ROLE OF CASPASE-3 ON OXIDATIVE STRESS IN THE AGING MOUSE ORGANS

BY

MITAL YOGESHBHAI PATEL

(Under the Direction of James L. Franklin)

ABSTRACT

Mitochondrial dysfunction could be a cause or consequence of apoptotic death. This effect may be facilitated by a substantial mitochondrial-dependent increase in the generation of superoxide free radicals and other downstream reactive species (RS). Electron leakage from the respiratory complexes causes reduction of molecular oxygen to form the superoxide free radical. Caspase-3 may induce this leakage of electrons within mitochondria by attacking the respiratory complex proteins. The cellular damage caused by the accumulation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) is enhanced with aging. This damage may have a role in the development of age-related neurodegenerative disorders, such as Alzheimer's and Parkinson's diseases, and may also contribute to the pathologies in other organ systems such as heart, kidney and liver. Neurons are extremely susceptible to oxidative stress because they have high oxygen demand, and relatively low antioxidant capacity. An abnormal production of superoxide free radical by mitochondria, and the downstream ROS and RNS renders neurons vulnerable to damage from these RS. Previous studies from our laboratory show that the depletion of Bax or caspase-3 from mouse sympathetic neurons in cell culture

significantly decreased the levels of ROS produced during apoptosis. Here we utilized oxidative stress markers to determine whether caspase-3 deletion reduces oxidative damage in the brains of female mice at 12 months of age. Oxidative stress markers such as lipid peroxides and 8-hydroxy-2'-deoxyguanosine (8-OHdG) levels were reduced in the brains from mice with genetically depleted levels of caspase-3. Nitration of protein tyrosine residues caused by RNS was also decreased in the brains of caspase-3 null mice compared to that of wild-type animals of the same age. Caspase-3 deletion had similar effects on lipid peroxidation, oxidative DNA damage (as assessed by 8-OHdG levels), and protein tyrosine nitration in the hearts, livers, and kidneys of these animals. The findings of this study indicate that caspase-3 plays a vital role in the generation of ROS, and hence contributing to the oxidative stress in the mouse brain as well as in other organs.

INDEX WORDS: Aging; oxidative stress; reactive species; apoptosis; caspase-3

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DEDICATION

I would whole-heartedly like to dedicate this to my amazing and caring family: I could not have fulfilled this without having your support and love. This is for my parents Yogeshbhai Patel and Induben Patel who have always been there for me and encouraging me throughout my academia. This is for my wife, Bhavika Patel; who walked this six-year PhD journey with me. All throughout of me being stressed-out, miserable, she has been through it too. With her love, care, and motivation I have undoubtedly been through the course of seeking this degree – we will make it through anything. I love you. This is for my brothers Jaimin Patel, Sandip Patel, Chirag Patel and Pintu Patel who are always there for me and are proud of me – it goes both ways. This is for my in-law (my parents), Narindrakumar Patel and Niruben Patel who have always been very loving and supportive by pushing me the extra mile.

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INTRODUCTION

Neuronal apoptosis, oxidative stress, and neurodegeneration

Programmed cell death or apoptosis is an important feature of normal development and homeostasis in multi-cellular organisms, so as to retain the normal anatomical and physiological function of different organs and tissues, including the brain. In the developing brain, the excessive number of neurons produced is removed by apoptosis during embryonal and postnatal refinement of the central nervous system [1-3]. This process of developmental cell death plays a vital role in establishing neuronal and glial populations of the correct size. In the developing peripheral nervous system (PNS), neuronal apoptosis has been shown to be important in the adjustment of the number of innervating neurons to the size of the final target cell population that they innervate. This programmed neuronal death also relieves the nervous system of inappropriate connections [4]. The survival of neurons is regulated by the availability of sufficient neurotrophic factors such as nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), and neurotrophin 3, provided by their target tissues. Neurons do not have the ability to multiply or divide, as they are post-mitotic but continue to mature and interact with each other. As a result, there is formation of synapses, pruning of dendrites and the myelination of neurons.

Generally, there is no replacement of dead neurons. Therefore, it is essential to understand how and why neuronal death occurs. Primarily, the two major forms of cell death are apoptosis and necrosis. Some of the other forms of cell death include autophagy, regulated necrosis, pyroptosis, ferroptosis, NETosis, pyronecrosis and necroptosis. Kerr, Wyllie, and Currie first used the term apoptosis in 1972 to characterize a morphologically different form of cell death. The term apoptosis was chosen from a Greek word meaning “falling off” as in leaves of a tree [5]. Apoptosis is a well-conserved and highly regulated mechanism of cell death. It is involved in the removal of unnecessary, surplus, aged or damaged cells, and thus it is of widespread biologically significant in the development, differentiation and immune system regulation [6, 7]. The characteristics of apoptotic death include cell shrinkage with intact organelles, nuclear condensation, aggregation of mitochondria and ribosomes, chromatin aggregation, fragmentation of the DNA into oligonucleosomes, and blebbing of the plasma membrane. Other hallmarks of apoptosis are a reduction in the mitochondrial membrane potential, production of free radicals, and externalization of phosphatidylserine residues [5, 8-10]. As a result of this programmed cell death, the cell fragments into apoptotic bodies that are then engulfed by phagocytes, thus avoiding inflammation and damage to the neighboring cells and tissues [11]. In contrast to apoptosis, necrotic death is normally not isolated to a single cell but is a more traumatic form of cell death that results from acute injury. Mitochondrial and nuclear swelling, dissolution of organelles, and condensation of chromatin around the nucleus histologically describe necrotic cell death. The rupture of nuclear and cytoplasmic membranes, and enzymatic degradation of DNA follow this that ultimately results into inflammation [5, 12]. Apoptosis is generally physiologically

beneficial to the organism but is also known to be involved in several pathological conditions as well. Deregulation of the apoptotic pathway contributes to the pathologies of several disorders; either by suppressing apoptosis (e.g. autoimmune diseases and cancer) and/or by augmenting apoptotic processes (e.g. neurodegenerative diseases) [11].

There are various signals triggering apoptosis, including the withdrawal of survival factors, exposure to pro-apoptotic stimuli such as DNA damage, decreased ATP synthesis, or specific ligand-receptor interactions in the plasma membrane. Apoptosis occurs via two pathways, the extrinsic pathway and the intrinsic pathway. The ultimate step in both pathways is the proteolytic activation of the executor caspases (cysteine-dependent, aspartate-specific proteases) such as caspase-3, 6, and 7 by the initiator caspases (i.e. caspase 8, 9, and 10) [13]. Caspases induce the cell death in part by activating DNases, cleaving DNA repair enzymes and degrading nuclear proteins. Stimulation of death receptors such as the tumor necrosis factor (TNF) receptor superfamily, p75, and Fas/APO-1 has been involved in regulating the extrinsic pathway of apoptosis (Figure 1.1). Signal transduction from these receptors (e.g. TNFR or Fas) is initiated through interaction with its specific ligand (e.g. TNF-α or Fas-ligand). Binding of the ligand to the death receptor, Fas/APO-1 causes receptor oligomerization and subsequent binding of the Fas associating death domain protein, FADD. This in turn recruits FLICE (FADD-like ICE; Interleukin-1β-converting enzyme/caspase 1) triggering the formation of the death-inducing signaling complex (DISC), which recruits procaspase 8. This then undergoes autoproteolytic cleavage to form active caspase 8 that then activates effector caspases such as caspase-3, 6, and 7 [14, 15]. The intrinsic pathway of apoptosis is mediated by mitochondria and is initiated from within the cell.

Mitochondria act as the power plants of the cell by providing them the energy in the form of adenosine triphosphate (ATP). Glucose is primarily the major source for energy production in the brain, and it gets metabolized in the cytosol by glycolysis to produce a small quantity of ATP, NADH, and pyruvate. After being transported into the mitochondrial matrix through the inner mitochondrial membrane, pyruvate then gets broken down by pyruvate dehydrogenase into acetyl-CoA which enters into the tricarboxylic acid (TCA) cycle. The primary function of TCA cycle is to reduce NAD^+ and FAD^+ to NADH and $FADH_2$ that then serves as electron donors. Most of the ATP is synthesized by oxidative phosphorylation that takes place in the mitochondria. The electron transport chain is a mitochondrial pathway in which electrons move across a redox span from one complex to the next and this plays a vital role in oxidative phosphorylation. Complex I (NADH dehydrogenase) accept fuel from the citric acid (TCA) cycle in the form of NADH, which donates electrons from hydrogen to the chain while complex II (succinate dehydrogenase) accepts electrons from $FADH_2$. The electrons from either complex I or II are then transported to coenzyme Q_{10} reducing it to form ubiquinol (QH_2). Reduced coenzyme Q_{10} passes the electrons to Complex III (cytochrome c reductase), which then reduces cytochrome c. The electrons are then transferred to complex IV (cytochrome c oxidase) via cytochrome c. With the help of these oxidation-reduction reactions occurring across the respiratory complexes, a proton gradient is generated across the inner mitochondrial membrane, creating the mitochondrial membrane potential ($\Delta\psi_m$). Protons (H^+) then go down their electrochemical gradient into the mitochondrial matrix, and this acts as the driving force of the motor in complex V (ATP synthase) that causes phosphorylation of ADP to ATP.

The four electrons removed from cytochrome c are transferred to O_2 producing two molecules of water ($O_2 + 4e^- + 4H^+ \rightarrow 2H_2O$) [16-18]. This energy in the form of ATP obtained from mitochondria is essential for functioning of the electrogenic activity of neurons [19]. It is also important in sustaining axonal transport, ionic balance, neuronal excitability, and survival. Mitochondria also play a vital role in neurotransmission and synaptic activation through maintaining Ca^{2+} homeostasis and ATP generation [20].

The intrinsic apoptotic cascade is activated by mitochondrial outer membrane permeabilization that occurs either due to the formation of pores mediated by Bax and Bak (pro-apoptotic members of the Bcl-2 family) or to mitochondrial permeability transition that takes place in response to opening of the permeability transition pore complex [21, 22]. This Bcl-2 family of apoptotic regulators can either have pro-apoptotic or anti-apoptotic functions. On stimulation by death signaling factors, the pro-apoptotic Bcl-2-related proteins Bax, Bad, Bid, and Bim that are localized in the cytosol translocate to mitochondria, where they induce the release of cytochrome c and other apoptotic factors from the mitochondrial intermembrane space. Anti-apoptotic proteins Bcl-2 and Bcl-xL are localized in the outer mitochondrial membrane (OMM) and they suppress cytochrome c release. The pro-apoptotic members can further be subdivided in two groups; the Bax subfamily of pro-apoptotic proteins (e.g. Bax, Bak and Bok), and the limited homology BH3-only subfamily of pro-apoptotic proteins (e.g. Bad, Bid, Bim, Bik, Puma, Noxa, Hrk and Blk). Bcl-2 homology domains present in the anti-apoptotic proteins (Bcl-2, Bcl-xL, and Mcl-1) is known as BH1-4, which is essential for binding to BH3-only proteins, and forming heterodimers with the Bax subfamily of pro-apoptotic members, thus inactivating the pro-apoptotic proteins [23-25].

The mechanism of action of Bcl-2 family members is not clearly understood, but previous studies suggest that it is the heterodimerization process and the antagonistic balance between anti-apoptotic Bcl-2/Bcl-xL and pro-apoptotic Bax/Bad that regulates the cellular decision to live or commit suicide [26]. On activation by apoptotic stimulus such as DNA damage, p53 induces the transcription of Bax, Noxa, and Puma, which then translocate to the OMM. This translocation of Bax and Bad is inhibited through its phosphorylation induced by survival factors, leading to its cytosolic sequestration. The members of Bcl-2 family can homodimerize as well as can form heterodimers between pro- and anti-apoptotic proteins [9, 24]. Heterodimerization purposes to counteract the opposing proteins. Initiation of the intrinsic apoptotic cascade occurs when the number of pro-apoptotic proteins on the OMM exceeds the anti-apoptotic proteins. After exposure of cells to apoptotic stimuli, the pro-apoptotic proteins of Bax subfamily gets inserted into the OMM. The membrane insertion of Bax is facilitated by its conformational changes and oligomerization [27-29]. Although it is clear that Bax induces permeabilization of OMM, the mechanism by which this occurs is under debate. It is postulated that oligomerized Bax has the ability to form pores through which cytochrome c transits into the cytosol [30]. Other apoptotic proteins that are released from the mitochondrial intermembrane space into cytosol include apoptosis-inducing factor (AIF) [31], endonuclease G [32], and second mitochondrial activator of caspases (Smac) [33]. Bax may also influence the voltage-dependent anion channel (VDAC) and/or the adenine nucleotide translocator (ANT), which may be important in controlling the release of cytochrome c [34, 35]. Once cytochrome c translocate into the cytoplasm, it initiates the intrinsic apoptotic cascade (Figure 1.1) by interacting with the adaptor protein Apaf-1

(Apoptotic protease activating factor-1) and triggering its oligomerization. Apaf-1 heptomer, along with cytochrome c and the cofactor dATP then recruits procaspase 9, resulting in the formation of a structure known as the apoptosome. The formation of an apoptosome facilitates the dimerization of procaspase 9 into active caspase 9, an upstream initiator of apoptosis. Caspase 9 then causes the activation of the effector caspases such as caspase-3, which induces apoptotic cell death [36-38].

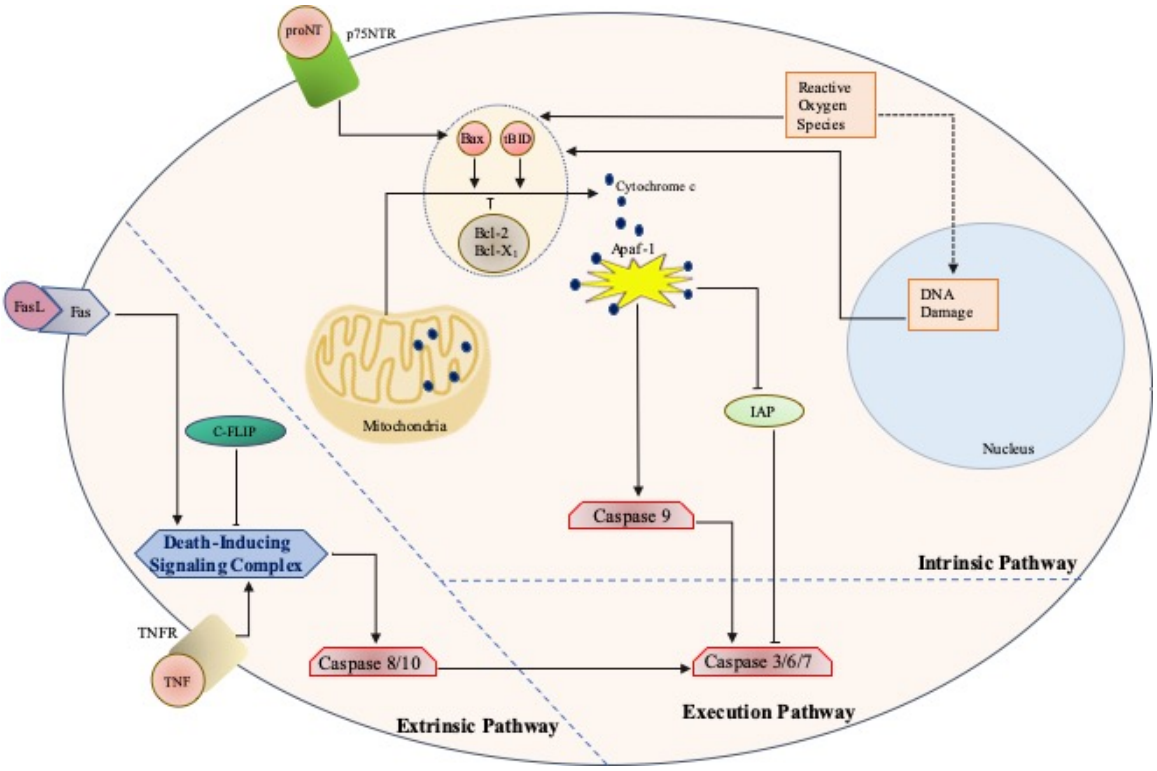


Figure 1.1: Mechanisms of neuronal apoptosis

Two mechanisms by which apoptosis occur are the intrinsic pathway and the extrinsic pathway, which ultimately results into proteolytic activation of executor caspases that mediate apoptotic cell death. Some of the signaling factors triggering the intrinsic pathway include reactive oxygen species [39], DNA damage [40], or stimulation of the p75NTR neurotrophin receptor by pro-neurotrophins [41]. As a result, the pro-apoptotic members of Bcl-2 protein family such as Bax translocate to the outer mitochondrial membrane and mediate the release of cytochrome c into the cytosol. This then binds with

Apaf-1 and mediates activation of initiator caspase 9 that ultimately results into activation of effector caspases such as caspase-3, 6 and 7. The extrinsic pathway is mediated through stimulation of ligand-bound death receptors such as Fas and TNFR. This is associated with the activation of initiator caspase 8 and 10 that ultimately causes the activation of effector caspases [42, 43].

In addition to the role of neuronal apoptosis in the development of nervous system, it is believed to also contribute to the loss of neurons occurring in a number of acute and chronic neurologic diseases such as stroke, Alzheimer's disease, and amyotrophic lateral sclerosis (ALS) [44, 45]. Neuronal apoptosis in these neurodegenerative diseases may not be due to trophic factor withdrawal, but it could be caused by pathogenic stimuli like abnormal protein aggregates and generation of reactive species [46, 47]. An excessive release of glutamate neurotransmitter also serves as a toxic insult triggering excitotoxic neuronal cell death in the mature nervous system [48, 49]. The formation of β-amyloid peptide serves as an extrinsic pathogenic inducer of neuronal apoptosis [50]. Intracellular trigger such as aggregation of misfolded proteins also cause apoptosis in neurologic diseases [47]. Many neurotoxic stimuli including excitotoxicity and exposure to β-amyloid ultimately results into oxidative stress intracellularly [51]. Oxidative stress characterized by enhanced ROS levels seems to have a vital role in triggering apoptosis and has been implicated in many acute and chronic neurodegenerative diseases including stroke, PD, and ALS [46, 52]. The involvement of ROS in the death of motor neurons has been shown in familial ALS due to mutations in copper/zinc superoxide dismutase (SOD) [53, 54]. ROS are thought to be involved in the damage of cellular components including lipids, proteins, and nucleic acids that eventually causes apoptosis [55]. The primary targets of ROS are the nuclear and mitochondrial DNA. Interestingly, DNA damage, in

part, is responsible for neuronal loss in many neurodegenerative diseases associated with oxidative stress, for example in Huntington's disease [56] and Parkinson's disease [57].

Free Radicals and Reactive Species

Free radicals are defined as the chemical species possessing a single unpaired electron that is highly reactive. Thus, the free radical produced is unstable and can undergo a reaction with another molecule to generate yet another free radical. This series of reactions leads to more and more damaging reactions. Most of the free radicals that induce damage to biological systems are the oxygen radicals and other ROS that are continuously generated as a byproduct of aerobic respiration as well as from other endogenous sources, such as NADPH oxidases [58]. RS are involved in the initiation of apoptosis [6, 59]. Being the primary source of $O_2^{\bullet-}$, mitochondria are highly susceptible to oxidative damage. The RS produced not only initiate a series of lipid peroxidation reactions within the membrane bilayer but also cause damage to vital inner mitochondrial membrane (IMM) proteins. Cardiolipin, a phospholipid component of the IMM serves a critical role in intrinsic apoptotic pathway. The high degree of unsaturation in cardiolipin makes it particularly prone to oxidation, and this causes its dissociation from and further inducing release of cytochrome c into the cytosol [60]. Damage to cytochrome c by nitration and oxidation also results in its activation for apoptosis [61, 62]. This indicates that cytochrome c serves as a possible connection between mitochondrial ROS and apoptosis [63]. Along with mitochondrial electron transport chain (ETC), other sites by which ROS are produced include peroxisomal fatty acid, cytochrome P450, and phagocytic cells [64].

ROS Generation in the Mitochondria

Mitochondria are the major source of ROS [65]. The production of mitochondrial ROS occurs during the process of oxidative phosphorylation. In this process, the electrons transported across the mitochondrial ETC can leak out of the respiratory complexes and cause a one-electron reduction of O_2 to form superoxide free radical ($O_2^{\bullet -}$) (Figure 1.2). This is then converted into other RS that comprises of both ROS and RNS, and can include free radicals as well as non-radicals.

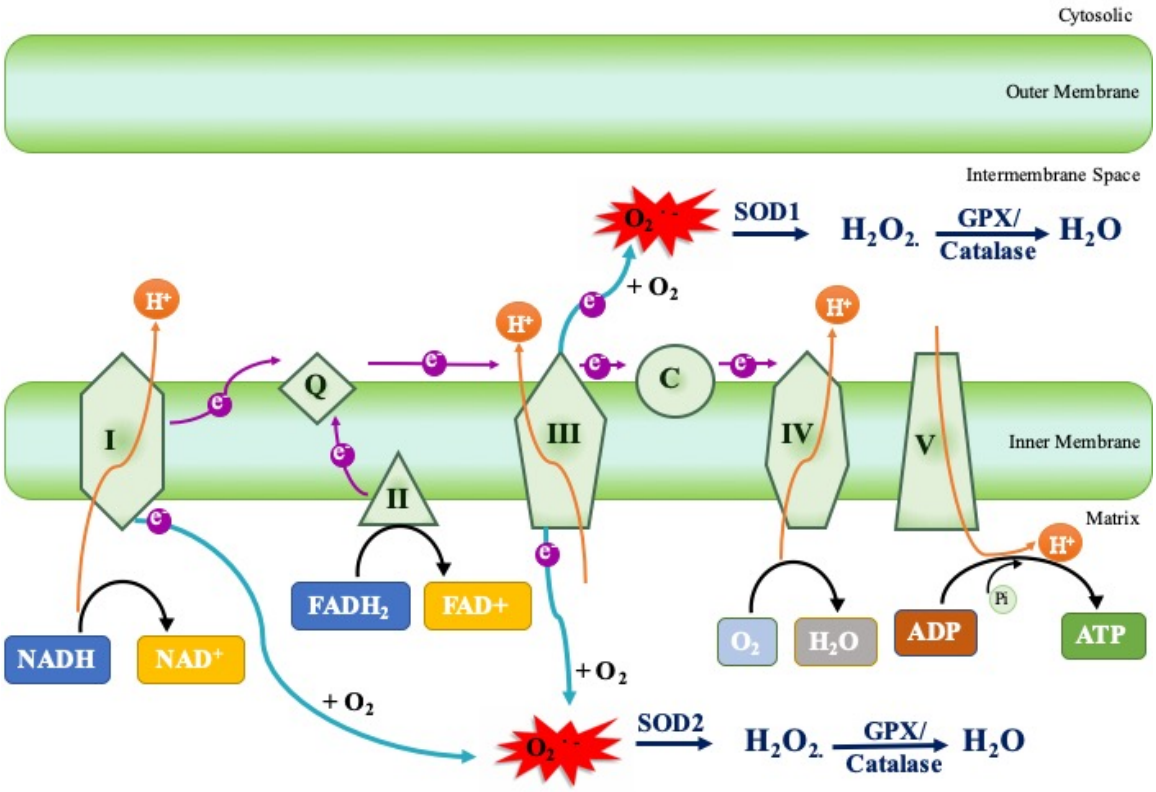


Figure 1.2: Mitochondrial production of ROS

The electrons (e^-) donated to complex I and II by NADH and $FADH_2$ respectively, pass through the electron transport chain and reduce molecular O_2 to H_2O at complex IV. During this process, the leakage of electrons occurring particularly at complex I and III

causes the formation of superoxide free radical ($O_2^{\bullet-}$). Production of ATP occurs by phosphorylation of ADP at complex V. The $O_2^{\bullet-}$ produced at complex I is within mitochondrial matrix while from complex III, it is released both in the matrix and intermembrane space. SOD1 (Cu/Zn SOD) and SOD2 (MnSOD) dismutate $O_2^{\bullet-}$ into H_2O_2, which is then metabolized by glutathione peroxidase (GPX) or catalase into H_2O [66].

Along with $O_2^{\bullet-}$, other ROS that are produced within the body include the hydroxyl radical ($OH^{\bullet}$), singlet oxygen ($^1O_2$), and hydrogen peroxide (H_2O_2). RNS generated are nitric oxide ($NO^{\bullet}$) and the RS derived from it such as peroxynitrite ($ONOO^-$) and nitrogen dioxide (NO_2). These RS have both physiological and pathological role. They are physiologically important in enzymatic reactions, gene expression, and signal transduction [67]. The RS formed also induces negative effects in the body by causing oxidative damage to biomolecules, cells, and tissues. They cause attenuation of long-term potentiation and synaptic neurotransmission in the neurons. RS are involved in the activation of MAP (mitogen-activated protein) kinases, excitotoxic calcium mobilization as well as in the release of cytochrome c through cardiolipin peroxidation, and thus inducing apoptosis [68-70]. Additionally, the RS generated are also implicated in aging and disease [19, 71].

Non-mitochondrial ROS Generation

Peroxisomal β-oxidation of fatty acids leads to production of ROS in the form of H_2O_2 as a by-product. Phagocytic cells are yet another vital source of oxidants, where they generate an oxidative burst of $NO^{\bullet}$, H_2O_2 and $O_2^{\bullet-}$ so as to defend the central nervous system from invading microbes and to get rid of the debris from damaged cells. The cytochrome P450 enzymes also stimulate the ROS burst during the defense process

against natural toxic chemicals [72]. In the cytosol, NADPH oxidase and xanthine oxidase are the main producers of superoxide free radical as well as other ROS such as H_2O_2 [73].

Reactions with superoxide free radical

An approximately 2-5% of the total oxygen consumed gets converted into $O_2^{\bullet-}$. The acceptance of one electron by oxygen results in multiple ROS through the formation of $O_2^{\bullet-}$ (Figure 1.3). Chemically, the superoxide free radical dismutates with itself to form H_2O_2 and oxygen with the help of superoxide dismutases (SODs) [74]. Further addition of electrons to H_2O_2 converts it into a highly reactive ¨ in the presence of transition metals via the Fenton/Haber-Weiss reaction. $OH^{\bullet}$ is known to be one of the most destructive free radical as it reacts spontaneously with any molecule from which it can accept a hydrogen atom. Thus, it has the ability to cause lipid peroxidation, and induce damage to DNA and proteins. H_2O_2 can also be further detoxified into water by the enzymes such as catalase, glutathione peroxidase and peroxiredoxins. The possibility of downstream reactions of $O_2^{\bullet-}$ depends on the competency of the target to out-compete the most obvious reaction of $O_2^{\bullet-}$ with micromolar levels of SOD2 in the matrix. One such biological target that competes with SOD2 for the $O_2^{\bullet-}$ is $NO^{\bullet}$ (Nitric oxide radical). The concentrations of $NO^{\bullet}$ are elevated in neuropathological conditions, and its reaction with $O_2^{\bullet-}$ takes place at a rapid rate than that of between $O_2^{\bullet-}$ and SOD2 (6.7×10^9 $M^{-1}s^{-1}$ and 1×10^9 $M^{-1}s^{-1}$, respectively) [75, 76]. By competing with endogenous SOD, the $NO^{\bullet}$ produced from l-arginine by nitric oxide synthases (NOS) reacts with $O_2^{\bullet-}$ to generate a toxic $ONOO^-$ [77]. Since the permeability of $O_2^{\bullet-}$ through the lipophilic membrane bilayer

is constrained by its electric charge, the deterioration induced by $O_2^{\bullet-}$ is more likely to occur at the site of its production. On the other hand, $NO^{\bullet}$ is barely polar and therefore readily diffuses across the mitochondrial membranes. $NO^{\bullet}$ has relatively a long half-life among the RS and this also favors its permeabilization into the mitochondria from cytoplasmic sources [78]. This toxic $ONOO^-$ produced ultimately decomposes to generate highly oxidizing and damaging intermediates such as $OH^{\bullet}$, $NO^{\bullet}$, $CO_3^{\bullet-}$ (Carbonate) as well as NO_3^- (Nitrate). $ONOO^-$ can lead to lipid peroxidation as well as other cellular damage by causing nitroxidative modifications of biomolecules [79].

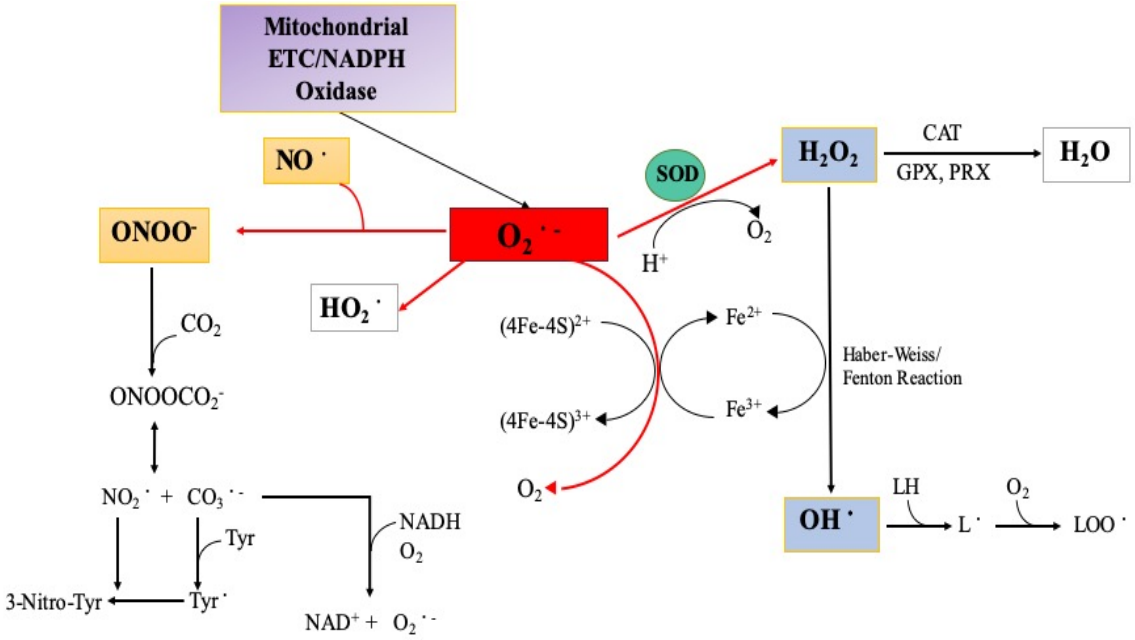


Figure 1.3: Reactions of superoxide free radical

Dismutation of superoxide ($O_2^{\bullet-}$) by SOD enzymes so as to produce hydrogen peroxide (H_2O_2) that is then converted into water by catalase (CAT), glutathione peroxidases (GPXs), and peroxiredoxins (PRXs). H_2O_2 via the Fenton/Haber-Weiss reaction forms the hydroxyl radical ($OH^{\bullet}$). $O_2^{\bullet-}$ reacts with nitric oxide ($NO^{\bullet}$) to produce peroxynitrite ($ONOO^-$). The CO_2 present within mitochondria reacts with $ONOO^-$ to form carbonate radicals ($CO_3^{\bullet-}$) and nitrogen dioxide radical ($NO_2^{\bullet}$) through homolysis of $ONOOCO_2^-$.

$CO_3^{\bullet-}$ induces oxidation of tyrosine (Tyr) residues of proteins to form tyrosyl radicals ($Tyr^{\bullet}$), which may result in their nitration to 3-nitrotyrosine (3-NT). Carbonate radicals also bring about oxidative modifications to other biomolecules such as NADH. Lipid peroxidation reactions are initiated through hydrogen removal of LH caused by $OH^{\bullet}$ and other RS to produce lipid radicals ($LOO^{\bullet}$). Hydroperoxyl radical ($HO_2^{\bullet}$) is also very reactive and is formed by the protonation of $O_2^{\bullet-}$ [80].

Caspases and RS

Caspases are the cysteine dependent aspartate-specific proteases that are activated by proteolysis and dimerization. The major role of caspases is the execution of apoptotic cell death by activating DNases, cleaving DNA repair enzymes and degrading cellular proteins. Caspases cleave their substrates at the aspartate residues. The family of caspases is also important in regulating immunity, neurogenesis, synaptic activity, cell proliferation and differentiation [81, 82]. They are also equally important in the regulation of RS. An outburst of RS production is observed during apoptotic death [59, 83]. Among the executioner caspases, it has been reported that caspase-3 is necessary for the enhanced production of $O_2^{\bullet-}$ both in the apoptotic and nonapoptotic neurons [84]. In addition to increased ROS generation, caspase-3 also affects electron transport chain and causes loss of mitochondrial transmembrane potential, reduction in ATP levels and loss of mitochondrial structural integrity [85]. In several cell types, including HeLa cells, mouse embryonic fibroblasts, sympathetic and cerebellar granule neurons, it has been reported that increased ROS production lies downstream of caspase activity and that depletion of caspase-3 suppresses both apoptotic cell death and the elevation in ROS levels [84-87].

Antioxidants

Due to the high reactivity and toxicity caused by RS particularly the mitochondrial ROS, there are a number of antioxidant enzyme systems developed in the mammalian cells, that helps in neutralizing the ROS formed. The brain primarily consists of SOD and glutathione peroxidase (GPx) antioxidant systems. Non-enzymatic antioxidants like vitamin E, glutathione, and metal-binding proteins also play an important role in scavenging ROS.

Antioxidant Defenses

SOD

Superoxide dismutases (SOD1 and SOD2) convert the highly toxic superoxide free radical into hydrogen peroxide, which is then metabolized into water plus oxygen by the enzymes catalase and GPx [66]. SOD1 is located in the mitochondrial intermembrane space where it detoxifies the superoxide free radical generated by the mitochondrial respiratory complexes, and thus attenuating oxidative stress. Copper and zinc are the cofactors required for active SOD1. Deficiency of SOD1 is associated with ALS, age-related muscle loss, early development of cataracts, and reduced lifespan [88]. SOD2 is localized only in the mitochondrial matrix and contains manganese at its reactive center [89]. Impairment of SOD2 is linked with early neonatal death in the mutant mice [90] and causes endothelial dysfunction in carotid artery of proatherogenic apolipoprotein E (ApoE)-deficient mice [91]. SOD3 is located in the extracellular space, and contains copper and zinc in its active site. SOD3 offers protection against vascular dysfunction and its deficiency causes coronary artery disease and myocardial infarction [92].

Glutathione (GSH) system

The synthesis of GSH is catalyzed by γ-glutamylcysteine synthetase followed by addition of glycine by the enzyme glutathione synthetase. GSH combats oxidative stress by reducing the levels of hydrogen peroxide or oxidized lipids through the oxidation of GSH to GS-SG adduct that is catalyzed by the antioxidant enzyme, GPx. Glutathione reductase causes recycling of GSH by transforming the oxidized GSH (GS-SG) back to reduced form (GSH) by consuming NADPH [93, 94]. GPx is beneficial in reducing vascular inflammation and atherosclerosis [95].

Iron Chelators and Vitamins

Iron chelators such as ferritin, transferrin and ceruloplasmin suppress the production of $OH^{\bullet}$ by binding iron and preventing it from participating in Fenton reaction. Desferrioxamine, an iron chelator has been shown to be beneficial in the treatment of brain edema [96]. Natural antioxidants like vitamin E have been indicated to reduce mitochondrial ROS levels but their effect is limited as they cannot penetrate through mitochondria or cross the blood-brain barrier efficiently [97, 98]. Therefore, synthetic ROS scavengers such as MitoVit-E were developed, in which vitamin E is covalently bonded to a triphenylphosphonium cation [99]. MitoVit-E suppresses ROS generation and oxidative stress-induced apoptosis in aortic endothelial cells [100]. Vitamin E also neutralizes the intermediate peroxyl radical that is generated during the lipid peroxidation process occurring as a result of impaired antioxidant systems in fibrosarcoma conditions of rats [101].

Oxidative stress and Aging

Aging is a complex phenomenon characterized by the gradual loss of physiological functions and increase in mortality that is associated with many pathological diseases. A number of theories of aging have been suggested, for example the free radical and mitochondrial theories of aging. According to both of these theories, one of the common causes of aging is the summative damage to mitochondria and mitochondrial DNA (mtDNA) caused by ROS [102]. The free radical theory proposes that aging occurs as a consequence of accumulation of detrimental effects induced by free radicals [103]. This free radical theory has been contradicted by an increasing number of studies that see oxidative damage as just one type of cellular damage, which could be associated with the aging process, and does not represent the cause of aging. Instead, the true root of aging may be defined by biological imperfectness leading to unavoidable accretion of myriad damage forms of molecular species [104]. The possible mechanism by which ROS causes aging is through induction of cellular senescence, a physiological process characterized by inhibition of cellular proliferation in response to damages occurring during replication [105, 106]. An imbalance between the generation and detoxification of ROS/RNS results into oxidative stress. The high oxygen consumption, approximately 20% of the total oxygen, by the brain along with long lifespan of neurons makes it particularly vulnerable to oxidative stress-induced damage [107]. In addition, the brain is excessively rich in lipids, and contains high amount of iron and copper as well as relatively low levels of antioxidants [19]. All these factors provide a suitable environment for the likely occurrence of oxidative damage in the most common neurodegenerative diseases.

Lipid peroxidation refers to oxidative degradation of polyunsaturated fatty acids (PUFAs) such as arachidonic acid to form malondialdehyde (MDA), which is a widely used biomarker of oxidative stress. At least 20% of the total fatty acids in the brain are comprised of unsaturated fatty acids. The MDA produced interacts with amino acids in the protein to form adducts that interfere with DNA base-pairing, and thus causing DNA damage [64]. Another lipid peroxidation product, hydroxy-2-nonenal (HNE) also exerts damaging effect by forming covalent adducts with histidine, lysine, and cysteine residues in proteins, thus affecting the activity of cellular proteins [108]. HNE-modified proteins have been detected along with neurofibrillary tangles in the senile plaques in aged dogs [109]. Oxidative stress also causes multiple changes in DNA, including base and sugar fragmentation products, strand breaks, and DNA-protein crosslinks. Several studies have shown that oxidative DNA damage is very crucial in various pathological diseases, such as cancer and neurodegenerative diseases. An increase in the levels of 8-OHdG, a biomarker of oxidative DNA damage has been detected in the mtDNA of aged tissues [110, 111]. This suggests that gradual augmentation of oxidative DNA damage is one of the types of cellular damage that may increase as a function of age. DNA has low vulnerability to oxidative damage when compared to lipids and this damage can be easily taken care of by endogenous repair mechanisms [112]. The RS produced as well induces oxidation of cellular proteins, which has been assessed by detecting the levels of protein carbonyl groups that is found to be elevated in the human cerebral cortex with age [113]. The formation of protein carbonyl groups occurs via direct oxidation of amino acid side chains and oxidation-induced peptide cleavage. The oxidative modification of proteins can also be demonstrated by measuring the levels of protein 3-nitrotyrosine (3-NT). The

cerebellum in the Purkinje cell layer, the molecular layer, and in the cerebellar nuclei of aged rats have shown immunoreactivity to 3-NT [114]. Thus, in addition to several diseases, oxidative damage could also be associated with the normal aging process as well as with the age-related disorders.

The Aging Brain

The fundamental manifestation of the aging process is a gradual loss in sustaining the physiology of tissue homeostasis and developing susceptibility to degenerative diseases and death. It has been widely stated that aging is a multifactorial process that is genetically influenced and altered epigenetically by the environment [115]. In the CNS, neurons serve as the processors and messengers of information, and are meant to survive throughout the lifetime of an organism. This long-term retention ability of neurons helps them to achieve their function of containing the information for a lifelong [116]. After the development of new neurons in the adult brain, their survival is solely based on their potentiality to form physiological branches with the existing neurons. But not all the newly formed adult neurons do so, and about half of them die [117]. It is necessary for the neurons to be anatomically and physiologically stable for longer periods of time so as to have a good life expectancy. This also helps individuals be free from age-related neurodegenerative disorders such as Alzheimer and Parkinson's diseases that mainly affects people over the age of 65. Due to the potential of RS to cause oxidative degradation of DNA, proteins, lipids as well as other biomolecules, they could be one of the etiological factors of these neurological diseases. In the neurons, the RS are produced primarily as the by-products of mitochondrial respiration, and therefore mitochondria are considered to be the main target of oxidative stress and play a vital role in aging. This

age-dependent diseases also show pathologies that corresponds to a role of apoptosis and oxidative stress as their causative factors [118]. Thus, elucidating the precise relationship between ROS-induced damage and apoptosis in aged animals may help in understanding the cause of these diseases. This is essential from a medical, social and economical perspective as it may broaden our knowledge that could be useful in developing future therapies for such age-related disorders.

CHAPTER 2

CASPASE-3 AND OXIDATIVE STRESS IN THE AGING BRAIN

Introduction

Aging is characterized by the gradual decline in anatomical and physiological functions, and by the enhanced vulnerability to pathogenic stimuli and death. There are several factors that play a vital role in mediating the aging process, and these include genetic, environmental and dietary factors. The etiological factors involved in normal aging are also associated with age-related disorders [119]. It has been postulated that free radicals and reactive species (RS), by causing oxidative damage to tissues and organs, induce deleterious effects and therefore facilitates aging of the organism [103]. In the aging brain, as well as in various neurodegenerative diseases, the normal antioxidant defense mechanisms deteriorate, and this augments the susceptibility of brain to the detrimental effects of oxidative damage [120]. RS such as reactive oxygen (ROS) or reactive nitrogen (RNS) are chemically reactive molecules that are naturally produced within biological systems. They have both physiological and pathological functions [121, 122]. RS are involved in regulating many cellular responses such as signal transduction, immune response, enzyme-catalyzed reactions, cell growth and other physiological responses [123, 124]. An imbalance between the generation and detoxification of ROS/RNS causes oxidative stress (OS). This can lead to lipid peroxidation, oxidative damage to DNA and proteins, and even induce cell death [125, 126].

Under normal cell physiology, ROS such as superoxide free radical ($O_2^{\bullet -}$) are generated as a byproduct of mitochondrial respiration and from NADPH oxidases [127, 128]. The leakage of electrons from the mitochondrial electron transport chain during oxidative phosphorylation reduces molecular O_2 to $O_2^{\bullet -}$ that is then converted into hydrogen peroxide (H_2O_2) by superoxide dismutase [129]. Through the iron-catalyzed Fenton reaction, $O_2^{\bullet -}$ can be converted into the highly toxic hydroxyl radical ($OH^{\bullet}$). The $O_2^{\bullet -}$ also reacts with nitric oxide (NO) to produce RNS such as peroxynitrite ($ONOO^-$) [73, 130]. By causing oxidative damage to cellular proteins, lipids and DNA, these RS may induce neurodegeneration such as in Parkinson's disease, Alzheimer's disease and stroke. Some studies have demonstrated that age-dependent increase in oxidative damage to mitochondrial DNA is higher as compared to the oxidative damage occurring to nuclear DNA in rodents [131]. Oxidative stress-induced cellular damage also attenuates long-term potentiation and synaptic neurotransmission [68, 132] as well as activation of MAP kinases, excitotoxic calcium mobilization, and apoptosis [133]. The aging of the brain could also be accelerated by oxidative modifications such as protein aggregation, DNA damage, and lipid peroxidation as well as by reduced mitochondrial function [134-138].

ROS levels are increased during apoptotic cell death, including the apoptotic death of neurons [139, 140]. The ROS produced by the mitochondrial electron transport chain lie downstream of the proapoptotic protein, Bax, and these ROS appear to play a vital role in the apoptotic cascade in both superior cervical ganglion (SCG) sympathetic neurons and cerebellar granule (CG) neurons [59, 84]. Bax also has a potent effect on the generation of ROS in non-apoptotic SCG and CG neurons. The activation of caspases

particularly caspase-3 that is mediated by Bax, is required for increased $O_2^{\bullet -}$ production during neuronal apoptosis, as well as in nonapoptotic neurons [84]. Hence, we proposed that genetic deletion of caspase-3 would suppress oxidative damage in the brains of aged mice.

Activation of caspase-3 causes disruption of electron transport, loss of mitochondrial transmembrane potential, decrease in ATP levels, production of ROS, and loss of mitochondrial structural integrity [86, 141]. In this study, we demonstrate that genetic ablation of caspase-3 in 12-month old female mice (C57BL/6 background) significantly decreased lipid peroxidation, oxidative DNA damage, and protein tyrosine nitration in the brain compared to the wild-type mice of the same age. Hence, determining the relationship between ROS, Bax and caspases will help in understanding the etiology of many pathological conditions and will further explore the mechanisms by which caspase-3 regulate RS production in the neuronal mitochondria.

Materials and Methods

Materials

DNA/RNA oxidative damage ELISA kit (Catalog # 589320) was purchased from Cayman Chemical (Michigan, USA). 3-Nitrotyrosine (3-NT) antibody (Catalog # ab61392) was from Abcam (Massachusetts, USA). All other reagents were purchased from Sigma (Missouri, USA) or Thermo Fisher Scientific (Massachusetts, USA) unless otherwise indicated.

Mouse breeding and genotyping

The Animal Studies Committee at the University of Georgia reviewed and approved all animal procedures. Mice were housed in an AAALAC-approved facility under supervision of two ACLAM certified veterinarians. All mice were kept on a 12 h light/dark cycle and given access to food and water *ad libitum*.

Mice hemizygous for the caspase-3 allele (caspase-$3^{+/-}$; C57BL/6 genetic background) were mated to generate caspase-$3^{+/+}$, caspase-$3^{+/-}$, and caspase-$3^{-/-}$ offspring. Founding breeders were obtained from The Jackson Laboratory (Maine, USA). Genotyping for caspase-3 wild-type, hemizygous and mutant mice were performed by a single PCR using isolated DNA from mouse pups or weanlings. Genomic DNA was extracted from each mouse from a tail snip using QuickExtract DNA extraction solution (Epicentre Biotechnologies, Madison, WI). In a 0.5 ml microcentrifuge tube, 25 µl of reaction mixture (8.61 µl ddH_2O, 0.63 µl primers, 12.5 µl 2X MangoMix solution) and 2 µl of extracted DNA were combined. PCR was performed using a Techne Genius thermocycler. PCR for Caspase-3 was performed as described by The Jackson Laboratory. The primers used were: wild-type forward 5'-GCG AGT GAG AAT GTG CAT AAA TTC-3' and wild-type reverse 5'-GGG AAA CCA ACA GTA GTC AGT CCT-3' to amplify a 320 bp sequence of the wild-type allele; neo/pgk forward 5'-TGC TAA AGC GCA TGC TCC AGA CTG-3' and same wild-type reverse to amplify a 300 bp sequence of the mutant allele. Cycling conditions were 94^0 C for 2 min followed by 10 cycles of 94^0 C for 20 sec, 65^o C for 15 sec, and 68^o C for 10 sec. This was followed by another set of 28 cycles of 94^o C for 15 sec, 60^o C for 15 sec, and 72^o C for 10 sec. PCR products were separated on a 3% (w/v) agarose gel and visualized with ethidium bromide

using a Fotodyne Foto/Analyst Dual-Light Luminary Workstation with TotalLab software (Fotodyne Inc., Wisconsin, USA).

Brain harvesting

Mice were anaesthetized by CO_2 exposure and rapidly killed by decapitation. Cerebrums were dissected out in a weigh boat chilled on ice and immediately snap-frozen in a 70% ethanol/dry ice bath and stored at -80^{o}C until analysis.

Thiobarbituric acid reactive substances (TBARS) assay

Excessive generation of ROS induces the formation of highly reactive and unstable lipid peroxides that ultimately degrade into more stable products such as malondialdehyde (MDA). MDA can be quantified using the thiobarbituric acid reactive substances (TBARS) assay, a technique based on the reaction of MDA with thiobarbituric acid (TBA) [142]. Frozen tissues were homogenized in 10% w/v in ice-cold PBS and centrifuged at 800 x g for 10 min at 4^{o} C. The Bradford Assay using the Pierce Coomassie Plus Bradford Assay and TBARS assay used the supernatant for protein determination. To the supernatant, 8.1% sodium dodecyl sulfate (SDS), 20% acetic acid, 10M sodium hydroxide (for pH 3.5) and 0.8% TBA was added. The volume of the mixture was then made up to 1 mL with dH_2O, and heated in a boiling water bath for 1 hour. After cooling, 1 mL of dH_2O and 1 mL of butanol/pyridine (15:1, v:v) mixture was added, and 200 µl aliquot was used to measure the absorbance at 532 nm using a Molecular Devices SpectraMax M2 spectrophotometer (Molecular devices, California, USA). Each sample was performed in triplicate. The concentration of lipid peroxides was

expressed as µM MDA per mg protein, calculated by a MDA standard curve, prepared from 0-10µM of tetraethoxypropane.

DNA damage

Oxidative damage to nucleic acids has been found to be associated with a variety of diseases including cancer and aging. Among all the purine and pyrimidine bases, guanine is the base that is highly prone to oxidation. The DNA was extracted from different organs including brain, heart, liver and kidney of the aged wild-type and caspase-3 mutant mice. Any oxidative damage to DNA was then analyzed using DNA/RNA oxidative damage ELISA kit. This kit detects all three oxidized guanine species; 8-hydroxy-2'-deoxyguanosine from DNA, 8-hydroxyguanosine from RNA, and 8-hydroxyguanine from either DNA or RNA. The principle of this ELISA is based on the binding of DNA/RNA oxidative damage monoclonal antibody-oxidatively damaged guanine complex to the goat polyclonal anti-mouse IgG that has been previously attached to the well. After addition of the Ellman's Reagent [143] to each well, an enzymatic reaction occurs producing a yellow colored product whose absorbance was read at 410 nm using a Molecular Devices SpectraMax M2 spectrophotometer. Each sample was performed in duplicate. The concentration of oxidized DNA was expressed as pg/mL, calculated by a standard curve.

Immunoblotting

Frozen mouse tissue was homogenized in ice-cold Modified RIPA lysis buffer containing protease inhibitors using a glass dounce homogenizer. Homogenates were then

centrifuged for 10 min at 10000 x g. The supernatant was transferred to a new 1.5 ml centrifuge tube for analysis. Protein concentration was determined using the Pierce Coomassie Plus - The Better Bradford Assay kit (Thermo Fisher Scientific, Rockford, IL). A molecular marker was added to one lane of a 12% Tris-HCl pre-cast gel (Bio-Rad). The remaining lanes were filled with a mixture of sample containing 50µg protein, sample buffer (100 mM Tris-HCl, 4% SDS, 10% bromophenol blue, 30% glycerol), and protease inhibitor cocktail (Calbiochem) and the gel was run for 1 h at 100V. Proteins were transferred onto PVDF membranes (Millipore) at 100 V for 1 h, dried, and blocked with blocking buffer for 30 mins at room temperature with gentle shaking. The membrane was then incubated in mouse anti-3-nitrotyrosine (3-NT) monoclonal antibody (1:1000 dilution) overnight at 4^{o} C with gentle shaking. The next day, the membrane was rinsed with 3 changes of wash buffer (TBS-T), with shaking. Secondary anti-mouse IgG (1:2000 dilution) was added for 1 h at room temperature. Washes were repeated, followed by incubation in detection solution (ECL Western Blotting Substrate, Pierce) for 2 min. Blot visualization and analysis were performed using a Fotodyne Foto/Analyst Dual-Light Luminary Workstation with TotalLab software. The immunoblot band density of 3-nitrotyrosine for each sample was normalized to the respective β-actin levels, and the data was represented as relative intensity to β-actin.

Statistical analysis

Graphical design and statistical significance measures were carried out using SigmaPlot 11.1 (Systat Software, Inc., California, USA). The distribution of the data was analyzed

by Kruskal-Wallis one-way ANOVA on ranks followed by Dunn's multiple comparisons post-*hoc* test or by the Mann-Whitney rank-sum test. Error bars represent ± SEM.

Results

Confirmation of caspase-3 gene deletion in the female mice by PCR genotyping

Genomic DNA was isolated from mouse pups or weanlings. Single PCR was carried out to confirm the genetic deletion of caspase-3 through replacement of the caspase protease-conserved catalytic site of the endogenous gene with a PGK-neo cassette. While primers wild-type forward and wild-type reverse detect the wild-type allele (320 bp sequence), primers mutant forward and wild-type reverse amplify the mutant allele (300 bp sequence). The DNA extracted from a heterozygous mouse shows both bands, 300 bp and 320 bp sequences (Fig. 2.1).

Genetic ablation of caspase-3 attenuated oxidative DNA damage in the aged mouse brain

The effect of caspase-3 on the production of RS was examined by determining oxidative stress markers in the brains of wild-type and caspase-3 mutant mice at 12 months of age. This middle age of mice was chosen based on its physiological relevance to the human lifespan of around 43 years [144, 145]. The free radicals generated from mitochondria are known to be one of the main causes of mitochondrial DNA (mtDNA) damage. Studies in the aged brain have shown increased levels of 8-hydroxy-2'-deoxyguanosine (8-OHdG), a biomarker of oxidative DNA damage, in mitochondrial DNA [146]. The levels of 8-OHdG are also elevated in both nuclear DNA and mtDNA of the post mortem brains of

aged subjects [147]. Based on previous studies from our laboratory; one possible mechanism by which Bax regulates mitochondrial superoxide production is through activation of caspases [84]. During apoptosis, Bax inserts into the outer mitochondrial membrane (OMM) and therefore causing its permeabilization and the release of cytochrome c into the cytoplasm where it induces the activation of caspases and subsequent cell death by causing the formation of an apoptosome [38, 148-150]. An elevation in ROS occurring in apoptotic SCG, CG and other types of neurons is suppressed by the caspase inhibitors indicating a vital role of caspases in the production of ROS in these cells [151], [152]. ELISA was used to assess oxidative DNA damage in the aged brain. Our results show increased brain DNA damage in the middle-aged, wild-type mice and these increases were nullified by the genetic ablation of caspase-3 (Fig. 2.2). This indicates that caspase-3 deletion has a significant effect in suppressing the oxidative stress in normally aged mice.

Effect of caspase-3 deletion on lipid peroxidation in the aged mouse brain

Aging is also associated with the changes in membrane fatty acid composition. In the inferior temporal cortex of the aged human brain, the levels of MDA, a product of lipid peroxidation has been found to be elevated [153]. It is also enhanced in the hippocampus and cerebellum of aged rodents [154, 155]. Hence, lipid peroxidation is considered to be a well-established approach for assessing oxidative stress in the brain. The TBARS assay was used to determine the levels of MDA in the aged mouse brain. The increase in the levels of MDA was significantly inhibited in the 12-month old mouse brain on genetic depletion of caspase-3 (Fig. 2.3).

Caspase-3 deletion reduces nitration of protein tyrosine residues in the aged mouse brain

The relationship between caspase-3 and the production of ROS/RNS was further characterized by studying the nitration of protein tyrosine residues by immunoblot analysis using a 3-nitrotyrosine monoclonal antibody. Several studies have proposed that protein oxidation is responsible for the progressive decline in physiological functioning that is associated with aging. An increase in the levels of 3-nitrotyrosine has been detected in the hippocampus and cerebral cortex of aged rats [156]. The nitration of protein tyrosine was significantly reduced in the brains of caspase-3 mutant mice as compared to the brains of wild-type mice of the same age (Fig. 2.4), suggesting that caspase-3 deletion reduces protein oxidation in the mouse brain.

Discussion

Cellular proteins involved in apoptotic pathway such as Bcl-2 family proteins also play a role in oxidative stress, as it has been previously reported that anti-apoptotic protein Bcl-2 exhibits antioxidant effects in cells [157]. Other studies have also shown that the members of Bcl-2 family can exert either antioxidant or pro-oxidant effects [158, 159]. We previously demonstrated that the proapoptotic protein Bax lies upstream of increased mitochondria-mediated ROS generation that takes place in the apoptotic mouse SCG and CG neurons in cell culture [59]. The studies from our laboratory also reported that Bax deletion decreases the levels of mitochondrial $O_2^{\bullet-}$ and other ROS produced in both apoptotic and nonapoptotic SCG and CG neurons [59, 84]. During this investigation, we

found that caspase-3 was involved in the pro-oxidant effect of Bax both in the apoptotic and nonapoptotic neurons and that the production of RS lies downstream of caspase-3 activation [59, 84]. The objective of this study was to assess the role of caspase-3 on oxidative stress associated with aging. And this is the first study looking at the effects of caspase-3 on oxidative damage caused by endogenous ROS in the aged brain.

The effect of caspase-3 on oxidative damage was assessed *in vivo* by determining oxidative stress markers such as lipid peroxidation, oxidative DNA damage, and nitration of protein tyrosine residues in the brains from female mice at the age of 12 months. Female C57BL/6 mice were chosen for this study because they show a greater increase in ROS levels with age than males as indicated by enhanced dihydroethidium (DHE) oxidation in the female brain. Older females also exhibit lower levels of antioxidants such as superoxide dismutase 1 (SOD1) and glutathione peroxidase 1(GPx1), thus indicating higher chances of developing oxidative stress [160]. Here we report an investigation demonstrating that DNA damage consistent with oxidative stress was augmented in wild-type mouse brains of 12 months of age. This oxidative DNA damage as detected by increased 8-OHdG levels was attenuated by genetic depletion of caspase-3. Lipid peroxides were also higher in the wild-type mouse brains. This peroxidation was suppressed by caspase-3 deletion in the brains of wild-type animals. Protein oxidation causes loss of protein function, cellular dysfunction, and eventually causes cell death [161]. Tyrosine nitration is a form of protein oxidation that is linked with age-related neurodegenerative diseases such as Alzheimer's disease [162]. The results showed that at middle age (12 months), removal of the executor caspase-3 leads to decreased levels of 3-nitrotyrosine in the mouse brain, as assessed by western blot analysis. This proposes that

caspase-3 deletion, in part, does assist in the mitigation of cellular damage induced by endogenous oxidative stress.

A regulated ROS production maintains an optimal redox state for the activation of intracellular signal transduction pathways. For example, ROS serves an important role in controlling intracellular signaling that mediates synaptic plasticity within the CNS [163]. But an increased level of ROS can react with proteins and nucleic acids and abolish their cellular functions and thus this may cause oxidative damage in the brain. Mitochondrial $O_2^{\bullet-}$ and the downstream RS such as $ONOO^-$ can cause oxidative and nitrosative damage to mitochondrial proteins. An excessive $O_2^{\bullet-}$ production occurring as a result of disruption in the activity of respiratory complexes could possibly be mediated by the effect of caspase-3. These ROS may also induce oxidative modifications to various protein components of mitochondrial complexes, for example the Fe-S centers of complex I, and this could be involved in the pathogenesis of neurologic diseases. A study reported a reduction in the levels of Ndufs7 (NADH:ubiquinone oxidoreductase core subunit S7) and complex I activity in patients with bipolar disorder (BD) [164]. They also found a negative correlation between complex I activity and the levels of protein carbonyls or 3-nitrotyrosine in the postmortem prefrontal cortex of BD patients. Not only by ROS-induced damage, caspase-3 may also reduce the expression levels of mitochondrial membrane proteins by directly cleaving them and therefore contributing to mitochondrial impairment. Oxidative modifications can have a negative impact on the enzymatic activity of complexes and further facilitate its loss by proteasomal degradation. Decreased expression of two catalytic subunits of complex I, Ndufv2 and Ndufv1 in the prefrontal cortex has been shown to be associated with schizophrenia [165]. ROS can

induce post-translational modifications and processing of peptides such as β-amyloid (Aβ) and tau which then self-aggregate to form intercellular plaques that is observed in the pathology of Alzheimer's disease (AD) [166]. Caspase-3 induced oxidative damage can also affect mitochondrial DNA (mtDNA) and this may cause its mutation. It could as well contribute to mtDNA deletions, and errors in the replication, transcription and sequencing of mtDNA. An age-dependent increases in oxidative mtDNA damage has been observed in the brains of sporadic Parkinson's disease (PD) patients. Their brain mitochondria also showed decreased levels of mitochondrial proteins in complexes I-V [167]. Along with disruption of complex I activity in dopaminergic neurons, formation of Lewy bodies in PD could also be mediated by ROS. Lipid peroxidations caused by mitochondrial ROS can as well induce cytotoxicity within the neurons. 4-Hydroxynonenal, a lipid peroxidation product has been shown to be neurotoxic by covalently binding with proteins, and thus enhancing expression and activity of β-secretase (β-site amyloid precursor protein cleaving enzyme; BACE) that consequently produces Aβ in AD [168]. Additionally, caspase-3 directly causes proteolytic cleavage of amyloid precursor protein (APP) resulting in the accumulation Aβ [169]. The ROS produced can also affect mitochondrial fission and fusion proteins. Mutation or oxidative changes in these proteins disturbs their cellular function by giving rise to mislocalization of mitochondria to the cell body, decreased ATP levels, synaptic loss, and neurodegeneration [170]. Hence, caspase-3 not only by its direct effect on cellular biomolecules but also by inducing mitochondrial $O_2^{\bullet-}$ generation could prove to be fatal in various neuropathological conditions.

The literature indicates that RS-induced oxidative damage may partly be linked with age-related cellular changes and its associated diseases [171, 172]. Since the production of ROS is essential to the progression of oxidative damage, it is a reasonable outcome that caspase-3 deletion would help in the reduction of this damage possibly through diminishing the production of $O_2^{\bullet -}$ within the mitochondria. Taken together, these data suggests that caspase-3 activation lies upstream of ROS generation in mouse brains *in vivo*, and that its depletion provides a beneficial effect on oxidative stress in the brain tissue.

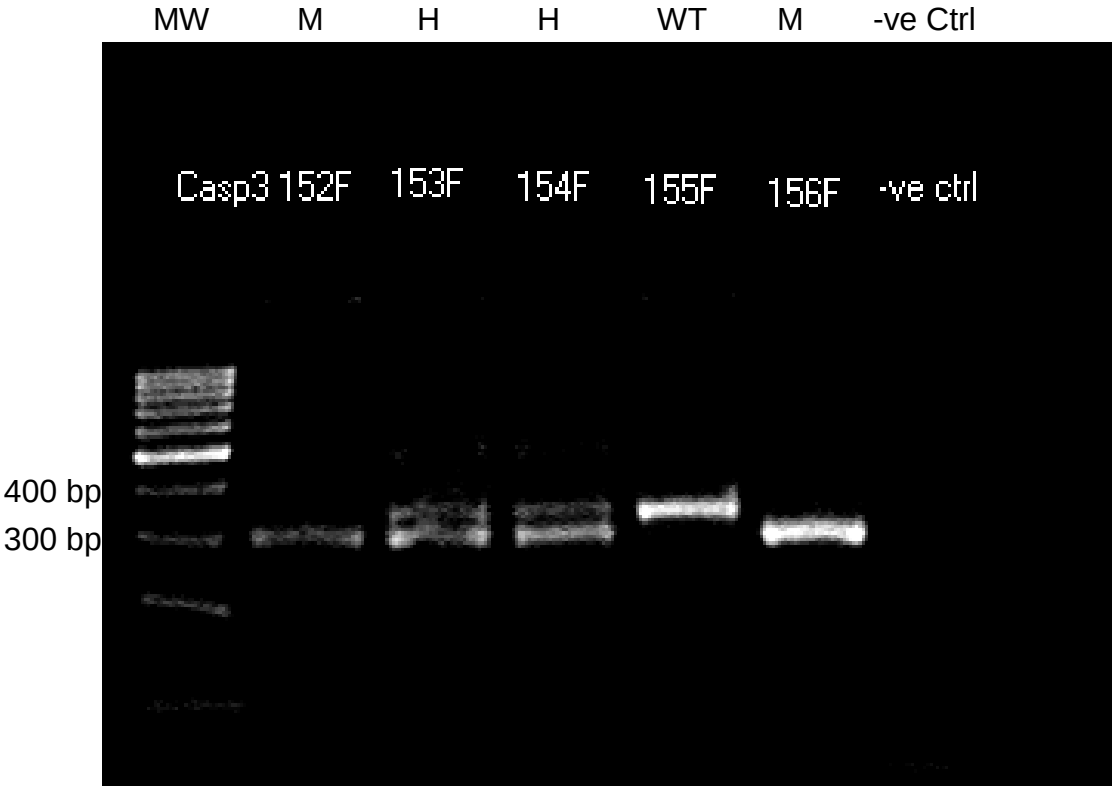


Figure 2.1: DNA gel electrophoresis of caspase-3 mice. Representative agarose gel electrophoresis showing DNA laddering that is illustrating the different genotypes of caspase-3. MW represents the DNA molecular weight marker. Caspase-3 mutant (M) mice show 300 bp band, wild-type (WT) shows 320 bp band, while the caspase-3 heterozygote (H) shows both the 300 bp and 320 bp bands.

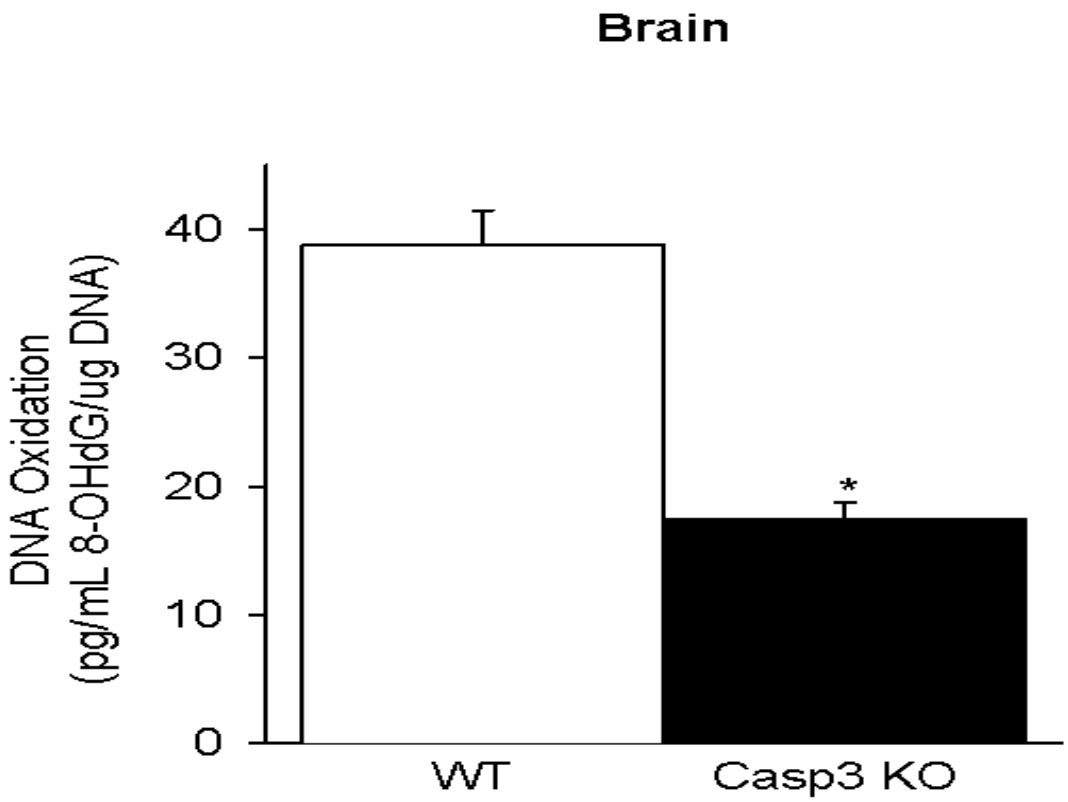


Figure 2.2: Caspase-3 deletion inhibited oxidative DNA damage in the aged mouse brains. The increase in the levels of oxidized DNA in the wild-type mouse brain was significantly ($p < 0.01$) suppressed by genetic ablation of caspase-3. These mice were 12-months old and the data is shown as pg/mL Oxidized DNA. n = 7-10 separate mouse brain tissue per genotype for oxidative DNA damage ELISA.

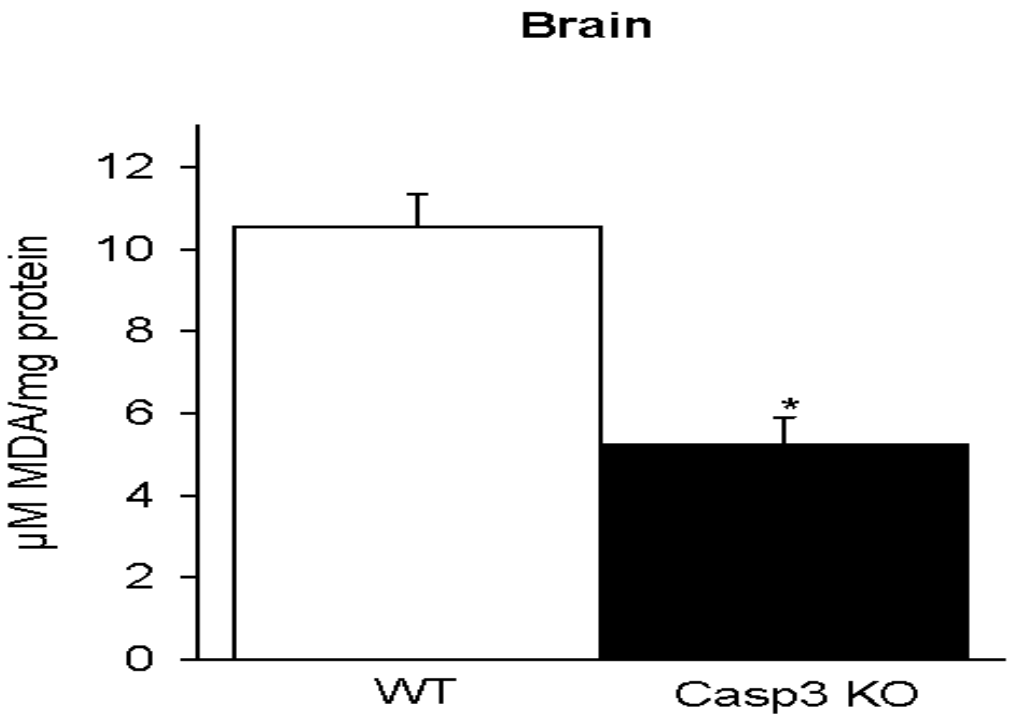
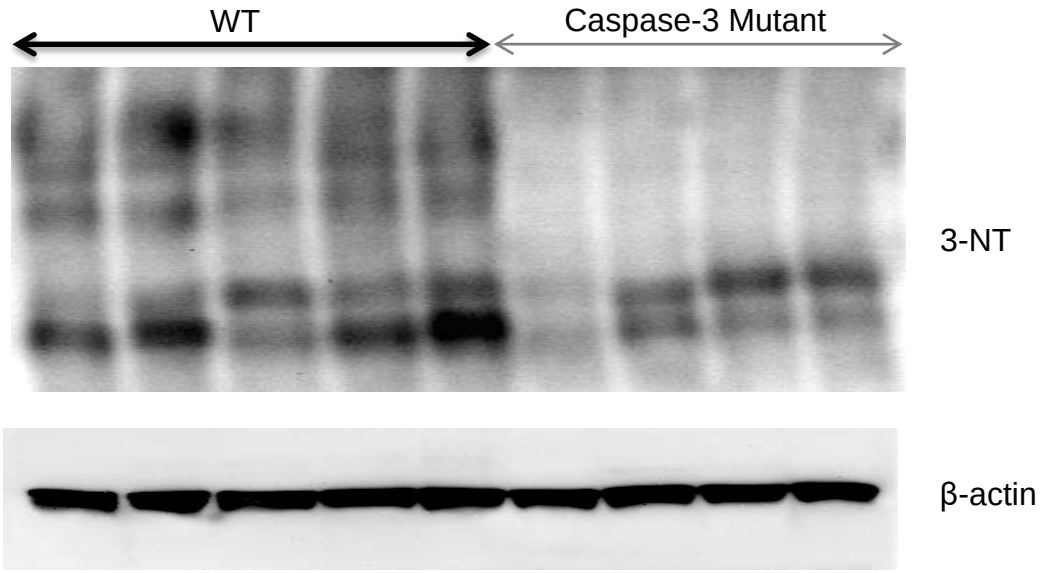


Figure 2.3: Depletion of caspase-3 reduced lipid peroxidation in the whole brains of 12-month-old mice. The levels of MDA were assessed by performing TBARS assay on fresh tissues from mice with caspase-3$^{+/+}$ and caspase-3$^{-/-}$ genotypes. An increase in lipid peroxidation was observed in the wild-type mice. Caspase-3 deletion inhibited this increase and significantly ($p \leq 0.01$) reduced lipid peroxidation. TBARS levels were calculated as µM MDA/mg protein. n = 9-12 separate mouse brain tissue per genotype for TBARS assay.

(A)



(B)

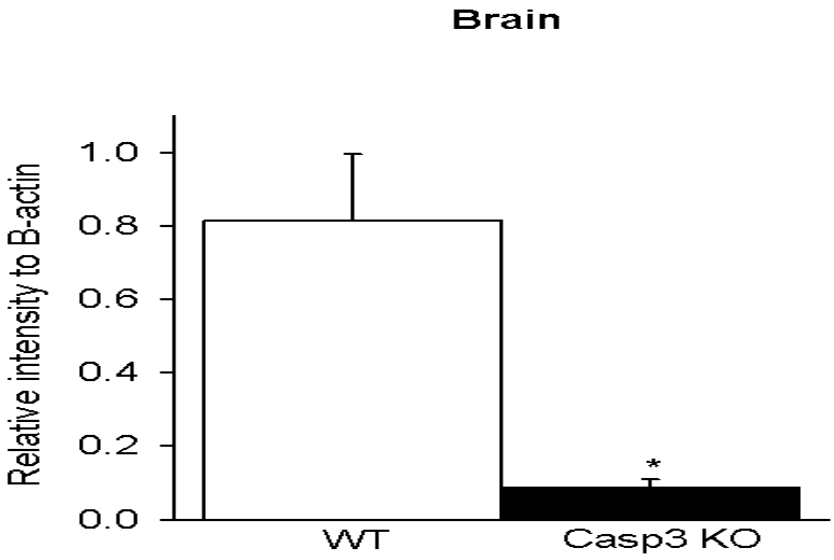


Figure 2.4: 3-NT protein levels in the aged mouse brains. There was a decrease of 3-NT protein levels in the mouse brain (A, B) on caspase-3 deletion ($p < 0.01$). Data is normalized to the β-actin levels (immunoblot band density). n = 5-10 separate mouse brain tissue per genotype for western blot analysis.

CHAPTER 3

EFFECT OF CASPASE-3 DELETION ON OXIDATIVE STRESS IN AGING TISSUES

Introduction

As per the poet Ralph Waldo Emerson, “The surest poison is time”. Biology has indeed explained that many functions of the body deteriorate with age. Aging is a process delineated by the progressive loss of tissue and organ function. Normal aging in different animal models and in humans is characterized by an accumulation of damage to DNA, lipids, and proteins [173, 174]. The oxidative stress theory of aging is based on the assumption that age-related functional losses are due to the augmentation of ROS/RNS-induced damages to these macromolecules [72]. An imbalance between RS generation and antioxidant defenses induces oxidative stress. Numerous studies have shown that the capability of heart to withstand oxidative stress decreases with age due to a reduction in the levels of antioxidant enzymes (e.g. GSH-Px and SOD), leading to the development of cardiovascular diseases [175]. Oxidative stress is also involved in several other age-related pathologies such as acute and chronic kidney disease, neurodegenerative diseases, macular degeneration, biliary diseases, and cancer [176].

Caspases are a family of cysteinyl aspartate-specific proteases that act as the executioners of apoptotic cell death. Some caspases also serve as regulatory molecules for immunity, cell differentiation and cell-fate determination [177]. Previous studies from our laboratory have reported that caspase-3 is necessary for increased production of $O_2^{\bullet-}$

and downstream ROS in the apoptotic SCG neurons [84]. In this study, our laboratory also showed that long-term treatment with caspase inhibitor, BAF greatly attenuated the levels of $O_2^{\bullet-}$ in nonapoptotic SCG neurons when compared to caspase-3 deletion, and this may indicate that BAF has lots of nonspecific effects. This also demonstrates that other caspases are as well involved in the production of mitochondrial $O_2^{\bullet-}$ in these nonapoptotic cells. The genetic ablation of caspase-3 also has a positive effect in diminishing the oxidative damage to DNA, lipids and proteins in the aged brain (Chapter 2). This is possibly mediated by the reduction in ROS generation due to caspase-3 deletion in the mouse brain. In addition to caspase-3, the activity of other caspases is also high in the liver, heart, and kidneys. Caspase 6 activity is enhanced with aging in the liver and kidney. The activity of caspase-3/7 is also increased in the liver with aging. An increase in caspase 8 activity is seen with aging in the kidney and heart [178]. Additionally, the amount of cellular turnover is greater in these organs compared to the brain. Therefore, the aim of this study is to investigate whether the removal of caspase-3 would aid in the reduction of oxidative damage induced by endogenous ROS production in these aging organs. In the current study we looked at the effect of caspase-3 depletion on oxidative DNA damage, lipid peroxidation, and nitration of protein tyrosine residues in the liver, heart and kidneys of aged C57Bl/6 mice.

Materials and Methods

Materials

The ELISA kit for assessing oxidative DNA damage (Catalog # 589320) was obtained from Cayman Chemical (Michigan, USA). An antibody to 3-Nitrotyrosine (3-NT)

(Catalog # ab61392) was purchased from Abcam (Massachusetts, USA). All other chemicals were obtained either from Sigma (Missouri, USA) or Thermo Fisher Scientific (Massachusetts, USA) unless otherwise mentioned.

Mice mating and genotyping

All animal procedures were audited and approved by the Animal Studies Committee at the University of Georgia. Under the supervision of two ACLAM certified veterinarians in an AAALAC-approved facility, all mice were placed on a 12-hour light/dark cycle and given access to food and water *ad libitum*.

Mice hemizygous for the caspase-3, caspase-$3^{+/-}$ (The Jackson Laboratory; Bar Harbor, ME) were mated resulting in the following 3 genotypes: caspase-$3^{+/+}$, caspase-$3^{+/-}$, and caspase-$3^{-/-}$ which are of the C57BL/6 genetic background. We chose one of these three genotypes (i.e. caspase-$3^{-/-}$) for our study, as this would give the most interesting results. In this study, female mice at the age of 12 months were used to evaluate the oxidative damage *in vivo.* The age selected represents the middle-aged animals and is based on the physiological relevance to the human lifespan.

DNA extraction:

The extraction of genomic DNA was carried out from a tail snip of each mouse using a QuickExtract DNA extraction solution (Epicentre Biotechnologies, Madison, WI). By using this DNA prepared from mouse pups or weanlings, they were then sorted into caspase-3 wild-type, hemizygous and mutant mice by performing genotyping with the help of a single PCR.

Polymerase chain reaction (PCR):

This was performed using a Techne Genius thermocycler. PCR for caspase-3 was carried out as per the directions given by The Jackson Laboratory. In this experiment, 23 µl of reaction mixture consisting of 8.61 µl ddH_2O, 0.63 µl of each primer, and 12.5 µl 2X MangoMix solution was mixed with 2 µl of extracted DNA. The primers used in this reaction include: wild-type forward 5'-GCG AGT GAG AAT GTG CAT AAA TTC-3' and wild-type reverse 5'-GGG AAA CCA ACA GTA GTC AGT CCT-3' for amplification of a 320 bp sequence of the wild-type allele; neo/pgk forward 5'-TGC TAA AGC GCA TGC TCC AGA CTG-3' and wild-type exon reverse to amplify a 300 bp sequence of the mutant allele. The PCR mixture was then subjected to different thermocycling conditions of 94^0 C for 2 min followed by 10 cycles of 94^0 C for 20 sec, 65^o C for 15 sec, and 68^o C for 10 sec. This was continued with another 28 cycles of 94^o C for 15 sec, 60^o C for 15 sec, and 72^o C for 10 sec. The PCR products formed were then separated by 3% (w/v) agarose gel electrophoresis. By using a Fotodyne Foto/Analyst Dual-Light Luminary Workstation with TotalLab software (Fotodyne Inc., Wisconsin, USA), DNA base pairs were visualized with the help of ethidium bromide present in the agarose gel.

Organ harvesting

After anaesthetizing the mice on exposure to CO_2, they were immediately decapitated. The organs such as heart, liver and kidney were dissected out and kept in a microcentrifuge tube chilled on ice, and instantly snap-frozen in a 70% ethanol/dry ice bath and stored at -80^{o}C until use.

Thiobarbituric acid reactive substances (TBARS) assay

Oxidative stress caused by increased production of ROS can modify membrane lipids into highly reactive and unstable lipid peroxides that eventually break down into more stable products like malondialdehyde (MDA). RS can oxidize PUFA such as arachidonic acid into MDA. This can be estimated by the TBARS assay, a technique based on the reaction of MDA with thiobarbituric acid (TBA) [142]. Frozen tissues were thawed on ice and then homogenized in a 10% w/v KCl, and centrifuged at 800x g for 10 min at 4^{o} C. The supernatant obtained was used for measuring the protein concentration using the Bradford Assay. For the TBARS assay, 8.1% sodium dodecyl sulfate (SDS), 20% acetic acid, 10M sodium hydroxide (for pH 3.5) and 0.8% TBA was added to the appropriate amount of supernatant. This mixture was then diluted to 1 mL with dH_2O, and placed in a water bath at 100°C for 1 hour. After cooling, 1 mL of dH_2O and 1 mL of butanol/pyridine (15:1, v:v) mixture was added to this reaction mixture and shaken gently. From this, 200 µl aliquot was pipetted out in a 96-well plate, and the levels of MDA were detected by measuring the absorbance at 532 nm using a Molecular Devices SpectraMax M2 spectrophotometer (Molecular devices, California, USA). Each sample was performed in triplicate. The concentration of lipid peroxides in the sample was calculated from a MDA standard curve that is prepared using 0-10µM strength of tetraethoxypropane, and was expressed as µM MDA per mg protein.

DNA damage

Oxidative damage to DNA is characterized by the changes in purine and pyrimidine bases, single and double-stranded breaks, and cross-linking with other biomolecules

[179]. Among these bases, guanine is highly sensitive to oxidation. A damage to DNA was quantified using DNA/RNA oxidative damage ELISA kit that measures all three oxidized guanine species; 8-hydroxy-2'-deoxyguanosine from DNA, 8-hydroxyguanosine from RNA, and 8-hydroxyguanine from either DNA or RNA. The DNA extracted from different organs including heart, liver and kidney of the aged wild-type and caspase-3 mutant mice was individually added into each well that were previously attached with goat polyclonal anti-mouse IgG. On addition of DNA/RNA oxidative damage monoclonal antibody, it forms a complex with the oxidatively damaged guanine within DNA, and this then binds with the attached antibody. An enzymatic reaction takes place on addition of Ellman's Reagent [143] to each well, resulting into a yellow colored product whose absorbance was measured at 410 nm using a Molecular Devices SpectraMax M2 spectrophotometer. Analysis of each sample was done in duplicate. The amount of oxidized DNA was measured as pg/mL, with the help of a standard curve.

3-Nitrotyrosine Western Blot

After defrosting the mouse tissue, it was homogenized with a glass dounce homogenizer in the ice-cold modified RIPA lysis buffer containing the protease inhibitor cocktail (Calbiochem #539134, La Jolla, CA). Homogenates were then centrifuged for 10 min at 10000x g. The supernatant was used for determining protein concentration using the Bradford reagent (Thermo Fisher Scientific, Rockford, IL). Each sample containing 50 µg protein and sample buffer (100 mM Tris-HCl, 4% SDS, 10% bromophenol blue, 30% glycerol) was loaded into individual wells of 12% Tris-HCl pre-cast gel (Bio-Rad). A

protein molecular weight marker was added in one of the lanes. After loading, SDS-PAGE (Polyacrylamide gel electrophoresis) was carried out by running the gel at 100V for 1 hour. The proteins separated based on their molecular weight were then transferred onto PVDF membranes (Millipore, Bedford, MA) at 100 V for 1-2 hours. This was followed by incubating the membrane with the blocking buffer for 30 mins at room temperature with gentle shaking. Mouse anti-3-nitrotyrosine (3-NT) monoclonal antibody (1:1000 dilution) was then added into the blocking buffer and incubated the membrane for overnight at 4^{o} C with gentle shaking. After overnight incubation, the membrane was washed three times with the wash buffer (TBS-T), with shaking. Each rinse was for 5-10 mins. After incubating the membrane with the secondary antibody, anti-mouse IgG (1:2000 dilution) for 1 h at room temperature with gentle shaking, the washes with TBS-T were repeated. Following the washes, incubation of the membrane in the detection solution, Pierce ECL Western Blotting Substrate (ThermoFisher Scientific, Waltham, MA) was done for 2 mins. Visualization of western blot and its analysis was done using a Fotodyne Foto/Analyst Dual-Light Luminary Workstation with TotalLab software. The protein band density representing 3-nitrotyrosine for each sample was normalized to the respective β-actin levels, and the data was represented as relative intensity to β-actin.

Statistics

Statistical analysis and graph representation were done with SigmaPlot 11.1 (Systat Software, Inc., California, USA). The analysis of data distribution was based on setting up the relevant statistical measures for each experiment. Kruskal-Wallis one-way ANOVA on ranks followed by Dunn's multiple comparisons post-*hoc* test or by the

Mann-Whitney rank-sum test was carried out unless otherwise indicated. All error bars are ± SEM.

Results

Deletion of caspase-3 reduced oxidative damage to lipids in the aged mouse heart, kidney and liver

The effect of caspase-3 deletion on the development of oxidative stress in aging organs was investigated by assessing the levels of lipid peroxides. The thiobarbituric acid reactive substances (TBARS) assay was performed to study lipid peroxidation in these organs. The same groups of female mice (12 months old) were used as in the brain study. Our results showed that caspase-3 depletion significantly reduced the levels of MDA in the mouse heart (Fig. 3.1), kidney (Fig. 3.4), and liver (Fig. 3.7).

Caspase-3 deletion reduced oxidative DNA damage in aged mouse organs

A gradual and irreversible accumulation of free radical-induced damage to cellular macromolecules could lead loss of physiological function and other age-related biological changes [180]. This theory also postulates that the augmentation of this oxidative damage is due to the inability of endogenous anti-oxidant defenses to counteract these changes. Oxidative damage to DNA was investigated by detecting the levels of 8-OHdG in the mouse heart, kidney, and liver at the age of 12 months. Previous studies have shown an age-related increase in the levels of oxidized DNA, 8-hydroxy-2'-deoxyguanosine (8-OHdG) in nuclear DNA extracted from liver, heart, brain, kidney, skeletal muscle, and spleen isolated from the C57Bl/6 mice studied. The levels of 8-OHdG in mitochondrial

DNA extracted from the livers of rats and mice were also elevated with age [181]. In the current study, ablation of caspase-3 gene significantly attenuated the increased oxidative damage to DNA in the heart (Fig. 3.2), kidney (Fig. 3.5), and liver (Fig. 3.8) of 12-month old female mice.

Caspase-3 deletion reduces nitration of protein tyrosine residues in the aged mouse organs

To discern the differences in protein tyrosine nitration in the heart, kidney, and liver between the wild-type and caspase-3 mutant mice, the proteins were probed for 3-nitrotyrosine (3-NT) using an antibody that specifically recognizes this posttranslational modification. The western blot of mouse heart (Fig. 3.3), kidney (Fig. 3.6), and liver (Fig. 3.9) clearly demonstrated a decrease in the nitrotyrosine content of proteins in the caspase-3 mutant mice when compared to the respective organs of the wild-type mice. This indicates that deletion of caspase-3 not only reduces oxidative stress in the aged brain but also suppresses oxidative DNA damage, lipid peroxidation, and nitration of protein tyrosine in the heart, liver, and kidney possibly by attenuating the production of mitochondrial ROS.

Discussion

The objective of the current study was to examine whether caspase-3 deletion only exerts beneficial effect on oxidative stress in the aging mouse brain or is a global phenomenon exhibiting in a widespread of organs. The free radical theory of aging was originally proposed by Harman in 1956, and was later termed as oxidative stress theory of aging.

This theory is based on the structural damage-based hypothesis suggesting that free radicals generated during oxidative phosphorylation induce detrimental effects on cellular components and connective tissues. This accumulation of oxidative damage may increase as a function of age. Though there has been a great deal of controversy over this theory because the inadequacy of specific endogenous antioxidants in mice did not reduce their survival [182]. And also another study contradicting this theory found that the longevity of anaerobically grown yeast cells was shorter than the cells grown aerobically [183]. The RS produced plays a major role in the aging process as well as in various age-related conditions such as cardiovascular diseases, chronic obstructive pulmonary disease, chronic kidney disease, neurodegenerative diseases, and cancer [176]. Previous studies have shown that the cells or tissues isolated from older animals are more susceptible to oxidative stress-induced damage *in vitro* as compared to the cells or tissues from younger animals [184, 185]. In the current study, we assessed the oxidative damage to DNA, lipids, and proteins in 12-month old female wild-type mice and investigated whether or not genetic ablation of caspase-3 would attenuate this deterioration in the heart, liver and kidneys.

Is it possible that the caspase-3-dependent impairment of DNA and proteins, and oxidative stress in these organs are a consequence of apoptosis? This cannot be ruled out. Though, our previous work has clearly demonstrated that apoptotic cell death is not required for caspase-3 to produce pro-oxidant effects [84]. In the neurons studied, caspase inhibitors largely decrease RS levels in apoptotic and nonapoptotic CT and other neurons [84, 151]. Low levels of basal (non-apoptotic) caspase activity exist in numerous cell types and mediate normal physiological functions [186]. Genetic depletion of

caspase-3 led to decreased oxidative damage to DNA, lipids, and proteins in the mouse brain *in vivo* (Chapter 2). The difference between these organs (i.e. liver and kidneys) and the mouse brain is that they have greater cellular turnover rate that aid in lowering the accumulation of oxidative damage. These organs, in addition to caspase-3, also express high levels of other executioner caspases that are equally important in the apoptotic cell death, and may also counteract the beneficial effect of caspase-3 deletion on oxidative stress. However, this was not the case in our study. Removal of caspase-3 greatly reduced the oxidative damage to DNA in all the tissues examined and these results were similar to that of the aged brain tissue. The increases in lipid peroxidation in these organs were also eliminated by the genetic deletion of caspase-3. The nitration of protein tyrosine residues caused by peroxynitrite and other RNS was as well decreased in the heart, liver and kidneys of caspase-3 mutant mice when compared to the wild-type mice. This suggests that caspase-3 deletion partly does help in the alleviation of oxidative damage in the aged mice.

Oxidative stress can initiate pathological conditions in the peripheral organs such as heart, liver and kidney. By disrupting cellular lipids, DNA and proteins, the ROS induces necrosis and apoptosis of hepatocytes, and this could produce liver damage. In response to oxidative stress in hepatocytes, the inflammatory cells such as macrophages and lymphocytes get activated and produce inflammatory mediators. ROS can also initiate the progression of liver fibrosis by inducing the synthesis and release of profibrogenic mediators from Kupffer cells and systemic inflammatory cells. The oxidative stress-related molecules may directly stimulate hepatic stellate cells, resulting in hepatic fibrogenesis [187]. Mitochondrial ROS produced may cause an impairment of

hepatic metabolism by oxidatively altering cytochrome P450 enzymes. This could result in an increase or decrease in the therapeutic activity of the pharmacological drugs consumed. An alteration in the activity of cytochrome P450 enzymes would also influence the endogenous biomolecules and their physiological effects. Oxidative stress within mitochondria is also linked with an inflammatory response in alcoholic liver disease. An elevated ROS levels can stimulate the expression of hypoxia-inducible factor-1 alpha that enhances the secretion of inflammatory mediators such as TNF-α, and thus resulting in immune response that further aggravates the hepatic injury [188]. The ability of heart to pump blood depends on the functional activity of ATP-dependent pump system such as Na^+/K^+-ATPase present within the cardiac cells. Any disruption in the enzymatic activity of respiratory complexes caused either directly by the cleaving effect of caspase-3 or indirectly through caspase-3 mediated oxidative damage could lead to a reduction in the synthesis of ATP. This would result in dysfunctioning of the energy dependent pump system that ultimately gives rise to cardiovascular diseases. Loss of ATP production results into accumulation of Na^+ within the cardiac cells causing depolarization of the resting membrane potential. This alters the impulse formation and/or impulse conduction leading to cardiac arrhythmias. By preventing ROS-induced changes in the expression and activity of Ca^{2+} channels, deletion of caspase-3 could reduce intracellular Ca^{2+}-overload in cardiomyocytes, and thus may exert a beneficial effect in hypertension, ischemic heart disease and heart failure. Oxidative damage can also affect renal function by interfering with the reabsorption of Na^+ in tubular cells of the nephron through the effect of ROS on the activity of renal Na^+/K^+-ATPase pump. Increased ROS production directly augments renal vascular resistance causing

vasoconstriction of renal blood vessels [189] and this may as well impact the functions of cardiovascular system. Genetic deletion of caspase-3 may also prevent impairment in renal membrane transport and metabolism by suppressing oxidative damage to renal cytochrome P450s. Thus, by studying the effects of caspase-3 on oxidative stress markers will broaden our knowledge not only on the likely mechanisms of mitochondrial $O_2^{\bullet-}$ generation but also on the possibility of exerting beneficial effects from oxidative damage by caspase-3 deletion. And this is not only specific to aged mouse brain but is widespread throughout the body.

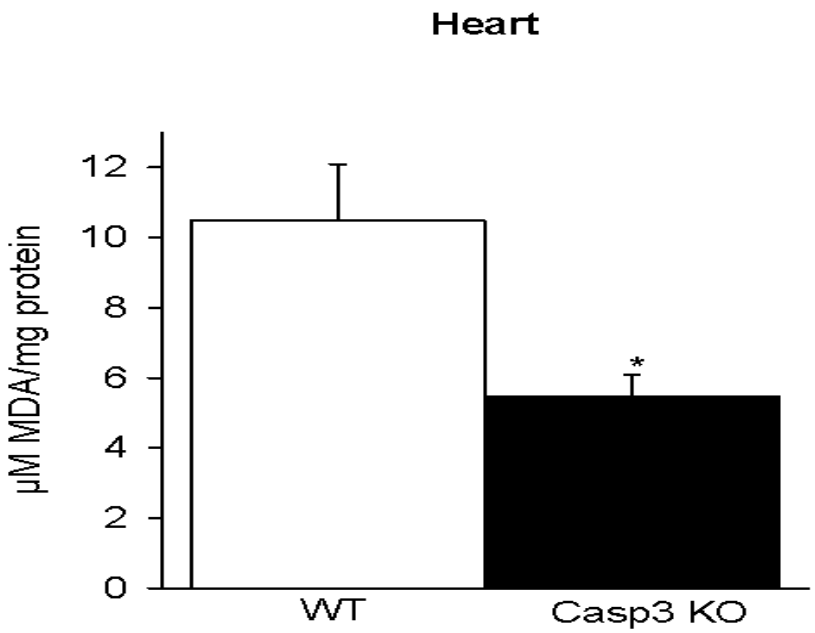


Figure 3.1: Caspase-3 depletion reduced lipid peroxidation in the whole heart of 12-month-old female mice. The levels of MDA were detected by performing TBARS assay on fresh tissues from mice with caspase-3$^{+/+}$ and caspase-3$^{-/-}$ genotypes. An increase in lipid peroxidation was observed in the wild-type mice. Caspase-3 deletion inhibited this increase and significantly ($p \leq 0.01$) reduced lipid peroxidation. TBARS levels were calculated as µM MDA/mg protein. n = 9-12 separate mouse heart per genotype for TBARS assay.

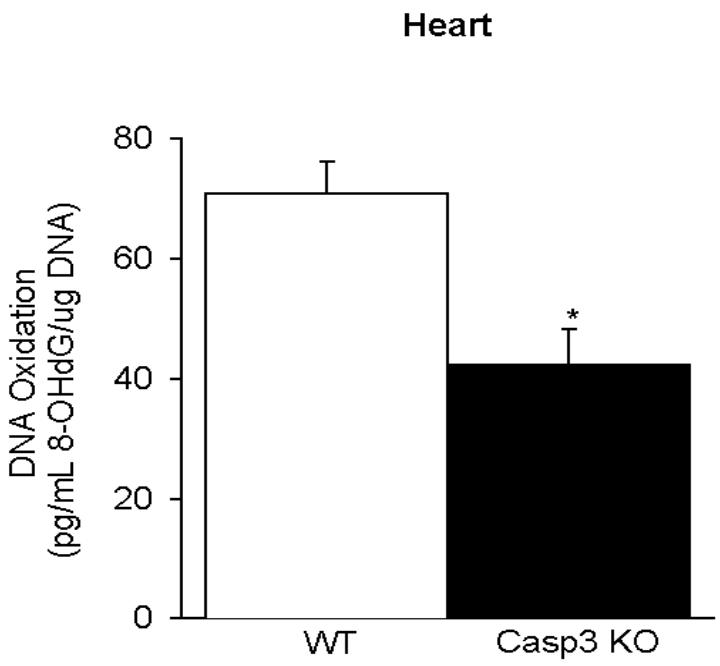
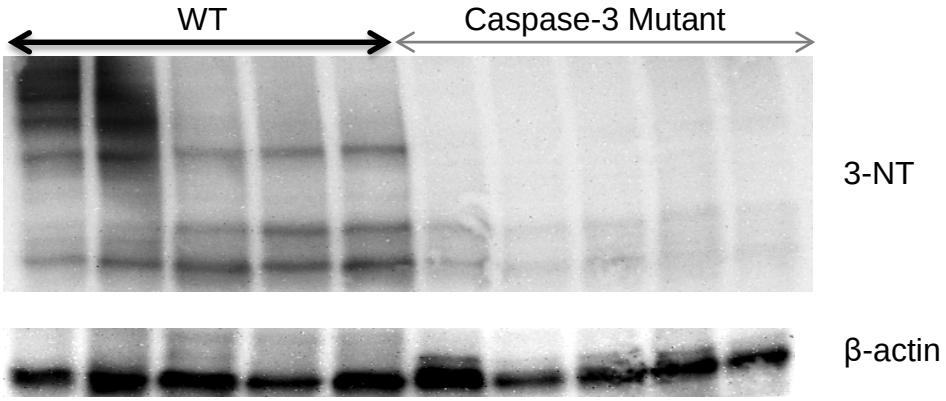


Figure 3.2: Caspase-3 deletion inhibited oxidative DNA damage in the aged mouse heart. The increase in the levels of oxidized DNA in the wild-type mouse heart was significantly ($p < 0.01$) suppressed by genetic ablation of caspase-3. These mice were 12-months old and the data is shown as pg/mL Oxidized DNA. n = 7-10 separate mouse heart per genotype for oxidative DNA damage ELISA.

(A)



(B)

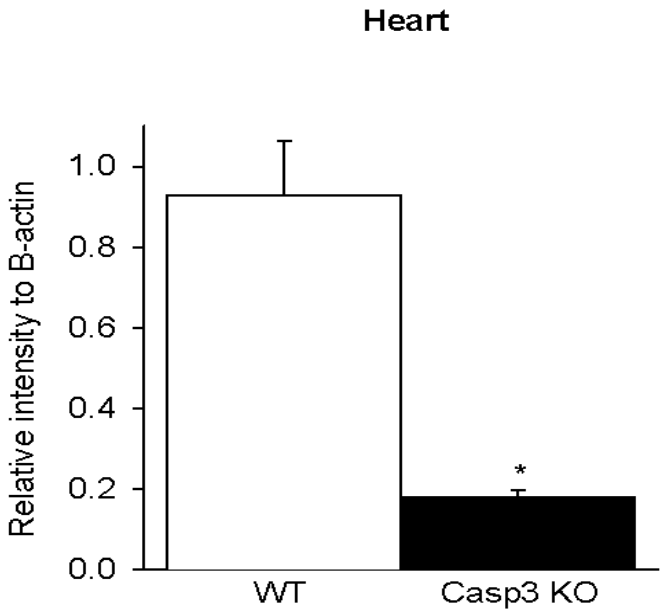


Figure 3.3: 3-NT protein levels in the aged mouse heart. There was a decrease of 3-NT protein levels in the mouse heart (A, B) on caspase-3 deletion ($p < 0.01$). Data is normalized to the β-actin levels (immunoblot band density). n = 5-10 separate mouse heart per genotype for western blot analysis.

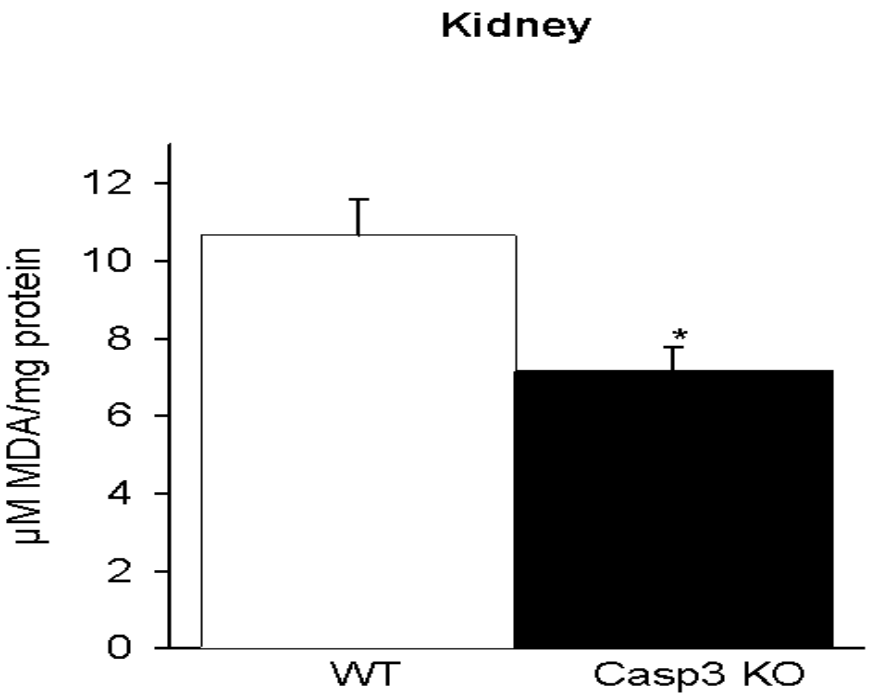


Figure 3.4: Depletion of caspase-3 suppressed lipid peroxidation in the kidneys of 12-month-old mice. The levels of MDA were assessed by carrying out TBARS assay on fresh tissues from mice with caspase-3$^{+/+}$ and caspase-3$^{-/-}$ genotypes. An elevation in lipid peroxidation was seen in the wild-type mice. Caspase-3 deletion reduced this increase and significantly ($p \leq 0.01$) decreased lipid peroxidation. TBARS levels were calculated as µM MDA/mg protein. n = 9-12 separate mouse kidney per genotype for TBARS assay.

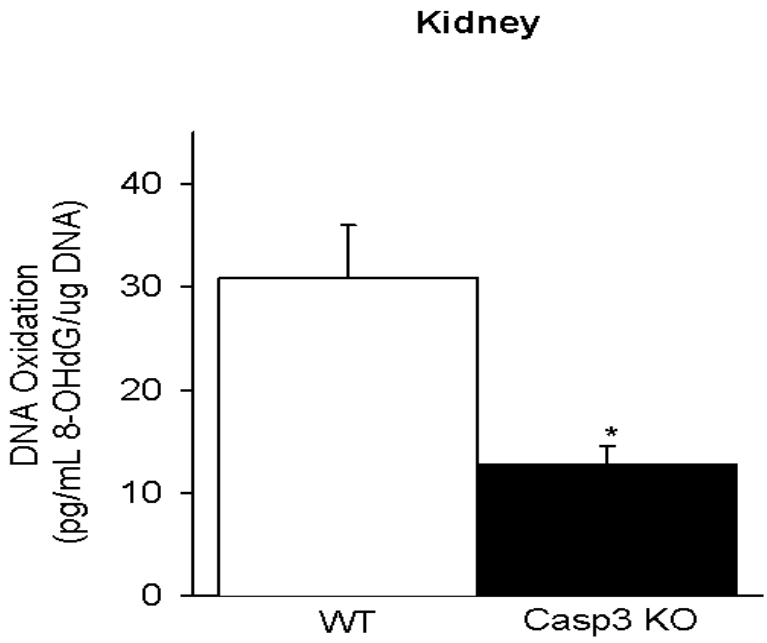
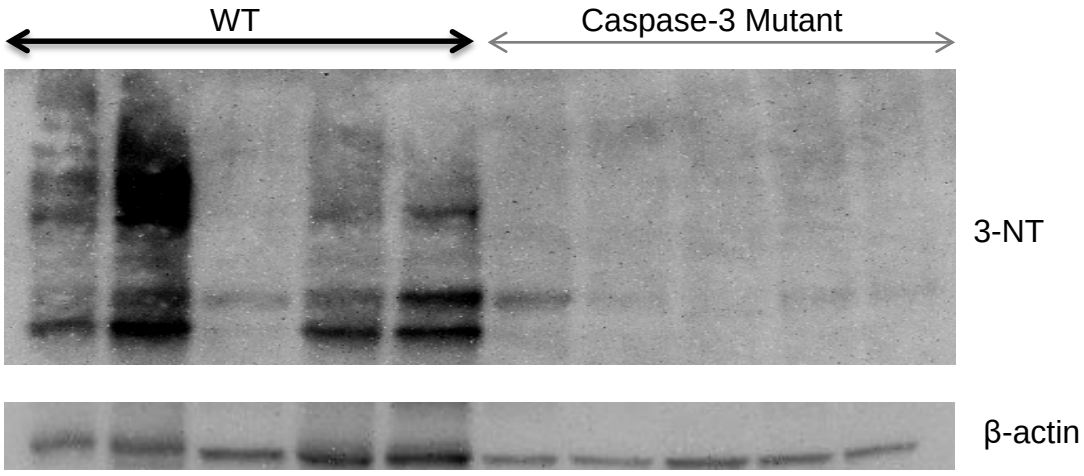


Figure 3.5: Genetic ablation of caspase-3 decreased oxidative DNA damage in the aged mouse kidneys. The increase in the levels of 8-OHdG in the wild-type mouse kidney was significantly ($p < 0.01$) suppressed by genetic deletion of caspase-3. These mice were 12-months old and the data is shown as pg/mL Oxidized DNA. n = 7-10 separate mouse kidney per genotype for oxidative DNA damage ELISA.

(A)



(B)

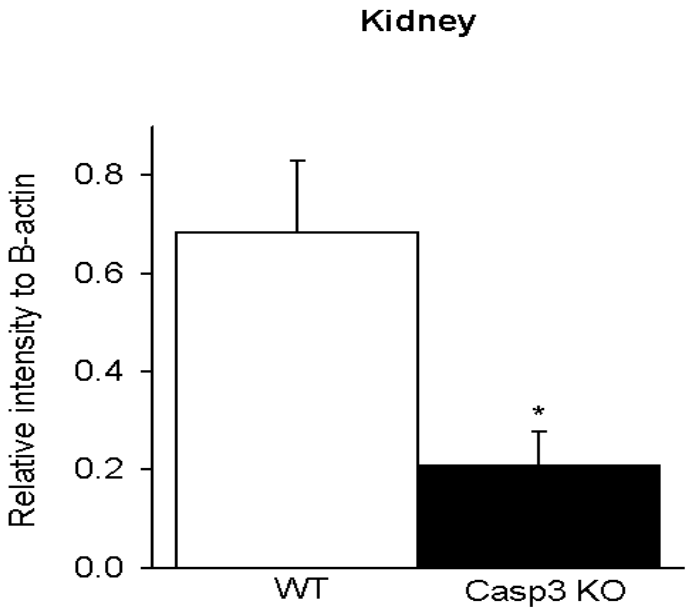


Figure 3.6: 3-NT levels in the aged mouse kidneys. There was a reduction in the 3-NT protein content in the mouse kidney (A, B) on caspase-3 deletion ($p < 0.05$). Data is normalized to the β-actin levels (immunoblot band density). n = 5-10 separate mouse kidney per genotype for western blot analysis.

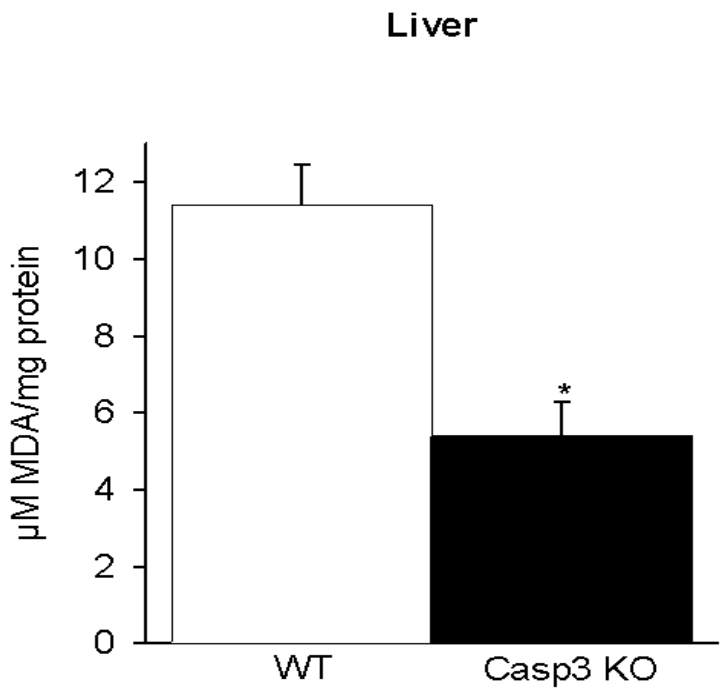


Figure 3.7: Depletion of caspase-3 attenuated lipid peroxidation in the livers of 12-month-old mice. The levels of MDA as assessed by TBARS assay performed using fresh tissues from caspase-$3^{+/+}$ and caspase-$3^{-/-}$ mice were significantly ($p \leq 0.01$) decreased in the mouse liver on caspase-3 depletion. TBARS levels were calculated as µM MDA/mg protein. n = 9-12 separate mouse liver per genotype for TBARS assay.

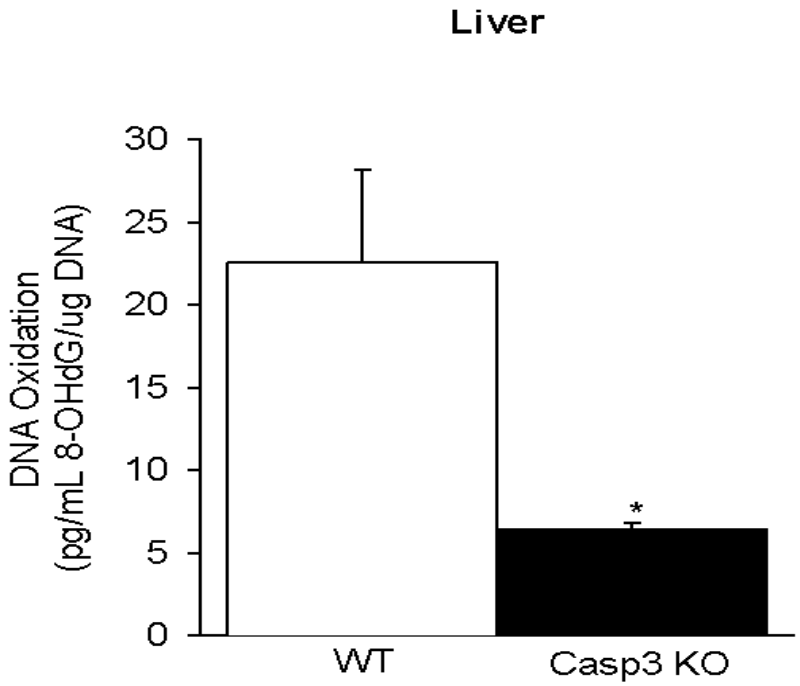
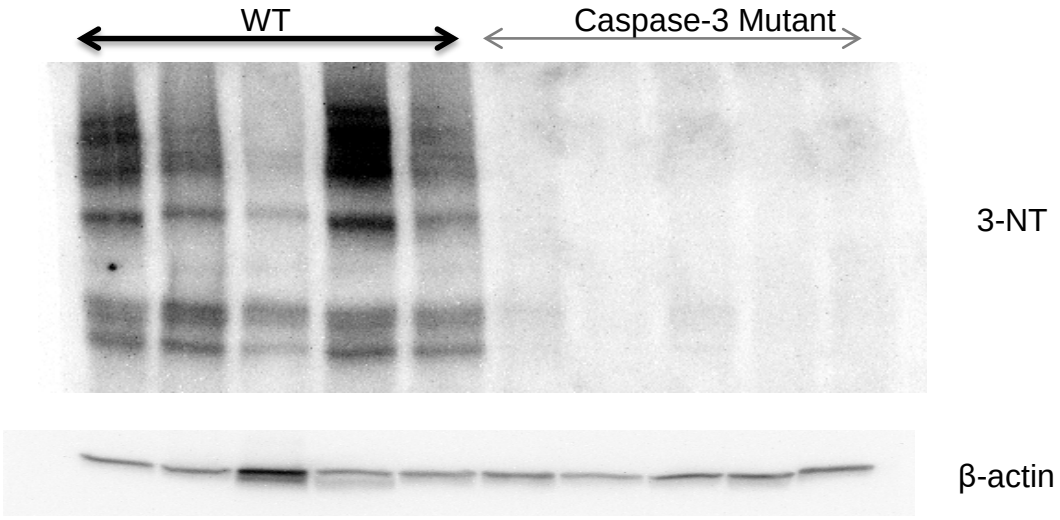


Figure 3.8: Caspase-3 deletion suppressed RS-induced DNA damage in the aged mouse livers. The increase in the levels of oxidized DNA in the wild-type mouse liver was significantly ($p < 0.01$) decreased by genetic deletion of caspase-3. These mice were 12-months old and the data is shown as pg/mL Oxidized DNA. n = 7-10 separate mouse liver per genotype for oxidative DNA damage ELISA.

(A)



(B)

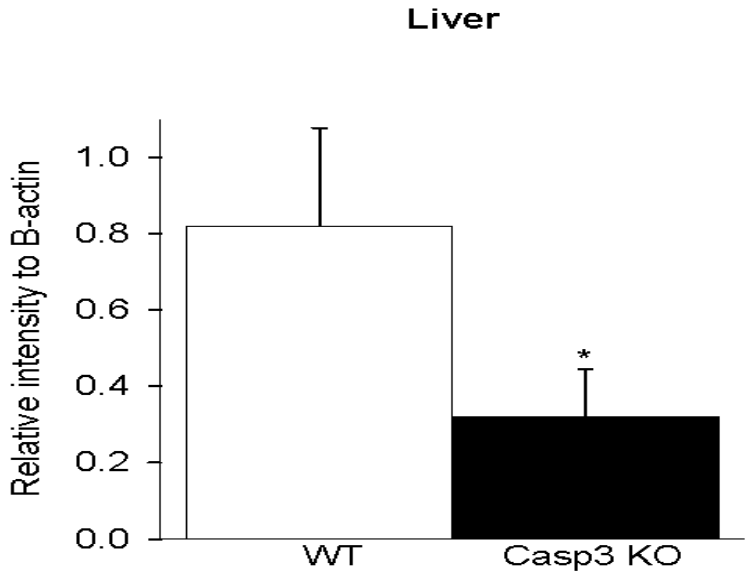


Figure 3.9: Nitration of protein tyrosine residues in the aged mouse livers. There was a significant ($p < 0.05$) reduction in the levels of 3-NT in the mouse liver (A, B) on caspase-3 deletion. Data is normalized to the β-actin levels (immunoblot band density). n = 5-10 separate mouse liver per genotype for western blot analysis.

CHAPTER 4

CONCLUSION

The current data demonstrates that the executioner caspases such as caspase-3 are not only important in apoptotic cell death but are also involved in inducing oxidative stress in aged mouse tissues *in vivo*, suggesting a role of caspase-3 in the production of ROS. This was accomplished by determining the markers of oxidative damage in the brain, kidney, heart and liver of aged wild-type and caspase-3 mutant mice. Oxidative stress is linked with the pathology of many diseases and therefore it is of utmost important to study the proteins involved in oxidative damage within tissues. Caspase-3(p32) is a member of the cysteine-aspartic acid protease family that gets activated by cleavage into p20 and p12 fragments, and causes degradation of various intracellular substrates [190]. It was discovered that the increase in DNA damage, lipid peroxidation, and nitration of protein tyrosine in different tissues of middle aged, wild-type female mice was eliminated by the deletion of caspase-3. This suggests the effect of caspase-3 in the process of oxidative stress and that caspase-3 deficiency in the mouse suppresses RS-induced damage in the aged brain, heart, liver, and kidney.

The ROS can be produced by caspase-3 through multiple mechanisms. One mechanism is that caspase-3 causes cleavage of p75 subunit (Ndufs1, NADH-ubiquinone oxidoreductase subunit) of respiratory complex I resulting into disruption of electron transport and mitochondrial membrane potential. This ultimately leads to generation of ROS, decrease in ATP levels, and subsequently mitochondrial damage [141]. This

mechanism of mitochondrial ROS production was further supported by another study. They clearly demonstrated that ROS formation occurs downstream of Bax- and Bak-mediated mitochondrial outer membrane permeabilization and is induced by the caspase-3-mediated cleavage of Ndufs1 subunit in response to TNFα plus cycloheximide [87]. A study in rat hippocampus showed protein carbonylation of 75 kDa subunit (possibly Ndufs1) of complex I, leading to impairment of its enzymatic activity during epilepsy development [191]. This suggests that disruption of Ndufs1 subunit inhibits complex I activity and this could further increase the generation of $O_2^{\bullet-}$. Another mechanism that strongly engenders the spontaneous production of mitochondrial ROS is through disruption of the interaction between cytochrome c1 subunit of complex III and cytochrome c. This disruption is mediated by caspase-3 through cleavage of cytochrome c1 at D106 site that is essential for binding with cytochrome c, and thus ultimately resulting into loss of oxidative phosphorylation and $O_2^{\bullet-}$ generation [192].

Stimulation of peripheral inflammatory response induced by oxidative stress not only affects heart, liver and kidney but could also influence physiological functions of neuronal and glial cells in the brain. The immune cells activated by ROS and inflammatory mediators can infiltrate into the brain tissue through the blood-brain barrier (BBB) and this may induce microgliosis and astrogliosis. This gliosis further promotes death of dopaminergic neurons in the substantia nigra by releasing various pro-inflammatory mediators such as interleukins, TNF-α, and chemokines, thus resulting in neuropathologies like PD. Impairment of cardiovascular function may as well have an impact on brain pathologies. Development of cardiac arrhythmias and disrupted excitation-contraction coupling engendered by excessive mitochondrial ROS production

leads to the pathogenesis of myocardial ischemia/reperfusion (I/R) injury and stroke. Prolonged ischemia causes death of neurons that are fed by blood vessels downstream of obstruction. Overproduction of mitochondrial $O_2^{\bullet -}$ deteriorates endothelium-dependent vasodilator mechanisms, possibly by reacting with NO and facilitating $ONOO^-$ formation, resulting in ischemia. If this vascular obstruction occurs in the cerebral circulation, it induces stroke [193]. Hence, by finding out the mechanisms by which caspase-3 causes production of mitochondrial $O_2^{\bullet -}$, one could possibly design novel therapeutic approaches that either suppress the effect of caspase-3 or prevent its binding to complex I and complex III subunits. This may be beneficial in reducing ROS-induced postischemic tissue injury, and abolishing neurocognitive deficits and neuronal cell death in stroke.

In conclusion, our results prompted the hypothesis that caspase-3 deletion may cause attenuation of oxidative stress markers in the aged brain as well as in other organs. This is exactly what was observed in the liver, heart, and kidneys of the aged mouse. This overall suggests that caspase-3 lies upstream of production of ROS and that the effect of caspase-3 deletion on the regulation of oxidative damage is more globally directed.

CHAPTER 5

FUTURE STUDIES

The electrons transported across the mitochondrial respiratory complexes not only serve as an energy source for ATP synthesis, but also induces ROS formation by monovalently reducing molecular oxygen to form the superoxide radical anion ($O_2^{\bullet -}$) [194]. ROS are comprised of both oxygen-centered radicals (unpaired electrons), as well as non-radicals that serve as oxidizing agents or are easily transformed into radicals [195]. The $O_2^{\bullet -}$ produced within mitochondria is mostly dismutated by the manganese superoxide dismutase (MnSOD/SOD2) to hydrogen peroxide (H_2O_2). This is then reduced to H_2O by oxidation of the tripeptide, glutathione (GSH). Oxidative stress arises when the $O_2^{\bullet -}$ levels surpass the defense of the intramitochondrial antioxidants, and this ensues damage to the vital mito-enzymes by oxidizing iron-sulfur (Fe-S) centers of non-heme proteins of the TCA cycle and mitochondrial electron transport chain. In addition, the free ferrous iron (Fe^{2+}) released from this reaction will produce highly reactive hydroxyl radical ($OH^{\bullet}$) via Fenton pathway. This $OH^{\bullet}$ is very toxic as it triggers a series of lipid peroxidation and oxidative damage to proteins and DNA [19]. Hence, not only $O_2^{\bullet -}$ but also its downstream reaction byproducts such as other ROS and RNS (Fig. 5.1) can be cytotoxic to the cell and its components.

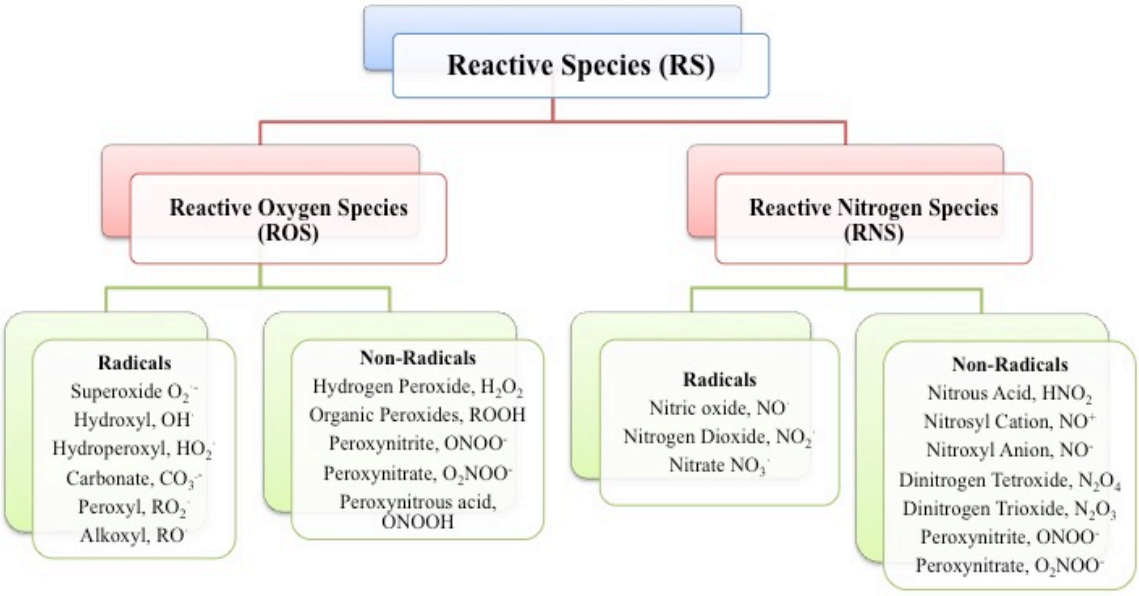


Figure 5.1: Biologically active ROS and RNS.

The ROS generated have a very short half-life, making it difficult to directly measure them *in vivo*. Hence, determination of ROS levels is mostly based on the molecular footprints left in their wake of destruction [19]. In the current study, we used the redox-sensitive dye 5-(and-6)-chloromethyl-2', 7'-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA) to assess the RS levels in cortical neurons *in vitro*. On oxidation by RS, this dye becomes fluorescent when excited by the 488 nm laser-line, and it mainly detects H_2O_2-associated ROS, and RNS [196]. This dye has been extensively used to measure RS levels in the rat and mouse sympathetic neurons [59, 197]. So far, we have determined the RS levels in the apoptotic and non-apoptotic cortical neuronal cultures from wild-type pups (0-1 day-old). Apoptosis was induced in

cortical neurons by treating them with staurosporine (STS), a bacterial alkaloid that inhibits various protein kinases [198, 199], as well as stimulates the mitochondrial apoptotic pathway. Studies have demonstrated that STS engenders cytochrome c release, activation of caspases [200, 201], production of intracellular ROS, and causes an elevation in the intracellular calcium levels [200, 202, 203]. In the present study, we also evaluated the effect of rotenone (mitochondrial complex I inhibitor) and/or antimycin-A (complex III inhibitor) treatment on the RS levels in apoptotic and non-apoptotic neuronal cultures from wild-type pups (0-1 day-old). The detailed procedure for culturing cortical neurons *in vitro* and the treatment protocol is described below (Fig. 5.2).

Cortical cell culture: Primary cortical neuronal cultures were prepared from the cerebrums of neonatal (0-1 day-old) C57Bl/6 mouse pups using the method of Xiang, *et al*. (1996) [204] with few changes. These include decreased trypsinization time, using poly-L-lysine in place of poly-D-lysine to coat the coverslips, and segregation of cells done using a 1 mL eppendorf pipette tip instead of fire-polished pasteur pipette. After killing the mouse pups on post-natal day 0/1 by decapitation, the dissected cerebrums were instantly placed in the ice-cold Leibovitz (L-15) medium. This was followed by the removal of meninges and chopping up the tissue before centrifuging it for 3 minutes at 4°C. Following extraction, the cortices were digested by incubation with trypsin (1 mg/mL) in Hank's Balanced Salt Solution without Ca^{2+} and Mg^{2+} (HBSS; Sigma, St. Louis, MO) for 15 minutes at 37°C. The tissue was then rinsed with trypsin inhibitor (2 mg/mL) in HBSS with Ca^{2+} and Mg^{2+} (Sigma) containing 30 units of DNase (Sigma) so as to inactivate the trypsin, centrifuged, and the supernatant discarded. To ensure that the tissue is totally free of trypsin, it was again washed 1x with 1 mg/mL trypsin inhibitor

and 1x with conditioned growth medium. The neurons were further dissociated by trituration in growth media. The neuronal cells were plated on poly-L-lysine-coated, #1 glass coverslips and maintained in 35 mm culture dishes. Cortical cultures were then incubated in Neurobasal-A growth medium that was supplemented with 2% B-27 serum-free supplement, 1% Penicillin/Streptomycin, 0.1% L-glutamine, and 20 mM fluorodeoxyuridine. The neuronal cultures were maintained at 37°C in an atmosphere of 5% CO_2 and 95% air. All the experiments were carried out at 4-5 days *in vitro* (DIV).

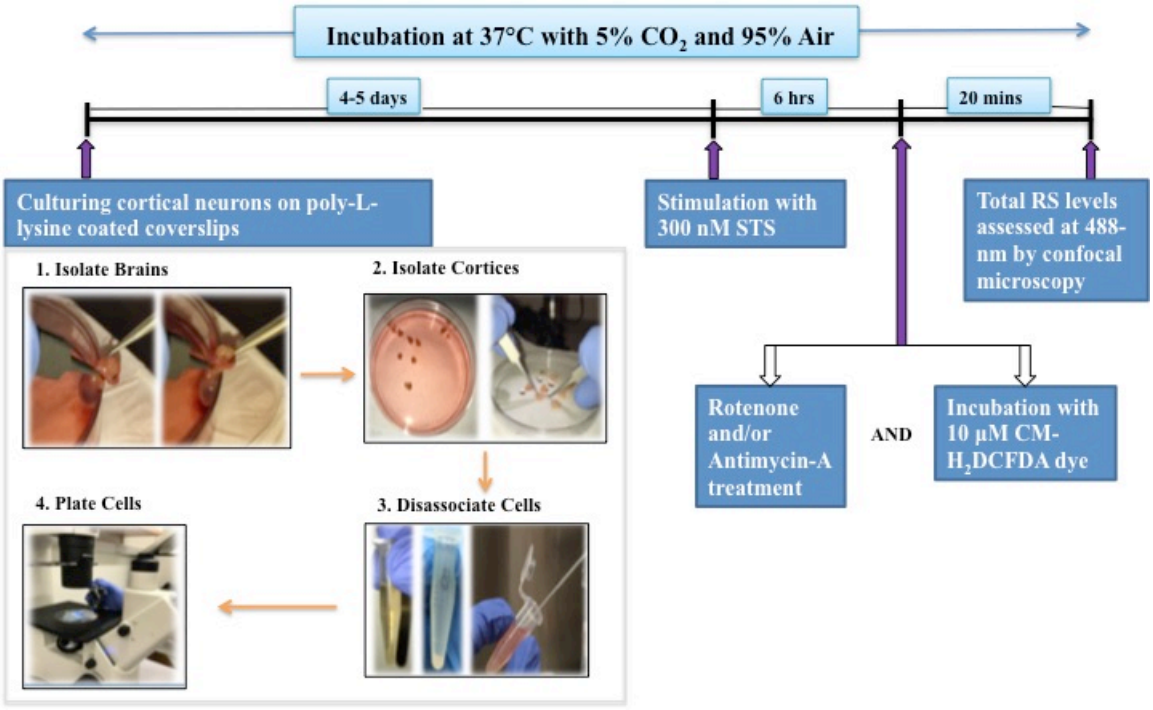


Figure 5.2: Illustration of primary cortical neuronal culture and treatment protocol.
Images adopted from: https://bio-protocol.org/e2501 [205].

Treatment conditions: At 4-5 DIV, Bax-dependent apoptosis was induced in the cortical cultures by treating them with 300 nM STS for 6 hrs. The apoptotic and non-apoptotic cultures were then incubated for 20 minutes at 37°C in a suitable experimental medium containing either rotenone and/or antimycin-A, along with 10 mM CM-H_2DCFDA fluorescent dye. Control (untreated) and only STS-treated cells were also incubated in the medium containing 10 mM CM-H_2DCFDA. The neuronal cultures were then washed twice with L-15 medium and kept in the second wash for confocal microscopy. The excitation of the CM-H_2DCFDA dye was done using a 488 nm laser-line of the confocal. All microscopy was carried out at room temperature.

In our *in vivo* studies (Chapter 2 and 3), we demonstrated that genetic deletion of caspase-3 reduces the markers of oxidative stress in the brain, heart, liver and kidney of 12-month old mice. Our future studies are focused on determining the mechanism(s) by which caspase-3 induces the generation of $O_2^{\bullet-}$ through its effect on either mitochondrial complex I and/or complex III in the cortical neurons *in vitro*. The objective for the initial part of this study is to localize the respiratory complexes involved in the production of $O_2^{\bullet-}$ by the use of specific substrates and inhibitors of these complexes. We have already looked at the effect of STS treatment on the total ROS levels in the cortical cultures isolated from wild-type mouse pups. On stimulation with STS, the total ROS levels as detected by CM-H_2DCFDA intensity were significantly increased in the cortical neurons (Fig. 5.3). This is in agreement with previous studies that have shown an elevation in the levels of ROS in various neuronal cell types on treatment with STS [25, 206].

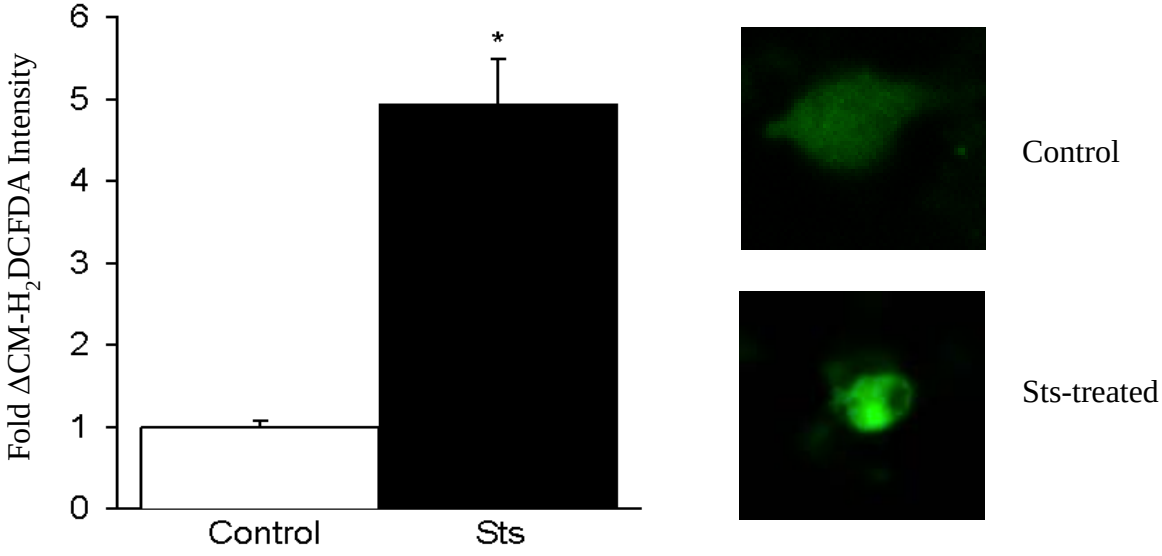


Figure 5.3: RS production after staurosporine treatment.

*Left: Wild-type (caspase$^{+/+}$) cortical neurons were treated with 300 nM STS for 6 hrs. STS-treatment significantly increased the RS levels when compared to the control neurons. The results are presented as the mean ± SEM. Data is calculated as fold change relative to untreated (control) cultures. *illustrates statistically significant difference (ANOVA on Ranks with Dunn's multiple comparisons test; $p < 0.01$).*

Right: Representative confocal microscopy images of untreated (top) and STS-treated (bottom) neurons. On incubating the cells with CM-H$_2$DCFDA, an increase in fluorescence intensity is seen in the STS-treated cells.

A number of studies have shown that rotenone, a mitochondrial complex I inhibitor induces the production of mitochondrial ROS [207, 208]. In our *in vitro* studies, we

found a slight increase in the total ROS in the non-apoptotic cortical neurons treated with rotenone (Fig. 5.4A).

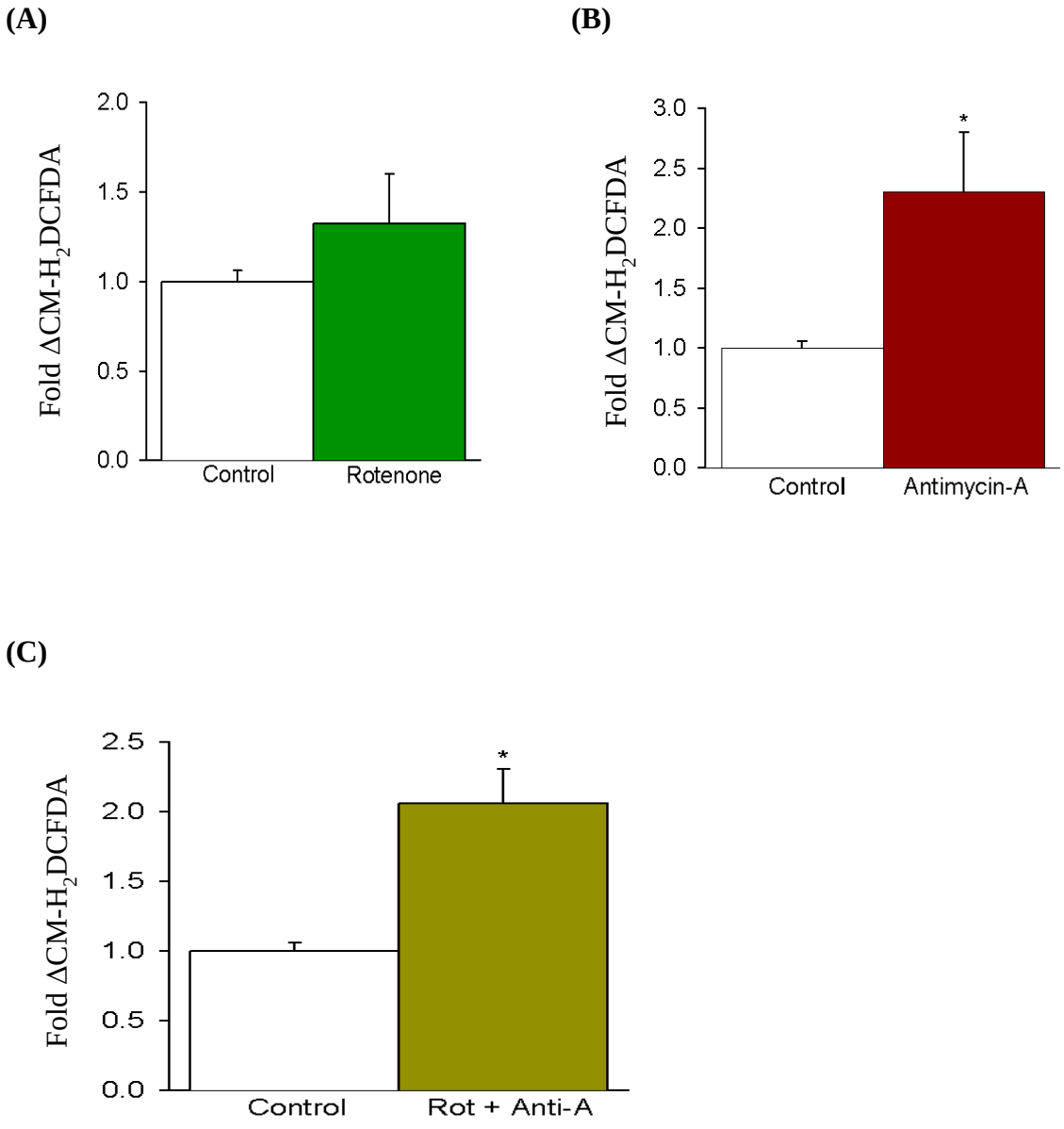
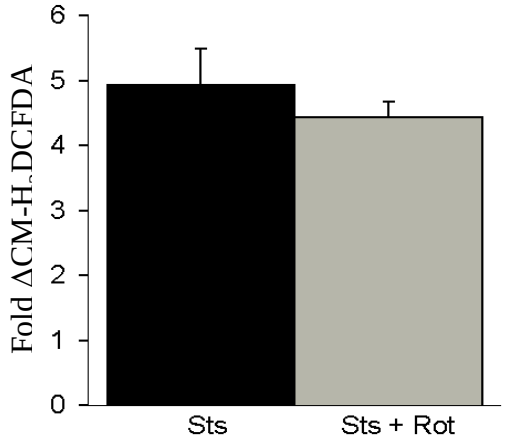


Figure 5.4: Effect of rotenone (Rot) and/or antimycin-A (Anti-A) treatment on RS levels in wild-type cortical neurons. Rotenone (10 μM) treatment for 20 minutes led to a slight

*increase in the RS levels as compared to untreated cells (A). Treating the cells with antimycin-A (1 μM, 20 minutes) significantly elevated the RS (B; $p < 0.05$). The combined treatment of rotenone plus antimycin-A (C) also augmented the levels of RS in comparison to controls ($p < 0.01$). The results are presented as the mean ± SEM. Data is calculated as fold change relative to untreated (control) cultures. * represents statistically significant difference (ANOVA on Ranks with Dunn's multiple comparisons test).*

Antimycin-A, a complex III inhibitor has been demonstrated to promote the generation of mitochondrial ROS [209]. A significant increase in the levels of ROS was observed in the neuronal cultures treated with only antimycin-A (Fig. 5.4B). The combination treatment of rotenone plus antimycin-A also enhanced the production of ROS in the non-apoptotic neurons (Fig. 5.4C). In the apoptotic cortical neurons, addition of rotenone marginally decreased the ROS levels (Fig. 5.5A). Previous studies from our laboratory have shown a reduction of ROS levels in the rotenone-treated apoptotic (NGF-deprived) sympathetic neurons [59, 210]. The likely explanation for this observation is that rotenone, by inhibiting the forward electron flow, could possibly suppress the electron leakage from complex III and thereby reducing the production of $O_2^{\bullet-}$, and hence the downstream ROS. On treating the apoptotic cortical cultures with antimycin-A, the generation of ROS was significantly enhanced when compared to the untreated apoptotic neurons (Fig. 5.5B). This indicates that antimycin-A induces the production of $O_2^{\bullet-}$ both in the apoptotic and non-apoptotic cortical neurons.

(A)



(B)

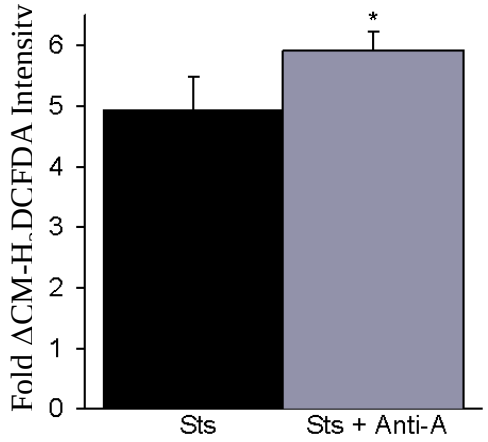


Figure 5.5: Effect of rotenone (Rot) or antimycin-A (Anti-A) treatment on RS levels in the apoptotic (STS-treated) wild-type neurons. Rotenone (10 μM) treatment for 20 minutes led to a slight reduction in the RS levels as compared to STS-treated cells (A). Treating the apoptotic cells with antimycin-A (1 μM, 20 minutes) significantly increased the RS (B; $p < 0.05$). The results are presented as the mean ± SEM. Data is calculated as fold

*change relative to untreated (control) cultures. * represents statistically significant difference (ANOVA on Ranks with Dunn's multiple comparisons test).*

Treatment with STS stimulates the intrinsic apoptotic pathway by causing induction of pro-apoptotic proteins such as Bax, and further activating the executioner caspases like caspase-3. Studies from our laboratory have shown an important role of Bax and caspase-3 in the production of mitochondrial ROS [84]. A schematic illustration of the effect of rotenone or antimycin-A on total ROS levels in the apoptotic and non-apoptotic cortical cultures from wild-type and caspase-3 mutant pups, is shown in Figure 5.6.

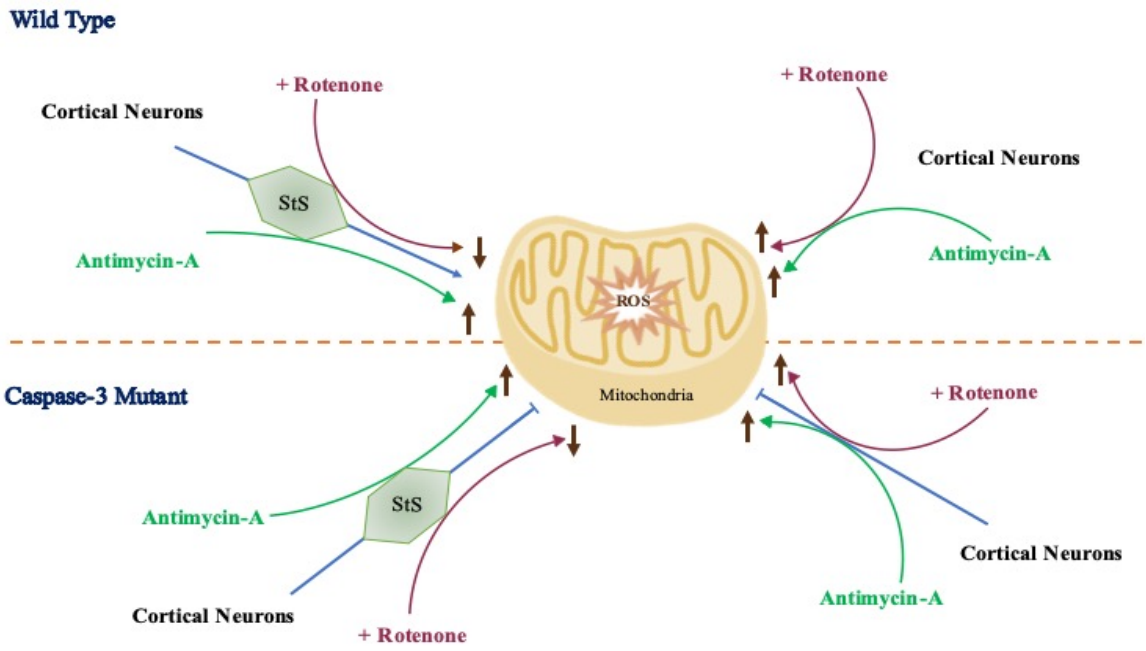


Figure 5.6: Illustration of the possible effects of rotenone or antimycin-A treatment on ROS levels in cortical neurons.

The focus of our next experiments is to localize the respiratory complexes involved in $O_2^{\bullet-}$ generation in the apoptotic and non-apoptotic wild-type (caspase-$3^{+/+}$) cortical neurons by using specific substrates and inhibitors of these complexes. MitoSOX, a mitochondria targeted redox-sensitive dye will be used to detect $O_2^{\bullet-}$ levels. We will then study the effect of caspase-3 deletion on RS levels in the apoptotic and non-apoptotic cortical neurons isolated from newborn (0-1 day-old) caspase-3 mutant mice. Then the effect of rotenone and/or antimycin-A treatment on the production of ROS will be determined in both the apoptotic and non-apoptotic caspase-3 mutant (caspase-$3^{-/-}$) neuronal cultures. After identifying the relevant complexes, we shall assess whether exposing digitonin-permeabilized caspase-$3^{+/+}$ and caspase-$3^{-/-}$ neurons to Bax protein

and/or active caspase-3 protein would influence RS production by the mitochondrial complexes. Ricci, *et al.* (2004) [141] have shown Ndufs1 subunit of complex I as a critical caspase-3 substrate in mitochondria. They also demonstrated that the cells expressing a noncleavable mutant form of Ndufs1 maintains mitochondrial membrane potential and ATP levels, as well as shows diminished production of mitochondrial ROS in response to apoptotic stimuli. Hence, we will also be looking at the expression of Ndufs1 protein in apoptotic and non-apoptotic caspase-$3^{+/+}$ and caspase-$3^{-/-}$ cortical neurons. Overall, this *in vitro* study will greatly extend our knowledge about the possible molecular mechanisms of mitochondrial $O_2^{\bullet-}$ production in neurons. This information could further be useful in designing the potential therapeutic approaches for combating excessive RS generation, and oxidative damage and its associated neuropathologies in the brain.

ABBREVIATIONS

ROS – Reactive oxygen species

RNS – Reactive nitrogen species

RS – Reactive species

OS – Oxidative stress

8-OHdG – 8-hydroxy-2'-deoxyguanosine

PNS – Peripheral nervous system

NGF – Nerve growth factor

BDNF – Brain derived neurotrophic factor

ATP – Adenosine triphosphate

dATP – Deoxyadenosine triphosphate

TNF-α - Tumor necrosis factor-α

Fas/APO-1 – Apoptosis antigen 1

FADD – Fas-associated death domain protein

FLICE – FADD-like IL-1β-converting enzyme

DISC – Death-inducing signaling complex

NADH – Nicotinamide adenine dinucleotide

$FADH_2$ – Flavin adenine dinucleotide

TCA – Tricarboxylic acid

BH3 – Bcl-2 homology

Bcl-2 – B-cell lymphoma 2

Bad – Bcl2 associated agonist of cell death

Bid – BH3 interacting-domain death agonist

Puma – p53 upregulated modulator of apoptosis

Hrk – Harakiri

Blk – B lymphocyte kinase

Mcl-1 – Myeloid cell leukemia sequence 1

PD – Parkinson's disease

AD – Alzheimer's disease

NADPH – Nicotinamide adenine dinucleotide phosphate

SOD – Superoxide dismutase

GPX – Glutathione peroxidase

$O_2^{\bullet-}$ - Superoxide free radical

$OH^{\bullet}$ – Hydroxyl radical

H_2O_2 – Hydrogen peroxide

$NO^{\bullet}$ – Nitric oxide

$ONOO^-$ - Peroxynitrite

GSH – Glutathione

mtDNA – Mitochondrial DNA

TBARS – Thiobarbituric acid reactive substances

PUFA – Polyunsaturated fatty acids

MDA – Malondialdehyde

3-NT – 3-Nitrotyrosine

SCG – Superior cervical ganglion

CG – Cerebellar granule

CT neurons – Cortical neurons

ELISA – Enzyme-linked immunosorbent assay

AAALAC – Association for Assessment and Accreditation of Laboratory Animal Care

ACLAM – American College of Laboratory Animal Medicine

PCR – Polymerase chain reaction

PBS – Phosphate-buffered saline

RIPA – Radioimmunoprecipitation assay

PVDF – Polyvinylidene difluoride

TBS-T – Tri-buffered saline and Tween 20

IgG – Immunoglobulin G

ECL – Enhanced chemiluminescence

ANOVA – Analysis of variance

SEM – Standard error of the mean

PGK-neo – Phosphoglycerate kinase-neomycin resistance gene

Fe-S – Iron-sulfur

Ndufs1 – NADH:Ubiquinone oxidoreductase core subunit S1

BAF – Boc-Asp(OMe)-fluoromethyl ketone

SDS-PAGE – Sodium dodecyl sulfate-Polyacrylamide gel electrophoresis

CM-H_2DCFDA – 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate

MitoSOX – Mitochondrial superoxide

STS (Sts) – Staurosporine

HBSS – Hank's balanced salt solution

DNase – Deoxyribonuclease

REFERENCES

1. Kerr, J.F.R., A.H. Wyllie, and A.R. Currie, *Apoptosis: a basic biological phenomenon with wide ranging implications in tissue kinetics.* Br J Cancer, 1972. **26**: p. 239-257.
2. Oppenheim, R.W., *Cell death during development of the nervous system.* Annu Rev Neurosci, 1991. **14**: p. 453-501.
3. Wright, L.L., T.J. Cunningham, and A.J. Smolen, *Developmental neuron death in the rat superior cervical sympathetic ganglion: cell counts and ultrastructure.* J Neurocytol, 1983. **12**(5): p. 727-38.
4. Becker, E.B.E. and A. Bonni, *Cell cycle regulation of neuronal apoptosis in development and disease*. Progress in Neurobiology, 2004. **72**(1): p. 1-25.
5. Kerr, J.F., A.H. Wyllie, and A.R. Currie, *Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics.* Br J Cancer, 1972. **26**(4): p. 239-57.
6. Sanchez, A., et al., *Apoptosis induced by transforming growth factor-beta in fetal hepatocyte primary cultures: involvement of reactive oxygen intermediates.* J Biol Chem, 1996. **271**(13): p. 7416-22.
7. Papaliagkas, V., et al., *The proteins and the mechanisms of apoptosis: a mini-review of the fundamentals.* Hippokratia, 2007. **11**(3): p. 108-13.

8. Wyllie, A.H., J.F. Kerr, and A.R. Currie, *Cell death: the significance of apoptosis.* Int Rev Cytol, 1980. **68**: p. 251-306.

9. Hengartner, M.O., *The biochemistry of apoptosis.* . Nature, 2000. **407**: p. 770-6.

10. Liu, X., et al., *DFF, a heterodimeric protein that functions down- stream of caspase-3 to trigger DNA fragmentation during apoptosis.* Cell, 1997. **89**: p. 175-84.

11. Hetts, S.W., *To Die or Not to Die: An Overview of Apoptosis and Its Role in Disease.* JAMA, 1998. **279**: p. 300-307.

12. Martin, L.J., *Neuronal cell death in nervous system development, disease, and injury.* Int J Mol Med 2001. **7**: p. 455-78.

13. Riedl, S.J. and G.S. Salvesen, *The apoptosome: signalling platform of cell death.* Nat Rev Mol Cell Biol, 2007. **8**(5): p. 405-13.

14. Nagata, S., *Apoptosis by death factor.* Cell, 1997. **88**: p. 355-65.

15. Putcha, G.V., et al., *Intrinsic and extrinsic pathway signaling during neuronal apoptosis: lessons from the analysis of mutant mice.* J Cell Biol, 2002. **157**(3): p. 441-53.

16. Hamanaka, R.B. and N.S. Chandel, *Mitochondrial reactive oxygen species regulate cellular signaling and dictate biological outcomes.* Trends Biochem Sci, 2010. **35**(9): p. 505-13.

17. Cadenas, E. and K.J. Davies, *Mitochondrial free radical generation, oxidative stress, and aging.* Free Radic Biol Med, 2000. **29**(3-4): p. 222-30.

18. Wallace, D.C., W. Fan, and V. Procaccio, *Mitochondrial energetics and therapeutics.* Annu Rev Pathol, 2010. **5**: p. 297-348.

19. Halliwell, B. and J.M.C. Gutteridge, *Free radicals in biology and medicine*. 4th Edition ed. 2007: Oxford ; New York: Oxford University Press.

20. Kroemer, G., L. Galluzzi, and C. Brenner, *Mitochondrial membrane permeabilization in cell death.* Physiol Rev, 2007. **87**(1): p. 99-163.

21. Galluzzi, L., et al., *Molecular definitions of cell death subroutines: recommendations of the Nomenclature Committee on Cell Death 2012.* Cell Death and Differentiation, 2012. **19**(1): p. 107-120.

22. Green, D.R. and G. Kroemer, *The pathophysiology of mitochondrial cell death.* Science, 2004. **305**(5684): p. 626-9.

23. Chalah, A. and R. Khosravi-Far, *The mitochondrial death pathway.* Adv Exp Med Biol, 2008. **615**: p. 25-45.

24. Lindsay, J., M.D. Esposti, and A.P. Gilmore, *Bcl-2 proteins and mitochondria-Specificity in membrane targeting for death.* Biochimica Et Biophysica Acta-Molecular Cell Research, 2011. **1813**(4): p. 532-539.

25. Pong, K., et al., *Attenuation of staurosporine-induced apoptosis, oxidative stress, and mitochondrial dysfunction by synthetic superoxide dismutase and catalase mimetics, in cultured cortical neurons.* Exp Neurol, 2001. **171**(1): p. 84-97.

26. Sedlak, T.W., et al., *Multiple Bcl-2 family members demonstrate selective dimerizations with Bax.* Proc Natl Acad Sci U S A, 1995. **92**(17): p. 7834-8.

27. Desagher, S., A. Osen-Sand, and A. Nichols, et al, *Bid-induced conformational change of Bax is responsible for mitochondrial cytochrome C release during apoptosis.* . J Cell Biol 1999. **144**(5): p. 891-901.

28. Eskes, R., et al., *Bid induces the oligomerization and insertion of Bax into the outer mitochondrial membrane.* Mol Cell Biol, 2000. **20**: p. 929-35.

29. Makin, G.W., et al., *Damage-induced Bax N-terminal change, translocation to mitochondria and formation of Bax dimers/complexes occur regardless of cell fate.* EMBO J, 2001. **20**(22): p. 6306-15.

30. Antonsson, B., et al., *Inhibition of Bax channel-forming activity by Bcl-2.* Science, 1997. **277**(5324): p. 370-2.

31. Susin, S.A., et al., *Molecular characterization of mitochondrial apoptosis-inducing factor.* Nature, 1999. **397**(6718): p. 441-6.

32. Li, L.Y., X. Luo, and X. Wang, *Endonuclease G is an apoptotic DNase when released from mitochondria.* Nature, 2001. **412**: p. 95-9.

33. Kandasamy, K., et al., *Involvement of proapoptotic molecules Bax and Bak in tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced mitochondrial disruption and apoptosis: differential regulation of cytochrome c and Smac/DIABLO release.* Cancer Res, 2003. **63**(7): p. 1712-21.

34. Marzo, I., et al., *Bax and adenine nucleotide translocator cooperate in the mitochondrial control of apoptosis.* Science, 1998. **281**(5385): p. 2027-31.

35. Shimizu, S., M. Narita, and Y. Tsujimoto, *Bcl-2 family proteins regulate the release of apoptogenic cytochrome c by the mitochondrial channel VDAC.* Nature, 1999. **399**(6735): p. 483-7.

36. Acehan, D., et al., *Three-dimensional structure of the apoptosome: implications for assembly, procaspase-9 binding, and activation.* Mol Cell, 2002. **9**(2): p. 423-32.

37. Shiozaki, E.N., J. Chai, and Y. Shi, *Oligomerization and activation of caspase-9, induced by Apaf-1 CARD.* Proc Natl Acad Sci U S A, 2002. **99**(7): p. 4197-202.

38. Danial, N.N. and S.J. Korsmeyer, *Cell death: critical control points.* Cell, 2004. **116**(2): p. 205-19.

39. Franklin, J.L., *Redox regulation of the intrinsic pathway in neuronal apoptosis.* Antioxid Redox Signal, 2011. **14**(8): p. 1437-48.

40. Sperka, T., J. Wang, and K.L. Rudolph, *DNA damage checkpoints in stem cells, ageing and cancer.* Nat. Rev. Mol. Cell Biol. , 2012. **13**: p. 579-590.

41. Nykjaer, A., T.E. Willnow, and C.M. Petersen, *p75NTR—live or let die.* Curr. Opin. Neurobiol., 2005. **15**: p. 49-57.

42. Dekkers, M.P., V. Nikoletopoulou, and Y.A. Barde, *Cell biology in neuroscience: Death of developing neurons: new insights and implications for connectivity.* J Cell Biol, 2013. **203**(3): p. 385-93.

43. Zhou, J.X. and X. Li, *Apoptosis in Polycystic Kidney Disease: From Pathogenesis to Treatment*, in *Polycystic Kidney Disease*, X. Li, Editor. 2015, Codon Publications: Brisbane (AU).

44. Friedlander, R.M., *Apoptosis and caspases in neurodegenerative diseases.* N Engl J Med, 2003. **348**(14): p. 1365-75.

45. Honig, L.S. and R.N. Rosenberg, *Apoptosis and neurologic disease.* Am J Med, 2000. **108**(4): p. 317-30.

46. Klein, J.A. and S.L. Ackerman, *Oxidative stress, cell cycle, and neurodegeneration.* J Clin Invest, 2003. **111**(6): p. 785-93.

47. Taylor, J.P., J. Hardy, and K.H. Fischbeck, *Toxic proteins in neurodegenerative disease.* Science, 2002. **296**(5575): p. 1991-5.

48. Choi, D.W., *Calcium and excitotoxic neuronal injury.* Ann N Y Acad Sci, 1994. **747**: p. 162-71.

49. Zipfel, G.J., et al., *Neuronal apoptosis after CNS injury: the roles of glutamate and calcium.* J Neurotrauma, 2000. **17**(10): p. 857-69.

50. Yankner, B.A., et al., *Neurotoxicity of a fragment of the amyloid precursor associated with Alzheimer's disease.* Science, 1989. **245**(4916): p. 417-20.

51. Lewen, A., P. Matz, and P.H. Chan, *Free radical pathways in CNS injury.* J. Neurotrauma, 2000. **17**: p. 871-890.

52. Jenner, P., *Oxidative stress in Parkinson's disease.* Ann Neurol, 2003. **53 Suppl 3**(3): p. S26-36; discussion S36-8.

53. Rosen, D.R., et al., *Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis.* Nature, 1993. **362**: p. 59-62.

54. Deng, H.X., et al., *Amyotrophic lateral sclerosis and structural defects in Cu,Zn superoxide dismutase.* Science, 1993. **261**(5124): p. 1047-51.

55. Kannan, K. and S.K. Jain, *Oxidative stress and apoptosis.* Pathophysiology, 2000. **7**(3): p. 153-163.

56. Brouillet, E., et al., *Replicating Huntington's disease phenotype in experimental animals.* Prog Neurobiol, 1999. **59**(5): p. 427-68.

57. Gu, G., et al., *Mitochondrial DNA deletions/rearrangements in parkinson disease and related neurodegenerative disorders.* J Neuropathol Exp Neurol, 2002. **61**(7): p. 634-9.

58. Shelat, P.B., et al., *Amyloid beta peptide and NMDA induce ROS from NADPH oxidase and AA release from cytosolic phospholipase A₂ in cortical neurons.* . Journal of Neurochemistry, 2008. **106**: p. 45-55.

59. Kirkland, R.A., et al., *A Bax-induced pro-oxidant state is critical for cytochrome c release during programmed neuronal death.* J Neurosci, 2002. **22**(15): p. 6480-90.

60. Shidoji, Y., et al., *Loss of Molecular Interaction between Cytochrome c and Cardiolipin Due to Lipid Peroxidation.* Biochemical and biophysical research communications, 1999. **264**: p. 343-347.

61. Vaughn, A.E. and M. Deshmukh, *Glucose metabolism inhibits apoptosis in neurons and cancer cells by redox inactivation of cytochrome c.* Nat Cell Biol, 2008. **10**(12): p. 1477-83.

62. Hortelano, S., A.M. Alvarez, and L. Bosca, *Nitric oxide induces tyrosine nitration and release of cytochrome c preceding an increase of mitochondrial transmembrane potential in macrophages.* FASEB J, 1999. **13**(15): p. 2311-7.

63. Deshmukh, M. and E.M. Johnson, Jr., *Evidence of a novel event during neuronal death: development of competence-to-die in response to cytoplasmic cytochrome c.* Neuron, 1998. **21**(4): p. 695-705.

64. Gemma, C., et al., *Oxidative Stress and the Aging Brain: From Theory to Prevention,* in *Brain Aging: Models, Methods, and Mechanisms*, D.R. Riddle, Editor. 2007: Boca Raton (FL).

65. Balaban, R.S., S. Nemoto, and T. Finkel, *Mitochondria, oxidants, and aging.* Cell, 2005. **120**(4): p. 483-95.

66. Xinyuan Li, P.F., Jietang Mai, Eric T Choi, Hong Wang and Xiao-feng Yang, *Targeting mitochondrial reactive oxygen species as novel therapy for inflammatory diseases and cancers.* Journal of Hematology & Oncology, 2013. **6**: p. 19.

67. Hancock, J.T., R. Desikan, and S.J. Neill, *Role of reactive oxygen species in cell signalling pathways.* Biochem Soc Trans, 2001. **29**(Pt 2): p. 345-50.

68. Steullet, P., et al., *Synaptic plasticity impairment and hypofunction of NMDA receptors induced by glutathione deficit: relevance to schizophrenia.* Neuroscience, 2006. **137**(3): p. 807-19.

69. Hyman, M.S., *Redox neurology: Visions of an emerging subspecialty.* Ann. N.Y. Acad. Sci. , 2004. **1012**: p. 342-355.

70. Iwata, S., et al., *Complete structure of the 11-subunit bovine mitochondrial cytochrome bc1 complex.* Science, 1998. **281**(5373): p. 64-71.

71. Lee, H.C. and Y.H. Wei, *Oxidative stress, mitochondrial DNA mutation, and apoptosis in aging.* Exp Biol Med (Maywood), 2007. **232**(5): p. 592-606.

72. Beckman, K.B. and B.N. Ames, *The free radical theory of aging matures.* Physiol Rev, 1998. **78**(2): p. 547-81.

73. Schieber, M. and N.S. Chandel, *ROS function in redox signaling and oxidative stress.* Curr Biol, 2014. **24**(10): p. R453-62.

74. Fridovich, I., *Superoxide anion radical (O2-.), superoxide dismutases, and related matters.* J Biol Chem, 1997. **272**(30): p. 18515-7.

75. Malinski, T., et al., *Nitric oxice measured by a porphyrinic microsensor in rat brain after transient middle cerebral artery occlusion.* Journal of cerebral blood flow and metabolism, 1993. **13**: p. 355-358.

76. Beckman, J.S. and W.H. Koppenol, *Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly.* Am J Physiol, 1996. **271**(5 Pt 1): p. C1424-37.

77. Huie RE, P.S., *The Reaction of NO With Superoxide.* Free Radic Res, 1993. **18**: p. 195-199.

78. Ferrer-Sueta, G. and R. Radi, *Chemical biology of peroxynitrite: kinetics, diffusion, and radicals.* ACS Chem Biol, 2009. **4**(3): p. 161-77.

79. Radi, R., et al., *Peroxynitrite-induced membrane lipid peroxidation: the cytotoxic potential of superoxide and nitric oxide.* Arch Biochem Biophys, 1991. **288**(2): p. 481-7.

80. Wang, Y., et al., *Superoxide dismutases: Dual roles in controlling ROS damage and regulating ROS signaling.* Journal of Cell Biology, 2018. **217**(6): p. 1915-1928.

81. D'Amelio, M., V. Cavallucci, and F. Cecconi., *Neuronal caspase-3 signaling: not only cell death.* Cell Death Differ, 2010. **17**: p. 1104-14.

82. Kuranaga, E. and M. Miura, *Nonapoptotic functions of caspases: caspases as regulatory molecules for immunity and cell-fate determination.* Trends in Cell Biology, 2007. **17**(3): p. 135-144.

83. Valencia, A. and J. Moran, *Role of oxidative stress in the apoptotic cell death of cultured cerebellar granule neurons.* J Neurosci Res, 2001. **64**(3): p. 284-97.

84. Kirkland, R.A., et al., *Bax regulates production of superoxide in both apoptotic and nonapoptotic neurons: role of caspases.* J Neurosci, 2010. **30**(48): p. 16114-27.

85. Ricci, J.E., et al., *Disruption of mitochondrial function during apoptosis is mediated by caspase cleavage of the p75 subunit of complex I of the electron transport chain.* Cell, 2004. **117**(6): p. 773-786.

86. Ricci, J.E., R.A. Gottlieb, and D.R. Green, *Caspase-mediated loss of mitochondrial function and generation of reactive oxygen species during apoptosis.* J Cell Biol, 2003. **160**(1): p. 65-75.

87. Huai, J., et al., *TNFa-induced lysosomal membrane permeability is downstream of MOMP and triggered by caspase- mediated NDUFS1 cleavage and ROS formation.* Journal of Cell Science, 2013. **126**: p. 4015-4025.

88. Muller, F.L., et al., *Trends in oxidative aging theories.* Free Radic Biol Med, 2007. **43**(4): p. 477-503.

89. Okado-Matsumoto, A. and I. Fridovich, *Subcellular distribution of superoxide dismutases (SOD) in rat liver: Cu,Zn-SOD in mitochondria.* J Biol Chem, 2001. **276**(42): p. 38388-93.

90. Li, Y., et al., *Dilated cardiomyopathy and neonatal lethality in mutant mice lacking manganese superoxide dismutase.* Nat Genet, 1995. **11**(4): p. 376-81.

91. Ohashi, M., et al., *MnSOD deficiency increases endothelial dysfunction in ApoE-deficient mice.* Arterioscler Thromb Vasc Biol, 2006. **26**(10): p. 2331-6.

92. Grammer, T.B., et al., *SOD3 R231G polymorphism associated with coronary artery disease and myocardial infarction. The Ludwigshafen Risk and Cardiovascular Health (LURIC) study.* Free Radic Res, 2009. **43**(7): p. 677-84.

93. Zhanga, M., et al., *Emerging Roles of Nrf2 and Phase II Antioxidant Enzymes in Neuroprotection.* Prog Neurobiol., 2013. **100**: p. 30-47.

94. Handy, D.E. and J. Loscalzo, *Redox regulation of mitochondrial function.* Antioxid Redox Signal, 2012. **16**(11): p. 1323-67.

95. Torzewski, M., et al., *Deficiency of glutathione peroxidase-1 accelerates the progression of atherosclerosis in apolipoprotein E-deficient mice.* Arterioscler Thromb Vasc Biol, 2007. **27**(4): p. 850-7.

96. Okauchi, M., et al., *Effects of deferoxamine on intracerebral hemorrhage-induced brain injury in aged rats.* Stroke, 2009. **40**(5): p. 1858-63.

97. Sokol, R.J., et al., *Vitamin E reduces oxidant injury to mitochondria and the hepatotoxicity of taurochenodeoxycholic acid in the rat.* Gastroenterology, 1998. **114**(1): p. 164-74.

98. Gilgun-Sherki, Y., E. Melamed, and D. Offen, *Oxidative stress induced-neurodegenerative diseases: the need for antioxidants that penetrate the blood brain barrier.* Neuropharmacology, 2001. **40**: p. 959-975.

99. Smith, R.A., et al., *Selective targeting of an antioxidant to mitochondria.* Eur J Biochem, 1999. **263**(3): p. 709-16.

100. Davidson, S.M., *Endothelial mitochondria and heart disease.* Cardiovasc Res., 2010. **88**: p. 58-66.

101. Vasavi, H., M. Thangaraju, and P. Sachdanandam, *Effect of alpha-tocopherol on lipid peroxidation and antioxidant system in fibrosarcoma bearing rats.* Mol Cell Biochem, 1994. **131**(2): p. 125-9.

102. Hang Cui, Y.K., and Hong Zhang, *Oxidative Stress, Mitochondrial Dysfunction, and Aging.* J Signal Transduct., 2011. **2012**.

103. Harman, D., *Aging: a theory based on free radical and radiation chemistry.* J Geront., 1956. **11**.

104. Gladyshev, V.N., *The Free Radical Theory of Aging Is Dead. Long Live the Damage Theory!* Antioxid. Redox Signal., 2014. **20**(4): p. 727-731.

105. Chandrasekaran, A., M. Idelchik, and J.A. Melendez, *Redox control of senescence and age-related disease.* Redox Biol, 2017. **11**: p. 91-102.

106. Pole, A., M. Dimri, and G. Dimri, *Oxidative stress, cellular senescence and ageing.* AIMS Mol Sci. , 2016. **3**(3): p. 300-324.

107. Lau, F.C., B. Shukitt-Hale, and J.A. Joseph, *The beneficial effects of fruit polyphenols on brain aging.* Neurobiol Aging, 2005. **26 Suppl 1**: p. 128-32.

108. Butterfield, D.A., et al., *Oxidatively induced structural alteration of glutamine synthetase assessed by analysis of spin label incorporation kinetics: relevance to Alzheimer's disease.* J Neurochem, 1997. **68**(6): p. 2451-7.

109. Papaioannou, N., et al., *Immunohistochemical investigation of the brain of aged dogs. I. Detection of neurofibrillary tangles and of 4-hydroxynonenal protein, an oxidative damage product, in senile plaques.* Amyloid, 2001. **8**(1): p. 11-21.

110. Maynard, S., et al., *Base excision repair of oxidative DNA damage and association with cancer and aging.* Carcinogenesis, 2009. **30**(1): p. 2-10.

111. Sawada, M. and J.C. Carlson, *Changes in superoxide radical and lipid peroxide formation in the brain, heart and liver during the lifetime of the rat.* Mech Ageing Dev, 1987. **41**(1-2): p. 125-37.

112. Duthie, S.J., et al., *DNA stability and lipid peroxidation in vitamin E-deficient rats in vivo and colon cells in vitro--modulation by the dietary anthocyanin, cyanidin-3-glycoside.* Eur J Nutr, 2005. **44**(4): p. 195-203.

113. Smith, C.D., et al., *Excess brain protein oxidation and enzyme dysfunction in normal aging and in Alzheimer disease.* Proc Natl Acad Sci U S A, 1991. **88**(23): p. 10540-3.

114. Chung, Y.H., et al., *Immunohistochemical study on the distribution of nitrotyrosine and neuronal nitric oxide synthase in aged rat cerebellum.* Brain Res, 2002. **951**(2): p. 316-21.

115. Kirkwood, T.B.L., *Understanding the odd science of aging.* Cell, 2005. **120**(4).

116. Nowakowski, R.S., *Stable neuron numbers from cradle to grave.* Proceedings of the National Academy of Sciences 2006. **103**: p. 12219-12220.

117. Dayer, A.G., et al., *Short-term and long-term survival of new neurons in the rat dentate gyrus.* J Comp Neurol, 2003. **460**(4): p. 563-72.

118. Antonsson, B., *Bax and other pro-apoptotic Bcl-2 family "killer-proteins" and their victim the mitochondrion.* Cell Tissue Res 2001. **306**: p. 347-361.

119. Halliwell, B., J.M. Gutteridge, and C.E. Cross, *Free radicals, antioxidants, and human disease: where are we now?* J Lab Clin Med. , 1992. **119**(6): p. 598-620.

120. Finkel, T. and N.J. Holbrook, *Oxidants, oxidative stress and the biology of ageing.* Nature, 2000. **408**(6809): p. 239-47.

121. Dillard, C.J., et al., *Effects of exercise, vitamin E, and ozone on pulmonary function and lipid peroxidation.* J Appl Physiol Respir Environ Exerc Physiol, 1978. **45**(6): p. 927-32.

122. Allen, R.G. and M. Tresini, *Oxidative stress and gene regulation.* Free Radic Biol Med, 2000. **28**(3): p. 463-99.

123. Valko, M., et al., *Free radicals, metals and antioxidants in oxidative stress-induced cancer.* Chem Biol Interact, 2006. **160**(1): p. 1-40.

124. Valko, M., et al., *Free radicals and antioxidants in normal physiological functions and human disease.* Int J Biochem Cell Biol, 2007. **39**(1): p. 44-84.

125. Halliwell, B., *Oxidative stress and neurodegeneration: where are we now?* J Neurochem, 2006. **97**(6): p. 1634-58.

126. Brieger, K., et al., *Reactive oxygen species: from health to disease.* Swiss Med Wkly, 2012. **142**: p. w13659.

127. Sagi, M. and R. Fluhr, *Production of reactive oxygen species by plant NADPH oxidases.* Plant Physiol, 2006. **141**(2): p. 336-40.

128. Madamanchi, N.R. and M.S. Runge, *Mitochondrial dysfunction in atherosclerosis.* Circ Res, 2007. **100**(4): p. 460-73.

129. Turrens, J.F., *Superoxide production by the mitochondrial respiratory chain.* Biosci Rep, 1997. **17**(1): p. 3-8.

130. Jourd'heuil, D., et al., *Reaction of superoxide and nitric oxide with peroxynitrite. Implications for peroxynitrite-mediated oxidation reactions in vivo.* J Biol Chem, 2001. **276**(31): p. 28799-805.

131. Ames, B.N., M.K. Shigenaga, and T.M. Hagen, *Oxidants, antioxidants, and the degenerative diseases of aging.* Proc Natl Acad Sci U S A, 1993. **90**(17): p. 7915-22.

132. Avshalumov, M.V., B.T. Chen, and M.E. Rice, *Mechanisms underlying H(2)O(2)-mediated inhibition of synaptic transmission in rat hippocampal slices.* Brain Res, 2000. **882**(1-2): p. 86-94.

133. Schipper, H.M., *Redox neurology: visions of an emerging subspecialty.* Ann N Y Acad Sci, 2004. **1012**: p. 342-55.

134. Harman, D., *Role of free radicals in aging and disease.* Ann N Y Acad Sci, 1992. **673**: p. 126-41.

135. Melov, S., et al., *Extension of life-span with superoxide dismutase/catalase mimetics.* Science, 2000. **289**(5484): p. 1567-9.

136. Drew, B. and C. Leeuwenburgh, *Aging and the role of reactive nitrogen species.* Ann N Y Acad Sci, 2002. **959**: p. 66-81.

137. Wang, D., et al., *Antioxidants protect PINK1-dependent dopaminergic neurons in Drosophila.* Proc Natl Acad Sci U S A, 2006. **103**(36): p. 13520-5.

138. Tripathi, A., *New cellular and molecular approaches to ageing brain.* Ann Neurosci, 2012. **19**(4): p. 177-82.

139. Dugan, L.L., et al., *Rapid suppression of free radical formation by nerve growth factor involves the mitogen-activated protein kinase pathway.* Proc Natl Acad Sci U S A, 1997. **94**(8): p. 4086-91.

140. Greenlund, L.J., T.L. Deckwerth, and E.M. Johnson, Jr., *Superoxide dismutase delays neuronal apoptosis: a role for reactive oxygen species in programmed neuronal death.* Neuron, 1995. **14**(2): p. 303-15.

141. Ricci, J.E., et al., *Disruption of mitochondrial function during apoptosis is mediated by caspase cleavage of the p75 subunit of complex I of the electron transport chain.* Cell, 2004. **117**(6): p. 773-86.

142. Armstrong, D. and R. Browne, *The analysis of free radicals, lipid peroxides, antioxidant enzymes and compounds related to oxidative stress as applied to the clinical chemistry laboratory.* Adv Exp Med Biol, 1994. **366**: p. 43-58.

143. Gan, W., et al., *Age-dependent increases in the oxidative damage of DNA, RNA, and their metabolites in normal and senescence-accelerated mice analyzed by LC-MS/MS: Urinary 8-oxoguanosine as a novel biomarker of aging.* Free Radical Biology and Medicine, 2012. **52**(9): p. 1700-1707.

144. Flurkey, Currer, and Harrison., *The Mouse in Biomedical Research*, ed. J.F.S.B.M.D.C.N.F.Q.A. Smith. Vol. 4. 2007.

145. Dutta, S. and P. Sengupta, *Men and mice: Relating their ages.* Life Sciences, 2016. **152**: p. 244-248.

146. Richter, C., J.W. Park, and B.N. Ames, *Normal oxidative damage to mitochondrial and nuclear DNA is extensive.* Proc Natl Acad Sci USA., 1988. **85**.

147. Mecocci, P., et al, *Oxidative damage to mitochondrial DNA shows marked age-dependent increases in human brain.* Ann Neurol., 1993. **34**.

148. Li, P., et al., *Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade.* Cell, 1997. **91**(4): p. 479-89.

149. Goldstein, J.C., et al., *The coordinate release of cytochrome c during apoptosis is rapid, complete and kinetically invariant.* Nat Cell Biol, 2000. **2**(3): p. 156-62.

150. Lucken-Ardjomande, S., S. Montessuit, and J.C. Martinou, *Contributions to Bax insertion and oligomerization of lipids of the mitochondrial outer membrane.* Cell Death Differ, 2008. **15**(5): p. 929-37.

151. Tan, S., et al., *The regulation of reactive oxygen species production during programmed cell death.* J Cell Biol, 1998. **141**(6): p. 1423-32.

152. Kirkland, R.A. and J.L. Franklin, *Bax affects production of reactive oxygen by the mitochondria of non-apoptotic neurons.* Exp Neurol, 2007. **204**(1): p. 458-61.

153. Dei, R., et al., *Lipid peroxidation and advanced glycation end products in the brain in normal aging and in Alzheimer's disease.* Acta Neuropathol, 2002. **104**(2): p. 113-22.

154. Gemma, C., et al., *Diets enriched in foods with high antioxidant activity reverse age-induced decreases in cerebellar beta-adrenergic function and increases in proinflammatory cytokines.* J Neurosci, 2002. **22**(14): p. 6114-20.

155. Cini, M. and A. Moretti, *Studies on lipid peroxidation and protein oxidation in the aging brain.* Neurobiol Aging, 1995. **16**(1): p. 53-7.

156. Shin, C.M., et al., *Age-related changes in the distribution of nitrotyrosine in the cerebral cortex and hippocampus of rats.* Brain Res, 2002. **931**(2): p. 194-9.

157. Hockenbery, D.M., et al., *Bcl-2 functions in an antioxidant pathway to prevent apoptosis.* Cell, 1993. **75**(2): p. 241-51.

158. Kowaltowski, A.J., A.E. Vercesi, and G. Fiskum, *Bcl-2 prevents mitochondrial permeability transition and cytochrome c release via maintenance of reduced pyridine nucleotides.* Cell Death Differ, 2000. **7**(10): p. 903-10.

159. Kowaltowski, A.J. and G. Fiskum, *Redox mechanisms of cytoprotection by Bcl-2.* Antioxid Redox Signal, 2005. **7**(3-4): p. 508-14.

160. Ali, S.S., et al., *Gender differences in free radical homeostasis during aging: shorter-lived female C57BL6 mice have increased oxidative stress.* Aging Cell 2006. **5**: p. 565-574.

161. Butterfield DA, S.E., *Protein oxidation processes in aging brain.* Adv Cell Aging Gerontol., 1997. **2**: p. 161-191.

162. Smith, M.A., et al., *Widespread peroxynitrite-mediated damage in Alzheimer's disease.* J Neurosci, 1997. **17**(8): p. 2653-7.

163. Li, Z., G. Ji, and V. Neugebauer, *Mitochondrial reactive oxygen species are activated by mGluR5 through IP3 and activate ERK and PKA to increase excitability of amygdala neurons and pain behavior.* J Neurosci., 2011. **31**(3): p. 1114-1127.

164. Andreazza, A.C., et al., *Mitochondrial complex I activity and oxidative damage to mitochondrial proteins in the prefrontal cortex of patients with bipolar disorder.* Arch Gen Psychiatry, 2010. **67**(4): p. 360-8.

165. Karry, R., E. Klein, and D. Ben Shachar, *Mitochondrial complex I subunits expression is altered in schizophrenia: a postmortem study.* Biol Psychiatry, 2004. **55**(7): p. 676-684.

166. Mattson, M.P., et al., *beta-Amyloid peptides destabilize calcium homeostasis and render human cortical neurons vulnerable to excitotoxicity.* J Neurosci, 1992. **12**(2): p. 376-89.

167. Charles R Arthur, S.L.M., Lisa D Dunham, Paula M Keeney and James P Bennett Jr, *Parkinson's disease brain mitochondria have impaired respirasome assembly, age-related increases in distribution of oxidative damage to mtDNA and no differences in heteroplasmic mtDNA mutation abundance.* Molecular Neurodegeneration 2009. **4**(37).

168. Tamagno E, P.M., Bardini P, Piccini A, Borghi R, Guglielmotto M, Santoro G, Davit A, Danni O, Smith M *-site APP cleaving enzyme up-regulation induced by 4- hydroxynonenal is mediated by stress-activated protein kinases pathways.* Journal of Neurochemistry 2005. **92**: p. 628-636.

169. Tesco, G., Y.H. Koh, and R.E. Tanzi, *Caspase activation increases beta-amyloid generation independently of caspase cleavage of the beta-amyloid precursor protein (APP).* J Biol Chem, 2003. **278**(46): p. 46074-80.

170. Cho, D.H., et al., *S-nitrosylation of Drp1 mediates beta-amyloid-related mitochondrial fission and neuronal injury.* Science, 2009. **324**(5923): p. 102-5.

171. Wallace, D.C., *Mitochondrial diseases in man and mouse.* Science, 1999. **283**(5407): p. 1482-8.

172. Simonian, N.A. and J.T. Coyle, *Oxidative stress in neurodegenerative diseases.* Annu Rev Pharmacol Toxicol, 1996. **36**: p. 83-106.

173. Miró, Ò., et al., *Aging is associated with increased lipid peroxidation in human hearts, but not with mitochondrial respiratory chain enzyme defects.* Cardiovasc. Res., 2000. **47**: p. 624-631.

174. Uysal, M., et al., *Increased hepatic lipid peroxidation in aged mice.* Mech Ageing Dev, 1989. **48**(1): p. 85-9.

175. Abete, P., et al., *Age-related decrease in cardiac tolerance to oxidative stress.* J Mol Cell Cardiol, 1999. **31**(1): p. 227-36.

176. Liguori, I., et al., *Oxidative stress, aging, and diseases.* Clin Interv Aging, 2018. **13**: p. 757-772.

177. Venero, J.L., M.A. Burguillos, and B. Joseph, *Caspases playing in the field of neuroinflammation: old and new players.* Developmental Neuroscience, 2012. **35**: p. 88-101.

178. Lessard-Beaudoin, M., et al., *Organ-specific alteration in caspase expression and STK3 proteolysis during the aging process.* Neurobiol Aging, 2016. **47**: p. 50-62.

179. Croteau, D.L., R.H. Stierum, and V.A. Bohr, *Mitochondrial DNA repair pathways.* Mutat Res, 1999. **434**(3): p. 137-48.

180. Stadtman, E.R., *Protein oxidation and aging.* Science, 1992. **257**: p. 1220-1224.

181. Michelle L. Hamilton, H.V.R., Jessica A. Drake, Hong Yang, Zhong Mao Guo, Kristen Kewitt, Christi A. Walter, and Arlan Richardson, *Does oxidative damage to DNA increase with age?* PNAS 2001. **98**(18): p. 10469-10474.

182. Perez, V.I., et al., *Is the oxidative stress theory of aging dead?* Biochim Biophys Acta., 2009.

183. Koc, A., et al., *Methionine sulfoxide reductase regulation of yeast lifespan reveals reactive oxygen species-dependent and -in- dependent components of aging.* Proc Natl Acad Sci USA 2004. **101**: p. 7999-8004.

184. Zahn, R.K., et al., *DNA status in brain and heart as prominent co-determinant for life span? Assessing the different degrees of DNA damage, damage susceptibility, and repair capability in different organs of young and old mice.* Mech Ageing Dev 1996. **89**(2): p. 79-94.

185. Rikans, L.E. and Y. Cai, *Age-associated enhancement of diquat-induced lipid peroxidation and cytotoxicity in isolated rat hepatocytes.* J Pharmacol Exp Ther, 1992. **262**(1): p. 271-8.

186. Nhan TQ, L.W., Schwartz SM *Physiological functions of caspases beyond cell death.* Am J Pathol 2006. **169**: p. 729-737.

187. Sanchez-Valle, V., et al., *Role of oxidative stress and molecular changes in liver fibrosis: a review.* Curr Med Chem, 2012. **19**(28): p. 4850-60.

188. Cichoż-Lach, H. and A. Michalak, *Oxidative stress as a crucial factor in liver diseases.* World J Gastroenterol, 2014. **20**(25): p. 8082-8091.

189. Welch, W.J., et al., *Nephron pO2 and renal oxygen usage in the hypertensive rat kidney.* Kidney Int, 2001. **59**(1): p. 230-7.

190. Nicholson, D.W. and N.A. Thornberry, *Caspases: killer proteases.* Trends Biochem Sci, 1997. **22**(8): p. 299-306.

191. Kristen Ryan, D.S.B., Philip Reigan, and Manisha Patel, *Post-Translational Oxidative Modification and Inactivation of Mitochondrial Complex I in Epileptogenesis.* The Journal of Neuroscience, 2012. **32**(33): p. 11250-11258.

192. Zhu, Y.S., et al., *Caspase cleavage of cytochrome c1 disrupts mitochondrial function and enhances cytochrome c release.* Cell Research, 2012. **22**(1): p. 127-141.

193. Kalogeris, T., Y. Bao, and R.J. Korthuis, *Mitochondrial reactive oxygen species: a double edged sword in ischemia/reperfusion vs preconditioning.* Redox Biol, 2014. **2**: p. 702-14.

194. Murphy, M., *How mitochondria produce reactive oxygen species.* Biochemical Journal, 2009. **417**(1).

195. Turrens, J.F., *Mitochondrial formation of reactive oxygen species.* The Journal of Physiology, 2003. **552**: p. 335-344.

196. Royall, J.A. and H. Ischiropoulos, *Evaluation of 2',7'-Dichlorofluorescin and Dihydrorhodamine 123 as Fluorescent Probes for Intracellular H2O2 in Cultured Endothelial Cells.* Arch. Biochem. Biophys., 1993. **302**: p. 348-355.

197. Kirkland, R.A. and J.L. Franklin, *Evidence for redox regulation of cytochrome C release during programmed neuronal death: antioxidant effects of protein synthesis and caspase inhibition.* J Neurosci, 2001. **21**(6): p. 1949-63.

198. Herbert, J.M., E. Seban, and J.P. Maffrand, *Characterization of specific binding sites for [3H]-staurosporine on vari- ous protein kinases.* Biochem. Biophys. Res. Commun., 1990. **171**: p. 189-195.

199. Ruegg, U.T. and G.M. Burgess, *Staurosporine, K-252 and UCN-01: potent but nonspecific inhibitors of protein kinases.* Trends Pharmacol Sci, 1989. **10**(6): p. 218-20.

200. Krohn, A.J.P., E.; Prehn, J. H. M., *Staurosporine-induced apoptosis of cultured rat hippocampal neurons involves caspase-1 like proteases as upstream initiators and increased production of superoxide as a main downstream effector.* J. Neurosci. , 1998. **18**: p. 8186-8197.

201. Krohn, A.J., T. Wahlbrink, and J.H.M. Prehn, *Mitochondrial depolarization is not required for neuronal apoptosis.* J. Neurosci., 1999. **19**: p. 7394-7404.

202. Prehn, J.H., et al., *Ca2+ and reactive oxygen species in staurosporine-induced neuronal apoptosis.* J Neurochem, 1997. **68**(4): p. 1679-85.

203. Kruman, I., Q. Guo, and M.P. Mattson, *Calcium and reactive oxygen species mediate staurosporine-induced mitochondrial dysfunction and apoptosis in PC12 cells.* J Neurosci Res, 1998. **51**(3): p. 293-308.

204. Xiang, J., D.T. Chao, and S.J. Korsmeyer, *BAX-induced cell death may not require interleukin 1β converting enzyme-like proteases.* Proc Natl Acad Sci U S A 1996. **93**: p. 14559-14563.

205. Alterman, J.F., et al., *A High-throughput Assay for mRNA Silencing in Primary Cortical Neurons in vitro with Oligonucleotide Therapeutics.* Bio Protoc, 2017. **7**(16).

206. Gil, J., et al., *Cytosolic and mitochondrial ROS in staurosporine-induced retinal cell apoptosis.* Free Radic Biol Med, 2003. **35**(11): p. 1500-14.

207. Li, N., et al., *Mitochondrial Complex I Inhibitor Rotenone Induces Apoptosis through Enhancing Mitochondrial Reactive Oxygen Species Production.* The Journal of Biological Chemistry, 2003. **278**(10): p. 8516-8525.

208. Lambert, A.J. and M.D. Brand, *Inhibitors of the Quinone-binding Site Allow Rapid Superoxide Production from Mitochondrial NADH:Ubiquinone Oxidoreductase (Complex I).* The Journal of Biological Chemistry, 2004. **279**(38): p. 39414-39420.

209. Chen, Q., et al., *Production of Reactive Oxygen Species by Mitochondria; CENTRAL ROLE OF COMPLEX III.* The Journal of Biological Chemistry, 2003. **278**(38): p. 36027-36031.

210. Kirkland, R.A., G.M. Saavedra, and J.L. Franklin, *Rapid activation of antioxidant defenses by nerve growth factor suppresses reactive oxygen species during neuronal apoptosis: evidence for a role in cytochrome c redistribution.* J Neurosci, 2007. **27**(42): p. 11315-26.