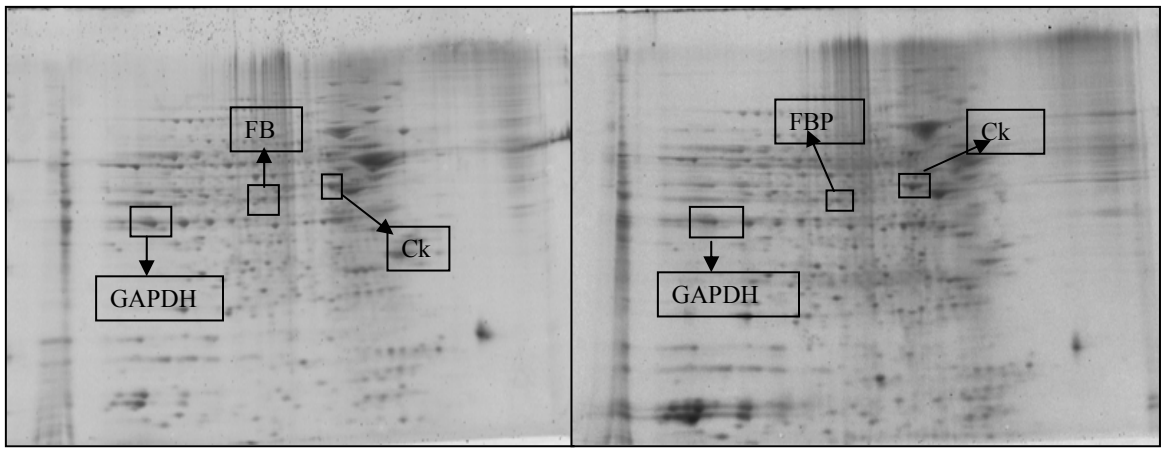
Figure 5.1

a)



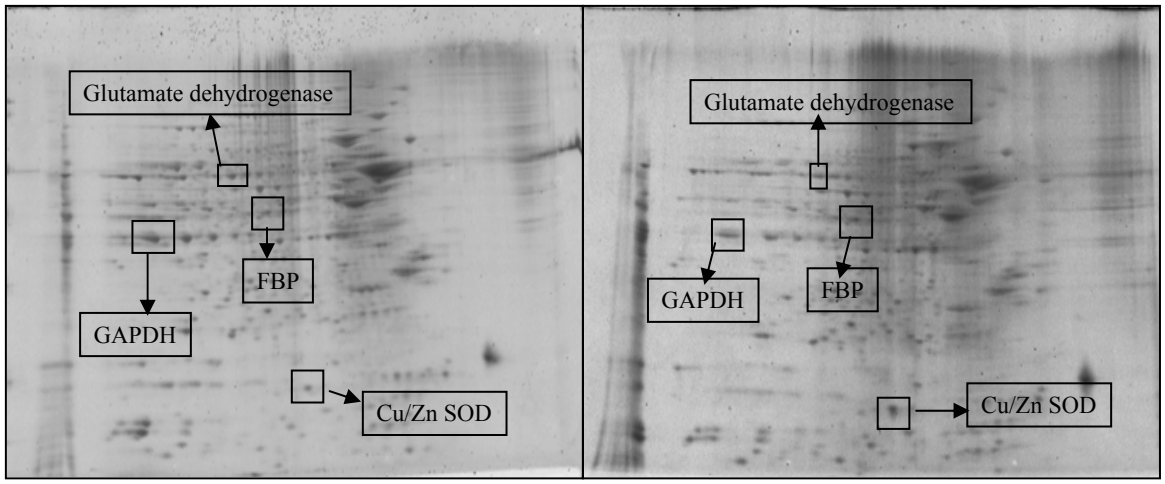
CC **EA**

b)



CC **CA**

c)



CC **CE**

Figure 5.1 SYPRO Ruby-stained 2D-gels maps, a) CC vs. EA b) CC vs. CA, and c) CC vs. CE of canine parietal cortex homogenates samples from the CC, CA, CE and EA treated animals are presented. Proteins identified by mass spectrometry are presented as the boxed spots.

Figure 5.2 Validation of protein identified by proteomics

Same as figure 4.4

Table 5.1 Summary of the proteins identified showing the MOWSE score, no of peptides matched molecular weight and pI

Identified Protein	GI accession number	Number of peptide matches identified	% Coverage of matched peptides	pI, MrW	Mowse score	Probability of a random identification
Cu/Zn Superoxide dismutase	gi|50978674	5	45	5.69,16074	69	1.3×10^{-7}
Fructose-Bisphosphate aldolase C	gi|56748614	10	37	6.46,39665	108	1.6×10^{-11}
Creatine Kinase B Chain	gi|320114	10	37	5.47,42960	115	3.2×10^{-12}
Glutamate dehydrogenase [NAD (P)]	gi|2494097	10	20	7.66,61701	95	3.2×10^{-10}
Glyceraldehyde-3-phosphate dehydrogenase (Phosphorylating)	gi|65987	8	23	6.90,35914	92	6.3×10^{-10}

Table 5.2 Canine brain proteins differentially expressed by different treatment paradigms

Identified Protein	%Control ± S.E.M	CC vs. CE	CC vs. CA	CC vs. EA	P value
Cu/Zn SOD	204 ± 44	√		√	< 0.02
FBP	139 ± 28	√	√		< 0.03
CK	171 ± 19		√		< 0.04
GLUD	152 ± 23	√			< 0.01
GAPDH	234 ± 42	√	√	√	< 0.05

Figure 5.3

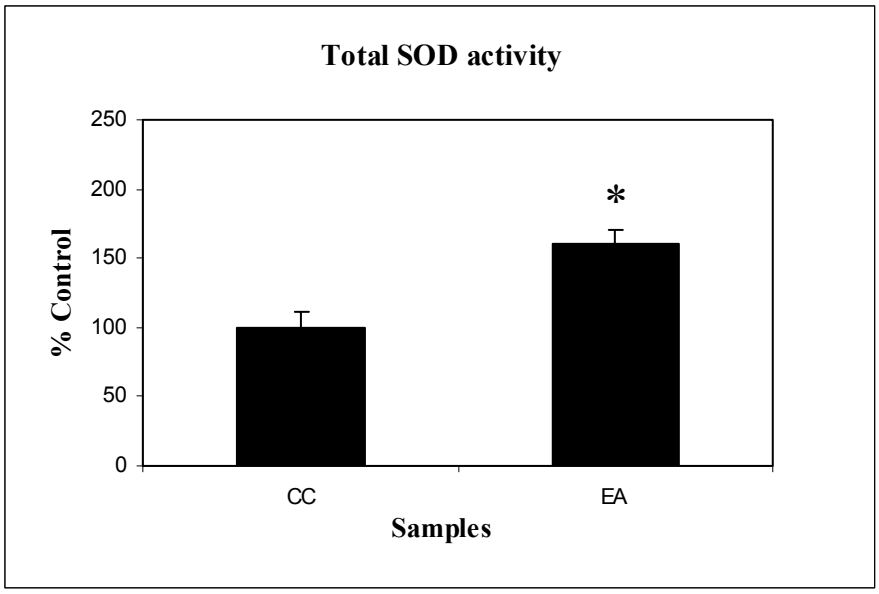


Figure 5.3 Comparison of the specific activity of SOD enzyme measured in canine brains obtained from animals on a control food- control environment treatment (CC) and canine on an enriched environment - antioxidant fortified food programme EA. There is a significant increase in SOD activity in EA compared to CC and is expressed as units per milligram of protein. Data are represented as % control ± SEM for animals in each treatment group, (n=6) * p <0.05.

Figure 5.4

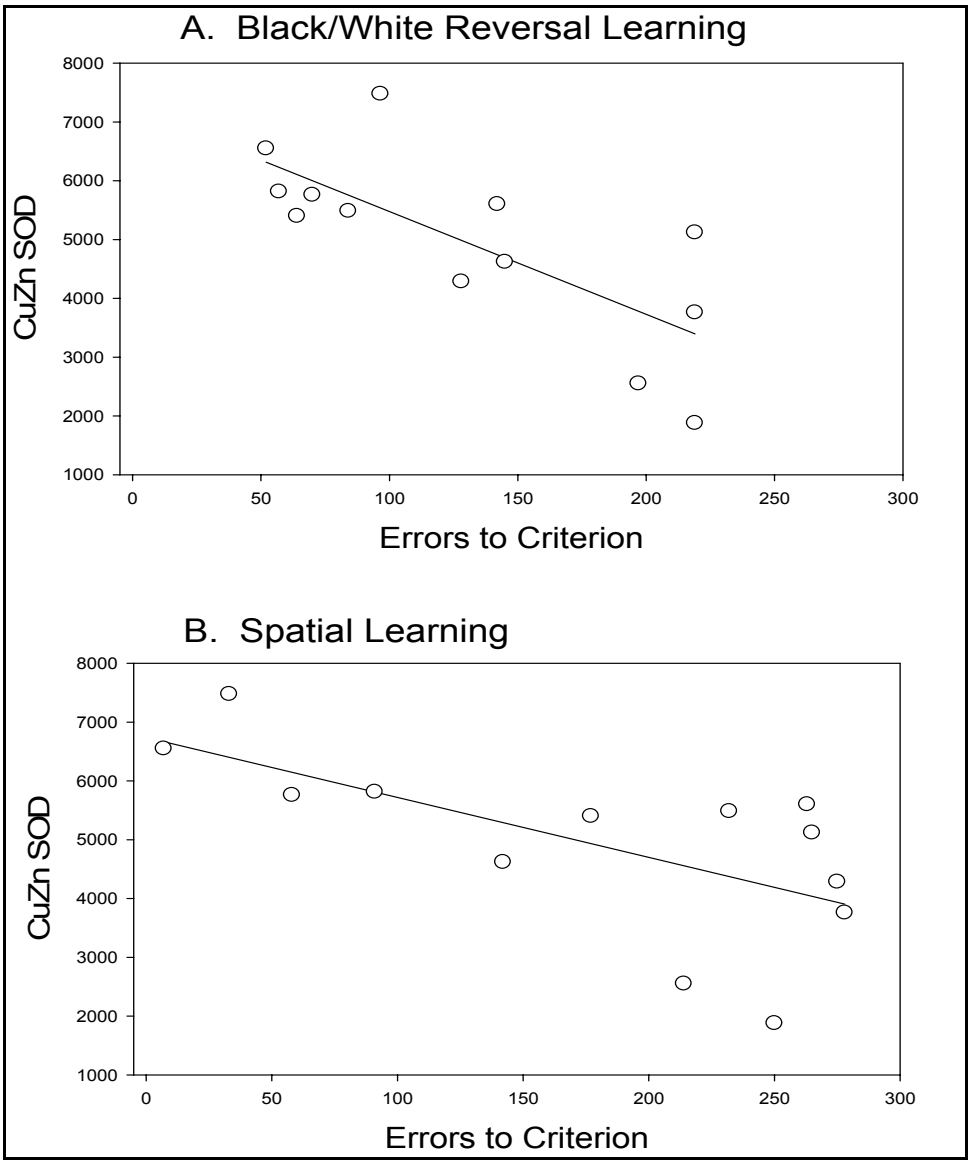


Figure 5.4 Correlation between measures of protein levels and cognitive function. Individual error scores are plotted as a function of CuZnSOD protein levels in the parietal cortex. (A). Black/white reversal learning was poorer in animals with lower levels of SOD. (B). Spatial learning was also impaired in animals with lower SOD protein levels. Line represents the results of a linear regression analysis

Figure 5.5

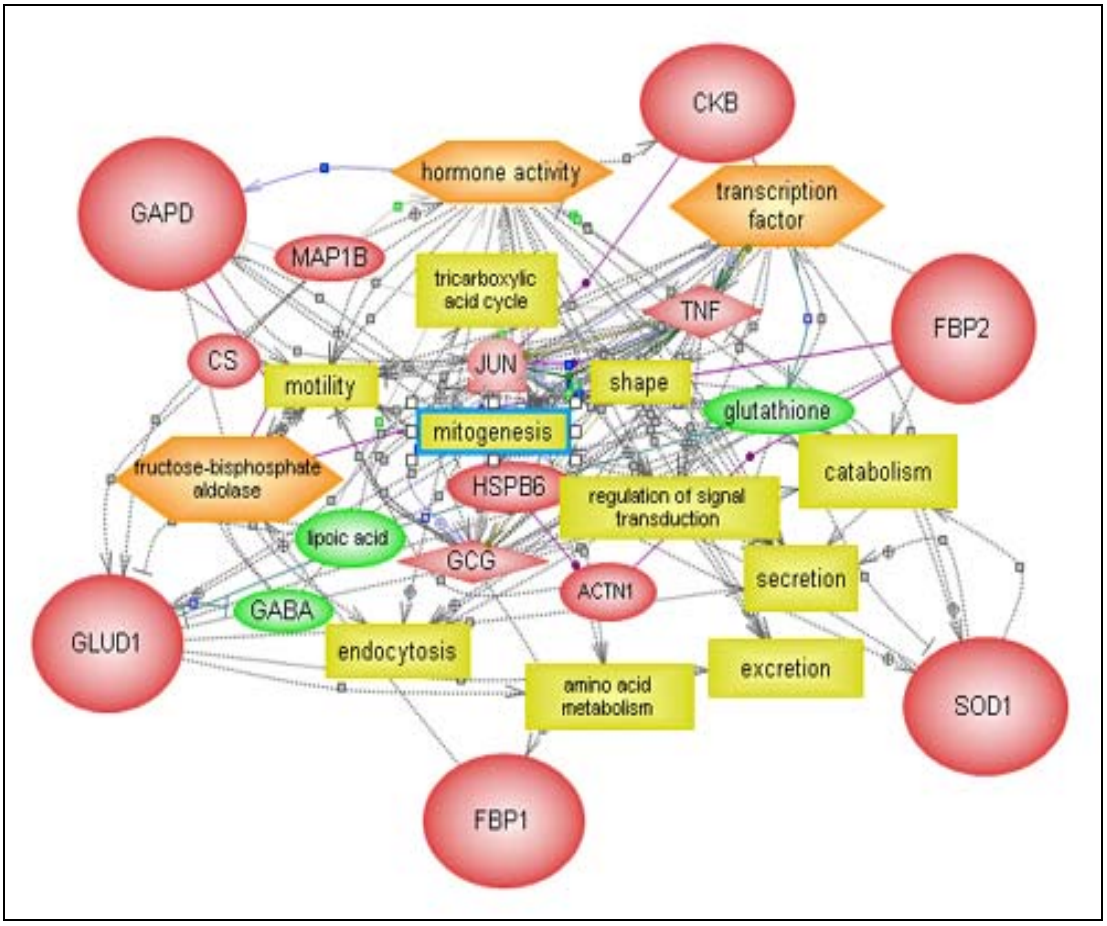


Figure 5.5 Schematic diagram of a functional interacteome of all parietal cortex proteins identified whose expression levels are significantly increased following treatment with and antioxidant-fortified food and a program of behavioral enrichment. This diagram was generated by the interaction explorer ™ Pathway Module (Stratagene), indicating that all of the proteins are directly or indirectly associated with cellular process shown.

5.5 Discussion

Long-term treatment with antioxidants and a program of behavioral enrichment in the canine model of human aging improves cognition (Milgram et al., 2002c; Milgram et al., 2004; Milgram et al., 2005). Age-related disorders are often accompanied by an increase in oxidative stress, reduction in antioxidant reserves and loss in energy metabolism among other pathological features (Hensley et al., 1996; Markesbery, 1997; Butterfield and Lauderback, 2002). In many animal models of aging, interventions with antioxidants are beneficial and result in a reduction of oxidative stress and improvements in cognitive function, however, the mechanisms that underlie these improvements are still not well understood (Joseph et al., 1998a; Joseph et al., 1998b; Farr et al., 2003). In the present study, we used brain tissue from a higher mammalian species provided with a long treatment protocol (2.8 years), typically not possible in aged rodent models, to elucidate biochemical pathways and mechanisms for the improvement of cognitive function observed. Using proteomics, we were able to identify key proteins from the canine parietal cortex whose expressions were significantly increased following a program of behavioral enrichment and treatment with a diet fortified with antioxidants. Four proteins related to energy metabolism, i.e., fructose-bisphosphate, creatine kinase, glutamate dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase were increased in expression as was the protein level and activity of a key antioxidant protein, Cu/Zn superoxide dismutase that also showed a significant association with cognition.

Fructose bisphosphate aldolase C (FBP) is a glycolytic enzyme that catalyses the reversible aldol cleavage or condensation of fructose-1, 6-bisphosphate into dihydroxyacetone-

phosphate and glyceraldehyde 3-phosphate (Perham, 1990). In vertebrates, three forms of this enzyme are found: aldolase A is expressed in muscle, aldolase B in liver, kidney, stomach and intestine, and aldolase C in brain, heart and ovary. The different isozymes have different catalytic functions: aldolases A and C are mainly involved in glycolysis, while aldolase B is involved in both glycolysis and gluconeogenesis (Perham, 1990). On the other hand, Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) catalyzes the oxidation of glyceraldehyde-3-phosphate to 1, 3-bisphosphoglycerate and NADH (Boyd-Kimball et al., 2005c). In addition to glycolysis GAPDH plays a role in membrane fusion and transport, accumulation of glutamate into presynaptic vesicles, and acting as a cellular sensor of oxidative stress (Chuang et al., 2005). Recent studies suggest GAPDH acts as a NO sensor (Hara et al., 2006). In AD, there is a reduced activity of glyceraldehyde-3-phosphate dehydrogenase (Mazzola and Sirover, 2001), and our laboratory has shown that GAPDH is significantly oxidized in AD and in animal models of AD (Boyd-Kimball et al., 2005a; Boyd-Kimball et al., 2005c; Sultana et al., 2006b). In the present study the expression of these glycolytic proteins was significantly increased following a program of enriched environment and a diet of antioxidants. This increased expression of FBP and GAPDH, proteins that are decreased in patients with AD (Bigl et al., 1999), may lead to increased glycolytic activity and function and when coupled to other bioenergetics pathways would further lead to an increase and maintenance of ATP production. Increased ATP production may be important for neuron survival and thus may serve as one mechanism underlying observed improvements in cognitive functions in the canine model of human aging.

The creatine kinase (CK) system is the most important immediate energy buffering and transport system especially in muscle and neuronal tissue (Wallimann et al.,

1998). CK consists of a cytosolic and a mitochondrial isoform (MtCK) with their substrates creatine and phosphocreatine. Creatine is typically phosphorylated to phosphocreatine in the intermembrane space of mitochondria where mitochondrial CK is located and is then transported into the cytosol (Schlattner et al., 1998). In the cytosol, the energy pool can be regenerated by transphosphorylation of phosphocreatine to ATP, which is catalyzed by cytosolic CK in close proximity to cellular ATPases. Moreover, the mitochondrial synthesis of creatine phosphate is restricted to uMiCK expressing neurons, suggesting uMiCK protects neurons under situations of compromised cellular energy state, which are often linked to oxidative stress and calcium overload through compensatory up-regulation of gene expression (Boero et al., 2003). CKs are prime targets of oxidative damage, MtCK in particular is a principal target of such damage, not only because if its sensitivity (Stachowiak et al., 1998; Koufen and Stark, 2000), but also due to its mitochondrial localization. Our laboratory has shown that though there is increased expression of CK in AD, it is significantly oxidized and its activity significantly reduced (Aksenov et al., 2000; Castegna et al., 2002b). In the brain of old brown Norway rats, CK is oxidatively modified and its activity significantly decreased (Aksenova et al., 1998). Also in aging neuronal cultures, there is a gradual increase in CK content but decreased activity of the enzyme. These changes in CK expression have been considered to be an early indicator of oxidative stress in aging neurons (Aksenova et al., 1999). In the present study, following exposure of the aging beagle dogs to a program of environmental enrichment and a diet fortified with anti-oxidants, we observed a significant increase in the expression of CK. This is in agreement with a previous study from our laboratory that showed a significant increase in the expression of CK in the senescence accelerated prone mouse strain 8 (SAMP8) mice

after intervention with alpha-lipoic acid, a mitochondrial co-factor and antioxidant included in the diet used in the present study (Poon et al., 2005e). This increased expression we posit is a compensatory mechanism for restoration of ATP production in the aging canine, thus leading to maintenance of energy reserves and possibly cognitive function

Glutamate dehydrogenase (GDH) is an enzyme located in the mitochondrial matrix that acts in either a metabolic or a catabolic pathway. In the biosynthetic pathway, GDH catalyzes the reductive amination of α-ketoglutarate with NADPH to yield glutamate. Alternatively, GDH can catalyze the formation of α-ketoglutarate from glutamate with NAD^+ and ammonium ion (Boyd-Kimball et al., 2005d). The catabolic activity of GDH is particularly important for the elimination of excitotoxic glutamate. We have previously shown using proteomics that when synaptosomes are treated with Aβ (1-42) (Boyd-Kimball et al., 2005d), GDH is oxidized and loses its function, a phenomenon observed in aging. In the present study, we observed that after treatment with antioxidants and exposure to a program of behavioral enrichment, the canine brain showed an increased expression of GDH. Increased expression of GDH could lead to increased elimination of excitotoxic glutamate by increasing the conversion of glutamate to α-ketoglutarate. The conversion of excess glutamate to α-ketoglutarate would desensitize NMDA receptors leading to a reduction in Ca^{2+} influx. Improved calcium homeostasis would in turn lead to maintenance of the long-term potentiation (LTP) and consequently, improved learning and memory (Michaelis, 1997), as observed in the canine study.

Cu/Zn Superoxide dismutase (CuZnSOD, SOD1 protein) is an abundant copper- and zinc-containing protein that is present in the cytosol, nucleus, peroxisomes, and mitochondrial intermembrane space of human cells and acts as an antioxidant enzyme by

lowering the steady-state concentration of superoxide (Selverstone Valentine et al., 2005). When mutated, SOD can also cause disease as in the case of the neurodegenerative disorder, familial amyotrophic lateral sclerosis (fALS) (Selverstone Valentine et al., 2005). The toxic gain of function of mutant SOD (mSOD) leads to the generation of reactive oxygen/nitrogen species (Valentine, 2002; Perluigi et al., 2005a; Poon et al., 2005f). Some researchers believe that the elevated oxidative activity associated with mSOD occurs by enzymes acting as peroxidases (Valentine, 2002) or as superoxide reductases (Liochev and Fridovich, 2000) or by producing $O_2^{\cdot -}$ to form peroxynitrite (Rakhit et al., 2002). In the wild type form, SOD dismutates superoxide to oxygen and water, hence reducing the levels of oxidative stress and protecting proteins, lipid and DNA from the toxic superoxide molecule (Gutteridge and Halliwell, 2000). In the present study a significant increase in the expression of SOD1 and significant increase in SOD enzymatic activity in the brain from canines that had undergone a combination of both treatment with antioxidant diet and a program of behavioral enrichment compared to age matched controls were found. This finding shows that a diet of antioxidants and a program of behavioral enrichment provided to the aging canine are able to increase the expression and activity of a key antioxidant protein, in turn protecting the cells form oxidative stress and improving memory and cognitive function. Consistent with this hypothesis is that of the measures of protein expression that distinguished the treatment groups in the current study. SOD1 protein level was the best predictor of a frontal-cortex dependent learning task and a measure of spatial memory. Animals that were showing improved error scores on these two tasks also had higher SOD1 protein.

In the current study, we demonstrated that following a long-term treatment with a diet fortified with antioxidants and a program of behavioral enrichment in the aging canine, there is a significant increase in the expression of key proteins related to energy metabolism and antioxidant activity. Increased protein expression does not necessarily directly translate to increased enzyme activity as reported for creatine kinase in AD (Aksenov et al., 1998a); however, we have shown here both an increase in expression and activity of Cu/Zn SOD in response to treatment. In addition, this increase in protein levels was found to be a good predictor of frontal-cortex dependent learning and a measure of spatial memory. The present findings therefore provide a neurobiological basis for improved neuronal function and cognition in canines treated with either or both an antioxidant enriched diet and behavioral enrichment. The inclusion of vitamin E, alpha-lipoic acid L-carnitine flavanoids in the diet not only provide improvements in antioxidant reserves but also plays a role in increasing the expression of key energy metabolism proteins that help in the maintenance of ATP levels, maintenance of cellular pathways and functions dependent on ATP eventually leading to an improvement in cognitive function and memory. Fig 5.6 provides a summary of the functional consequence of increased expression and reduced oxidation of proteins identified in the aging canine following treatment with an antioxidants fortified diet and a program of behavioral enrichment.

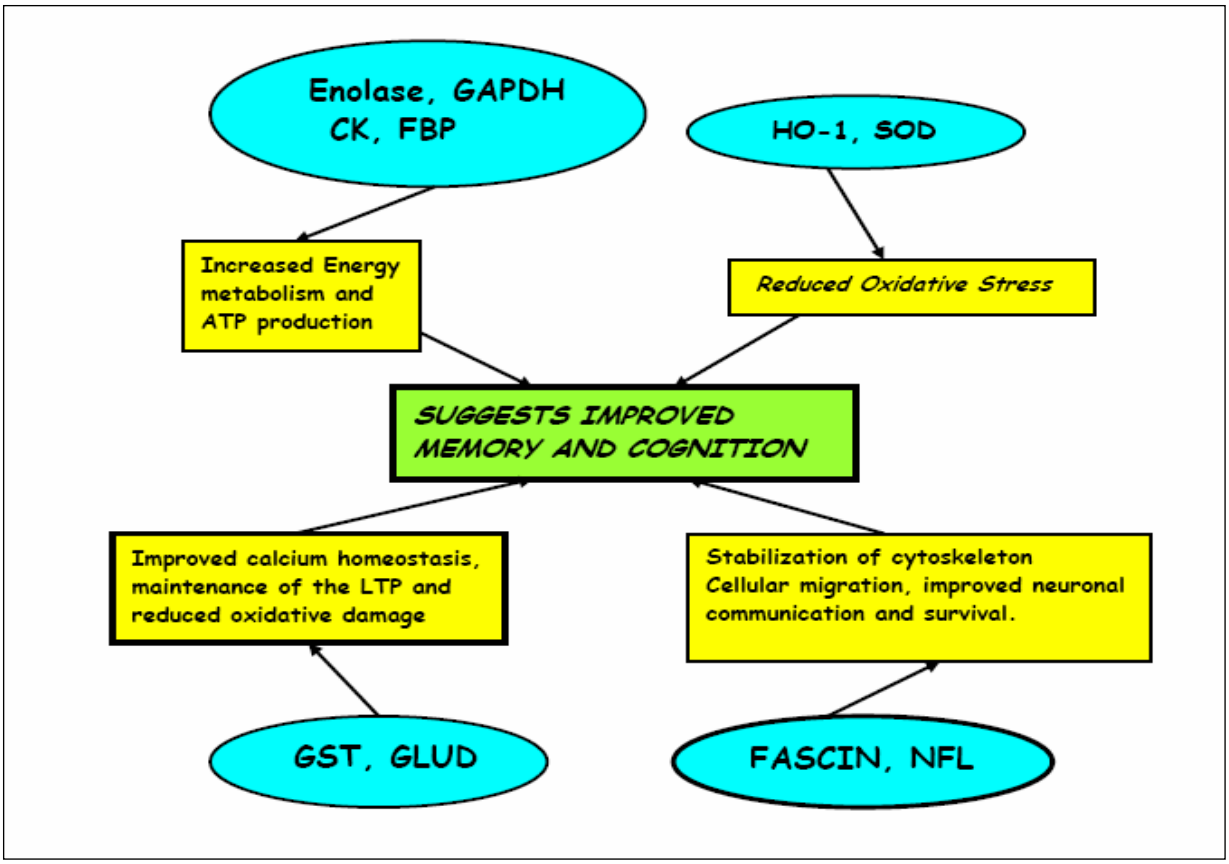


Figure 5.6 A summary of the functional consequence of increased expression and reduced oxidation of proteins identified in the aging canine following treatment with an antioxidants fortified diet and a program of behavioral enrichment.

5.6 Acknowledgement

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CHAPTER SIX

Proteomic Expression Analysis of Brain Proteins from Dementia-free Nonagenarians: Relevance to Successful Aging and Alzheimer's disease

6.1 Overview

Aging is a unique phenomenon that affects all animal species. In humans, aging is usually accompanied by impairments in the functional capacity of the brain leading to its susceptibility to neurodegenerative disorders such as Alzheimer's disease (AD) among others. In spite of functional declines in aging, recently, there has been a rise in the number of nonagenarian and centenarians who are cognitively intact and who demonstrate no evidence of neurodegenerative diseases despite their advanced ages; this phenomenon is usually referred to as successful aging. The mechanisms underlying this phenomenon of successful aging are still under investigation. Various studies have identified several genes thought to be responsible for dementia-free aging and extended longevity, but since no single gene can be found responsible for modulating successful human aging, it is thought that this process occurs possibly through multiple routes. To further investigate this phenomenon, we have carried out a proteomic expression profile analysis on brain proteins in the frontal cortex of 5 nonagenarian (mean age ± SD, 94.8 ± 2.9) with AD and 4 normal non-demented age matched control nonagenarians (mean age ± SD, 94.5 ± 1.3). In addition, we have evaluated biomarkers of oxidative stress as measured by the levels of protein oxidation and lipid peroxidation, i.e., protein carbonyls and protein-bound 4-hydroxynonenal (HNE), isoprostanes (F_2-IsoP) and neuroprostanes (F_4-NP). There was no significant difference in the levels of protein carbonyls, HNE, F_2-IsoP and F_4-NP when the two groups were compared. However, using proteomics we

were able to identify six proteins that were differentially expressed. Among these, we observed a significant decrease in the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), ATP synthase H^+ transporting, human peroxiredoxin 5 (PRDX5) and malate dehydrogenase (MDH) in the non-demented nonagenarian compared to those with AD. However, the levels of protein expression for fructose bisphosphate aldolase (FBP1) and ubiquitin carboxyl-terminal hydrolase L1 (UCHL1) were found to be significantly increased when the same groups were compared. In addition, the severity of cognitive decline (MMSE) and amyloid beta-peptide (Aβ) levels correlated with these changes in protein expression. These results show that though the levels of oxidative stress were comparable between age-matched non-demented and demented nonagenarians, the differential expression of proteins related to energy metabolism and antioxidant systems could contribute to the mechanisms that result in extended longevity and maintenance of cognitive reserve and functional capacity in this rising population of non-demented nonagenarians and centenarians.

6.2 Introduction

Aging is defined as the gradual alteration in structure and function that occurs over time, eventually leading to an increased probability of death not associated with disease or trauma, and it can be considered to be a product of an interaction between genetic, environmental and lifestyle factors (Ashok and Ali, 1999; Gonos, 2000). Aging is usually characterized by impairments in physiological functions, such as impairments in the brain, increased susceptibility to dementia and neurodegenerative disorders, i.e., Alzheimer's disease (AD), Parkinson disease (PD), Huntington's disease HD, and amyotrophic lateral sclerosis (ALS), among others (Poon et al., 2004b). Centenarians are the fastest growing age-group in the USA, and it is projected that there will be more than 400,000 in the next 30 years (Silver et al., 2002). Studies have shown that there is an increase in the incidence of dementia among people aged 85 years and above (Silver et al., 2001; Perls, 2004b, a), and most people believe that dementia is inevitable to people in this age group (Blansjaar et al., 2000; Snowdon, 2003; Perls, 2004a). However, various studies on nonagenarians and centenarians have shown that approximately 30% of centenarians are cognitively intact and that among those who are demented, 90% show a delay in the time they present signs of cognitive impairments well into their 90's (Hagberg et al., 2001; Silver et al., 2001; Perls, 2004b, a). As a result, the increasing number of non-demented nonagenarians and centenarians disputes the notion that dementia is inevitable as one becomes older (Green et al., 2000; Perls, 2006). This rising number of the oldest-old provides a unique resource for the study of dementia-free aging and related disorders.

The ability to achieve extreme old age while still maintaining intact cognitive function appears to be the result of a complex combination of factors such as genetics, environment, and lifestyle, among others, showing the complexity of understanding the mechanisms for successful aging process in the human population (Perls, 2005; Perls, 2006). Various studies have shown the gene expression profiles in the aging brain (Galvin and Ginsberg, 2005), but few have been able to show the protein expression profile in the cognitively intact nonagenarians. Therefore, to better understand the cellular and biochemical mechanisms involved in the brains of cognitively intact nonagenarians, a differential expressional proteomic study on brain from nonagenarians who had undergone successful aging and were non-demented compared to their demented aged-matched controls was performed in the current study.

Our laboratory has previously used proteomics to identify proteins that have undergone oxidative modifications or significant differential expression in AD subjects compared to age-matched controls. These studies among others have continued to provide evidence that energy metabolism and maintenance of ATP levels plays an integral role in the pathological and biochemical pathways of age-related neurodegenerative disorders (Castegna et al., 2002b; Castegna et al., 2002a; Castegna et al., 2003; Sultana et al., 2006d; Sultana et al., 2006c; Sultana et al., 2006a; Sultana et al., 2006b). Though we have been successful in these studies, having non-demented nonagenarians and age-matched subjects with AD is a unique resource that was previously not available to us. Hence, this current study is the first report using proteomics to investigate possible mechanisms that are involved in successful aging of cognitively intact nonagenarians. We identified six brain proteins that were significantly

differentially expressed. We observed a significant decrease in the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), ATP synthase, H^+ transporting protein, human peroxiredoxin 5 (PRDX5), and malate dehydrogenase (MDH) in the non-demented nonagenarian compared to those with AD. On the other hand, the levels of protein expression of fructose bisphosphate aldolase (FBP1) and ubiquitin carboxyl-terminal hydrolase L1 (UCHL1) were found to be significantly increased when the same groups were compared. Further, we found that the severity of cognitive decline (MMSE) correlated with these changes in protein expression and that the extent of Aβ was also linked to protein expression. These results show that though the levels of oxidative stress were comparable, the increased expression of proteins related to energy metabolism and antioxidant systems in the demented brain of nonagenarians compared to their non-demented aged-matched nonagenarians could contribute to the mechanisms that result in extended longevity and maintenance of cognitive reserve in the successful aging population.

6.3 Experimental procedures

6.3.1 Subjects

Subjects in the study were participants in the 90+ study, a population based investigation of aging and dementia in the oldest old. In the early 1980s, residents of Leisure World, a retirement community in southern California, were mailed a health questionnaire. Those who completed and returned the questionnaire became members of the Leisure World Cohort Study. Of the members of the Leisure World Cohort, those who were alive and aged 90 years or older on January 1, 2003 were eligible to participate in The 90+ Study. Participants in the 90+ study were given neuropsychological testing every 6 months until their death. A subset of these participants agreed to brain autopsy and tissue donation. The subjects in this study were selected from the first 19 cases to come to autopsy. Subjects were selected based on their clinical and neuropathological diagnoses. The subject demographics are summarized in Table 6.1. The neuropsychological testing and neuropathology diagnosis were performed by Dr Daniel J. Berlau at the Institute for Brain Aging and Dementia, Department of Neurology, University of California, Irvine, California.

Table 6.1 Subject demographics and neuropathological cases

	CLIN DX	NP DX	Tangle Stage	Plaque Stage	PMI	Brain Weight	Sex	Age at Death
1	AD	Alzheimer's Disease, Lewy Bodies within Amygdala	V	B	5.5	987.9	Female	95
2	Probable AD	Alzheimer's Disease	VI	B	2.3	1289.6	Female	100
3	Probable AD	Alzheimer's Disease	VI	C	2.8	901.5	Female	92
4	Dementia – AD	Alzheimer's Disease; Hippocampal Sclerosis	V	A	4.5	1156.3	Female	92
5	Dementia - unspecified	Alzheimer's Disease	VI	C	4.4	1015.9	Female	95
6	Normal	Senile Degenerative Changes	IV	0	5.3	1261.5	Male	94
7	Normal	Senile Degenerative Changes	III	0	2.2	1128.5	Female	93
8	Normal	Senile Degenerative Changes	III	B	3.8	1390.0	Male	96
9	Normal	Senile Degenerative Changes	III	B	3.2	1192.9	Female	95

6.3.2 Neuropsychological testing

The Mini-Mental State Exam (MMSE) was administered to the participants in the 90+ study every 6 months as a measure of overall cognitive status. Only the data from their final examination was used for statistical analysis in this study. The MMSE score is out of 30 possible points and includes the following sections: temporal orientation, spatial orientation, attention, mental reversal, recall, naming, repetition, writing, reading and obeying a command, constructional praxis, and visual construction (Folstein, 1975).

6.3.3 Neuropathology diagnosis

Each brain was weighed at autopsy and one cerebral hemisphere (together with the brainstem and cerebellum) was fixed in 4% paraformaldehyde for 2 weeks, and the contralateral hemisphere was coronally sectioned and frozen at -80°C. Cortical and subcortical regions were dissected and paraffin embedded for histological and immunohistochemical examination. Modified Bielschowsky, hematoxylin-eosin and immunostains for tau, α-synuclein, ubiquitin, glial fibrillary acidic protein (GFAP-astrocytosis) and CD68 (activated microglial cells) were applied. Microscopically, neuritic plaque formation and neurofibrillary degeneration was categorized according to Braak & Braak staging (Braak and Braak, 1991; Braak and Braak, 1995). The final neuropathology diagnosis was established using NIA/Reagan criteria (disease, 1997).

6.3.4 Sample preparation

Brain samples were minced and suspended in 10 mM HEPES buffer (pH 7.4) containing 137 mM NaCl, 4.6 mM KCl, 1.1 mM KH_2PO_4, 0.1 mM EDTA, and 0.6 mM

$MgSO_4$ as well as proteinase inhibitors: leupeptin (0.5 mg/mL), pepstatin (0.7 μg/mL), type II S soybean trypsin inhibitor (0.5 μg/mL), and PMSF (40 μg/mL). Homogenates were centrifuged at 14,000 × *g* for 10 min to remove debris. Protein concentration in the supernatant was determined by the BCA method (Pierce, Rockford, IL, USA).

6.3.5 Measurement of protein carbonyls

Protein carbonyls are an index of protein oxidation and were determined as described previously (Butterfield and Stadtman, 1997)**.** Briefly, samples (5 μg of protein) were derivatized with 10 mM 2, 4-dinitrophenylhydrazine (DNPH) in the presence of 5 μL of 12% sodium dodecyl sulfate for 20 min at room temperature (23°C). The samples were then neutralized with 7.5 μL of the neutralization solution (2 M Tris in 30% glycerol). Derivatized protein samples were then blotted onto a nitrocellulose membrane with a slot-blot apparatus (250 ng per lane). The membrane was then washed with wash buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20) and blocked by incubation in the presence of 5% bovine serum albumin, followed by incubation with rabbit polyclonal anti-DNPH antibody (1: 100 dilution) as the primary antibody for 1 h. The membranes were washed with wash buffer and further incubated with alkaline phosphatase-conjugated goat anti-rabbit antibody as the secondary antibody for 1 h. Blots were developed using fast tablet (BCIP/NBT; Sigma-Aldrich) and quantified using Scion Image (PC version of Macintosh-compatible NIH Image) software.

6.3.6 Lipid peroxidation

6.3.6.1 Measurement of protein-bound 4-hydoxynonenal (HNE)

HNE is a marker of lipid oxidation and the assay was performed as previously described (Lauderback et al., 2001). Briefly, 10 µl of sample were incubated with 10 µl of Laemmli buffer containing 0.125 M Tris base pH 6.8, 4 % (v/v) SDS, and 20% (v/v) glycerol. The resulting sample (250 ng) was loaded per well in the slot blot apparatus containing a nitrocellulose membrane under vacuum pressure. The membrane was blocked with 3% (w/v) bovine serum albumin (BSA) in phosphate buffered saline containing 0.01% (w/v) sodium azide and 0.2% (v/v) Tween 20 (PBST) for 1 h and incubated with a 1:5000 dilution of anti-4-hydroxynonenal (HNE) polyclonal antibody in PBST for 90 min. Following completion of the primary antibody incubation, the membranes were washed three times in PBST. An anti-rabbit IgG alkaline phosphatase secondary antibody was diluted 1:8000 in PBST and added to the membrane. The membrane was washed in PBST three times and developed using Sigmafast Tablets (BCIP/NBT substrate). Blots were dried, scanned with Adobe Photoshop, and quantified by Scion Image.

6.3.6.2 Measurement of Neuroprostanes and Isoprostanes

Dr. J.D Morrow at the Vanderbilt University School of Medicine quantified the levels of Isoprostanes (F_2-IsoPs) and Neuroprostanes (F_4-NPs) in brain specimens of the oldest old. This was done using highly precise and accurate mass spectrometric assays applying stable isotope dilution techniques, as described previously (Morrow and

Roberts, 1999; Musiek et al., 2004). Briefly, specimens were dissected from frozen middle frontal gyrus of individual cases and lipids were extracted from specimens by the method of Folch and colleagues (Folch et al., 1957). F_2-IsoP and F_4-NPs were esterified in tissue and hydrolyzed by chemical saponification, extracted using C_{18} and silica Sep-Pak cartridges (Waters Corporation, Milford, MA), purified by thin-layer chromatography, converted to pentafluorobenzyl ester trimethylsilyl ether derivatives, and quantified using gas chromatography/negative ion chemical ionization/mass spectrometry. The stable isotope dilution techniques used [$^2H_4$]-8-iso-PGF_{2a} as an internal standard for F_2-IsoPs and [$^{18}O_2$]17-F_{4c}-NP as an internal standard for F_4-NPs.

6.3.7 Two-dimensional electrophoresis

Brain samples (200 μg) were incubated with 4 volumes of 2N HCL at room for electrophoresis. Proteins were then precipitated by the addition of ice-cold 100% trichloroacetic acid (TCA) to obtain a final concentration of 15% TCA. Samples were then placed on ice for 10 min and precipitate centrifuged at 16,000 g for 3 min. The resulting pellet was then washed three times with a 1:1(v/v) ethanol/ethyl acetate solution. The samples were then suspended in 200 μl of rehydration buffer composed of a 1:1 ratio (v/v) of the zwittergent solubilization buffer (7M urea, 2M thiourea, 2% Chaps, 65 mM DTT, 1% zwittergent 0.8% 3-10 ampholytes and bromophenol blue) and ASB-14 solubilization buffer (7M urea, 2M thiourea 5Mn TCEP, 1% (w/v) ASB-14, 1% (v/v) Triton X-100, 0.5% Chaps, 0.5% 3-10 ampholytes) for 1 h.

6.3.8 First dimension electrophoresis

For the first-dimension electrophoresis, 200 μL of sample solution was applied to a 110-mm pH 3–10 ReadyStrip™ IPG strips (Bio-Rad, Hercules CA). The strips were then actively rehydrated in the protean IEF cell (Bio-Rad) at 50 V for 18 h. The isoelectric focusing was performed in increasing voltages as follows; 300 V for 1 h, then linear gradient to 8000 V for 5 h and finally 20 000 V/h. Strips were then stored at –80 °C until the 2nd dimension electrophoresis was to be performed.

6.3.9 Second dimension electrophoresis

For the second dimension, the IPG® Strips, pH 3–10, were equilibrated for 10 min in 50 mM Tris–HCl (pH 6.8) containing 6 M urea, 1% (w/v) sodium dodecyl sulfate (SDS), 30% (v/v) glycerol, and 0.5% dithiothreitol, and then re-equilibrated for 15 min in same buffer containing 4.5% iodoacetamide instead of dithiothreitol. Linear gradient precast criterion Tris–HCl gels (8–16%) (Bio-Rad) were used to perform second dimension electrophoresis. Precision Protein™ Standards (Bio-Rad, CA) were run along with the sample at 200 V for 65 min.

6.3.10 SYPRO ruby staining

After the second dimension electrophoresis, the gels were incubated in fixing solution (7% acetic acid, 10% methanol) for 20 min and stained overnight at room temperature with 50 ml SYPRO Ruby gel stains (Bio-Rad). The SYPRO ruby gel stain was then removed and gels stored in deionized water.

6.3.11 Image analysis

The nitrocellulose blots (oxyblots) were scanned and saved in TIFF format using Scan jet 3300C scanner (Hewlett Packard, CA). SYPRO ruby-stained gel images were obtained using a STORM phosphoimager (Ex. 470 nm, Em. 618 nm, Molecular Dynamics, Sunnyvale, CA, USA) and also saved in TIFF format. PD-Quest (Bio-Rad) imaging software was then used to match and analyze visualized protein spots among differential 2D gels for each group of subjects.

6.3.12 In-gel trypsin digestion

Samples were digested by trypsin using protocols previously described (Thongboonkerd et al., 2002). Briefly, spots of interest were excised using a clean blade and placed in Eppendorf tubes, which were then washed with 0.1 M ammonium bicarbonate (NH_4HCO_3) at room temperature for 15 min. Acetonitrile was then added to the gel pieces and incubated at room temperature for 15 min. This solvent mixture was then removed and gel pieces dried. The protein spots were then incubated with 20 μL of 20 mM DTT in 0.1 M NH_4HCO_3 at 56 °C for 45 min. The DTT solution was removed and replaced with 20 μL of 55 mM iodoacetamide in 0.1 M NH_4HCO_3. The solution was then incubated at room temperature for 30 min. The iodoacetamide was removed and replaced with 0.2 mL of 50 mM NH_4HCO_3 and incubated at room temperature for 15 min. Acetonitrile (200 μL) was added. After 15 min incubation, the solvent was removed, and the gel spots were dried in a flow hood for 30 min. The gel pieces were rehydrated with 20 ng/μL modified trypsin (Promega, Madison, WI) in 50 mM

NH_4HCO_3, with the minimal volume enough to cover the gel pieces. The gel pieces were incubated overnight at 37 °C in a shaking incubator.

6.3.13 Mass spectrometry

A MALDI-TOF mass spectrometer in the reflectron mode was used to generate peptide mass fingerprints. Peptides resulting from in-gel digestion with trypsin were analyzed on a 384 position, 600 μm AnchorChip™ Target (Bruker Daltonics, Bremen, Germany) and prepared according to AnchorChip recommendations (AnchorChip Technology, Rev. 2, and Bruker Daltonics, Bremen, Germany). Briefly, 1 μL of digestate was mixed with 1 μL of alpha-cyano-4-hydroxycinnamic acid (0.3 mg/mL in ethanol: acetone, 2:1 ratio) directly on the target and allowed to dry at room temperature. The sample spot was washed with 1 μL of a 1% TFA solution for approximately 60 seconds. The TFA droplet was gently blown off the sample spot with compressed air. The resulting diffuse sample spot was recrystallized (refocused) using 1 μL of a solution of ethanol: acetone: 0. 1 % TFA (6:3:1 ratio). Reported spectra are a summation of 100 laser shots. External calibration of the mass axis was used for acquisition and internal calibration using either trypsin autolysis ions or matrix clusters and was applied post acquisition for accurate mass determination.

6.3.14 Analysis of peptide sequences

Peptide mass fingerprinting was used to identify proteins from tryptic peptide fragments by utilizing the MASCOT search engine based on the entire NCBI and SwissProt protein databases. Database searches were conducted allowing for up to one

missed trypsin cleavage and using the assumption that the peptides were monoisotopic, oxidized at methionine residues, and carbamidomethylated at cysteine residues. Mass tolerance of 150 ppm, 0.1 Da peptide tolerances and 0.2 Da fragmentation tolerances was the window of error allowed for matching the peptide mass values. Probability-based MOWSE scores were estimated by comparison of search results against estimated random match population and were reported as -10*log10 (*p*), where *p* is the probability that the identification of the protein is a random event. MOWSE scores greater than 63 were considered to be significant ($P < 0.05$). All protein identifications were in the expected size and isoelectric point (pI) range based on the position in the gel.

6.3.15 Aβ ELISA

Dr. Elizabeth Head of the University of California Irvine performed the Aβ ELISA assay described here. Aβ was sequentially extracted from approximately 200 mg of frozen midfrontal cortex taken adjacent to cortex for proteomics experiments in 0.1M Tris pH = 6.8 with 1% SDS and a Protease inhibitor Cocktail Kit (MP Biochemicals Inc.) containing 0.4 mg/ml AEBSF, 1mg/ml EDTA-Na2, 1μg/ml Leupeptin and Pepstatin A) at 1 mL buffer/150mg wet weight tissue using Potter Elevehjem 10 ml Wheaton glass tube and centrifuged at 4° C at 100 000 x *g* for 1 hour. The pellet was resuspended in 70% formic acid and sonicated on ice. After centrifugation at 4° C at 100 000 x *g* for 1 hour, the supernatant was collected and stored at –80°C until assayed. Brain samples were run in triplicate on ELISA plates coated with a monoclonal anti-Aβ1-16 antibody (kindly provided by Dr. William Van Nostrand, Stony Brook University, Stony Brook, NY) and detection was by monoclonal HRP conjugated anti-$A\beta_{1\text{-}40}$ (MM32-13.1.1) and

anti-$A\beta_{1-42}$ (MM40-21.3.1) antibodies (kindly provided by Dr. Christopher Eckman, Mayo Clinic Jacksonville, Jacksonville, CA) (Das et al., 2003; Kukar et al., 2005; McGowan et al., 2005). For standards, Aβ1-40 and Aβ1-42 (Bachem California, Inc., Torrance, CA) were used after a pretreatment with HFIP to prevent fibril formation.

6.3.16 Statistical analysis

Statistical analysis of differentially expressed protein levels matched with spots on 2D-gels from brain samples from cognitive intact nonagenarians and demented age-matched subjects were used and carried out using Student's *t*-tests. A value of $p < 0.05$ was considered statistically significant. Only proteins that are considered significantly different by Student's *t*-test in the brains of the old normal versus the old with AD were subjected to in-gel trypsin digestion and subsequent proteomic analysis. This is the normal procedure for proteomics studies, since sophisticated statistical analysis used for microarray studies are not applicable for proteomics studies (Maurer et al., 2005). To analyze the association between proteomic outcome measures and clinical and neuropathological characteristics of autopsy cases used in the current study, we calculated Spearman Rho's correlation co-efficients.

6.3.17 Protein Interacteome

The functional protein interacteome was obtained by using the Interaction Explorer ^{TM} Software Pathway Assist software package (Stratagene, La Jolla, CA). Pathway Assist is software for functional interaction analysis. It allows for the identification and visualization of pathways, gene regulation networks and protein interaction maps (Donninger et al., 2004). The proteins are first imported as the gene

symbols as a set of data. This data set is then searched against ResNet, a database containing over 500,000 biological interactions built by applying the MedScan text-mining algorithms to all PubMed abstracts. These interactions are then visualized by building interaction networks with shortest-path algorithms. This process can graphically identify all known interaction among the proteins. The information of the function of these proteins and their relevance to diseases are then obtained by using the BIOBASE's Proteome BioKnowledge Library form Incyte Corporation (Incyte, Wilmington, DE) (Hodges et al., 2002).

6.4 Results

6.4.1 Pathology of brain from oldest-old cognitively normal and AD subjects

As shown in Table 6.2, nondemented and demented individuals were not different in education level (t (7) =1.38 p=0.21), age (t (7) =0.28 p=0.79), post mortem interval (t(7)=0.33 p=0.75) or interval between last clinic assessment and death (t(7)=1.25 p=0.25). Nondemented subjects selected for this study had Braak & Braak tangle stages between III and IV whereas those with dementia had Braak & Braak tangle stages V and VI. Individuals with AD had significantly lower MMSE scores (t (7)=6.19 p<. 0005) but not brain weights (t (7)=1.89 p=0.10). The short post-mortem intervals employed suggests little oxidative damage or degradation occurred following death prior to harvesting the brain (Butterfield et al., 2003; Butterfield, 2004; Poon et al., 2005a; Poon et al., 2005b).

6.4.2 Protein oxidation levels in the demented and nonagenarians

Oxidative stress and the resulting damage has been shown to be one of the main mechanisms contributing to neurodegeneration and cognitive decline in a number of age-related neurodegenerative disorders such as AD (Aksenov et al., 1998b; Aksenov et al., 2000; Aksenov et al., 2001; Butterfield et al., 2001; Butterfield and Lauderback, 2002). Protein carbonylation is one of the markers of protein oxidation (Butterfield and Stadtman, 1997). There was no significant difference in both the levels of protein carbonyls among the subjects investigated (Fig.6.1).

6.4.3 Levels of lipid peroxidation in the demented and nonagenarians

4-hydroxynonenal HNE is a marker of lipid oxidation (Lauderback et al., 2001). HNE binds to proteins by Michael addition (Butterfield and Stadtman, 1997), altering their conformation, and thereby reducing there activity (Subramaniam et al., 1997; Lauderback et al., 2001). In the current study there was no significant differences observed in the levels of lipid peroxidation markers as measured by HNE, isoprostanes and neuroprostanes between the non-demented nonagenarians and the demented aged-matched nonagenarians (Fig.6.2) This is consistent with a previous study that showed no significant increase in the levels of malondialdehyde (MDA), another measure of lipid peroxidation in nondemented nonagenarians compared to septo/octogenarians (Rea et al., 2004).

6.4.4 Differential expression of protein levels in the demented and non-demented nonagenarians

Two-dimensional electrophoresis offers an excellent tool for the screening of abundant protein changes in various disease states (Butterfield et al., 2003; Butterfield, 2004; Poon et al., 2006b; Poon et al., 2006a). To assess whether there were any changes in the proteomic profile in the brains of cognitively intact nonagenarians in the present study, we investigated the pattern of protein expression in the frontal cortex from the demented nonagenarians compared to the age-matched cognitively intact nonagenarians. Comparing the densitometric intensities of individual spots on the gels, four proteins were expressed at significantly lower levels, and two proteins were expressed at significantly higher levels in the brains of the nonagenarians with AD compared to the non-demented nonagenarians. Fig 6.3 shows SYPRO ruby stained 2D gels of the old

normal (A) vs. old AD (B) groups mentioned above, with identified protein boxed and labeled. The brain proteins identified with decreased expression in the non-demented nonagenarians compared to aged-matched AD were: peroxiredoxin 5 (PRDX5), malate dehydrogenase (MDH), ATP Synthase H^+ transporting protein, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). In contrast, two proteins, ubiquitin carboxyl-terminal hydrolase L1 (UCHL1) and fructose bisphosphate aldolase (FBP1) were identified by proteomics to have increased expression in the non-demented nonagenarians compared to the demented nonagenarians. These proteins identified by mass spectrometry are shown in Table 6.3. Table 6.4 provides the changes in protein levels expressed as percent control ± S.E.M.

6.4.5 Correlations between changes in protein expression, Aβ and cognitive status.

Table 6.5 shows that several significant correlations were observed between Aβ, MMSE scores and protein expression levels. Overall, Aβ 40 either in the soluble or insoluble fractions was associated with protein expression of GAPDH, MDH and FBP. In contrast, the amount of soluble or insoluble Aβ 42 did not correlate with any of the observed changes in protein expression. Further, MMSE scores were significantly associated with MDH such that higher MMSE scores (better cognition) was linked to lower MDH, which is consistent with overall lower levels of MDH in nondemented nonagenarians. Despite the small sample size used for these correlation analyses, the general trends appear to be promising.

6.4.6 Protein interacteome

Fig 6.4 shows the protein interacteome of proteomics-identified proteins differentially expressed in the brains of cognitively intact and demented nonagenarians. The Interaction Explorer PathwayAssist (Stratagene) software shows that the proteins identified in this study are related to regulation of signal transduction, energy metabolism, and chaperone activity among others. As a result, the present findings suggest that in brains of non-demented nonagenarians, cognitive functions and memory are preserved in part via pathways associated with these functions.

Table 6.2 Characteristic of AD and non-demented nonagenarians		
Characteristic	**Controls (n=4)**	**AD (n=5)**
	Number/Total	
Females	2/4	5/5
> High school education	4/4	2/5
	Mean (Range)	
Age	94.5 (93-96)	95 (92-100)
Interval between last MMSE and death (Days)	71.0 (50-109)	41.8 (6-104)
Post-Mortem Interval (hours)	3.6 (2.2-5.3)	3.9 (2.3-5.5)
MMSE Total Score	25.0 (21-28)	4.6 (0-12)

Figure 6.1

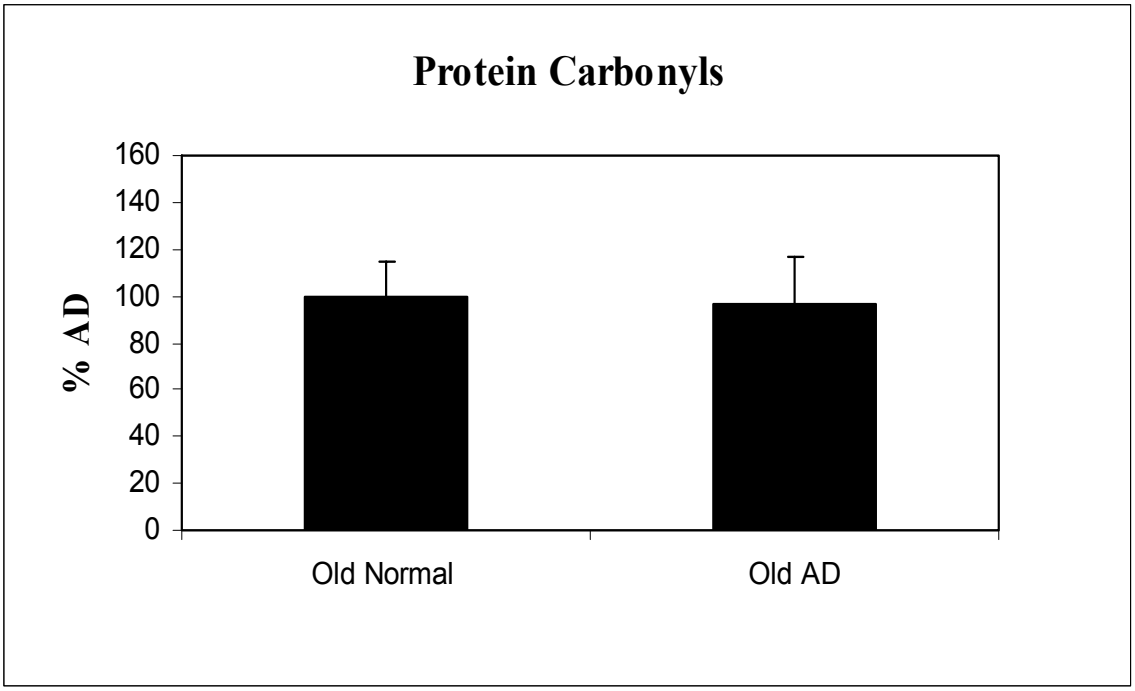

Figure 6.1 Shows protein carbonyl levels. There was no significant difference in the levels of protein carbonyls measured in both the old nonagenarians with AD compared to their age-matched normal nonagenarians.

Figure 6.2

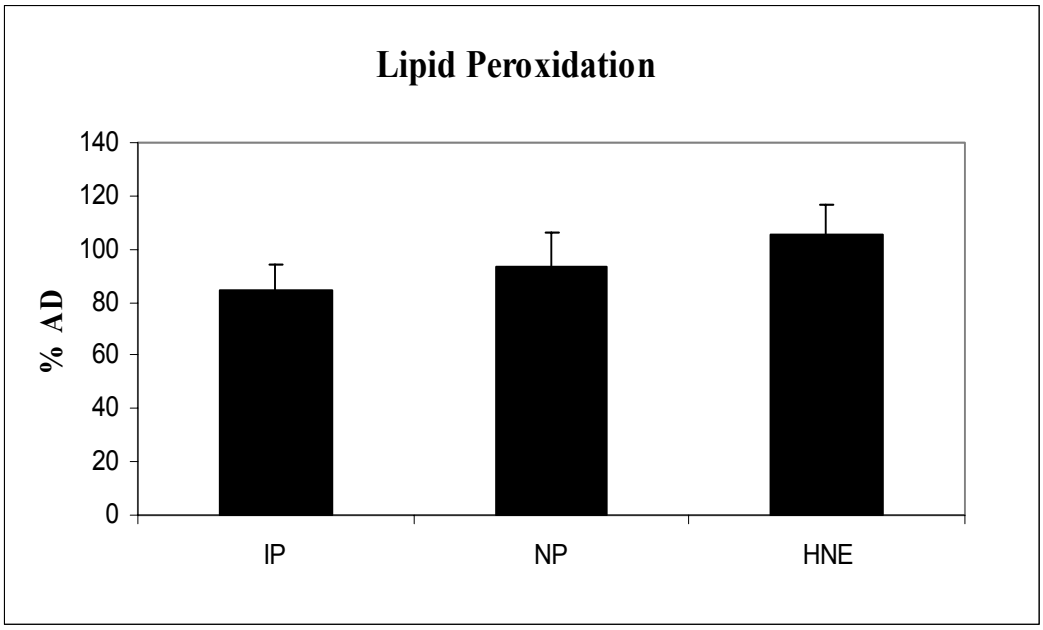

Figure 6.2 Shows lipid peroxidation levels as measured by 4-hydroxynonenal (HNE), isoprostanes (IP) and neuroprostanes (NP). There were no significant differences in the levels of all lipid peroxidation biomarkers in the old nonagenarians with AD compared to their age-matched normal old nonagenarians.

Figure 6.3

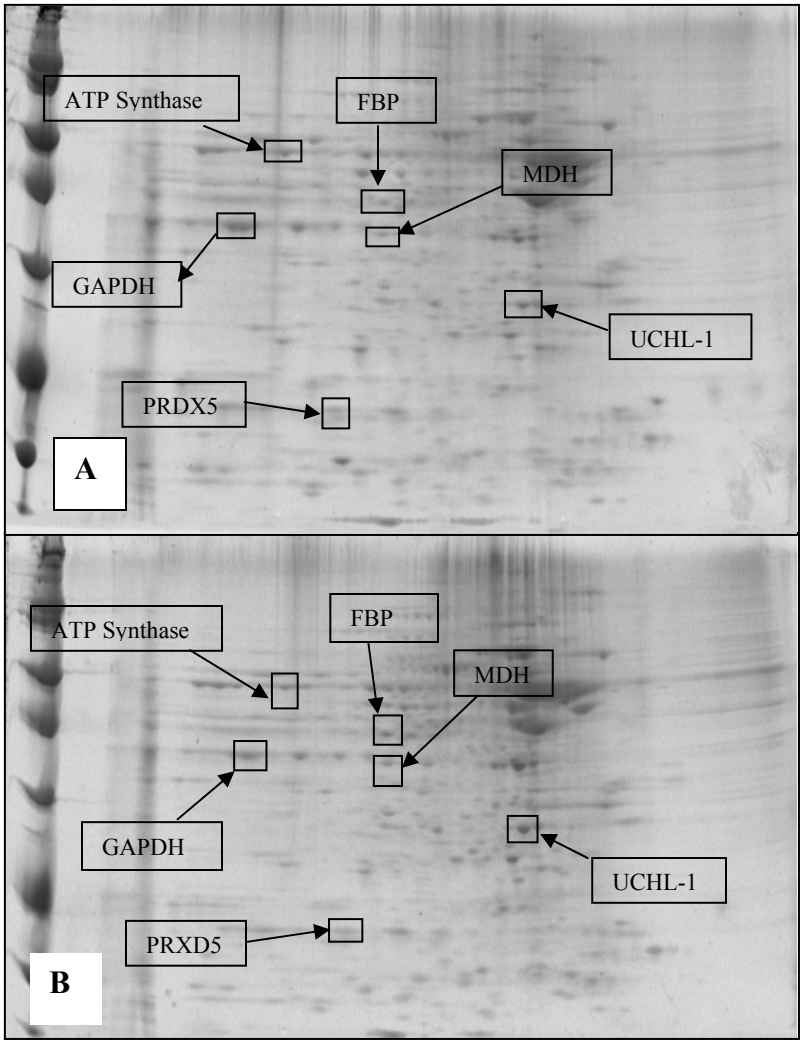

Figure 6.3 Shows SYPRO Ruby-stained 2D-gels maps of brain samples from normal old (A) nonagenarians vs. old nonagenarians with AD (B). Proteins identified by mass spectrometry showing differential expression are presented as the boxed spots.

Table 6.3 Proteomic characterizations of differentially expressed human brain proteins identified

Identified Protein	GI accession number	Number of peptide matches identified	% Coverage of matched peptides	pI, MrW	Mowse score	Probability of a random identification
GAPDH	gi|120649	8	20	8.58,36070	65	3.2×10^{-7}
ATP Synthase, H+ transporting, mitochondrial F1 complex	gi|114517	13	25	9.07,59886	133	5.0×10^{-14}
Human Peroxiredoxin 5 PRDX5	gi|16975162	5	40	6.96,17060	60*	1.0×10^{-6}
Malate dehydrogenase cytoplasmic	gi|1708967	12	32	6.89,36500	115	3.2×10^{-12}
Fructose Bisphosphate aldolase FBP1	gi|312137	16	49	6.41,39816	204	4.0×10^{-21}
Ubiquitin carboxyl-terminal hydrolase	gi|21361091	12	59	5.33,25151	134	4.0×10^{-14}

Table 6.4 Summary of differentially expressed proteins

Identified Protein	%Control ± S.E.M	P value
GAPDH	56 ± 18	0.03
ATP Synthase	35 ± 17	0.07*
Peroxiredoxin	51 ± 15	0.05
Malate dehydrogenase	61 ± 37	0.05
Fructose bisphosphate	141 ± 21	0.01
UCHL1	167 ± 19	0.002

Table 6.5 Correlations between proteomic analysis results and other markers

	MMSE	Insoluble Aβ		Soluble Aβ	
		1-40	1-42	1-40	1-42
GAPDH	-0.67	0.57	-0.19	0.79*	0.26
ATP5A1	-0.36	0.62	0.40	0.40	0.38
Prdx	-0.67	0.64	-0.07	0.69	0.02
MDH	-0.88**	0.81*	0.14	0.81*	0.14
FBP	0.64	-0.83*	-0.38	-0.67	-0.38
UCHL1	0.67	-0.69	-0.1	-0.69	-0.26

* Represents significant correlation

Figure 6.4

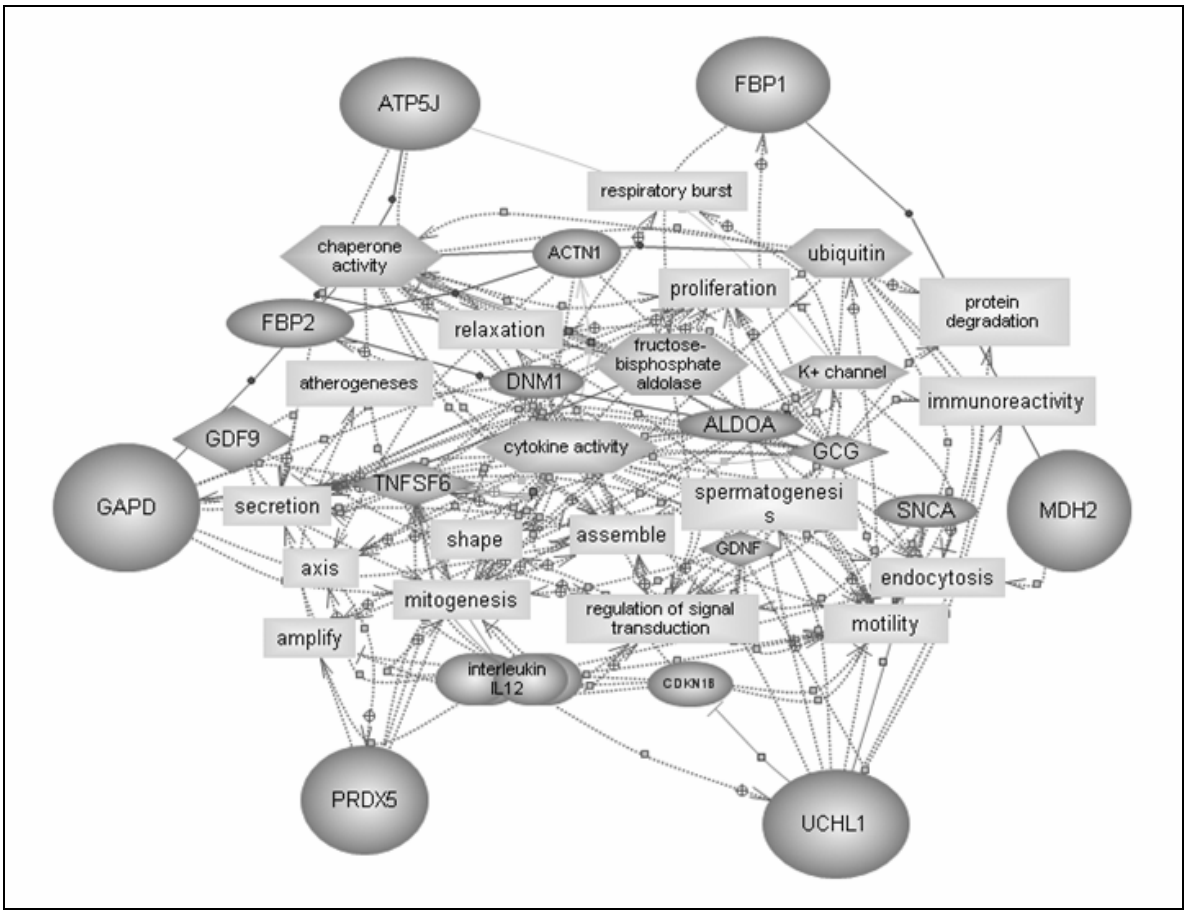

Figure 6.4 Schematic diagram of a functional interacteome of all proteins identified to be differentially expressed in the brains of cognitively intact nonagenarians compared to their demented age-matched controls. This diagram was generated by the interaction explorer ™ Pathway Module (Stratagene), indicating that all the proteins are directly or indirectly associated with cellular process shown.

6.5 Discussion

Some centenarians and nonagenarians escape or delay the onset of age-related disorders such as stroke, diabetes, cancer and AD (Perls et al., 2002a). The mechanism for the delay or escape of cognitive impairments in this group of individuals is still not well understood. Genetic predisposition seems to play a significant role in the mechanisms of dementia-free longevity, but the identification of a single gene responsible for this is still far from being archived (Gonos, 2000; Perls et al., 2000; Perls, 2001). As a result, the mechanism for cognitive preservation in the oldest-old is thought to follow multiple routes (Perls, 2004a; Galvin and Ginsberg, 2005). To better understand the mechanisms through which the oldest-old remain cognitively intact and maintain functional reserve, we analyzed the levels of oxidative damage by measuring the levels of protein carbonyls and protein-bound 4-hydroxynonenal (HNE), F_2-IsoP and F_4-NPs, all being biomarkers of oxidative stress. We also carried out a proteomic study to investigate differentially expressed proteins in the brains of age-matched non-demented and demented nonagenarians. There were no significant changes in the levels of protein oxidation as measured by the levels of protein carbonyls. In addition, there also were no significant changes observed in the levels of lipid peroxidation as measured by the levels of HNE, F_2-IsoP and F_4-NPs between these two groups. These results are consistent with a previous study that showed no changes in the levels of malondialdehyde (MDA) in nonagenarians (Rea et al., 2004). We speculate that elevated oxidative stress in brains of subjects with cognitive normality is the same as that from subjects with elevated oxidative stress in AD brains (Butterfield and Lauderback, 2002). However, using proteomics, we were able to identify four proteins whose expressions were significantly

decreased in the non-demented nonagenarians compared to nonagenarians with AD; these included: PRDX5, MDH, ATP synthase, and GAPDH. We also identified two proteins UCHL1 and FBP1 whose expressions were significantly increased in the cognitively intact nonagenarians compared to frontal cortex in age-matched AD individuals. The proteins identified were related to the antioxidant system, proteosome function and energy metabolism. The consequences of their differential expressions are discussed here with relevance to successful aging.

Peroxiredoxins (Prdxs) are a family of peroxidases that act as antioxidants by reducing hydrogen peroxide (H_2O_2), and alkyl hydroperoxides (Kim et al., 2000a). This is achieved with the use of reducing equivalents derived from thiol-containing donor molecules, such as thioredoxin. Prdxs have been identified in a variety of organisms ranging from prokaryotes to mammals (Chae et al., 1994), and to date, six distinct groups of mammalian prdxs (I–VI) have been identified. Prdxs I, II and VI are localized predominantly to the cytosol, while Prdxs III and V are largely detected in the organelles (Kang et al., 1998; Seo et al., 2000). Prdx2 expression has been shown to be significantly increased in AD, ALS, Down's syndrome and Parkinson's disease (PD) most probably as a compensatory mechanism (Kim et al., 2001; Allen et al., 2003; Krapfenbauer et al., 2003). Our laboratory has previously shown that prdx2 is significantly oxidized in the brain of the gracile axonal dystrophy (gad) mouse and is implicated as a possible mechanism of oxidative stress-induced neurodegeneration (Castegna et al., 2004). In the brain of senescence-accelerated mice prone 8 (SAMP8) mice, we have shown that following administration of antisense oligonucleotide directed at the Aβ region of amyloid precursor protein (APP), there is a significant decrease in the protein carbonyl

levels of prxd2, which could elevate antioxidant levels, thereby contributing to the observed improvements in learning and memory in the mice (Poon et al., 2005d). From the present study, we have observed a significant decrease in the expression levels of prdx5 in the brains of non-demented nonagenarians compared to the aged-matched demented nonagenarians. This results continues to support our previous study that showed an increase in expression in AD patients as a possible compensatory mechanism (Krapfenbauer et al., 2003), this in effect would provide protection against apoptosis by blocking of TNF-induced AP-1 activation (Shau et al., 1998) and in essence lead to improved cellular and biological functions contributing to maintenance of cognitive function observed in the normal nonagenarians.

Malate dehydrogenase (MDH) is a mitochondrial metabolic enzyme involved in the malate-aspartate shuttle that links glycolysis to the mitochondria electron transport chain (ETC), by transferring NADH to the electron transport complex 1, resulting into increased ATP synthesis (Poon et al., 2005a). The activity of MDH has previously been shown to be decreased during aging, probably through oxidative modification (Hrachovina and Mourek, 1990). Our laboratory has previously shown that MDH expression is significantly increased in the olfactory bulb of old mice possibly to compensate for reduced activity of ATP synthase seen in this model (Poon et al., 2006a). We have also shown that there is reduced nitration of MDH in the hippocampus of aged rats on caloric restriction (Poon et al., 2006b). In the present study, we observed a significant decrease in the levels of MDH in the brains of successfully aged nonagenarians. This decrease in expression conceivably would mean that normal nonagenarians compared to their demented controls have sufficient levels of MDH

resulting in an increased supply of NADH to the ETC leading to more production of ATP, maintaining energy metabolism, improved neuronal function and maintenance of cognitive function and reserve in these successfully aged individuals. This is consistent with our additional observations that higher MMSE scores were associated with lower MDH and similarly, higher amounts of Aβ 40 are linked to lower MDH protein expression.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a glycolytic enzyme located in the cytosol that catalyzes the conversion of glyceraldehyde-3-phosphate to 1, 3-phosphoglycerate, the first oxidation-reduction reaction in the glycolytic pathway. The activity of GAPDH has been found to be reduced in AD (Mazzola and Sirover, 2001) and its accumulation together with other glycolytic enzymes such as α-enolase and γ-enolase has been shown in AD brain (Schonberger et al., 2001), possibly as compensation for altered energy metabolism seen in AD (Vanhanen and Soininen, 1998; Messier and Gagnon, 2000; Scheltens and Korf, 2000). GAPDH has been identified as one of the nitrated proteins in AD (Sultana et al., 2005b). GAPDH was also identified to be oxidized by intracerebral injection of amyloid beta-peptide Aβ (1-42) into rat brain, likely explaining the possible loss or reduced activity of the enzyme in AD (Boyd-Kimball et al., 2005c). In the present study, we report GAPDH as one of the proteins whose expression is significantly decreased in the brain of cognitively intact nonagenarians and further, we observed a significant positive correlation between GAPDH and the amount of soluble Aβ1-40 extracted from the brain. As a result, normal nonagenarians possibly have enough levels and activity of GAPDH thus not necessitating increased expression which would lead to increased ATP production consequently leading to improved

function of ATP dependent cellular pathways related to maintenance of memory and cognitive function for the demented nonagenarians.

ATP synthase F1 subunit (ATP5A1) is a component of the mitochondrial ATP synthase complex. ATP synthase uses the electrochemical proton gradient established across the mitochondria membrane for synthesis of ATP from ADP and inorganic phosphate P_i (Leyva et al., 2003). Our laboratory has previously shown that ATP synthase is significantly oxidized in the gracile axonal dystrophy (*gad*) mouse brain (Castegna et al., 2004), a condition resulting from defective ubiquitin carboxyl hydrolase (UCHL-1) enzyme and proteosomal dysfunction. ATP synthase deficiency has been associated with an increase in oxidative stress, as observed in PD (Basso et al., 2004). In the aging human brains, the protein levels of ATP5A1 have been shown to be down-regulated (Lu et al., 2004) and its expression has been shown to be decreased in the olfactory bulb of the aging mice (Poon et al., 2006a). In the present study, we identified ATP5A1 as one of the proteins whose expression was significantly decreased in the brains of non-demented nonagenarians. We believe that this decrease in protein expression of ATP5A1 just like the rest of the proteins discussed above shows that due to successful aging cognitively intact nonagenarians don't require compensation for ATP synthesis like the aged-matched demented nonagenarians thus, the decrease in the expression of the above proteins in the cognitively intact nonagenarians compared to the age-matched demented nonagenarians should not seem unusual. The presence of Aβ in AD potentially necessitates for a compensatory mechanism to counter the effects of Aβ induced oxidative stress and possible cognitive decline. On the other hand, in the normal nonagenarians though there could be Aβ, its levels might not be sufficient to warrant for

this compensation mechanism hence the observed decrease in their expression during successful aging.

In the present study, we also identified UCHL1 and FBP1 as two proteins that were significantly increased in the brains of cognitively intact nonagenarians compared to aged-matched AD. UCH L-1 is an enzyme involved in the process of proteolytic degradation of misfolded or damaged proteins by the enzymatic complex, the proteosome (Kornitzer and Ciechanover, 2000). Proteolytic degradation is usually signaled by the covalent conjugation of ubiquitin to damaged proteins and is catalyzed by specific ubiquitin-transferring enzymes (Hershko and Ciechanover, 1998). As aging occurs, there is accumulation of misfolded, damaged or oxidized proteins and hence there is a need for their degradation since their accumulation could contribute to the increase in the number of oxidized proteins observed in AD among other deleterious effects (Keller et al., 2000; Castegna et al., 2004). UCHL1 has previously been shown to be oxidatively modified in AD (Castegna et al., 2002b), and point mutations in its gene cause familial PD (Leroy et al., 1998; Zhang et al., 2000). In addition, mutations in a gene encoding UCHL1 in the gracile axonal dystrophy (gad) mutant mouse results in brain axonal degeneration, severe motor neuronal impairment and Aβ deposition in the brain (Saigoh et al., 1999; Kurihara et al., 2001). The increased expression of UCHL-1 in successful aging observed here may reflect the observation that proteosomal activity is elevated in normal controls compared to AD brains (Keller et al., 2000; Castegna et al., 2004), and we speculate that though there could be possibly less oligomeric Aβ (1-42) in the normal nonagenarians relative to AD brain where copious amounts of oligomeric Aβ (1-42) occur (Butterfield and Lauderback, 2002), there could still be a relatively large amount of oxidized or damaged

proteins in the normal non-demented nonagenarians brain thereby necessitating for more expression of UCHL1 thus contributing to successful aging.

Fructose bisphosphate aldolase C (FBP) on the other hand is a glycolytic enzyme that catalyses the reversible aldol cleavage or condensation of fructose-1, 6-bisphosphate into dihydroxyacetone-phosphate and glyceraldehyde 3-phosphate (Perham, 1990). The role of FBP in aging and age-related disorders is still not well established, though we speculate that an increased expression would result into possibly increased activity and an increase in energy metabolism. FBP protein expression may be at least partially linked to the extent of Aβ as we observed a significant correlation between higher levels of insoluble Aβ 40 and lower FBP. The identification of these differentially expressed proteins in the brains of cognitively intact nonagenarians seems unusual. However, with advanced age and the presence of neurodegenerative disorders such as AD, there is a decrease in energy metabolism and accumulation of damaged proteins as previously mentioned (Vanhanen and Soininen, 1998; Messier and Gagnon, 2000) and as a compensatory response, we expect to observe a global increase expressions of proteins related to these functions. However, as observed in the present study this is not necessarily the case adding to the complexities in the mechanisms involved in successful aging.

In an extreme case from the Nuns study in which despite genetic predisposition to AD and presence of AD pathology in the neocortex, this part of the brain remained perfectly preserved, suggesting that her neocortex was resistant to Alzheimer-related neurodegeneration (Snowdon et al., 1997; Snowdon, 2003). In addition, a study on neuropathology on cognitively normal older adults has shown that brains of many

nondemented elderly individuals contain variable numbers of senile plaques. This has been known as pathologic aging, referring to a form of senile cerebral amyloid not associated with clinical manifestations of AD (Crystal et al., 1988; Dickson et al., 1992; Crystal et al., 1993). Therefore, the presence of neurofibrillary tangles NFT and senile plaques and other AD-related pathology and the lack of a correlation between dementia and neuropathology in the brain of non-demented centenarians and nonagenarians, could possibly suggest impairment in the proteosome functions in the brain. Hence, we expect an increase in the expression of proteins related to proteosomal function such as UCHL1. Thus, despite an increase in damaged proteins as observed in the pathology, cognition is still maintained. In addition, it has been established that the role of AD pathology on cognition is a discontinuous process and until a certain threshold is reached, this pathology has no significant effect on cognition (Knopman et al., 2003) thus, this findings could not be so unusual. Most recently, it has been established that UCHL1 is required for normal synaptic and cognitive function (Gong et al., 2006). As a result, the increased expression of UCHL-1 in successful aging observed here may reflect that one, the proteosomal activity is elevated in normal controls compared to AD brains (Keller et al., 2000; Castegna et al., 2004), and two that synaptic function is well maintained leading to improvements and maintenance of cognitive function. On the other hand, the increase in expression of FBP might have a significant but subtle effect in the increment of energy levels, especially since we have also seen a significant decrease in the expression of more proteins related to energy metabolism in the present study.

This study therefore, has provided us with possible additional mechanisms and insights on how the oldest-old are able to remain cognitive intact. It has previously been

established that there is a significant increase in the levels of oxidative stress associated with advanced age; we have also shown that key proteins undergo significant oxidative modification leading to a loss in function in most neurodegenerative disorders(Butterfield and Castegna, 2003a; Boyd-Kimball et al., 2005c; Poon et al., 2005d; Butterfield et al., 2006d; Poon et al., 2006b; Poon et al., 2006a; Sultana et al., 2006a). In the present study there was no significant difference observed in the levels of oxidative stress biomarkers, protein carbonyl, HNE, F_2-IsoP and F_4-NPs in the brains of age-matched cognitively intact and demented nonagenarians. This finding was consistent with a previous study that showed no significant increase in the levels of MDA, another measure of lipid peroxidation in cognitively intact nonagenarians compared to septo/octogenarians (Rea et al., 2004). As a result, we can conclude that oxidative stress did not play a significant role in the maintenance of cognitive function in the non-demented nonagenarians. In addition, it has been reported that there is altered energy metabolism in AD (Vanhanen and Soininen, 1998; Messier and Gagnon, 2000; Scheltens and Korf, 2000). The reduction in the levels of ATP could potentially lead to dysfunction in electrochemical gradients, ion pumps, and ion channels among others. This would eventually lead to loss in synaptic plasticity, neuronal dysfunction and loss in long-term potentiation and eventual loss in memory and cognition (Michaelis, 1997; Boyd-Kimball et al., 2005c). In the present study, we have shown that there is a significant increase in the expression of proteins related to energy metabolism and antioxidant system in the demented nonagenarians compared to the age-matched normal nonagenarians and we believe that one mechanism for the maintenance of neuronal function hence cognitive and memory function is through, but not exclusively, maintenance of ATP levels. Also using correlation analyses,

we have shown that there is a general trend of association between protein expression levels and intact cognition. In particular, MMSE scores were significantly associated with MDH, i.e., higher MMSE scores (better cognition) was linked to lower MDH, which is consistent with overall lower levels of MDH in nondemented nonagenarians. On analyzing our current results using the Interaction Explorer Software PathwayAssist (Stratagene), as shown in (Fig 6.4), the proteins identified in this study are related to regulation of signal transduction, energy metabolism, and chaperone activity among other cellular process relevant to the maintenance or improvement of cognitive function. Since we have previously seen that there is altered ATP production in the aging brain, the increased expression of energy metabolism related proteins can improve ATP production leading to neuronal recovery and improved cognitive function as seen in the cognitive intact nonagenarians and centenarians. Therefore, in addition to genetic, environmental and other factors in playing a role in achieving dementia-free aging, we believe that by maintaining sufficient ATP levels through efficient energy metabolism and improving antioxidant reserves, one is capable of maintaining synaptic plasticity, neuronal function through long-term potentiation and eventually improving memory and cognitive function (Michaelis, 1997). Thus, centenarians/nonagenarians have the potential to represent a model of resistance to age-related diseases and successful aging.

A possible caveat in this study could be the number of subjects used. We had four non-demented and five aged-matched demented nonagenarians; this may be a small data set to provide global interpretations. Also the short post mortem intervals (PMI) used in obtaining tissues and the low standard deviations obtained within each group provide additional confidence that the number of subject used is sufficient enough to provide a

model to study and understand events taking place in disease-free aging. In our previous proteomics studies, the use of five subjects per group was acceptable for statistical analysis, considering also that in most proteomic studies, the use of sophisticated statistical analysis like those used for micro array studies are not applicable. Therefore, we have confidence in the observations we have made and we believe that our present findings are able to provide a good representation of events and mechanisms taking place in successful aging.

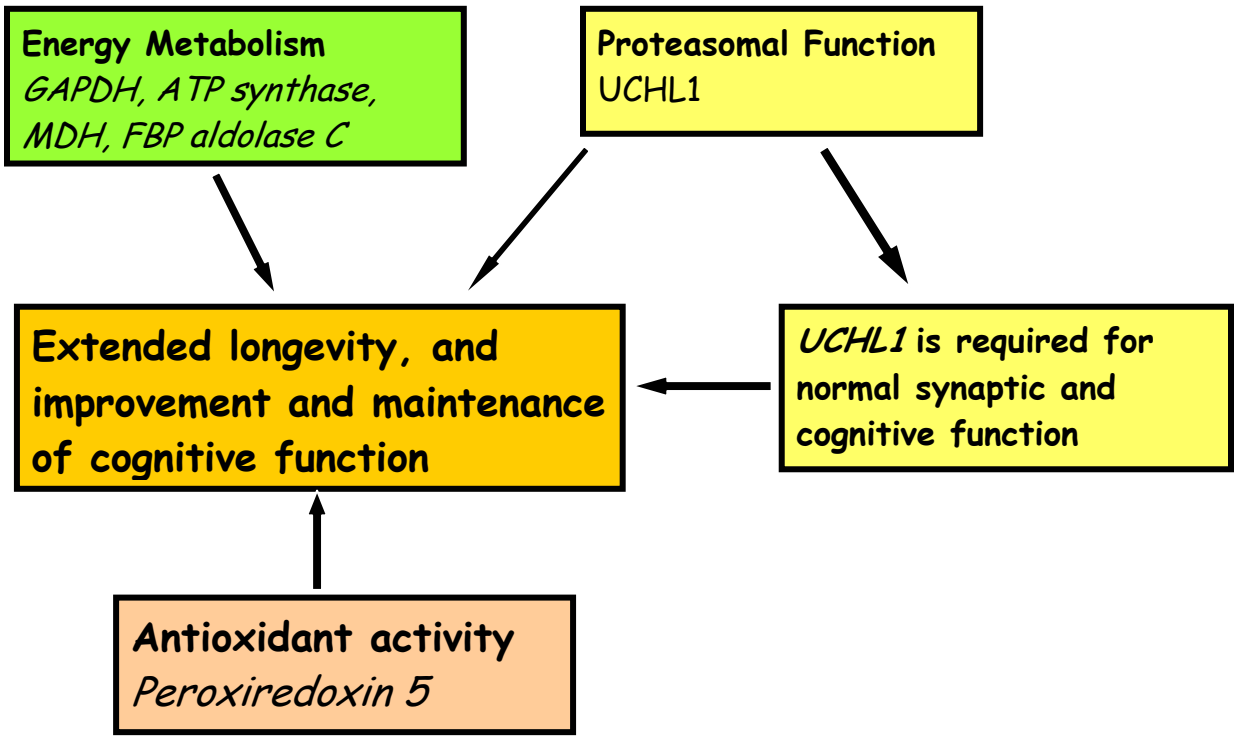

Figure 6.5 Summary of the functional consequence for the differential expression of proteins in the cognitively intact nonagenarians.

6.6 Acknowledgements

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CHAPTER SEVEN

Proteomic Identification of Oxidized Mitochondrial-related Proteins Following Experimental Traumatic Brain Injury (TBI)

7.1 Overview

Experimental traumatic brain injury (TBI) has been know to result into a significant loss of cortical tissue at the site of injury and in the ensuing hours and days a secondary injury exacerbates this primary injury resulting into a massive loss of tissue leading into significant neurological dysfunction. The mechanism of the secondary injury is not well understood, but is thought to be mediated by the mitochondria. This mitochondrial dysfunction is believed to involve excitatory amino acids, disruption of Ca^{2+} homeostasis, production of reactive oxygen species (ROS), ATP depletion, oxidative damage of mitochondrial proteins and an overall breakdown of mitochondrial energetics and eventual cell death. The identity of proteins that undergo oxidative modification after TBI has not been known. As a result, in the present study we utilized a 3h post-injury controlled cortical impact (CCI) model of TBI, coupled with proteomics to identify these specific mitochondrial fraction proteins from the cortex and hippocampus that were oxidatively modified after injury. We were able to identify from the Cortex; Pyruvate dehydrogenase (lipoamide), Voltage dependent anion channel (VDAC), Fumarate hydratase 1, ATP synthase H^+ transporting and prohibitin. From the hippocampus we identified; Cytochrome C Oxidase Va, Isovaleryl Coenzyme A Dehydrogenase, alpha enolase and Glyceraldehyde-3-phosphate dehydrogenase as proteins that had undergone oxidative modification following experimental TBI. In addition, we have also shown that following TBI, there is a reduction in the consumption of oxygen. Also, the activities of

complex I, complex IV, and pyruvate dehydrogenase (PDH), have been shown to be decreased. The present findings show that following TBI, the proteins of the electron transport chain and related ATP production mechanisms are highly oxidatively modified, leading to a massive breakdown of mitochondrial energetics and eventual cell death. The identification of these proteins provides new insights into the mechanisms that ensue following TBI and also provide avenues of possible therapeutic interventions after TBI.

7.2 Introduction

Traumatic brain injury (TBI) is a serious health care problem in the United States, with more than 400,000 individuals hospitalized each year with an estimated annual cost of greater than 25 billion dollars. (Tibbs et al., 1998; McNair, 1999). As a result, there is an urgent need of therapeutic interventions to curb this growing epidemic. The discovery and development of possible therapeutics will only be achieved the mechanism involving the pathogenesis of TBI are completely or well understood.

Traumatic brain injury has been known to result in a rapid a significant loss cortical tissue through necrosis. In the ensuing hours and days, a secondary injury exacerbates this primary injury resulting in a significant neurological dysfunction.(Sullivan et al., 1998b; Sullivan et al., 1999) The neuropathology of TBI is believed to involve excitatory amino acids, a disruption in Ca^{2+} homeostasis, production of reactive oxygen species (ROS), depletion of ATP and an overall breakdown in mitochondria bioenergetics (Sullivan et al., 1998b; Scheff and Sullivan, 1999)

Mitochondria are known to serve a number of functions. Its principle role is that is acts as the powerhouse of the cell, generating the much need ATP that is necessary for normal physiological functions to occur.(Wallace, 1999; Szewczyk and Wojtczak, 2002). Other than its role in energy metabolism, the mitochondria also play a role in cell survival or cell death (apoptosis) process. The mitochondria act as Ca^{2+} sinks, maintaining Ca^{2+} homeostasis for normal cell functions. In the event of an excessive uptake of $Ca^{2+}$and eventual overload, the mitochondria has been shown to increase the production of reactive oxygen species (ROS), that induces the opening of the mitochondrial

permeability pore (mPTP), release of cytochrome C, inhibition of ATP production and cell death.(Sullivan et al., 2005)

Reactive oxygen species (ROS) are always produced as byproducts of ATP synthesis. It has been shown that following TBI there is an increase in the production of (ROS). (Zhang et al., 2005) The generation of these ROS and eventual oxidative stress has been reported in many animal models and human disorders to have effects in inactivation of various enzymes. This is believed to occur through oxidative modifications of DNA, RNA, Lipids, proteins, among others (Butterfield and Lauderback, 2002). Mitochondrial enzymes in particular have been found to be targets of oxidative stress in most neurodegenerative diseases(Gibson and Huang, 2004). A recent study of brain ischemia showed that Pyruvate dehydrogenase (PDH) is inactivated by an increase ROS (Martin et al., 2005), α-ketoglutarate dehydrogenase complex (KGDHC) activity has also been shown to be inhibited under conditions of oxidative stress in AD (Gibson et al., 1998). In TBI, complex I respiration has been shown to be dramatically decreased, with a possible involvement of inhibition of PDH being evoked leading to reduction in ATP production (Sullivan et al., 2005).

All these studies have shown the inhibition or inactivation of these enzymes, but the mechanisms that ensue leading to inactivation are still speculative. Proteomics has recently been used with a lot of success in identifying various proteins that have been oxidatively modified hence inactivated in various neurodegenerative diseases and disorders such as, ALS, AD, Parkinson's, among others. (Castegna et al., 2002a; Castegna et al., 2003; Boyd-Kimball et al., 2005b; Poon et al., 2005f). Gene expression analysis studies in the rat hippocampus following TBI have shown that there is

differential expression at both mRNA and protein levels of genes associated with energy metabolism (Li et al., 2004). Using proteomics, we report for the first time the significant oxidation of Pyruvate dehydrogenase (lipoamide), Voltage dependent anion channel (VDAC), Fumarate hydratase 1,ATP synthase H+ transporting, Prohibitin, Cytochrome C Oxidase Va, Isovaleryl Coenzyme A Dehydrogenase, alpha enolase and Glyceraldehyde-3-phosphate dehydrogenase following experimental TBI. In addition, we also report the decreased activity of PDH, complex I and IV. These findings are discussed here to provide new insights into the mechanisms of TBI and also provide possible new targets for therapeutic interventions.

7.3 Experimental Procedures

7.3.1 Animal and surgical procedures

All procedures using animals were approved by the University of Kentucky Animal Use and Care Committee. Approximately 20 young adult (250–300 g), male Sprague–Dawley rats (Harlan Laboratories, IN) were pooled giving n=5 with each animal acting as its own control. All animals were housed in group cages on a 12-h light/dark cycle with free access to water and food. Animals were subjected to a moderate unilateral cortical contusion as previously described (Sullivan et al., 1999; Sullivan et al., 2000). This cortical contusion results in severe behavioral deficits, significant loss of cortical tissue, blood–brain barrier disruption and a loss of hippocampal neurons (Sullivan et al., 2002), mimicking some of the events that take place in human closed-head injury. Briefly, the subjects were anesthetized using isoflurane (2%) and placed in a stereotaxic frame (Kopf Instruments, Tujunga, CA). The head was positioned in the horizontal plane with the nose bar set at negative 5. Using sterile procedures, the skin was retracted and a craniotomy made with a hand-held trephine lateral to the sagittal suture and centered between Bregma and lambda. The skullcap was carefully removed without disrupting the underlying dura. The exposed brain was injured using a pneumatically controlled impacting device with a 5-mm beveled tip, which compressed the cortex at 3.5 m/s to a depth of 1.5 mm (Baldwin and Scheff, 1996; Sullivan et al., 1999). After injury, Surgicel (Johnson and Johnson, Arlington, TX) was laid on the dura and the skullcap replaced. A thin coat of dental acrylic was then spread over the craniotomy site and allowed to dry before the wound was stapled closed. After 3 h animals were then anesthetized and samples harvested.

7.3.2 Mitochondria isolation.

Isolation of total mitochondria was carried out as previously described (Sullivan et al., 2003; Sullivan et al., 2004b; Sullivan et al., 2004a). At 3 h post injury, the injured cortex and hippocampi from the ipsilateral and corresponding contralateral regions were dissected using a cork punch (d=8 mm) that was centered over the impact area, the tissue minced, and then homogenized separately in a glass dounce homogenizer containing approximately five times the volume of isolation buffer with 1 mM EGTA (215 mM mannitol, 75 mM sucrose, 0.1% BSA, 20 mM HEPES, 1 mM EGTA, pH 7.2). The homogenate was spun twice at 1300 × g for 3 min in an eppendorf microcentrifuge at 4◦C. The supernatant were then transferred to new tubes, topped off with isolation buffer with EGTA and spun at 13,000 × g for 10 min. The resulting supernatant was discarded and the pellet resuspended in 500 μL of isolation buffer with EGTA. The resulting pellet was then resuspended in 0.5 ml of mitochondrial isolation buffer (215 mM mannitol, 75 mM sucrose, 0.1% bovine serum albumin, 1 mM EGTA, 20 mM HEPES, pH 7.2), and the plasma membranes were ruptured by nitrogen decompression (Parr Cell Disruption Bomb) at 1000 p.s.i. for 5 min (Brown et al., 2004). The homogenized tissue and an equal volume of 30 % Percoll in isolation buffer was added (~4 ml). The resultant homogenate was layered on a discontinuous Percoll gradient with the bottom layer containing 40 % Percoll solution in isolation buffer, followed by a 24 % Percoll solution, and finally the sample in a 15 % Percoll solution. The density gradients were spun in a Sorvall RC-5C plus super speed refrigerated centrifuge (Asheville, NC) in a fixed angle SE-12 rotor at 30,400 x g for 10 minutes. Following centrifugation, band 3 (Sims, 1990) were separately removed from the density gradient. The samples were washed by

centrifugation at 16,700 x g for 15 minutes. The supernatant was discarded and the loose pellet was resuspended in the 1 ml of isolation buffer. The mitochondrial fractions were then placed in separate 15 ml conical tubes and an equal volume of 30 % Percoll was added to each sample and discontinuous Percoll density gradient centrifugation was performed again as described above. Band 3 was obtained from the gradients and 10 ml of isolation buffer without EGTA (215 mM mannitol, 75 mM sucrose, 0.1 % BSA, 20 mM HEPES, pH is adjusted to 7.2 with KOH) was added. The fractions were centrifuged at 16,700 x g for 15 minutes and subsequently at 11,000 x g for 10 minutes. The resultant pellet was resuspended in 1 ml of isolation buffer without EGTA and centrifuged at 10,000 x g for 10 minutes. The final mitochondrial pellet was resuspended in isolation buffer without EGTA to yield a protein concentration of ~10mg/ml and stored on ice for respiration assays or resuspended in lysis buffer (10 mM HEPES, 137 mM NaCl, 4.6 mM KCl, 1.1 mM KH_2PO_4, 0.6 mM $MgSO_4$ and 0.5 mg/mL leupeptin, 0.7 μg/mL pepstatin, 0.5 μg/mL trypsin inhibitor, and 40 μg/mL PMSF) for subsequent assays. Protein concentration was assayed using the Pierce BCA method.

7.3.3 Mitochondria respiration assay

Mitochondrial oxygen consumption was measured by using a Clark-type electrode in a continuously stirred, thermostated sealed chamber (Oxytherm System; Hansatech Instruments Ltd.) at 37°C as previously described (Brown et al., 2004; Jin et al., 2004; Sullivan et al., 2004b). Isolated mitochondrial protein (~30 μg) was suspended in respiration buffer (215 mM mannitol, 75 mM sucrose, 20 mM HEPES, 2 mM $MgCl_2$, 2.5 mM inorganic phosphate, 0.1% BSA, pH 7.2) in a final volume of 0.25 ml. The respiratory control ratio (RCR) was calculated as the ratio of oxygen consumption in the

presence of 10 mM pyruvate and 5 mM malate or 10 mM succinate in the presence of ADP (state III) and in the absence of ADP (State IV).

7.3.4 Measurement of protein carbonyls

Protein carbonyls are an index of protein oxidation and were determined as described previously (Butterfield and Stadtman, 1997). Briefly, the mitochondrial samples n=5 (5 μg of protein) were derivatized with 10 mM 2, 4-dinitrophenylhydrazine (DNPH) in the presence of 5 μL of 12% sodium dodecyl sulfate for 20 min at room temperature (23°C). The samples were then neutralized with 7.5 μL of the neutralization solution (2 M Tris in 30% glycerol). Derivatized protein samples were then blotted onto a nitrocellulose membrane with a slot-blot apparatus (250 ng per lane). The membrane was then washed with wash buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20) and blocked by incubation in the presence of 5% bovine serum albumin, followed by incubation with rabbit polyclonal anti-DNPH antibody (1: 100 dilution) as the primary antibody for 1 h. The membranes were washed with wash buffer and further incubated with alkaline phosphatase-conjugated goat anti-rabbit antibody as the secondary antibody for 1 h. Blots were developed using Sigma-fast tablet (BCIP/NBT; Sigma-Aldrich) and quantified using Scion Image (PC version of Macintosh-compatible NIH Image) software.

7.3.5 Measurement of 3-nitrotyrosine (3-NT)

Nitration of proteins is another form of protein oxidation (Castegna et al., 2003). The nitrotyrosine content was determined immunochemically as previously described (Drake et al., 2003b). Briefly, samples were incubated with Laemmli sample buffer in a

1:2 ratio (0.125 M Trizma base, pH 6.8, 4% sodium dodecyl sulfate, 20% glycerol) for 20 min. Protein (250 ng) was then blotted onto the nitrocellulose paper using the slot-blot apparatus and immunochemical methods as described above for protein carbonyls. The mouse anti-nitrotyrosine antibody (5: 1000 dilutions) was used as the primary antibody and alkaline phosphatase-conjugated anti-mouse secondary antibody was used for detection. Blots were then scanned using scion imaging and densitometric analysis of bands in images of the blots was used to calculate levels of 3-NT.

7.3.6 Measurement of 4-hydoxynonenal (HNE)

HNE is a marker of lipid oxidation and the assay was performed as previously described (Lauderback et al., 2001). Briefly, 10 µl of sample were incubated with 10 µl of Laemmli buffer containing 0.125 M Tris base pH 6.8, 4 % (v/v) SDS, and 20% (v/v) Glycerol. The resulting sample (250 ng) was loaded per well in the slot blot apparatus containing a nitrocellulose membrane under vacuum pressure. The membrane was blocked with 3% (w/v) bovine serum albumin (BSA) in phosphate buffered saline containing 0.01% (w/v) sodium azide and 0.2% (v/v) Tween 20 (PBST) for 1 h and incubated with a 1:5000 dilution of anti-4-hydroxynonenal (HNE) polyclonal antibody in PBST for 90 min. Following completion of the primary antibody incubation, the membranes were washed three times in PBST. An anti-rabbit IgG alkaline phosphatase secondary antibody was diluted 1:8000 in PBST and added to the membrane. The membrane was washed in PBST three times and developed using Sigmafast Tablets (BCIP/NBT substrate). Blots were dried, scanned with Adobe Photoshop, and quantitated with Scion Image.

7.3.7 Two-dimensional electrophoresis

Mitochondrial proteins (200 μg) were incubated with 4 volumes of 2N HCL at room for electrophoresis or 20mM DNPH for western blotting at room temperature for 20 min. Proteins were concentrated through precipitation by the addition of ice-cold 100% trichloroacetic acid (TCA) to obtain a final concentration of 15% TCA. Samples were then placed on ice for 10 min and precipitate centrifuged at 16,000 g for 3min. The resulting pellet was then washed three times with a 1:1(v/v) ethanol/ethyl acetate solution. The samples were then suspended in 200 μl of rehydration buffer composed of a 1:1 ratio (v/v) of the Zwittergent solubilization buffer (7M urea, 2M thiourea, 2% Chaps, 65 mM DTT, 1% Zwittergent 0.8% 3-10 ampholytes and bromophenol blue) and ASB-14 solubilization buffer (7M urea, 2M thiourea 5Mn TCEP, 1% (w/v) ASB-14, 1% (v/v) Triton X-100, 0.5% Chaps, 0.5% 3-10 ampholytes) for 1 h.

7.3.7.1 First dimension electrophoresis

For the first-dimension electrophoresis, 200 μl of sample solution was applied to a 110-mm pH 3–10 ReadyStrip™ IPG strips (Bio-Rad, Hercules CA). The strips were then actively rehydrated in the protean IEF cell (Bio-Rad) at 50 V for 18 h. The isoelectric focusing was performed in increasing voltages as follows; 300 V for 1 h, then linear gradient to 8000 V for 5 h and finally 20 000 V/h. Strips were then stored at –80 °C until the 2nd dimension electrophoresis was to be preformed.

7.3.7.2 Second dimension electrophoresis

For the second dimension, the IPG® Strips, pH 3–10, were equilibrated for 10 min in 50 mM Tris–HCl (pH 6.8) containing 6 M urea, 1% (w/v) sodium dodecyl sulfate (SDS), 30% (v/v) glycerol, and 0.5% dithiothreitol, and then re-equilibrated for 15 min in the same buffer containing 4.5% iodoacetamide instead of dithiothreitol. Linear gradient precast criterion Tris–HCl gels (8–16%), 13.3 x 8.7 cm (W x L) (Bio-Rad) were used to perform second dimension electrophoresis. Precision Protein™ Standards (Bio-Rad, CA) were run along with the sample at 200 V for 65 min.

7.3.8 SYPRO ruby staining

After the second dimension electrophoresis, the gels were incubated in fixing solution (7% acetic acid, 10% methanol) for 20 min and stained overnight at room temperature with 50ml SYPRO Ruby gel stain (Bio-Rad). The SYPRO ruby gel stain was then removed and gels stored in DI water.

7.3.9 Western Blotting

Mitochondrial proteins (200 μg) incubated with 20mM DNPH were used for western blotting. The strips and gels were run as described (Sections 7.3.7.1 and 7.3.7.2) above. After the second dimension, the proteins from the gels were transferred onto nitrocellulose papers (Bio-Rad) using the Transblot-Blot® SD semi-Dry Transfer Cell (Bio-Rad), at 15 V for 4 h. The 2,4-dinitrophenyl hydrazone (DNP) adduct of the carbonyls of the proteins was detected on the nitrocellulose paper using a primary rabbit antibody (Chemicon, CA) specific for DNP-protein adducts (1:100), and then a

secondary goat anti-rabbit IgG (Sigma, MO) antibody was applied. The resulting stain was developed by application of Sigma-Fast (BCIP/NBT) tablets.

7.3.10 Image analysis

The nitrocellulose blots (oxyblots) were scanned and saved in TIFF format using Scanjet 3300C (Hewlett Packard, CA). SYPRO ruby-stained gel images were obtained using a STORM phosphoimager (Ex. 470 nm, Em. 618 nm, Molecular Dynamics, Sunnyvale, CA, USA) and also saved in TIFF format. PD-Quest (Bio-Rad) imaging software was then used to normalize the gels and blots using the total spot density option, match and analyze visualized protein spots among differential 2D gels and 2D oxyblots, with one blot and one gel for each individual sample. The spot intensity of the gel corresponded to the protein levels, while the spot intensity if the blot corresponded to the carbonyl levels.

7.3.11 In-gel trypsin digestion

Samples were digested by trypsin using protocols previously described and modified by (Thongboonkerd et al., 2002). Briefly, spots of interest were excised using a clean blade and placed in Eppendorf tubes, which were then washed with 0.1 M ammonium bicarbonate (NH_4HCO_3) at room temperature for 15 min. Acetonitrile was then added to the gel pieces and incubated at room temperature for 15 min. This solvent mixture was then removed and gel pieces dried. The protein spots were then incubated with 20 μL of 20 mM DTT in 0.1 M NH_4HCO_3 at 56 °C for 45 min. The DTT solution was removed and replaced with 20 μl of 55 mM iodoacetamide in 0.1 M NH_4HCO_3. The

solution was then incubated at room temperature for 30 min. The iodoacetamide was removed and replaced with 0.2 mL of 50 mM NH_4HCO_3 and incubated at room temperature for 15 min. Acetonitrile (200 μL) was added. After 15 min incubation, the solvent was removed, and the gel spots were dried in a flow hood for 30 min. The gel pieces were rehydrated with 20 ng/μL-modified trypsin (Promega, Madison, WI) in 50 mM NH_4HCO_3 with the minimal volume enough to cover the gel pieces. The gel pieces were incubated overnight at 37 °C in a shaking incubator.

7.3.12 Mass spectrometry

A MALDI-TOF mass spectrometer in the reflectron mode was used to generate peptide mass fingerprints. Peptides resulting from in-gel digestion with trypsin were analyzed on a 384 position, 600 μm AnchorChip™ Target (Bruker Daltonics, Bremen, Germany) and prepared according to AnchorChip recommendations (AnchorChip Technology, Rev. 2, Bruker Daltonics, Bremen, Germany). Briefly, 1 μL of digestate was mixed with 1 μL of alpha-cyano-4-hydroxycinnamic acid (0.3 mg/mL in ethanol: acetone, 2:1 ratio) directly on the target and allowed to dry at room temperature. The sample spot was washed with 1 μL of a 1% TFA solution for approximately 60 seconds. The TFA droplet was gently blown off the sample spot with compressed air. The resulting diffuse sample spot was recrystallized (refocused) using 1 μL of a solution of ethanol: acetone: 0. 1 % TFA (6:3:1 ratio). Reported spectra are a summation of 100 laser shots. External calibration of the mass axis was used for acquisition and internal calibration using either trypsin autolysis ions or matrix clusters and was applied post acquisition for accurate mass determination.

7.3.13 Analysis of peptide sequences

Peptide mass fingerprinting was used to identify proteins from tryptic peptide fragments by utilizing the MASCOT search engine based on the entire NCBI and SwissProt protein databases. Database searches were conducted allowing for up to one missed trypsin cleavage and using the assumption that the peptides were monoisotopic, oxidized at methionine residues, and carbamidomethylated at cysteine residues. Mass tolerance of 150 ppm, 0.1 Da peptide tolerance and 0.2 Da fragmentation tolerance was the window of error allowed for matching the peptide mass values. Probability-based MOWSE scores were estimated by comparison of search results against estimated random match population and were reported as -10*log10 (p), where p is the probability that the identification of the protein is a random event. MOWSE scores greater than 63 were considered to be significant ($P < 0.05$). All protein identifications were in the expected size and isoelectric point (pI) range based on the position in the gel.

7.3.14 Immunoprecipitation

Immunoprecipitation of specific proteins was performed as previously described (Sultana and Butterfield, 2004). Mitochondria samples (200 μg) from control or injured animals were incubated overnight at 4 ^{o}C with respective antibodies of identified proteins. The antibody–antigen complexes were collected with protein A/G–Sepharose-conjugated beads. This was followed by three washing steps with buffer B (50Mm Tris Hcl (pH-8.0), 150 Mm NaCl, and 1% NP40). The proteins were resolved by SDS-PAGE followed by immunoblotting on a nitrocellulose membrane (BioRad). The proteins were then detected with alkaline phosphate labeled secondary antibody (Sigma).

7.3.15 Statistical analysis

Statistical analysis of specific protein carbonyl levels matched with anti-DNP-positive spots on 2D-oxyblots from mitochondria from the ipsilateral cortex (IC) and hippocampus (IH) with contralateral cortex (CC) and hippocampus (CH), was carried out using Student's *t*-tests. That is, each animal served as its own control, the contralateral side being the control for the ipsilateral side. A value of $p < 0.05$ was considered statistically significant. Only proteins that are considered significantly different by Student's *t*-test were subjected to in-gel trypsin digestion and subsequent proteomic analysis. This is the normal procedure for proteomics studies, sophisticated statistical analysis used for micro array studies are not applicable for proteomics studies.(Maurer et al., 2005)

7.3.16 Protein Interacteome

The functional protein interacteome was obtained by using Interaction Explorer ™ Software Pathway Assist software package (Stratagene, La Jolla, CA). Pathway Assist is software for functional interaction analysis. It allows for the identification and visualization of pathways, gene regulation networks and protein interaction maps (Donninger et al., 2004). The proteins are first imported as the gene symbols as a set of data. This data set is then searched against ResNet, a database containing over 500,000 biological interactions built by applying the MedScan text-mining algorithms to all PubMed abstracts. These interactions are then visualized by building interaction networks with shortest-path algorithms. This process can graphically identify all known interaction among the proteins. The information of the function of these proteins and their relevance

to diseases are then obtained by using the BIOBASE's Proteome BioKnowledge Library form Incyte Corporation (Incyte, Wilmington, DE) (Hodges et al., 2002).

7.3.17 Complex I assay:

Complex I activity was measured in isolated mitochondria as the rotenone-sensitive decrease in NADH absorption at 340 nm with ubiquinone-1 as the final acceptor, as previously described (Sriram et al., 1998) with some slight modifications (Sullivan et al., 2004b). Briefly, mitochondria were freeze-thawed and sonicated three times and diluted 1 µg/µl in 10 mM KPO_4 buffer. The assay was performed in 25 mM KPO_4 buffer (pH 7.2) containing mitochondrial protein (6 µg), 5 mM $MgCl_2$, 1 mM KCN, 1 mg/ml BSA, and 150 µM NADH. The reaction was preincubated for 2 minutes at 30°C, the baseline established, and the reaction initiated by addition of coenzyme Q-1 (50 µM). The activity was measured by monitoring NADH fluorescence (340 nm excitation, > 450 nm emission) over time under the same conditions as described above. The assay was also performed in the presence of rotenone (10 µM) to determine the rotenone-insensitive activity and the rotenone-sensitive complex I enzyme activity calculated by subtracting the rotenone-insensitive activity from the total activity. All the assay protocol for a 96-well plate performed with BioTek Synergy HT plate reader (Winooski, VT, USA).

7.3.18 Pyruvate Dehydrogenase complex (PDHC) assay:

PDHC activity was measured with previously protocol previously described (Starkov et al., 2004) with slight modification using increment in fluorescence measurement of NADH with BioTek Synergy HT plate reader (Winooski, VT, USA).

Briefly, mitochondria were freeze thawed and sonicated for 3 cycles. About 8 µg of double ficoll gradient purified mitochondrial protein were added into the buffer containing final concentration of 50mM KCl, 10mM HEPES pH 7.4, 0.3mM thiamine pyrophosphate (TPP), 10µM $CaCl_2$, 0.2mM $MgCl_2$, 5mM pyruvate, 1 µM rotenone and 0.2mM NAD^+. Reaction started by addition of 0.14mM CoASH and assay was performed at Ex_λ 340 nm/ Em_λ 460nm and increase in fluorescence was observed at 1 min interval at 30^0C. The PDHC activity calculated and expressed as nmol/mg protein.

7.3.19 Complex IV assay:

Cytochrome C Oxidase (Complex IV) of mitochondrial electron transport chain component activity was as previously described (Wharton and Tzagoloff, 1967) with slight modification using BioTek Synergy HT plate reader (Winooski, VT, USA). Briefly, mitochondria were freeze thawed and sonicated for 3 cycles. About 8 µg of double ficoll gradient purified mitochondrial protein were added into the 10mM KPO_4 buffer pH 8.0, 50µM reduced cytochrome c and the decrease in absorbance was observed with difference with and without addition of Cytochrome C at 1 min interval at 37^0C at 550nm. First the rate constant k was calculated for oxidation reaction of ferricytochrome c calculated and the specific activity were expressed as (k/mg protein) for cytochrome c oxidase.

7.4 Results

7.4.1 Reduction in mitochondrial bioenergetics following TBI.

Mitochondrial bioenergetics was measured following TBI. As illustrated in Figure 7.1, mitochondria isolated 3h post injury from the ipsilateral cortex of TBI animals show a reduction in the rate of respiration in the presence of ADP (state III) or FCCP (state V) compared to mitochondria isolated from contralateral cortex or sham-operated animals. Mitochondria from TBI animals demonstrated a significant reduction in state III respiration and a significant loss in the electron transport chain (ETC) capacity, as measured by the rate of respiration in the presence of the uncoupler FCCP (state V; in excess) when driven by the complex-I substrates pyruvate/malate or the complex-II substrate succinate (Figure 7.1B). As demonstrated in Figure 7.1 C, there was also a significant decline in the respiratory control ratio (RCR) in the mitochondria isolated from the ipsilateral cortex compared to those from the contralateral and sham operated animals. No difference in respiration rates were observed between contralateral mitochondria or mitochondria isolated from sham-operated animals (Figure 7.1B). It is also apparent that the loss bioenergetics was not due to a disruption in the inner membrane since states II and IV (absence of ADP) are not significantly altered, indicating that a loss of ADP phosphorylation and ETS activity was the underlying cause.

7.4.2 Increase in oxidative stress levels from mitochondria isolated after TBI

To determine if the cause of this loss of ETS activity and bioenergetics could be related to oxidative damage to the mitochondrial proteins, the level of protein carbonyls, 3NT and HNE as indicators of oxidative stress were measured in mitochondrial proteins

isolated from the cortex and hippocampus of TBI animals. Fig.7.2 shows that, following TBI, there is a significant increase in the levels of 3NT, HNE and protein carbonyls measured from mitochondria isolated from the ipsilateral hippocampus (IH) compared to the contralateral (CH) hippocampus. Fig.7.3 shows that the levels of 3NT, HNE and protein carbonyl from the cortical IC vs. CC were increased, but these changes were not significant. Together, these results extend previous findings of an increase protein and lipid oxidation following TBI (Braughler and Hall, 1992; Sullivan et al., 1998a; Sullivan et al., 1999; Sullivan et al., 2000; Lifshitz et al., 2004b).

7.4.3 Mass spectrometry analysis of mitochondrial proteins from the hippocampus and cortex.

Mass spectrometry analysis and data base searches allowed for the identification of these protein spots from the cortex (Table 7.1) and hippocampus (Table 7.2). From the cortex, the following mitochondrial proteins were identified as being oxidatively modified in TBI: pyruvate dehydrogenase, voltage dependent anion channel-2 (VDAC-2), fumarate hydratase 1, ATP synthase H^+ transporting F1 alpha subunit, all of which are proteins involved in ATP production, transport or the activity of the ETS, and prohibitin. From the hippocampus, cytochrome C oxidase Va, Isovaleryl coenzyme A dehydrogenase, enolase-1 and glyceraldehyde-3-phosphate dehydrogenase were identified as being oxidatively modified in TBI. As can be seen in tables 7.1 and 7.2, the probability of a random identification is exceedingly low.

7.4.4 Redox proteomics identification of mitochondrial proteins from the hippocampus and cortex following TBI

Proteomics has previously been used to identify brain proteins that have undergone oxidative modifications in different human diseases and models thereof (Butterfield et al., 2003; Butterfield and Castegna, 2003a; Butterfield, 2004; Butterfield and Boyd-Kimball, 2004b)**.** Here, we used a similar approach, with slight modifications, to identify oxidized mitochondrial proteins following TBI. Protein oxidation levels in the mitochondria isolated from the cortex and hippocampus were measured by identifying carbonylated proteins using anti-DNPH immunochemical detection of 2D-oxyblots from the cortex (Fig 7.4) and hippocampus (Fig 7.5). Using PD-Quest (Bio-Rad) imaging software, protein spots were matched between 2D-PAGE maps and the 2D-oxyblots. The immunoreactivity of each spot was normalized using total spot density option to the protein content in the 2D-PAGE. Only protein spots that showed a significant increase in protein carbonylation in the ipsilateral side compared to the contralateral side were excised from the gels and subjected to in-gel trypsin digestion. Tables 7.3 and 7.4 provide a summary of the levels of oxidation for individual proteins.

7.4.5 Validation of identified protein spots

Prior experience from our laboratory has demonstrated validation of each proteomic-identified protein by immunochemical means with no exceptions. In this study, VDAC-2 was probed with anti-VDAC antibody. Figure 7.6 shows validation of VDAC-2 through western blot analysis Figure 7.6 (b) and immunoprecipitation Figure 7.6(c). Thus, we are confident that the protein identifications reported are correct.

7.4.6 Decreased Complex I, Complex IV and PDH activities following TBI

The activities of complex I, complex IV and pyruvate dehydrogenase (PDH) were measured following 3 h post TBI. As shown in Figure 7.7 (a) and 7.7 (b), there is a significant decline in the activities of complex I and complex IV respectively, from the mitochondria isolated from ipsilateral cortex compared to those isolated from contralateral cortex. Figure 7.7 (c) on the other hand, illustrates the enzymatic activity of PDH. As shown, there was a decline in the activities of PDH following TBI from mitochondria isolated from the ipsilateral cortex compared to mitochondria isolated from the contralateral cortex; however this decline in activity was not significant. These results confirm our previous findings showing that the loss in mitochondrial bioenergetics observed is most likely due to a decline in the activities of the ETC complexes.

Figure 7.1

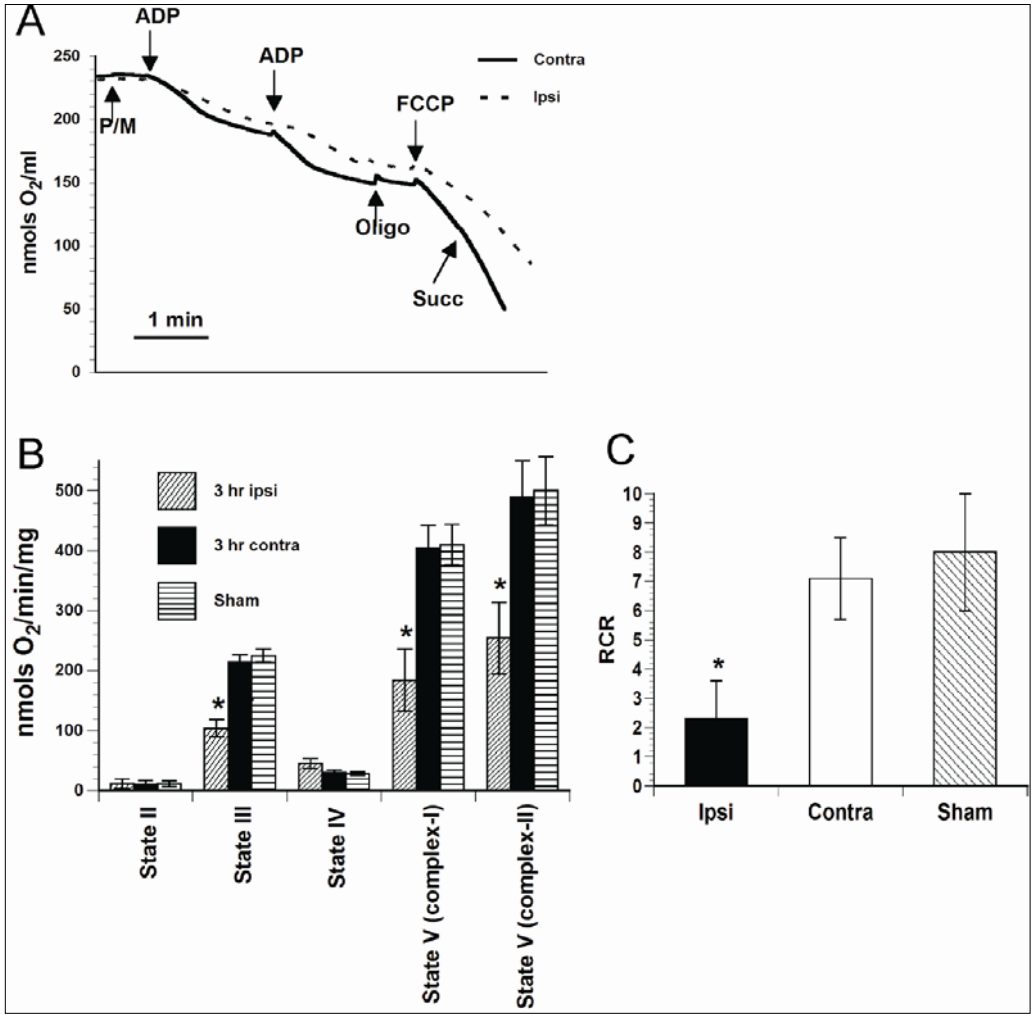

Figure 7.1 Traumatic brain injury (TBI) significantly impairs mitochondrial bioenergetics. As illustrated in panel A, TBI results in a loss of mitochondrial function demonstrated by reduction in oxygen consumption. Animals were injured (1.5 mm) and mitochondria isolated 3 hrs post-injury from the ipsilateral or contralateral cortex as well as sham-operated animals. Respiration rates were quantified and expressed as nmols O_2/min/mg consumed under various experimental conditions (panel B). TBI significantly reduced mitochondrial oxygen consumption in the presence of ADP (state III), indicating

a reduction in the ability to phosphorylate ADP. Significant differences were also apparent when the mitochondrial electron transport system (ETS) was uncoupled (excess FCCP) from the ATP synthase, a measure of maximum ETS capacity. This loss in ETS capacity was demonstrated when the ETS was driven via NADH-linked, complex-I substrates (pyruvate/malate; P/M) or the FADH-linked, complex-II, substrate (succinate; Succ). Panel C demonstrate the reduction in RCR ratio in mitochondria isolated from the ispi lateral relative to those isolated from sham and contralateral. Bars are group means, SEM. *$p < 0.05$ using a Neuman-Keuls post hoc.

Figure 7.2

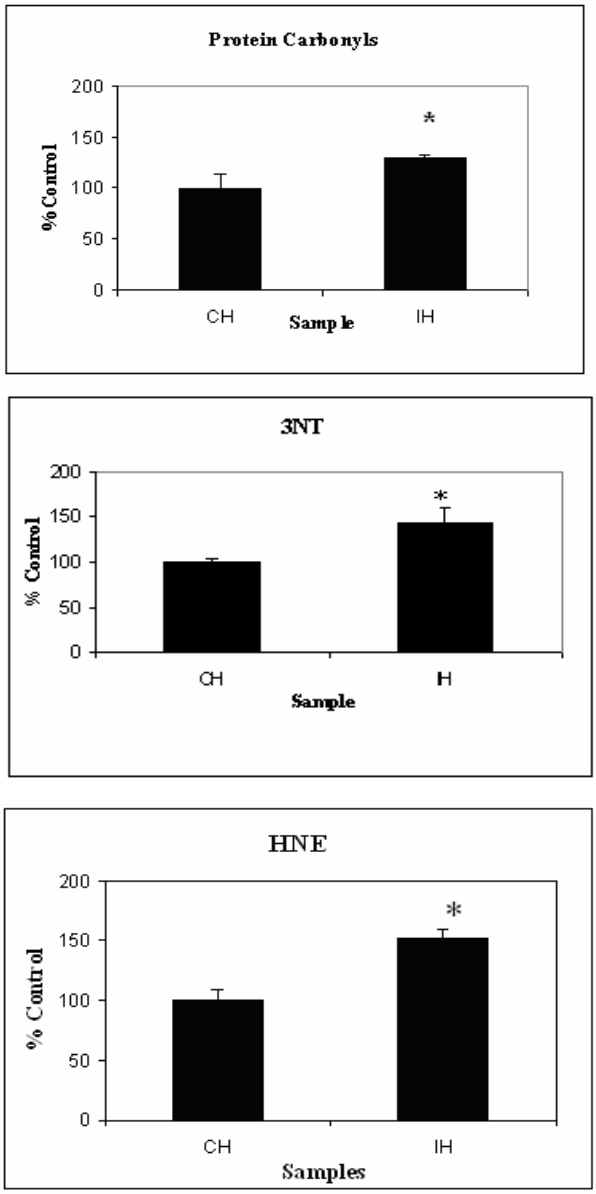

Figure 7.2 Protein carbonyls, 3NT and HNE levels of hippocampal mitochondria. There was a significant increase in the levels of all parameters measured from the contralateral and ipsilateral regions (CH vs. IH) of rats that had undergone TBI. Data are represented

as % control; error bars indicate the SEM for animals in each group. Measured values are normalized to the CC values (n=5) * p <0.05.

Figure 7.3

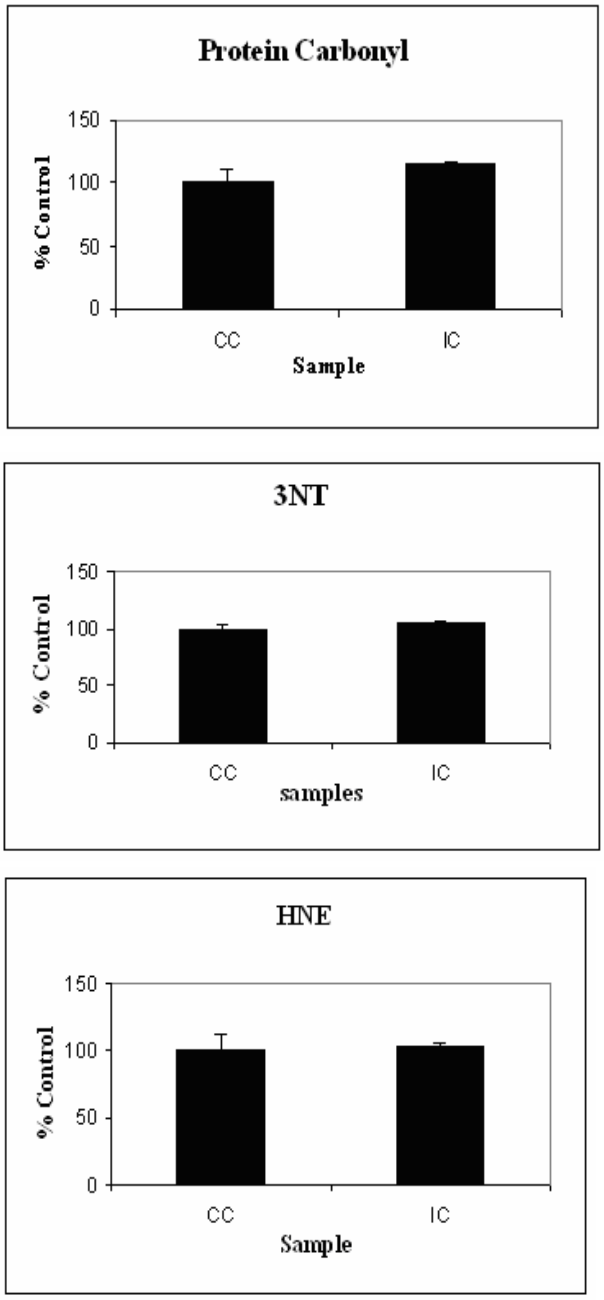

Figure 7.3 Protein carbonyls, 3NT and HNE levels of cortical mitochondria. There was an increase in the levels of all parameters measured from the contralateral and ipsilateral regions (CC vs. IC) of rats that had undergone TBI, but these levels were not significant. Data are represented as % control; error bars indicate the SEM for animals in each group. Measured values are normalized to the CC values.

Table 7.1 Summary of cortical mitochondrial-related proteins identified following TBI

Identified Protein	GI accession number	Number of peptide matches identified	% Coverage of matched peptides	pI, MrW	Mowse score	Probability of a random identification
Pyruvate dehydrogenase (lipoamide) E2	gi|50925725	11	38	6.2,38.9	243	5.0×10^{-25}
Prohibitin	gi|6679299	13	58	5.57,29.9	329	1.3×10^{-33}
Voltage dependent anion channel 2	gi|33243895	4	13	7.44,31.7	90	1.0×10^{-9}
Fumarate hydratase 1	gi|8393358	7	17	9.06,54.4	136	2.5×10^{-14}
ATP synthase, H+ transporting F1 alpha subunit	gi|111745	14	30	9.22,59.8	330	1.0×10^{-33}

Table 7.2 Summary of hippocampal mitochondrial-related proteins identified following TBI

Identified Protein	GI accession number	Number of peptide matches identified	% Coverage matched peptides	pI, MrW	Mowse score	Probability of a random hit
Enolase-1	gi|50926833	7	22	6.16,47.1	93	$5.0x10^{-10}$
Isovaleryl Coenzyme A dehydrogenase	gi|56970429	8	18	8.03,46.4	111	$7.9x10^{-12}$
Glyceraldehyde-3-phosphate dehydrogenase	gi|51766262	3	11	8.66,28.1	87	$2.0x10^{-9}$
Cytochrome Oxidase subunit Va	gi|6680986	5	22	6.08,16.0	135	$3.2x10^{-14}$

Figure 7.4

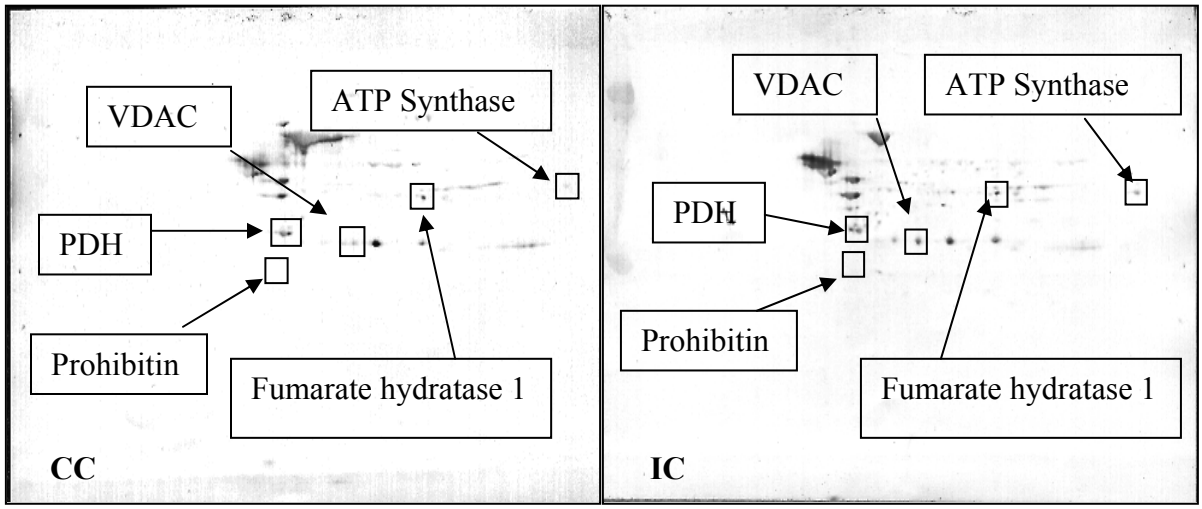

Figure 7.4 2D-oxyblots of cortical mitochondrial samples CC vs. IC. Proteins identified by mass spectrometry are presented as the boxed spots.

Table 7.3 Specific carbonyl levels of oxidized cortical proteins		
Identified Protein	**Specific protein carbonyl levels of IC vs. CC (n=5) (%Control ±SEM)**	**P Value**
Pyruvate dehydrogenase (lipoamide) E2	367 ± 47	< 0.04
Prohibitin	2042 ± 74	< 0.04
Voltage dependent anion chanel-2	962 ± 68	< 0.04
Fumarate hydratase 1	2742 ± 85	0.06
ATP synthase, H+ transporting FI alpha subunit	318 ± 30	< 0.01

Figure 7.5

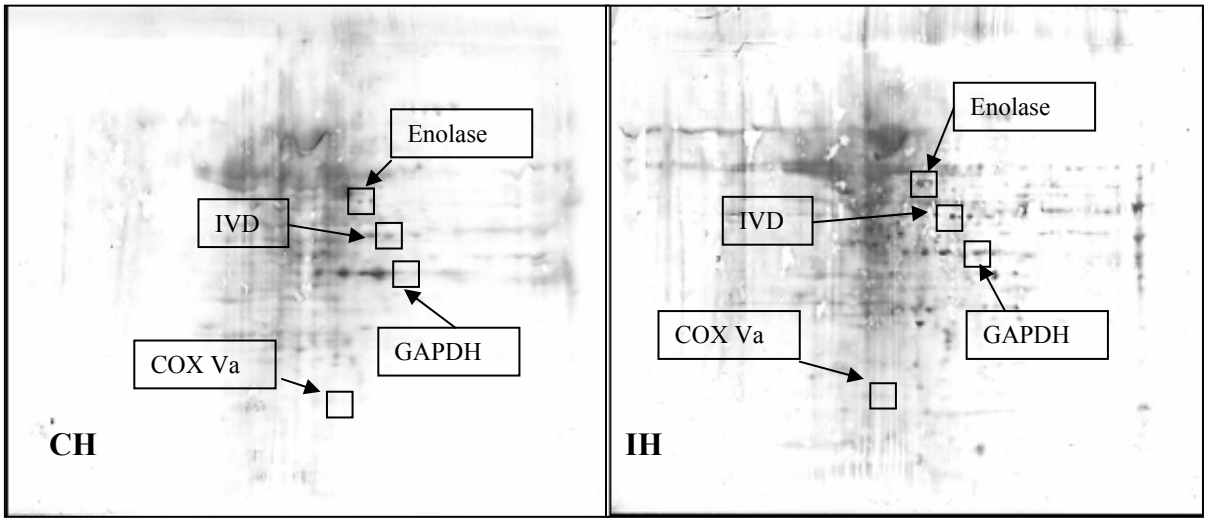

Figure 7.5 2D-oxyblots of hippocampal mitochondrial samples CH vs. IH. Proteins identified by mass spectrometry are presented as the boxed spots

Table 7.4 Specific carbonyl levels of oxidized hippocampal proteins		
Identified Protein	**Specific protein carbonyl levels of IH vs. CH (n=5) (% Control ± SEM)**	**P Value**
Enolase-1	588 ± 38	0.07
Isovaleryl Coenzyme A dehydrogenase	1220 ± 32	< 0.03
Glyceraldehyde-3-phosphate dehydrogenase	518 ± 27	< 0.03
Cytochrome Oxidase subunit Va	1362 ± 39	0.05

Figure 7.6

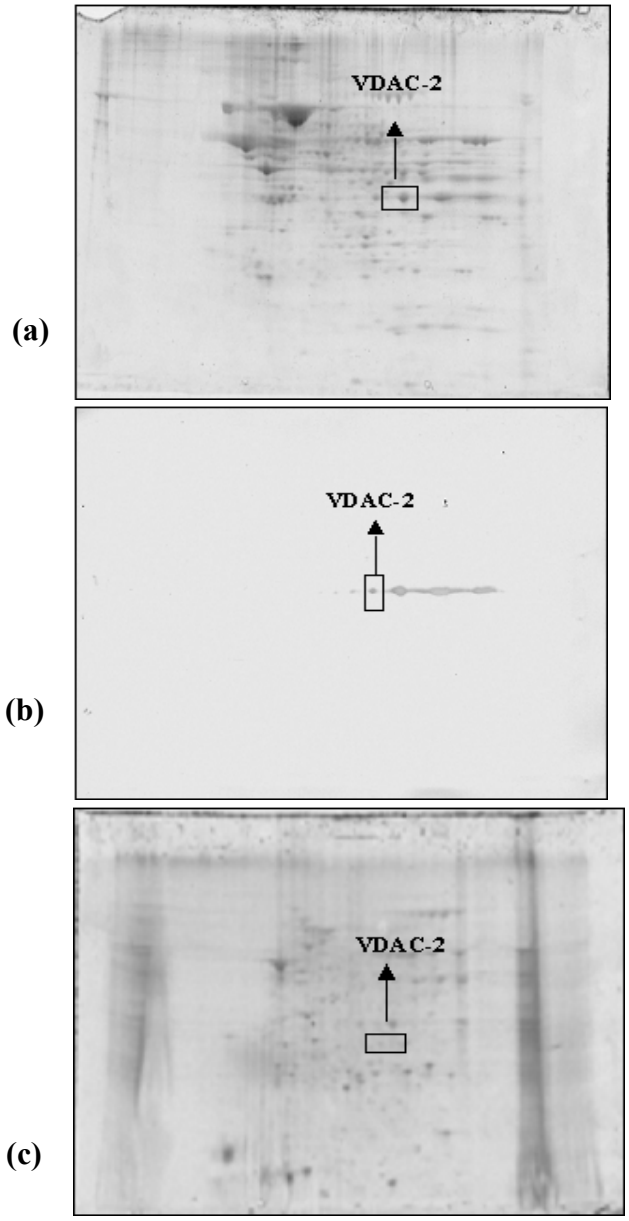

Figure 7.6 Validation of protein identified by MS. (a) Cortex mitochondria gel showing position of VDAC (b) blots probed with anti VDAC antibody (c) shows gel of cortex mitochondria immunoprecipitated with anti-VDAC antibody.

Figure 7.7

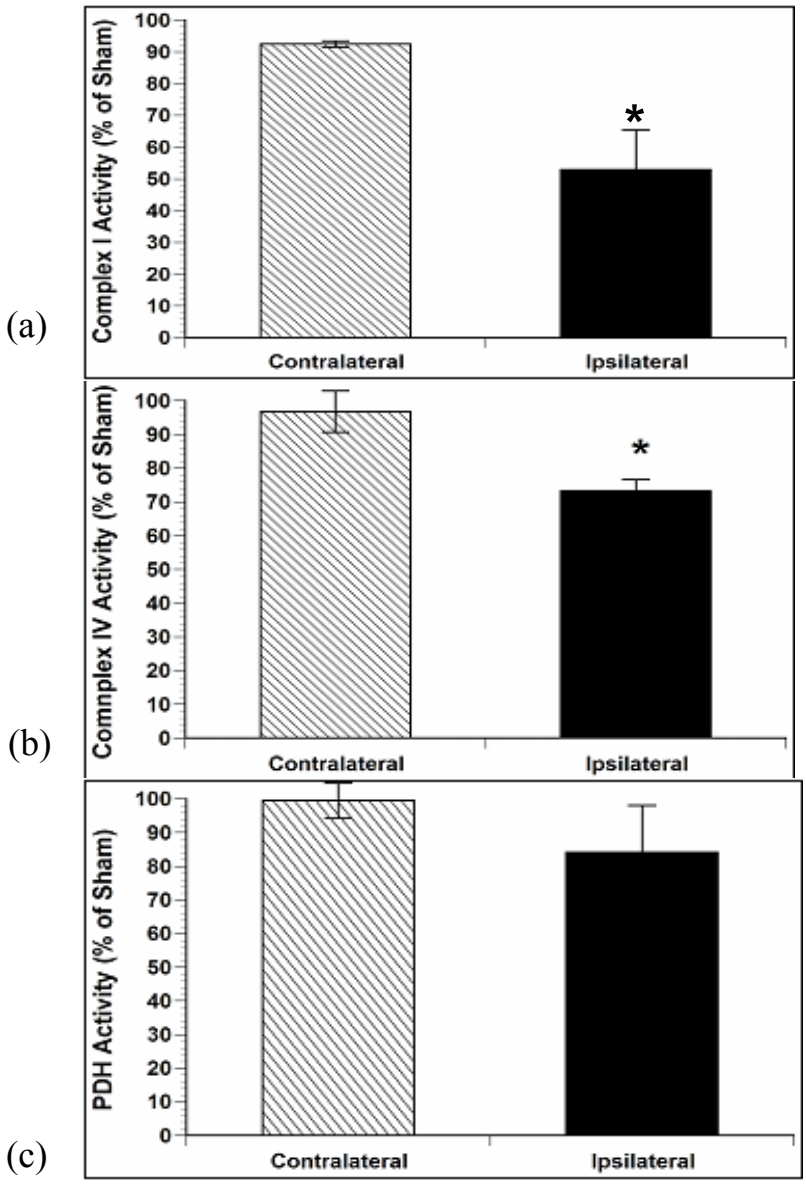

Figure 7.7 Shows the activity of Complex I (a), Complex IV (b) and Pyruvate dehydrogenase (PDH) (c) as a percentage of sham. There was a significant decline in the activities of Complex IV following TBI in the IC compared to the CC. There was also a reduction in the activities of complex I and the activity of PDH though not significant.

7.5 Discussion

The present study demonstrates that following TBI, critical mitochondrial fraction proteins from the cortex and hippocampus involved in energy metabolism are susceptible to oxidative damage, and hence inactivation. We demonstrate that these proteins are significantly and rapidly oxidized, presenting a possible mechanism for the decreased mitochondrial bioenergetics as seen particularly in the cortical mitochondria following TBI. Following TBI, there is an increase in the production of ROS (Braughler and Hall, 1992; Sullivan et al., 1998a; Sullivan et al., 1999; Sullivan et al., 2000; Lewen et al., 2001; Lifshitz et al., 2004b), which has been established to contribute to neuropathological events observed following TBI and other brain injury models (Sullivan et al., 1998b; Sullivan et al., 2002; Fiskum et al., 2003; Martin et al., 2005; Sullivan et al., 2005). In addition to increase in ROS, there is reduced mitochondrial bioenergetics following experimental TBI. This loss in the capacity of the ETC to consume oxygen has been shown to be an etiology observed in most CNS injuries (Lifshitz et al., 2004b; Sullivan et al., 2005). The present study extends these findings to show elevated oxidative stress levels following TBI and is the first to report the identification of specific mitochondrial-related proteins that are significantly oxidized following TBI and following this modification there is decreased activity of key enzymes and ETC complexes. The implications for the identification of these proteins are discussed below.

Pyruvate dehydrogenase (PDH) is a mitochondrial multienzyme complex composed of pyruvate dehydrogenase (E1), a dihydrolipoamide S-acetyltransferase (E2) and dihydrolipoamide dehydrogenase (E3). It catalyses the irreversible conversion of pyruvate, NAD^+ and CoA to acetyl CoA, NADH and CO_2 making it the central link

between glycolysis and TCA cycle (Zaidan et al., 1998; Martin et al., 2005). The activity of PDH is usually regulated through events of continuous phosphorylation (inactivation) and dephosphorylation (activation) by the enzymes pyruvate dehydrogenase kinases and phosphatases respectively (Holness and Sugden, 2003). Inactivation of PDH or its deficiency has been reported in several studies of models of ischemia-reperfusion and human diseases (De Meirleir et al., 1993; Zaidan et al., 1998; Hard et al., 2001). Leading to metabolic failure, secondary lactic acidosis (Hard et al., 2001), increase in ROS production (oxidative stress) leading to cellular damage and death (Bogaert et al., 2000). Following canine cardiac arrest and resuscitation, it has been reported that there is a selective loss of PDH activity in different neuronal subclasses (Bogaert et al., 2000), short-term forebrain ischemia in rats, causes a partial inactivation of PDH activity (Zaidan et al., 1998) and low levels of PDH have been associated with various neurodegenerative disorders such as Alzheimer's disease (Sheu et al., 1985), Huntington's disease (Sorbi et al., 1983), Wernicke-korsakoffsyndrome (Lavoie and Butterworth, 1995) and other hereditary ataxias (Sheu et al., 1989).

One mechanism for inactivation of PDH has been hypothesized to be the oxidative modification of the enzyme, involving possible site-specific protein oxidation of critical sulfhydryl groups (Tabatabaie et al., 1996). Or possibly due to metal-catalyzed oxidation of susceptible amino acids (Bogaert et al., 1994). The activity of PDH in different models of TBI has been extensively studied. In a recent micro array study of gene expression in the hippocampus following experimental TBI, PDH E1 α-subunit gene was found to be down regulated after moderate injury but unregulated in severe injury (Li et al., 2004). A study of rat hippocampus after chronic antidepressant

treatment, showed that PDH EI complex had undergone modulation (Khawaja et al., 2004). While in another proteomic study of an animal model for schizophrenia, protein levels of PDH complex E2 were found to be decreased (Paulson et al., 2003; Paulson et al., 2004). These studies provide evidence that different subunits of the PDH complex are susceptible to modulation during conditions of oxidative stress, and as had been established, inactivation of one subunit of the complex renders the whole enzyme complex inactive (Zaidan et al., 1998).

In the present proteomic study we found PDH lipoamide beta complex to be significantly oxidized following TBI. In addition, we have also shown that following experimental TBI the activity of PDH is also decreased though not significantly. As a consequence of these events, the activity of complex I was also found to be decreased, this would therefore disrupt the supply of NADH, a required substrate for complex I, contributing to the NADH-linked impairment of mitochondrial bioenergetics as seen in Fig. 7.1.

Voltage dependent anion channel-2 (VDAC) are pore-forming proteins (porins) found in the outer mitochondrial membrane of all eukayotes (Yoo et al., 2001). VDAC in conjunction with adenine nucleotide translocator (ANT), mediate the exchange of adenine nucleotides between the cytosol and the matrix (Vander Heiden et al., 2000). VDAC has also been known to form part of a mega pore known as the mitochondrial permeability transition pore mPTP, which mediates the release of cytochrome c from the mitochondria to the cytosol in early events of apoptosis leading to activation of the caspase cascade (Sullivan et al., 2002). VDAC has also been found to interact with Bcl-2 family of proteins regulating mitochondrial potential and release of cytochrome c

(Vander Heiden et al., 2001). Pro-apoptotic proteins Bax and Bid have been shown to accelerate the opening of the pore while anti-apoptotic protein BcL-2 binds directly to VDAC hence closing it (Yoo et al., 2001).

Impairment in ATP-ADP exchange by the closure of VDAC inhibits the activity of F_1F_0-ATPase. This leads to the inhibition of proton H^+ re-entry, hyperpolarization of the inner mitochondrial membrane, which promotes osmotic matrix swelling, rupturing the outer membrane releasing cytochrome c and activation of the caspase cascade. In a recent report (Liberatori et al., 2004), VDAC proteins were found to be involved in the loss of mitochondrial potential and neurodegeneration during ischemia reperfusion. In patients with AD and Downs syndrome, the expression of VDAC proteins has been found to be significantly reduced (Yoo et al., 2001). Other than its role in being part of mega pore mPTP, the activity of VDAC in brain injury models has not been extensively studied, the only reports provided confirm the presence of VDAC as part of the that is involved in release of cytochrome c.

In the present study, we show for the first time that VDAC-2 is significantly oxidized following TBI. The oxidation of VDAC posses a number of possible consequences. The conformational changes of the porin after oxidation could leave the channel in an open state; this could lead to entry of solutes into the mitochondria leading to swelling and eventual rupture of the outer mitochondrial membrane and release of cytochrome c. The conformational change might also leave VDAC in a closed state, preventing the exchange of ATP-ADP leading to hyperpolarization and activation of caspase cascade and eventual cell death. As a result oxidation of VDAC after TBI can be one of the causes for injury induced cell death following TBI.

ATP synthase (Complex V) is a multicatalytic subunit enzyme made up of two portions, the F_0, an integral membrane complex responsible for translocation of protons and the larger extra membrane complex, F_I, which is composed of five subunits ($\alpha_3\beta_3\gamma\delta\varepsilon$) (Kanski et al., 2005). ATP synthase uses the electrochemical proton gradient established across the mitochondria membrane during respiration for the synthesis of ATP from ADP and inorganic phosphate P_i (Leyva et al., 2003). The mechanism of ATP synthesis involves sequences of coordinated conformational changes of the α and β subunits.

Human diseases associated with a deficiency in ATP synthase show that there is no complete loss but a partial loss of the ATP synthase enzyme activity. This deficiency results from mutations in MTATP6, a mitochondrial encoded component (De Meirleir et al., 2004). ATP synthase deficiency has been associated with and increase in oxidative stress as seen in Parkinson' disease (Basso et al., 2004). Our laboratory has shown that ATP synthase is significantly oxidized in gracile axonal dystrophy (*gad*) mouse (Castegna et al., 2004). ATP synthase has also been found to be a target for cysteine oxidation of proteins during renal oxidative stress (Eaton et al., 2003). A study of rat cardiac proteins, recently identified ATP synthase as one of the proteins significantly nitrated as a function of age (Kanski et al., 2005). In a proteomic study after an inflammatory challenge, ATP synthase was again identified as one of the proteins that's was significantly nitrated (Aulak et al., 2001). A proteomic study of Muscles of A Diabetes Model Otsuka Long-Evans Tokushima Fatty (OLETF) rat (Oh-Ishi et al., 2003) found ATP synthase to be significantly oxidized. These studies have shown that ATP synthase is susceptible to oxidation and hence inactivation under conditions of oxidative stress.

In the present study, we identified ATP synthase as one of the mitochondrial proteins that were significantly oxidized following TBI. The oxidation of this subunit will as a result inhibit the activity of this enzyme compromising a crucial cellular process of ATP generation after brain injury eventually leading to neuronal cell death.

Cytochrome C Oxidase Va (COX) is also known as Complex IV. This is the terminal enzyme complex of the electron transport chain (ETC). It transfers reducing equivalents from cytochrome c to oxygen that is then reduced to water (Harris et al., 2001). COX is composed of 13 subunits. Three subunits form the catalytic core of the enzyme and are encoded by the mtDNA, while the rest are nuclear encoded and are involved in the assembly and regulation of the enzyme (Mootha et al., 2003). Human COX deficiency results into a condition known as Leigh syndrome, French-Canadian type (LSFC). It is characterized by a subacute neurodegeneration of the brainstem and basal ganglia and recurrent episodes of acute and often fatal metabolic acidosis and coma (Mootha et al., 2003).

The activity of COX has been shown to be reduced in drosophila, as an effect of oxidative stress and age (Schwarze et al., 1998). A recent review found a possible role for COX in stress induced apoptosis and degenerative diseases (Kadenbach et al., 2004). In Alzheimer's disease, various studies have shown an impairment of cytochrome c oxidase activity and depletion of ATP (Kim et al., 2000c). The activity of COX in various models of brain injury has also been extensively established. In one study, it was shown that following TBI there is a reduction of gene expression and activity of COX (Harris et al., 2001). Earlier fluid percussion injury studies of concussive brain injury in rats, found that after concussion, the cerebral cortex ipsilateral to the site of injury showed a decrease in

the activity of COX, this began as soon as one day and lasting for up to 10 days after the injury. The ipsilateral dorsal hippocampus also showed a decrease in COX activity, but not as severe as in the cortex (Hovda et al., 1991). COX activity in injured animals 24 h following an ablation of rat sensorimotor cortex was found to be significantly reduced throughout the cerebral cortex and particularly at the hemisphere ipsilateral to the ablation (Sutton et al., 2000). The above studies among others have provided evidence for the reduction in activity of COX after brain injury. These studies have though come short at presenting mechanisms that ensue in the inactivation of this enzyme. In the present study, we identify a subunit of COX as one of the proteins that was significantly oxidized following TBI, and as was discussed, oxidation of a single subunit could render the whole enzyme inactive. COX is the terminal complex of the ETC and as we have seen, it is a key site in the regulation of energy metabolism. Its oxidation would therefore lead to a reduction in the membrane potential; a compromise in ATP production and the generation of more ROS. This could in part be of the possible mechanisms of neurodegeneration observed after TBI.

Prohibitin is an evolutionarily conserved protein with homologues from yeast to man. Its exact physiological function has been difficult to define. Despite this, it has been demonstrated that prohibitin is involved in many cellular processes, such as cell cycle regulation, senescence, transcription regulation and tumor suppression (Coates et al., 2001). Recently, it has been established that PHB is located in the inner mitochondria membrane and that it acts as a chaperone in the assembly of subunits of mitochondrial respiratory chain complexes and stabilization of membrane proteins (Nijtmans et al., 2000; Nijtmans et al., 2002). Impaired assembly of ATP synthase is associated with

neurological muscle weakness, ataxia and retinitis pigmentosa (NARP) syndrome, a possible role of disruption of PHB (Nijtmans et al., 2001). A disruption on the PHB genes has been shown to have a decreased replicative lifespan in yeast, due to a gradual decline in cellular metabolic capacity (Coates et al., 1997) and a larval arrest in fruit fly (Eveleth and Marsh, 1986). In higher organism therefore, disruption of PHB could possibly have greater metabolic effect. It should be noted however that its involvement in TBI is still speculative.

It has recently been demonstrated that prohibitin can be induced by metabolic stress (Liu et al., 2004), its expression is increased in apoptotic cells and that it might play a role in regulation of the mitochondrial respiratory activity (Liu et al., 2004). These roles suggest that PHB is a critical mitochondrial protein required to maintain normal mitochondrial homeostasis. In the present study, we identified prohibitin as one of the proteins that were significantly oxidized following TBI. Oxidation of prohibitin may disrupt its functions of the assembly of ETC, leading to a breakdown in energy metabolism.

Isovaleryl Coenzyme A dehydrogenase (IVD). This is a nucleus-encoded, homotetrameric mitochondrial flavoprotein, which is found in the third step of the leucine catabolism pathway. It is a member of the acyl-CoA dehydrogenase (ACD) family of enzymes, all of which employ a similar enzyme mechanism for the α, β-dehydrogenation of acyl-CoA substrates (Mohsen et al., 2001). IVD catalyzes the conversion isovaleryl-CoA to 3-methylcrotonyl-CoA and transfers electrons to the electron-transferring flavoprotein (ETF). A specific ETF dehydrogenase then shifts the electrons to coenzyme

Q in the electron-transport chain for ATP synthesis (Mohsen et al., 1998; Volchenboum and Vockley, 2000).

A common disorder associated with a deficiency of this enzyme is Isovaleric academia (Mohsen et al., 1998). Isovaleric acidemia is a rare inborn error of metabolism caused by a deficiency of isovaleryl-CoA dehydrogenase (IVD). The activity of IVD has been extensively studied in both humans and plant physiology (Daschner et al., 1999; Daschner et al., 2001) but no study has reported its role in TBI or any other brain injury models.

IVD was one of the mitochondrial proteins that were found to be significantly oxidized following TBI. We report here for the first time its involvement in brain injury. It is possible that following TBI there is a reduced amount of ATP being generated from the ETC, leading to the dependence on other pathways for the supply of substrates for energy production. Here we see the possible involvement of the leucine catabolism pathway, which also gets inhibited due to the oxidation of IVD a key enzyme in this pathway. The oxidation of IVD following brain injury could as a result lead to a critical compromise of the total bioenergetics homeostasis and eventual cell death following TBI.

Fumarate hydratase (FH) also known as fumarase is an enzyme of the Tricarboxylic Acid Cycle (TCA), which catalyzes the conversion of Fumarate to L-malate. FH is also found in the glyoxylate and urea cycles (Pollard et al., 2003). The intracellular localization of this enzyme is not limited to the mitochondria, it can also be found in the cytosol as was found in rat liver, its function here is still unknown (Tuboi et al., 1990). A deficiency of this enzyme or mutations in the genes encoding this enzyme have been known to be the cause of Multiple cutaneous and uterine leiomyomata

MCUL1 (uterine fibroids), skin leiomyoma, and papillary type II renal cell cancer (Tomlinson et al., 2002). Recent proteomic study on mitochondrial proteins in cardiomyocytes from chronic stressed rat, showed a decrease in expression of Fumarate hydratase-1 after chronic restrain stress and a decrease in energy metabolism (Liu et al., 2004).

In the present study, we found Fumarate hydratase-1 as one of the key mitochondrial proteins that were significantly oxidized after TBI. The resulting consequence of this would be a breakdown of the TCA cycle and its inability to supply necessary substrates required for energy metabolism leading into an overall breakdown of bioenergetics and eventual cell death as observed in TBI.

Enolase-1 and Glyceraldehyde-3-phosphate dehydrogenase GAPDH

Enolase and GAPDH are glycolytic enzymes known to be located in the cytosol. Enolase interconverts 2-phosphoglycerate and phosphoenolpyruvate while GAPDH catalyzes the NAD^+ dependent oxidation of glyceraldehyde-3-phosphate to 1,3-bisphosphoglycerate and NADH in the second phase of glucose catabolism. GADPH has been typically known for its role in glycolysis but recent studies are now supporting the idea that it has diverse activities. Possible roles include regulation of the cytosol, membrane fusion and transport, accumulation of glutamate into presynaptic vesicles a, binding to low molecular weight G-protein and acting as a cellular sensor of oxidative stress. It is also thought that GADPH forms an integral part of apoptosis and may participate in neuronal death in some age related neurodegenerative diseases (Chuang et al., 2004). These emerging roles of GADPH raise questions on its cellular localization.

Enolase and GADPH are not typical mitochondrial proteins, but evidence has it that some glycolytic proteins can be localized in the mitochondria (Liaud et al., 2000; Taylor et al., 2003). A well-known glycolytic enzyme found in the mitochondria is hexokinase. Hexokinase has been found to be associated with the outer mitochondrial membrane where its activity has been implicated in regulation of ATP synthesis. It has also been found to promote closure of VDAC and prevent apoptosis (Azoulay-Zohar et al., 2004). In a recent proteomic study of the human heart mitochondrial proteome, (Taylor et al., 2003) 4% of total proteins identified were found to be associated with glycolysis. In another study (Liaud et al., 2000), GADPH and TPI were shown to be imported to the mitochondria of diatom *Phaeodactylum tricornutum* as a GADPH-TPI fusion. Another plant mitochondrial proteomic study GADPH and enolase were among the glycolytic proteins identified from the mitochondria, this study was even able to show that these protein were attached to the outer membrane of the mitochondria (Giege et al., 2003).

Taken together, the above studies continue to provide increasing evidence for the mitochondrial localization of glycolytic proteins. The purpose for this localization is not well known, but studies speculate that the whole glycolytic pathway could be associated with the OMM or IMS. This could be possible because most glycolytic enzymes have hydrophobic regions that could form membrane-spanning domains (Giege et al., 2003). This localization would be to provide pyruvate directly and at high concentrations where it can be taken up as substrate for respiration and ATP generation. When levels of ATP are reduced the mitochondria might initiate the interaction of glycolysis and the OMM to compensate for the reduction in energy production.

There is increasing evidence that GADPH and enolase –dependent pathways are being associated with different pathologies that involve oxidative stress. Our lab has previously identified α-enolase, as one of the enzymes one that was significantly oxidized in AD (Castegna et al., 2002a) and SAMP8 mice (Poon et al., 2004d). Our lab has also identified GAPDH as one of the proteins found to be significantly oxidized by Aβ(1–42)(Boyd-Kimball et al., 2005b). In recent proteomics study of *Staphylococcus aureus* (Weber et al., 2004), it was shown that oxidative stress triggers thiol oxidation of GADPH consistent with previous reports of the oxidation of glycolytic enzymes . Recently enolase was also found to be oxidatively modified in rat skeletal muscle (Kanski et al., 2005), this same study also identified GADPH as a target for protein nitration another example of protein oxidation.

In the present study we identified two glycolytic proteins enolase and GADPH from a mitochondrial preparation, as "mitochondrial" proteins that had been significantly oxidized following TBI. Oxidation of these proteins will most probably inactivate these enzymes hence inhibiting their glycolytic functions, reducing the amount of pyruvate supplied to the TCA leading to a reduction in ATP production and eventual cell death.

Following TBI, alterations in excitatory amino acids, increased ROS and disruption of Ca^{2+} homeostasis have been found to contribute to the cellular damage and eventual neurological dysfunction. The sequel of events that ensue following TBI have though not been well understood though the mechanism of this neurodegeneration is believed to be mediated in part by the mitochondria via an oxidative stress pathway. Mitochondrial bioenergetics plays a critical role in the normal physiology of cells. With a loss in the respiration capacity of the ETC and production of ROS after TBI (Sullivan et

al., 2005), it has been hypothesized that various mitochondrial proteins could be oxidatively modified and inactivated (Bogaert et al., 2000; Fiskum et al., 2004; Martin et al., 2005). This proteomics study is the first to provided new insights into the mechanisms of oxidative cellular damage after TBI.

We have demonstrated that following TBI and isolation of total mitochondria, key components of the ETC are oxidatively modified. Specifically, we have shown that the activities of PDH, complex I, and complex IV are reduced following TBI. This continues to support the notion that oxidative modification of proteins tends to lead to their inactivation. Additionally, we also have identified other proteins associated with energy metabolism that are significantly oxidized following TBI, which interact directly or indirectly with cellular bioenergetics pathways. The Stratagene software to determine the "interacteome" of the proteomics identified oxidatively modified proteins following TBI was determined. The oxidation of these identified proteins are of significant importance, due to their involvement with the ETS and/or apoptotic pathways, as well as their roles in the maturation and assembly process of key mitochondrial proteins. The identification of these oxidatively modified proteins present the first causal evidence for the significant loss of mitochondrial function that has been demonstrated to occur after TBI (Figure 7.1). They may also provide new insights into the mechanism of cellular damage after TBI and also provide novel targets for potential therapeutic interventions in this significant health problem. A summary of our working hypothesis involving oxidative damage to mitochondrial-related proteins is provided in Fig 7.8 below.

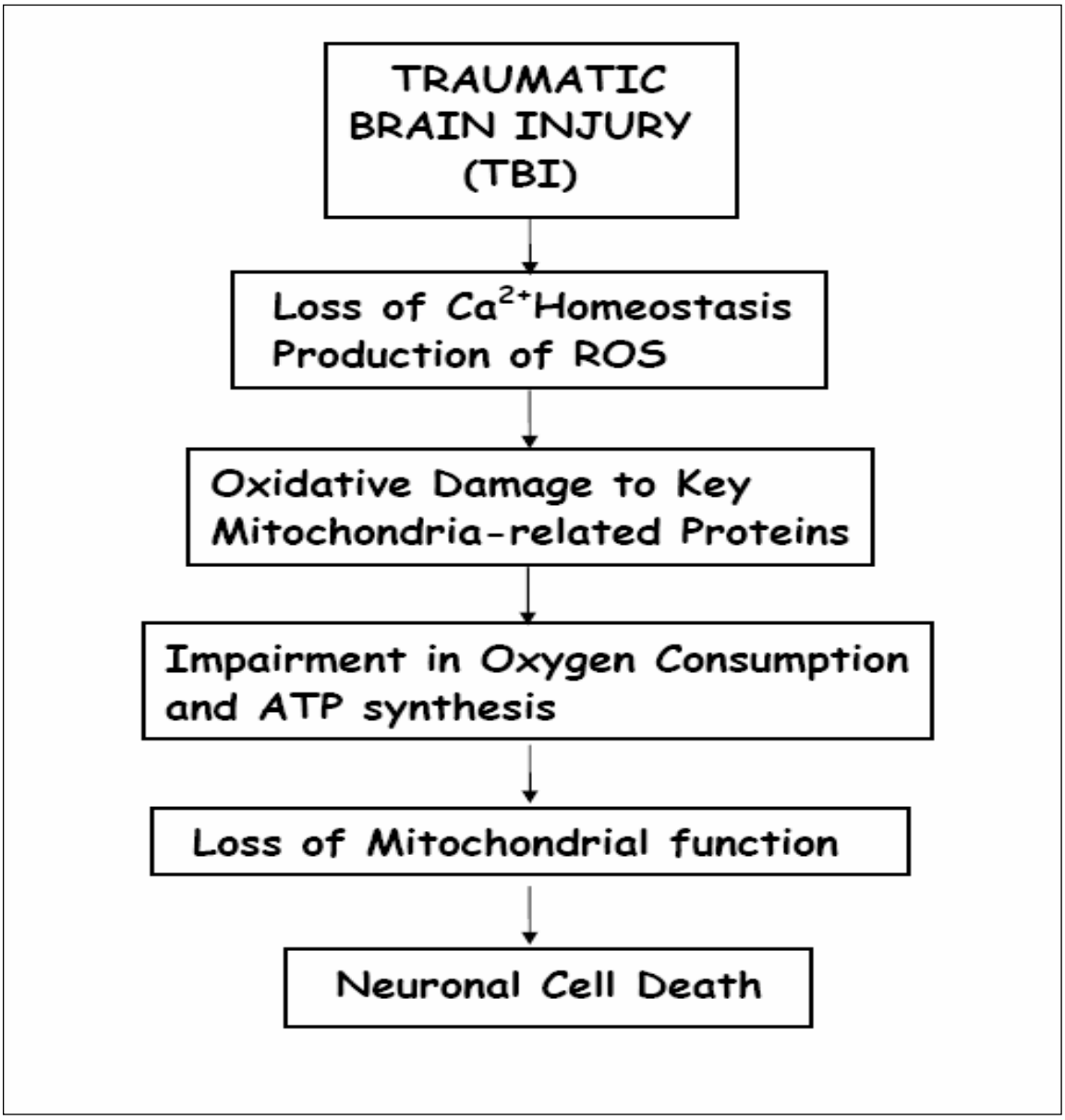

Figure 7.8 Summary of mechanisms that ensue following experimental TBI

7.6 Acknowledgments

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CHAPTER EIGHT

Conclusions and Future Studies

8.1 Conclusions

The work presented in this dissertation examined the mechanisms of improved cognitive function following intervention with a diet rich in antioxidants and a program of behavioral enrichment in a canine model of human aging. Additionally, the mechanisms of successful aging were also investigated. Through a proteomic analysis, we also examined the role of oxidative stress in traumatic brain injury specifically looking at oxidatively modified mitochondrial proteins.

Investigation on the role of an antioxidant fortified diet and a program of behavioral enrichment in the improvement of memory and cognitive function in the aging canine was examined. It was demonstrated that there was a significant decrease in the levels of oxidative stress as measured by protein carbonyls and 3NT in the parietal cortex of aged dogs that had a combination of these interventions (EA) compared to the other paradigms used, i.e., CC, EC and CA. Additionally, a proteomic analysis showed that there was a significant increase in the expression of Cu/Zn superoxide dismutase, fructose-bisphosphate aldolase C, creatine kinase, glutamate dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase, proteins related to energy metabolism, antioxidant system and detoxification mechanisms. Further redox proteomic analysis identified glutamate dehydrogenase [NAD (P)], glyceraldehyde-3-phosphate dehydrogenase (GAPDH), α-enolase, neurofilament triplet L protein, glutathione S-transferase (GST) and fascin actin bundling protein, as proteins that were significantly less oxidized following an examination of the EA and CC treated canine. An examination of the enzymatic activity of some of these identified proteins determined that there was a significant increase in

the activities of glutathione-S-transferase (GST) and total superoxide dismutase (SOD following the EA treatment compared to control. A significant correlation was also obtained between the levels of protein expression, oxidative damage, antioxidant activity and the improvement in cognitive function. We conclude that the combination of an antioxidant fortified diet and a program of behavioral enrichment in the aging canines may be translated to humans, providing a possible intervention for those at risk for developing age-related neurodegenerative disorders, including Alzheimer's disease.

The possible mechanisms involved in successful aging were explored. Frontal cortex samples from cognitively intact nonagenarian and those of demented nonagenarians were examined. Analysis of the level of oxidative stress in these groups of individual determined that there were no significant differences in protein oxidation as measured by the levels of protein carbonyls and lipid peroxidation as indexed by the levels of isoprostanes, neuroprostanes and HNE. However, proteomic analysis showed that there was a significant increase in the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), ATP synthase H^+ transporting, human peroxiredoxin 5 (PRDX5) and malate dehydrogenase (MDH) in the brains of demented nonagenarians compared to the cognitively intact nonagenarians. On the other hand the protein levels of fructose bisphosphate aldolase (FBP1) and ubiquitin carboxyl-terminal hydrolase L1 (UCHL1) were found to be increased in the cognitively intact nonagenarians compared to the demented age matched control. The consequence of this is still unknown; however, a recent study supports the role of UCHL-1 in normal synaptic and cognitive function. As a result, the increased differential expression of these proteins and in particular UCHL-1 observed here may reflect the following, one, the proteosomal activity is elevated in

normal controls compared to AD brains, and two that synaptic function is well maintained leading to improvements and maintenance of cognitive function in the normal nonagenarians. In addition, using correlation analyses, we have shown that there is a general trend of association between protein expression levels and intact cognition. In particular, MMSE scores were significantly associated with MDH, i.e., higher MMSE scores (better cognition) was linked to lower MDH, which is consistent with overall lower levels of MDH in nondemented nonagenarians. We conclude that this increase in the expression of key proteins may play a key role in the mechanisms leading to successful aging.

Lastly, the role of oxidative stress in experimental traumatic brain injury (TBI) was examined. Analysis of the oxygen consumption capacity of these mitochondria revealed that there was a significant decline in the respiratory capacity of brain mitochondria isolated from the ipsilateral cortex (IP) compared to those isolated from the contralateral cortex (CC) 3 h following experimental TBI. There were no differences in the respiratory capacities between mitochondria isolated from sham-operated animals compared to those from the CC animals. This investigation demonstrated that there was a significant increase in the levels of oxidative stress in the brain mitochondria isolated from the IP compared to those isolated from the contralateral cortex CC of rat after 3h following controlled cortical impact injury. We further explored the identity of key mitochondrial proteins that would have undergone possible oxidative modification following TBI from the cortex and hippocampus. We identified Cytochrome C Oxidase Va, Isovaleryl Coenzyme A Dehydrogenase, alpha enolase and Glyceraldehyde-3-phosphate dehydrogenase from the hippocampus and Pyruvate dehydrogenase

(lipoamide), Voltage dependent anion channel (VDAC), Fumarate hydratase 1, ATP synthase H^+ transporting and prohibitin from the cortex. Further examination of enzymatic activities demonstrated that there was a decline in the activities of pyruvate dehydrogenase and a significant decline in the activities of complex I and IV of the electron transport chain. We conclude that oxidative stress plays a significant role in the pathogenesis of TBI and it contributes to the oxidative modification of key mitochondrial-related proteins leading to a loss in mitochondrial bioenergetics and possible cell death.

Taken together, these studies continue to support the role of oxidative stress in neurodegenerative disorders and that intervention with antioxidant contribute to the maintenance of memory and cognitive function providing a possible avenue for the development of therapeutic interventions for oxidative stress mediated disorders and in particular to the aging population at risk for developing AD and those prone to CNS injury.

8.2 Future Studies

Based on the findings in this dissertation, the following experiments may warrant further exploration:

1. Based on the findings of increased oxidative stress following TBI and also the significant oxidative modification of key mitochondrial-related proteins, an expressional proteomic analysis of brain proteins and/or plasma following TBI would provide further insight into on the disorder and possible development of potential biomarkers.

2. Also since oxidative stress plays a key role in TBI, intervention with possible antioxidants or antioxidant related compounds such as brain soluble D609 and the identity of mitochondrial proteins protected should be further examined.
3. The findings of differentially expressed proteins in the cognitively intact nonagenarians though promising were not complete due to the limited number of subjects. With the increase in the number of subjects, this would provide further insight into the biochemical mechanisms that lead to successful aging.
4. Though there were no differences in the levels of oxidative stress between the cognitively intact nonagenarians and the demented controls, a redox proteomics analysis would be able to identify the redox status of key proteins hence providing additional insight into the successful aging process.
5. The availability of plasma extracted from the aging canines undergoing the combined therapy of antioxidant diet and a behavioral enrichment program is a unique resource that warrants further examination with a possibility of developing potential biomarkers for age-related disorders AND in particular AD.

Appendix A

Nucleoside Reverse Transcriptase Inhibitor (NRTI) - 2', 3'-dideoxycytidine (ddC) Induce Oxidative Stress: Relevance to HIV Dementia

A1. Overview

Human immunodeficiency virus dementia (HIVD) is the most common form of dementia occurring among young adults. In HIVD, neuronal cell loss occurs in the absence of neuronal infection. With the advent of highly active anti-retroviral therapy (HAART), the incidence of HIVD has drastically reduced, though prevalence of milder forms of HIVD continues to rise. Though these agents have been used successfully in suppressing viral production, they have also been associated with a number of side effects. Here, we examine the possible role of NRTI's, in particular 2', 3'-dideoxycytidine (ddC), in the neuropathology of HIVD. Synaptosomes and isolated mitochondria treated and incubated for 6 h with CSF-achievable concentrations of ddC, i.e., 6-11ng/ml, were found to show a significant increase in oxidative stress with 40 nM ddC as measured by protein carbonyls and 3-nitrotyrosine (3NT). Protection against protein oxidation induced by ddC was observed when mitochondria isolated from gerbil brain 1 h after injection i.p with the brain accessible antioxidant and glutathione mimetic tricyclodecan-9-yl-xanthogenate (D609). In addition, there is a significant reduction in the levels of anti-apoptotic protein Bcl-2 and a significant increase in cytochrome-c release and also a significant increase in the expression of pro-apoptotic protein caspase-3 were observed after mitochondria were treated with 40 nM ddC. The results reported show that ddC at 40 nM can induce oxidative stress, cause the release of cytochrome c,

and in addition, reduce the levels of anti-apoptotic proteins leading to a induction of apoptosis. These findings provide evidence for a possible role of the NRTI's, and in particular, ddC, in the mechanisms involved in HIVD.

A2. Introduction

Infection of the brain with HIV often results in a dementing disorder known as HIV-dementia (HIVD), the most common form of dementia occurring in human populations of less than 60 years of age. This dementia is marked by loss in memory and impaired cognitive function, among other indices of dementia (Navia and Price, 1987). Pathologically, reactive astrocytosis, myelin pallor, infiltration by macrophages and multinucleated giant cells, among others, characterize HIVD (Nath et al., 998). Since the neuronal loss and dysfunction observed in HIVD occurs, though the virus rarely infects neurons (Mattson et al., 2005), the mechanism of this neurodegeneration is still not known. Several studies have implicated the involvement of HIV viral proteins such as Tat, gp120 and gp 41, as well as neurotoxins that are produced from activated astrocytes and microglia, in the pathways leading to neurodegeneration (Adamson et al., 1996; Cheng et al., 1998; Nath et al., 2000; Pocernich et al., 2005).

In recent years, the use of highly active antiretroviral therapies (HAART) has revolutionized the treatment of AIDS, with a suppression of viral load and consequent reduction in complications observed in the late-stages of the disease (Gray et al., 2003). HAART can suppress the replication of the virus in the long term, but this is often accompanied by significant toxicities that can compromise treatment (Egger et al., 1997; Brinkman and Kakuda, 2000). Among the toxicities reported with the NRTI's include: hepatic steatosis, lactic acidosis, encephalopathy, lipodystrophy, peripheral neuropathy, and myopathy (Scalfaro et al., 1998; Blanche et al., 1999). Anti-retrovirals such as the nucleoside reverse transcriptase inhibitors (NRTIs), which include, 2', 3'-dideoxycytidine (ddC), are of great significance and are an important component of HAART (White,

2001).The mechanisms of these toxicities has been found to involve inhibition of neurite regeneration and inhibition of DNA polymerase gamma, leading to inhibition of mitochondria DNA synthesis, resulting in oxidative stress and eventual mitochondrial dysfunction (Cui et al., 1997). But recently, Mallon et al. (2005) have shown that NRTIs could cause mitochondrial dysfunction through other mechanisms other than inhibition of DNA polymerase gamma (Mallon et al., 2005).

With the advent of HAART, the incidence of HIVD has fallen, while the cumulative prevalence of HIVD has risen (Neuenburg et al., 2002). Despite the decline of HIVD incidence, neurological complications still remain an important cause of disability and death associated with AIDS (Kandanearatchi et al., 2003; McArthur et al., 2003). The rise in the prevalence of HIVD despite the use of HAART is consistent with the poor penetration of some of these anti-retrovirals into the CNS, hence increasing the longevity of the virus in the CNS (Deutsch et al., 2001). However, it has recently been reported that these drugs can effectively cross into the CNS, therefore, raising the question of their efficacy in the CNS and their possible role in HIVD (Enting et al., 1998; Sawchuk and Yang, 1999; Gibbs et al., 2003a; Gibbs et al., 2003b). For example, one treatment study showed variable results on the efficacy of NRTIs in the brain: one group of patients on HAART and having HIVD remained stable from cognitive impairment, while the other group showed signs of progressive neurological impairment even with treatment (Kandanearatchi et al., 2003). This disparity could possibly be due to the increased survival of the virus in the brain or could possibly be due to the NRTIs on their own being neurotoxic as has recently been shown with the increase in damage to brain mitochondria in HIV positive patients using NRTI's (Schweinsburg et al., 2005). The

latter may be of particular concern, since several experimental strategies are currently being considered to increase the transport of NRTIs across the blood brain barrier.

Therefore, in the current study, we have investigated these NRTIs, and specifically ddC, with the hypothesis that NRTIs could in themselves be neurotoxic and thus play a role in the progression of HIVD through an oxidative stress mechanism, possibly involving mitochondria. We report here that ddC, at concentrations achievable in the CSF, induces oxidative stress when treated to synaptosomes and isolated mitochondria. The levels of oxidative stress as measured by protein carbonyls on synaptosomes were significantly reduced upon the use of a known brain accessible antioxidant and glutathione mimetic [D609] (Zhou et al., 2001; Lauderback et al., 2003; Sultana et al., 2004). In addition, we observed a significant reduction in the levels of the anti-apoptotic protein Bcl-2, a significant release of cytochrome c and an equally significant increase in caspase-3 protein levels upon treatment of mitochondria with ddC. These results provide a possible mechanistic pathway for cell death through oxidative stress-induced apoptosis and are consistent with the notion of the possible contribution of ddC to HIVD.

A3. Experimental procedures

Material and Methods

Unless stated otherwise, all chemicals and antibodies were purchased from Sigma-Aldrich (St. Loius, MO). The oxidized protein detection kit was purchased from Intergen (Purchase, NY). The nucleoside reverse transcriptase inhibitor (NRTI), 2', 3'-dideoxycytidine (ddC), was obtained from NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH (NSC 606170). Assays for protein oxidation, lipid peroxidation, cytochrome c release and Bcl-2 levels were performed 6 h after NRTI treatment with some modifications as previously described (Butterfield et al., 1999a; Yatin et al., 1999; Butterfield and Lauderback, 2002).

Animals

The animal protocols were approved by the University of Kentucky Animal Care and Use Committee. For all studies in this paper, male Mongolian gerbils 3 months of age and approximately 100 g in weight were used. The rodents were housed in the University of Kentucky Central Animal Facility under 12-h light/dark conditions and fed standard Purina rodent laboratory chow.

Isolation of synaptosomes

Synaptosomes were isolated from three-month old male Mongolian gerbils as previously described (Whittaker, 1993). Briefly, the gerbils (n=5) were sacrificed by decapitation and the brain immediately isolated and dissected. The cortex was placed in 0.32 M sucrose isolation buffer containing 4 μg/ml leupeptin, 4 μg/ml pepstatin, 5 μg/ml

aprotinin, 2 mM ethylene di-amine tetra-acetic acid (EDTA), 2 mM ethylene glycol-bis-tetraacetic acid (EGTA), 20 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES), 20 μg/ml trypsin inhibitor, and 0.2 mM phenylmethanesulfonyl fluoride (PMSF), pH 7.4. The tissue was homogenized by 20 passes with a Wheaton tissue homogenizer. The homogenate was centrifuged at 1500 *g* for 10 minutes. The supernatant was retained and centrifuged at 20,000 *g* for 10 minutes. The resulting pellet was resuspended in ~1 ml of 0.32 M sucrose isolation buffer and layered over discontinuous sucrose gradients (0.85 M pH 8.0, 1.0 M pH 8.0, 1.18 M pH 8.5 sucrose solutions each containing 2 mM EDTA, 2 mM EGTA, and 10 mM HEPES) and spun at 82,500 *g* for 1 hr at 4^{o}C. Synaptosomes were collected from the 1.0/1.18M sucrose interfaces, and washed in Locke's buffer (154 mM NaCl, 5.6 mM KCl, 2.3 mM $CaCl_2$, 1.0 mM $MgCl_2$, 3.6 mM $NaHCO_3$, 5 mM glucose, 5 mM HEPES) twice for 10 minutes at 32,000 *g*. The resulting synaptosomal membranes were assayed for protein concentration by the Pierce BCA method. The synaptosomal preparation was relatively free of non-synaptic moieties (Whittaker, 1993).

Isolation of Mitochondria

Mitochondria were isolated from gerbil brain following i.p. injection with saline (control) or with D609 (50 mg/ kg body wt), 1 h after injection as previously described (Sultana et al., 2004). The brain mitochondria were isolated as previously described with minor modifications (Sims, 1990). Briefly gerbils, (n=6) were decapitated and the whole brain was isolated on ice. Whole brain was homogenized in ice-cold isolation buffer (250 mM sucrose, 10 mM HEPES, and 1 mM potassium EDTA, pH 7.2, 4 μg/ml leupeptin, 4

μg/ml pepstatin, 5 μg/ml aprotinin 20 μg/ml trypsin inhibitor) with 6 passes of a Wheaton tissue homogenizer. The homogenate was centrifuged for 3 min at 1,330× *g* at 4°C, and the resulting pellet was resuspended in isolation buffer and centrifuged at 1,330× *g* for 3 min. The supernatants from both spins were combined and spun at 21,200x *g* for 10 min at 4°C. The pellet was resuspended in 15% Percoll solution (v/v in isolation buffer) and layered onto discontinuous Percoll gradients of 23 and 40% Percoll (v/v in isolation buffer). Gradients were centrifuged at 30,700× *g* for 5 min at 4°C. At the 23-40% Percoll interface, mitochondria were isolated and resuspended in respiration buffer (250 mM sucrose, 2 mM magnesium chloride, 20 mM HEPES, and 2.5 mM phosphate buffer, pH 7.2) and centrifuged at 16,700× *g* for 10 min at 4°C. The pellet was resuspended in respiration buffer, centrifuged at 6,900× *g* for 10 min at 4°C, and the resulting pellet was washed in PBS at 6,900× *g* for 10 min at 4°C.

Incubation of synaptosomes and mitochondria with ddC

Stock solutions of ddC were diluted in phosphate buffered saline (PBS). Synaptosomal and mitochondrial concentrations of 4mg/ml were then treated with the ddC to a final concentration based on the CSF-achievable concentration range of 6-11ng/ml (Sawchuk and Yang, 1999). These samples were then incubated for 6 h at 37^{o}C with frequent vortexing. Controls were incubated with PBS. These samples were then frozen for subsequent assays.

Oxidation of synaptosomes with Fe^{2+}/H_2O_2

Protein oxidation was initiated by addition of hydroxyl radical generating mixture (30 μM $FeSO_4$/3 mM H_2O_2) as previously described (Pocernich et al., 2000). Briefly,

synaptosomes (4 mg proteins/ml) were suspended in 0.987 ml of lysing buffer and treated with 30 μM Fe^{2+} ($FeSO_4 \cdot 7H_2O$) and 3 mM H_2O_2 (diluted from 30% H_2O_2), bringing the total volume to 1 ml, for 30 min at room temperature. Fe^{2+} reacting with H_2O_2 produce hydroxyl free radicals (Butterfield and Stadtman, 1997). The protein suspension was then washed four times with lysing buffer at 14,000 rpm for 4 min. This sample was used as a positive control for protein oxidation.

Measurement of protein carbonyls

Protein carbonyls are an index of protein oxidation and were determined as described previously (Butterfield and Stadtman, 1997). Briefly, 5μl of sample were derivatized with 10mM 2, 4-dinitrophenylhydrazine in the presence of 5μl of 12% SDS for 20 min at room temperature. The samples were neutralized with 7.5 μL of the neutralization solution (2 M Tris in 30% glycerol). Derivatized protein samples were blotted onto nitrocellulose membrane with a slot-blot apparatus (250 ng/lane). The membrane then was washed with wash buffer (10mM Tris-HCl (pH 7.5), 150mM NaCl, 0.05% Tween 20), blocked by incubation of 5% BSA, followed by incubation with rabbit polyclonal anti-DNPH antibody as primary antibody for 1 h. The membranes were washed with wash buffer and further incubated with alkaline phosphatase (ALP)-conjugated goat anti-rabbit antibody as the secondary antibody for 1 h. Blots were developed using Sigma fast tablets (BCIP/NBT) and were quantified using Scion Image (PC version of Macintosh compatible NIH Image) software.

Measurement of lipid peroxidation

4-Hydroxynonenal (HNE) levels

HNE is a marker of lipid peroxidation, and this assay was performed as previously described (Lauderback et al., 2001). Briefly, 10 μl of sample both from cell culture or synaptosomes were incubated with 10 μl of Laemmli buffer containing 0.125 M Tris base pH 6.8, 4 % (v/v) SDS, and 20% (v/v) Glycerol. The resulting sample (250 ng) was loaded per well in the slot blot apparatus. Samples were loaded onto a nitrocellulose membrane under vacuum pressure. The membrane was blocked with 3% (w/v) BSA in phosphate buffered saline containing 0.01% (w/v) sodium azide and 0.2% (v/v) Tween 20 (PBST) for 1 hr and incubated with a 1:5000 dilution of anti-4-hydroxynonenal (HNE) polyclonal antibody in PBST for 90 min. Following completion of the primary antibody incubation, the membranes were washed three times in PBST. An anti-rabbit IgG alkaline phosphatase secondary antibody was diluted 1:8000 in PBST and added to the membrane. The membrane was washed in PBST three times and developed using Sigma fast Tablets (BCIP/NBT substrate). Blots were dried, scanned with Adobe Photoshop, and quantified with Scion Image.

Measurement of cytochrome c release

Cytochrome c release was detected as previously described with slight modifications (Yang et al., 1997). Briefly, after incubation and centrifugation of mitochondrial samples, the supernatant was used for Western blot analysis for cytochrome c release. The membrane was blocked in blocking buffer (3% bovine serum albumin) in PBS/Tween for 2 h and incubated with a 1:2000 dilution of anti-cytochrome

c polyclonal antibody (C-5723; anti-sheep; Sigma) in PBS/Tween for 2 h. The membrane then was washed in PBS/Tween for 5 min three times after incubation. The membrane was incubated for 1 h, after washing, with an anti-sheep IgG alkaline phosphatase secondary antibody diluted in PBS/Tween in a 1:8000 ratio. The membrane then was washed three times in PBS/Tween for 5 min and developed in Sigma Fast tablets. Blots were dried, scanned with Adobe PhotoShop, and quantified using Scion Image software.

Measurement of Bcl-2 levels

The levels of anti-apoptotic protein Bcl-2 were detected as previously described (Yang et al., 1997) with slight modification as described above, except a 1:2000 dilution of anti-Bcl-2 monoclonal antibody (AAM-072; anti- mouse; stressgen) in PBS/Tween for 2 h was used.

Statistical analysis

Student's t-test was used to assess statistical significance; p values < 0.05 were considered significant.

A4 Results

Increased oxidative stress in synaptosomes following treatment with ddC

Synaptosomes are a good model for studying neuronal synaptic function at nerve terminals and display markers of neurodegeneration upon treatment with various oxidants such as amyloid β-peptide (Aβ) (Butterfield and Boyd-Kimball, 2005). Hence, synaptosomes were used for the present study to determine if ddC caused oxidative damage**.** Oxidative stress can lead to a variety of detrimental effects on neurons including protein cross linking, decreased protein turnover, loss of protein function, altered redox potential, disruption of Ca^{2+} homeostasis, and ultimately cell death (Butterfield and Stadtman, 1997). As shown in Fig A1a, synaptosomes were exposed to a known hydroxyl radical generating mixture (30 μM $FeSO_4$/1 mM H_2O_2) as a positive control and a range of ddC concentrations (29 nM- 52 nM). There was a significant increase in protein carbonyls levels on synaptosomes treated with 40 nM ddC $p < 0.05$. These levels were comparable to those of the synaptosomes treated with Fe^{2+}/H_2O_2**.** Fig A1b shows levels 3-nitrotyrosine (3NT), another marker of protein oxidation, in ddC treated synaptosomes. A significant increase in the levels of 3NT with 40nM ddC was observed; however, there was no significant change in the levels of lipid peroxidation as indexed by HNE when synaptosomes were treated with ddC in all the concentration ranges Fig A1c.

Increased oxidative stress in mitochondria following treatment with ddC

Fig A2 shows the levels of protein carbonyls and HNE in mitochondria treated with ddC. Fig A2a shows a significant increase in the levels of protein oxidation as indexed by protein carbonyls. These results were significant at 40 nM ddC, seen

previously with synaptosomes. Fig A2b shows the levels of lipid peroxidation as indexed by HNE was unaffected when mitochondria were treated with a varying range of concentrations of ddC, consistent with the results with synaptosomes.

D609 protects mitochondria from oxidative stress mediated by ddC

The use of antioxidants has been shown to provide protection against oxidative insult in a number of models of neurodegenerative disorders (DeAtley et al., 1999; Calabrese et al., 2003a; Farr et al., 2003; Lauderback et al., 2003; Sultana et al., 2005a).The levels of protein oxidation as indexed by protein carbonyls in mitochondria isolated from brain of gerbils injected 1 h before sacrifice with saline or D609 are shown in Fig. A3. These brain mitochondrial samples were subsequently treated with 40 nM ddC, the concentration that showed the most effect. The levels of protein carbonyls were significantly increased in mitochondria isolated from brain of saline-injected gerbils and treated with ddC compared to control. Mitochondria isolated from brain of D609-injected gerbils and subsequently treated with 40 nM ddC showed a significant decrease in the levels of protein carbonyl levels compared to mitochondria isolated from brain of saline-injected gerbils and subsequently treated with 40 nM ddC.

Increased cytochrome c release from mitochondria following treatment with ddC

Mitochondria are the main source for the generation of reactive oxygen species (ROS), which has been known to contribute to the release of cytochrome c and subsequent induction of apoptosis (Sullivan et al., 1999). Fig. A4 shows the level of cytochrome c released from mitochondria isolated from gerbil brain and treated with

various CSF concentrations of ddC. There was a significant increase in the amount of cytochrome c released by mitochondria treated with 40nM ddC compared to control.

Decreased levels of anti-apoptotic protein Bcl-2 following treatment with ddC

Bcl-2 is a human proto-oncogene that modulates cell death or apoptotic pathways by regulating the release of pro-apoptotic molecules from the mitochondria. Fig A5 shows the level of Bcl-2 protein in mitochondria isolated from gerbil brain and treated with various CSF concentrations of ddC. Though there was a decrease in the levels of Bcl-2 with all concentrations of ddC used, there was a significant decrease in the levels of Bcl-2 with 52nM ddC treatment compared to control.

Increased expression of pro-apoptotic protein caspase-3 after treatment with ddC

As an additional evidence of apoptotic events talking place after the treatment of mitochondria with ddC, we measured the levels of caspase-3 protein levels as shown in figure A6. There was an observable increase in the levels of caspase- 3 in all treatment but there was a higher increase in the 40nm ddC treated mitochondria. It should be noted that since this was just additional proof of apoptotic events taking place, and n = 4 was used. The changes observed are about 40% of control with statistical significance $p < 0.05$.

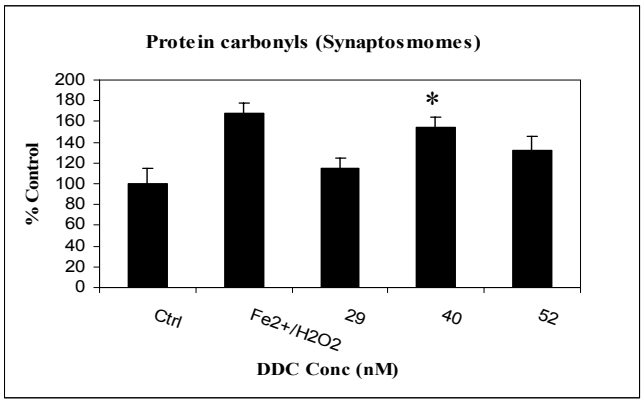
No effect on protein carbonyl levels following treatment with 3TC

As additional evidence for the oxidative stress-induced toxicities of NRTI's we have shown that 3TC the most tolerable NRTI in HIV therapy did not induce oxidative stress in synaptosomes treated using concentrations achievable in the CSF compared to DDC Figure A7. As a result the oxidative stress-related effects we are observing in the

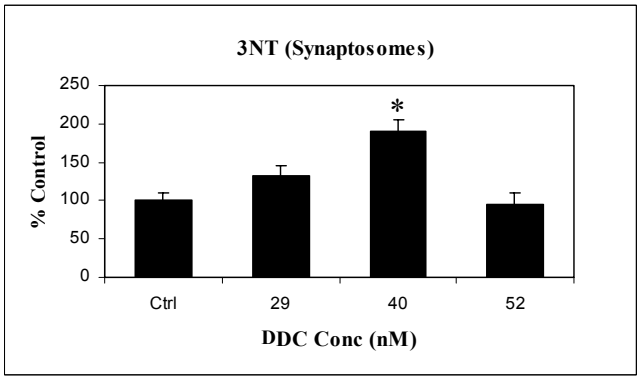
present study are specific to only ddC and not any other NRTI. It should be noted that n=4

Figure A1

(A)



(B)



(C)

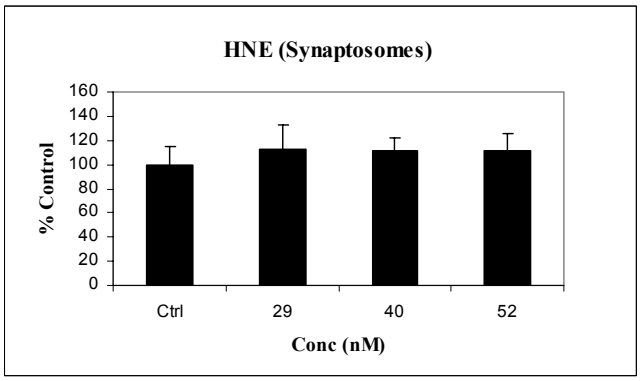


Figure A1 Protein oxidation and lipid peroxidation as indexed by protein carbonyls, 3NT and HNE in ddC treated synaptosomes. There is an increase in the levels of protein carbonyls (Fig A1a) and 3NT (Fig A1b) on synaptosomes treated with 40nM ddC. The levels of protein carbonyls were comparable to those of synaptosomes treated with the

positive control of the hydroxyl radical generating mixture of Fe^{2+}/H_2O_2. Fig A1C shows the levels of lipid peroxidation as indexed by HNE; there was no significant difference among the treatments. Results here are given as the mean ± S.E.M. expressed as percentage of control values. (*) $p < 0.05$ n=5.

Figure A2

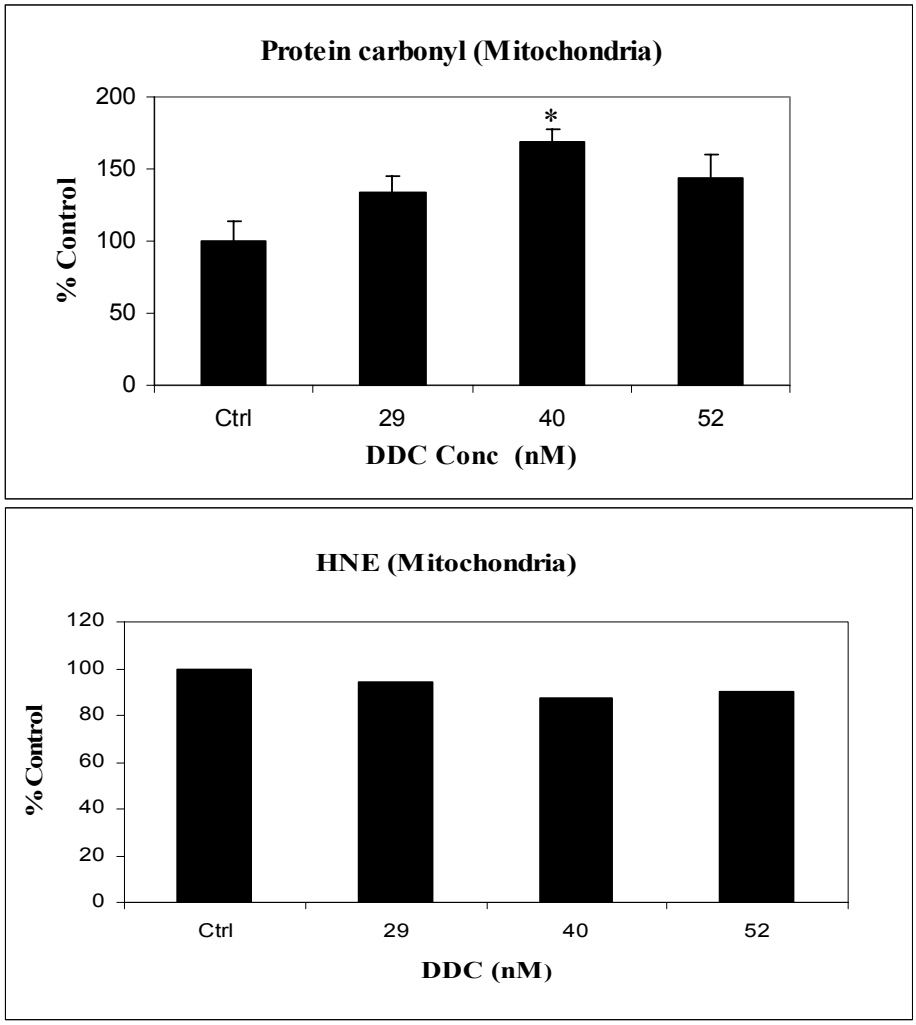


Figure A2 Protein oxidation and lipid peroxidation as indexed by protein carbonyls and HNE respectively on ddC treatment of isolated mitochondria. There is an increase in the levels of protein carbonyls (Fig A2a) on mitochondria treated with 40nM ddC. Fig A2b shows the levels of lipid peroxidation as indexed by HNE; there was no significant difference among the treatments. Results here are given as the mean ± S.E.M. expressed as percentage of control values. (*) $p < 0.05$ n=6.

Figure A3

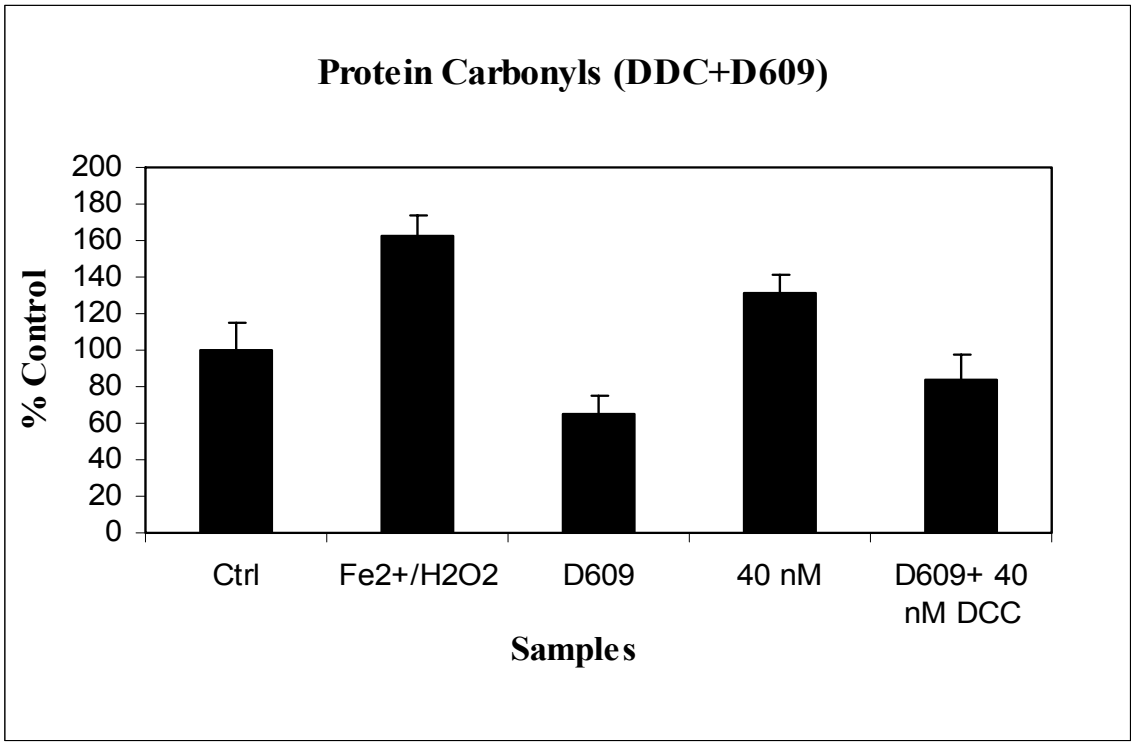


Figure A3. The levels of protein oxidation as indexed by protein carbonyl in mitochondria isolated from brain of gerbils injected with saline or D609. The levels of protein carbonyls were found to be significantly increased in mitochondria isolated from brain of saline- injected gerbils and treated with ddC compared to control. Mitochondria isolated from brain of D609-injected gerbils and treated with 52nM of ddC showed a significant decrease in the levels of protein carbonyl levels as compared to mitochondria isolated from brain of saline-injected gerbils brain and subsequently treated with 52nM ddC. Results here are presented as the mean ± S.E.M. expressed as percentage of control values. (*) $p < 0.05$ n=6. D609 concentration used was 50 μM, chosen based on prior studies of this agent

Figure A4

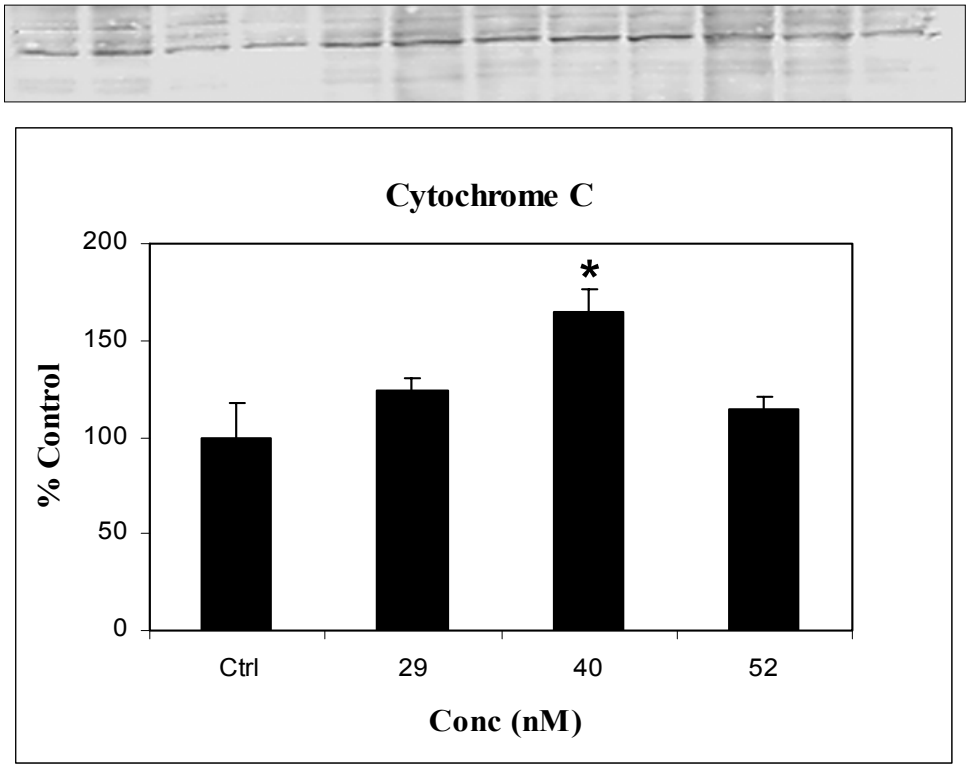


Figure A4. The level of cytochrome-c release from mitochondria isolated from gerbil brain and treated with different concentrations of ddC. There was a significant release in cytochrome-c when 40 nM ddC was added to isolated mitochondria. Data here are presented as the mean ± S.E.M. expressed as percentage of control values. (*) $p < 0.05$ n=4.

Figure A5

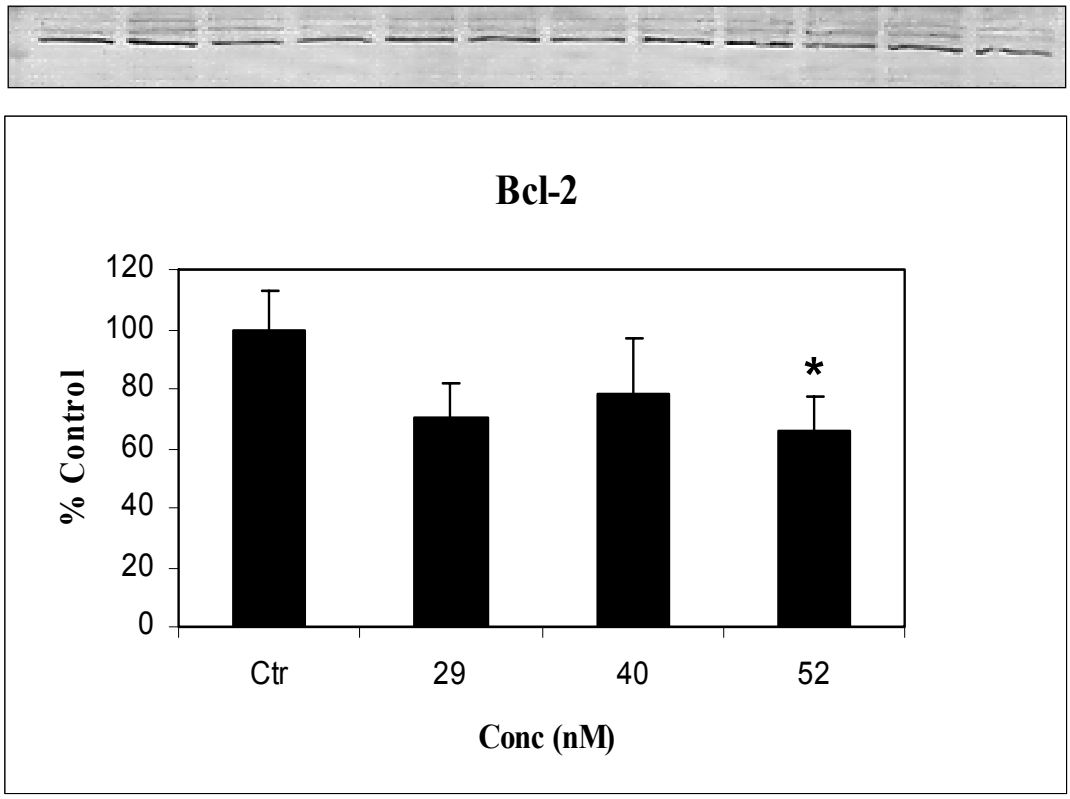


Figure A5. The protein levels of anti-apoptotic protein Bcl-2 in brain mitochondria isolated from gerbil brain and treated with ddC. There was a reduction in the levels of Bcl-2 in with all ddC concentrations used, but a significant reduction was observed with 52 nM ddC. Data here are presented as the mean ± S.E.M. expressed as percentage of control values. (*) $p < 0.05$ n=4.

Figure A6

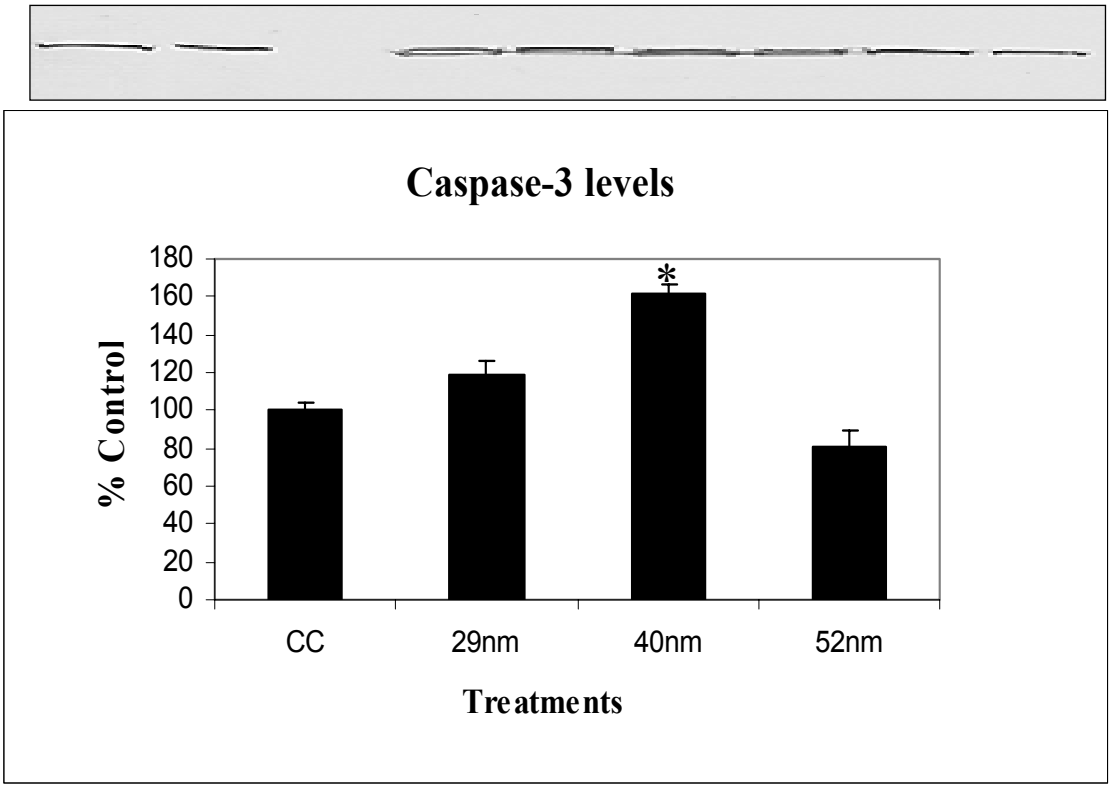


Figure A6 The protein levels of pro-apoptotic protein Caspase-3 in brain mitochondria isolated f from gerbil brain and treated with ddC. There was an increase in the levels of caspase 3 in with all ddC concentrations used, with 40 nM ddC showing the highest level. Data here are presented as the expressed as percentage of control values. n =4 $p < 0.05$

Figure A7

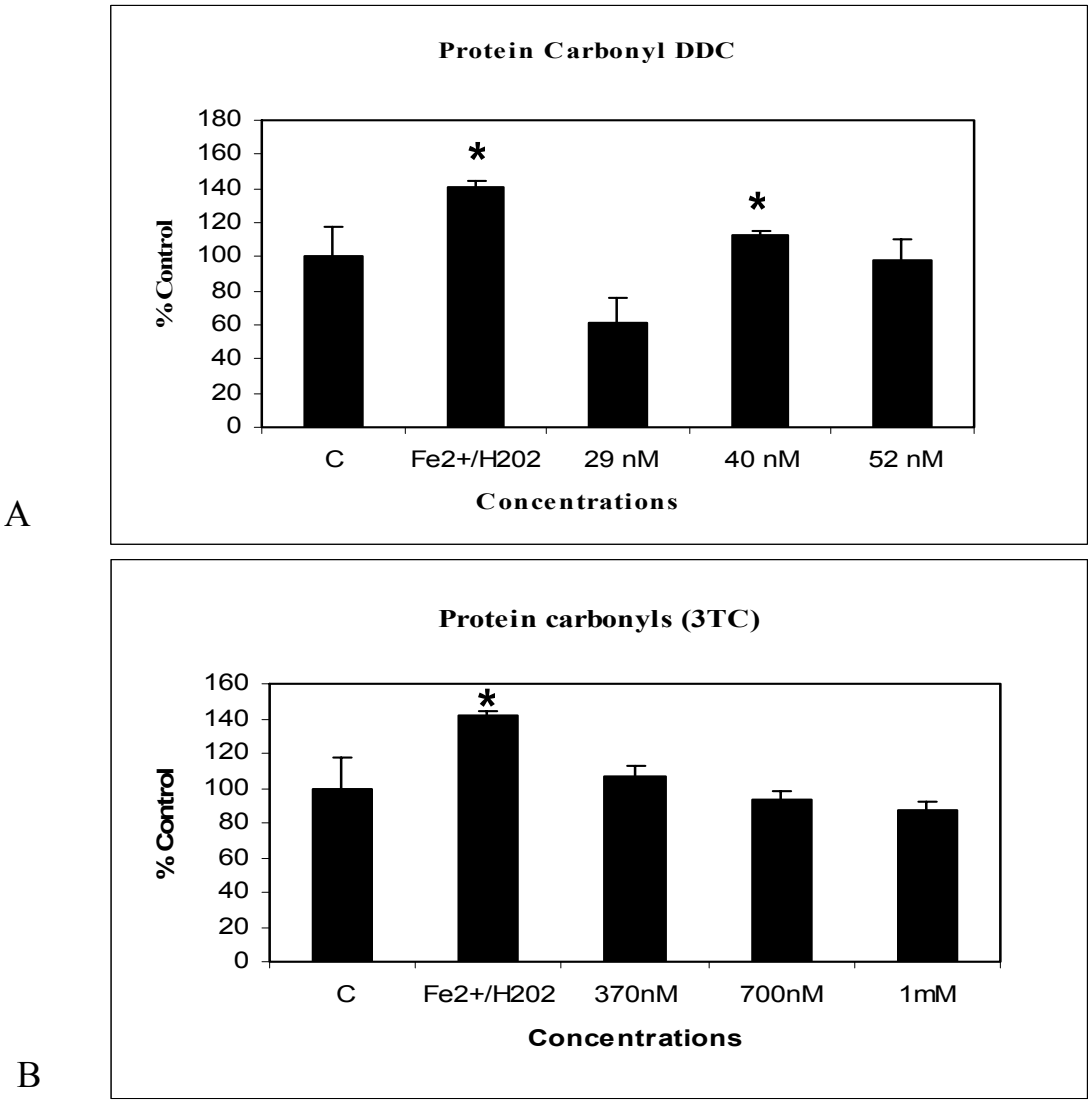


Figure A7 Protein oxidation as indexed by protein carbonyls in ddC and 3TC treated synaptosomes. There is an increase in the levels of protein carbonyls (Fig A7a) on synaptosomes treated with 40nM ddC but no significant changes are observed in 3TC treated synaptosomes (Fig A7b). Results here are given as the mean ± S.E.M. expressed as percentage of control values. n = 4(*) $p < 0.05$

A5 Discussion

The findings reported here show that ddC, at physiologically relevant concentrations achievable in the CSF and especially at 40nM, is able to induce oxidative stress in both synaptosomes and isolated mitochondria, as measured by the elevated levels of protein oxidation. DDC also reduced the levels of the anti-apoptotic protein Bcl-2, slightly increased the levels of Bax, induced the release of cytochrome c, and increased caspase-3 protein levels. These results implicate oxidative stress and the mitochondria as possible pathways involved in NRTI-induced neuronal death.

Infection of the brain with HIV often results in a decline of neuropsychological performance, neurological impairments (McArthur et al., 1989; Karlsen et al., 1993, 1995; Baldewicz et al., 2004), and a dementing disorder known as HIV-dementia (HIVD). HIVD is the most common form of dementia in young adults, and it is estimated that 30% of adults infected with HIV usually develop HIVD (Janssen et al., 1992; Nath and Geiger, 1998). The mechanism of this neurodegeneration remains unknown, since HIV rarely infects neurons (Nath and Geiger, 1998). Recent studies have proposed various mechanisms for HIVD, for example, HIV over-activates immune cells within the brain to produce cytokines or chemokines, which can inhibit the growth and survival of brain cells or trigger cell death and low levels of transforming growth factor β 1(TGF β 1) may enhance brain cell damage in people with HIV infection (Ensoli et al., 1999; Letendre et al., 1999). HIV proteins such as Tat, nef, gp120, and gp 41, have also been implicated in the pathways leading to this neurological dysfunction (Adamson et al., 1996; Cheng et al., 1998; Nath et al., 2000; Pocernich et al., 2004; Pocernich et al., 2005). Oxidative stress has been found to play a major role in most neurodegenerative

disorders (Farr et al., 2003; Butterfield and Boyd-Kimball, 2005; Keller et al., 2005; Pocernich et al., 2005). This could be another possible mechanism involved in HIVD induced neurodegeneration, since recent studies have shown that there is increased oxidative stress in the brain and CSF of HIVD patients (Chauhan et al., 2003; Turchan et al., 2003).

The NRTIs, including ddC, form the basis of the HAART therapy (White, 2001). These molecules are derivatives of nucleoside bases with varying modifications that give alternative substrates for the DNA polymerases. The most common chemical modification is the lack of 3'OH group of the sugar bases (Kakuda, 2000). The mechanism for action of ddC is through inhibiting the attachment of nucleic acids and at the same time terminating DNA chain elongation once incorporated (Balzarini, 1994). Just like endogenous nucleic acids, NRTIs have to undergo phosphorylation by a number of kinases to give the active triphosphorylated form (Kakuda, 2000). It is known that the concentrations of NRTIs decrease progressively as they move from the plasma to the CSF (Sawchuk and Yang, 1999; Kandanearatchi et al., 2003). The penetration of NRTIs into the CNS has been an issue to date (Enting et al., 1998; Sawchuk and Yang, 1999). An increase in the incidence of HIV dementia has been thought to be due to the prolonged survival of the HIV virus in the brain resulting from the poor penetration of the antiretroviral used in the therapy (Deutsch et al., 2001), but studies now suggest that some of these drugs do effectively penetrate the CNS (Enting et al., 1998; Arendt et al., 2001; Gibbs et al., 2003a). DDC is transported to the CNS through organic anion transporters (Hedaya and Sawchuk, 1989). This could be a possible explanation for the lack of any significant difference observed in the levels of lipid peroxidation product

HNE measured in both synaptosomes and isolated mitochondria treated with ddC in the current study. For HNE to be generated, the oxidant must be capable of inserting itself into the lipid bilayer to trigger the cascade for lipid peroxidation (Butterfield and Boyd-Kimball, 2005). Since the transport of ddC into the CNS is through organic anion transporters ddC may not get into the bilayer and thus not induce lipid peroxidation, though this is speculative at present.

Though NRTIs are capable of controlling the replication of the HIV virus, they also have adverse effects during therapy (Scalfaro et al., 1998; Blanche et al., 1999). Mitochondrial dysfunction has been previously observed with NRTIs, and extensive studies on the mechanism of NRTI toxicity has been linked to mitochondrial dysfunction, mutations in mtDNA and oxidative stress through inhibition of DNA polymerase gamma, which encodes 13 oxidative phosphorylation (OXPHOS) genes (Lewis et al., 2001; Lewis et al., 2003). It has recently been suggested that NRTIs could cause mitochondrial dysfunction through mechanisms other than inhibition of DNA polymerase gamma (Mallon et al., 2005). Of the above mechanisms of toxicity, oxidative stress seems to play a major role. Oxidative stress is an imbalance between reactive oxygen species and cellular antioxidant defenses (Butterfield and Stadtman, 1997). The role of HIV infection and NRTI on induction of oxidative stress has been known for a while, but has not been well explored (Droge, 2002). We have shown in the present study that, on treatment of synaptosomes and isolated mitochondria with CSF-achievable concentrations of ddC, there is a significant increase in the oxidative stress as indexed by the levels of protein oxidation biomarkers, i.e., protein carbonyls and 3NT. These results support the role NRTI induction of oxidative stress. DDC at lower concentrations had very little effect of

the induction of protein oxidation; however at 40nM of ddC there was a significant increase in the levels of protein oxidation, which on further increase in the concentration of ddC had no effect. This observed concentration-effect relationship could be possibly due the saturation of ddC transporters at 40 nM ddC.

The use of antioxidants has been found to be protective in various models neurodegenerative disorders (DeAtley et al., 1999; Calabrese et al., 2003a; Farr et al., 2003; Lauderback et al., 2003). Tricyclodecan-9-yl-xanthogenate (D609) is an inhibitor of phosphatidylcholine-specific phospholipase C, an antioxidant, anti-tumor and a glutathione (GSH) mimetic (Zhou et al., 2001; Lauderback et al., 2003; Sultana et al., 2004). Mitochondria isolated from brain of D609-injected gerbils and treated with 52 nM ddC showed decreased oxidative stress as measured by the levels of protein carbonyls, demonstrating that though ddC can induce oxidative stress, these effects can be modulated when a brain accessible glutathione mimetic antioxidant is used. We previously suggested that D609 possibly could have therapeutic relevance in treating HIVD (Pocernich et al., 2005).

Mitochondria are not only an essential component of the cell for energy generating capacity but are targets of oxidative stress because of their ability to generate ROS. In addition mitochondria also are prodigious regulators of cell death (Melov, 2000). Several molecules contained within the mitochondria, such as cytochrome c, have a pro-apoptotic influence. In contrast, Bcl-2 members regulate the pores in the inner membrane of the mitochondria, which when opened lead to release of pro-apoptotic molecules and induction of apoptosis (Stefanis, 2005). The inhibition of mtDNA replication can result in acquired defects that leads to most mitochondrial genetic diseases (Lewis et al., 2003). As

a result, NRTIs could thus generate phenotypic OXPHOS defects that would eventually compromise the electron transport chain (ETC), resulting in energy depletion and eventually cellular dysfunction. It is from this consideration that the phrase "mitochondrial dysfunction hypothesis" was introduced (Lewis et al., 2003), linking NRTI toxicity to their effects on the mitochondria (Moyle, 2000; Dalakas et al., 2001). In the present study, we have observed a significant decrease in the levels of anti-apoptotic protein Bcl-2 and consequently a significant increase in the release of cytochrome c, upon treatment of mitochondria with ddC. We also observed an increase in the protein levels of caspase-3. These results present a possible mechanism through which NRTIs could induce cell death and neuronal loss in HIV-related dementia. The decrease of Bcl-2 protein, increase in cytochrome c and increase in caspase-3 provides a synergistic environment for the induction of apoptosis. The down regulation of Bcl-2 levels, leads to the release of cytochrome c, which binds to Apaf-1and pro-caspase 9 forming the apoptosome. Also the slight observation of the increase in Bax levels would lead to an increase in the permeability of the mitochondrial membrane possibly leading to the release of cytochrome c (Susin et al., 2000). These mediators then activate caspase 9, which triggers the cascade of "effectors" caspases and eventual cell death (Stefanis, 2005). Our findings are consistent with a recent report that showed that other than ddC, 2', 3'-didehydro-3'-deoxythymidine (d4T), also caused apoptosis in rat dorsal root ganglion neuronal cell cultures in HIV free environments (Bodner et al., 2004).

Despite the use of HAART, neurological complications still remain an important cause of disability and death associated with AIDS (Kandanearatchi et al., 2003; McArthur et al., 2003). The efficacy of these drugs in the brain is still controversial and

research into the effect of HAART on the signs and symptoms of HIVD has resulted into a number of conflicting findings. For example, there are reports showing that HAART is capable of reducing HIV encephalitis and leukoencephalopathy, improving neurocognitive function, mental concentration, speed of mental processing, memory, and visuo-spatial and constructional abilities (Gendelman et al., 1998; Dougherty et al., 2002; Vago et al., 2002). Another study reports a partial improvement of clinical abnormalities after patients were on HAART (Stankoff et al., 2001). A study by (Bouwman et al., 1998)) has reported that after HAART treatment some patients remained free of cognitive impairment with prolonged life span, while others become neurologically impaired. Dougherty et al. (2002) treated 96 HIV- demented patients with HAART, and of the 30 patients that had adequate follow-up data, 60% showed neurological improvement (Dougherty et al., 2002). These studies among others continue to raise the question on the effect of the antiretroviral therapy on the incidence and prevalence of HIVD and the efficacy of the NRTIs in the brain, therefore supporting the possibility that these drugs could themselves contribute to the neurological impairments. In a recent study, it has been shown that there is brain mitochondrial injury in HIV positive patients who are on NRTI therapy (Schweinsburg et al., 2005), this provides additional support for the present study of the possible involvement of NRTI's in HIV-dementia.

As a result, the current study was an effort at studying a possible toxic effect of NRTI's and in particular ddC, with oxidative stress and mitochondrial dysfunction as the key mechanism of toxicity. Synaptosomes provide a good model of the synapse and display markers of neurodegeneration upon treatment with various oxidants. Equally, isolated mitochondria provide a good model to study mechanisms of neurodegeneration

since they are a source of ROS and control cell death through regulation of apoptosis (Butterfield et al., 1999a; Lauderback et al., 2001; Lauderback et al., 2003; Brito et al., 2004; Pocernich et al., 2004; Butterfield and Boyd-Kimball, 2005). As noted above, despite the reduction of late stage HIV complications with the use of antiretroviral treatment, neurological complications are still being reported, questioning the efficacy of these drugs in the brain (Bouwman et al., 1998). It is therefore our view that in addition to the prolonged survival of the HIV virus in the brain (Deutsch et al., 2001) and increased penetration of the antiretrovirals to the CNS (Enting et al., 1998; Sawchuk and Yang, 1999), these NRTIs could by themselves contribute to this neurological complication through increased oxidative stress as shown by the increase in oxidative stress biomarkers measured here. It is also possible that ddC could potentiate and act synergistically with other compounds such as Tat, gp120 and gp41 in inducing mechanisms implicated in HIVD (Pocernich et al., 2005). This is particularly possible since Bonder et al (2004) have recently reported that there exists a possible synergy in toxicity to DRG neuronal cell cultures by gp120 and another NRTI, d4T. In addition, a recent study has also provided evidence for the injury to brain mitochondria from patients taking NRTI's (Schweinsburg et al., 2005) and that NRTI-oxidative stress-induced DNA damage through inhibition of repair mechanism could lead to oxidative stress-induces apoptosis in neurons (Hashiguchi et al., 2004; Harrison et al., 2005). Therefore, evidence provided in the present study suggests that ddC could possibly contribute to the mechanisms involved in HIVD through induction of oxidative stress, reduction in anti-apoptotic protein Bcl-2, release of cytochrome c and increase in caspase 3 and bax

proteins making this study the first to establish the possible involvement of NRTIs in HIV related dementia.

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Appendix B

Analysis of Protein Expression Levels in Brain of NF-Kβ P50 Heterozygous Knockout Mice

B1 Overview

Nuclear factor kappa B (NF-κB) is an important transcriptional factor. Its role in oxidative stress, and most recently in pro- and anti-apoptotic related mechanistic pathways, have well been established, however, there still lacks a complete understanding of the roles its various subunits play in normal human physiology or disease. Because of the dual nature of NF-κB, the wide range of genes it regulates and the plethora of stimulus that activates it, various studies addressing the functional role of NF-κB proteins have resulted in a number of differing findings. As a result, in this dissertation, we undertook to examine the effect of a stimulus-free environment on the frontal cortex of mice brain with the p50 subunit of NF-κB partially knocked p50 (-/+). Heterozygous p50 mice knockout (KO) and wild type (WT) were used, and at 7-9 weeks they were sacrificed and various brain regions dissected. We analyzed the levels of oxidative damage in the frontal cortex of both the p50 (-/+) and WT mice; measuring the levels of 4-hydroxynonenal (HNE), 3-nitrotyrosine (3NT) and protein carbonyls. There was a significant reduction in the levels of all oxidative stress parameters analyzed in the p50 (-/+) mice when compared to the WT. We further carried out a proteomic profile analysis to identify proteins that were differentially expressed in these two groups of animals. We identified proteins whose expressions were significantly increased in the p50 (-/+) mice compared to the WT. These identified proteins were: ATP synthase gamma chain,

Ubiquinol-cyt-C reductase, Heat shock protein 10 (Hsp10), Fructose Bisphosphate aldolase C, and NADH-ubiquinone oxidoreductase. We have therefore established that partial knock out of the p50 subunit of NF-κB results in a reduction in the levels of oxidative stress. In addition, there is a significant increase in the expression of key proteins in the p50 (-/+) brain compared to that of the WT. These findings suggest that the p50 subunit is an essential component of NF-κB transcription factor that potentially can be targeted for the development of therapeutic interventions in disorders where oxidative stress plays a key role.

B2 Introduction

Nuclear factor kappa B (NF-κB) is a key transcription factor that was earlier known to regulate the immune and inflammatory processes and viral replication (Baeuerle and Henkel, 1994; Baldwin, 1996). The NF-κB family of transcription factors plays key roles in the regulation of cell growth, activation, differentiation, and survival (Santoro et al., 2003). In addition, an increasing amount of evidence suggests that NF-κB plays an important role in synaptic plasticity and long-term memory formation (Albensi and Mattson, 2000; Mattson et al., 2000). The activation of NF-κB is responsible for transcription of various genes like: TNFα, interleukins, such as IL1, IL2, and IL6; chemokines, adhesion molecules, such as ICAM-1 VCAM, and E-selectin; enzymes such as iNOS, COX-2 and proliferation-related proteins such as Cyclin D1 among many others (Karin et al., 2002; Ferrucci et al., 2004; Chung et al., 2005). The dysregulation of NF-κB activity has been shown to play a critical role in many inflammatory diseases and in various neurodegenerative disorders such as Alzheimer's and Parkinson's disease (Hunot et al., 1997; Kaltschmidt et al., 1997; Yoshidome et al., 1999).

NF-κB transcription factor represents a group of both homodimeric and heterodimeric complexes. In mammals, this family of NF-κB consists of several proteins, which include NF-*κ*B1 (p50), NF-*κ*B2 (p52), RelA (p65), c-Rel (Rel), and RelB (Hayden and Ghosh, 2004). A hallmark of this family of proteins is that they contain a highly conserved domain of ~300 amino acids, termed the *Rel* homology domain, which contains sequences important for dimerization, DNA binding, and nuclear localization (Grilli et al., 1993; Hayden and Ghosh, 2004). The classical and well studied NF-*κ*B

heterodimer, which is also a potent activator of gene expression, is composed of p50 and p65 subunits. Unlike p65, p50 and p52 subunits lack transactivation domains and are produced either by the proteolytic processing of the precursor molecules p105 and p100 in the presence of ATP (Ghosh et al., 1998; Sun and Andersson, 2002) or as has been suggested, cotranslationally from incompletely synthesized molecules by the proteosome (Moorthy et al., 2006).

NF-κB is expressed in many cell types in the nervous system and is constitutively active in subsets of cells in the cortex and hippocampus of the rodent brain at comparably low levels (Kaltschmidt et al., 1994; O'Neill and Kaltschmidt, 1997). In resting cells, the classical NF-κB heterodimer p65/p50 is complexed with the inhibitor protein IκB and sequestered as an inactive complex in the cytoplasm. Activation or stimulation of NF-κB in the cells leads to the phosphorylation and immediate proteosomal-mediated degradation of IκB. This leads to the nuclear translocation of NF-κB which then binds to the regulatory region and transcription of various responsive genes (May and Ghosh, 1998; Santoro et al., 2003; O'Donnell et al., 2005). NF-κB can be activated in both transcriptional activating and repressing forms. In systems in which NF-κB is activated during apoptosis, NF-κB can either prevent or potentiate cell death signaling (Beg and Baltimore, 1996; May and Ghosh, 1998). For example, a pro-apoptotic role for NF-κB in ischemia or glutamate-related damage has been reported (Grilli et al., 1996), while on the other hand, there is evidence from cell culture models that NF-κB may counteract cell death in neurons induced by β-amyloid (Mattson et al., 1997; Lezoualc'h et al., 1998). Hence, depending on the experimental settings NF-κB and can either be pro- or anti-apoptotic. This dual role of NF-κB has indeed presented considerable complexities and

difficulties in studies that try to examine the mechanisms of the action of this transcription factor. In addition, the wide range of stimuli capable of activating NF-κB, and also the large number of genes it regulates, differing results have been reported on the roles of specific subunits of NF-κB. As a result the precise role of NF-κB and its related mechanistic pathways have been difficult to study and have not been completely established.

Although classical NF-κB is a heterodimer composed of the p50 and p65 subunits, little is known about gene regulation involving other hetero- and homodimeric forms of NF-κB which contain trans-activation domains and can act as activators or inhibitors of transcription (Gadjeva et al., 2004). In an effort to determine the various roles of the subunits that mediate the observed regulation of NF-κB activity, various mice with certain subunits knocked out have been used. In particular, since the p65/p50 heterodimeric form is the most predominant and the indispensable component of NF-κB, knockouts of these two subunits have been developed (Flohe et al., 1997). However, p65 knockouts have been found to have defects during development and they die prematurely of liver apoptosis (Beg et al., 1995; Hayden and Ghosh, 2004), hence not many studies of p65 knockouts have been carried out. On the other hand, although p50 deficient mice have been shown to display functional defects in immune response, they develop normally (Beg et al., 1995), and also, since neurons from animals genetically ablated for p50 survive well in culture and are no more sensitive or resistant to neuronal death than wild type neurons (Aleyasin et al., 2004), they provide the best avenue to study various mechanisms of NF-κB-related activity. Of the two subunits, less is known regarding the precise role of p50 in NF-κB-mediated inhibition of apoptosis, and at present, the

importance of the NF-κB signaling pathway is still in question especially since various studies of p50 knockout mice carried out so far have presented contradictory findings. For example, in one study, the absence of the p50 subunit of NF-κB resulted in a significant reduction in infarct size in parts of the vascular territory of the middle cerebral artery (Kawano et al., 2006). Also, in an initial report of mice with targeted disruption of the NF-κB1 subunit, p50 (p50 −/− mice), it was discovered that while these mice are more susceptible to certain bacterial pathogens, they are resistant to murine encephalomyocarditis virus (EMCV) infections causing myocarditis and dilated cardiomyopathy that kills normal healthy mice (Schwarz et al., 1998). On the other hand, it has been shown that mice depleted of the p50 subunit have been more susceptible to neuronal injury when exposed to a wide range of toxins and stimulants (Yu et al., 1999; Pennypacker et al., 2001; Kassed et al., 2002).

Considering the above, the present study involved further analysis of the p50 subunit of NF-κB. Since most studies on p50 knockout mice have so far been carried out using a wide range of stimuli (Weih et al., 1997; Iimuro et al., 1998; Pennypacker et al., 2001; Mabley et al., 2002; Gadjeva et al., 2004; Kassed and Herkenham, 2004), the present study provided a unique opportunity for investigating the role of p50 subunit in a stimulus-free experimental environment hopefully providiing us with additional evidence on the mechanism of NF-κB activation. We hypothesized that the partial knock out of the p50 (-/+) subunit could potentially be protective in a stimulus-free environment through an oxidative stress mechanism. To test our hypothesis, we measured the levels of oxidative stress biomarkers in the brain of WT and p50 (-/+) mice and observed a significant reduction in the levels of lipid peroxidation as measured by HNE and the

levels of protein oxidation as measured by the levels of 3NT and protein carbonyls in the p50 (-/+) mice compared to the WT. We further carried out a differential expression proteomic analysis on the p50 (-/+) mice. Proteomics has recently been successfully used in our laboratory to identify various proteins that have undergone modifications in various disease states and models thereof (Butterfield, 2004; Butterfield et al., 2006a; Butterfield et al., 2006c; Sultana et al., 2006b). As a result, using proteomics we identified five proteins to be significantly increased in expression in the p50 (-/+) mice when compared to the WT. Therefore, the lack of the p50 subunit makes the brain of mice less susceptible to oxidative stress and also activates the expression of key proteins involved in energy metabolism and antioxidant activity. Hence, this study has provided insights into the role of NF-κB and especially the role of the p50 subunit of NF-κB.

B3 Experimental procedures

Animals and experimental protocols

Male wild type and NF-κB p50 -/+ mice were received from Jackson Laboratory at 4-6 weeks and were sacrificed at 7-9 weeks. They were housed in a temperature and humidity controlled room with a 12:12 h light: dark cycle, with food and water available *ad libitum*. All procedures and protocols were approved by the Institutional Animal Care and Use Committee. These were performed in Dr Charles Rammasamy's laboratory at INRS-Institut Armand-Frappier, and INAF, University Laval, Québec, Canada.

Measurement of protein carbonyls

Protein carbonyls are an index of protein oxidation and were determined as described previously (Butterfield and Stadtman, 1997). Briefly, samples (5 µg of protein) were derivatized with 10 mM 2, 4-dinitrophenylhydrazine (DNPH) in the presence of 5 µL of 12% sodium dodecyl sulfate for 20 min at room temperature (23°C). The samples were then neutralized with 7.5 µL of the neutralization solution (2 M Tris in 30% glycerol). Derivatized protein samples were then blotted onto a nitrocellulose membrane with a slot-blot apparatus (250 ng per lane). The membrane was then washed with wash buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20) and blocked by incubation in the presence of 5% bovine serum albumin, followed by incubation with rabbit polyclonal anti-DNPH antibody (1: 100 dilution) as the primary antibody for 1 h. The membranes were washed with wash buffer and further incubated with alkaline phosphatase-conjugated goat anti-rabbit antibody as the secondary antibody for 1 h. Blots were developed using fast tablet (BCIP/NBT; Sigma-Aldrich) and quantified using Scion

Image (PC version of Macintosh-compatible NIH Image) software. No non-specific background binding of the primary or secondary antibodies was found.

Measurement of 3-nitrotyrosine (3-NT)

Nitration of proteins is another form of protein oxidation (Castegna et al., 2003). The nitrotyrosine content was determined immunochemically as previously described (Drake et al., 2003b). Briefly, samples were incubated with Laemmli sample buffer in a 1:2 ratio (0.125 M Trizma base, pH 6.8, 4% sodium dodecyl sulfate, 20% glycerol) for 20 min. Protein (250 ng) was then blotted onto the nitrocellulose paper using the slot-blot apparatus and immunochemical methods as described above for protein carbonyls. The mouse anti-nitrotyrosine antibody (5: 1000 dilutions) was used as the primary antibody and alkaline phosphatase-conjugated anti-mouse secondary antibody was used for detection. Blots were then scanned using scion imaging and densitometric analysis of bands in images of the blots was used to calculate levels of 3-NT. No non-specific binding of the primary or secondary antibodies was found.

Measurement of 4-hydoxynonenal (HNE)

HNE is a marker of lipid oxidation and the assay was performed as previously described (Lauderback et al., 2001). Briefly, 10 µl of sample were incubated with 10 µl of Laemmli buffer containing 0.125 M Tris base pH 6.8, 4 % (v/v) SDS, and 20% (v/v) Glycerol. The resulting sample (250 ng) was loaded per well in the slot blot apparatus containing a nitrocellulose membrane under vacuum pressure. The membrane was blocked with 3% (w/v) bovine serum albumin (BSA) in phosphate buffered saline

containing 0.01% (w/v) sodium azide and 0.2% (v/v) Tween 20 (PBST) for 1 h and incubated with a 1:5000 dilution of anti-4-hydroxynonenal (HNE) polyclonal antibody in PBST for 90 min. Following completion of the primary antibody incubation, the membranes were washed three times in PBST. An anti-rabbit IgG alkaline phosphatase secondary antibody was diluted 1:8000 in PBST and added to the membrane. The membrane was washed in PBST three times and developed using Sigmafast Tablets (BCIP/NBT substrate). Blots were dried, scanned with Adobe Photoshop, and quantified by Scion Image. A small background of the primary antibody binding to the membrane was found, but this was the same in both control and subject blots.

Two-dimensional electrophoresis

Brain samples (200 μg) were incubated with 4 volumes of 2N HCl at room for electrophoresis. Proteins were then precipitated by the addition of ice-cold 100% trichloroacetic acid (TCA) to obtain a final concentration of 15% TCA. Samples were then placed on ice for 10 min and precipitate centrifuged at 16,000 g for 3min. The resulting pellet was then washed three times with a 1:1(v/v) ethanol/ethyl acetate solution. The samples were then suspended in 200 μl of rehydration buffer composed of a 1:1 ratio (v/v) of the Zwittergent solubilization buffer (7M urea, 2M thiourea, 2% Chaps, 65 mM DTT, 1% Zwittergent 0.8% 3-10 ampholytes and bromophenol blue) and ASB-14 solubilization buffer (7M urea, 2M thiourea 5Mn TCEP, 1% (w/v) ASB-14, 1% (v/v) Triton X-100, 0.5% Chaps, 0.5% 3-10 ampholytes) for 1 h.

First dimension electrophoresis

For the first-dimension electrophoresis, 200 µL of sample solution was applied to a 110-mm pH 3–10 ReadyStrip™ IPG strips (Bio-Rad, Hercules CA). The strips were then actively rehydrated in the protean IEF cell (Bio-Rad) at 50 V for 18 h. The isoelectric focusing was performed in increasing voltages as follows; 300 V for 1 h, then linear gradient to 8000 V for 5 h and finally 20 000 V/h. Strips were then stored at –80 °C until the 2nd dimension electrophoresis was to be performed.

Second dimension electrophoresis

For the second dimension, the IPG® Strips, pH 3–10, were equilibrated for 10 min in 50 mM Tris–HCl (pH 6.8) containing 6 M urea, 1% (w/v) sodium dodecyl sulfate (SDS), 30% (v/v) glycerol, and 0.5% dithiothreitol, and then re-equilibrated for 15 min in the same buffer containing 4.5% iodoacetamide instead of dithiothreitol. Linear gradient precast criterion Tris–HCl gels (8–16%) (Bio-Rad) were used to perform second dimension electrophoresis. Precision Protein™ Standards (Bio-Rad, CA) were run along with the sample at 200 V for 65 min.

SYPRO ruby staining

After the second dimension electrophoresis, the gels were incubated in fixing solution (7% acetic acid, 10% methanol) for 20 min and stained overnight at room temperature with 50ml SYPRO Ruby gel stain (Bio-Rad). The SYPRO ruby gel stain was then removed and gels stored in DI water.

Image analysis

SYPRO ruby-stained gel images were obtained using a STORM phosphoimager (Ex. 470 nm, Em. 618 nm, Molecular Dynamics, Sunnyvale, CA, USA) and also saved in TIFF format. PD-Quest (Bio-Rad) imaging software was then used to match and analyze visualized protein spots among differential 2D gels with one gel for each individual sample.

In-gel trypsin digestion

Protein spots statistically different that controls were digested in-gel by trypsin using protocols previously described and modified by (Thongboonkerd et al., 2002). Briefly, spots of interest were excised using a clean blade and placed in Eppendorf tubes, which were then washed with 0.1 M ammonium bicarbonate (NH_4HCO_3) at room temperature for 15 min. Acetonitrile was then added to the gel pieces and incubated at room temperature for 15 min. This solvent mixture was then removed and gel pieces dried. The protein spots were then incubated with 20 μL of 20 mM DTT in 0.1 M NH_4HCO_3 at 56 °C for 45 min. The DTT solution was removed and replaced with 20 μL of 55 mM iodoacetamide in 0.1 M NH_4HCO_3. The solution was then incubated at room temperature for 30 min. The iodoacetamide was removed and replaced with 0.2 mL of 50 mM NH_4HCO_3 and incubated at room temperature for 15 min. Acetonitrile (200 μL) was added. After 15 min incubation, the solvent was removed, and the gel spots were dried in a flow hood for 30 min. The gel pieces were rehydrated with 20 ng/μL-modified trypsin (Promega, Madison, WI) in 50 mM NH_4HCO_3 with the minimal volume enough

to cover the gel pieces. The gel pieces were incubated overnight at 37 °C in a shaking incubator.

Mass spectrometry

A MALDI-TOF mass spectrometer in the reflectron mode was used to generate peptide mass fingerprints. Peptides resulting from in-gel digestion with trypsin were analyzed on a 384 position, 600 μm AnchorChip^{TM} Target (Bruker Daltonics, Bremen, Germany) and prepared according to AnchorChip recommendations (AnchorChip Technology, Rev. 2, Bruker Daltonics, Bremen, Germany). Briefly, 1 μL of digestate was mixed with 1 μL of alpha-cyano-4-hydroxycinnamic acid (0.3 mg/mL in ethanol: acetone, 2:1 ratio) directly on the target and allowed to dry at room temperature. The sample spot was washed with 1 μL of a 1% TFA solution for approximately 60 seconds. The TFA droplet was gently blown off the sample spot with compressed air. The resulting diffuse sample spot was recrystallized (refocused) using 1 μL of a solution of ethanol: acetone: 0. 1 % TFA (6:3:1 ratio). Reported spectra are a summation of 100 laser shots. External calibration of the mass axis was used for acquisition and internal calibration using either trypsin autolysis ions or matrix clusters and was applied post acquisition for accurate mass determination.

Analysis of peptide sequences

Peptide mass fingerprinting was used to identify proteins from tryptic peptide fragments by utilizing the MASCOT search engine based on the entire NCBI and SwissProt protein databases. Database searches were conducted allowing for up to one

missed trypsin cleavage and using the assumption that the peptides were monoisotopic, oxidized at methionine residues, and carbamidomethylated at cysteine residues. Mass tolerance of 150 ppm, 0.1 Da peptide tolerance and 0.2 Da fragmentation tolerance was the window of error allowed for matching the peptide mass values (Butterfield and Castegna, 2003b). Probability-based MOWSE scores were estimated by comparison of search results against estimated random match population and were reported as -10*log10 (p), where p is the probability that the identification of the protein is a random event. MOWSE scores greater than 63 were considered to be significant ($p < 0.05$). All protein identifications were in the expected size and isoelectric point (pI) range based on the position in the gel.

Statistical analysis

Statistical analysis of differentially expressed protein levels matched with spots on 2D-gels from frontal cortex brain samples of the p50 (-/+) and wild type (WT) were used and carried out using Student's *t*-tests. Here we report a comparative proteomics analysis of p50 (-/+) vs. WT. A value of $p < 0.05$ was considered statistically significant. Only proteins that are considered significantly different by Student's *t*-test were subjected to in-gel trypsin digestion and subsequent proteomic analysis. This is the normal procedure for proteomics studies, since sophisticated statistical analysis used for micro array studies are not applicable for proteomics studies (Maurer et al., 2005).

Protein Interacteome

The functional protein interacteome was obtained by using Interaction Explorer ™ Software Pathway Assist software package (Stratagene, La Jolla, CA). Pathway Assist is software for functional interaction analysis. It allows for the identification and

visualization of pathways, gene regulation networks and protein interaction maps (Donninger et al., 2004). The proteins are first imported as the gene symbols as a set of data. This data set is then searched against ResNet, a database containing over 500,000 biological interactions built by applying the MedScan text-mining algorithms to all PubMed abstracts. These interactions are then visualized by building interaction networks with shortest-path algorithms. This process can graphically identify all known interaction among the proteins. The information of the function of these proteins and their relevance to diseases are then obtained by using the BIOBASE's Proteome BioKnowledge Library form Incyte Corporation (Incyte, Wilmington, DE) (Hodges et al., 2002).

B4 Results

Decreased levels of oxidative stressing p50 (-/+) mice and compared to WT

The levels of protein carbonyls, 3NT and HNE as indicators of oxidative stress were measured in the frontal cortex of p50 (-/+) mice and compared to WT. Fig.B1 shows total protein oxidation measured by the accumulation of protein carbonyls and 3NT and also the levels of lipid peroxidation as measured by protein-bound HNE. There was a significant decrease in the levels of all oxidative stress parameter measured in the p50 (-/+) mice compared to WT.

Increased protein expression levels in p50 (-/+) mice compared to the wild type

Two-dimensional electrophoresis offers an excellent tool for the screening of abundant protein changes in various disease states (Butterfield, 2004). To assess whether there were any changes in the proteomic profile in the brain of p50 (-/+) mice in the present study, we investigated the pattern of protein expression in the frontal cortex from the p50 (-/+) mice compared to the wild type. Comparing the densitometric intensities of individual spots on the gels, five proteins were expressed at significantly higher levels in the frontal cortex of p50 (-/+) mice compared to the wild type. Fig A2 shows SYPRO ruby stained 2D gels of the p50 (-/+) mice (A) vs. wild type (B) groups, with identified protein boxed and labeled. The brain proteins identified with increased expression in the p50 (-/+) mice were: ATP synthase gamma chain, Ubiquinol-cyt-C reductase, Heat shock protein (Hsp10), Fructose Bisphosphate aldolase C, and NADH-ubiquinone oxidoreductase. These proteins identified by mass spectrometry are shown in Table A1. Table A2 provides the changes in protein levels expressed as arbitrary units (A.U) ±

S.E.M. Table A1 shows that the mowse scores obtained are all highly significant and that the probability of a random identification using proteomics is exceedingly small in this study.

Protein interacteome

Fig B3 shows the protein interacteome of proteomics-identified proteins differentially expressed in the frontal cortex of p50 (-/+) mice. The Interaction Explorer PathwayAssist (Stratagene) software shows that the proteins identified in this study are related to regulation of signal transduction, energy metabolism, and chaperone activity among others. As a result, the present findings suggest that the partial knock out p50 subunit of NF-κB leads to the expression of proteins that are involved in various pathways that provide normal development and protection against oxidative stress.

Figure B1

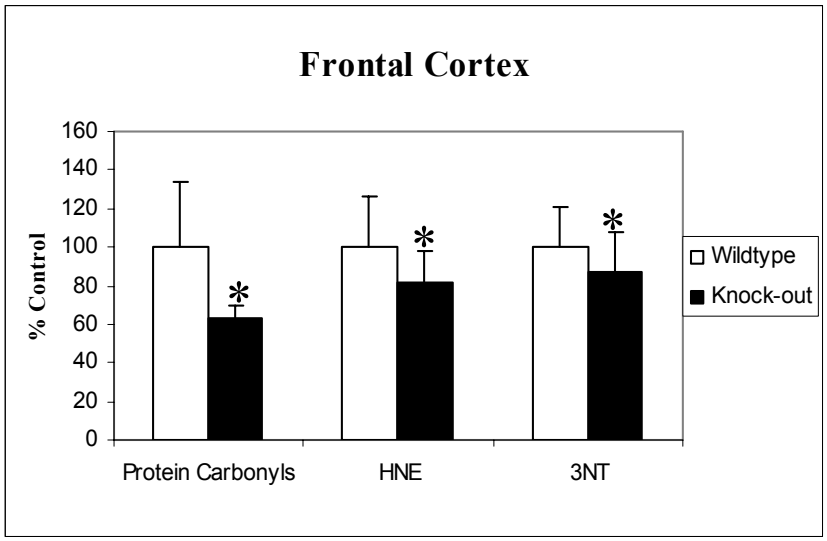
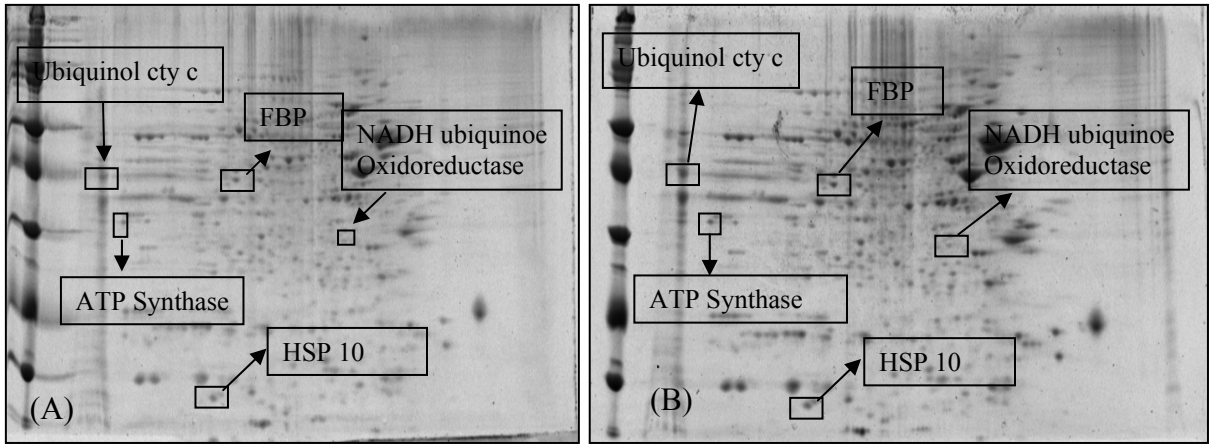


Figure B1 Shows protein carbonyl, 3NT and HNE levels in the frontal cortex of p50 (-/+) compared to wild type. There was a significant reduction in the levels of protein carbonyls, 3NT and HNE in the p50 (-/+) mice compared to the wild type. Data are represented as % control; error bars indicate the SEM for each group measured (* $p<0.05$ n=5).

Figure B2



WT KO

Figure B2 Shows SYPRO Ruby-stained 2D-gels maps of frontal cortex brain samples from Wild-type (A) vs. p50 (-/+) mice (B). Proteins identified by mass spectrometry showing differential expression are presented as the boxed spots.

Table B1. Differentially expressed protein levels from the frontal cortex of NF-κβ p50 $^{-/+}$ Heterozygous Knockout Mice

Identified Protein	GI accession number	Number of peptide matches identified	Percent Coverage of matched peptides	pI, MrW	Mowse score	Probability of a random identification
ATP Synthase gamma chain	gi|21263432	8	20	9.06,32900	61	8.0×10^{-7}
Ubiquinol-cyt-C reductase core protein 2	gi|14548302	8	23	9.26,48200	77	2.0×10^{-8}
Heat shock protein, mitochondrial (Hsp10)	gi|2493662	14	43	8.18,10800	74	4.0×10^{-8}
Fructose Bisphosphate aldolase C	gi|32470593	9	22	6.79,39600	162	6.3×10^{-17}
NADH-ubiquinone oxidoreductase	gi|23396786|	8	28	6.40,30300	102	6.3×10^{-11}

Table B2. Proteomic characterizations of differentially expressed NF-κB knockout mice brain proteins identified

Identified Protein	WT (A.U. ± S.E.M)	KO (A.U. ± .E.M)	Fold Change	P-Value
ATP Synthase	959 ± 160	1367 ± 88	1.4	0.05
Ubiquinol cyt C reductase	3514 ± 1482	8008 ± 1393	2.3	0.01
HSP 10	1341 ± 542	2671 ± 155	1.9	0.04
FBP	1344.± 84	1609 ± 35	1.2	0.01
NADH-Ubiquinone oxidoreductase	346 ± 137	781 ± 133	2.3	0.05

Figure B3

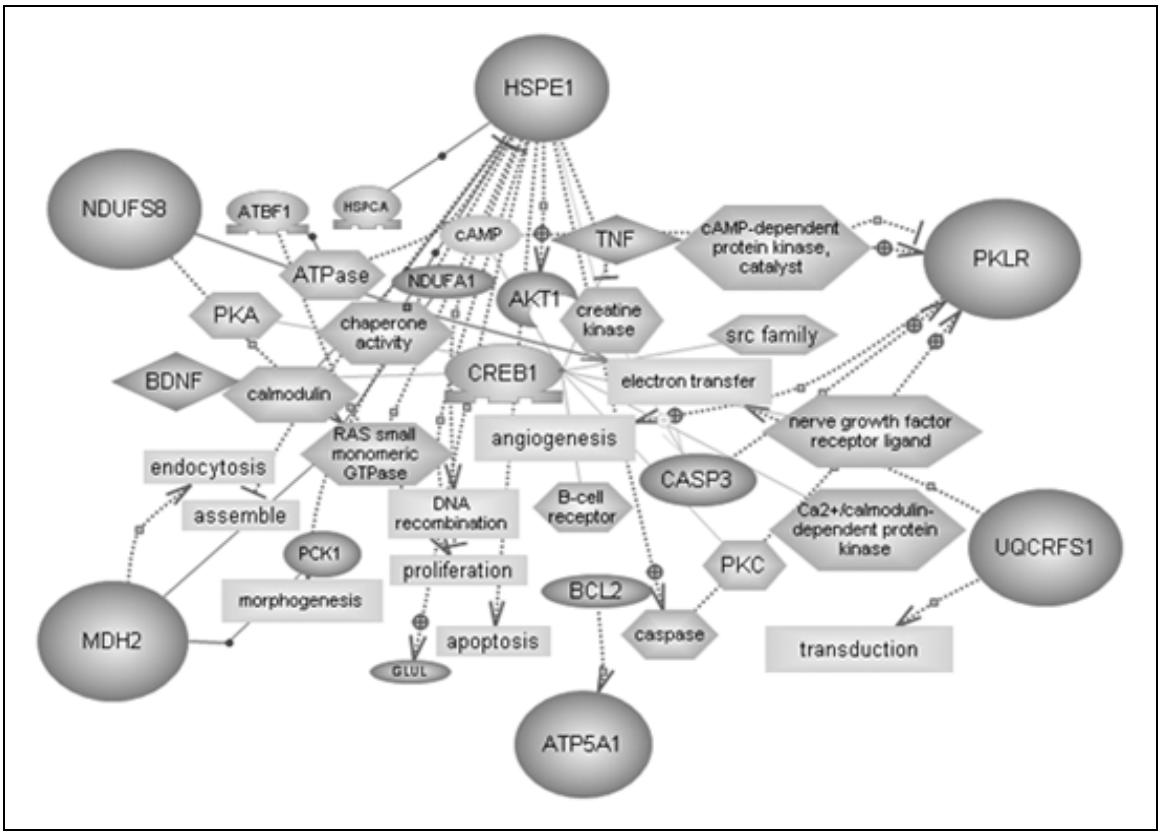


Figure B3 Schematic diagram of a functional interacteome of all proteins identified to be differentially expressed in the brains of p50 (-/+) compared to wild type. This diagram was generated by the interaction explorer ™ Pathway Module (Stratagene), indicating that all the proteins are directly or indirectly associated with cellular process shown.

B5 Discussion

In the present study we have found that the partial knock out of the p50 subunit and a stimulus-free experimental set-up, lead to a reduction in the levels of oxidative damage in the p50 (-/+) mice brain when compared to that of the wild type mouse. We observed a significant reduction in the levels of oxidative stress biomarkers, i.e., protein carbonyls, 3NT and lipid peroxidation product HNE. In addition, we carried out a proteomic profile analysis of the frontal cortex of the p50 (-/+) mice brain and compared it to the wild type. We found that there was a significant increase in the expression of proteins related to energy metabolism and antioxidant activity in the p50 (-/+) mice brain compared to the wild type. These results present an intriguing finding and are discussed here with relevance to the mechanism of NF-κB transcription factor, and the role of the p50 subunit in oxidative stress.

NF-κB has been known to transcribe a wide range of genes with different and sometimes opposite functions. NF-κB is involved in transcribing both pro-apoptotic genes such as p53, Fas and FasL (Matsui et al., 1998; Kirch et al., 1999) and anti-apoptotic genes such as, Bcl-2, *Bcl-x* and *SOD2* (Lipton, 1997; Mattson et al., 1997; Tamatani et al., 1999). NF-κB activity is regulated by various factors, such as stress and injury related stimuli, cell type, and organ type among others. The wide range of stress- and injury-related stimuli regulated by NF-κB present investigators with extreme difficulties in trying to elucidate mechanistic pathways of this transcription factor and has led to the generation of numerous differing findings. An understanding of its dual action might therefore be possible if we consider its various subunits and their role in regulating its activation. As mentioned earlier, NF-κB consists of several proteins, which include

NF-κB1 (p50), NF-κB2 (p52), RelA (p65), c-Rel (Rel), with the heterodimer p50/p65 being the most predominant (Grilli et al., 1993; Ghosh et al., 1998; Santoro et al., 2003). Usually located in the cytosol, the presence of a stimulus triggers the translocation to the nucleus where it binds to promoter region of specific genes activating its transcription (Grilli et al., 1993).

The role of its various subunits and in particular p50 in oxidative stress related mechanisms is still at question. Though the p50 subunit generated from precursor molecule p105 lacks a transactivation domain, its disruption in various experimental settings has generated a number of interesting findings. For example, in a model of Huntington's disease p50 (-/-) mice have been shown to have increased oxidative stress, calcium dysregulation and increased damage to striatal neurons following treatment with mitochondrial toxin 3-nitropropionic acid (3NP) (Yu et al., 1999). It has also been shown that lack of p50 negatively modulates learning ability and hippocampal response to brain injury after chemical-induced lesion (Kassed et al., 2002). In addition, a higher constitutive expression of cyclooxygenase-2 (COX-2) in p50-/- mice following an acute inhalation of pulmonary irritant ozone has been observed (Mabley et al., 2002; Fakhrzadeh et al., 2004). It has also been reported that p50 is increased in neurons surviving hippocampal injury after middle cerebral artery occlusion (Pennypacker et al., 2001) and that overexpression of p50 inhibits TNF- α gene expression following lipopolysaccharide (LPS) on p50-/- splenocytes (Gadjeva et al., 2004). On the other hand, it has been shown that NF-κB p50-deficient mice show; reduced anxiety-like behaviors in tests involving exploratory drive and anxiety (Kassed and Herkenham, 2004); reduced ischemic damage (Schneider et al., 1999); resistance to murine

enecephalomyocarditis virus (EMCV) infections that cause myocarditis and dilated cardiomyopathy which kill normal healthy mice (Schwarz et al., 1998); and reduced ventricular rupture and improved cardiac function and survival after myocardial infarction (MI) (Kawano et al., 2005). In addition to the above, a study by O'Donnell et al., 2005 showed that following reovirus infection in the CNS, NF-κB p50 (+/+) mice exhibited significant neuronal apoptosis, while p50 (-/-) mice displayed a minimal apoptotic response. On the other hand, reovirus infection in the heart induced minimal apoptosis in the NF-κB $p50^{+/+}$ mice, while extensive apoptosis occurred in the heart of $p50^{-/-}$ mice (O'Donnell et al., 2005). This study provided two distinct roles for the NF-κB p50 subunit in the brain and heart, continuing to confirm the organ and cell type specificity of the NF-κB activity. The studies mentioned above provide two differing effects resulting from the disruption of the p50 subunit of NF-κB, hence, presenting researches with a difficult task of trying to conclusively elucidate the exact mechanisms of this key transcription factor (Lipton, 1997).

It has been shown that NF-κB is redox regulated and that oxidative stress is an activator of NF-κB and a known regulator of gene transcription since most agents that that activate NF-κB, trigger the formation of reactive oxygen species (ROS) or are oxidants themselves, e.g. superoxide, hydrogen peroxide, hydroxyl radicals and peroxynitrite (Laskin and Pendino, 1995; Kim et al., 2000b; Chung et al., 2005). In addition, NF-κB activation can also be triggered by ROS in the absence of any stimulus, as is the case in the current study. The upstream processes for the activation of NF-κB have also been shown to involve a series of serine/threonine kinases and phosphatases that are regulated by the redox status of the cell, further emphasizing the interaction of

NF-κB and oxidative stress (Flohe et al., 1997). In the present study, we observed a significant decrease in the levels of oxidative stress as indexed by oxidative stress biomarkers, i.e., protein carbonyls, 3NT and lipid peroxidation product HNE in the brains of partially knocked out p50 (-/+) mice compared to those of the WT. It has previously been shown that 4-hydroxyhexenal (HHE), a reactive aldehdye similar to HNE, activates NF-κB which further activates p38, MAPK and extracellular signal regulated kinase (ERK) leading to various signaling cascades (Je et al., 2004). As a result, the reduced levels of HNE in the p50 (-/+) mice observed in the present study could thus inhibit the activation of NF-κB leading to a possible protective mechanism.

The partial knock out of p50, i.e., p50 (-/+), generates a dysfunctional subunit, which as a result could lead to a possible compensatory mechanism from the other heterodimer member of the classical NF-κB transcription factor, p65. Since p65 has a transactivation domain and is responsible for the transcriptional activation of target genes, its compensatory role would therefore bring about a more functional NF-κB transcription factor and enhance activity (Kato et al., 2002). This proposed compensatory mechanism phenomenon has previously been documented in cytokine knockout mice where the redundancy of the cytokine cascade compensates for the deletion of a single cytokine gene (Kato et al., 2002). Various studies of p50 knockouts also continue to put forth the possible compensational role of p65. In a study of partial hepatectomy in p50 (-/-) mice, there was an increased expression of the p65 protein in the nucleus which was believed to provide compensation for the absence of p50 leading to normal liver regeneration and repair following liver injury (Iimuro et al., 1998; Kato et al., 2002). It has also been shown that gene deletion of p50 does not alter hepatic inflammatory

response since the increased nuclei level of p65 protein in the p50 (-/-) mice compared to the p50 (+/+) mice was believed to compensate for sufficient transcriptional activation of relevant genes (Beg et al., 1995). It is also suggested that both p50 and p65 may have critical inhibitory functions and that the inhibitory functions of p65 are more clearly demonstrated in the absence of p50 (Gadjeva et al., 2004). In the same fashion, we hypothesis that the partial knock out of p50 leads to a dysfunctional heterodimer hence necessitating a compensatory response from p65 which then leads to the transcriptional of antioxidant genes and hence a reduction in the levels of oxidative stress.

Other than the compensatory response mechanisms suggested for the reduction in oxidative stress levels in the p50 (-/+) mice, it is also possible that other pathways could be involved in activating the NF-κB leading to the observed reduction in the levels of oxidative stress observed in the present study. One potential pathway could be the involvement of nuclear factor-erythroid 2–related factor 2 (Nrf2). Just like NF-κB, Nrf2 is also involved in the innate immune response and survival. In particular, Nrf2 regulates the basal and inducible expression of various antioxidant and other cytoprotective genes by binding to the antioxidant response element of DNA in conditions of oxidative stress (Motohashi and Yamamoto, 2004). It has been reported that there could be a possible cross-talk among redox-sensitive transcription factors NF-κB and Nrf2, in that there is dependency of key family members of AP-1 and NF-κB on Nrf2 (Yang et al., 2005). Thus, Nrf2 regulates NF-κB activation largely by modulating its upstream signaling components (Thimmulappa et al., 2006). Under normal conditions, there are low nuclear levels of Nrf2; under stress conditions there is an increase in the nuclear accumulation of Nrf2 resulting in its binding to the antioxidant response element leading to enhanced

transcriptional activation of antioxidant target genes (Thimmulappa et al., 2006). A broad range of antioxidants has been known to inhibit the activation of NF-κB (Flohe et al., 1997); however, oxidative stress induced activation of NF-κB leads to the up-regulation of antioxidant enzymes such as glutathione peroxidase and catalase among others (Messina et al., 2006). For the present study we believe that with the compensatory effect of p65, NF-κB could be activated and in turn it would activate Nrf2 leading to the up-regulation of antioxidant enzymes resulting into the reduction in the level of oxidative stress observed. Downstream effects of Nrf2 and NF-κB activity in the p50 (-/+) mice can be observed in the present study from the proteomics results obtained. In addition to a reduction in the levels oxidative damage in the p50 (-/+) mice in the current study, we also observed a significant increase in the expression of key proteins related to energy metabolism and cytoprotective activities. These proteins identified and their possible roles in survival are discussed as follows;

Heat shock proteins (HSP) are a family of molecules that are highly conserved during evolution and involved in many cellular functions, such as protein folding. Some examples include HSP10, -27, -60, -70, -72 and -90. Mammalian heat shock protein 10 (Hsp10) also known as chaperonin 10 is a mitochondrial protein involved in protein folding. It has been shown that HSP 10 forms a heptameric lid, which binds to a double-ring toroidal structure that is composed of seven Hsp subunit rings (Xu et al., 1997). Increased expression of HSPs has recently been found to be associated with a number of neurodegenerative disorders, immunomodulatory activities among others (Flohe et al., 2003; Poon et al., 2004b). It is believed that HSPs are exported to the plasma membrane and are released from apoptotic cells as a source of signaling to the innate and adaptive

immune system (Beg, 2002; Flohe et al., 2003). HSP10 and another chaperone HSP60 interact in a two-step folding mechanism in the mitochondria of prokaryotic and eukaryotic cells (Beg, 2002) and it is hypothesized that HSP60 and HSP10 could possibly be a new diagnostic and prognostic tools for certain cancers (Triantafilou and Triantafilou, 2004). In the present study, we have shown that there is a significant increase in the expression of HSP10 in the p50 (-/+) mice compared to the wild type. The increased expression could mean enhanced activity leading to proper folding of proteins and more protection against oxidative stress and this could possibly contribute to the significant reduction of oxidative stress observed in these p50 (-/+) mice.

The *NADH: ubiquinone oxidoreductase* also known as Complex I, couples the oxidation of NADH and the reduction of ubiquinone, to the generation of a proton gradient which is then used for generation of ATP in the mitochondria (Weiss et al., 1991). This complex provides the input to the electron transport chain from the NAD-linked dehydrogenases of the citric acid cycle. Dysregulation of this complex has been associated with various neurodegenerative disorders such as AD among others (Robinson, 1998; Loeffen et al., 2000). In the current study, we observed a significant increase in the expression of NADH: ubiquinone oxidoreductase in the p50 (-/+) mice compared to the wild type. The significance of this could imply increased activity and also provide an efficient mechanism for the generation of proton gradient that eventually leads to an increase in the production of ATP.

Fructose Bisphosphate aldolase C (FBP) is a glycolytic enzyme that catalyses the reversible aldol cleavage or condensation of fructose-1, 6-bisphosphate into

dihydroxyacetone-phosphate and glyceraldehyde 3-phosphate (Perham, 1990). In vertebrates, three forms of this enzyme are found: aldolase A is expressed in muscle, aldolase B in liver, kidney, stomach and intestine, and aldolase C in brain, heart and ovary. The different isozymes have different catalytic functions: aldolases A and C are mainly involved in glycolysis, while aldolase B is involved in both glycolysis and gluconeogenesis (Perham, 1990). In the current study we observed a significant increase in the expression of FBP in the p50 (-/+) mice compared to the wild type that would lead to improved glycolytic function and enhanced energy metabolism.

ATP synthase (Complex V) is a mitochondrial enzyme that utilizes the electrochemical proton gradient established across the inner mitochondrial membrane by the ETC for synthesis of ATP (Leyva et al., 2003). ATP synthase deficiency has been associated with an increase in oxidative stress (Basso et al., 2004). Our laboratory has shown that ATP synthase is oxidatively modified and is inactivated in the gracile axonal dystrophy (*gad*) mouse brain (Castegna et al., 2004). In the present study, ATP synthase γ chain expression was significantly increased in the p50 (-/+) mice compared to the wild type. The increased expression of this enzyme could presumably result in increased activity, hence enhancing the crucial cellular process of ATP generation.

Ubiquinol-cytochrome-C reductase also known as the bc_1 complex or complex III is the central redox enzyme in the ETC. This complex catalyzes the oxidation of diverse quinols by high potential redox-carriers, in the process generating a proton gradient that is utilized for the generation of ATP (Mulkidjanian, 2005). In the current study the expression of the Ubiquinol-cytochrome-C reductase core protein 2 also known as complex III subunit II was found to be significantly increased. This is a component of the

Ubiquinol-cytochrome c reductase complex and the core protein 2 is required for the assembly of the Complex III. As a result, the increase in expression of three key proteins of the ETC, a glycolytic enzyme and a chaperone protein in the present study is very significant, in that this ensures not only sufficient generation of adequate ATP that would lead to improved cellular activities and hence survival in the p50 (-/+) mice, but also the proper folding of proteins.

The present study and those discussed in this dissertation research underscore the complexity of the mechanism through which NF-κB is activated or repressed. It has been suggest that the ultimate biological effect of NF-κB activation will be dependent on a number of reasons; these include the stimulus responsible for this activation or repression, the subunit composition of the NF-κB, gene targets, the cell type and organ among many others (Culmsee et al., 2003). With these many variables to consider and different experimental approaches available, a wide variation in the interpretation of results will be expected. It should therefore be noted that whereas all these studies mentioned in this manuscript had various stimuli and the knockouts were homozygous, the present study had heterozygous knockout mice and there was no stimulus given. We believe that the differences in the knockout models are very subtle with the major difference being only on the lack of stimulus, which therefore should not undermine the importance of the present findings. Though speculative, we believe that the partial knock out of p50 subunit of NF-κB might be a pre-conditioning step preparing the mice against oxidative stress induced effects. The effect of various stimuli on the p50 (-/+) mice showing a reduced oxidative stress warrants further investigation since this might be a potential avenue for therapeutic interventions. We therefore provide additional insights

on the complexities of NF-κB activity especially in a stimulus-free environment. We also suggest that the p50 subunit of NF-κB plays a significant role in the oxidative stress-related neurodegeneration and that it should be considered as a potential target in the development of therapeutics strategies involving oxidative stress and nuclear factor kappa B (NF-κB) activity.

B6 Acknowledgement

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Appendix C

Data supporting figures and Tables

Figure 4.1A

n	CC	EC	CA	EA
1	415	485	260.5	320.5
2	560	494	272	243
3	393.5	343.5	405.5	360.5
4	357.5	353.5	315	185
5	360.5	361	458.5	289.5
6	480.5		395	255
Averages	427.833	407.4	351.083	275.583
SEM	32.1796	33.6621	32.7565	25.2406
% Control	100	95.224	82.0608	64.4137

Figure 4.1B

n	CC	EC	CA	EA
1	491.667	319	371.5	379
2	389.333	349	343.5	341
3	407	389.5	347.5	308
4	381	341	310	325
5	320	332	323.5	321
6	377		347	292
Averages	394.333	346.1	340.5	327.667
SEM	22.8494	11.9398	8.71875	12.2792
% Control	100	87.7684	86.3483	83.0938

Figure 4.1C

n	CC	EC	CA	EA
1	381.5	469	361.5	434.5
2	452	381.5	428	363
3	371	348.5	398.5	352.5
4	404.5	430.5	372.5	368.5
5	449	361.5	343.5	388.5
6	427		372	361.5
Averages	414.16667	398.2	379.33333	378.08333
SEM	13.944334	22.529758	12.163242	12.302044
% Control	100	96.14487	91.58954	91.28773

Table 4.3

	CC	EA	%Control	Sem
1301	52.7658	9.295329	17.6162	7.592398
2504	23.42602	6.361421	27.15537	5.339302
3101	30.12821	6.159792	20.44526	5.697713
4501	30.41586	7.046319	23.1666	6.583777
9701	3.533036	0.562666	15.92585	0.325476

Figure 4.5

	Read 10 Avg	Read1 Avg	Change OD	mg/ml	Activity	Specific activity
CC1	1.003	0.8335	0.1695	8.8589552	0.1765625	0.0199304
CC2	1.2705	1.064	0.2065	10.515477	0.2151042	0.020456
CC3	1.334	1.1575	0.1765	9.5210958	0.1838542	0.0193102
CC4	1.0765	0.958	0.1185	8.7279309	0.1234375	0.0141428
CC5	1.049	0.8575	0.1915	7.4340659	0.1994792	0.0268331
						0.0201345
EA1	1.3435	1.075	0.2685	8.2108528	0.2796875	0.0340631
EA2	1.525	1.2655	0.2595	9.6427613	0.2703125	0.0280327
EA3	1.734	1.474	0.26	8.0213356	0.2708333	0.0337641
EA4	1.472	1.2895	0.1825	7.8388374	0.1901042	0.0242516
EA5	1.467	1.262	0.205	7.951144	0.2135417	0.0268567
						0.0293937

					CC 100	EA 145.98653
			GST Change in OD	% Control mg/ml	Activity	Specific activity
CC1	0.059	0.0725	0.0135	0.3129096	0.0047647	0.0696962
CC2	0.07	0.0835	0.0135	0.1507208	0.0047647	0.1446953
CC3	0.0535	0.0655	0.012	0.3211009	0.0042353	0.0603718
CC4	0.061	0.074	0.013	0.3112713	0.0045882	0.0674681
CC5	0.0605	0.0715	0.011	0.2621232	0.0038824	0.0677924
						0.082005
EA1	0.0755	0.1025	0.027	0.1982307	0.0095294	0.2200326
EA2	0.064	0.091	0.027	0.3079948	0.0095294	0.1416167
EA3	0.06	0.0785	0.0185	0.3964613	0.0065294	0.0753815
EA4	0.059	0.0875	0.0285	0.2588467	0.0100588	0.1778674
EA5	0.0645	0.08	0.0155	0.1965924	0.0054706	0.1273676
						0.148453

			SOD % control	CC 100	EA 181.0299

Figure 4.6

	CC	EA
	250	342
	146	360
	231	340
	191	362
	226	331
	311	409
Average	225.83333	357.33333
SEM	22.694223	11.441639
%Control	100	158.2288

Table 5.2

	CU/ZN SOD		FBP		CK	
	CC	CE	Control	CE	Control	CA
	5121	5601	4134	3455	6586	5470
	2436	5762	2093	6386	4777	8022
	1880	5401	2004	5531	1088	5448
	1718	4621	2785	4075	4545	10010
	2553	6549	1979	3243	4769	8252
Average	2741.6	5586.8	2599	4538	4353	7440.4
Fold change		2.037788		1.746056		1.709258

	GLUD		GAPDH	
	Control	CE	Control	EA
	3337	3448	4289	7248
	1615	3779	3902	12762
	1905	3528	2668	9710
	2412	3118	4189	7737
	1757	2864	3306	5581
Average	2205.2	3347.4	3670.8	8607.6
Fold change		1.517958		2.344884

Figure 5.3

			Change in OD	mg/ml	Activity	Specific activity
CC1	0.059	0.0725	0.0135	0.3129096	0.0047647	0.0696962
CC2	0.07	0.0835	0.0135	0.1507208	0.0047647	0.1446953
CC3	0.0535	0.0655	0.012	0.3211009	0.0042353	0.0603718
CC4	0.061	0.074	0.013	0.3112713	0.0045882	0.0674681
CC5	0.0605	0.0715	0.011	0.2621232	0.0038824	0.0677924
						0.082005
EA1	0.0755	0.1025	0.027	0.1982307	0.0095294	0.2200326
EA2	0.064	0.091	0.027	0.3079948	0.0095294	0.1416167
EA3	0.06	0.0785	0.0185	0.3964613	0.0065294	0.0753815
EA4	0.059	0.0875	0.0285	0.2588467	0.0100588	0.1778674
EA5	0.0645	0.08	0.0155	0.1965924	0.0054706	0.1273676
						0.148453

	SOD	CC	EA
% Control		100	181.0299

Figure 5.4

Case	Treatment Group	Aox Diet	Env Enrich	Black/White discrimination errors	Black/white Reversal Errors	Spatial errors	CuZnSOD
1521S	1	1	1	N/A	N/A	N/A	1718
1510A	1	1	1	154	219	250	1880
1508U	1	1	1	N/A	N/A	N/A	2436
1543S	1	1	1	50	197	214	2553
1494D	1	1	1	83	219	265	5121
1506B	2	1	2	8	219	278	3761
1529S	2	1	2	12	128	275	4286
1523U	2	1	2	28	84	232	5486
1542S	2	1	2	8	57	91	5815
1518D	2	1	2	79	96.5	33	7477
1491B	3	2	1	74	202	255	N/A
1508A	3	2	1	74	146	60	N/A
1509U	3	2	1	131	182	N/A	N/A
1523B	3	2	1	77	137	120	N/A
1532S	3	2	1	108.5	202	223	N/A
1542T	4	2	2	85	145	142	4621
1541B	4	2	2	21	64	177	5401
1502S	4	2	2	82	142	263	5601
1521B	4	2	2	60	70	58	5762
1581T	4	2	2	44	52	7	6549

Figure 6.1

	Normal	AD
	596	413
	511.5	462
	448	552
	487.5	489.5
		542.5
Average	510.75	491.8
Stdev	62.57329	57.65696
SEM	31.286645	23.062784

Figure 6.2

	IsoP (ng/ml)		NeuroP (ng/ml)	
	Normal	AD	Normal	AD
	1.98	1.34	128.56	131.64
	2.25	1.99	136.54	233.18
	1.95	1.22	170.41	127.66
	2.17	1.41	108.07	53.21
		2.84		89.92
Average	2.0875	1.76	135.895	127.122
Stdev	0.145688	0.3424422	25.946959	73.905317
%Control	100	84.311377	100	93.544281

Table 6.4

		MDH		FBP		UCHL1
	Normal	AD	Normal	AD	Normal	AD
	1711.2	1185.6	1632	3021.8	2124.8	3549.2
	1898.5	1025.4	2037.1	2932	1915.2	3765.3
	1754.2	1202.5	1884.2	2339.7	2792.8	4202.5
	1954.2	1085.2	1896.1	2242.8	1951.7	3178.7
Average	1829.53	1124.68	1862.35	2634.075	2196.125	3673.925
Fold Change		0.6147371		1.4143824		1.6729125

	GAPDH			ATP		PRDX
	Normal	AD	Normal	AD	Normal	AD
	4734.3	10993.3	4305.8	45.3	3625.3	2113.6
	6273.3	6132.1	2308.6	1072.4	3339	2708.3
	4740.9	8798.7	2947.5	10.6	6012.8	1409.1
	2614.2	6700.2	1772.2	2881.5	2516.3	1701.5
Average	4590.675	8156.075	2833.525	1002.45	3873.35	1983.125
Fold Change		0.5628535		0.353782		0.5119922

Figure 7.1

	RCR	SD
Ipsi	2.30	1.30
Contra	7.10	1.40
Sham	8.00	2.00

Figure 7.2

	HNE		3NT		Carbonyl	
	CH	IH	CH	IH	CH	IH
	368.5	802	394.25	604	736.66667	1003.3333
	502	779	460.5	565	698.66667	1142.6667
	481.5	753.5	448	659.5	754	926.233
	595.5	626.5	409	612	680.66667	890.66667
Average	486.875	740.25	427.9375	610.125	717.5	990.72492
% Control	100	152.04108	100	142.57339	100	138.08013
Sem	46.609805	39.188912	15.695798	19.397568	16.859715	55.83915

Table 7.3

Cortex	Contra blots/gels	Ipsi blot/gels		
SSP	Average	Average	% Control	Sem
3402	62.2	228.3	367.1	47.2
5404	16.9	162.7	962.8	68.8
6606	1.3	36.7	2742.7	85.9
6609	0.02	0.06	318.8	30.0
3303	0.06	1.28	2042.5	74.3

Table 7.4

Hippo	Contra Blots/gels	Ispi Blots/gels		
SSP	Average	Average	% Control	Sem
3303	0.062485	0.851117	1362.103	38.55067
4204	0.028807	0.351442	1220.004	31.80999
6511	7.174938	42.21875	588.4197	37.7746
6609	0.021081	0.109313	518.536	27.16987

Figure 7.7

Complex I	Mean	SEM
Contralateral	92.50	0.925
Ipsilateral	52.90	12.5

Complex IV	Mean	SEM
Contralateral	96.76	6.11
Ipsilateral	73.18	3.41

PDH activity	Mean	SEM
Contralateral	99.50	5.22
Ipsilateral	84.10	13.97

Figure A1

	Ctrl	Fe2+/H2O2	29	40	52
	336	589	448	540	509
	381	611	412	569	472.5
	358.5	600	386	554.5	423
	321	599	342	509	462
	354	606	378	512	412
	365.2	576	404	523	445
Average	352.6167	596.8333	395	534.5833	453.9167
Stdev	21.33639	12.6082	35.6595	24.12139	35.31914
Sem	8.534557	5.043279	14.2638	9.648558	14.12766

Figure A3

	Ctrl	Fe2+/H2O2	D609	40 nM	D609+ 40 nM DCC
	336	589	225	436	306
	381	611	242	509	293
	358.5	600	233.5	472.5	299.5
	321	599	221.5	462	311
	354	606	263.2	421	285.1
	365.2	576	241	488	279
Average	352.6167	596.8333	237.7	464.75	295.6
Stdev	21.33639	12.6082	14.97331	32.57875	12.27273
Sem	8.534557	5.043279	5.989324	13.0315	4.909094

Figure A4

	Ctrl	29	40	52
	104	107	137	114
	124	125	171	121
	65	130	175	99
Average	97.66667	120.6667	161	111.3333
% Control	100	123.5495	164.8464	113.9932
Stdev	30.00556	12.09683	20.88061	11.23981
SEM	20.0037	8.064554	13.92041	7.493207

Figure A5

	Ctrl	29	40	52
	182	99	84	97
	184	146	151	139
	133	107	157	93
Average	166.3333	117.3333	130.6667	109.6667
% Control	100	70.54108	78.55711	65.93186
Stdev	28.88483	25.14624	40.52571	25.48202
Sem	16.69643	14.5354	23.42527	14.72949

Figure A6

	CC	29nm	40nm	52nm
	203	228	309	183
	196	247	326	142
	191	236	301	153
	186	214	316	146
Average	194	231.25	313	156
%Control	100	119.201	161.3402	80.41237
Stdev	7.25718	13.88944	10.61446	18.5652
Sem	3.62859	6.944722	5.307228	9.2826

Figure A7

	C	Fe2+/H202	370nM	700nM	1mM
	401	538	380	333	307
	328	512	371	326	306
	353	498	375	329	298
	336	502	362	336	310
Average	354.5	512.5	372	331	305.25
% Control	100	144.5698	104.9365	93.37094	86.10719
Stdev	32.70576	17.99074	7.615773	4.396969	5.123475
Sem	16.35288	8.995369	3.807887	2.198484	2.561738

Figure B1

	Carbonyl		HNE		3NT	
	wt	ko	wt	ko	wt	ko
	232.5	171	379.5	301.5	534.33333	365.66667
	367.5	154	389.5	341.5	419	336.33333
	271	191	355	269	423	432.33333
	166	170.5	357.5	351.5	443.33333	429.33333
	298.5	157	496.5	353	476.33333	434.66667
Average	267.1	168.7	395.6	323.3	459.2	399.66667
% Control	100	63.159865	100	81.723964	100	87.035424
Stdev	74.9995	14.652645	58.260621	36.852069	47.740968	45.659975
Sem	34.090682	6.6602931	26.482101	16.750941	21.70044	20.754534

Figure B2

	WT	KO	Fold change	p value
ATP Synthase	959.14	1367.02	1.42526	0.0562
Ubiquinol cyt c reductase	2113.84	8008.46	3.78858	0.01624
HSP10	1340.64	2671.24	1.99251	0.04613
FBP	1343.66	1609.08	1.19754	0.01913
NADH-ubiquinone	345.9	780.54	2.25655	0.05324

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List of Abbreviations

AD Alzheimer's disease, ALS amyotrophic lateral sclerosis, Aβ amyloid-beta peptide, Apaf-1 apoptosis protease-activating factor 1, AIF apoptosis inducing factor, ATP adenosine tri-phosphate, AIDS acquired immunodeficiency syndrome, AA arachidonic, BCA Bicinchoninic acid, BSA Bovine serum albumin, BDNF brain derived neurotrophic factor, CNS central nervous system, CSF cerebral spinal fluid, CsA cyclosporine A, CDNB 1-chloro-2, 4-dinitrobenzene, CC contralateral cortex, DNA deoxyribonucleic acid, DCH decosahexaenoic acid, DHLA Dihydrolipoic acid, DTT dithiothreitol, DNPH 2, 4-dinitrophenyl hydrazine, DDC 3'-dideoxycytidine, ESI electrospray ionization, ETC electron transport chain, EDTA ethylene di-amine tetra-acetic acid, EGTA ethylene glycol-bis-tetraacetic acid, GST Glutathione-S-transferase, GSH Glutathione (GSH), GCEE γ-glutamylcysteine ethyl ester, GR glutathione reductase, GPx glutathione peroxidase, GAPDH glyceraldehyde-3-phosphate dehydrogenase, GDH glutamate dehydrogenase, HD Huntington's disease, HIV Human Immunodeficiency Virus, HIVD HIV-Dementia, HO-1 Heme-oxygenase-1, HNE 4-Hydroxy-2-nonenal, HEPES 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid, HAART highly active antiretroviral therapies, 8-OHdG 8-hydroxy-2'-deoxyguanosine, IEF isoelectric focusing (IEF), IsoPs isoprostanes, IPG immobilized pH gradients, IC ipsilateral cortex, MDA malondialdehyde, MALDI Matrix-Assisted Laser Desorption/Ionization, mPTP Mitochondria permeability transition pore, 3NT 3-Nitrotyrosine, NOS nitric oxide synthase, NP neuroprostanes, NAC N-acetyl-L-cysteine, NFT neurofibrillary tangles, NRTI nucleoside reverse transcriptase inhibitors, NF-κB Nuclear factor kappa B, OMM outer mitochondrial membrane, PDH Pyruvate dehydrogenase, PDHC Pyruvate

Dehydrogenase complex, PAGE polyacrylamide gel electrophoresis, PUFA polyunsaturated fatty acids, PD Parkinson disease, PLA_2 phospholipase A_2, PMSF phenylmethanesulfonyl fluoride, PBS phosphate buffered saline, ROS Reactive oxygen species, RNS Reactive nitrogen species, SOD Superoxide dismutase, SP senile plaques, SCI spinal cord injury, SDS sodium dodecyl sulfate

TBI traumatic brain injury, TOH α- tocopherol, TNFα tumor necrosis factor α, TCA trichloroacetic acid, VDAC voltage-dependent anion channel (VDAC)

VITA

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Refereed Papers Resulting from this Dissertation Research

1. Antony, J.M, Guido van Marle**, Opii, W.O**, D Allan Butterfield, Mallet, F Yong, V.W Wallace, J.L Deacon, R.M, Warren, K, Power, C (2004). Human endogenous retrovirus glycoprotein-mediated induction of redox reactants causes oligodendrocyte death and demyelination. *Nature Neuroscience* 7, 1088 - 1095.

2. Zhu, Y, Jones, G, Tsutsui, S, **Opii, W.O**, Liu, S, Silva, C, D. Allan Butterfield and Power, C (2005) Lentivirus Infection Causes Neuroinflammation and Neuronal Injury in Dorsal Root Ganglia: Pathogenic Effects of STAT-1 and Inducible Nitric Oxide Synthase *The Journal of Immunology*, 175: 1118-1126

3. Yumin Chen, Chotiros Daosukho, **Wycliffe O. Opii**, Delano M. Turner, William M. Pierce, Jon B. Klein, Mary Vore, D. Allan Butterfield, and Daret K. St. Clair (2006) Cardiac metabolic proteins are specifically oxidized in Adriamycin treated mice. Free Radic Biol Med. 41(9): 1470-7.

4. Butterfield DA, Abdul HM, **Opii W.O**, Newman SF, Joshi G, Ansari MA, Sultana R. (2006) Pin1in Alzheimer's disease. J Neurochem. 98(6): 1697-706.

5. Mubeen Ahmad Ansari, Joshi G, Huang, Q, **Opii, W.O**, Hafiz Mohmmad Abdul, Sultana R, Butterfield, D.A (2006). *In vivo* effect of D609 in brain mitochondria against the free radical mediated oxidant, Fe2+/H2O2, 2,2-azobis-(2-amidinopropane) and amyloid beta peptide: Relevance to Alzheimer's disease and

other oxidative stress-related neurodegenerative disorders in which mitochondria are involved (In Press *Free Radical Biol Med*)

6. **Opii, W.O**, Joshi G, Head, E, M, Milgram, N.W, Muggenburg, B.A, Klein, J.B, Pierce W.M, Cotman, C.W, and Butterfield, D.A. (2006). Proteomic identification of brain proteins in the canine model of human aging following a long-term treatment with antioxidants and a program of behavioral enrichment: Relevance to Alzheimer's disease *(In press, Neurobiology of Aging).*

7. **Opii, W.O**, Sultana R, Hafiz Mohmmad Abdul [1], Mubeen-Ahmad Ansari, Nath, A and D. Allan Butterfield (2006) Oxidative Stress and Toxicity Induced by the Nucleoside Reverse Transcriptase Inhibitor (NRTI) - 2', 3'-dideoxycytidine (ddC): Relevance to HIV Dementia. (Article in press, *Experimental Neurology*).

8. **Opii, W.O**, Nukala, V.N, Sultana R, Day, K.M, Merchant, M.L, Klein, J.B, Sullivan, P.G and D. Allan Butterfield. Proteomic Identification of Oxidized Mitochondrial Proteins Following Experimental Traumatic Brain Injury (Under review *Journal of Neurotrauma*)

9. **Opii, W.O**, Turner D.M, Pierce W.M. Ramassamy C and Butterfield, D.A; Proteomic Analysis of Protein Expression Levels in Brain of NF-Kβ Heterozygous Knockout Mice (Submitted to Biochimica et Biophysica Acta)

10. **Opii, W.O,** Head, E, Turner D.M, Pierce W.M, Cotman, C.W and Butterfield, D.A. Proteomic expression analysis of brain proteins from dementia-free nonagenarians: relevance to successful aging and Alzheimer's disease. (Submitted to Journal of *Gerontology)*

11. Joshi G, **Opii W.O**, Sultana R, St Clair D and Butterfield DA: Proteomic identification of differentially expressed proteins in Adriamycin injected mice brain. (Manuscript to be submitted to *Neurobiology of Disease*)

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