The examination of age-related changes to motoneurons in ad libitum fed and caloric restricted rats

by

Jeremy Chopek

A thesis submitted to the Faculty of Graduate Studies of

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Abstract

The objective of this study was two fold. The first purpose was to determine the effect of aging on the number of motoneurons and the amount of apoptosis and reactive oxygen species (ROS) damage present in the ventral horn of the lumbar spinal cord in rat. The second purpose was to determine if caloric restriction (CR) would attenuate any observed age-related changes in motoneuron numbers or biomarkers of apoptosis and ROS damage. Three groups of female Fisher355/Norway Brown rats were used. A young (8 month) control group fed *ad libitum* (youngAL), an old (30-31 months) group fed *ad libitum* (oldAL) and an old (30-31 months) group fed a 40% calorie restricted diet starting at 14 weeks of age (oldCR). Using immunohistochemistry, Choline acetyltransferase (ChAT) – positive neurons in the ventrolateral horn larger than 15μm in diameter and having a clear soma and nucleus were sized and counted as motoneurons in the lumbar enlargement of the lumbar spinal cord. Western blots were used to quantify several biomarkers of apoptosis and ROS damage present in the ventral horn of the lumbar enlargement. Results demonstrated a significant increase ($p < 0.05$) in body weight for the oldAL compared to the youngAL, while the increase was attenuated in the oldCR group. No age-related loss of motoneurons was found. An age-related decrease in cytochrome c and OGG1 was found ($p < 0.05$), while CR had no effect on attenuating this loss. The presents results demonstrate that motoneurons located within the lumbar enlargement, which are responsible for innervating the majority of the hindlimb muscles, are not lost with age, suggesting that the previously reported age-related loss of motoneurons is muscle specific. Furthermore, the beneficial effect of CR in attenuating apoptosis and ROS damage is not demonstrated in the motoneuron.

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Abbreviations

5-HT	serotonin
8-OHdg	8-hydroxy-deoxyguanosine
8-OHG	8-hydroxy-guanosine
AD	Alzheimer's disease
ADL	Adductor Longus
AIF	apoptosis inducing factor
AL	ad libitum
ALS	Amyotrophic lateral sclerosis
ANOVA	analysis of variance
ARC	apoptosis repressor with a CARD
Bcl-2	B cell lymphoma 2
BDNF	brain derived neurotrophic factor
BH	B cell lymphoma homologue
CARD	caspase recruitment domain
ChAT	choline acetyl transferase
CNS	central nervous system
CON	control
CR	caloric restriction
DED	death effector domain
EDL	Extensor Digitorium Longus
FADD	Fas-ligand associated death domain
FBNF	Fisher 355/ Brown Norway

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Introduction

In 1956, Harman proposed the idea that free radicals produced in the normal course of metabolism would react with cell constituents and lead to deleterious side effects. The theory was expanded in 1969 to include the idea that if one could reduce or inhibit free radical production and damage, then one could increase one's life span (Harman, 1969).

In 1972 it became apparent that the majority of free radicals were created in the mitochondria (Harman, 1972). This lead to an explosion of studies on the topic of mitochondria and the effect it has on aging (for reviews see Navarro and Boveris, 2007;Lee and Wei, 2007). In short, studies now confirm that mitochondria are the major sources of reactive oxygen species (ROS) production and that the ROS will primarily attack the mitochondria, given its close proximity to the ROS (Barja, 2004b). This will lead to mitochondrial DNA (mtDNA) damage and depending on the amount of damage present; either DNA repair or cell death via apoptosis will occur (Lee and Wei, 2007;Higami and Shimokawa, 2000;Papazoglu and Mills, 2007).

To date, only one non-genetic intervention exists to reduce the creation of ROS – caloric restriction (Barja, 2004b;Bua et al., 2004;Dirks Naylor and Leeuwenburgh, 2008;Drew et al., 2003;Duffy et al., 1997;Hepple et al., 2006). The exact mechanism of how CR reduces ROS and how CR can prolong life span by 40% remains elusive.

There are ample studies that describe the age-related changes in the brain and muscle (for reviews see: Marzetti and Leeuwenburgh, 2006;Esiri, 2007) and the attenuating effects CR has on the aging process in these particular areas (Mayhew et al., 1998;Monti et al., 2004). However, little attention has been focused on the possible

beneficial effects CR has on the aging process in the alpha motoneuron. The importance of understanding the effects of CR on the aging process of the alpha motoneuron is highlighted by the fact that the alpha motoneuron is linked to progressive motoneuron diseases that mimic or show signs of an accelerated aging process (for reviews see: Reddy et al., 2004;Lee and Wei, 2007;Papazoglu and Mills, 2007).

Review of Literature

1.0 Theories of aging

A brief review on the theories of aging is needed to understand the direction of this thesis. A review by Medvedev (1990) concluded that there are over 300 theories on aging. Given the fact that so many theories overlap each other and that the current consensus is to combine theories or take the best of each theory instead of seeing each theory as opposing one another (Vina et al., 2007;Muller et al., 2007) four main theories will be discussed. Included are the free radical theory of aging (FRTA); gene regulation theory of aging; telomere theory of aging and the inflammation theory of aging. Each of these theories was selected based on the fact they are the current, relevant, and studies exist that provide evidence for their role in aging. For reviews on these theories see Medvedev (1990), Vina *et al.* (2007) and Tosato; Zamboni; Ferrini & Cesari (2007b).

1.1 Free radical theory of aging

The free radical theory of aging (FRTA) may be the most accepted theory and most studied theory to date. The FRTA was first introduced by Harman in 1956 and the theory stated that aging and degenerative diseases are attributable to the "deleterious side attacks of free radicals on cell constituents and on the connective tissues. The free

radicals probably arise largely through reactions involving molecular oxygen catalyzed in the cell by the oxidative enzymes and in the connective tissues by traces of metals such as iron, cobalt, and manganese (Harman, 1956 pg 299)."

In 1972, the theory was expanded to include the mitochondria as the major producer of free radicals. Harman suggested that the life span was determined by the rate of oxygen utilization. This in turn determined the rate of damage accumulation produced by free radicals in the mitochondria (Harman, 1972).

The importance of the mitochondria in the production of ROS led to the extension of Harman's FRTA to the theory called the Mitochondrial Theory of Free Radicals proposed by Miquel et al. in (1980). The theory suggested that aging is the result of ROS damage specifically to the mtDNA in post-mitotic cells (Vina et al., 2007). It is thought that ROS are produced through normal cellular respiration in the mitochondria and they have the ability to damage cell constituents. The damage that occurs to the mitochondria will lead to impaired respiratory function which leads to a greater production of ROS. This increase in ROS leads to further impaired respiratory function, eventually leading to a decrease in ATP production and eventual cell death thought to occur via apoptosis (which will be discussed in the next section) (Thompson, 2006).

Sufficient evidence exists that supports the use of the FRTA and a brief selection of these findings is mentioned. First, approximately 1-2% of all oxygen used by the mitochondria is converted to a free radical instead of water (Chance et al., 1979;Boveris and Chance, 1973). Second, *Drosophila* (fruit fly) over expressing the Copper/Zinc – superoxide dismutase (SOD) show increases in both average and maximal life spans, a decrease in protein oxidation and a delay in the loss of motor function (Orr and Sohal,

1994). Third, old animals show a greater amount of oxidative damage to proteins, DNA and lipids (Sohal et al., 1993). Fourth, the rate of ROS production is faster in short lived species compared to long lived species (Barja, 2004a;Barja et al., 1994).

1.2 Gene regulation theory of aging

The gene regulation theory of aging states that senescence is the result of changes occurring in gene expression (Kanungo, 1975). To date, there is no direct evidence that supports a programmed mechanism for aging encoded in DNA. However, certain gene manipulations in yeast, fruit flies, nematodes and mice have been able to extend the life of these species (Tosato et al., 2007b). As well, a locus on chromosome 4 of human centenaries has been found that aids in the longevity of these people. Although this theory is beginning to make advances, direct evidence stills needs be found that would explain a programmed aging mechanism encoded with in the genes and therefore will not be discussed further (Tosato et al., 2007b).

1.3 Telomere theory of aging

The theory originated from Hayflick in which he described cell aging as a process that involves a limited number of cell divisions and that after each cell division there is a loss in telomeres (Hayflick, 1998). Hayflick originally described the difference between normal cells and immortal (cancer) cells and attributed the difference to the enzyme telomerase (Hayflick, 1998;Tosato et al., 2007a). After each cell division, telomeres are lost to the replication process but can be regained by the presence of telomerase.

Hayflick demonstrated that cancer cells or immortal cells have unlimited amounts of telomerase activity and suggested that is why cancer cells can continue to proliferate indefinitely (Hayflick, 1998). This has recently been expanded to show other immortal

cells such as stem cells, germ cells and T lymphocytes also express telomerase and have the ability to maintain their telomere length (Tosato et al., 2007b). As well, when cells are supplied with exogenous telomerase, the cell is able to maintain its normal telomere length and proliferate. This theory has gained acceptance but the mechanism of how telomere loss will lead to senescence and evidence in a greater variety of species is still needed (Tosato et al., 2007b).

1.4 Inflammation theory of aging

An area that is relatively new is the potential role inflammation has on the aging process. "Inflammation is a complex host's normal defense reaction to insult and stress, both physiological and non physiological, like chemicals, drugs, oxidants, or a variety of microbial entities" (Chung et al., 2001 pg. 327). Although it is not known what mechanism leads to inflammation in the aging process, evidence shows that ROS and reactive nitrogen species (RNS) are created during the inflammation process and can have deleterious effects, such as described in the FRTA which ultimately leads to tissue death. The investigation of the pathways and mechanisms that are vital for the inflammation theory of aging are currently underway and will lead to a better understanding of its potential in aging (Tosato et al., 2007b).

2.0 Aging and apoptosis

"Apoptosis is an evolutionary, orchestrated cell-death process characterized by membrane-blebbing, DNA fragmentation, and the formation of distinct apoptotic bodies that contain components of the dead cell" (Crighton et al., 2006 pg. 121). It is different from necrosis, which is thought to be a passive form of cell death that is due to ATP depletion. Necrosis results in vacuolization, breakdown of the cellular membrane, and

the release of inflammatory mediators and the cellular content (Edinger and Thompson, 2004).

Apoptosis has been linked to cell loss during development, differentiation, aging and neurological disorders (Lee and Wei, 2007;Martin, 2000;Eve et al., 2007). Typically, apoptosis is described as occurring through either an extrinsic or intrinsic pathway. The extrinsic pathway involves a ligand/receptor interaction with the *death receptors* located on the cell's plasma membrane. The two main extrinsic pathways involved are the tumuor necrosis factor alpha (TNFα) pathway and the FAS ligand FAS receptor pathway (Pollack and Leeuwenburgh, 2001;Lee and Wei, 2007;Hengartner, 2000).

Once the ligand is bound to the death receptor, a conformational change occurs in the receptor resulting in a death inducing signaling complex. This complex recruits the Fas-associated Death Domain (FADD) which in turn recruits and cleaves pro-caspase-8 to its activated form caspase-8. Caspase-8 in return, cleaves and activates caspase-3 which leads to cleaving of DNA and proteins resulting in the cell's death (Hengartner, 2000). This pathway will not be discussed further since most studies on aging and caloric restriction have focused on the intrinsic pathway which refers to apoptosis induced by the mitochondria.

2.1 Intrinsic pathway of apoptosis

Following the FRTA, Lee And Wei (2007) in a recent review concluded that the accumulation of mtDNA mutations may promote apoptosis and play an important role in mammalian aging. Furthermore, markers for apoptosis have been found to be increased in advanced age neurological disorders such as Alzheimer's Disease (AD) and Parkinson's Disease (PD) (Martin, 2000;Eve et al., 2007;Gonzalez de Aguilar et al.,

2000). As well, when the initiators or executors responsible for apoptosis are damaged, cancerous cells are allowed to proliferate (Papazoglu and Mills, 2007).

One of the main proteins concerned with point mutations in mtDNA is transcription factor p53, also known as tumor suppressor p53 or simply - p53 (Papazoglu and Mills, 2007;Lee and Wei, 2007;Kuntz et al., 2000;Zhu et al., 2002). P53 functions to arrest cellular proliferation in response to DNA damage, oxidative damage, hypoxia and activated oncogenes (Papazoglu and Mills, 2007). Initially p53 will try to repair the damaged DNA, but if the DNA damage is beyond repair, p53 will initiate apoptosis (Lee and Wei, 2007;Chung and Ng, 2006;Culmsee and Mattson, 2005).

Once p53 has initiated apoptosis, the mitochondria are targeted. P53 functions to increase the expression of pro-apoptotic genes and decrease the expression of anti-apoptotic genes located on the mitochondrial membrane. Collectively, these proteins are referred to as the B-cell lymphoma 2 family (Bcl-2) which consists of three sub classes that are both pro and anti-apoptotic. Group 1 includes the Bcl-2 gene, and the Bcl-2 homologue (BH) domains BH1-BH4. This class of genes are considered to be inhibitors or anti-apoptotic (Hengartner, 2000). Group 2 includes Bax, Bad and Bak which are pro-apoptotic. Group 3 contains both pro and anti-apoptotic genes with the two most commonly expressed genes being Bid (inhibitor of apoptosis) and Bik (pro-apoptosis) (Hengartner, 2000).

The ratio between the pro and anti-apoptotic proteins found on the mitochondrial membrane will determine if mitochondrial (intrinsic) apoptosis will occur (Chung and Ng, 2006;Oltvai et al., 1993;Green and Reed, 1998;Gonzalez de Aguilar et al., 2000;Hetz et

al., 2007). Recently it has been found that the ratio changes in favor of pro-apoptotic genes with age (Bazhanova et al., 2008;for review seeLee and Wei, 2007).

Upon activation of the pro-apoptotic genes and their subsequent proteins, cytochrome c is released from the inner membrane of the mitochondria. It is unclear how exactly pro-apoptotic proteins (Bax, Bad) cause the release of cytochrome c but it is thought to increase the membrane permeability transition (MPT) pore. The increase in permeability for cytochrome c is believed to occur in one of several ways: 1) the formation of a pore; 2) regulation of caspase molecules via adaptor proteins; 3) oligomerization to form a weak ion channel pore (Hengartner, 2000).

Cytochrome c is a peripheral protein found in the intermembrane space of the mitochondria which is responsible for shuttling electrons from complex III to complex IV during normal cellular respiration (Navarro and Boveris, 2007). Once apoptosis has been initiated, cytochrome c is leaked out of the mitochondria and into the cytosol where it binds with apoptosis protease activating factor-1 (Apaf-1) and caspase- 9 to form an apotosome. The apotosome will then cleave procaspase- 3 to its activated caspase- 3 form. This leads to a caspase cascade were other members of the caspase family are activated and cleave proteins and DNA to cause the death of the cell (Hengartner, 2000).

2.2 Caspase cascade

Caspases (cysteinyl aspartic acid-protease) are proteolytic proteins that are generally found in their zymogen form – procaspase (Fan et al., 2005;Rupinder et al., 2007). Fourteen different caspases have been identified and can be classified according to their main function. Caspases- 2, -8, -9 and -10 are referred to as apoptosis activators and caspases- 3, -6 and -7 are referred to as apoptosis executioners. Another class of

caspases exists and is referred to as inflammatory mediators. Caspases- 1, -4, -5, -11, -12, -13 and -14 comprise the inflammatory mediator class (Fan et al., 2005).

All procaspases have a proteolytic domain and a prodomain. The initiator caspases have a long prodomain that contains a death effector domain (DED) and a caspase recruitment domain (CARD). These two domains help recruit more initiator caspases and to activate executioner caspases (Rupinder et al., 2007).

Caspases can be activated either by death receptors on the cell membrane or by the formation of an apotosome in the cytosol. Once the initiator caspases are activated, executioner procaspases are cleaved to their active form and initiate cell death by destroying fundamental cellular infrastructure and activating factors which further damage the cell (Rupinder et al., 2007).

3.0 The aging process

Aging is defined as the "progressive, more-or-less random, accumulation of diverse, deleterious changes...that increase the chance of disease and death with age (Harman, 2006 pg 10-11)." Factors such as genetics, lifestyle, environment, diet, stress, medical care, etcetera, can lead to an accelerated aging process for a particular person or species. Given the fact that living conditions, nutrition and medicine have improved, the chance for death from these factors in developed countries is minimal and the main factor, especially after the age of 28 is the *inborn aging process* (IAP) (Harman, 2006).

The IAPs are the developments, changes or deletions that occur in the body as we grow older. The IAP is what has kept the average life expectancy to age 85 and that allows for a maximum life span of 122 years of age in humans (Harman, 2006). For the

purpose of this thesis, the review of literature will deal with what is known about the IAP and specifically the IAP on the brain, the motoneuron and skeletal muscle.

3.1 Aging and the brain

Aging in the brain is thought to occur through the accumulation of ROS which leads to apoptosis and the eventual loss of cells and tissue. The central nervous system (CNS) is more susceptible to ROS compared to other organs because the tissue is post mitotic and because of its large energy requirement (Esiri, 2007;Xu et al., 2005).

In humans, the volume of the brain decreases with age at an approximate rate of 0.1-0.2% per year between the ages of 30-50 and between 0.3-0.5% per year after the age of 70 (Esiri, 2007). The primary loss is largely cortical and sub cortical white matter (Mrak et al., 1997). Controversy exists on whether or not neuronal numbers are decreased with age. A recent review by Esiri (2007) found that if neuron loss does occur, only mild or undetectable losses occur. Esiri attributed this recent conflicting finding between earlier studies and newer studies to the fact that new stereological counting methods exist that give an un-biased count of neuron numbers. What is known for certain is that there is a decrease in neuron size with age and that neuron size reflects the dendritic and axonal arborizations of a particular neuron (Mrak et al., 1997;Esiri, 2007).

Two prominent neurotransmitter systems - the cholinergic and the GABAergic systems have been shown to be decreased with age in rats (Virgili et al., 2001;Monti et al., 2004). Choline Acetyl Transferase (ChAT), a marker for cholinergic neurons, was reduced by 15-25% in the hippocampus, spinal cord and the striatum (Virgili et al., 2001;Monti et al., 2004). Glutamate decarboxylase (GAD), a marker for GABAergic neurons had a widespread loss in the CNS including the cortex, olfactory cortex,

hippocampus, habenula, interpeduncular nucleus and the spinal cord (Virgili et al., 2001). The loss in neurotransmitters could explain the possible loss of neurons with age.

Furthermore, markers for ROS, apoptosis and DNA fragmentation have been shown to be increased with age in rat (Monti and Contestabile, 2003). Smith et al. (1991) found an increase in protein oxidation in the human brain with age when compared to a young group. Mandavilli and Rao (1996) examined single and double stranded DNA breaks in the rat brain. A steady increase in DNA breaks was found as age increased and that the cerebral cortex was the most vulnerable region of the brain. Meccoci (1993) found that in humans, an increase in oxidative damage to both nuclearDNA and mtDNA occurs with age with a greater damage occurring to the mtDNA. For a further review on oxidative damage in the brain, see Poon, Calabrese, Scapagnini & Butterfield (2004).

3.2 Aging and the spinal cord

It is well known that motoneurons are lost with age in both rodents (Hashizume et al., 1988;Kanda, 2002;Jacob, 1998;Ishihara and Araki, 1988) and humans (Tomlinson and Irving, 1977;Kawamura et al., 1977). A marked decrease in motoneuron numbers in the rat is seen between 26 months *(old rat)* to 31 months of age *(very old rat)* when compared to an 8 month (young) control group. A marked decrease in motoneuron numbers in human lumbar spinal cord is seen after the age of 60 (Tomlinson and Irving, 1977). As well, Kawamura (1977) states the loss of motoneuron numbers in humans is between 175-260 per decade.

Hashizume (1988) found that with age, medial gastrocnemius (MG) alpha motoneurons decreased by 30%. Ishihara (1988) found similar age-related changes in Extensor Digitorum Longus (EDL) motoneurons when comparing 120 week old rats to

10 week old rats. Ishihara also found a decrease in the oxidative capacity of the EDL motoneurons at week 120 compared to the week 10 and week 60 groups. Jacob (1998) quantified the number and sizes of motoneurons in the lumbar spinal cord segment L4/L5 between 6 month old and 22 month old rats. A decrease in the number of motoneurones (728 vs. 407) and the average soma size was found in the old group compared to the young group. However, when Soleus motoneurons were examined in rat and cat, no age-related loss of motoneurons was found (Gutmann and Hanzlikova, 1966;Ishihara et al., 1987). This may suggest that the loss of motoneurons is muscle type specific.

Despite the knowledge that there is an age-related loss of motoneurons sparse evidence exists on whether motoneuron death is from the accumulation of ROS damage and ultimately apoptosis. Primarily, studies have examined the effects of ROS and apoptosis in the spinal cord after induced spinal cord trauma, during development or in a motor neuron disease model (Martin et al., 2005;Eve et al., 2007;Bigini et al., 2007;Al Abdulla and Martin, 1998). The only study that was found that has looked at DNA fragmentation and markers for apoptosis is from Monti and Contestabile (2003). Monti and Contestabile (2003) examined DNA fragmentation, caspase- 1 and caspase- 3 activation in aged and CR rats (CR finding will be discussed in Caloric Restriction and Aged Spinal Cord section). It was found that DNA fragmentation and caspase- 1 activation increased significantly with age but caspase- 3 did not. It was hypothesized that caspase- 3 did not change because caspase- 3 has a short activation period and that they may not have had a sensitive enough assay to measure its activation.

3.3 Aging and skeletal muscle

Age is a significant factor in the loss of skeletal muscle as skeletal muscle mass progressively declines after the age of 45 in both men and women (Janssen et al., 2000). One of the reasons that muscle mass declines with age is because there is a loss of muscle fibers, preferentially the type 2 fibers and atrophy in the remaining muscle fibers (Ishihara and Araki, 1988;Larsson et al., 1978;for review see Marzetti and Leeuwenburgh, 2006;Dirks and Leeuwenburgh, 2006;Lexell, 1995). It has been shown that by the age of 80, between 30-40% of skeletal muscle fibres are lost (Lexell, 1995). The exact mechanism for the loss of skeletal muscle fibres or skeletal muscle mass is not known but is thought to occur via apoptosis (Chung and Ng, 2006;Dirks and Leeuwenburgh, 2006;Dirks and Leeuwenburgh, 2005;Dirks Naylor and Leeuwenburgh, 2008;Seo et al., 2008).

As with apoptosis in the brain and the spinal cord, it is believed that ROS production causes mtDNA mutations within the skeletal muscle that leads to the eventual initiation of apoptosis. Drew *et al.* (2003), found that the rate of ATP production in rat gastrocnemius declined in the old (24 months) versus the young (12 months) by 50%. A decline in ATP production is an indicator of mitochondrial dysfunction caused by oxidative damage.

Further support that apoptosis plays a role in sarcopenia is from Chung and Ng (2006). Chung and Ng examined markers for oxidative DNA damage and apoptosis in the gastrocnemius of middle aged (16 months) and old (29 months) aged rats. It was found that DNA fragmentation increased by 42%, p53 increased by 90% (pro-apoptotic), Bax (pro-apoptotic) increased by 226%, and Bcl-2 (anti-apoptotic) increased by 65%.

Previous studies have shown that a very strong indicator of whether or not apoptosis will initiate is the ratio between the levels of Bax/Bcl-2. In this case, the ratio went from 1.02 in the middle aged rats to 1.96 in the old aged rats. Furthermore, it was found that Apaf-1 increased 880% and the level of cleaved caspase-9 increased 209.8%.

4.0 Caloric restriction

Caloric restriction refers to a diet with a restriction in caloric intake without undernutrition (Koubova and Guarente, 2003). CR is also described as an experimental procedure that increases the longevity of many species and decreases the incidence of degenerative diseases (Gomez et al., 2007). There are two popular methods to achieve CR in animals. The first method, animals are on a caloric restricted diet everyday. The restriction can be anywhere between 25-50% of normal intake. The second method, referred to as intermittent fasting (IF) or every other day eating allows the animal to eat freely on alternate days and on the other day, food is withheld (Martin et al., 2006). This method works out to be a 25-30% restriction in calories.

CR is the only non genetic experimental intervention that has been found to increase the life span by up to 50% in certain species. CR has been proven to delay age-related changes in several species including yeasts, rotifers, spiders, worms, fish, fruit flies, mice and rats (Koubova and Guarente, 2003;Sohal and Weindruch, 1996). Recent studies suggest that CR is also effective in non human primates (rhesus monkey), showing similar results as seen in rodents (reviewed in Rao, 2003).

It would be beneficial to determine if the results seen in rodents can be extrapolated to humans. Unfortunately, one cannot simply test CR on humans. However, one form of a CR study exists in humans. Humans that were sealed in biosphere 2 did

not have sufficient food to last the two year period and had to restrict their caloric intake to approximately 1750-2100 cal/day. It was found that the CR humans had similar physiological, hematological, hormonal and biochemical changes seen in rodents and monkeys although the paper did not report if there was any negative side-effects associated with the CR (Rao, 2003).

4.1 Mechanisms of caloric restriction

The exact mechanisms by which CR retards the aging process is currently unknown. Given the fact that CR has such global physiological responses such as decreased oxygen consumption, reduced blood glucose levels, increase in insulin sensitivity and a more efficient immune system, it is hard to describe the mechanisms of CR with one particular theory (Prolla and Mattson, 2001;Martin et al., 2006).

One idea that has gained favor is that by withholding or reducing food intake, a mild stress response is created. By inducing a mild stress response, an increase in stress proteins such as the heat shock proteins (hsps) are activated and can refold misfolded proteins and degrade damaged proteins in a more efficient manner (Martin et al., 2006). Other beneficial factors that would be seen by a mild stress response would include an increase in neurotrophic factors, an increase in insulin sensitivity and an increase in circulating hormones such as cortisol (Martin et al., 2006).

For the purpose of this thesis, a brief review of the beneficial effects CR has on mitochondria, the brain, the motoneuron and skeletal muscle will be included. This review will only focus on observable alterations in neuron sizes, neuron numbers, oxidative markers and apoptotic markers seen in these tissues.

4.2 Caloric restriction and the mitochondria

One of the most studied areas and most important areas regarding CR is the mitochondria since it is the main producer of ROS. In animals, CR reduces the amount of ROS produced by the mitochondria in the liver, the heart, skeletal muscle and the brain (Hepple et al., 2006;Barja, 2004a;Drew et al., 2003;Gomez et al., 2007;Pamplona and Barja, 2006). This reduction in ROS is directly related to the reduction in mtDNA damage and ultimately the increased longevity seen in several species (for reviews see Martin et al., 2006;Lee and Wei, 2007;Barja, 2004a;Bua et al., 2004;Navarro and Boveris, 2007). As stated earlier, the rate of ROS production determines the longevity or the rate of aging of a particular species (Barja, 2004a).

4.3 Caloric restriction and the brain

At the functional level, CR has been shown to increase memory and learning in rodents (Carter et al., 2007;Martin et al., 2006). At the molecular level, CR has been shown to attenuate the loss of brain derived neurotrophic factor (BDNF) and serotonin (5-HT) (Duan et al., 2003;Lee et al., 2002). However, another study examining CR and BDNF in aged rats found that CR did not attenuate the changes in BDNF expression with age (Newton et al., 2005). Therefore if CR does attenuate the loss of BDNF, this could explain the attenuation of the age-related loss of neurons and the increase in neuroplasticity of CR rodents (Mattson et al., 2004).

Shelke and Leeuwenburgh (2003) examined the effect age and CR had on DNA fragmentation, caspase activation and apoptosis activity in old (26 month old) compared to young (12 month old) rats. It was found that DNA fragmentation increased with age and that the increase was attenuated by CR. Furthermore, the level of ARC (apoptosis

repressor with a CARD) which functions to inhibit caspase- 2 and the release of cytochrome c were decreased in age but the loss was attenuated with CR. CR also attenuated the age-related increase in cytochrome c and caspase- 2 and but had no effect on caspase- 3 activity.

Hiona and Leeuwenburgh (2004) found that the levels of PARP (marker for apoptosis) increased with age and the increase was less in the CR rats compared to the *ad libitum* (AL) rats. As well, XIAP (apoptosis inhibitor) increased with age and that the increase was significantly higher in the CR group. However, Hiona and Leeuwenburgh (2004) were unable to find a decrease in DNA fragmentation with CR. They attributed this to the fact that they used whole tissue homogenates which would make detecting DNA fragmentation very difficult.

Further support that CR attenuates oxidative damage is from Wolf et al. (2005), where the levels of 8-hydroxy-deoxyguanosine adducts (8-OHdG) in the brain, skeletal muscle, heart, liver and lymphocytes in aged rats were examined. It was found that the level of 8-OHdG increased in all tissues with age and the age-related increase was attenuated by CR. CR returned the 8-OH-dG levels in the 12 and 24 month old rats to the same level seen in the 2 month old rats.

Monti et al. (2004) examined the effect CR had on the cholinergic and GABAergic systems in various regions of the brain and the spinal cord. It was found that a decrease in choline acetyl transferase (ChAT) and glutamate decarboxylase (GAD) was found in all regions of the aged brain and spinal cord and that CR did not attenuate the loss. This finding may indicate that CR will not attenuate the loss of motoneurons due to

age. However, since whole tissue homogenates were used, it was not able to determine where the loss of ChAT occurred in the spinal cord.

4.4 Caloric restriction and the spinal cord

To date, only two studies have examined CR in the rat spinal cord. The first, from Kanda (2002) studied the effect of age and CR on the number of motoneurons innervating the medial gastrocnemius (MG). The study used 16 and 28 month old rats and divided each age group into an AL and CR group. The CR group was subjected to the IF diet regime and was only fed on Mondays, Wednesdays and Fridays. Kanda found that there was a decrease in MG motoneuron numbers with age and that the loss was greater in the AL group compared to the CR group (12.6% versus 5.7%).

This was the only study found that examined CR and motoneuron numbers in the aged rat. However, it has several limitations that need to be addressed. First, retro-grade labeling was used by injecting horseradish peroxidase (HRP) solution into the MG nerve. Therefore, only neurons innervating the MG were counted which does not give a full representation of lumbar spinal cord motoneuron loss. Second, there is a potential that not all of the motoneurons were labeled in the aged rats due to the retraction of the motoneuron axon from the muscle.

Kanda addressed this issue and states not all motoneurons were labeled when examined and that a greater number of unlabeled motoneurons were seen in the aged rats. The other limitation of the study that should be addressed is the age of the animals. It would be beneficial to use rats at a more advanced age such as 32-36 months as opposed to 28 months.

The only other article that studied age and CR in spinal cord is from Monti and Contestable (2003). Old age rats (30 months) divided into a control group and an IF group (fed every other day) were compared to young rats (4 months) for markers of apoptosis and DNA fragmentation. An age-related increase in DNA fragmentation and caspase- 1 activity was found but no increase in caspase- 3 activity was found in the spinal cord. CR was found to only attenuate the age-related increase in caspase- 1 but not the increase in DNA fragmentation.

4.5 Caloric restriction and muscle

CR has been shown to attenuate the age-related loss of muscle (Payne et al., 2003), with CR having a greater effect on attenuating the loss of type 2 muscle fibres (Boreham et al., 1988). CR also appears to delay the conversion of the remaining type 2 muscle fibres to type 1 muscle fibres (Boreham et al., 1988;Lee et al., 1998;Lee et al., 1998;Payne et al., 2003). CR not only attenuates the loss of muscle mass but it also retards the loss of muscle force and strength associated with age (Payne et al., 2003;Mayhew et al., 1998). One mechanism by which CR prevents the loss of muscle mass and strength is by maintaining the expression of dihydropyridine receptors and ryanonide receptors which are voltage gated calcium channels needed for the release of calcium and muscle contractions to occur (Mayhew et al., 1998).

CR has been found to reduce apoptosis in muscle, which is presumed to be a leading cause of sarcopenia (Phillips and Leeuwenburgh, 2005;Dirks and Leeuwenburgh, 2004;for review see Dirks Naylor and Leeuwenburgh, 2008). Edwards *et al.* (2007) examined the gene expression of apoptosis markers in young versus old and AL versus CR mice. Edwards found that in the gastrocnemius, 712 genes had an age-effect, with

311 genes showing a higher expression in the young and 401 genes showing a higher expression in the old. CR was found to attenuate 87% of the age-related changes in gene expression.

Of the genes examined, 5.2% were found to influence the cell cycle. In particular, two of these genes (p21 and GADD454) that are mediated by p53 and responsible for cell cycle arrest were significantly increased. Therefore four other known apoptosis genes mediated by p53 were examined – puma, noxa, tnfrsf10b and bok. All four genes were found to increase significantly with age and that the age-related increase was significantly attenuated by CR.

CR has also been found to reduce mtDNA damage in the rat soleus muscle. *Cassano et al.* (2006) found that CR in 28 month old rats attenuated mtDNA mutations to the levels found in 12 month old rats. Aspnes et al. (1997) examined mtDNA mutations in the Soleus, EDL, Adductor Longus (ADL) and the Vastus Lateralis in young (3-4 months) and very old rats (30-32 months) on either a 35% or 50% CR diet. It was found that both CR diets reduced mtDNA mutations, with the 50% CR diet having a significantly greater effect than the 35% CR diet in the soleus and ADL.

Rationale for Project

While it is well known that motoneurons are lost with age, little is known about the effects of age on the biomarkers for apoptosis and ROS damage in the motoneuron. The purpose of this study is to examine the effect of age on motoneuron numbers and diameters and biomarkers for apoptosis and ROS damage. Furthermore, the reported

beneficial effects of CR will be examined on any observed age-related changes in motoneuron numbers and diameters and biomarkers for apoptosis and ROS damage.

Hypotheses

1. Alpha motoneurons will be decreased in the old rats (30-32 month old) compared to young rats (8 months).

   This is hypothesized because it has been previously shown that spinal motoneurons in both humans and rats are lost with age.

2. Alpha motoneuron loss will be higher in the oldAL group compared to the oldCR group.

   This is hypothesized because it has been previously shown that CR prevents motoneuron loss in the rat MG.

3. Markers of ROS damage and apoptosis will be higher in the old rats compared to the young rats.

   This is hypothesized because it has been previously shown that part of the aging process in the brain and muscle is an increase in ROS damage and the number of apoptotic cell bodies.

4. Markers of ROS damage and apoptosis will be higher in the oldAL group compared to the oldCR group.

   This is hypothesized because in both brain and muscle, CR has been previously shown to decrease ROS and the number of apoptotic bodies compared to free eating species.

.Methods

Animal dietary regimen

Twenty female Fisher 355/ Brown Norway (FBNF1) rats were obtained from the National Institute of Aging (NIA) at an age of 24 months (Harlan Sprague Dawley Inc., Indianapolis, Indiana). Ten of these animals were fed *ad libitum* (oldAL) with NIH-31 rat chow and 10 of these rats were fed a calorie restricted diet of NIA fortified chow beginning at 14 weeks of age (oldCR). Appendix 1 shows the composition of the NIH-31 regular chow fed to the AL group and the NIH-31 fortified rat chow fed to the CR group. The nutrient content is virtually identical between the two rat chows. CR for the oldCR rats was slowly phased in over a 3 week period to a daily intake of 60% of the AL rats.

At arrival to our facility, oldAL animals continued to feed *ad libitum* whereas the oldCR animals were maintained on a restricted diet of 15g of NIH-31 fortified rat chow per day according to previously published protocols of dietary restriction for the FBNF1 breed of rat (Turturro et al., 1999). This static diet of 15g approximates to 60% of what the oldAL group would eat. Animals were housed individually in standard plastic cages in an environmentally-controlled room maintained at 23° C with a 12:12 h light:dark cycle. Water was provided *ad libitum* for both groups of rats.

Ten young (8.4±4.6 mo) FBNF1 animals fed *ad libitum* were used as a control group (youngAL). OldAL and oldCR rats were kept in our facility until the age range of 30-32 months old at which time they were used in electrophysiology experiments conducted in the lab.

Experimental procedure and spinal cord extraction

Animals were anesthetized with ketamine and xylazine for a period of 8-12 hours, during which time electrical recordings from the lumbar spinal motoneurons were taken. Upon completion of the experiment, the animals were euthanized with an overdose of ketamine and xylazine followed by decapitation. The lumbar enlargement portion of the spinal cord was immediately removed, placed on cork and fresh frozen in isopentane and then stored in a freezer at -70° C until further processing.

Tissue Sectioning

Tissue was cut on a cryostat at 15μm and every sixth section was mounted on glass slides for immunohistochemistry. This resulted in approximately 25-35 sections per animal. To normalize the results, the total number of neurons was multiplied by 35/x, where x equals the number of sections cut for that animal. Normalizing to 35 sections will equal 3.15mm of lumbar spinal cord (**35** sections x every **6**th section x **15** micron per section).

Every sixth section was selected because it prevented the possibility of double counting neurons and is a reliable method for predicting motoneuron numbers for a given area (Coggeshall and Lekan, 1996;West, 1999). This was also confirmed in a previous pilot study that showed every sixth section provided an accurate number of overall neuronal numbers (see appendix 2).

Each time the tissue was processed and immunohistochemistry was performed, one animal from each group (oldAL, oldCR and youngAL) was included to ensure that each group was processed under the same conditions. The person performing the

immunohistochemistry and the person counting the neurons were blinded to what group each animal belonged to. The technician responsible for cryo-sectioning the tissue was the only person aware of what group each animal belonged to. It is believed that this is the first double blinded study conducted on motoneuron quantification in aged rats.

Immunohistochemistry

A double labeled immunohistochemistry protocol for identifying neurons in the ventro-lateral portion of the ventral horn was developed using a ChAT and **Neu**ronal **N**uclear (NeuN) antibody (appendix 3). Briefly, slides were removed from the -70°C freezer and placed in cold 4% paraformaldehyde for 15 minutes. The slides were then removed and washed in a phosphate buffer solution with triton (PBS-T). A blocking solution consisting of PBS-T, bovine serum albumin and goat serum was then applied for one hour. The slides were then removed and the ChAT primary antibody was applied and left to incubate for 72 hours in a 4°C refrigerator. The slides were then washed in PBS-T and then the anti-goat secondary antibody was applied for two hours followed by another wash in PBS-T. The NeuN primary antibody was then applied and left to incubate overnight. The slides were then washed in PBS-T and then the anti-mouse secondary antibody was applied for two hours. The slides were washed and then vector shield and coverslips were applied.

The ChAT and NeuN antibodies were obtained from Chemicon (AB 144P and MAB377, respectively) and were used at 1:50 and 1:1000 respectively. The secondary antibodies, FITC donkey anti-goat and Cy3 donkey anti-mouse were obtained from Jackson laboratories (705-095-003 and 715-165-151) and were used at 1:100 and 1:300.

Choline Acetyltransferase

ChAT is the enzyme responsible for the synthesis of acetylcholine (ACh) from acetyl CoA and choline. ChAT is synthesized in the perikaryon of cholinergic neurons and is transported to the nerve terminals where it is used in the reaction described above to create Ach. Currently, it is the most specific marker for the functional state of cholinergic neurons located in the central and peripheral nervous system (Oda, 1999). Specifically, in the spinal cord ChAT positive neurons are only located in the ventral horn and stain the cell body (Oda and Nakanishi, 2000).

NeuN

The NeuN antibody specifically identifies a neuron specific nuclear protein found in the nervous system. In the spinal cord, it is specific only to neurons and will not identify glial cells or ogliodendrocytes in the white matter (Mullen et al., 1992).

Motoneuron identification

Each slide was viewed under a fluorescent microscope (Nikon) equipped with a camera (SnapPRO). Pictures were taken in ImagePRO which was capable of measuring cell diameter, area, perimeter and etcetera. For each section, only the ventro-lateral portion of the ventral horn was analyzed (see Figure 1). A picture at 10x magnification was taken under the FITC fluorescence spectrum to identify cell bodies labeled with ChAT and then a picture was taken under the Cy3 fluorescence spectrum to identify the cell nucleus with NeuN. The pictures were then merged together and the merged picture was used to count motoneurons. In order for a cell to be labeled a motoneuron it must be double labeled (expressing both ChAT and NeuN) and the nucleus must be clearly visible.

Below are examples of photos taken using ChAT (green), NeuN (red) and the merged picture (Figures 2-4). Since no antibody exists that is specific to alpha motoneurons, motoneurons were classified by their cell body size. Neurons with a cell body diameter between 15 and 25μm were considered to be gamma motoneurons and neurons with a cell body diameter larger than 25μm were considered to be alpha motoneurons (Ishihara et al., 1988;Hashizume et al., 1988;Jacob, 1998). For each cell determined to be a motoneuron, the cell's size, area, diameter and perimeter were recorded in Microsoft Excel.

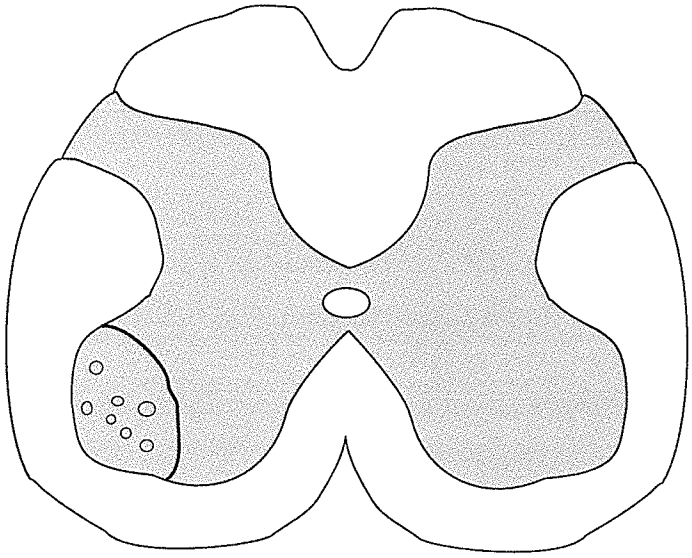

Figure 1 Schematic representation of the rat lumbar spinal cord
A cross section depiction of a rat lumbar spinal cord, illustrating the ventral horn. Within the ventral horn only the ventro-lateral portion was examined for ChAT positive neurons.

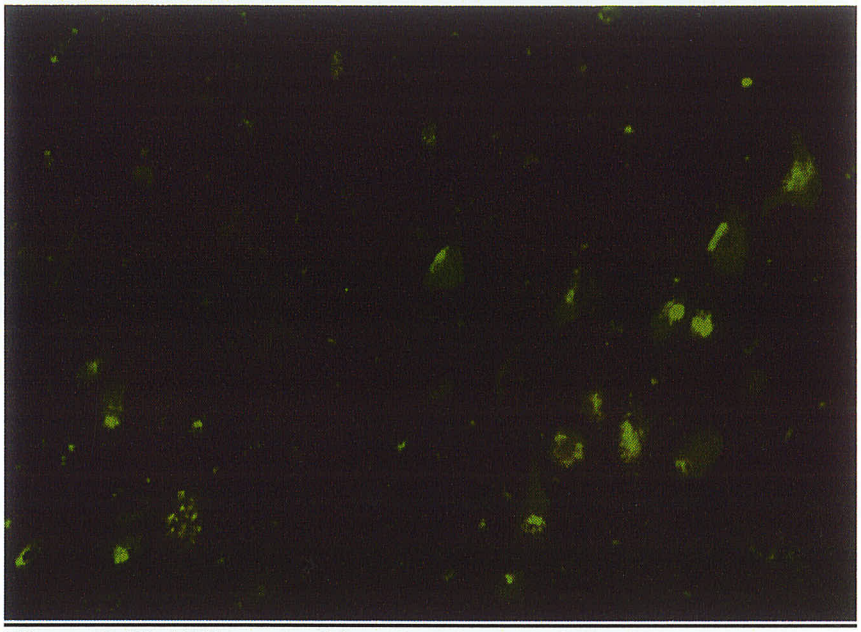

Figure 2 ChAT immunohistochemistry staining
Image taken under 10x magnification. ChAT staining is seen throughout the cell body with some areas of dense localization seen via the intense green fluorescence.

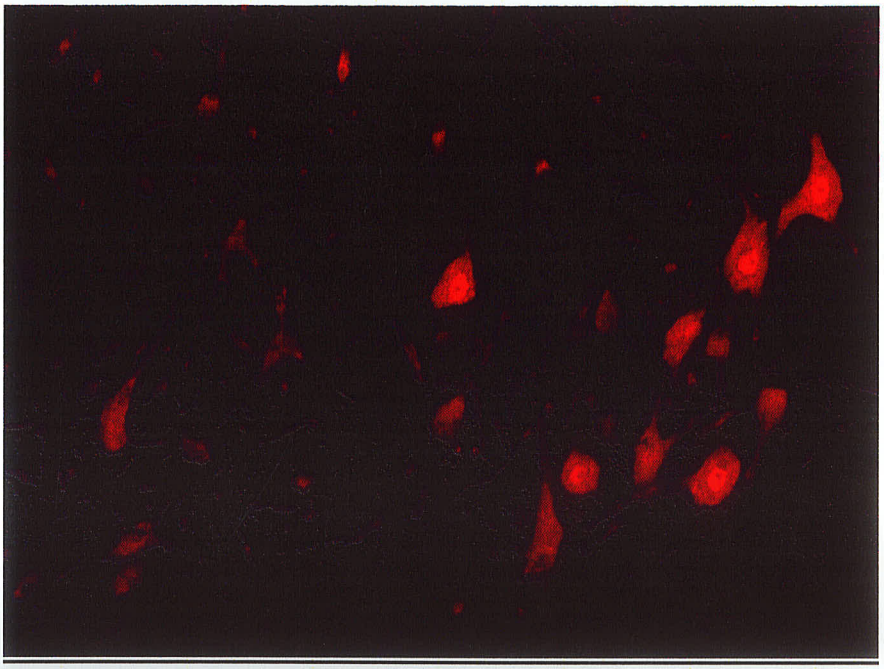

Figure 3 NeuN immunohistochemistry staining
Image taken under 10x magnification. NeuN staining is seen through out cell body with greater fluorescence seen in the cell nucleus. NeuN provides a very good outline of the neuron's morphology.

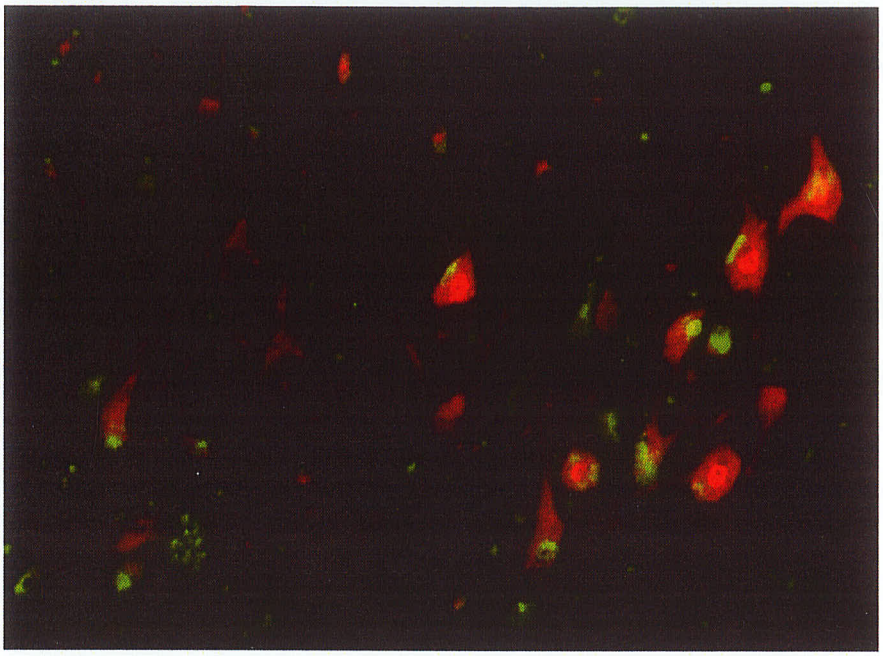

Figure 4 Merged photograph of ChAT and NeuN immunohistochemistry
Cells expressing both ChAT and NeuN with a visible nucleus are identified as motoneurons.

Western Blots

Protein concentrations for various markers of ROS and apoptosis were analyzed using immunoblots (Western Blots). The tissue samples were extracted from the ventral horn of the lumbar enlargement from four to five animals for each group. A detailed protocol for the Western blots can be found in appendix 4.

Briefly, the process involved homogenizing the ventral horn of lumbar spinal cord on ice in a Radioimmunoprecipitation (RIPA) buffer solution. The homogenized tissue was then centrifuged at 13000 revolutions per minute for 15 minutes. Protein densities were determined using the BioRad protein dye reagent which is then used to determine the sample size to be used. Samples were run on a 10-15% SDS gel and then transferred to a polyvinylidene fluoride (PVDF) membrane. The PVDF membrane was then stained

in Ponceau Red for five minutes followed by three washes in a tris-phosphate buffer solution (TBS). The PVDF membrane was then blocked in TBS with triton (TBST) and 5% milk for one hour. The various primary antibodies described below were applied with TBST and a 5%milk solution. After this, the PVDF membrane was washed in TBST and the appropriate horseradish peroxidase secondary antibody was then applied for one hour. Detection of the protein band was performed using a Pierce Super Signal kit and imaging analysis was performed by the Bio Rad Fluor-S-Multi-Imager.

Antibodies for Western Blots

Caspase- 3 is a pro-apoptotic protein found in the cytosol upon activation of the caspase cascade (Hengartner, 2000). The antibody against caspase- 3 was purchased from Cell Signaling (product #4272). The anti-caspase- 3 antibody was used at 1:1000 and detects a band at 14 kilodaltons (kDa). Apoptosis-inducing factor (AIF) is a protein that is released from the mitochondria from either the internal or external signaling of apoptosis and translocates to the nucleus to activate endonucleases (Krantic et al., 2007). The antibody against AIF was purchased from Cell Signaling (product #4642). The anti-AIF antibody was used at 1:500 and detects bands at 57 and 67 kDa. Cytochrome C is a protein that is released from the mitochondria from internal signaling and forms a component of the apotosome which is responsible for the activation of the caspase cascade (Hengartner, 2000). The antibody against cytochrome c was purchased from Cell Signaling (product# 4272). The anti cytochrome c antibody was used at 1:1000 and detects a band at 14kDa. Hsp27 is a chaperone protein found in the cytosol that has been linked to CNS tissue protection during times of stress (Concannon et al., 2003). The

antibody again Hsp27 was purchased from Santa Cruz (product #sc-1048). The anti-Hsp27 antibody was used at 1:150 and detects a band at 27kDa. Oxoguanine glcosylase 1 (Ogg1) is the enzyme that repairs 8-hdroxyguanine, a form of oxidative DNA damage (Wong et al., 2008). The antibody against Ogg1 was purchased from Novus Biologicals (product # NB100-106). The anti Ogg1 antibody was used at 1:1000 and detects bands at 39kDa.

Statistical Analyses

Main effects for dietary regimen and age were determined using one-way analyses of variance (ANOVAs) detecting potential differences for:

1. The total number of ChAT positive neurons
2. The total number of presumed alpha motoneurons
3. The total number of presumed gamma motoneurons
4. The average diameter of the presumed alpha motoneurons
5. The average diameter of the presumed gamma motoneurons
6. The total amount of Ogg1 present in the ventral horn of the lumbar enlargement
7. The total amount of AIF present in the ventral horn of the lumbar enlargement
8. The amount of nuclear AIF present in the ventral horn of the lumbar enlargement
9. The total amount of Caspase- 3 present in the ventral horn of the lumbar enlargement
10. The total amount of cytochrome c present in the ventral horn of the lumbar enlargement
11. The total amount of Hsp27 present in the ventral horn of the lumbar enlargement

Tukey's post hoc analysis was utilized when indicated to test for differences among the groups. Significance level was set to $p < 0.05$ for all analyses. As well, a test for homogeneity of variance was performed for each ANOVA. If a difference in variance was found, a Kruskal Wallis test was performed.

Results

Table 1 provides a summary of the mean age, diet, body weight and body weight relative to muscle mass for each group. A significant age difference ($p<0.05$) between the young and old was found. Body weight for the oldAL was significantly elevated compared to the youngAL and oldCR ($p < 0.05$). When body weight was compared relative to the weight of 11 hindlimb muscle for each rat, the ratio of body weight to muscle mass in the oldAL was significantly larger compared to the youngAL and oldCR ($p< 0.05$). As well, the ratio of body weight to muscle mass for the oldCR group was significantly larger compared to the youngAL group ($p < 0.05$). The muscle weight (grams) used to compare to body weight was the combined weight (grams) of the red and white vastus lateralis and rectus femoris of both the left and right hindlimbs and tibialis anterior, soleus, plantaris, medial and lateral gastrocnemius of the left hindlimb.

Table 2 provides a summary of the results. For each variable, a one way ANOVA was performed. Tukey's post hoc analysis was utilized when indicated to test for among the groups.

Table 1 Animal Characteristics

Animal Characteristics	youngAL	oldAL	oldCR
Age (mo)	8.4 ± 4.6	30.8 ± 1.3*	31.04 ± 1.04*
Diet	Ad libitum	Ad libitum	40% calorie-restricted
Body wt (g)	233.3 ± 31.6	346.9 ± 46.8**	262.7 ± 14.4
Body wt (g) divided by hindlimb muscle mass §	38.7 ± 2.9	54.6 ± 5.7**	45.1 ± 12.1*

*Different from the youngAL, **different from the oldCR and youngAL (one-way ANOVA and Tukey's Post Hoc Analysis, $p < 0.01$). § = wet muscle weight of 11 hindlimb muscles (red and white vastus lateralis and rectus femoris from both the left and right hindlimbs and soleus, tibialis anterior, plantaris, medial and lateral gastrocnemius from the left hindlimb).
Results have been previously reported in Kalmar *et al.* 2009.

Table 2 Summary of results

	youngAL	oldAL	oldCR
Body wt (g)		↑	
Body wt relative to muscle mass		↑↑	↑
Motoneuron numbers		Not significant	
Motoneuron diameters		Not significant	
Caspase 3		Not significant	
AIF - total		Not significant	
AIF - Nuclear fraction		Not significant	
Cytochrome c		↓	↓
OGG1		↓	↓
Hsp27			↓

Single arrow indicates either a significant increase or decrease compared to the youngAL. Double arrow indicates a significant difference compared to both youngAL and oldCR.

Motoneuron Numbers

Eighteen rats were used for the identification of ChAT-positive neurons located in the ventro-lateral region of the lumbar enlargement of the spinal cord. Six rats from each group were analyzed. Table 3 provides a summary of the average number of ChAT-positive neurons found for each group. Appendix 5 provides a detailed report of the total number of ChAT-positive neurons counted for each rat.

A one-way ANOVA revealed there was no significant difference in the total number of ChAT-positive neurons among groups (Figure 5). No difference was found in the total number of ChAT-positive presumed alpha motoneurons between groups (Figure 6). Although the difference among the groups was not significant, the difference was large. Compared to the youngAL group, the oldAL and oldCR had a 23% and 30% difference in the mean total amount of ChAT-positive presumed alpha motoneurons. There was no difference in the mean total number of ChAT-positive presumed gamma motoneurons among groups.

As well, a Chi square test was performed since it can be argued that the data collected on motoneuron numbers are fact non-parametric data. For the Chi square analysis the youngAL group was used as the expected number and the oldAL and oldCR were the observed numbers. The Chi square analysis revealed no significant difference between the observed and expected numbers ($p = 0.35$) indicating no difference in motoneuron numbers between the young and old groups.

Table 3 ChAT positive neurons per 3.15mm of lumbar spinal cord

Group	Total number of ChAT-positive neurons	Presumed alpha motoneurons	Presumed gamma motoneurons
YoungAL (n=6)	8298 ± 2086	5010 ± 1667	3294 ± 1165
OldAL (n=6)	7620 ± 853	3870 ± 937	3750 ± 535
OldCR (n=6)	7290 ± 1953	3480 ± 1462	3810 ± 1452

Presumed alpha motoneurons refer to ChAT-positive neurons with a diameter greater than 25μm and presumed gamma motoneurons refer to ChAT-positive neurons with a diameter between 15μm and 24.99μm. Mean ± SD. The number of neurons for each animal was normalized to a 3.15mm length of the lumbar enlargement.

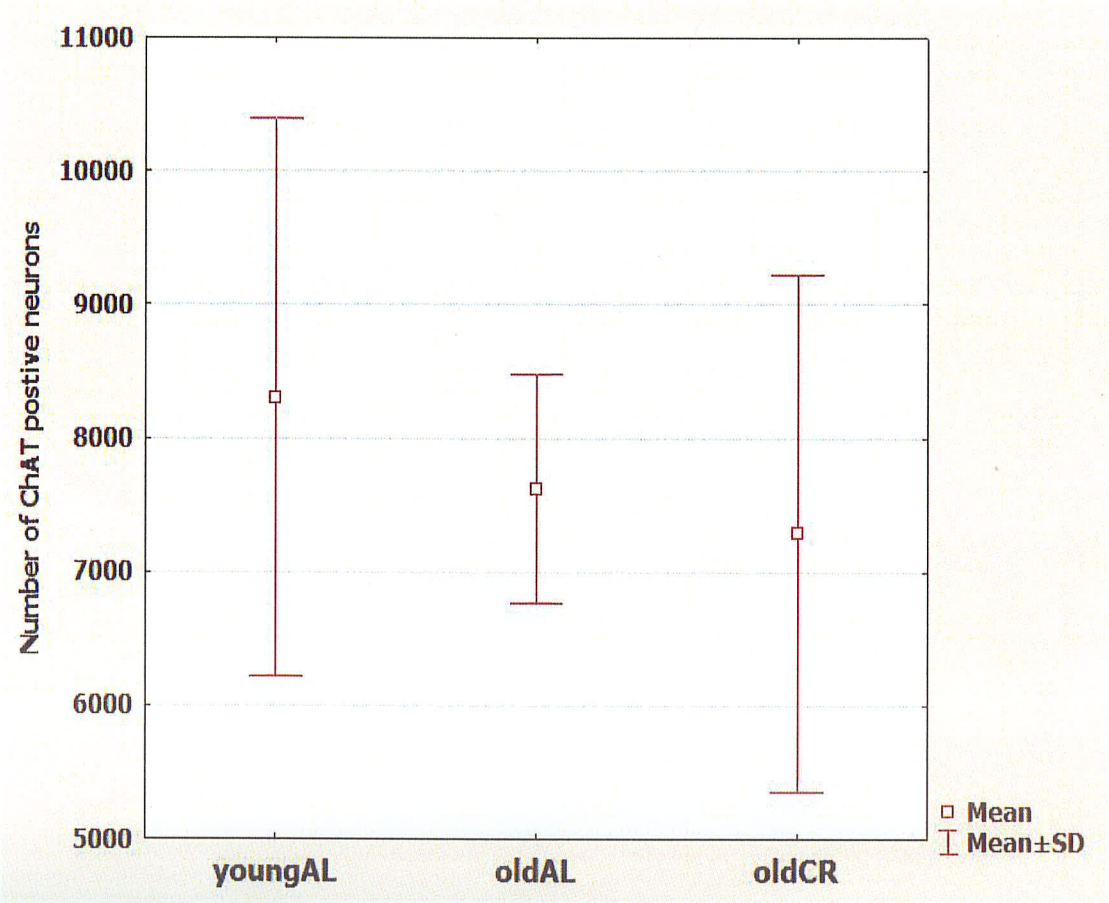

Figure 5 Effect of diet and age on ChAT-positive neurons
Average number of ChAT-positive motoneurons located in the ventro-lateral horn of rat lumbar spinal cord (n=6). No significant difference between groups was found.

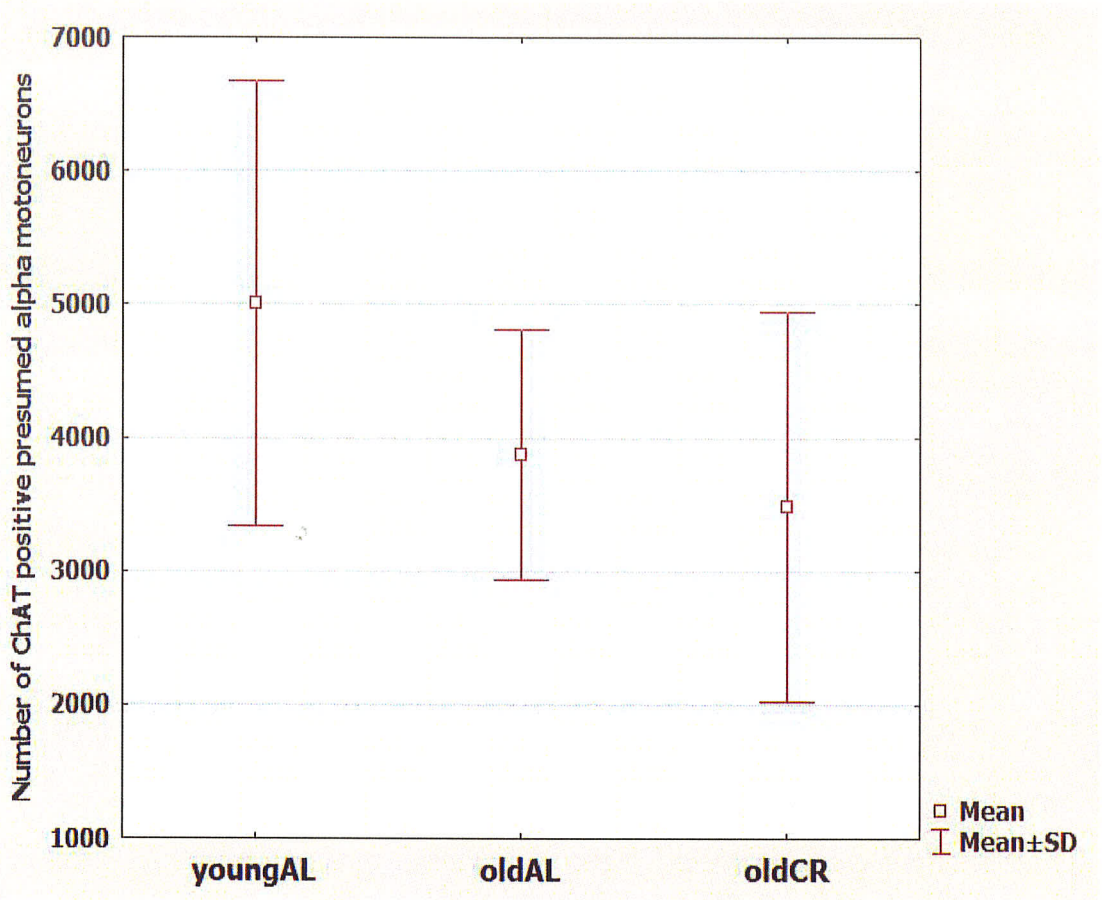

Figure 6 Effect of diet and age on ChAT-positive presumed alpha motoneurons
Average number of presumed ChAT-positive alpha motoneurons (>25um) located in the ventro-lateral horn of rat lumbar spinal cord (n=6). No significant difference between groups was found.

Motoneuron Diameters

For each ChAT-positive neuron identified under the fluorescent microscope the diameter of the neuron was recorded using ImagePRO software. Table 4 provides a brief overview of the average diameters for the neurons recorded. Presumed gamma motoneurons were classified as ChAT-positive neurons with diameters between 15μm and 24.99μm. Presumed alpha motoneurons were classified as ChAT-positive neurons with diameters greater than 25μm. Appendix 6 provides the results of each rat analyzed.

The motoneuron diameters were placed into 3.99μm bins from the smallest neuron diameter recorded (15μm) to the largest neuron diameter recorded (66μm) (Figure 7). A two way ANOVA was performed to determine if there was an interaction between group and bins. A bin effect ($p < 0.001$) was found but no difference among groups was found. A one-way ANOVA was also performed for the diameters for presumed ChAT-positive alpha motoneurons (> 25μm). No significant difference was found.

Table 4 ChAT-positive neuron diameters (μm)

Group	Total	Alpha	Gamma
youngAL	29.64 ± 3.07	36.22 ± 1.99	19.51 ± 0.53
oldAL	28.04 ± 1.98	36.65 ± 1.70	19.30 ± 0.27
oldCR	27.29 ± 3.40	35.93 ± 1.83	19.15 ± 0.40

Mean ± SD (n=6). No difference between groups was found for the average diameter for the total, presumed alpha or presumed gamma ChAT-positive neurons.

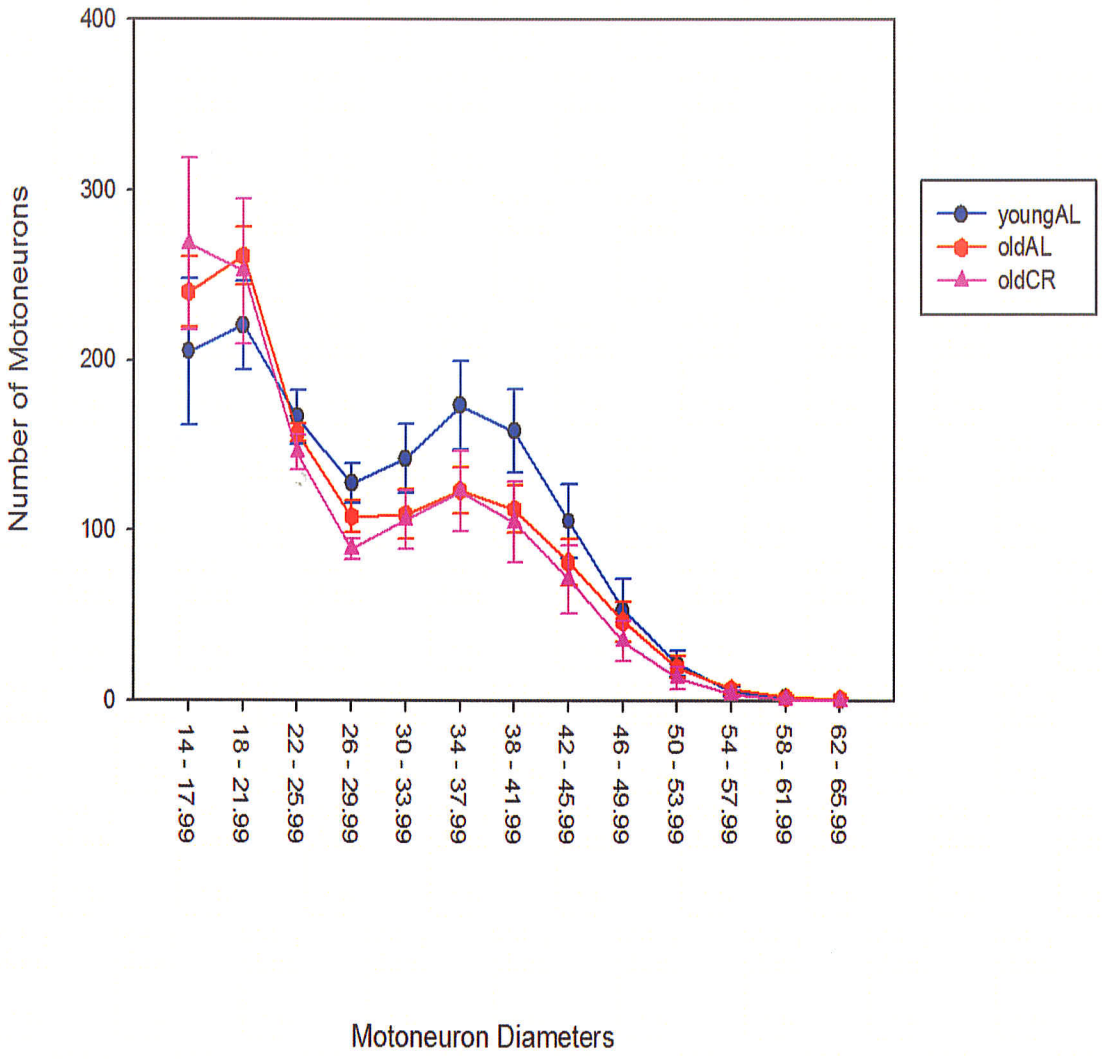

Figure 7 Distribution of ChAT-positive neuron diameters.
13 bins of 3.99μm each were created. Bin 1 represents diameters from 14-17.99μm and bin 13 represents diameters from 62-65.99μm. Square and error bars represent the mean and SD (n=6). No group effect was found.

A histogram was created to examine the ChAT-positive neuron size distribution for each rat. For each rat a bimodal distribution was found which is consistent with previously published papers (Hashizume et al., 1988;Ishihara et al., 2003). The two peaks of a bimodal distribution represent the mode for presumed gamma and alpha motoneurons. Figure 8 shows a histogram from an individual rat representing each group. The distribution for the individual rat is consistent with the distribution seen from every rat belonging to that particular group. The *trough* or the lowest point between the peaks of the bimodal distribution for each particular rat was consistent between rats and between groups.

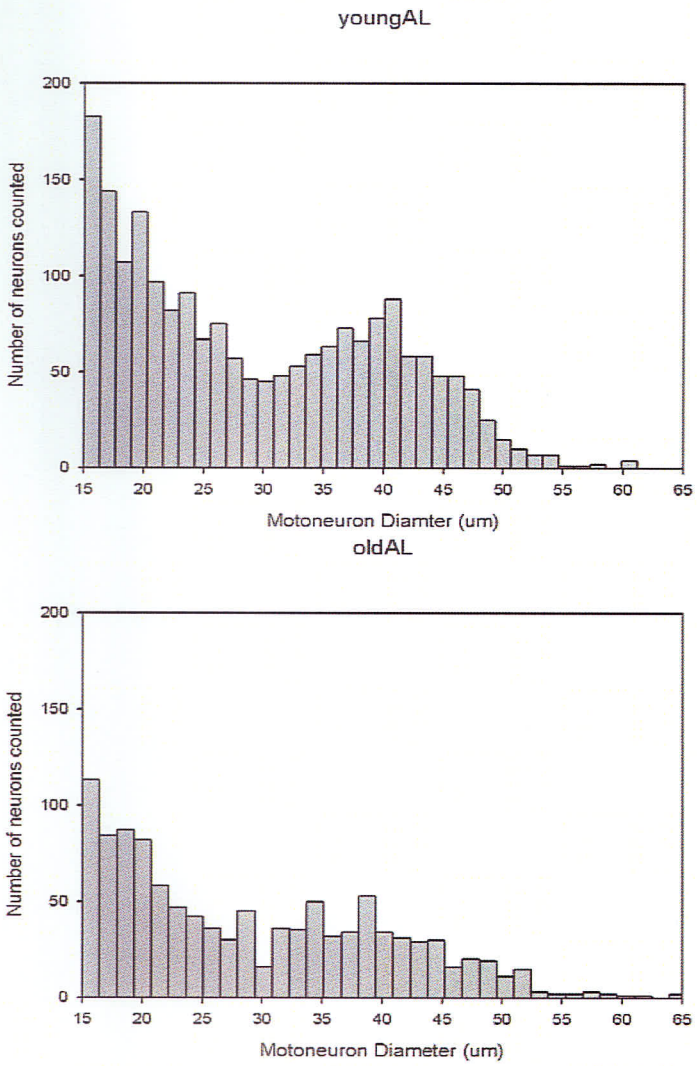

Figure 8 ChAT-positive neuron histograms
The motoneuron distribution of lumbar motoneurons from an individual rat representing each group. For each group, the location of the trough is located near the same motoneuron diameter (approxiamately 29-30μm).

Apoptosis and ROS damage markers

The examination of apoptotic and ROS markers was done by analyzing protein from the ventral horn of the lumbar enlargement using Western blots. Depending on the amount of tissue available, four to five animals from each group were analyzed for various proteins known to be involved in apoptosis or ROS damage repair.

Caspase- 3

The antibody used was specific for the activated caspase- 3 form and not the inactivated pro-caspase- 3 form. Figures 9A and 9B represent the Western blot analysis and graph illustrating caspase- 3 activation. No difference in the level of caspase- 3 was found among groups. A test for homogeneity of variance indicated that the variability among groups was not equal. Therefore a Kruskal Wallis test was performed which takes into account difference in variability. The results still did not reach significance.

Apoptosis-Inducing Factor

For the analysis of AIF two different Western blots were used. The first analyzed the total protein concentration of AIF in the ventral horn and the second analyzed AIF that was bound to the membrane which would indicate AIF had translocated to the nucleus to initiate apoptosis (nuclear AIF). Figures 10A and 10B show the total amount of AIF protein present in ventral horn. No group effect was found. Figures 11A and 11B show nuclear AIF protein levels. No group effect was found.

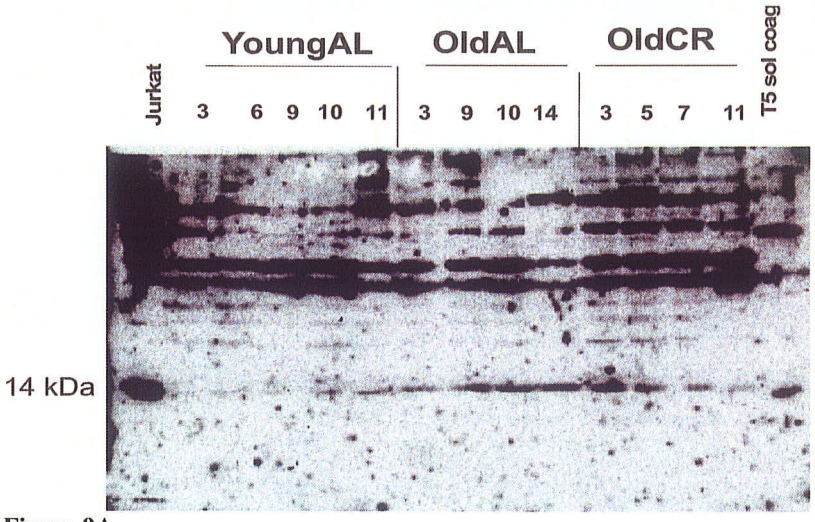

Figure 9A

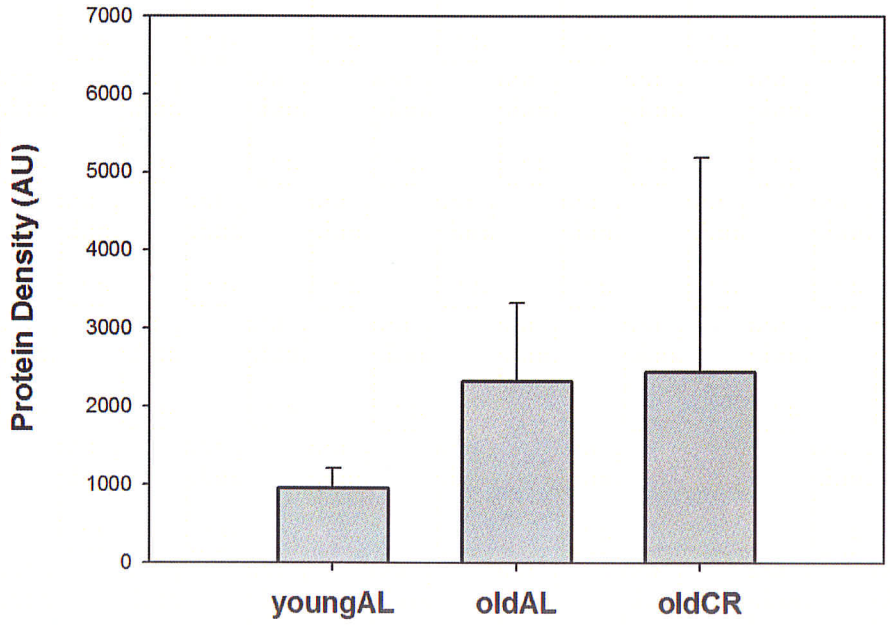

Figure 9B

Figure 9A and 9B Western blot analysis of caspase- 3 activation
(A) Western blot showing the levels of caspase- 3 in the ventral horn of the lumbar spinal cord for each animal. Numbers under the group heading refer to the rat the tissue sample was obtained from. (B) Graph representing the mean (n=6) and SD of the level of caspase-3 in the lumbar ventral horn for each group. No difference was found between groups. AU= arbitrary units.

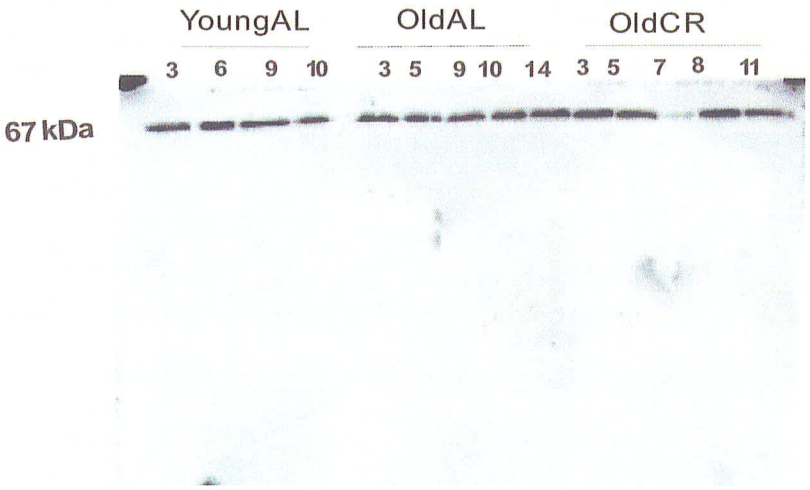

Figure 10A

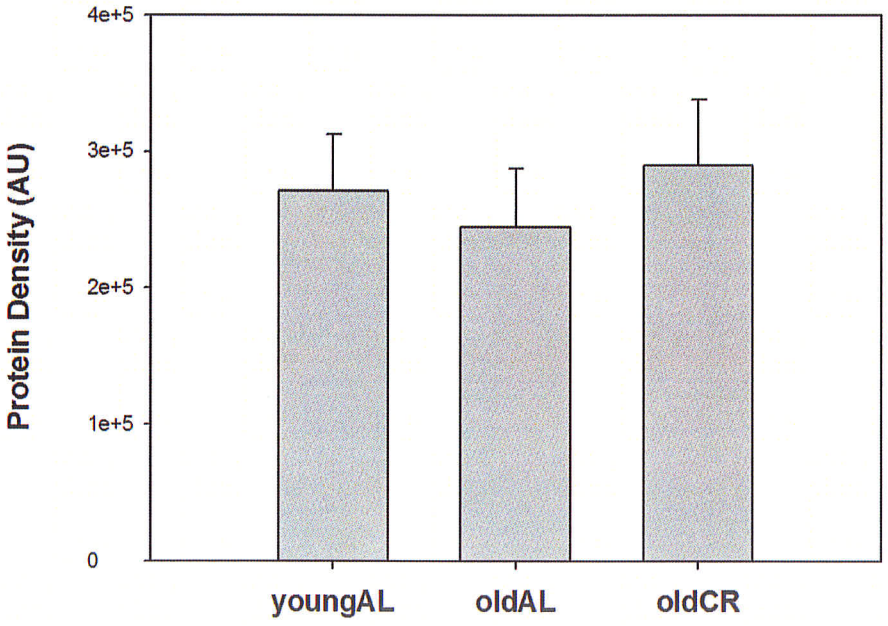

Figure 10B

Figure 10A and 10B Western blot analysis of the total amount of AIF
(A) Western blot showing the levels for the total amount of AIF present in the ventral horn of the lumbar spinal cord for each animal. (B) Graph representing the mean (n=6) and SD for the quantity of AIF present in the ventral horn of the lumbar spinal cord for each group. No difference between groups was found. AU= arbitrary units.

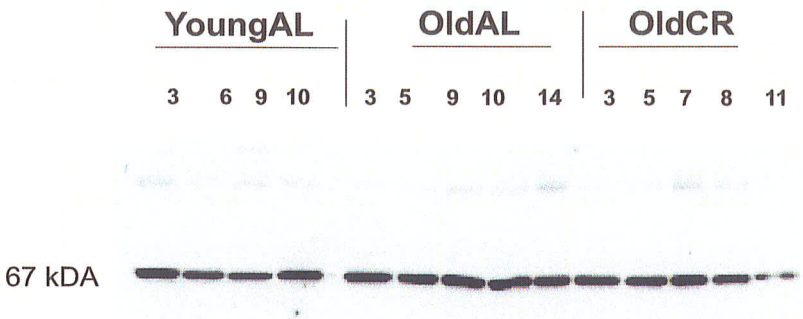

Figure 11A

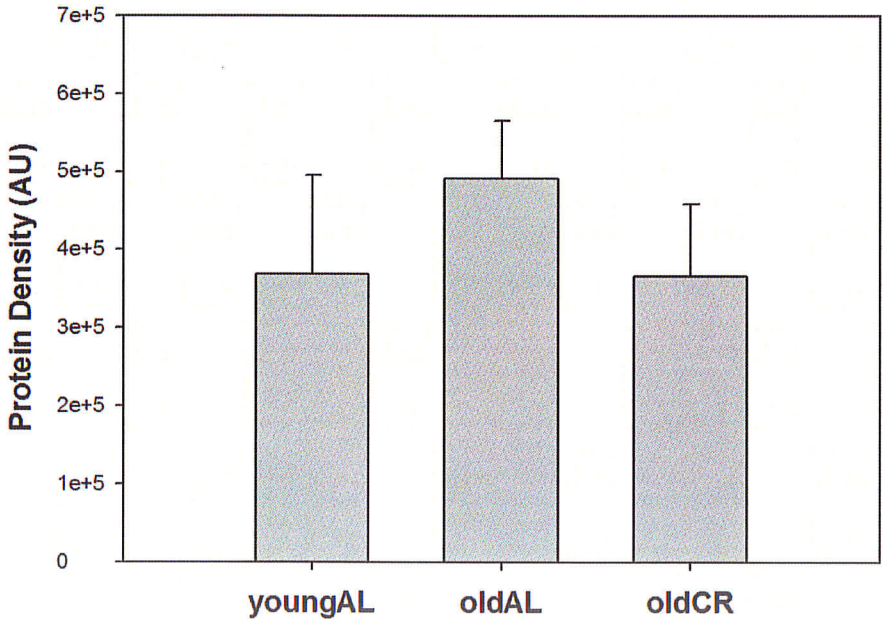

Figure 11B

Figure 11A and 11B Western blot analysis of nuclear AIF
(A) Western blot showing the levels of nuclear AIF present in the ventral horn of the lumbar spinal cord for each animal. (B) Graph representing the mean (n=6) and SD for the quantity of nuclear AIF present in the ventral horn of the lumbar spinal cord for each group. No difference between groups was found. AU= arbitrary units.

Cytochrome C

Figures 12A and 12B show the Western blot and graph of cytochrome c levels for each group. A one-way ANOVA revealed a significant group effect was found ($p < 0.001$). Tukey's post-hoc analysis revealed that the difference was between the youngAL compared to the oldAL ($p < 0.001$) and the youngAL compared to the oldCR ($p < 0.01$). No difference existed between the oldAL compared to the oldCR.

OGG1

OGG1 is an enzyme that is responsible for the repair of oxidative DNA damage. Figures 13A and 13B show the Western blot and graph of OGG1 levels found in the ventral horn of the lumbar spinal cord for each group. A one-way ANOVA revealed a group effect was found ($p < 0.001$). Tukey's post-hoc analysis revealed that the difference was between the youngAL compared to the oldAL ($p < 0.001$) and the youngAL compared to the oldCR ($p < 0.001$). No difference existed between the oldAL and oldCR.

Hsp27

Figures 14A and 14B show the Western blot and graph for the level of Hsp27 found in the ventral horn of the lumbar spinal cord for each group. A one-way ANOVA revealed that a significant group effect was found ($p < 0.05$). Tukey's post-hoc analysis revealed a significant difference between the youngAL and oldCR ($p < 0.02$). No difference was found between oldCR compared to oldAL or the youngAL compared to the oldAL.

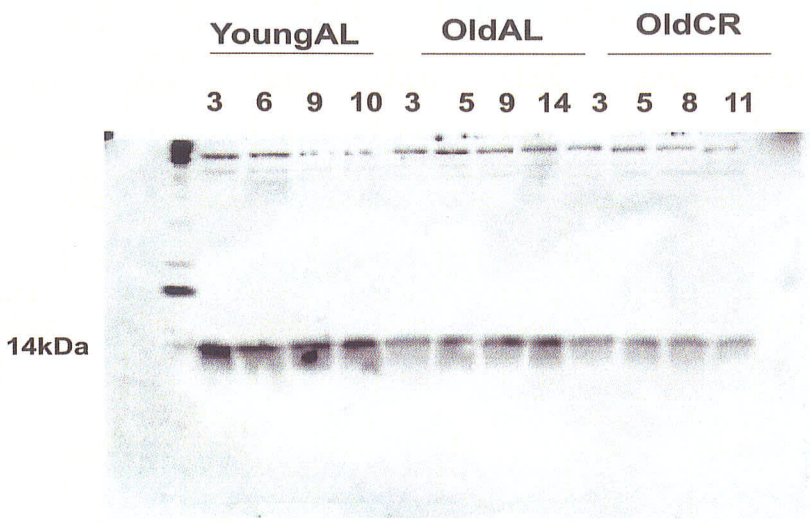

Figure 12A

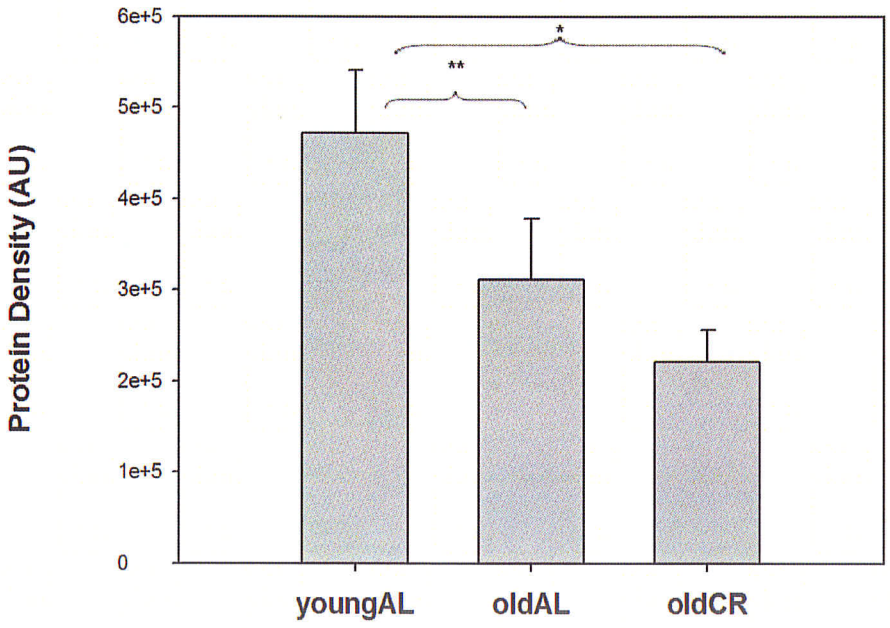

Figure 12B

Figure 12A and 12B Western blot analysis of cytochrome c

(A) Western blot showing the levels of cytochrome c present in the ventral horn of the lumbar spinal cord of each rat. (B) Graph representing the mean (n=4) and SD for the quantity of cytochrome c for each group. * Significant difference between youngAL and oldCR ($p < 0.001$). ** Significant difference between the youngAL and oldAL ($p < 0.01$). AU = arbitrary units.

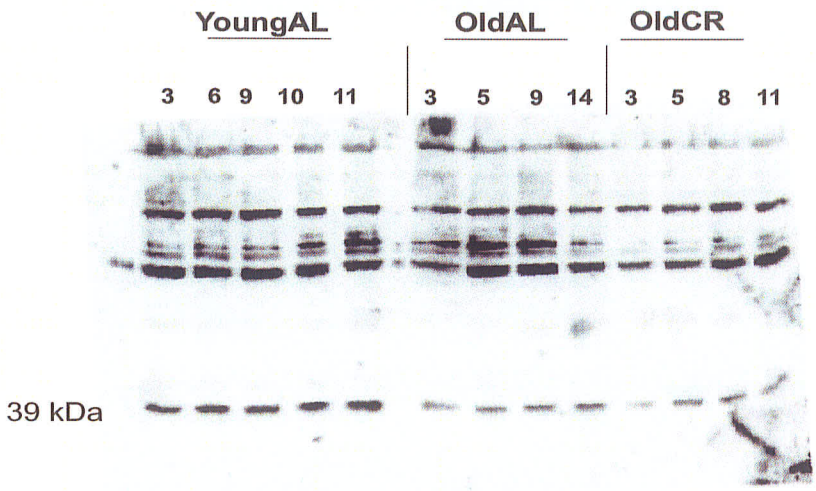

Figure 13A

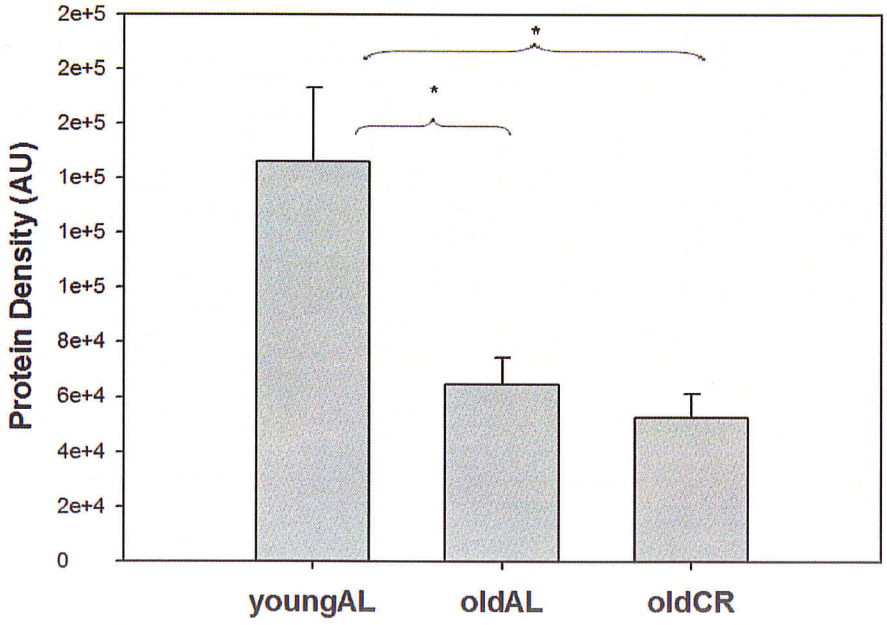

Figure 13A and 13B Western blot analysis of OGG1

(A) Western blot showing the levels of OGG1 present in the ventral horn of the lumbar spinal cord for each rat. Arrow indicates the OGG1 band. (B) Graph representing the mean (n=4) and SD for the quantity of OGG1 for each group. * Significant difference between the youngAL and oldAL and the youngAL and oldCR ($p < 0.001$). AU= arbitrary units.

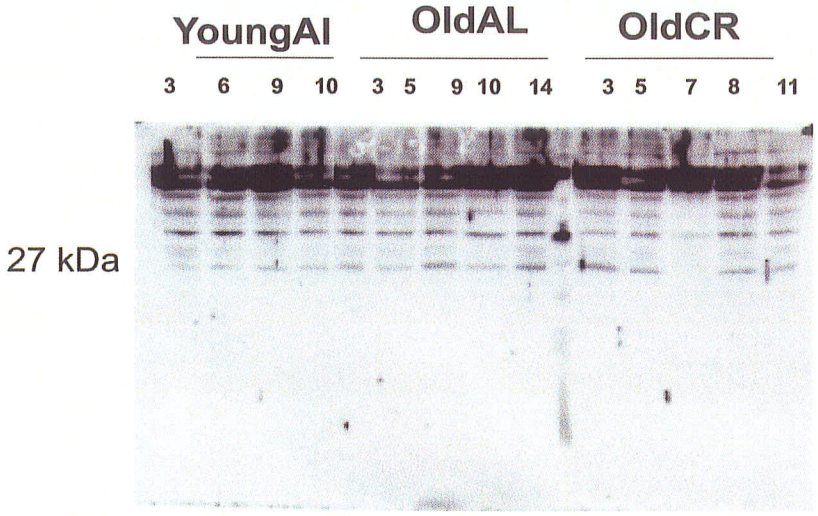

Figure 14A

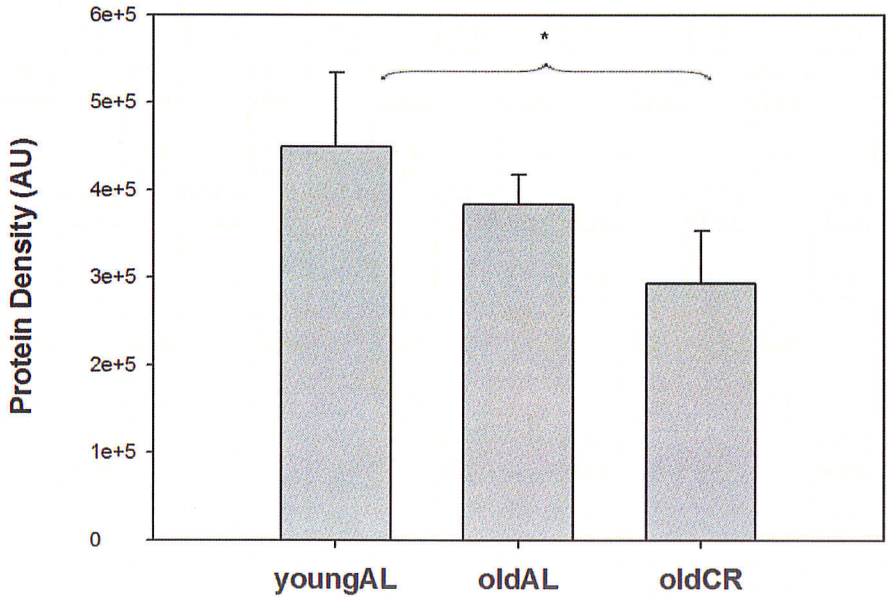

Figure 14B

Figure 14A and 14B Western blot analysis of Hsp27

(A) Western blot showing the level of Hsp27 present in the ventral horn of the lumbar spinal cord for each rat. (B) Graph representing the mean (n=6) and SD for the quantity of Hsp27 for each group. * Significant difference between the youngAL and oldCR ($p < 0.02$). AU= arbitrary units.

Discussion

The purpose of this study was twofold. The first purpose was to determine the effects of aging on the number of motoneurons, the diameter of motoneurons and biomarkers for apoptosis and ROS damage. The second purpose was to determine if CR would attenuate any observed age-related changes in motoneuron numbers, diameters and biomarkers for apoptosis and ROS damage. It was believed that we would see a significant loss of motoneurons with age based on previous literature demonstrating an age-associated loss of motoneurons in humans and rats (Tomlinson and Irving, 1977;Kanda, 2002;Hashizume et al., 1988).

It was believed CR would have a beneficial effect based on substantial evidence obtained from studies examining the effects of CR in muscle fibers. It has been concluded that CR attenuates age-related increases in apoptotic and ROS markers in muscle as well as prevents muscle fiber loss with age (Dirks and Leeuwenburgh, 2004;Dirks Naylor and Leeuwenburgh, 2008). However, in the present study no significant age-related loss of ChAT positive neurons was found nor did it appear CR had a beneficial effect on motoneurons.

It is necessary before the results are discussed to clarify why ChAT was used in the present study. An argument can be made that the reason no age-related loss of motoneurons was found is because ChAT stains all cholinergic neurons in the nervous system and is not specific to motoneurons. Therefore a possibility of staining other neurons such as interneurons exists and the results found may not accurately represent motoneuron loss in the lumbar spinal cord.

This statement is true, but currently ChAT is the most specific immunoflouresecent marker for motoneurons. When examining ChAT positive neurons in the spinal cord it has been previously shown that ChAT only stains neurons in the ventral horn of the spinal cord (Oda and Nakanishi, 2000). Although other neurons exist in the ventral horn besides motoneurons, it is believed neurons located in the ventro-lateral region of the ventral horn of the lumbar spinal cord are predominately motoneurons that innervate the hindlimbs.

As well, a border that has been previously described that separates the presumed motoneurons in the ventro-lateral portion (Lamina IX) of the spinal cord from the rest of the ventral horn of the spinal cord was noticeable in the present study (Sickles and McLendon, 1983;Kawamura et al., 1977). It has been shown that Renshaw cells (a type of interneuron) and Ia inhibitory interneuron are located just outside of this border in lamina VII but do not enter lamina IX (Alvarez et al., 1997;Jankowska and Lindstrom, 1972).

To enhance the chances that only motoneurons were counted in the present study, only ChAT positive neurons with a diameter greater than 15μm were counted. Although the average diameters of all the specific interneurons are not known, it has been shown in the cat, the average diameter of Renshaw cells is between 10 -15μm and the soma area of the Ia inhibitory interneurons is 600μm^2 or a diameter of 25μm (Jankowska and Lindstrom, 1972;Jankowska and Lindstrom, 1971).

Neurons in the cat are larger then neurons in the rat. When motoneurons in the soleus were compared between cat and rat the average diameter in the cat was 51.5μm

(Cullheim et al., 1987) and 35.2μm in the rat (Chen and Wolpaw, 1994). Based on this, the 32% difference between the cat's average soleus motoneuron diameter and the rat's average soleus motoneuron diameter was applied to the cat's average Ia inhibitory interneuron diameter to hypothetically determine the rat's average Ia inhibitory interneuron diameter. It was assumed based on the 32% difference, that the average interneuron diameter in the rat was equal to 17μm. Although this diameter is slightly larger than the cutoff point of 15μm used in the present study, Ia inhibitory interneurons are assumed to be the largest interneuron class in the spinal cord (Jankowska and Lindstrom, 1972) and when the location and strict boundary of the interneurons is also taken into consideration it is believed very few interneurons above 15μm were counted in this study.

As well, based on the available methods to label and quantify motoneurons, using a ChAT antibody was believed to be the best available method for aged animals. As pointed out earlier, HRP injected into muscles has been found not to label all motoneurons in aged animals and axoplasmic transport is slowed in aged animals (McMartin and O'Connor, 1979;Kanda, 2002;Frolkis et al., 1985). Also, when HRP was used to detail motoneuron pools in the lumbar spinal cord of young healthy rats, it was not transported properly in 30% of the rats used, meaning no motoneurons were seen under a microscope. Based on the limited number of rats available, it could not be risked that some of the animals would not transport the enzyme to the soma. Furthermore, when the HRP was successfully injected and transported back to the soma, it was found to label only 70% of motoneurons (Nicolopoulos-Stournaras and Iles, 1983).

Therefore, since a border was visible between lamina IX and lamina VII, we are certain only neurons located in lamina IX were counted in the present study. As well, since only neurons with a diameter of 15μm were counted, it limited the chances of counting spurious interneurons that may somehow be present in lamina IX. Overall, a very strong case can be made that by counting ChAT-positive neurons located in lamina IX, only gamma and alpha motoneurons were counted in the present study. Based on this, for the rest of the discussion, ChAT-positive neurons will be referred to motoneurons.

The effect of age on the alpha motoneuron

The first portion of this study was to determine if there was an age-associated loss of presumed alpha motoneurons. It was believed based on previous research that a statistically significant loss of alpha motoneurons with age would be found (Tomlinson and Irving, 1977;Hashizume et al., 1988;Jacob, 1998). However this was not the case in the present study. No statistically significant difference between the youngAL and oldAL group in presumed alpha motoneurons was found.

Although the difference in presumed alpha motoneurons was not statistically significant the percentage of motoneurons lost is consistent with what is reported in the literature. In the present study, there was a loss of approximately 23% when the oldAL was compared to the youngAL. This difference is approximately the same when examining previous studies on motoneuron loss in rats (Hashizume et al., 1988;Kanda, 2002;Hashizume and Kanda, 1995;Ishihara and Araki, 1988).

Based on the fact that the loss of motoneurons in the current study is similar to that previously reported but the current finding that the loss of motoneurons with age was

not significant, may indicate a statistical type 2 error occurred. This would imply that although the motoneuron loss was found not to be significant, in fact the difference maybe significant. However the significance maybe lost due to the high variability in motoneuron numbers counted and the low sample number. For the current study, the power (which is based on the means, standard deviation and sample size of the study) was found to be 0.179 (calculated with Sigma Stat). This power level is extremely low compared to the minimal ideal power of 0.80 and much lower than the recommended power of 0.90. In order to achieve a higher level of power (0.80) and be certain that a type 2 error was not committed a minimum of 122 rats would be needed. Unfortunately, this number is unrealistic to use for a study like this and therefore the finding that there was no significant difference in motoneuron numbers between the young and old must be interpreted with caution.

To my knowledge, this is the first study that has examined in the rat, the lumbar enlargement of the spinal cord which is responsible for innervating the majority of the hindlimb muscles and the first study to use immunoflouresence to label motoneurons. The studies by Hashizume and Kanda (Hashizume et al., 1988;Kanda, 2002) using HRP injection examined only the motoneurons innervating the MG and the study by Ishihara (Ishihara and Araki, 1988) also using HRP injection examined only motoneurons innervating the EDL. In these studies, age-related motoneuron loss was found to be 30% in the Hashizume study, approximately 23% in the Kanda study and 21% in the Ishihara study.

A more concise study from Jacob (1998) examined the number of motoneurons in the lumbar sections L4/L5 in Fisher 344 rats using light microscopy. When 22 month old rats were compared to young 6 month old rats a significant decrease of approximately 44% was found. This finding is much larger than any other study examining age-related changes in motoneuron numbers. A possible explanation for this is that longitudinal sections of 6μm were examined and not every motoneuron per mm^3 was counted. Instead the authors used Abercrombie's formula which is a valid stereological method but is not as accurate as newer methods for histological counts (Hedreen, 1998;Hedreen, 1998;Mandarim-de-Lacerda, 2003;Pover and Coggeshall, 1991).

The problem Jacob may have faced in counting the motoneurons per area of tissue and then estimating from his counts is the possibility that the distribution of neurons is not uniform throughout every cut of tissue. Therefore, depending on what section was counted it may under- or overestimate the number of motoneurons in a given tissue sample. If Jacob had used the fractionator method, which is a newer more valid method for histological counts (Lyck et al., 2007;Mandarim-de-Lacerda, 2003) and which was used in the present study, the actual counts would have been different and a more accurate count of motoneurons in a particular portion of tissue.

It is difficult based on these studies to decide whether or not an actual age-associated loss of presumed alpha motoneurons exists in the lumbar spinal cord or whether it is a preferential loss of specific motoneuron pools in the lumbar spinal cord. If the latter statement is correct, then it can be argued that the reason no difference was found in the number of motoneurons between the youngAL and oldAL is because the loss

of motoneurons innervating type 2 muscle fibres are negated by a possible lack of an age-related decrease in motoneurons innervating type 1 muscle fibres. This hypothesis is based on the fact it is well known that type 2 muscle fibres are preferentially lost with age (Lexell, 1995) and therefore motoneurons innervating type 2 muscle fibres may also be preferentially lost. Unfortunately, there is not one study that has specifically looked at motoneuron pools from various muscle groups (i.e. fast twitch versus slow twitch) throughout the life span of a rat and quantified motoneuron losses for each motoneuron pool. Therefore, to support the finding that overall in the entire lumbar enlargement there is no loss of motoneurons with age, studies that have quantified specific motoneuron pool losses and muscle fibre losses will be discussed.

Evidence shows that muscle fibre loss in the EDL (type 2 muscle) begins at 60 weeks of age in the rat (Boreham et al., 1988;Ishihara and Araki, 1988). Specifically, the loss of the EDL's fast glycolytic (FG) fibres is seen at 60 weeks of age, whereas the loss of fast oxidative glycolytic (FOG) fibres is seen at 120 weeks and no age-related loss of slow oxidative (SO) fibres is seen (Ishihara and Araki, 1988). Consistent with this, it has also been shown that in the Tibialis Anterior ((TA), type 2 muscle), the loss of FG fibres begins at 65 weeks of age and there is no loss of FOG fibres. When examining the Soleus (type 1 muscle) it was noted that fibre loss was not present until 103 to 130 weeks, depending on the particular study (Boreham et al., 1988;Ishihara et al., 1987;Gutmann and Hanzlikova, 1966).

Several of these studies that examined specific muscle fibre loss also looked at the loss of motoneurons with age (Gutmann and Hanzlikova, 1966;Ishihara and Araki,

1988;Ishihara et al., 1987). Gutmann and Hanzlikova found that at 27 months in rat, there was no loss of soleus motoneurons although there was a loss of soleus muscle fibres. Ishihara (1987) looked at both the soleus and TA as noted in the above paragraph. It was found that the motoneurons innervating TA significantly decreased with age at week 135, whereas there was no loss of motoneurons innervating the soleus muscle. This finding is important since it was noted earlier, TA fibre loss occurred at 65 weeks followed by the loss of TA motoneurons at 135 weeks. Therefore it was concluded that muscle fibre loss occurs before the reduction in motoneuron numbers. Further support that muscle fibre loss occurs before the loss of motoneurons is from Ishihara (1988) in which the loss of EDL fibres began at 60 weeks of age followed by the loss of motoneurons at 120 weeks. Therefore, if soleus muscle fibre loss is not seen until 130 weeks, a very strong possibility exists that motoneuron loss will not be seen before the rat dies due to the advanced age of the rat.

More evidence that suggests that age-related loss of motoneurons is muscle type specific is from Hashizume and Kanda (Hashizume and Kanda, 1990;Hashizume and Kanda, 1995). Both of these studies examined a forelimb motoneuron pool (Ulnar nerve) and a hindlimb motoneuron pool (MG nerve). It was found that there was a significant decrease in MG motoneuron numbers but not a significant decrease in forelimb motoneurons with age. Furthermore, the decrease in the number of myelinated fibres was less in the ulnar nerve than the MG nerve (Hashizume and Kanda, 1995).

Based on the above studies that examined specific motoneuron pools it appears that indeed specific muscles groups may show an age-related loss of motoneurons

whereas other muscle groups may not show an age-related loss of motoneurons. It appears that motoneuron pools that innervate primarily type 2 muscle fibres are affected by age whereas motoneuron pools that innervate primarily type 1 muscle fibres are not affected by age. Therefore since the present study examined the lumbar enlargement, which innervates the majority of the hindlimb muscles, there is a very good possibility that overall motoneurons in the lumbar spinal cord are not lost by 32 months of age in rat.

Another possible argument that can be made of why no age-related loss of motoneurons was found is because of the large variability in the number of motoneurons counted for each group. Indeed the coefficient of variation (CV) was high, with a SD for the presumed alpha motoneurons being 1667 for the youngAL and 937 for the oldAL this resulted in a CV of 33% and 24% for the youngAL and oldAL respectively. However, this CV is similar to the CV found in the study done by Tomlinson and Irving (1977) that examined motoneuron loss in humans. The CV for this particular study was 6.4% for the 61-70 year old age group, 13.5% for the 81-90 year old age group and 25% for the 91-95 year old age group, indicating that the CV increased with age. The CV from the present study was only compared to the Tomlinson and Irving study because it examined the entire lumbar segment versus one or two muscle groups as done in the rat.

As well, when examining the variability of previous studies, it is obvious a degree of variability exists between each study when examining the same motoneuron pool. At approximately the same age (13-16 months), using the same rat strain and using the same HRP method to count MG motoneurons, 133.8 ± 4.5 and 97.5 ± 4.2 motoneurons were found in the Hashizume *et al.* (1988) study and the Kanda (2002) study respectively. To

further highlight the variability in counting motoneurons, it has also been shown in rat using HRP injection that 61 motoneurons are responsible for innervating the MG which is much lower that the 134 and 98 motoneurons found in the above mentioned studies (Nicolopoulos-Stournaras and Iles, 1983).

Another possible source of variability in counting motoneurons arises from the individual differences in the amount of type 1 and type 2 muscle fibres that maybe expressed among animals and humans. In the rat hindlimb musculature, it has been demonstrated that approximately 76% of muscle fibres are FG, 19% are FOG and 5% are SO fibres (Armstrong and Phelps, 1984). If an individual has a greater portion of SO fibres, that person may have a higher number of motoneurons. This is based on the fact that in the MG, SO fibres comprise approximately 5- 7% of the muscle fibres however slow motoneurons (motoneurons that innervate SO fibres) comprise 10% of the motoneuron pool innervating the MG (Gardiner, 1993; Armstrong and Phelps, 1984). Therefore if a rat has a higher percentage of SO fibres in the hindlimbs, that rat will presumably have a larger number of motoneurons innervating the hindlimbs. The opposite is found in regards to FG fibres. If a rat has a higher percentage of FG fibres, that rat will have a lower amount of motoneurons innervating the hindlimbs. Therefore, the fibre type composition of the hindlimb musculature can help explain the high variability in motoneuron numbers found in the present study.

One last possible source that may have contributed to the variability in the motoneuron counts is the differences in physical activity. However, it is believed this did not influence the results of the present study since the animals were housed in identical

cages. As well, it has been shown that life-long exercise did not prevent MG motoneuron loss in the rat (Kanda and Hashizume, 1998). Therefore, even if a difference in physical activity levels was found in the present study, this should not impact the number of motoneurons innervating the hindlimbs.

Another aspect of this study was to determine if the loss of motoneurons with age is based on the diameter of the motoneuron. It was assumed that larger diameter sized motoneurons would be preferentially lost with age based on evidence that shows large motoneurons are preferentially lost in amyotrophic lateral sclerosis (ALS) (Sobue et al., 1983) and aging when examined in the MG (Hashizume et al., 1988).

When all the motoneurons for this study were placed in 2μm bins from the smallest sized motoneuron to the largest sized motoneuron counted, only a bin effect was noticed not a group effect. As well, the motoneuron diameter distribution was illustrated using histograms. The histograms showed bimodal distributions which represents the peak for both presumed gamma and alpha motoneurons. The trough which is the lowest point located between the presumed alpha and gamma motoneuron peaks was virtually equal between the groups. If a difference in motoneuron size distribution with age occurred, the trough for the oldAL would have been expected to shift either to the right or the left indicating a change in the size distribution of motoneurons. A one-way ANOVA indicated that there was no difference found between the troughs.

Based on the above reports, it is believed that this study is the first to thoroughly quantify and size motoneurons in the lumbar enlargement which is responsible for innervating the majority of the hindlimb muscles. Using a double-blinded

immunohistological protocol along with the fractionator method for histological counts, this study provided an unbiased and accurate method to quantify motoneurons in the rat lumbar enlargement. Therefore, it is safe to conclude that no age-related loss of motoneurons exist when the lumbar enlargement is examined versus a specific muscle group.

The effect of age on apoptosis and ROS markers in the ventral horn

Out of the six markers for apoptosis or ROS activity examined in the ventral horn of the lumbar spinal cord, only cytochrome c and OGG1 showed an age effect. Both cytochrome c and OGG1 were found to be decreased with age. This is an interesting finding since an increase in apoptosis and ROS markers has consistently been reported in skeletal muscle and brain (Dirks and Leeuwenburgh, 2004;Monti and Contestabile, 2003). Furthermore, motoneurons have also been reported to be the most oxidative stress susceptible cells in the CNS (Xu et al., 2005). Briefly each marker will be discussed below, except for Hsp27 which will be discussed in the CR section since it was decreased in the oldCR group but not the oldAL group.

Cytochrome c

In the present study, it was found that cytochrome c decreased with age. Under normal physiological conditions cytochrome c works in the inner mitochondria as an electron transporter between complexes 3 and 4 (Green and Reed, 1998). Once apoptosis has been initiated cytochrome c leaks out of the mitochondria and forms an apotosome with caspase- 9 and Apaf- 1 and initiates the caspase cascade (Hengartner, 2000). With age, cytochrome c has been found to decrease with age in both rat skeletal muscle (Dirks

and Leeuwenburgh, 2004;Chabi et al., 2008) and mice brain (Manczak et al., 2005). The decrease is believed to be a result of cytochrome c leaving the mitochondria and forming an apotosome (Manczak et al., 2005). The decrease in cytochrome c might also be reflective of the decrease in mitochondrial content with age (Chabi et al., 2008). Therefore as a result of both age and apoptosis, the concentration of cytochrome c will decrease. This is consistent with the present finding that cytochrome c in the ventral horn of the spinal cord decreased with age and this provides partial evidence that motoneurons die via the intrinsic or mitochondrial pathway of apoptosis.

OGG1

In the present study, OGG1 was decreased in the oldAL compared to the youngAL. OGG1 is the enzyme responsible for the repair of the specific DNA damage termed 8-hydroxyguanosine (8-OHG). Previous reports have shown that 8-OHG (marker for oxidative damage) has be found to be significantly increased in the hippocampus and cortex in middle-aged mice and then be significantly decreased in old-aged mice (Manczak et al., 2005). This suggests that oxidative damage increases with age and then once this damage is beyond repair, apoptosis and the loss of cells occurs and that is why the level of 8-OHG is then decreased with advanced age. However, since there was not a significant loss of motoneurons with age in the present study the above explanation is not suitable. Another possible explanation of why OGG1 is lost with age could simply be that the motoneuron's DNA repair systems are diminished with age.

Caspase- 3

It is not necessarily surprising that the level of caspase- 3 activation was not elevated with age in the present study since the activation period of caspase- 3 is less than six hours (Bigini et al., 2007). Therefore, with the small amount of tissue sampled, and the small number of motoneurons per volume of tissue, it would be virtually impossible to see an elevation in caspase- 3 activation. Furthermore, Bigini *et al.* (2007) also determined that in the cervical spine of wobbler mice, at the peak of motoneuron loss, only 0.3 cells per section would be stained immunohistologically for caspase-3.

Aside from this point, another possible reason caspase- 3 levels were not increased is because no age-related loss of motoneurons was present. Caspase- 3 would only become activated when apoptosis is initiated. Therefore, since there was no decrease in motoneurons with age, then there should be no increase in apoptosis which ultimately means there should be no increase in caspase- 3 expression.

Apoptosis-Inducing Factor

In the present study, no change in the total amount of AIF or the amount of nuclear AIF (indicating apoptosis) was found. Currently, the literature seems to support either a steady state of AIF expression, which would confirm our results or the literature supports an increase in AIF with age, which may indicate an increase in apoptosis.

Briefly, in skeletal muscle AIF protein has been shown to be increased (Dirks and Leeuwenburgh, 2004) or to be the exact same with age (Chung and Ng, 2006). When

gene expression was examined for AIF in aged skeletal muscle it was found to increase by 7- to 50- fold with age in rat (Baker and Hepple, 2006). In the nervous system AIF has recently been reviewed and it was found that under normal physiological conditions AIF levels stay the same but under pathological conditions AIF levels increase (Krantic et al., 2007). In this latter review a pathological condition included mitochondrial dysfunction. Since mitochondrial dysfunction is part of the aging process, one can conclude that AIF expression should increase with age.

The results in the present study can be viewed several ways. First, it can be argued that since motoneurons were not lost with age then neither the total amount of AIF nor the amount of nuclear bound AIF should increase. Second, if AIF does indeed increase with age, irrespective of an increase in apoptosis, then it can be argued that the reason no increase in AIF was found is because the purported increase in AIF would be offset by the decrease in mitochondrial content with age. AIF under normal conditions is found in the mitochondria (Krantic et al., 2007) so the age-related decrease in mitochondrial content may offset the age-related increase in AIF.

In conclusion, there appears to be a decrease in cytochrome c and OGG1 with age, indicating that with age it is possible that there is either an increase in cytochrome c leaving the mitochondria and forming an apotosome or a decrease in mitochondrial content. Furthermore it appears that with age there is a decrease in the DNA repair enzymes. It is possible that no increase in the other biomarkers of apoptosis were found with age because no age-related loss of motoneurons occurred.

Caloric restriction and the alpha motoneuron

It is difficult to determine if CR has a beneficial effect in the motoneuron since there were very few age-related changes found in this study. From the age-related changes found, it appears CR had an effect on attenuating the increase in body weight and preserving the ratio between body weight to muscle mass but no effect on attenuating the loss of cytochrome c and OGG1. As well a decrease in Hsp27 was found in the oldCR compared to the youngAL. This unique find will be discussed later.

It is clear that the CR protocol for this study was effective since the oldAL group weighed significantly more than the youngAL and oldCR group. The CR protocol also was effective in attenuating the increase in body weight to muscle mass ratio that was found in the oldAL group. The above results demonstrate the effectiveness of the CR protocol, therefore it can be concluded that the result that CR does not attenuate the loss of cytochrome c or OGG1 is in fact a true finding and not an error due to an ineffective CR protocol.

Support for the finding that CR in this study had no beneficial effect in the motoneuron is from Monti *et al.* (2004;2003). In the 2003 study by Monti *et al.*, 30 month old Wistar rats either fed ad libitum or a 30% CR diet were compared to young (4 month old) rats. It was shown that caspase- 1 and DNA fragmentation increased with age in the lumbar spinal cord and that CR did not reduce the level of DNA fragmentation.

In the 2004 study by Monti *et al.*, the effect of CR on synaptic function was examined since it had been previously shown that ChAT (marker for cholinergic neurons) and GAD (marker for GABAergic neurons) were decreased with age in the CNS and to a

greater extent in the spinal cord (Virgili et al., 2001). It was concluded that CR did not attenuate the loss of ChAT and GAD in the spinal cord.

Further evidence, both indirectly and directly, that supports the idea CR does not have a beneficial effect in motoneurons is from Newton *et al.* (2005) and Kalmar *et al.* (2009). Newton *et al.* (2005) found that CR did not attenuate the age-related decline in BDNF expression in hippocampus neurons. BDNF is a neurotrophic factor that helps promote motoneuron survival in adults (Copray and Kernell, 2000). Direct support is from Kalmar *et al.* (2009) that examined the effect CR had on the biophysical properties of alpha motoneurons. It was found that with age, the firing patterns and biophysical properties of alpha motoneurons changed and became more variable and that CR had no affect on attenuating these changes.

One of the most intriguing findings of the present study was that Hsp27, a chaperone protein that is up-regulated in motoneurons when the spinal cord is subjected to insult (Arya et al., 2007;Concannon et al., 2003;Sharp et al., 2006;Franklin et al., 2005;Wagstaff et al., 1999) was decreased in the oldCR group but not in the oldAL group. Hsp27 is believed to be anti-apoptotic by inhibiting the release of cytochrome c and various members of the pro apoptotic BH-3 family (Arya et al., 2007;Concannon et al., 2003). Why Hsp27 is decreased in the oldCR is difficult to determine especially since it has been shown that Hsp27 not only increases during an insult or stress but also during the normal aging process (Chung and Ng, 2006).

One possible explanation for the decrease of Hsp27 with age is evidence now suggesting that Hsp27 is specific to motoneurons in the ventral horn (Plumier et al.,

1997). Therefore since the number of motoneurons was decreased with age (although, not significant), the amount of Hsp27 detected through western blot analysis would be decreased. However, this could explain why the oldCR group was significantly decreased but does not explain why the oldAL group was not affected to the same extent. A possible reason for the difference in the level between the oldCR group and oldAL group is that CR is supposed to decrease the amount of protein damage or protein turnover which ultimately would decrease the need for Hsp27 to be up-regulated. Overall, it is a unique finding that Hsp27 would only be significantly decreased in the oldCR group and not the oldAL group.

Based on the above findings, it is safe to conclude that the results were not in error due to an inappropriate CR protocol but in fact true results in which CR did not attenuate the loss of cytochrome c or OGG1 in the ventral horn. Overall, the finding that CR is not effective in attenuating age-related changes in the motoneuron is very interesting and unique since CR has been shown to be beneficial in the heart, liver, muscle and brain (Wolf, 2005; Dirks *et al.*, 2007; Hiona & Leeuwenburgh, 2004).

Limitations

The main limitation to this study was the lack of a specific marker or antibody for alpha motoneurons. Presently, no antibody to distinguish between an alpha and gamma motoneuron exists. The most specific antibody is ChAT which interacts with both alpha and gamma motoneurons. Therefore a method of separating presumed alpha and gamma motoneurons based on diameter was used in this study and as previously reported in the

literature. Although it is believed to be an effective method to separate motoneurons based on diameter the strength of this study would increase dramatically if it were known for certain if the motoneuron of interest were an alpha or gamma motoneuron.

Another limitation to this study is that the western blots performed allowed for the quantification of protein concentrations in the ventral horn of the spinal cord and not necessarily motoneurons exclusively. Therefore, one cannot definitively make the statement that the protein concentrations quantified in our samples are exclusively from motoneurons. However, most studies do not separate the ventral horn from the dorsal horn as was performed in this study (Monti *et al.*, 2004). Therefore the results from this study are more conclusive than previously shown studies examining apoptotic markers in the motoneuron.

Conclusion

While it has been previously shown that motoneurons are lost with age, when a full quantification of all the motoneurons in the lumbar enlargement is performed – an age-related loss of motoneurons is not found. Furthermore it appears that very few markers for apoptosis or ROS damage are altered with age. As well, CR which has been shown to be beneficial in various other tissues does not appear to attenuate the age-related loss of cytochrome c or OGG1 in the motoneuron.

Future Directions

A study that should be conducted is to quantify specifically what hindlimb motoneuron pools are lost with age rather than examining only one or two motoneuron pools at a time. It appears each time a study is conducted, it uses the two extremes in muscle types, the gastrocnemius which is virtually all type 2 fibres and the soleus which is almost all type 1 fibres. A very important part of this study was demonstrating that an age-related loss of motoneurons is not present when all the motoneurons innervating the majority of the hindlimb muscles are taken into consideration. Therefore, it would be very beneficial to know exactly what motoneuron pools are lost with age and what motoneuron pools are not lost with age. As well, by quantifying motoneurons for each particular muscle group, it would provide a future reference for those studies looking at age-related losses by providing a known number of motoneurons for each muscle group.

Another possible study would be to look at other markers for apoptosis since it cannot be determined from this study if motoneurons die via apoptosis. Mainly it would be beneficial to quantify the protein concentrations of Bcl-2, Bax and Bid since it has been shown that the ratio between these pro- and anti-apoptotic markers determine whether or not apoptosis occurs. This ratio in other tissue such as skeletal muscle has been shown to favor apoptosis with age. Therefore it would be interesting to see if this is the case with the motoneuron and provide the evidence needed to conclude that in fact motoneurons die via apoptosis.

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Appendix 1 – Diet composition for the AL and CR rat chow

NIH-31/NIA Fortified Diet
&
NIH31 Average Nutrient Composition

Ingredients:

Ground wheat, ground corn, ground oats, wheat middlings, fish meal, soybean meal, corn gluten meal, dehydrated alfalfa meal, soybean oil, dicalcium phosphate, brewers dried yeast, salt, calcium carbonate, choline chloride, menadione sodium bisulfite complex (source of vitamin K activity), thiamine mononitrate, calcium pantothenate, vitamin E supplement, vitamin A acetate, riboflavin, vitamin B12 supplement, niacin, vitamin D3 supplement, pyridoxine HCL, folic acid, biotin, magnesium oxide, ferrous sulfate, manganous oxide, copper sulfate, zinc oxide, calcium iodate, cobalt carbonate.

Average Nutrient Composition

		NIH-31/NIA Fortified	NIH-31
Protein	%	18.74	18.42
Fat	%	4.41	4.47
Fiber	%	4.58	4.05
Ash	%	6.51	6.64
Nitrogen-Free Extract	%	55.04	55.91
Gross Energy	kcal/g	3.95	4.02
Digestible Energy	kcal/g	3.36	--
Metabolizable Energy	kcal/g	3.07	--
Linoleic Acid	%	1.79	--
Moisture	%	--	10.51

Amino Acids

		NIH-31/NIA Fortified		NIH-31
Arginine	%	1.10		1.06
Methionine	%	0.36		0.39
Histidine	%	0.42		0.41
Leucine	%	1.50		1.61
Lysine	%	0.96		0.95
Tryptophan	%	0.22		0.24
Valine	%	0.88		0.96
Cystine	%	0.26		0.28
Isoleucine	%	0.76		0.90
Threonine	%	0.71		0.71
Pherylalanine	%	1.53	Pherylalanine	0.92
+Tyrosine			Tyrosine	0.70

Appendix 1 continued

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Minerals

		NIH-31/NIA Fortified	NIH-31
Calcium	%	1.03	1.06
Phosphorus	%	0.93	0.92
Sodium	%	0.30	0.26
Chlorine	%	0.48	0.42
Potassium	%	0.59	0.59
Magnesium	%	0.20	0.20
Iron	mg/Kg	336.41	300.20
Maganese	mg/Kg	156.01	152.80
Zinc	mg/Kg	48.41	50.40
Copper	mg/Kg	13.28	13.20
Iodine	mg/Kg	2.01	1.94
Cobalt	mg/Kg	0.53	0.53
Selenium	mg/Kg	0.30	--

Vitamins

		NIH-31/NIA Fortified	NIH-31
Vitamin A	IU/g	40.49	--
Vitamin A3	IU/g	--	30.73
Vitamin D3	IU/g	7.00	4.19
Vitamin E	mg/Kg	52.15	38.30
Choline	mg/g	2.60	1.96
Niacin	mg/Kg	116.16	92.20
Pantothenic Acid	mg/Kg	55.07	39.50
Pyridoxine	mg/Kg	13.16	10.20
Riboflavin	mg/Kg	11.04	7.80
Thiamine	mg/Kg	123.44	77.30
Menadione	mg/Kg	111.01	22.00
Folic Acid	mg/Kg	2.13	1.70
Biotin	mg/Kg	0.38	0.13
Vitamin B12	mcg/Kg	93.80	53.00
Vitamin C	mg/g	--	--
Carotene	mg/Kg	--	--

Appendix 2 – Determination for counting every sixth section

Animal used: FBNC7
Sectioned at 15um
Total 198 Cuts
=2.970mm

Total Numbers (Every Third Section):
Cuts=66
Right = 331 Avg per section = 5.01
Left = 291 Avg per section = 4.41
Total = 622 Avg per section = 9.42

Total Number (Every Sixth Section):
Cuts= 33
Right = 166 x 2 = 332 Avg per section = 5.03
Left = 145 x 2 = 290 Avg per section = 4.39
Total = 311 x 2 = 622 Avg per section = 9.42

Total Numbers (Every Ninth Section):
Cuts= 22
Right = 106 x 3 = 318 Avg per section = 4.82
Left = 101 x 3 = 303 Avg per section = 4.59
Total = 207 x 3 = 621 Avg per section = 9.41

Total Numbers (Every Twelfth Section):
Cuts= 17
Right = 84 x 4 = 336 Avg per section = 4.94
Left = 77 x 4 = 308 Avg per section = 4.53
Total = 161 x 4 = 644 Avg per section = 9.47

Appendix 3- ChAT and NeuN immunohistochemistry protocol

ChAT 1:50	NeuN 1:1000
FITC antiGoat 1:100	Cy3 antimouse 1:300

1. Incubate 30min 4% Paraformaldehyde
2. Wash 2x10 min in PBST
3. Sodium Borohydride 3x15min (prepared fresh)
4. Wash 3x10 min in PBST
5. Incubate 60 min, agitated in 5% normal donkey serum, 1% BSA + 0.1M phosphate buffered saline with 0.3% Triton [PBST]
6. Incubate **72hours** @ 4°C in ChAT (made in goat) @1:50+PBST with 1% donkey serum and 1% BSA
7. Wash 3x10min in PBST
8. Incubate 2hours in dark in antiGoat FITC (made in Donkey) @1:100 +PBST with 1% donkey serum and 1% BSA
9. Wash 3x10 min in PBST
10. Incubate **24hours @room temp** in Neun (made in mouse) @1:1000 +PBST with 1% donkey serum and 1% BSA
11. Wash 3x10 min in PBST
12. Incubate 2 hours in dark in antiMouse Cy3 (made in donkey) @1:300 +PBST with 1% donkey serum and 1% BSA
13. Wash 1x10 min in PBST
14. Wash 2x10 min in 50mM Tris-HCL
15. Dry and Coverslip with Vectashield

Appendix 4 – Western Blot protocol

1. Homogenize the ventral horn of the lumbar spinal cord on ice in RIPA buffer solution.
2. Centrifuge at 13000RPM for 15 minutes.
3. Determine protein densities using the BIORAD protein dye reagent (determines sample size to use).
4. Pour SDS gels and let the gel set for one hour, remove the isopropanol with water, pour 1x separating gel buffer solution on top, cover with saran and leave over night.
5. Pour off buffer solution and add stacking gel. Then place the comb into place in the stacking gel. Let the gel set for 45 minutes.
6. Mix protein sample with solution (ddH20, 4x sample loading buffer solution) and boil for 5 minutes, cool for 5 minutes and then centrifuge. Pipette the remaining solution into the wells in the stacking gel. Place the gels into the buffer chamber and fill the inner chamber with buffer solution. Run chamber at 150V for 45 minutes or until dye front is at the bottom.
7. Remove the gel and place in buffer solution for 10 minutes. Prepare the PVDF transfer membrane. Place gel with brillo pads, whatman paper and PVDF in the black and red trans-blot apparatus. Insert apparatus into the tank, fill with cold transfer buffer and place icepack in the bottom of the tank. Transfer at 100V for 1.5 hours.
8. Remove PVDF membrane and soak in ponceau red for 5mins. Rinse membrane in water to remove excess stain. Scan the PVDF membrane to record the gel and size markers. Wash in TBS-T, then block in TBST/5%milk for 1 hour.
9. Apply the various antibody in TBST/5%milk for one hour. Wash in TBST 3X10.
10. Apply secondary antibody (HRP conjugated) for one hour. Wash in TBST 3X10.
11. Apply the Pierce Super Signal for 1 minute. Place the membrane into a clear plastic sheet and then place in the BioRad Fluor-S-Multi-Imager to expose image to light.
12. Determine of bands is then done using densitometery.

Appendix 5 – Number of motoneurons counted for each rat

Group	Total Neurons	**Normalized #*(35/x)**	Alpha Mn #s	**Normalized #*(35/x)**	Gamma Mn #s	**Normalized #*(35/x)**	Sections Counted
youngAL	613	**1073**	217	**380**	396	**693**	20
youngAL	859	**969**	566	**639**	293	**330**	31
youngAL	1794	**1652**	1085	**999**	709	**653**	38
youngAL	1456	**1699**	978	**1141**	478	**558**	30
youngAL	1980	**1733**	1109	**970**	871	**762**	40
youngAL	706	**1177**	528	**880**	178	**297**	21
oldAL	1057	**1480**	562	**787**	495	**693**	25
oldAL	1101	**1329**	589	**711**	512	**618**	29
oldAL	1285	**1153**	748	**671**	537	**482**	39
oldAL	1506	**1352**	879	**789**	627	**563**	39
oldAL	899	**1210**	373	**502**	526	**708**	26
oldAL	1223	**1098**	456	**409**	767	**688**	39
oldCR	1613	**1764**	831	**909**	782	**855**	32
oldCR	669	**807**	330	**398**	339	**409**	29
oldCR	1016	**1270**	665	**831**	351	**439**	28
oldCR	879	**1282**	256	**373**	623	**909**	24
oldCR	856	**1152**	267	**359**	589	**793**	26
oldCR	434	**1013**	262	**611**	172	**401**	15

Appendix 6 – Average motoneuron diameter's for each rat

Group	Total	Alpha	Gamma
youngAL	24.00 ± 8.01	33.08 ± 6.41	19.03 ± 2.65
youngAL	31.88 ± 8.59	35.71 ± 6.16	20.52 ± 2.79
youngAL	31.07 ± 9.98	36.90 ± 6.72	19.66 ± 2.93
youngAL	29.07 ± 9.44	35.40 ± 6.36	19.37 ± 2.88
youngAL	32.49 ± 11.25	38.92 ± 7.65	19.34 ± 2.84
youngAL	29.34 ± 10.69	37.32 ± 7.28	19.18 ± 2.85
oldAL	29.00 ± 11.00	37.58 ± 7.81	19.12 ± 2.78
oldAL	29.46 ± 11.60	38.73 ± 7.98	18.94 ± 2.63
oldAL	27.98 ± 8.34	33.95 ± 5.43	19.73 ± 2.81
oldAL	29.89 ± 10.79	37.54 ± 7.57	19.31 ± 2.86
oldAL	26.40 ± 9.87	36.49 ± 7.15	19.25 ± 2.65
oldAL	25.47 ± 9.19	35.59 ± 7.13	19.46 ± 2.72
oldCR	29.74 ± 10.00	36.63 ± 6.34	19.25 ± 2.84
oldCR	23.06 ± 7.63	32.98 ± 5.48	18.60 ± 2.52
oldCR	23.88 ± 9.27	36.08 ± 8.25	18.87 ± 2.65
oldCR	27.08 ± 9.08	34.91 ± 6.18	19.47 ± 2.83
oldCR	31.94 ± 10.91	38.41 ± 7.47	19.67 ± 2.99
oldCR	28.06 ± 10.09	36.56 ± 6.43	19.02 ± 2.72

Table 4 Diameter for each individual rat
Mean ± SD