

The Impact of Age on Microglial Activation, Glucose Uptake, and Functional
Recovery from Spinal Cord Injury

by

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DEDICATION

For Mom, who taught me that with hard work, I can do anything.

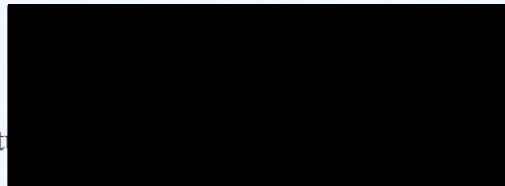
For Terry, who showed me that curiosity and learning have no limits.

And for Opa, the greatest scientist I will ever know and the original Dr. von Leden, who inspired me to dedicate my life to the pursuit of knowledge.

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Ramona Ellenbogen von Leden

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ABSTRACT

The Impact of Age on Microglial Activation, Glucose Uptake, and Functional Recovery from Spinal Cord Injury

Ramona Ellenbogen von Leden, PhD in Neuroscience, 2017

Thesis directed by: Dr. Kimberly R. Byrnes, PhD, Associate Professor,
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The number of spinal cord injury (SCI) cases among people middle-aged or older has been steadily increasing over the past 4 decades, and currently, the average of age of onset of SCI is 42. Increasing age at time of injury is correlated with worsened functional outcome and injury severity, and is associated with higher rates of co-morbidities and mortalities. DNA mutations are found in tissue by middle-age, leading to changes in cellular response genes. These include increased inflammatory genes which lead to increased oxidative damage and chronic inflammation. Aged microglia (the primary immune and inflammatory responder in the central nervous system (CNS)) show basally increased pro-inflammatory activity, considered a ‘primed’ state of activation, which is hyper-responsive, causing a more deleterious effect on injury severity. Metabolism and transport of glucose (the primary energy source for the CNS), including neurons and microglia, is decreased with age. However, the influence of age on glucose

transport and uptake after SCI is currently unknown. Though age-related changes to inflammation and glucose uptake have been documented, the mechanisms behind these changes are not well understood. This dissertation aimed to determine how age impacts microglial activation, glucose uptake, and functional recovery from SCI. Currently, there are limited pre-clinical models available for the study of middle-aged response to SCI. To address our aims, a middle-aged (12 month) male rodent model was developed and characterized for the study of the inflammatory response and glucose uptake pathway, both with and without SCI. This model was compared to a young adult (3 month old) male rodent to demonstrate differences by age. To investigate inflammation in our aged model, we used tissue analysis with markers of microglial activation, inflammatory gene expression, the NADPH oxidase (NOX)-2 enzyme expression (the major reactive oxygen species producer on microglia), oxidative damage and functional recovery. To investigate age-related impacts on glucose uptake and transport, we used [^{18}F]Fluorodeoxyglucose (^{18}FDG) PET imaging and tissue analysis of gene and protein expression of two glucose transporters (GLUT): neuronal-specific GLUT3 and insulin-sensitive GLUT4 (found on both neurons and microglia). Our naïve inflammation study demonstrated age-related increases in microglial activation, DNA oxidation, and NOX2 component and pro-inflammatory related gene expression. Our naïve glucose study showed age-related depression in glucose uptake, with increased GLUT3/4 gene expression but not protein levels. After SCI, our middle-aged rodents showed worsened functional recovery and increased lesion volume, with age-related increases in microglial activation,

oxidative stress, NOX2, and both pro and anti-inflammatory gene expression. ¹⁸FDG-PET imaging revealed age-related increased glucose uptake which matched the inflammatory response. Tissue analysis showed inconsistently altered GLUT3/4 gene expression and protein levels, suggesting impaired translation with age. Cumulatively, these results demonstrate that by middle-age, there are changes in both the inflammatory and glucose uptake pathways that have detrimental effects on recovery from SCI. Further, our work has identified two potential therapeutic targets for aging and SCI: modulation of the inflammatory response through inhibition of NOX2, and manipulation of glucose uptake and transport through intranasal insulin. Glucose and inflammation reciprocally interact, and play important roles in successful functionality of the CNS, therefore, modulation of either system could have a positive impact on the both systems. The work detailed here contributes to the understanding of age-related alterations to both inflammation and glucose uptake, and will aid in the development of therapeutic strategies for the growing aged SCI population.

Ethics Approval: All animal procedures were approved by the Uniformed Services University IACUC and complied fully with the principles set forth in the “Guide for the Care and Use of Laboratory Animals” prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (DHEW pub. No. (NIH) 85-23, 2985).

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CHAPTER 1: Introduction - Aging and Spinal Cord Injury (SCI)

STATISTICS AND PREVALENCE

There are approximately 282,000 people in the USA currently living with spinal cord injury (SCI), with more than 15,000 new cases every year (2, 80). Males account for the majority of new cases, and though a large percentage occur in the young adult population (16-30 years old), incidence of SCI has increased 5-fold in the last 40 years (55, 66, 168) among the middle-aged (30-65 years old) and elderly populations (65 and older). In the 1970's, the average age of onset was 29, but currently the average age of onset is 42 (83, 134). Incidence rates among the young adult male population have steadily declined in the last 25 years (annual percent change of -2.5%) while the incidence rates among the aging male population has seen a steady increase (annual percentage change of 2.7%) (83). Vehicle crashes are the leading cause of injury (almost 40%), but percentage of SCI caused by falls has increased to more than 30% with the increasing age of onset, as has overall in-hospital mortality, especially in the elderly population (83, 134). Co-morbidities and re-hospitalization occur in almost 30% of the population living with SCI, and ongoing cost of care is considerable (depending on age at onset and severity of injury, lifetime cost of care can range from \$1,000,000 to \$5,000,000) (134). Lifetime costs of SCI per person at older age of onset are lower than younger age of onset – cost at approximately 50 years old can range from \$1,100,000 to \$2,600,000 whereas cost at approximately 25 years old can range from \$1,500,000 to \$4,700,000 (83, 134). However, this discrepancy can be

attributed to shorter life expectancy and higher rates of co-morbidities and mortality in the aging population (83, 134). General life expectancy in persons living with SCI remains significantly lower than in persons without SCI (134). Cause of death is mainly due to co-morbidities – common co-morbidities include septicemia, pneumonia, endocrine, metabolic and nutritional diseases, nervous system diseases, cancer, stroke, urinary diseases, digestive diseases, musculoskeletal disorder and mental disorders (134).

Normal aging leads to a variety of physiologic changes to the body, including decline of the musculoskeletal system (reduced muscle mass, osteoporosis, degeneration of joints), cardiovascular and pulmonary function, gastrointestinal motility, endocrine production, and nervous system function (cognitive decline, neuronal decay, and decreased balance) (179). SCI can exacerbate these age-related physiological changes (84). Dysregulated immune and inflammatory responses (180) can lead to a state of chronic inflammation (4, 118), contributing significantly to age-related co-morbidities (55, 66). Inflammation is considered an important factor in aging and disease, and has been associated with co-morbidities, lengthened recovery time and reduced functional recovery after SCI (55, 66). Thus, research on age-related inflammation at the functional and cellular level is needed to effectively assess and treat this growing SCI risk group.

THE FREE RADICAL THEORY OF AGING

The free radical theory of aging states that the accumulation over time of free radicals produced during aerobic respiration – known as reactive oxygen species (ROS) – causes cumulative damage on cells resulting in aging and cell death (74). Basal levels of ROS molecules are necessary for normal cellular function, and under normal conditions, accumulating ROS are combated by antioxidant defenses which convert these harmful radicals into benign molecules such as oxygen and water (64, 176). However, when ROS production is increased as it is in aging, antioxidant reserves are not sufficient to mitigate their negative effects, leading to cell damage; this process is referred to as oxidative stress (64). It is suggested that mitochondria, the primary producers of and consumers of cellular energy (119), become less efficient and dysfunctional with aging due to accumulation of oxidative stress (64, 75, 99, 108, 183), ultimately leading to DNA damage (108). Accordingly, aging tissue shows progressive DNA damage beginning by the age of 40 and becoming most pronounced by 70 (108). This damage leads to increased expression of genes involved in stress response and repair including those crucial to recovery from SCI, the immune and inflammatory response (108). Clinical studies have demonstrated the effect of these increases, showing correlations of slower gait speed in aging individuals with overproduction of ROS like superoxide ($O_2^{\cdot-}$) anions (4), and the presence of pro-inflammatory cytokines with decreased muscle mass and bone density, morbidity, frailty and cognitive decline (118).

FUNCTIONAL OUTCOMES AND RECOVERY AFTER SCI

Mechanisms of SCI can be classified as primary and secondary; primary mechanisms are considered the greatest prognostic indicator, where secondary mechanisms can lead to exacerbation of damage and limit restoration, playing an important role in morbidity and mortality related to injury (50). The most common functional outcome from SCI is temporary or permanent paralysis below the initial impact site (24, 29). Recovery varies greatly depending on the severity of the injury, but the majority occurs within the first 3 months, and can include both sensory and limited motor function recovery (29). In animal models of SCI, outcome measures of injury largely focus on secondary mechanisms and resulting functional outcomes, as they serve a broader basis for development of therapeutic strategies (50).

Contusion or compression injury are commonly used models of SCI in rodents (61, 105). Contusion injury is caused by an impact that crushes the dorsal side of the spinal cord, which results in partial sparing of ventral nerve fibers that connect with the cord rostral and caudal to the injury site (10). This injury model in animals, created by impact with an accelerating rod, is considered to mimic human impact SCI fairly accurately (117). Contusion injuries can vary from mild to severe dependent on the force used for the contusion; injury severity is judged by tissue lost in the injury site and recovery of motor function (86). Compression injuries in animal models involve a multiple surface compression of the spinal cord. Compression is typically created with either forceps or a clip on the external surface of the cord, or a balloon inflated from inside the cord. These methods are

considered to result in a faithful model to human compression SCI (15, 145, 169). Injury severity is worsened with prolonged compression of the cord (44, 46, 58).

Common outcome measures for assessing severity of SCI are lesion volume and assessment of gross and fine motor function recovery. Motor function is controlled by a series of tracts distributed throughout the spinal cord; spinal networks are able to compensate for some loss of tissue which contributes to observable functional recovery in injury models (62). There is not an exact association between functional deficits and size of lesion, but once the proportion of lost tissue from a given tract reaches a threshold, functional loss is observable (106, 153). Lesion volume is indicative of injury severity due to cell loss (153). Gross motor function is determined by assessing the ability to walk, plantar step, and bend joints (most often determined by the Basso Beattie and Bresnahan (BBB) Locomotor Rating Scale in rats or Basso Mouse Scale (BMS) in mice) (6, 7). Fine motor function is determined most often by assessing gait and footprint analysis (which measure precise movements, stride patterns and weight bearing ability in limbs) (116, 117, 153). Gross and fine motor scores increase as rodents recover their ability to take weight bearing steps and regain normal gait with complete strides (9, 13, 81), and the combination of these assessments indicates the extent to which functional mobility has been impaired.

Current Animal Models of Aged SCI

Clinical studies have demonstrated that age at injury is correlated with decreased motor functional recovery, injury severity and overall decline of health (76, 146). Age at time of injury has been shown to increase functional disability,

as measured by the clinical Functional Independence Measure (146). Further, SCI has been associated with premature aging of other systems including the genitourinary, gastrointestinal, skin and subcutaneous systems, and increased age of onset at time of SCI has been shown to compound this effect (76, 146).

The established animal models of aging SCI have been largely performed in rodents at ages consistent with the elderly population (77, 148, 166). There is a need to expand the field to include rodent models of middle-age, which is more consistent with the current average age of onset of SCI (134). Unpublished pilot work in our lab found that geriatric rats show a greater number of co-morbidities and decreased survival rate after SCI compared to middle-aged rats. These findings indicate that these models may not be comparable to SCI at middle-age. Only one group to date has developed a middle-aged animal model of SCI, using mice (182), an increasingly popular animal model injury due to the ease of creation of transgenic models for the study of specific genes and proteins involved in repair (91). However, mice models of SCI do not demonstrate the same pathology as rats and humans; though inflammatory cells respond and are present at the site of injury, mice lack the formation of a cystic cavity that is a hallmark of SCI in rats and humans (22). As rats are a closer physiologic approximation to humans, outcomes from SCI in rats are more comparable to human SCI (91). Therefore, to further the translational possibilities for aging and SCI research, there is a need for the development of a middle-aged rat model of SCI.

Animal studies of aging and SCI are limited, but have begun to expand in recent studies (77, 148, 182, 183). Elderly female rats subjected to a moderate

contusion injury showed increased locomotor deficits by 7 days post injury that continued out to at least 28 days compared to young female rats, with increased lesion area and cell death outside the injury epicenter (77). Functional deficits included both gross and fine motor function – BBB scores were decreased in aged rats, and step analysis showed increased base of support and contact area in aged rats leading to increased crossing time on a catwalk (77). Elderly male rats subjected to a hemisection injury showed significantly impaired gross locomotor BBB score at 8 weeks post injury also compared to young adult rats, correlating with decreased neuronal health and axonal repair (148). Elderly mice subjected to a contusion injury showed decreased BMS score and plantar stepping (indicative of ability to place weight bearing steps) at 28 days post injury (dpi) compared to young adult mice, correlating with increased lesion volume (59). Importantly, middle-aged mice subjected to a mild-moderate contusion have shown a similar deficit, with decreased BMS score and impaired gait symmetry compared to young adult mice (182, 183).

SCI AND INFLAMMATION

Permanent sensory, autonomic, and motor function loss after SCI is caused by expansion of secondary tissue damage and cell death from the primary injury site (24, 92). Contusion SCI occurs in two phases – the acute phase (or ‘primary injury’), which is the actual mechanical insult/injury, and the chronic phase (or ‘secondary injury’), which occurs in the days to months following (92, 147). During the acute phase, there is a reduction in cell viability with neuron,

astrocyte and oligodendrocyte loss, laceration and contusion of axons, and an influx of inflammatory cells (147). The chronic stage includes white matter and axon loss, formation of a glial scar, and chronic low level inflammation (24, 147). Continued damage in the chronic phase is considered the ‘secondary injury’, and is largely mediated by the response of microglia, the resident macrophage of the central nervous system (CNS) (24, 103). These cells act as the primary immune and inflammatory responders to disruptions in the CNS (24, 103).

Microglia

The main function of microglia is to maintain homeostasis in the CNS by sensing and responding to environmental stimuli (95, 130). Microglia are found in uninjured tissue in a resting state with ramified morphology, becoming activated in response to insult. Activated microglia play a primary role in the inflammatory response to SCI, appearing in the spinal cord within 24 hours post injury, peaking in activity at 7 dpi and showing prolonged low-level chronic activation by 30 dpi (103, 150). When activated, microglia take on a classical pro-inflammatory activation phenotype (known as M1 microglia) or an alternative anti-inflammatory activation phenotype (known as M2 microglia). M1 microglia take on an amoeboid morphology, releasing cytotoxic and inflammatory mediators (80, 103, 138, 150). The release of these inflammatory mediators also produce ROS and a variety of pro-inflammatory factors and cytokines (80, 103, 138, 150), including cell surface marker CD86 (73), tumor-necrosis factor (TNF) α (73), nuclear factor (NF) κ B (101), and inducible nitric oxide synthase (iNOS) (165). M2 microglia take on phagocyte-like morphology, releasing neurotrophic factors

and cytokines (24, 80, 103), including the macrophage mannose receptor CD206 (141), nitric oxide blocking type-1 arginase enzyme (Arg-1) (28), interleukin (IL)-4 (141), and IL-10 (82). After SCI, M1 response is rapidly induced and maintains at the injury site; M2 response is smaller in comparison and transient (89). A high M1/M2 ratio of microglia is found by 7 dpi and plays an important role in cellular recovery from SCI - M1 microglia are neurotoxic whereas M2 microglia promote regeneration, therefore a higher ratio of M1 microglia indicates a more toxic, detrimental cellular environment for recovery (28, 89, 97).

Age-related Alterations to Microglia

Aged microglia show increased basal activation with upregulated pro-inflammatory cytokine mRNA expression and release (31, 142, 157, 178). Aged microglia take on a 'primed' hypertrophic morphology, appearing larger than resting microglia, with shorter processes, though not fully amoeboid like activated microglia (157). Previous work has demonstrated age-specific variance in basal gene expression of microglial activation, resulting in age-related alterations in response to a stimulus (36). Age-related 'priming' appears to be more pro-inflammatory, with a higher level of M1 pro-inflammatory microglia over M2 anti-inflammatory microglia (183). Impaired microglial regulation is associated with age-related priming, which has been suggested to compromise microglial functionality, making them more resistant to negative feedback (133). For example, 'primed' microglia are over-responsive to secondary challenges (131, 132), including exaggerated inflammatory response to trauma and accelerated neurodegeneration (133). Importantly, age-related alterations to basal microglial

activation has been found in middle-age rodent models (142), demonstrating that age-related altered cellular functionality occurs by middle-age.

Microglial Production of Reactive Oxygen Species (ROS)

Microglia produce ROS, contributing to oxidative stress in the cellular environment (16). Though mitochondria were once believed to be the principal source of ROS in oxidative stress, there is increasing evidence suggesting a crucial role for the NADPH oxidase (NOX) enzyme (103), present in numerous CNS cells including neurons (34), microglia and astrocytes (1, 112). Active NOX produces O₂ that can be converted into other oxidants like hydrogen peroxide, hydroxyl radicals and peroxynitrite, which can have severe cytotoxic effects on the cellular environment (172). NOX is the primary source of ROS in microglia and astrocytes (63). Physiologic processes involved in the activation of these cells are regulated by NOX-dependent ROS production, including the inflammatory response, and numerous studies have indicated that NOX plays a modulatory role in microglial activation, as reduction of NOX mitigates microglial response (29, 99, 139, 164).

There are multiple isoforms of the NOX enzyme with varying functions and components, and activation of NOX involves the upregulation of these components and assembly of the enzyme (176). The NOX1, NOX2, and NOX4 isoforms have all been found in neurons, microglia and astrocytes, but the NOX2 isoform is the best understood and is predominantly found on phagocytes, including microglia, where it is involved in regulation of microglial inflammatory processes (12, 159). The structure of NOX2 consists of gp91^{PHOX}, a conserved

membrane enzymatic core, two heme groups and FAD/ NADPH binding sites (176). To become activated, additional cytoplasmic subunits p47^{PHOX}, p40^{PHOX}, and p67^{PHOX} must be phosphorylated (68, 176). Together, these subunits form the NOX2 complex (3, 68, 167), which translocates to the cell membrane and binds to a flavocytochrome (cyt b558) via p22^{PHOX}, an accessory subunit protein (67, 68, 163). Once assembled, the components reduce oxygen to superoxide, a type of ROS (43). Chronic upregulation of activity and increased expression of NOX2 is found in both traumatic brain injury (TBI) (99) and SCI (24), indicating a role for NOX2 in injury response (176). Our lab has characterized the role of NOX2 in SCI in young rodent models and its potential as a therapeutic target (35), and shown that inhibition of NOX2 leads to decreased oxidative stress and improved functional recovery in a young adult mouse model of SCI (88).

Age-related Alterations to Microglial ROS Production

Increased basal NOX2 activity and expression is found in aging and has been associated with physical frailty (4, 118, 178). As a primary producer of ROS, age-related increases in NOX2 activity may lead to ROS overproduction. Overproduction can lead to accumulation of ROS and increased oxidative stress, contributing to the pro-inflammatory shift in microglial activation states in the aging CNS (108). In studies of TBI, NOX2 has been shown to mediate the M1/M2 ratio, and age-related increases in pro-inflammatory activation and NOX2 activity are found to contribute to worsened outcome (23, 99).

Aging, Inflammation and SCI

There is currently limited work on how age-related increases in oxidative stress and microglial activation influence the inflammatory response and functional recovery to SCI (59, 144, 182). Middle-age and elderly mouse models of SCI have demonstrated a shift with age in microglia/macrophage phenotype (59, 182, 183). In the injury site, middle-aged mice have increased presence of M1 macrophages, and an age-dependent shift towards decreased presence of M2 phenotype macrophages, compared to young adult mouse controls (182). These findings were associated with both impaired functional recovery and increased tissue damage, suggesting that secondary injury processes may be influenced by imbalances in microglial/macrophage phenotype ratios (182). An elderly mouse model of SCI showed impaired induction of the M2 microglia associated IL-4 receptor (IL-4R α , present in M2 phenotype microglia), which was associated with age-related reduced functional recovery (59). Lack of induction of IL-4R α resulted in attenuated expression of M2 associated factors Arg-1, IL-1 β , and chemokine ligand 2 (CCL2), leading to lower recruitment of anti-inflammatory M2 microglia to the injury site (59). These findings were verified using a transgenic knock-out (KO) mouse model (genetically modified so that target genes are inactivated by replacement or disruption with artificial DNA) where IL-4R α was the target gene; confirming that reduced IL-4R α induction led to reduced chemokines, and demonstrating age-related impairment to the anti-inflammatory related IL-4 signaling cascade (59). Interestingly, recent work has demonstrated the presence of a previously unreported age-specific M2 macrophage phenotype that produces ROS, suggesting that normally neuroprotective M2 macrophages

may take on an altered phenotype with age that is more neurotoxic (183).

The role NOX2 plays in this process is less well characterized. To date, only one study, in a middle-aged mouse model of SCI, has demonstrated age-related increased accumulation of ROS and expression of NOX2 (183). This study found increased lipid peroxidation, a downstream cytotoxic outcome of increased ROS and an indicator of increased oxidative stress (183). Though limited, these studies clearly demonstrate that by middle-age, there are age-related alterations to key players in the inflammatory response (NOX2 and microglia) that impact both functional recovery and injury severity.

GLUCOSE UTILIZATION IN THE CENTRAL NERVOUS SYSTEM (CNS)

Glucose is the primary energy source for the CNS (113, 115) and aids in maintenance and homeostasis of both neuronal and glial cell health (170). Glucose is a major component of dietary carbohydrates, and digestion and metabolism is required to break down glucose for absorption into organ systems (162). Glucose metabolism is regulated by insulin, the main anabolic hormone of the body (162). Insulin promotes the absorption of glucose from the blood into fat, liver and skeletal muscle cells, where it is processed, undergoing glycolysis to form ATP, the main usable form of energy for the body (162). To deliver glucose into the CNS from the blood, it must be transported across endothelial cells of the blood-brain barrier, and then transported into neurons and glial cells (162). The facilitative glucose transporter proteins (GLUTs) are responsible for the regulation of this process (49, 113, 149, 162).

Glucose Transporters (GLUTs)

There are 5 GLUT isoforms present in the CNS, each with different cell specificity (149). GLUT1 is insulin-insensitive and predominant in the blood-brain barrier endothelial cells and astrocytes, allowing facilitated diffusion of blood glucose into the CNS (170). GLUT2 is found in lower concentrations, and is specific to hypothalamic and hippocampal neurons, where it acts as the “glucose sensing” isoform (102). GLUT3 is highly expressed on neurons, and has the highest affinity for available glucose (87, 170). GLUT4 is intracellular, and present in a variety of cells including microglia, neurons, endothelial cells and astrocytes (25, 54, 129, 170). GLUT4 is ‘insulin sensitive’ – it requires insulin to translocate to the plasma membrane, allowing for glucose transport into cells (158). GLUT5 is exclusively expressed on microglia, and is primarily a fructose transporter (87).

Glucose and SCI

SCI related increases in inflammation are correlated with hyperglycolysis and systemic hyperglycemia (30, 78, 87, 140). Previous work in our lab and others has used positron emission tomography (PET)-based measurements of glucose uptake to demonstrate an altered post-injury glucose uptake pattern in the spinal cord (127, 175). Our study revealed an acute decrease in glucose uptake after injury which returned to baseline by two weeks post injury (175). As glucose is utilized by both neurons and glial cells, this post-injury glucose uptake pattern has been associated with both neuronal loss and inflammatory activation (52, 140, 155).

Glucose, Aging and SCI

Glucose metabolism and transport are impaired with age in the CNS (45, 48), and are decreased in the brain and fatty tissue (45, 57, 137). Further, aging and injury together have been shown to impair glucose metabolism through altered GLUT functionality in skeletal muscle and the brain (45, 48, 57, 72). These alterations with age and/or injury indicate an important interaction between glucose metabolism and inflammation, which is supported by the necessity of glucose for inflammatory cell function (54, 136). PET imaging of glucose uptake has been used in previous work to differentiate alterations in glucose uptake between normal aging and neurodegenerative diseases like Alzheimer's Disease (125). These studies demonstrate a clinically translational application for research in glucose metabolism in aging and/or injury (and related inflammation).

SIGNIFICANCE OF THIS DISSERTATION

The work included in this dissertation provides a characterization of a middle-aged rat SCI model for the study of age-related alteration of inflammation and glucose uptake. We demonstrate effects of middle-age on basal and post-SCI inflammation (oxidative stress, activation of microglia, gene expression of pro-inflammatory factors and the ROS producing NOX2 enzyme) and glucose uptake patterns (glucose uptake and GLUT3/4 gene and protein levels). As demonstrated by the literature, age has a palpable effect on inflammation and glucose utilization, systems crucial to homeostatic CNS maintenance as well as response to trauma (99). Age-related increases in basal activation of inflammatory cells has

been identified in the brain (132). Animal models of TBI have demonstrated altered cellular functionality related to age-related ‘priming’ of the inflammatory response (99, 121). TBI in aged mice show upregulated NOX expression, reduced antioxidant gene expression, increased lesion size and pro-inflammatory microglial activation when compared to young mice (99, 121). However, not only is there a higher ratio of glia to neurons in the spinal cord than in the brain, the spinal cord has been shown to have a larger overall inflammatory response to glial activation than the brain (152). Therefore, these findings cannot be literally translated to the spinal cord. Research interest in SCI and aging has been more limited, though it has begun to expand recently, demonstrating age-related increases in oxidative stress and a shift in M1/M2 ratio towards increased pro-inflammatory microglial/macrophage activation (59, 182, 183). Further, though it is established in the literature that glucose metabolism is affected by aging (57, 125), no work to date has examined glucose uptake and GLUT expression in the aging rodent spinal cord. As glucose is the primary energy source of the CNS, it is important to determine age-related alterations to this system in the context of injury, as glucose uptake and transport are relevant to both inflammation and response to injury.

With the increasing aging population in the US, it is likely that the number of SCI’s will continue to rise in older age groups. These groups are correlated with increased medical costs, greater co-morbidities and lowered quality of life due to extended recovery periods. The research detailed here contributes to a greater understanding of cellular mechanisms in aging and injury, providing a

basis for further research and will contribute to the development of future treatments and pharmacological targets for therapeutic interventions.

HYPOTHESIS

This literature review and our preliminary research led to the development of the following hypothesis: **middle-age related increases in pro-inflammatory microglial activation and oxidative stress lead to an exaggerated glial response, altered glucose utilization and diminished functional recovery to spinal cord injury.** This hypothesis was tested with three specific aims.

SPECIFIC AIMS

Aim 1. Characterize basal microglial activation states, inflammatory cytokine and NOX2 expression in the aging rodent spinal cord. (Chapter 2)

As aging in the middle-aged rat spinal cord has not been specifically characterized, determination of cellular localization and basal activation and expression of microglia, inflammatory cytokine and NOX2 gene expression is needed to better understand changes in inflammation at middle-age.

Aim 2. Demonstrate the effects of aging on microglial activation, inflammatory cytokine and NOX2 expression, lesion size and functional recovery after spinal cord injury. (Chapter 2)

Elevated glial response after SCI could affect severity of injury and recovery, leading to prolonged inflammation and worsened motor outcome.

Characterization of the impact of age on post injury inflammation and NOX2 expression is needed, and may contribute to the development of potential therapeutic targets.

Aim 3. Examine effect of age and injury on glucose uptake and transport, and determine correlation to the inflammatory response in the rodent spinal cord. (Chapter 3)

Previous work has demonstrated that ¹⁸FDG-PET based measurements of glucose uptake after SCI in 3 month old rats show altered glucose uptake acutely after injury. As age alters neuronal viability and glial response, it is necessary to determine how age alters this glucose uptake profile and associated cellular changes. This information will contribute to furthering the understanding of age-related interactions between glucose and inflammation and may provide important information that can contribute potential therapeutic avenues.

CHAPTER 2: Age exacerbates microglial activation, oxidative stress, inflammatory and NOX2 gene expression, and delays functional recovery in a middle-aged rodent model of spinal cord injury.

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ABSTRACT

Spinal cord injury (SCI) among people over age 40 has been steadily increasing since the 1980's and is associated with worsened outcome than injuries in young people. Age related increases in reactive oxygen species (ROS) are suggested to lead to chronic inflammation. The NADPH oxidase 2 (NOX2) enzyme is expressed by microglia and is a primary source of ROS. This study aimed to determine the effect of age on inflammation, oxidative damage, NOX2 gene expression and functional performance with and without SCI in young adult (3 months) and middle-aged (12 months) male rats. Briefly, young adult and middle-aged rats were assessed in two groups – naïve and moderate contusion SCI. Functional recovery was determined by weekly assessment with the Basso, Beattie, and Bresnahan (BBB) general motor score (analyzed by 2-way ANOVA) and footprint analysis (analyzed by Chi square analysis). Tissue was analyzed for markers of oxidative damage (8-OHdG, Oxyblot, and 3-NT), microglial related inflammation (Iba1), NOX2 component (p47^{PHOX}, p22^{PHOX}, and gp91^{PHOX}) and inflammatory (CD86, CD206, TNF α , and NF κ B) gene expression (all analyzed by unpaired student t-test). In both naïve and injured aged rats compared to young rats, tissue analysis revealed significant increases in 8-OHdG and Iba1, as well as inflammatory and NOX2 component gene expression. Further, injured aged rats showed greater lesion volume rostral and caudal to the injury epicenter. Finally, aged injured rats showed significantly reduced BBB scores and stride length after SCI. These results show that middle-aged rats demonstrate increased microglial activation, oxidative stress, and inflammatory gene expression, which may be related to elevated NOX2 expression, and

contribute to worsened functional outcome following injury. These findings are essential to elucidating the mechanisms of age-related differences in response to SCI and developing age appropriate therapeutics.

Keywords: Spinal cord injury, Aging, NOX2, Microglia, Inflammation

INTRODUCTION¹

There are over 250,000 people in the United States currently living with a spinal cord injury (SCI), and over 15,000 new cases are documented every year (135). These injuries cause permanent motor, autonomic and sensory function loss(50). Reduced cell viability and neuron, astrocyte and oligodendrocyte loss are seen acutely after SCI (147). White matter and axon loss, glial scar formation, and inflammation are seen chronically after injury, in the days to months following onset (147).

SCI is largely studied in the young adult population due to the high propensity of injuries from athletic and military injuries and car accidents, however, there has been a notable increase in age of onset in recent decades (83). In the 1970's, the average age at onset of injury was 29, but the average age has now increased to 42 (135). Aging tissue shows progressive DNA damage beginning by age 40, causing increased expression of genes involved in stress response and repair, including immune and inflammatory genes (108). Further, aging causes changes to hormones and cellular function associated with increased co-morbidities, lengthened recovery time, reduced functional recovery, and prolonged chronic inflammation after SCI (66).

One notable change to the cellular environment with aging is an increase in oxidative stress caused by the buildup of reactive oxygen species (ROS) over time (75, 94). The superoxide producing NADPH oxidase (NOX) enzyme is a

¹ Abbreviations: SCI – spinal cord injury, NOX – NADPH oxidase, ROS – reactive oxygen species, DPI – days post injury, CNS – central nervous system

major source of ROS (63). The NOX2 isoform has been shown to be most responsive to injury, and is present on microglia, the primary inflammatory cell type of the central nervous system (CNS) (24). Microglia are found in a resting state in uninjured tissue, with a ramified cell body and long processes. When activated, they take on a pro-inflammatory state with an amoeboid-like cell shape, releasing cytotoxic and inflammatory mediators (103). Activated microglia are found in the spinal cord cellular environment within 24 hours post-injury, and have a maintained, low-level activated presence at 60 days post injury (dpi) (103).

In animal models of traumatic brain injury (TBI), aged microglia show alterations to morphology and activation compared to younger microglia (133, 150, 157). In uninjured aged brain tissue, microglia demonstrate a hypertrophic or partially activated cell shape, considered a 'primed' activation state (133). When injured, these aged cells show a hyper-active response to injury compared to younger cells, resulting in extended chronic inflammation (133). Previous work has demonstrated that in the spinal cord, compared to the brain, there is a significantly greater inflammatory response and recruitment of macrophages and neutrophils to trauma (8, 152, 184). Therefore, findings in the aging brain may not accurately indicate what will occur in the spinal cord.

Few studies have examined how age influences microglial activation and/or oxidative stress in the spinal cord (59, 77, 144, 182), and the role NOX2 plays in this process (183). Work in our lab has characterized the role of NOX2 in SCI (35), demonstrating that inhibition of NOX2 improves functional recovery and decreases oxidative stress and inflammation in a young adult rodent model of

SCI (88). Previous work on age related cellular alterations after spinal cord injury have been performed in mice (183) and aged female rats (77), while in this paper we examine a middle-aged male rat model of SCI as the average age of onset of human SCI is 42 years old. Based on previous work showing age related alterations after injury in brain (99, 133, 157) and spinal cord (59, 70, 77, 182), we investigated microglia, NOX2 components, and ROS prior to and chronically after injury (30 dpi). We now show that microglial and NOX2 gene expression and ROS production are increased in middle-aged rats, demonstrating age related alterations in the spinal cord.

METHODS

Animals

Male Sprague Dawley rats were given free access to food and water and a 12h light/12h dark cycle. Young adult rats (3 months old: 300-500g; Taconic, Germantown, MD: n = 24; Harlan, Frederick, MD: n=6) from Harlan were used in addition to rats from Taconic in pilot naïve and 30 dpi injured study groups for immunohistochemistry and functional assessment. No significant difference was noted between groups from different vendors, therefore data from all vendors are presented together. Rats were housed for 1 week prior to undergoing surgery or assessment for naïve studies. Middle-aged rats (12 months old, retired breeders: 400-600g; Harlan, Frederick, MD: n=28) were acquired at 10 months of age, and gentled twice a week until they had matured to 12 months of age, at which point they underwent surgery or assessment for naïve studies. One 12 month old injured rat naturally perished at 20 dpi, and was removed from behavioral testing data

(final n = 17 behavioral analysis). Two 3 month old injured rats were not included in tissue analysis as tissue was damaged in processing (final n = 14 tissue analysis). All 12 month old injured rats were subjected to full behavioral battery, but the behavioral battery for the first cohort of 3 month old injured rats did not include footprint analysis testing, meaning total number of animals tested in 3 month old group was smaller than 12 month old (n = 5/10 of 3 month old rats were able to perform 28 dpi footprint analysis, n = 7/18 of 12 month old rats were able to perform 28 dpi footprint analysis). After functional testing, groups were divided amongst tissue analysis groups for histology, immunohistochemical or biochemical analysis as designated prior to injury. All animal procedures were approved by the Uniformed Services University IACUC and complied fully with the principles set forth in the “Guide for the Care and Use of Laboratory Animals” prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (DHEW pub. No. (NIH) 85-23, 2985); this manuscript has been written in accordance with the ARRIVE (Animal Research: Reporting In Vivo Experiments) guidelines (90).

Contusion Spinal Cord Injury

Contusion SCI was performed in rats (n = 35) (see Table 1 for breakdown of animal groups) as described previously (34). Briefly, rats were anesthetized with isoflurane (4% induction, 2.5% maintenance in 50% O₂), and a laminectomy was performed at vertebral level T9 to expose the spinal cord. Moderate injury (150 kDynes force, 1500μ displacement, 127 mm/s velocity, 1 s dwell time) was induced with the Infinite Horizons Impactor (Precision Systems Incorporated,

Natick, MA). Naïve animals (n = 22) received no surgery or anesthesia. Post injury care included dual housing on diamond soft bedding (Harlan Laboratories, Frederick, MD), twice daily bladder expressions until spontaneous micturition returned and analgesic supplementation to drinking water for 48 hours following injury (Children’s Tylenol; acetaminophen, 200mg/kg).

Number of Animals per Group and Experiment					
Age	Injured/Naïve	Function	Immunohistochemistry	Lesion volume	Biochemistry/PCR
3 months old	Naïve	12	4	0	4
12 months old	Naïve	10	4	0	4
3months old	Injured	18	4	6	4
12 months old	Injured	17	4	6	4

Table 1. Number of animals per group for all experiments in inflammation study. Animals were first divided by age (3 months old or 12 months old) and injury type (naïve or injured). Functional analysis was performed on all animals in study. Animals used in function experiments were sacrificed and divided into sub groups for tissue experiments (immunohistochemistry, lesion volume, and biochemistry/PCR).

Functional Assessment

The Basso–Beattie–Bresnahan (BBB) scale was used to assess functional neurological recovery, as detailed previously (6). Performance of left and right hindlimbs on a scale from 0 (no spontaneous movement) to 21 (normal locomotion) was averaged to obtain the total BBB on days 1, 7, 14, 21 and 28 dpi. More detailed locomotion was assessed using the ‘footprint analysis test’, modified from Kunkel-Bagden et. al (100), and performed on the same days as BBB assessment. Subjects’ hind paws were pressed to an inkpad to dye their hind feet, and were then allowed to walk across a sheet of paper lining a long and

narrow runway (6.5 inch wide, 76 inches long) to ensure animals walked in a straight line for analysis. Prints were analyzed for width of toe spread and length of stride length (in mm). Subjects' footprints were only analyzed if they had regained ability to walk with support on hindlimbs (a minimum BBB score of 11), and percentage of animals who had regained the ability to perform the task was analyzed by age group at each assessment day.

Histology and Lesion Volume

At 30 dpi, rats were anesthetized with Euthasol (pentobarbital sodium and phenytoin sodium; 200mg/kg, IP) and intracardially perfused with 10% buffered formalin (Fisher Scientific, Hampton, New Hampshire). A 1 cm block of spinal cord tissue was dissected, extending 5 mm rostral and caudal from the center of the lesion site (or from the T9 vertebral site for naïve animals). Tissue was sectioned into 20µm axial sections, and every third slice was saved for histological analyses. To determine lesion volume, tissue was processed with a standard H&E stain. Lesion volume from H&E images collected with NanoBrightfield Software (Hamamatsu Corporation, Middlesex, NJ) were quantified using the Cavalieri method as previously described (104, 122). Ten sections were analyzed per animal at even intervals throughout spinal cord from 360 microns pre lesion epicenter (0-360 microns on histogram in Figure 5A) through lesion epicenter (between 360-540 microns on histogram) to 360 microns post epicenter (540-900 microns on histogram) to provide an analysis of volume of lesion throughout the spinal cord.

Immunohistochemistry

Standard fluorescent immunohistochemistry was performed on tissue sections from animals 30 dpi obtained as described above. Iba1 (microglia, 1:100; Wako Bioproducts, RRID:AB_839504; Richmond, VA) and 8-OHdG (DNA oxidation, 1:500; Abcam, RRID:AB_940049; Cambridge, MA) were used as primary antibodies, with visualization by fluorescent secondary antibodies (Alexa-Fluor Secondaries, 1:500; Molecular Probes, Eugene, OR). Negative controls (sections not stained with primary antibody) were used to confirm specificity of secondary antibodies. Immunofluorescent images were captured under the same fluorescence conditions and exposure time on a NanoZoomer system (Hamamatsu, Bridgewater, NJ) or an Olympus BX43 microscope (Olympus America). Immunofluorescence was quantified using pixel density measurement in Scion Image as previously described (47). Ten axial sections were analyzed per animal starting caudally at the first sign of lesion, then at even intervals rostrally through the spinal cord for 1cm to provide analysis throughout the lesion. Analysis was performed on grey matter directly adjacent to the lesion site at 20X magnification. The average pixel density of all ten sections was reported for each animal.

Comparative RT-PCR

A 10mm section of the spinal cord, centered at the lesion epicenter, was dissected and immediately frozen on dry ice. The TRIzol® protein and mRNA extraction method (Invitrogen, Carlsbad, CA) was employed so that each sample could be used for both western blotting and mRNA quantification. Briefly, RNA

was extracted from each cord sample individually using TRIzol® reagent and chloroform, then purified with the Qiagen RNeasy kit (#74104, Qiagen, Hilden, Germany). RNA concentration was measured using the Nanodrop system (Thermo Scientific, Wilmington, DE). Complementary DNA (cDNA) conversion was performed from 1 µg of RNA using a Veriti thermal cycler (Applied Biosciences, Waltham, MA) and a high capacity cDNA conversion kit (Applied Biosciences, Waltham, MA) using the manufacturer's protocol. Quantitative Real Time PCR (qRT-PCR) was performed using the StepOnePlus Real-Time PCR System (Applied Biosciences, Waltham, MA) according to manufacturer's instructions. To determine changes in gene expression, primers were designed using the Primer-Blast tool, then obtained from Integrated DNA Technologies (IDT, Coralville, IA; See Table 2 for detailed primer information). Reported gene expression values were determined using the $\Delta\Delta$ CT method: raw value of each sample normalized first to internal control (GAPDH) and then to control group (3-month).

Gene	Sense	Antisense
CD86	5'-TGCTCATCTAAGCAAGGATACCCG-3'	5'-CGACTCGTCAACACCACTGTCCTG-3'
CD206	5'-GGATTGTGGAGCAGATGGAAG-3'	5'-CTTGAATGGAAATGCACAGAC-3'
p22 ^{PHOX}	5'-GACGCTTCACGCAGTGGTACT-3'	5'-CACGACCTCATCTGTCACTGG-3'
p47 ^{PHOX}	5'-CAGAATGTTGCCTGGTTG-3'	5'-GTCCCCTCCCTTAGATGA-3'
gp91 ^{PHOX}	5'-CTTCACACGGCCATTACAC-3'	5'-GTCATAGGAGGGTTTCCGGC-3'
TNF α	5'-GGCAGCCTTGTCCTTGAAGAG-3'	5'-GTAGCCACGTCGTAGCAAACC-3'
NF κ B	5'-GTGCAGAAAGAAGACATTGAGGTG-3'	5'-AGGCTAGGGTCAGCGTATGG-3'
iNOS	5'-CAGCCCTCAGAGTACAACGAT-3'	5'-CAGCAGGCACACGCAATGAT-3'
Arg-1	5'-CAGTATTCACCCGGCTACG-3'	5'-GCCTGGTTCTGTTCGGTTTG-3'
CD68	5'-TGTACCTGACCCAGGGTGGA-3'	5'-GAATCCAAAGGTAAGCTGTCCGTAA-3'
IL10	5'-CAAGGAGCATTTGAATTCCC-3'	5'-GGCCTGTAGACACCTTGGTC-3'
GAPDH	5'-GCTGGTCATCAACGGGAAA-3'	5'-ACGCCAGTAGACTCCACGACA-3'

Table 2. Gene primers tested by comparative RT-PCR in inflammation study. Table provides sense and antisense sequences for each gene tested in this study. Genes investigated that showed no significant difference by age in either naïve or injured animals were omitted from results.

Western Blot and Oxyblot

Protein from frozen tissue samples was isolated in TRIzol® reagent (Invitrogen, Carlsbad, CA). Detection of protein oxidation was performed by processing protein samples using the Oxyblot Protein Oxidation Detection Analysis Kit (Millipore, Billerica, MA) according to manufacturer's protocol. For both Oxyblot and western blotting, 15 µg of sample protein was run in a Mini-PROTEAN® TGX™ Precast Gel (Bio-Rad, Hercules, CA) and transferred to a Trans-Blot® Turbo™ (Bio-Rad) nitrocellulose membrane. Antibodies raised against 3-Nitrotyrosine (5µg/ml; Abcam, RRID:AB_942087) were probed overnight at 4°C. Immune complexes and oxidated proteins were detected with appropriate secondary antibodies and chemiluminescence reagents (Pierce).

For both Oxyblot and western blots, GAPDH (0.5µg/ml; Millipore, RRID:AB_2107445) was used as a control for gel loading and protein transfer. NIH Image J was used to assess pixel density of resultant blots for quantitation.

STATISTICS

Sample sizes were calculated based on our previous work(154). All assays were carried out by investigators blinded to subject group. Quantitative data are presented as mean +/- standard error of the mean (SEM). General motor performance using the BBB test was analyzed using a 2-way ANOVA with Tukey's multiple comparison's post hoc test. Ability to plantar step in footprint analysis task was analyzed using Chi square analysis. Footprint analysis test, immunohistochemistry, biochemistry and q-RT PCR were analyzed using the student t-test. All statistical tests were performed using the GraphPad Prism Program, Version 6.03 for Windows (GraphPad Software, San Diego, CA). A *p* value < 0.05 was considered statistically significant.

RESULTS

Uninjured middle-aged animals show altered stepping pattern in footprint analysis

Footprint analysis of toe spread and stride length in uninjured animals revealed an alteration in motor function in middle-aged animals compared to young animals (Figure 1A). Toe spread was measured from the thumb toe (toe 1) to the pinky toe (toe 5) in the hind paws. 12 month aged rats showed a

significantly wider toe spread than 3 month aged rats ($t(20)=4.98$, $p=0.0005$).

Stride length was measured as the length in mm between each consecutive step in the hind paws. Though not significant, 12 month aged rats showed a trend toward longer strides than 3 month aged rats (Figure 1B; $t(20)=1.97$, $p=0.0627$), indicating that overall stepping patterns are altered with age.

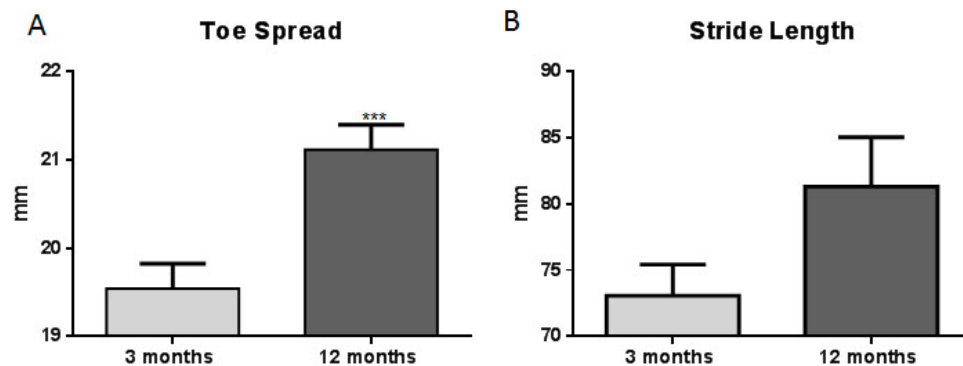


Figure 1. Uninjured middle-aged animals show altered stepping pattern in footprint analysis.

A) Uninjured 12 month old rats ($n=10$) have significantly wider toe spread than 3 month old rats ($n=12$). B) Uninjured 12 month old rats show a trend towards increased stride length compared to 3 month old rats, supporting toe spread findings. *** $p<0.001$. Bars represent mean $\pm$ SEM.

Microglial activation and pro-inflammatory gene expression is altered with age

To determine if aged tissue showed basal differences in microglial activation, uninjured tissue from 3 and 12 month old rats was immunostained with the microglial marker Iba-1 (Figure 2A) and processed for comparative RT-PCR for expression genes associated with microglial activation and NOX2 components. Our findings showed significantly increased Iba-1 staining ($t(6)=2.59$, $p=0.0496$) in 12 month aged rat tissue compared to 3 month aged rats

(Figure 2B). Comparative RT-PCR results (Figure 2C) showed significantly increased gene expression in 12 month aged rats of the pro-inflammatory phenotype marker CD86 ($t(6)=7.46$, $p=0.0050$) compared to 3 month aged rats. No significant difference by age was found in any other genes probed, including the anti-inflammatory phenotype marker CD206, transcription factor NF κ B or inflammatory cytokine TNF α (Table 3). Relative fold changes between 3 month and 12 month old rats for all genes tested listed in numerical form can be found in Table 3 under “naïve”.

Middle-aged rats show increased gene expression of NOX2 cytosolic subunit component p47^{PHOX}

To determine if NOX2 activity and expression were influenced by age, uninjured tissue from 3 and 12 month old rats was processed for comparative RT-PCR and analyzed for target gene expression. Gene expression of the activated cytosolic subunit p47^{PHOX} was significantly increased in 12 month aged rats compared to 3 month aged rats ($t(6)=3.47$, $p<0.0001$; Figure 2D). However, gene expression of the activated membrane bound NOX2 components p22^{PHOX} and gp91^{PHOX} did not reveal any significant difference by age.

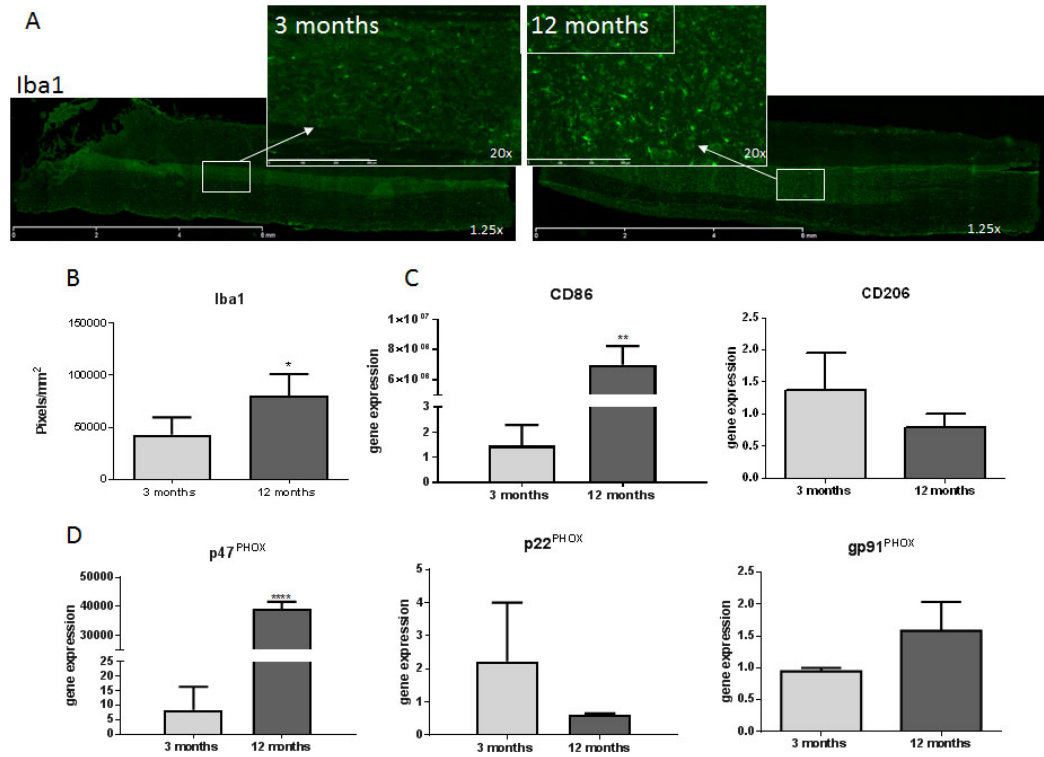


Figure 2. Microglia and NOX2 component activation and gene expression is altered with age.

A) Fluorescent microscope images of longitudinal sections of naïve 3 month and 12 month rat spinal cord. Sections were stained with Iba-1 (green). Sections were imaged at 1.25x magnification and encompass an entire 10mm section in the thoracic spinal cord. Inserts were taken at 20x magnification in the grey matter delineated by white boxes and arrows. B) Immunostaining shows age related differences in the microglial marker Iba1, showing significantly increased staining in 12 month old versus 3 month old rats (Size bar=100µm). N=4/group. C) Comparative qPCR reveals significant increase in gene expression of the pro-inflammatory phenotype marker CD86. D) Comparative RT-PCR reveals significant increase in p47^{PHOX} gene expression in 12 month aged rats compared to 3 month aged rats. No significant difference was seen in gene expression of p22^{PHOX} or gp91^{PHOX} between the two groups. N=4/group. *p<0.05, **p<0.005, ****<0.0005. Bars represent mean +/- SEM.

Comparative Fold Change by Injury Group				
Naïve		Gene Tested	30 dpi	
3 months old	12 months old		3 months old	12 months old
1.467 ± 0.8292	**6.993x10 ⁶ ± 1.258x10 ⁶	CD86	1.096 ± 0.2886	***1.253x10 ⁶ ± 145818
1.371 ± 0.5850	0.7830 ± 0.2167	CD206	1.104 ± 0.3465	**139991 ± 24361
2.217 ± 1.785	0.6052 ± 0.04079	p22 ^{PHOX}	1.030 ± 0.1825	*20678 ± 6017
8.352 ± 8.037	***39131 ± 2480	p47 ^{PHOX}	7.739 ± 5.672	*1.225x10 ⁶ ± 473918
0.9453 ± 0.05264	1.583 ± 0.4531	gp91 ^{PHOX}	0.9421 ± 0.08263	*1.948 ± 0.4574
3.229 ± 2.839	163.1 ± 141.8	TNFα	0.6954 ± 0.3801	*11.55 ± 2.502
1.226 ± 0.5548	0.8387 ± 0.6209	NFκB	1.027 ± 0.1751	*4.108 ± 0.7734
44.06 ± 22.85	0.01669 ± 0.01419	iNOS	1.109 ± 0.3358	1.069 ± 0.4000
3.118 ± 2.773	1230 ± 794.0	Arg-1	1.047 ± 0.2304	1.382 ± 0.03944
0.4958 ± 0.4531	0.3324 ± 0.02472	CD68	1.104 ± 0.3346	1.011 ± 0.3394
2.438 ± 1.521	7.806 ± 4.643	IL10	1.051 ± 0.2427	1.057 ± 0.1975

Table 3. Comparative gene fold changes for all genes tested by injury group. Table demonstrates how age effects gene expression before and after injury. The average of the 3 month group in both the naïve and injury group was considered the control mean value. Each individual sample's fold change was compared to the control mean. Fold changes are presented in table as mean values of all samples in each group +/- SEM. N=4/group. *p<0.05, **p<0.005, ***p<0.0005.

ROS production is altered with age

To determine if tissue showed basal differences in ROS production with age, uninjured tissue from 3 and 12 month old rats was immunostained with the DNA oxidation marker 8-OHdG (Figure 3A), Oxyblot analysis was performed to determine protein oxidation, and western blotting was performed to determine levels of 3-NT, a marker of protein nitrosylation. Our findings showed that 12 month aged rats compared to 3 month aged rats demonstrated a significant increase in 8-OHdG staining ($t(6)=3.06$, $p=0.0281$; Figure 3B) and a trend towards increased protein oxidation via Oxyblot ($t(6)=2.18$, $p=0.0946$; Figure

3C). Western blotting did not show any difference in protein nitrosylation between the two ages (Figure 3D).

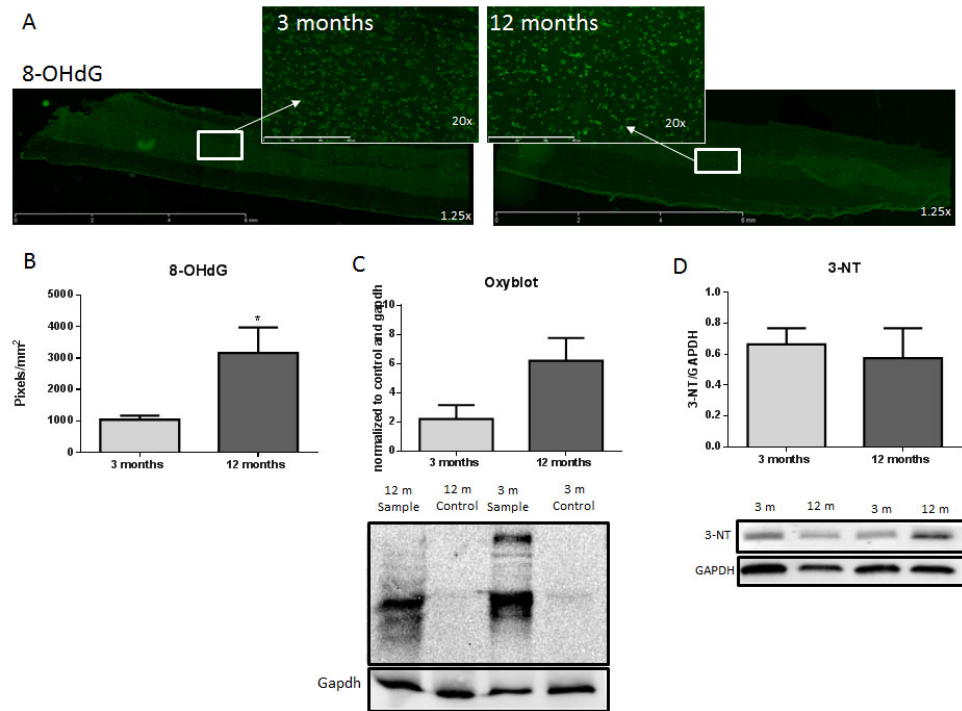


Figure 3. ROS production is increased with age.

A) Fluorescent microscope images of longitudinal sections of naïve 3 month and 12 month rat spinal cord. Sections were stained with 8-OHdG (green). Sections were imaged at 1.25x magnification and encompass an entire 10mm long section in the thoracic spinal cord. Inserts were taken at 20x magnification in the grey matter delineated by white boxes and arrows. B) 8-OHdG stain shows significantly greater oxidative damage in 12 month old versus 3 month old rats (Size bar=100µm). N=4/group. C) Oxyblot analysis shows a trend (p=0.0946) towards increased protein oxidation in 12 month aged rats compared to 3 months aged rats. D) Western blot analysis shows protein expression of 3-NT, a marker of nitrosylated proteins which is a product of oxidative stress, is not significantly different between 12 month and 3 month aged rats. N=4/group. *p<0.05. Bars represent mean +/- SEM.

Middle-aged rats show diminished motor function recovery after SCI

Next we examined both middle-aged and young animals after moderate contusion SCI to investigate how age alters response to injury. Our results from the BBB general motor test showed worsened performance by aged animals after injury (Figure 4). 12 month old rats showed worsened performance compared to 3 month old rats at all time points except 1 dpi, indicating significantly impaired recovery to injury [$F(1,33)=12.81$, $p<0.0001$; Figure 4A). At 1 dpi, 12 month and 3 month old rats averaged a score 0.471 ± 0.2827 and 1.000 ± 0.485 respectively, indicating no stepping ability with only slight movement of one joint on one limb. At 7 dpi, the 3 month old group has progressed to a mean of 6.583 ± 1.188 , indicating extensive movement in all multiple joints, while the 12 month old group remained at an average score of 0.529 ± 0.208 . By 14 dpi, the 3 month group continued to show steady improvement with a BBB score of 10.970 ± 1.217 , with occasional weight supported steps but no limb coordination, and the 12 month group showed sweeping of feet with no weight bearing steps, averaging 6.971 ± 1.309 . At 21 and 28 dpi, improvement in the 3 month old group was still steady (13.310 ± 1.009 and 14.42 ± 1.070 , respectively), showing consistent weight bearing plantar stepping with coordinated limb movements, while the 12 month old group reached a max performance of 9.618 ± 1.202 at 21 dpi, with weight bearing plantar stepping but only occasional coordinated limb movements, and never improved beyond that score. This comparison demonstrates not only that the 12 month old groups recovery was diminished in comparison with the 3 month old, but that the overall speed of recovery was delayed – the improvements in functional performance in the 12

month old group occurred at least one week after the same score had been achieved in the 3 month old group.

The number of rats that achieved plantar stepping, enabling them to complete footprint analysis, at each time point through 28 days was assessed and found to differ by age. The number of 12 month aged rats that achieved plantar stepping by 28 dpi was significantly lower than the 3 month aged rats [$\chi^2(3, N=31)=37.52, p<0.0001$; Figure 4B]. Rats that recovered the ability to take plantar steps with their hindlimbs were tested on the footprint analysis task. At 28 dpi, 12 month old rats that were able to plantar step demonstrated a significantly shorter stride length ($t(10)=2.53, p=0.0297$; Figure 4D) and a trend toward narrower toe spread ($t(10)=1.192, p=0.0967$; Figure 4C) in comparison to the 3 month old rats.

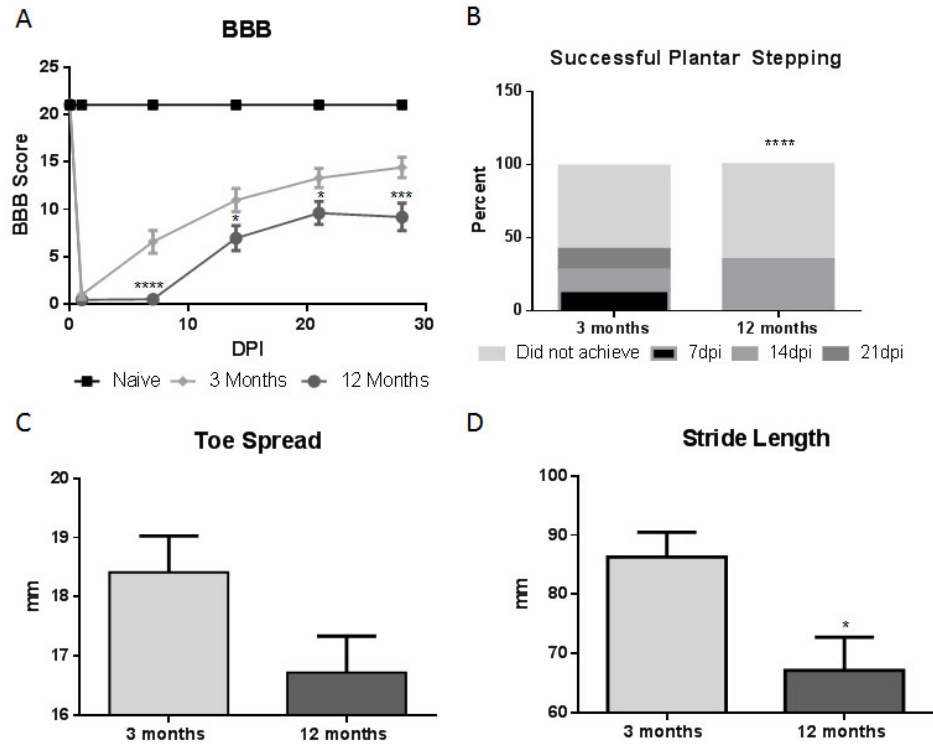


Figure 4. Middle-aged animals show diminished motor function recovery after SCI. A) At 1 dpi no difference in performance in BBB scores were observed in injured rats. At all other timepoints (7, 14, 21, and 28 dpi), 12 month old injured rats (n=17) showed significantly reduced BBB scores compared to 3 month old rats (n=18), indicating significantly impaired recovery to injury. B) Aged animals show impaired ability to plantar step and complete footprint analysis task. 12 month old rats (n=17) show significantly diminished ability to plantar step in order to complete the footprint analysis task compared to 3 month old rats (n=14), indicating a diminished ability to take steps or strides. C) At 28 dpi, toe spread analysis showed a trend in 12 month old rats (n=7) toward narrower toe spread compared to 3 month old rats (n=5). D) 12 month old rats (n=7) showed significantly decreased stride length compared to 3 month old rats (n=5). * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$, **** $p < 0.0001$. Bars represent mean $\pm$ SEM.

Middle-aged rats have significantly greater lesion volume rostral and caudal to the lesion epicenter

At 30 dpi, results from our lesion volume analysis showed a larger lesion volume in 12 month old rats compared to 3 month old rats, however, the volume of the lesion varied by proximity to lesion epicenter (Figure 5A). At the lesion epicenter (360-540 microns), both 3 month and 12 month old animals

demonstrated marked tissue damage and loss, with no significant difference in volume between age groups (Figure 5C). But both rostral (0-180 microns) and caudal (720-900 microns) lesion epicenter, 12 month old animals had significantly greater lesion volumes than 3 month old ($t(10)=6.09$, $p<0.0001$ /rostral, and $t(10)=4.64$, $p=0.0017$ /caudal; Figure 5B and D).

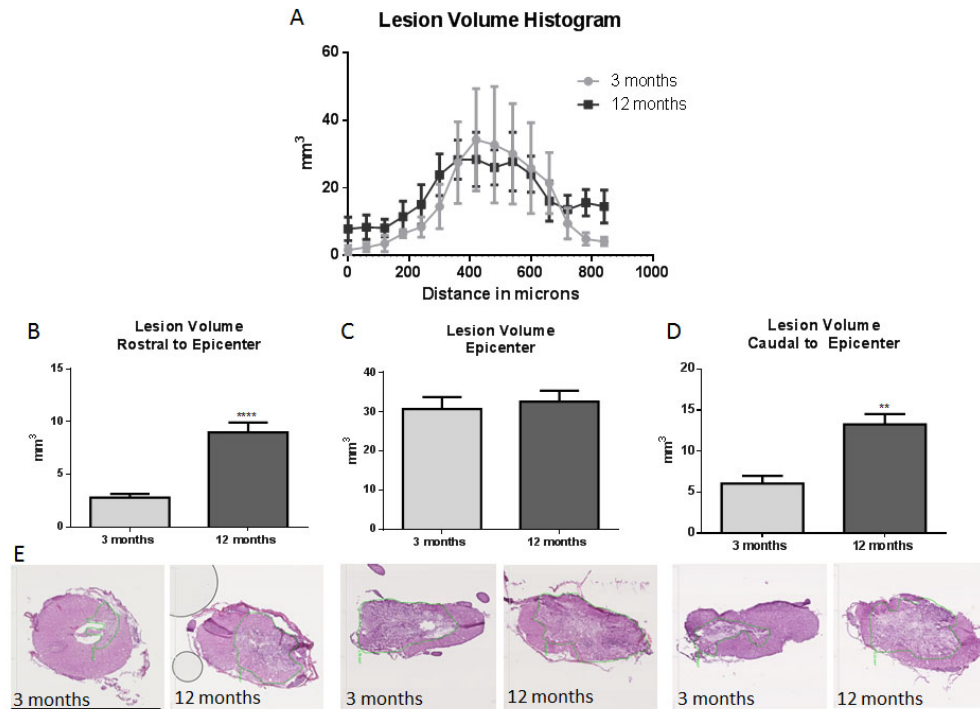


Figure 5. Middle-aged rats have significantly greater lesion volume rostral and caudal to the lesion.

A) Quantitative analysis of tissue stained with H&E reveals that at 30 dpi, 12 month old rats show an increased lesion volume compared to 3 month old rats both rostral and caudal to the injury epicenter, suggesting aged rats have an increased volume and length of lesion compared to young. Lesion volume histogram shows the pattern of lesion spread from the epicenter. B) Lesion volume rostral to the epicenter (0-180µ) was significantly greater in 12 month old rats compared to 3 month old rats. C) No significant difference was found in lesion volume at injury epicenter (360-540µ). D) Lesion volume caudal to the epicenter (720-900µ) was significantly greater in 12 month old aged rats compared to 3 month old rats. E) Photos depict sections of spinal cord at each segment through the lesion from both 3 and 12 month rats. Rostral image is from 120µ section, epicenter image is from 400µ section, and caudal image is from 820µ section). Size bar=3mm. N=6/group. ** $p<0.005$, **** $p<0.0001$. Bars represent mean $\pm$ SEM.

Microglial activation and gene expression at 30 dpi is altered with age

Immunostaining at 30 dpi in both 3 and 12 month aged tissue revealed increased microglial activation, as defined by cell size and number, and reflected in pixel density of Iba1 staining microglia in comparison to naïve tissue (Figure 6A). Aged tissue showed significantly greater Iba1 staining compared to young tissue ($t(6)=2.16$, $p=0.0487$; Figure 6B). Comparative RT-PCR results (Figure 6C) showed significantly increased gene expression in 12 month aged rat compared to 3 month aged rats of pro-inflammatory marker CD86 ($t(6)=7.26$, $p=0.0050$), anti-inflammatory marker CD206 ($t(6)=5.75$, $p=0.0045$), inflammatory cytokine TNF α ($t(6)=3.47$, $p=0.0127$) and transcription factor NF κ B ($t(6)=4.98$, $p=0.0156$). Relative fold changes between 3 month and 12 month old rats for all genes tested listed in numerical form can be found in Table 3 under “30 dpi”. An additional experiment comparing gene expression between naïve and injured groups within each age group was performed on selected genes to verify gene expression patterns (Table 4).

Middle-aged rats show increased NOX2 component gene expression at 30 dpi

To determine if NOX2 activity and expression after injury were influenced by age, tissue from injured 12 month aged rats and 3 month aged rats at 30 dpi was processed for comparative RT-PCR and analyzed for expression of target genes. Gene expression of the cytosolic subunit p47^{PHOX} and the membrane components p22^{PHOX} and gp91^{PHOX} were significantly increased in 12 month aged rats compared to 3 month aged rats ($t(6)=3.47$, $p=0.0404$; $t(6)=4.61$, $p=0.0182$ and $t(6)=2.94$; $p=0.0186$, respectively; Fig. 6D).

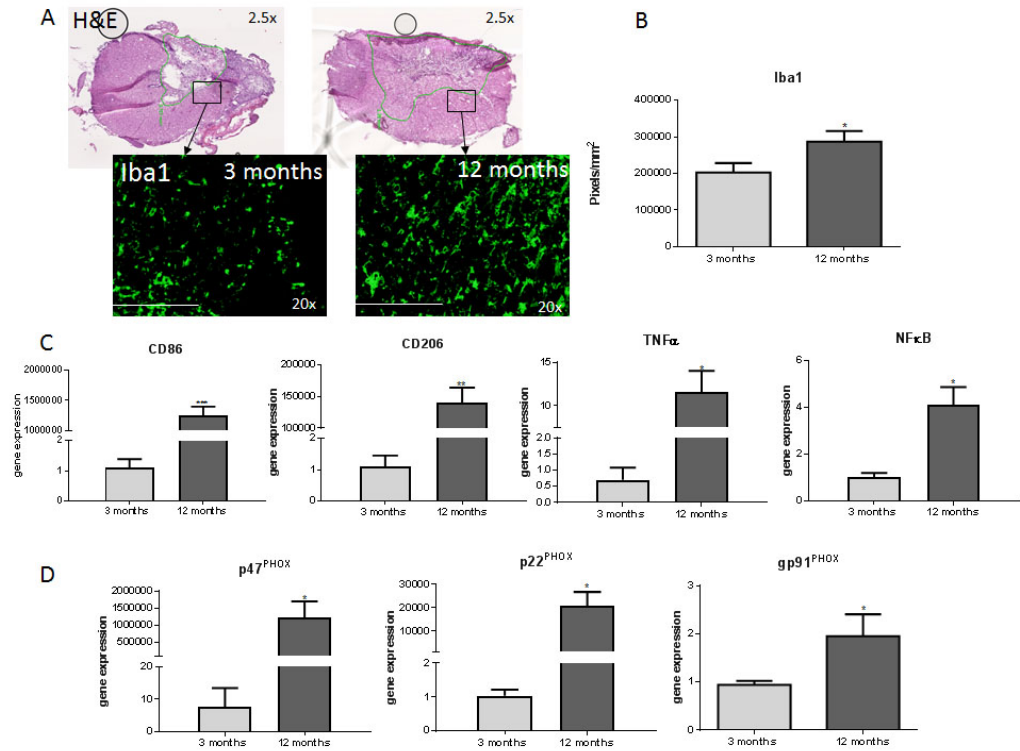


Figure 6. Middle-aged rats show increased microglial and NOX2 component activation and gene expression after SCI at 30 dpi.

A) 30 dpi H&E stained images of 3 month and 12 month old injured spinal cord. Black boxes depict location of Iba1 stained quantification, in the closest intact tissue outside the central cavity of the injury. B) Black arrows point to corresponding images of immunofluorescent staining of Iba1. 12 month aged rats showed significantly greater staining in comparison to 3 month aged rats at 30 dpi. (Size bar = 100 μ m, N=4/group). C) Comparative RT-PCR reveals significant increase in gene expression of the M1 phenotype marker cd86, M2 phenotype marker cd206, and inflammatory cytokines TNF α and NF κ B in 12 month aged rats compared to 3 month aged rats, suggesting an overall increase in microglial gene expression after injury with age. D) Comparative RT-PCR of NOX2 components p47^{PHOX}, p22^{PHOX}, and gp91^{PHOX} reveal a significant increase in gene expression of all three genes in 12 month aged rats compared to 3 month aged rats at 30 dpi. N=4/group. *p<0.05, **p<0.005, ***p<0.0005. Bars represent mean $\pm$ SEM.

Comparative Fold Change by Age Group				
3 months old		Gene Tested	12 months old	
Naïve	30 dpi		Naïve	30 dpi
1.467 ± 0.8292	4.481 ± 1.179	CD86	0.6781 ± 0.3546	0.7444 ± 0.08664
1.371 ± 0.5850	0.7586 ± 0.5561	CD206	1.094 ± 0.3027	111858 ± 67300
1.043 ± 0.2966	225.2 ± 76.85	p22 ^{PHOX}	1.005 ± 0.06771	218.9 ± 132.1
1.491 ± 0.8159	3.134 ± 2.551	p47 ^{PHOX}	1.232 ± 0.4360	**180416 ± 31468
1.001 ± 0.02864	28.02 ± 3.662	gp91 ^{PHOX}	1.154 ± 0.3588	**54.89 ± 21.85

Table 4. Comparative gene fold changes by age group for significantly impacted genes.

Table demonstrates the effect of injury on gene expression within age groups. The average of the naïve in both the 3 and 12 month age group was considered the control mean value. Each individual sample's fold change was compared to the control mean. Fold changes are presented in table as mean values of all samples in each group +/- SEM. N=4/group/. **p<0.005.

Middle-aged rats show increased ROS production and oxidative stress at 30 dpi

To determine if injured aged tissue showed differences in ROS production, tissue from 3 and 12 month old rats was processed for Oxyblot and western blotting at 30 dpi. Oxyblot analysis revealed that 12 month aged rats compared to 3 month aged rats demonstrated significantly increased protein oxidation ($t(6)=4.04$, $p=0.0156$; Figure 7A), and western blotting of 3-NT, a marker of protein nitrosylation, demonstrated significantly increased protein nitrosylation ($t(6)=1.758$, $p=0.0038$; Figure 7B).

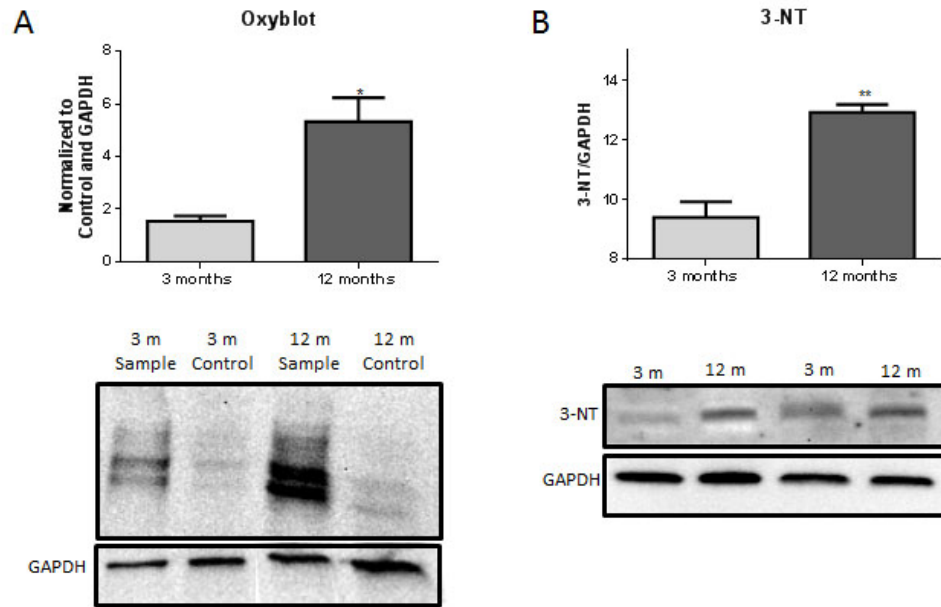


Figure 7. Middle-aged rats show increased ROS production and oxidative stress at 30 dpi.

A) Oxyblot analysis reveals a significant increase in protein oxidation at 12 month aged rats compared to 3 months aged rats at 30 dpi. B) Western blot analysis reveals a significant increase in 3-NT, a marker of nitrosylated proteins which is a product of oxidative stress, in 12 month aged rats compared to 3 month aged rats 30 dpi. N=4/group. * $p < 0.05$, ** $p < 0.005$. Bars represent mean $\pm$ SEM.

DISCUSSION

This study demonstrates that aging alters the inflammatory environment in the 12 month old rat spinal cord. Uninjured 12 month old, middle-aged rats show increased microglial activation, NOX2 cytosolic subunit and pro-inflammatory gene expression, and increased DNA oxidation when compared to 3 month old, young adult rats. At 30 dpi, middle-aged rats show decreased functional recovery and increased lesion volume, microglial activation, NOX2 component and inflammatory gene expression, and protein oxidation and nitrosylation compared to young adult rats.

Previous work has shown increasing oxidative stress with age (94). We now demonstrate that this increase is obvious in the CNS, with increased markers of oxidative stress in the middle-aged rat spinal cord both before and after injury. In naïve middle-aged rats compared to young adult, increases in the DNA peroxidation marker 8-OHdG, but not of protein carbonylation (Oxyblot) and nitrosylation (3-NT) were observed. As mutations in DNA have been found by early middle-age (108), DNA peroxidation may be one of the first signs of increasing ROS production, as part of a pro-inflammatory environment in aging tissue (133). As protein modifications occur further downstream than DNA, age-related increases in protein carbonylation and nitrosylation markers may be more indicative of a hyperactive inflammatory response to injury.

The increased ROS production found in middle-aged rats coincides with our results showing increased microglial activity with age. Increased staining of the microglial marker Iba1 was coupled with increased pro-inflammatory gene expression of CD86 in middle-aged rats, suggesting microglia are present in aged tissue in a pro-inflammatory state. Further, staining demonstrates alterations in microglial phenotype as defined previously (103), with 3 month rats showing a non-activated phenotype with lengthy processes and a small cell body, and the 12 month old rats showing a partially activated microglial phenotype, with an engorged, rounder shaped cell body. These findings in the spinal cord are similar to previous findings in the brain (133), suggesting an age-related pattern of the inflammatory response that is not impacted by regional differences between the spinal cord and the brain (184).

Interestingly, our gene expression experiments at 30 dpi showed an increase in both pro-inflammatory and anti-inflammatory related genes, suggesting that chronically after injury, all microglial activity is elevated, not only pro-inflammatory as seen in uninjured tissue. Previous work using aged mouse and female rat models of traumatic injury has shown a decrease in anti-inflammatory signaling and an increase in pro-inflammatory signaling at 24 hours and 7 dpi (59, 77, 99, 182). In a traumatic brain injury model with 23 month old mice, a similar increase in both pro- and anti-inflammatory gene expression was observed at 24 hours post-injury (121). Our findings at 30 dpi suggest a change in activation type may occur chronically post injury that leads to a more active overall inflammatory response. Further, Morganti et al. (121) found that acute gene expression alterations were strongly dependent on peripheral monocyte infiltration; it is possible that the 30 dpi data in our study are more microglial related due to the reduction in macrophage invasion at this later time point (85). Future work should evaluate these earlier time points and contribution of peripheral and central inflammatory cells in 12 month old rats to fully characterize our rodent model.

Increased gene expression of the activated NOX2 cytosolic subunit p47^{PHOX} but not the membrane components p22^{PHOX} or gp91^{PHOX} suggests NOX2 expression is increased in middle-aged naïve rats, corresponding with the observed pro-inflammatory microglia. At 30 dpi, gene expression of both NOX2 membrane components p22^{PHOX} and gp91^{PHOX} and the cytosolic subunit p47^{PHOX} are elevated in middle-aged rats compared to young adult, suggesting that

microglial NOX2 is 'primed' prior to injury and activated more fully with injury. This could play a role in the increased ROS production we see in middle-aged spinal cord. We and others have shown that NOX2 is involved in microglial ROS production (35, 88, 183).

Our lesion volume experiments at 30 dpi showed that at the epicenter of the injury, the lesion volume was not significantly different by age, but was significantly increased in middle-aged rats compared to young adult in sections of the spinal cord both rostral and caudal to the injury, demonstrating an increased volume farther from the lesion epicenter in middle-aged rats. Our results coincide with previous work showing increased lesion volume in aged female rats after SCI (77) and in a mouse model of TBI (99). These findings demonstrate that injury severity is increased in middle-aged rats, which may be a result of the exaggerated inflammatory response leading to increased oxidative stress on cells. Control of motor function is distributed throughout the spinal cord with multiple interacting tracts, and recovery of function observable in injury models is largely due to the ability of the spinal networks to compensate for some loss of tissue (62). Interestingly, previous work has shown that the increase of lesion volume and functional deficits is not completely linear, but with increasing injury severity, once a threshold proportion of tract is lost, loss of function is seen (106, 153). The increased lesion volume found in our middle-aged rats would impact a greater number of tracts in the spinal cord, likely surpassing that threshold and contributing to the delayed functional recovery we observed.

No general motor function difference was observed by age group in naïve rats. Interestingly, however, naïve middle-aged rats show altered stepping patterns compared to young adult rats, with wider toe spread and longer stride length, indicative of an increasing base of support with age. After contusion SCI, both general and fine motor function performance was more prominently affected in middle-aged rats than young adult at all time points examined out to 30 dpi. Middle-aged rats compared to young adult rats showed age related delays in motor function with delayed general functional recovery, as demonstrated by lower scores on the BBB test, and worsened ability to complete the footprint task. These results agree with previous work in mouse and male and female rat models of aged SCI (71, 77, 182). These previous studies demonstrate that 18 month old female rats showed elevated base of support during walk both before and after injury (77), similar to our studies, while 12 month old male rats consistently demonstrate impaired BBB function by 7 days post-injury (71). In contrast, 14 month old male mice did not demonstrate functional impairment before injury, although this may be due to the use of only the locomotor score, similar to the BBB; however, a significant reduction in locomotor score was noted after injury (182), as in our study.

Interestingly, footprint analysis in middle-aged rats after SCI are opposite of what was seen in naïve, showing decreased stride lengths and narrower toe spread at 30 dpi versus increased stride length and wider toe spread in naïve. Previous work in mouse models of SCI have shown that both stride length and toe spread decrease significantly after injury (9, 13, 81), and have suggested that the

decrease may be indicative of sensory motor function deficits in the hindlimbs due to injury. Thus, our findings of impaired recovery and altered stepping pattern in footprint analysis may be associated with an age related decrease in precise motor control in the hindlimbs.

CONCLUSION

Overall, the results of our study suggest that increasing age leads to a pro-inflammatory environment, with alterations to microglial activation, NOX2 enzyme component expression, and an increase in oxidative stress in the cellular environment that may contribute to worsened outcomes after SCI. The initiating factor in these effects and the cause/effect relationship between each remains unclear and is an important consideration for the field moving forward. It is clear that there are intimate relationships between inflammation, oxidative damage and NOX2, and the current work contributes to understanding that all three play a role in motor function and post-injury recovery. However, more work is needed to understand which, if not all 3, is the essential component. We currently hypothesize that age related changes to ROS production caused by increased microglial activation and NOX2 expression are associated with an exaggerated chronic inflammatory response to injury.

With the rise in SCI in the aging population, the results of this study suggest that increased inflammatory response may be an important factor to be considered in therapeutic interventions after injury, and that age should be considered when developing a therapeutic treatment plan. Further, previous work

has demonstrated that inhibition of the NOX enzyme can decrease oxidative stress and inflammation. Due to the association of NOX2 with both ROS production and microglial activity, our findings suggest a potential avenue for NOX2 inhibitory treatment options to quell the exaggerated inflammatory response, and should be a topic of interest for future research.

CHAPTER 3: Aging alters the glucose uptake pathway in the naïve and injured rodent spinal cord

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ABSTRACT

Aging results in increased activation of inflammation-related glial cells and decreased neuronal viability following spinal cord injury (SCI). Metabolism and transport of glucose is also decreased with age, although the influence of age on glucose transporter (GLUT) expression or glucose uptake in SCI is currently unknown. We performed [^{18}F]Fluorodeoxyglucose (^{18}FDG) PET imaging of young (3 month) and middle-aged (12 month) rats and found decreased glucose uptake in middle-aged rats compared to young rats at baseline, followed by increased uptake 14 days post contusion SCI. *qRT-PCR* of neuron-specific GLUT3 and non-specific GLUT4 (present on glial cells) reveals an inverse relationship between GLUT3/4 gene expression and glucose uptake patterns. Protein expression reveals increased GLUT3 in 3 month rats only, consistent with age related decreases in glucose uptake, and increased GLUT4 in 12 month rats only, consistent with age related increases in inflammatory activity and glucose uptake. Inconsistencies between gene and protein suggest an influence of age-related impaired translation and protein degradation. Overall, our findings show that age alters glucose uptake and GLUT3/4 expression profiles before and after SCI, and may suggest a therapeutic avenue in addressing glucose uptake in the aging population.

Keywords: Spinal cord injury, Aging, Glucose metabolism, Inflammation, FDG-PET Imaging

INTRODUCTION²

The average age of spinal cord injury (SCI) has been steadily increasing since the 1970's, with a current average age of onset of 42 (83, 135). Though SCI has largely been studied in the young adult population, age-related differences in response to SCI are important to consider, and recent work has begun to explore these differences (77, 133, 182, 183). SCI results in permanent sensory, motor, and autonomic deficits (50). Cellular changes include acute neuron, astrocyte and oligodendrocyte reduction of cell viability, followed by chronic white matter and axon loss, glial scar formation, and inflammation (147).

Work in our lab and work from other groups has demonstrated that aging increases glial cell activity and promotes an exaggerated response to SCI (121, 131, 133, 174, 182, 183). Aged spinal cord tissue shows increased basal inflammatory activity and oxidative stress (133, 142, 183), resulting in reduced neuronal viability and contributing to worsened outcome following SCI (70, 71, 77, 156). Further, aging results in changes to hormonal and cellular function which has been associated with increased co-morbidities, lengthened recovery time, reduced functional recovery, and prolonged chronic inflammation after SCI (66).

Glucose is the major energy source for the central nervous system (CNS) (113, 115) and is primarily consumed by neurons and glial cells (170), thus glucose uptake and transport is necessary for homeostatic maintenance. Aging

² Abbreviations: Spinal Cord Injury (SCI), Days Post Injury (DPI), Glucose Transporter (GLUT), [¹⁸F]Fluorodeoxyglucose (¹⁸F-FDG), positron emission tomography (PET), Region of Interest (ROI)

results in impairments of glucose metabolism and transport in brain and fatty tissue (45, 48, 57, 137). In addition, increased inflammation after SCI is correlated with hyperglycolysis and systemic hyperglycemia (30, 78, 87, 140).

Glucose is transported into cells by glucose transporters (GLUTs), a group of membrane proteins that facilitate the transport of glucose across the cell membranes of neurons, endothelial and glial cells (49, 113, 149). There are multiple isoforms of GLUTs specific to different cell types; most notably GLUT3 (abundant in neurons) (87, 170) and GLUT4 (insulin-sensitive and present on neurons, microglia, endothelial cells and astrocytes) (25, 54, 129, 170). Other isoforms include GLUT1 (predominant in the blood-brain-barrier, present on endothelial cells and astrocytes), GLUT2 (present on hypothalamic and hippocampal neurons), GLUT5 (fructose transporter present in microglia), and GLUT8 (insulin-sensitive and present on neurons) (87). Both age and injury lead to alterations in GLUT functionality in the brain and skeletal muscle, which can contribute to impaired glucose uptake and metabolism (45, 48, 57, 72).

Previous work in our lab has used positron emission tomography (PET)-based measurements of glucose uptake to demonstrate the post-injury glucose uptake pattern in the spinal cord after injury (175). We found that 3 month old rats showed acute decreased glucose uptake at 6 hours post-injury which returned to baseline levels by 14 days post-injury (dpi). This glucose pattern reflected the consumption of glucose by neurons and glial cells – the acute depression of glucose uptake may be associated with post-injury neuron loss in the lesion site (52, 155), and the return to baseline by 1 and out to 14 dpi coincided with

increased inflammatory glial activity during both the primary and secondary phases of injury (103), which would contribute to increased glucose consumption.

The purpose of this study was to determine how age and associated cellular changes alter the glucose uptake profile. Our first aim was to expand our knowledge of the primary and secondary post-injury glucose uptake pattern, specifically the relationship between glucose uptake and glucose transporter expression (specifically neuron-specific GLUT3 and glial cell present GLUT4). Our second aim was to investigate how aging impacts the post-injury glucose uptake pattern and cell specific glucose transport.

METHODS

Animals

Male Sprague Dawley rats were given free access to food and water and a 12h light/12h dark cycle. Young adult rats (3 months old: 300-500g; Harlan, Frederick, MD: n = 30) were housed for 1 week prior to undergoing surgery or assessment for naïve studies. Middle-aged rats (12 months old, retired breeders: 400-600g; Harlan, Frederick, MD: n = 24) were acquired at 10 months of age, and gentled twice a week until they had matured to 12 months of age, at which point they underwent surgery or assessment for naïve studies. All rats were divided amongst tissue analysis groups for immunohistochemical or biochemical analysis as needed by time-point. Division of animals by experimental group is outlined in Table 5. All animal procedures were approved by the Uniformed Services University IACUC and complied fully with the principles set forth in the “Guide for the Care and Use of Laboratory Animals” prepared by the Committee on Care

and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (DHEW pub. No. (NIH) 85-23, 2985); this manuscript has been written in accordance with the ARRIVE (Animal Research: Reporting In Vivo Experiments) guidelines (90).

Experiment Group	3 Month Rats			12 Month Rats		
	Naïve	Sham**	SCI	Naïve	Sham**	SCI
PET Imaging (Naïve/1/14 dpi)*	12	6	6	8	4	4
qRT-PCR/Western Blot (Naïve)***	4	NA	NA	4	NA	NA
qRT-PCR/Western Blot (1 dpi)	NA	NA	4	NA	NA	4
qRT-PCR/Western Blot (14 dpi)	NA	4	4	NA	4	4
Total n	10	10	10	8	8	8

Table 5. Number of rats per group for all experiments in glucose study. Rats were divided by age (3 months old or 12 months old) and injury type (naïve, sham or SCI). *All rats that underwent PET imaging were included in baseline quantification analysis. **Sham rats were processed for 14 dpi biochemistry. ***For biochemistry experiments, the 1 cm section of spinal cord encompassing the lesion site at T9 was divided – 5mm caudal of the injury epicenter for qRT-PCR, and 5 mm rostral of the injury epicenter for Western Blot.

Contusion Spinal Cord Injury

Contusion SCI was performed in rats (n=10 (3 month) and 8 (12 month)) as described previously (34). Briefly, rats were anesthetized with isoflurane (4% induction, 2.5% maintenance in 50% O₂), and a laminectomy was performed at vertebral level T9 to expose the spinal cord. Moderate injury (150 kDynes force, 1500 μ displacement, 127 mm/s velocity, 1 s dwell time) was induced with the Infinite Horizons Impactor (Precision Systems Incorporated, Natick, MA). Sham animals (n = 10 (3 month) and 8 (12 month)) received laminectomy but no

impact. Naïve animals (n = 10 (3 month) and 8 (12 month)) received no surgery or anesthesia. Post injury care included dual housing on diamond soft bedding (Harlan Laboratories, Frederick, MD), twice daily bladder expressions until spontaneous micturition returned and analgesic supplementation to drinking water for 48 hours following injury (Children's Tylenol; acetaminophen, 200 mg/kg).

microPET/CT Imaging and Analysis

Prior to injury and at 1 and 14 dpi, PET imaging was performed to assess FDG uptake, as previously described (175). Animals were anesthetized with isoflurane for tail vein injection (1.5 – 2 mCi FDG) and during the 70-minute FDG uptake period. After uptake, a static 30-minute PET scan was acquired in list mode using a Siemens Inveon Multimodality scanner (Siemens Medical Solutions, Erlangen, Germany). Immediately prior to the PET scan, rats underwent a micro-computed tomography (CT) scan for anatomical localization and attenuation correction. The field of view was centered at the injury site, the T9 vertebrae. The following parameters were used for PET reconstruction: reconstruction algorithm - OSEM3D/MAP (OSEM3D iterations – 2; MAP iterations – 18); corrections - attenuation and scatter; image size – 256 x 256; requested resolution (smoothing) - 0.5 mm. The intrinsic resolution of the scanner at the center of the field of view was 1.4 mm.

Images were analyzed using Siemens Inveon Research Workplace (IRW) software, version 4.2. Alignment of PET scan with CT ensured that the placement of the ROI was within the spinal column. Additionally, a manual rigid-body registration was used for select subjects in which the scanner transformation

matrix produced visibly misaligned images. This resulted in some datasets being co-registered based on the scanner transformation matrix and some by manual registration, which may be attributable as a source of some variation across subjects. Quantification of tracer uptake was obtained in regions of interest (ROIs) standardized in size to the injury site of the spinal cord as described previously (175).

Comparative RT-PCR

A 5 mm section of the spinal cord, centered at the lesion epicenter, encompassing the caudal half of the lesion site, was dissected from naïve rats or at 1 or 14 dpi and immediately frozen on dry ice. Briefly, RNA was extracted from each cord sample individually using TRIzol® reagent (Invitrogen, Carlsbad, CA) and chloroform, then purified with the Qiagen RNeasy kit (#74104, Qiagen, Hilden, Germany). RNA concentration was measured using the Nanodrop 2000 (Thermo Scientific, Wilmington, DE). Complementary DNA (cDNA) conversion was performed from 1 µg of RNA using a Veriti thermal cycler (Applied Biosciences, Waltham, MA) and a high capacity cDNA conversion kit (Applied Biosciences, Waltham, MA) using the manufacturer's protocol. Quantitative Real Time PCR (*q*RT-PCR) was performed using the StepOnePlus Real-Time PCR System (Applied Biosciences) according to manufacturer's instructions. To determine changes in gene expression, primers were designed using the Primer-Blast tool, then obtained from Integrated DNA Technologies (IDT, Coralville, IA). Primers were designed for glucose transporters GLUT3 (sense – 5'-CCATCTCTGGTGTTCGCTGT- 3', anti-sense – 5'-

GTCTTCCAACCGCTCTTCCA- 3'), GLUT4 (sense – 5'-
CAGGCCGGGACACTATACC- 3', anti-sense – 5'-
AATCACTTTCTGTGGGGCGT- 3'), and internal control (GAPDH, sense – 5'-
GCTGGTCATCAACGGGAAA-3', anti-sense – 5'-
ACGCCAGTAGACTCCACGACA-3'). Reported gene expression values were
determined using the $\Delta\Delta$ CT method: raw CT value of each sample normalized
first to CT value of the internal control (GAPDH) and then to control group
(either age matched naïve or 3 month injured, dependent on analysis performed).

Western Blot

A 5 mm section of the spinal cord was dissected from naïve animals or at
1 or 14 dpi, encompassing the rostral half of the lesion site, and immediately
frozen on dry ice. Protein from tissue samples was isolated in RIPA Buffer
(Pierce, Rockford, IL) containing protease inhibitor (1%, Halt Protease Inhibitor
Single-Use Cocktail, Thermo Scientific). 25 µg of sample protein was run in a
Mini-PROTEAN® TGX™ Precast Gel (Bio-Rad, Hercules, CA) and transferred
to a Trans-Blot® Turbo™ (Bio-Rad) nitrocellulose membrane. Antibodies raised
against GLUT3 (20µl/ml, Abcam, RRID AB_732609) and GLUT4 (10µl/ml,
Abcam, RRID AB_305554) were probed overnight at 4°C. Previous work has
verified predicted 60kD band for quantification of GLUT3 (96). Multiple bands
appear when probed with GLUT4; expected band at 54kD was quantified as
shown in previous work (107), while established extra bands near 45kD are of
unknown identity. Immune complexes were detected with appropriate secondary
antibodies and chemiluminescence reagents (Pierce). GAPDH (0.5µl/ml;

Millipore, RRID AB_2107445) was used as a control for gel loading and protein transfer. NIH Image J was used to assess pixel density of resultant blots for quantitation.

STATISTICS

Sample sizes were calculated based on our previous work(154, 175). All assays were carried out by investigators blinded to subject group. Quantitative data are presented as mean +/- standard error of the mean (SEM). Post injury PET imaging was analyzed using a 2-way ANOVA with Tukey's multiple comparison's post hoc test. Baseline PET imaging, western blots and q-RT PCR were analyzed using the student t-test. All statistical tests were performed using the GraphPad Prism Program, Version 6.03 for Windows (GraphPad Software, San Diego, CA). A p value < 0.05 was considered statistically significant.

RESULTS

Baseline FDG uptake is decreased in the middle-aged rat spinal cord

To determine glucose uptake in naïve 3 month and 12 month rats, ^{18}F FDG-PET imaging was performed and scans were analyzed using region of interest (ROI – T9 spinal cord injury site, see Figure 8A for representative images) analysis with reference region (cerebellum) normalization. 12 month rats have a significant decrease in glucose uptake compared to 3 month rats at baseline ($t(5.269)$, $p<0.0001$; Figure 1B). To verify that these differences in glucose uptake were not region specific, we analyzed an ROI in the cervical spine using the same

shape and size as at T9. Glucose uptake was significantly decreased in the 12 month rats compared to 3 month animals as well ($t(3.304)$, $p=0.0064$; Figure 8B).

Middle-aged rats show altered GLUT3/4 gene and protein expression

To determine if age altered the interaction of glucose transport and glucose uptake, we examined gene and protein expression of neuron-specific GLUT3 and non-specific GLUT4 in the naïve spinal cords of our 3 month and 12 month rats. Interestingly, gene expression of both GLUT3 and GLUT4 was significantly increased in the 12 month rats compared to the 3 month rats ($t(2.985)$, $p=0.0405$ and $t(7.05)$, $p=0.0021$, respectively; Figure 8C), showing an inverse relationship to glucose uptake. Protein levels of GLUT3 and GLUT4 were not different between age groups (Figure 8D).

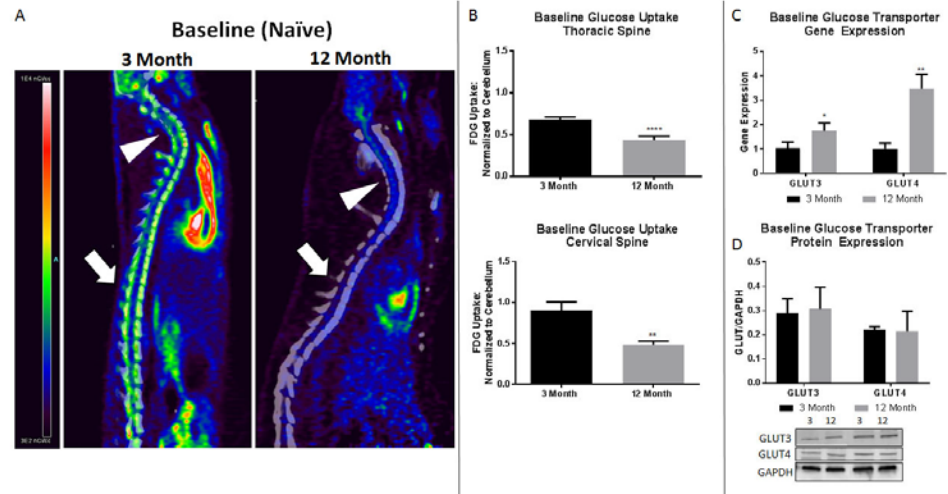


Figure 8. Middle-aged rats show altered glucose uptake pattern and GLUT3/4 gene expression compared to young adult rats.

A) Representative PET images of 3 and 12 month baseline (naïve) spinal cord. Arrowheads point to cervical spine region, block arrows points to thoracic injury site. Heatmap provided to left of images – black/blue represents lowest level of uptake, red/white represents highest level of uptake. B) Naïve 12 month rats show significant decreases in glucose uptake compared to naïve 3 month rats in both the thoracic and cervical regions of the spine. No difference in glucose uptake is found in cervical spine after injury, indicating post injury alterations in glucose uptake are specific to the injury site. C) Comparative RT-PCR between 3 month and 12 month rats reveal significantly increased gene expression in 12 month rats in both GLUT3 and GLUT4. D) No significant difference in GLUT3 or GLUT4 protein expression is found by age group. N=12/group 3 month, 8/group 12 month (PET imaging), 4/group (gene and protein expression); * $p < 0.05$, ** $p < 0.005$, **** $p < 0.0001$.

FDG uptake is increased in middle-aged rats at 14 dpi

To determine if glucose uptake was altered by age after SCI, FDG-PET was performed at 1 and 14 dpi in 3 and 12 month rats (Figure 9A – representative images of FDG-PET uptake at 1 and 14 dpi). Analysis with a two-way ANOVA revealed a significant difference between time post injury ($F=2,50=6.01$, $p=0.0046$) and age at injury ($F(4,50)=5.69$, $p=0.0007$), and there was a significant interaction effect ($F(8,50)=5.736$, $p<0.0001$), indicating that differences between age groups were dependent on time post injury. Post-hoc analysis revealed that at 14 dpi, 12 month injured rats showed significantly increased glucose uptake in comparison to 3 month injured rats ($**p=0.0038$; Figure 9B). No difference in uptake was observed at 1 dpi by age. Sham-injury in the 12 month old rats resulted in a significant increase in glucose uptake at 14 dpi compared to both 3 month sham and injured rats ($\#p=0.0174$ and $*p=0.0113$, respectively; Figure 9B); at 1 dpi, there was no significant difference between any age or injury group. Regardless, 12 month injured rats remained the highest glucose uptake group of all age and injury groups, showing significantly increased uptake compared to both the 12 month and 3 month sham rats ($\$p=0.0172$ and $\#p=0.0041$, respectively; Figure 9B).

We examined the same size ROI in the cervical spine (C5 vertebrae) to verify that these findings were region specific and are a result of injury – two-way ANOVA analysis revealed no significant difference by age or injury in sham or injured groups (Figure 9C).

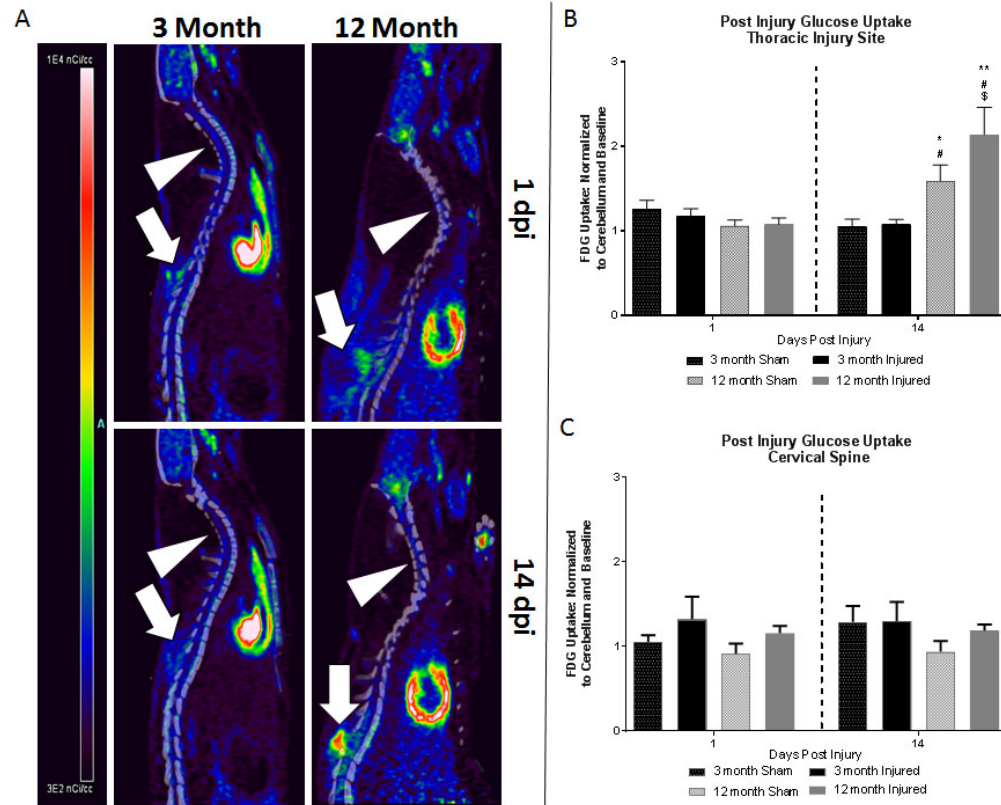


Figure 9. Post injury glucose uptake differs by age at 14 dpi.

A) Representative images of both 3 and 12 month injured rats at 1 and 14 dpi. Arrowheads points to cervical spine region, block arrows points to thoracic injury site. Heatmap provided to left of images – black/blue represent lowest level of uptake, red/white represents highest level of uptake. B) At 14 dpi, 12 month injured rats showed significantly higher glucose uptake than all other age or injury groups. 12 month injured rats showed significantly increased uptake compared to 3 month injured (**), 3 month sham (#) and 12 month sham rats (\$). 12 month sham rats also show significant increases in glucose uptake compared to 3 month injured (*) and sham (#) groups. C) At the cervical spine, no significant difference is found any group by age or injury. N=6/group 3 month, 4/group 12 month; *p<0.05, **p<0.005.

Gene and protein expression of GLUT3/4 is altered after SCI

Comparative *qRT-PCR* and Western Blot were performed to determine gene and protein expression levels of GLUT3 and GLUT4 post-injury. To characterize the post-injury gene and protein expression pattern of GLUT3/4 and determine if age alters that pattern, we compared all post-injury samples to the age matched naïve group as a control (Figure 10). GLUT3 gene expression was significantly decreased at 1 dpi, in both 3 month ($t(6.469)$, $p=0.0029$; Figure 10A) and 12 month rats ($t(8.58)$, $p=0.0010$; Figure 10B). Interestingly, GLUT3 was significantly increased at 14 dpi in 3 month ($t(3.772)$, $p=0.0196$; Figure 10A), however 12 month rats showed no significant difference compared to naïve. At both 1 and 14 dpi GLUT4 gene expression was significantly increased in both 3 ($t(4.958)$, $p=0.0158$ and $t(3.97)$, $p=0.0165$, respectively; Figure 10A) and 12 month rats ($t(9.922)$, $p=0.0006$ and $t(3.404)$, $p=0.0272$; Figure 10B).

Protein levels did not follow the same pattern as gene expression within age groups post injury. In 3 month rats, GLUT3 protein levels at both 1 and 14 dpi were significantly increased compared to naïves ($t(3.44)$, $p=0.0014$ and $t(3.907)$, $p=0.0298$; Figure 10C), and GLUT4 was significantly increased at 1 dpi ($t(18.69)$, $p=0.0001$; Figure 10C). 12 month rats also had significant increases in GLUT4 at both 1 and 14 dpi ($t(11.79)$, $p=0.0003$ and $t(2.684)$, $p=0.0500$, respectively; Figure 10D), however, they did not show any significant difference in GLUT3 protein expression at any time point post injury.

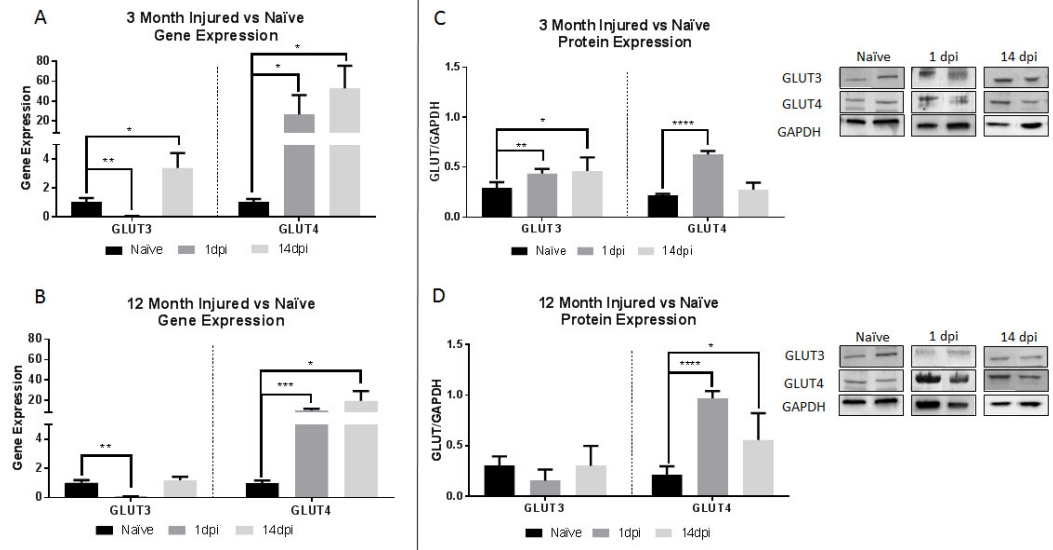


Figure 10. Comparative qRT-PCR of young adult and middle-aged rats show similar post injury GLUT3/4 gene expression pattern compared to age matched controls; protein expression is increased in both age groups.

A) 3 month rats show significantly decreased GLUT3 gene expression at 1 dpi; GLUT3 gene expression is significantly increased at 14dpi. At both time points GLUT4 is significantly increased. B) 12 month rats also show significantly decreased GLUT3 gene expression at 1 dpi and significantly increased GLUT4 at 1 and 14 dpi. C) In 3 month rats, GLUT3 protein expression is significantly increased at all time points post injury. GLUT4 protein expression is increased at 14 dpi. D) In 12 month rats, GLUT4 protein expression is significantly increased at both time points post-injury. No significant difference is seen in GLUT3 protein expression at any time point post injury. Representative images of Western Blot bands at each time point are listed by age group. N=4/group; * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$, **** $p < 0.0001$.

GLUT3/4 gene and protein expression differs by age

To determine if age effected the overall amount of gene and protein expression for GLUT3/4, analysis was performed comparing between age groups at each time post injury, with all data normalized to the 3 month group (as control). At 1 dpi, GLUT3 gene expression remained elevated in 12 month rats compared to 3 month rats ($t(6.425)$, $p = 0.0076$; Figure 11A), but by 14 dpi was significantly decreased ($t(2.909)$, $p = 0.0437$; Figure 11A). Though GLUT4 gene expression was also significantly increased in naïve 12 month rats ($t(7.05)$,

$p=0.0021$; Figure 8C), there was no significant difference between age groups at either 1 or 14 dpi.

Protein expression did not follow the same pattern as gene expression. Neither GLUT3 nor GLUT4 were significantly different by age in naïve groups (Figure 8D). At 1 dpi, however GLUT3 was significantly decreased in 12 month rats compared to 3 month rats (Figure 11B; $t(4.105)$, $p=0.0148$), and GLUT4 was significantly increased in 12 month rats ($t(7.333)$, $p=0.0018$; Figure 11B). By 14 dpi, no differences in protein levels were found between age groups (Figure 11B).

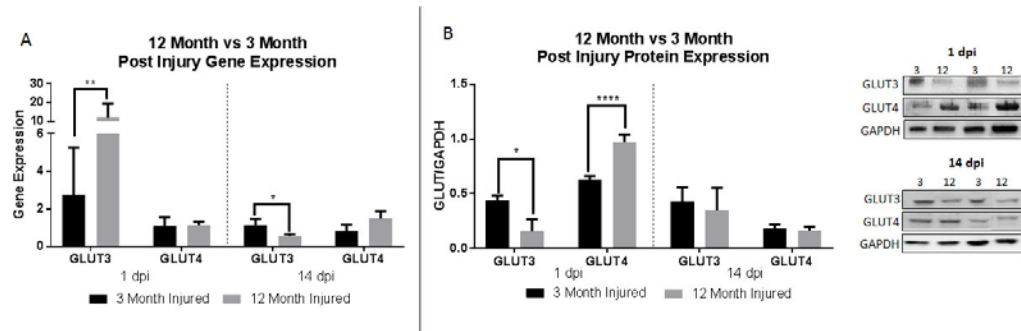


Figure 11. Middle-aged rats show altered gene and protein expression of GLUT3/4 compared to young adult rats.

A) Comparative qRT-PCR shows that GLUT3 gene expression significantly increases at 1 dpi and significantly decreases at 14 dpi in 12 month rats compared to 3 month rat. B) In protein expression, 12 month rats show significantly decreased GLUT3 and significantly increased GLUT4 at 1dpi. N=4/group, * $p<0.05$, ** $p<0.005$, **** $p<0.0001$.

Inflammatory marker gene expression is increased in 14 dpi injured and sham rats compared to naïve controls; middle-aged injured rats show greatest expression of inflammatory markers

Injured rats show increased inflammatory gene expression compared to sham and naïve rats (11, 24, 103), and age-related increases to the inflammatory response have been shown both before and after injury (99, 133, 160). To determine if the inflammatory response may be related to the increased glucose uptake observed at 14 dpi in both sham and injured 12 month old rats (Figure 12), we examined gene expression of two known pro-inflammatory markers, CD86 and iNOS. First, 14 dpi 3 and 12 month injured and sham rat tissue were compared to age matched naïve rats. Gene expression of CD86 was significantly increased in both 3 month ($t(5.264)$, $p=0.0062$; Figure 12A) and 12 month sham rats ($t(3.038)$, $p=0.0385$; Figure 12A) compared to age matched naïves; no difference was observed in iNOS gene expression between sham and naïve in either group. Gene expression of CD86 and iNOS was significantly increased in both 3 month ($t(4.662)$, $p=0.0096$ and $t(5.428)$, $p=0.0056$ respectively; Figure 12A) and 12 month ($t(18.12)$, $p<0.0001$ and $t(4.208)$, $p=0.0136$, respectively; Figure 12B) injured rats compared to age matched naïves. Injured rats also showed significantly increased CD86 and iNOS compared to age matched sham groups in both 3 month ($t(4.161)$, $p=0.0141$ and $t(18.24)$, $p<0.0001$, respectively; Figure 12A) and 12 month ($t(14.96)$, $p=0.0001$ and $t(5.318)$, $p=0.0060$, respectively; Figure 12B).

A second analysis was performed to determine the influence of age on inflammatory gene expression within injury groups. In 12 month injured rats, both CD86 and iNOS were significantly increased compared to 3 month rats ($t(4.172)$,

$p=0.0140$ and $t(4.835)$, $p=0.0084$, respectively; Figure 12C). In our 12 month sham rats, we found a significant increase in CD86 gene expression compared to 3 month rats ($t(4.233)$, $p=0.0133$; Figure 12D) but no significant difference was found in iNOS.

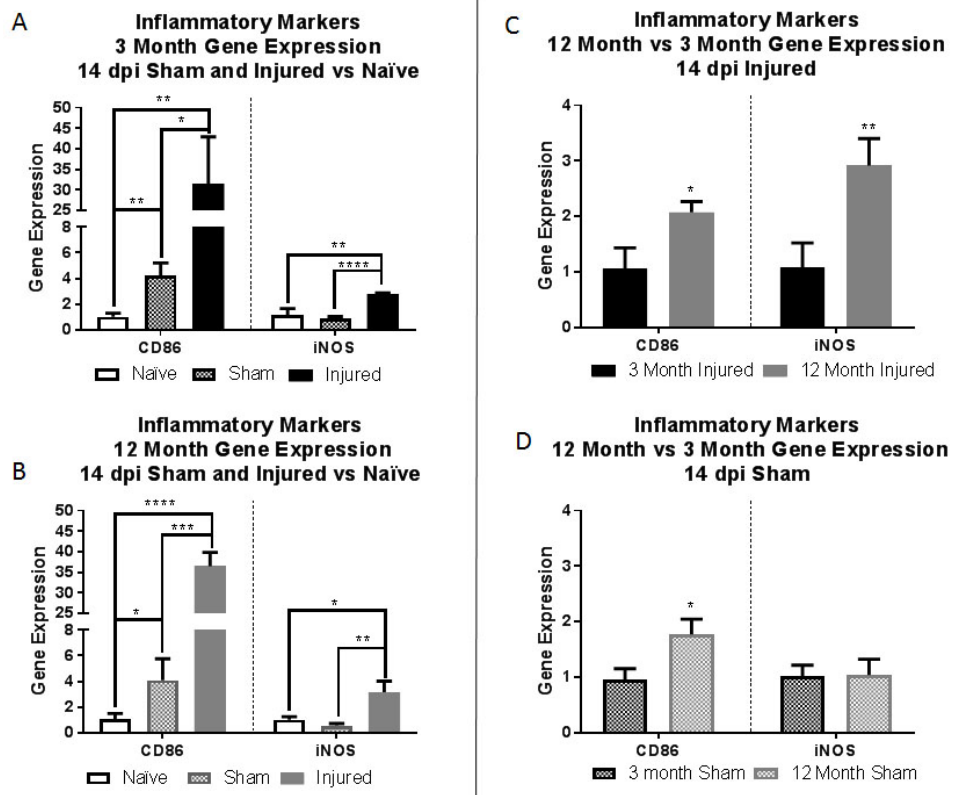


Figure 12. Inflammatory markers CD86 and iNOS are increased in 14 dpi injured and sham rats compared to naïve controls; middle-aged injured rats show increased expression of both CD86 and iNOS compared to young adult rats. A) At 14 dpi, 3 month injured rats showed a significant increase in CD86 and iNOS gene expression when compared to age matched naïves and 14 dpi shams. B) At 14 dpi, 12 month injured rats showed a significant increase in CD86 and iNOS gene expression when compared to age matched naïves and 14 dpi shams. C) When compared to 3 month rats, 12 month injured rats show significantly increased gene expression of both CD86 and iNOS. D) 12 month sham rats showed significantly higher CD86 gene expression than 3 month sham rats; No significant difference was seen in iNOS by age group. $N=4/\text{group}$; * $p<0.05$, ** $p<0.005$, *** $p<0.0005$, **** $p<0.0001$.

GLUT4 gene and protein expression is elevated in injured rats versus sham

Sham 12 month rats demonstrated increased glucose uptake at 14 dpi compared to 3 month sham rats, but showed decreased glucose uptake and inflammatory marker gene expression compared to 12 month injured rats. These findings suggest there is an age-related inflammatory response of surgery alone on glucose uptake, but not as great as age and injury. Differences were also found in GLUT expression between injured and naïve age matched rats. An additional analysis was performed to determine if surgery alone influenced GLUT expression in sham rats compared to age matched injured rats at 14 dpi (injured rats normalized to sham as control). 3 and 12 month sham 14 dpi tissue was analyzed for gene and protein expression using comparative *qRT-PCR* and Western Blot (Figure 13). Our findings show a similar alteration of GLUT expression between sham and injured as we found between naïve and injured, validating that the surgery alone did not majorly impact GLUT expression. GLUT4 gene and protein expression was significantly increased in injured rats compared to sham rats in both 3 month ($t(19.51)$, $p<0.0001$ and $t(5.232)$, $p=0.0258$, respectively; Figure 13A/B) and 12 month ($t(9.517)$, $p=0.0007$ and $t(2.963)$, $p=0.0252$, respectively; Figure 13A/B); GLUT3 gene expression was significantly increased in 3 month injured rats ($t(3.852)$, $p=0.0183$; Figure 13A), but no difference was found in protein expression (Figure 13B).

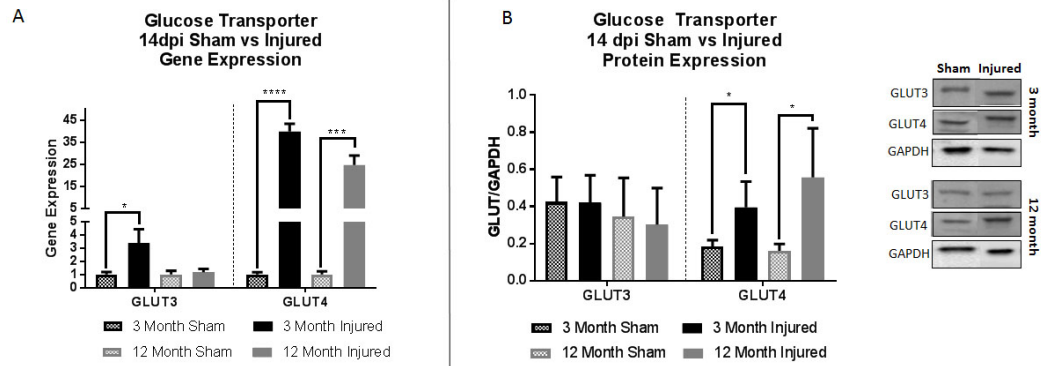


Figure 13. Middle-aged and young adult injured rats show increased GLUT4 gene and protein expression compared to sham at 14 dpi.

A) Glut4 is significantly increased in both 3 and 12 month rats compared to age matched sham group. No significant difference was found between age groups in GLUT3 gene expression. B) GLUT4 protein expression is significantly increased in both 3 and 12 month rats compared to age matched sham group. No significant difference was between age groups in GLUT3 protein expression. N=4/group; ** $p < 0.005$, *** $p < 0.0005$, **** $p < 0.0001$.

DISCUSSION

The results of this study establish the glucose uptake pathway pattern after SCI (Figure 14A) and the influence of age on this pattern (Figure 14B). Glucose uptake is depressed at baseline in 12 month rats compared to 3 month rats, followed by a notable increase during the secondary injury period post SCI (Baseline and 14 dpi glucose uptake; Figure 14B). An inverse relationship between baseline glucose uptake and gene expression suggests GLUT gene expression changes in response to glucose uptake and is influenced by age; increases in 12 month GLUT3 gene expression may be associated with age related decreases to neuronal viability, resulting in dysregulated glucose transport (124), while increased GLUT4 gene expression may indicate an attempt of the cellular environment to maintain balance in response to age-related depressed glucose uptake and increased inflammatory activity. Further, these gene expression

findings may reflect a response to changes in glucose uptake, with increasing or decreasing expression levels to encourage a return to cellular homeostasis through translation of proteins. Interestingly, GLUT3/4 protein levels between age groups do not differ at baseline, suggesting age-related impairments to gene translation and degradation may lead to inconsistent gene translation (171, 173).

A) Post Injury Glucose Pattern: 3 month Injured vs Baseline			
		1 dpi	14 dpi
Glucose Uptake		—	—
Gene Expression	GLUT3	↓	↑
	GLUT4	↑	↑
Protein Expression	GLUT3	↑	↑
	GLUT4	↑	—

B) Post Injury Glucose Pattern: 12 Month vs 3 Month				
		Baseline	1 dpi	14 dpi
Glucose Uptake		↓	—	↑
Gene Expression	GLUT3	↑	—	↓
	GLUT4	↑	—	—
Protein Expression	GLUT3	—	↓	—
	GLUT4	—	↑	—

Figure 14. Post Injury Glucose Pattern is altered by age.

A) Post injury glucose pattern in 3 month injured rats compared to 3 month naïve rats (control). B) Changes in post injury glucose pattern in 12 month rats compared to 3 month rats (control). Legend: Blue dash = no significant change from control. Red arrow = significant change.

Post SCI, the 3 month rat shows alterations to glucose transporter gene expression and protein levels. Acute depression of neuron specific GLUT3 gene

expression followed by increased expression by 14 dpi suggests acute neuronal loss leads to an attempt by the central nervous system to increase glucose transport at later time points to encourage glucose uptake through translation of GLUT proteins. GLUT3 gene expression, GLUT3 protein levels in 3 month rats remained elevated over baseline throughout both primary and secondary injury. Similar to 3 month rats, 12 month rats also showed an acute depression in GLUT3 gene expression at 1 dpi compared to naïve. However, at 14 dpi, 12 month GLUT3 gene expression did not significantly increase compared to naïve, as it did in 3 month rats. Further, 12 month GLUT3 protein levels do not increase over baseline, suggesting an age-related loss of neuronal viability leads to altered GLUT expression after SCI. Comparatively, GLUT3/4 gene expression and protein levels in 12 month rats differ from 3 months rats. Though both age groups showed depressed GLUT3 gene expression 1 dpi, 12 month rats maintained higher gene expression compared to 3 month rats. Conversely, though both age groups show a return to naïve expression or higher at 14 dpi, 12 month rats showed decreased GLUT3 gene expression compared to 3 month rats.

Increased nonspecific GLUT4 at both 1 and 14 dpi is consistent with the presence of GLUT4 on inflammatory cells (129), and agrees with increased inflammatory response post-injury. GLUT4 protein levels in 3 month rats were elevated at 1 dpi only, matching the post injury inflammatory activation pattern - an acute spike in macrophage/microglia infiltration followed by a second spike by 7 dpi, and chronic low level inflammation out to at least 30 dpi (103). Interestingly, GLUT4 protein levels in 12 month rats were elevated at both 1 and

14 dpi, matching age-related increases in chronic inflammation after SCI (133). Changes in protein levels between age groups were only found at 1 dpi, when GLUT3 levels were decreased and GLUT4 levels increased in 12 month rats compared to 3 month rats, consistent with both age-related loss of neuronal viability and increased inflammatory activity.

It is likely that inconsistencies found between GLUT3/4 gene and protein expression are due to age and injury related increases in mRNA turnover and dysregulation of protein translation and degradation (17, 171). It should be noted that half-life of genes varies by type, and degradation of gene mRNA may be responsible for differences in expression between genes and proteins (41, 173). However, the current study did not examine translocation of GLUTs to the membrane or functional activation of the transporters. Age and/or injury related impairments to transportation and translocation of GLUTs could lead to altered localization of the transporter and contribute to increased or decreased GLUT3/4 without any significant effect on uptake is seen (like at 1 dpi). To fully determine the impact of gene and protein expression on functional activity and transport of glucose into cells, this should be studied in future work.

The thoracic contusion injury site is superficial and causes mostly white matter damage with less impact on the neuron heavy grey matter regions of the spinal cord (52, 53). GLUT3 has been shown to be most abundant on neurons (170), and GLUT4, though present on a variety of cells including neurons, is present on inflammatory glial cells (microglia and astrocytes) that migrate to the injury site (129). In the peripheral nervous system, GLUT4 increases in response

to injury, where GLUT3 does not (65). Further, presence of GLUT4 mRNA has been shown to be region specific in both the brain and the spinal cord, with a strong presence in motor neurons and in the reticular fields and in the ventral horn of the spinal cord (54). However, previous work has shown that GLUT4 has inconsistent immunoreactivity in these regions, supporting that post-transcriptional regulation of GLUT4 expression is dependent on physiological conditions (54).

In the naïve cervical spine, we saw similar glucose uptake as at the thoracic injury site – 12 month rats show depressed glucose uptake compared to 3 month rats. This agrees with previous work on aging and glucose, which has shown that glucose metabolism is compromised and less efficient with age (48, 57, 137). Glucose uptake pattern from outside the injury site shows no significant differences between age groups post SCI, suggesting that glucose uptake in 12 month rats (which was depressed in baseline) has increased to similar levels of uptake as 3 month rats. As glucose metabolism is less efficient with age, this small increase in uptake could be related to a reduction in the metabolism of glucose by inflammatory cells and neurons. This small increase above baseline does not occur in 3 month rats (whose uptake outside the injury site appears similar to baseline levels), potentially related to normally functioning glucose metabolism. This finding supports our hypothesis that age alters glucose uptake pattern but is also injury specific and related to the post injury inflammatory response. Both 3 and 12 month rats show more variable uptake values after injury, and 12 month rats do not show a depression like at baseline. Variability in both

age groups is likely related to the cellular environment in the spinal cord – a small influence of the inflammatory response on the entire spinal cord may account for variability in uptake outside of the injury site, though not significant. Though inflammation at the injury site is most prominent, SCI related inflammation has been shown to increase inflammation in regions distant from the injury site, including the brain (181).

In our previous work using PET imaging of glucose uptake after SCI (175), we compared injured to sham rats, whose glucose uptake matched naïve controls, suggesting there is no influence of the surgery alone on glucose uptake. In this study we found that glucose uptake in 12 month sham rats was significantly increased at 14 dpi compared to 3 month shams. As inflammatory activity in aged rats is increased in naïve tissue and hyper-responsive to insult (133), it was important to determine if the laminectomy surgery alone caused an inflammatory response. No significant difference in gene expression of pro-inflammatory markers CD86 and iNOS was found in naïve or 14 dpi sham within each age group. However, there was a significant increase in CD86 and iNOS in 12 month rats compared to 3 month rats, most robustly in injured but also in sham rats. Whereas injured 12 month rats showed significant increases in both CD86 and iNOS compared to 3 month injured rats, 12 month sham rats only showed a significant increase in CD86 compared to 3 month sham rats. This age-related increase in one but not both of these inflammatory markers in 12 month sham rats indicates that age-related hyper-responsiveness to insult may cause a small inflammatory response to the spinal laminectomy surgery. This response may

contribute to the increased glucose uptake in 12 month old sham at 14 dpi. However, 12 month sham glucose uptake remains significantly decreased compared to 12 month injured glucose uptake, verifying that injury and age have a more profound effect on the inflammatory response and glucose uptake than age alone. Further, GLUT4 gene and protein expression is consistently increased in injured rats compared to sham regardless of age, supporting our inflammatory marker data.

CONCLUSION

We now show that alterations in glucose uptake profiles and expression in GLUT 3/4 are aligned with decreased neuronal health and increased inflammatory markers with injury and age. Inconsistent GLUT3/4 gene and protein expression both before and after injury between age groups suggests that the glucose transporter system may be dysfunctional and disconnected from glucose uptake patterns, and may play a role in impaired glucose metabolism after injury. Collectively, these alterations to glucose uptake, transport and metabolism could contribute to injury severity, chronic inflammation, and age-related impairments in functional recovery from SCI. As glucose is necessary for homeostatic function of all central nervous system cells, our findings suggest that manipulation of glucose uptake and metabolism could be a potential therapeutic avenue for aging and/or SCI. Specifically, our results in this study show that GLUT4, which is insulin-sensitive and present on both neurons and glial cells, is altered significantly with age alone and after SCI. Previous work in our lab and others

has demonstrated that therapeutic intervention with intranasal insulin increases cerebral glucose uptake, improves memory, and decreases neuroinflammation after traumatic brain injury, as well as improves cognitive function in neurodegeneration (18, 39). Thus, our current findings suggest a potential novel target (insulin) for therapeutic intervention in both aging and injury in the spinal cord.

CHAPTER 4: Discussion

The findings included in this dissertation contribute to the understanding of age-related alterations of cellular mechanisms of inflammation and glucose uptake involved in normal aging and recovery from SCI. This work characterizes a rat model of middle-age and middle-aged SCI, filling a gap in current animal models of aging and SCI. We demonstrate age-related alterations to inflammation and glucose uptake, and show a close interaction between post-injury glucose uptake and post-injury inflammatory markers. Glucose utilization and inflammation play a vital role in homeostatic maintenance of the CNS, and the two systems influence the performance of each other. Our findings that both systems are altered and associated with each other elucidate potential directions and therapeutics that will contribute to the treatment of aging and SCI.

CHARACTERIZATION OF INFLAMMATION IN A MIDDLE-AGED RAT MODEL OF SCI

The literature demonstrates a clear influence of age on inflammation and recovery from CNS injury. However, the majority of work in aging and trauma has been performed in elderly animal models (31, 59, 150, 157, 182, 183). The current average age of SCI is 42 (134), at which point age related DNA and cellular mutations have begun in the body, though they become more pronounced with more advanced age (32, 108). Suffering a SCI at middle-age has a higher cost of care and long-term effect on quality of life, as there is greater post-injury life expectancy in middle-age than elderly (134). Therefore, it is important to

differentiate cellular alterations and functional recovery to SCI at middle-age versus elderly.

Only one previous group has developed an animal model for investigation of middle-age SCI (182, 183). This model utilized middle-aged mice, and demonstrated that during the first week post SCI, there is a shift towards increased presence pro-inflammatory, M1 phenotype macrophages and decreased presence of anti-inflammatory M2 phenotype macrophages, associated with increased tissue damage and worsened functional recovery (182). These findings suggest that secondary injury processes may be influenced by imbalances in macrophage phenotype ratios caused by age (182). As this study was conducted in the first week post injury, our middle-aged rat model of SCI both supports and expands on these findings, demonstrating age-related alterations to inflammatory activity chronically after SCI. At 30 dpi, we found increased microglial activation and M1 pro-inflammatory related cytokine gene expression, NOX2 component gene expression and increased oxidative stress, associating with increased lesion volume and long-term impaired functional recovery.

Importantly, recent work using middle-aged mice has discovered the presence of a previously unreported ROS producing M2 microglia/macrophage phenotype, as well as increased NOX2 protein levels and ROS production in the spinal cord during the first week post SCI (183). Our findings at 30 dpi also demonstrate the presence of M2 microglia related inflammatory cytokines. Based on the finding of an alternative, ROS producing M2 microglial phenotype in middle-aged mice, the increased M2 inflammatory cytokine expression found in

our rats may show the chronic presence of both pro- and anti-inflammatory microglial activity, contributing to the observed age-related increases in oxidative stress. Cumulatively, the results found in middle-aged mice and our middle-aged rats demonstrate that age-related impairments to inflammatory response are not transient, and may have long-term effects on functional recovery from SCI in middle-age. Future work should investigate the presence of specific microglial phenotypes found in middle-aged rats to determine if imbalances in microglial phenotype seen in mouse models occur in rat models as well.

Though this model has contributed important data on aging and inflammation, mice do not share the same SCI pathology as humans, leaving a need in the field for an animal model that more closely mimics human SCI (91). Rats show similar pathology to humans, and our middle-aged rat model characterized here provides validating evidence to support previous work as well as expanding on the field to show that age-related alterations to inflammation after SCI are found across multiple species (91). Further, our animal model utilized middle-aged male rats, but other groups have utilized aged female rat models to examine age-related differences in SCI. Previous work in a female elderly model of SCI showed reduced functional recovery and increased lesion volume (77), but unpublished pilot studies from our lab found that middle-aged female rats showed no difference in functional recovery compared to young adult female rats. Female rats have higher levels of estrogen (which has been shown to be neuroprotective) than males (20, 151). Presence of high estrogen levels can influence recovery from SCI at different ages – elderly female rats show depleted

estrogen levels with the loss of reproductive capabilities (161), whereas middle-age female rats have been shown to retain estrogen levels (26). Estrogen has even been explored as a potential treatment in male rodent models of SCI, showing increased neuronal survival and reduced inflammatory activity (151). Therefore, female rat models of aging are not fully comparable to male models. Additionally, as occurrence of SCI is higher in males, it is more relevant to the clinical population to study SCI in males (134), making it necessary for more research to be performed in aged animal models of SCI that are both age and sex relevant.

It should be noted that our investigation of age-related inflammatory response is limited to basal inflammatory activity (naïve) and a chronic post-injury timepoint. These timepoints are relevant to normal aging, secondary injury and longitudinal outcomes, but in order to complete the characterization of the model, future work should investigate multiple post-injury timepoints, including the first 3 days post-injury, when elderly rats have shown age-related differences (77), and the first week post-injury, when middle-aged mice have demonstrated age related differences (59, 182, 183).

CHARACTERIZATION OF GLUCOSE UPTAKE PATHWAY IN A MIDDLE-AGED RAT

Our glucose study shows the use of our model for the examination of systems outside of the inflammatory response. Our model is the first to examine middle-aged alterations in glucose uptake in the rodent spinal cord both basally and longitudinally after SCI. Our exploration of neuronal and inflammatory related GLUT3/4 gene expression and protein levels show that glucose uptake and

transport are influenced by both age and injury, showing a compounded effect when injury occurs with age. Though there is an established connection between CNS glucose utilization and inflammation (87), our model is the first to associate age-related alterations in glucose uptake and GLUT transport specifically with inflammatory markers present in SCI. These findings suggest that the interaction of inflammation and glucose uptake and transport is important to consider in the functionality of both systems.

Effect of Oxidative Stress and Inflammation on Glucose Uptake Pathway

Our examination of basal pro-inflammatory activity in naïve middle-aged rats showed increased microglial activation, pro-inflammatory gene expression, NOX2 component gene expression, and DNA oxidation compared to young adult rats. Middle-aged rats also showed depression of glucose uptake with increased gene expression of GLUT3/4 (with no change in protein levels) compared to young adult rats. Glucose utilization by both neurons and inflammatory cells contribute to observed glucose uptake seen in PET imaging, and is useful for assessment of functional activity in both the brain and spinal cord (21, 110).

Normal aging has been shown to result in impairments in glucose metabolism and transport in the brain (45, 48). The observed depression of glucose uptake in our middle-aged rats may be caused by age-related impairments to the ability of neurons and inflammatory cells to transport glucose into cells through glucose transporters, including those we examined in this study, GLUT3/4 (2). The neuronal specific GLUT3 has been shown to have decreased protein levels with age, suggesting depression of glucose uptake seen in middle-

aged rats is influenced by age-related alterations to neuronal health and functionality (57). However, our results showed altered expression from these previous findings, with a significant increase in GLUT3 gene expression, and no difference in GLUT3 protein levels between age groups. The increase in GLUT3 gene expression in our middle-aged rats may be an attempt at the transcriptional level to compensate for age-related depression of glucose uptake, but the lack of a difference in protein levels indicates an age-related impairment in translation of gene to protein (33). The increase in oxidative stress found in aging may also play a role in our observed inconsistencies in GLUT translation to protein. It has been found that mRNA modified by 8-OHdG (a product of DNA oxidation) can result in impaired mRNA regulation and subsequent translation (33). Our study of basal oxidative stress markers identified increased 8-OHdG in middle-aged tissue, validating the presence of an increased oxidative environment that could impair GLUT translation. Our basal GLUT4 gene expression and protein level results mirrored GLUT3 – significantly increased gene expression with age but no difference in protein levels, indicating that impairments in protein translation may be playing a role in GLUT4 similar to GLUT3. GLUT4 deficient transgenic mice have been shown to have reduced brain glucose uptake, insulin resistance, and impaired neuronal functionality (143). GLUT4 is present on neurons, though not as abundantly as GLUT3, as well as on microglia (129). However, we have not determined the relative expression of GLUT4 on microglia and neurons, and future work should explore this expression in order to determine if neuronal GLUT4 is specifically effected by aging.

Influence of Inflammation on Insulin-Sensitive GLUT4 Functionality

Our basal middle-aged inflammatory study found age-related increases in microglial activation, pro-inflammatory cytokine CD86 and 8-OHdG, indicative of a ‘primed’, but not fully activated inflammatory environment. Previous work has demonstrated that high levels of inflammation can increase glucose uptake (2, 87, 131), however, chronic low-level activation of microglia has been associated with impaired neuronal health and glucose metabolism (42, 51). To become activated, GLUT4 requires insulin to translocate into the membrane (14, 60). Insulin resistance and downregulation of GLUT4 gene transcription and translocation are seen in aging (26, 37, 38), likely caused by the increased presence of pro-inflammatory cytokines, which have been found to inhibit insulin receptors (38). Thus, insulin inhibition by the observed ‘primed’ inflammatory environment with age may lead to altered GLUT4 gene transcription in both neurons and microglia, contributing to depressed basal glucose uptake.

INTERACTION OF GLUCOSE AND INFLAMMATION IN A MIDDLE-AGED RAT MODEL OF SCI

Our glucose uptake study showed that at 14 dpi, both glucose uptake and pro-inflammatory markers were significantly higher in middle-aged rats, indicative of a chronic hyper response to injury by middle-aged inflammatory cells. Our glucose uptake study went out only to 14 dpi, but in our inflammation studies, we saw significantly increased microglial activation, inflammatory markers, and increased oxidative stress markers out to 30 dpi. Based on the association between increased glucose uptake and inflammatory markers at 14

dpi, it is likely that glucose uptake in middle-aged rats would remain elevated at 30 dpi as well, and future work should explore the longitudinal impact of age-related inflammation on glucose uptake after SCI.

In vitro studies of activated macrophages have demonstrated that glucose utilization is vital for maintenance of inflammatory phenotypes, as attenuation of aerobic glycolysis in activated macrophages reduces bacterial killing and inflammatory cytokine production (37, 93). Further, there is evidence that inflammatory cell metabolism of glucose may be adaptable in environments with high levels of oxidative stress, leading to increased glucose uptake in regions with increased inflammatory cell presence and neuronal death, masking observable metabolic deficits caused by an insult (disease or injury) (2). ^{18}F -FDG-PET imaging of cerebral ischemia has shown normal glucose uptake and transport in ischemic regions where there is increased neuronal death (2). Decreased blood flow is found in ischemic regions, leading to insufficient oxygen supplies, which prevents cells like neurons from metabolizing glucose (2). These normal glucose levels have been attributed to the infiltration of inflammatory cells, as once inflammatory cells have migrated away from the site, glucose uptake decreases significantly (2). The survival and function of these inflammatory cells without normal oxygen suggests that inflammatory cells are able to utilize non-oxidative mechanisms to metabolize glucose and maintain functionality even when crucial metabolic conditions are altered (2).

Influence of Inflammation on Glucose Uptake Assessed By PET Imaging

Our model of glucose uptake and transport includes acute and chronic time points, but there is great potential for the use of PET imaging in long term longitudinal examination of post-injury functional recovery and related glucose uptake. Further, the altered glucose uptake pathway reported here with age using PET imaging demonstrates its potential as a functional diagnostic tool for SCI that is sensitive enough to differentiate glucose uptake between age groups. Previous work from our lab using PET imaging of Fluorodeoxyglucose (FDG) in a rat model of SCI glucose uptake showed an acute depression of glucose returns to baseline by 1 dpi and is maintained out to 14 dpi, due to increased inflammatory cell activity (175). Our current study using PET imaging of FDG in a middle-aged model of SCI shows observable alterations to glucose uptake caused by age on the post-injury glucose uptake pattern – with significant increases in uptake at 14 dpi. Previously, PET imaging of FDG uptake has been successfully used for age-related alterations to glucose uptake and inflammatory activity in models of Alzheimer's Disease (AD), TBI and autoimmune encephalomyelitis (EAE) in the spinal cord (19, 21). Longitudinal PET imaging studies in aging mice models have shown decreased glucose metabolism with age and alterations to FDG uptake in Alzheimer's Disease (AD) models (114, 177). In a rat model of TBI, FDG-PET imaging showed that neurons and glial cells have different patterns of glucose uptake post-injury (19). Brain regions with neuronal cell damage alone showed depression of glucose uptake, whereas regions with neuronal cell damage but increased glial activity showed increased glucose uptake (19). EAE related spinal cord lesions showed increased glucose uptake correlated with the density of

infiltrating microglia/macrophages. Though these studies have demonstrated injury and age related alterations to glucose uptake patterns, our current study is the first to show the impact of age on glucose uptake after SCI. Our findings support that age-related inflammation can alter post-injury glucose uptake pathways, important information to consider in using modalities like PET imaging for clinical diagnosis and prognosis of injury.

POTENTIAL THERAPEUTIC TARGETS: NOX2 AND INSULIN

The work included here identifies two potential therapeutic avenues for investigation that are influenced by age and impact inflammation and metabolism: inhibition of the microglial NOX2 enzyme and manipulation of the glucose uptake and transport pathway via insulin.

Inhibition of NOX2

NOX2 has shown promise as a potential therapeutic target in SCI in previous studies of young adult animal models (5, 29, 88, 98, 109), and may play an important role in age-related alterations to inflammation, as its production of ROS on microglia contributes to oxidative stress in the cellular environment. The potential for manipulation of inflammatory activity with NOX2 may aid in moderation of the heightened inflammatory response found in age.

The role of NOX in microglial activation state and response to injury remains unclear, however, there is evidence that NOX2 may be an important modulating factor. Previous work has found that activation of microglial NOX2 initiates ROS production that is culpable in neuronal damage as well as ‘primes’

microglia, leading to enhanced pro-inflammatory responses to stimuli (29). Transgenic NOX2 KO mice show altered inflammatory phenotype during the first 7 days after TBI, with an increased ratio of anti-inflammatory, M2 like microglia/macrophage activity (5). Further, bone marrow-derived macrophages from NOX2 KO mice showed reduced markers of pro-inflammatory activation after stimulation with lipopolysaccharide (a toll-like receptor agonist that can induce M1, pro-inflammatory activity)(5). In wild type mice, pre-treatment with nonspecific NOX inhibitors prior to TBI have shown marked decreases in NOX2 expression and attenuated blood-brain-barrier dysfunction, neurological deficits, and cortical apoptosis (109). Post-injury, specific inhibition of NOX2 in mouse models of TBI reduced deficits in cognitive function and increased anti-inflammatory, M2 microglia type activity (98).

Work in our laboratory in a mouse model of SCI has shown that specific inhibition of NOX2 results in significant suppression of pro-inflammatory cytokines and oxidative stress markers when administered immediately post-injury and assessed at 24 hours and 7 dpi, with a marked reduction in M1 polarization of microglia (88). This data confirms that inhibition of NOX2 resulted in changes in cytokine expression and ROS production, showing that NOX2 plays an important role in secondary damage after SCI, and may be a viable therapeutic approach for targeting in the aging population.

Manipulation of Glucose Uptake and Transport via Insulin

Insulin is a promising therapeutic target for modulation of impaired glucose uptake and transport pathways in aging and traumatic injury. In the CNS,

insulin plays a significant role in the maintenance of glucose and energy homeostasis, cognition and memory, and neuromodulatory systems (14, 27). Insulin-sensitivity is worsened by age, heavily influencing the ability of the body and CNS to metabolize its primary fuel source, glucose (60). With increasing presence of pro-inflammatory cytokines (seen in both aging and injury), insulin receptor availability drops, leading to insulin resistance (38, 60, 120, 126). Our glucose study demonstrates age-related alterations to expression of insulin-sensitive GLUT4 (present on both neurons and microglia), indicating that alterations to glucose uptake may be related by decreased insulin availability.

In both clinical and pre-clinical studies of neurodegeneration and TBI, increasing insulin availability leads to improved glucose uptake and functional outcomes. In animal models of TBI, longitudinal intranasal insulin leads to increased cerebral glucose uptake and reduced cognitive impairments (18, 39, 111). In clinical studies of early stage AD and mild cognitive decline, longitudinal treatment with intranasal and intravenous insulin improve learning, memory, and cognitive function (39, 123). These studies demonstrate that increasing insulin availability does improve glucose uptake and functional outcomes, and could be alternatively proposed for aging and SCI.

Intersection of NOX2 and Insulin: An Example of the Connection between Inflammation and Glucose Uptake

Work from our lab and others have demonstrated that the glucose uptake and inflammatory systems influence the functionality of each other (2, 25, 87). As we and others have demonstrated, age shows progressive pro-inflammatory

activation and altered glucose utilization which is exacerbated by CNS injury (both TBI and SCI) (77, 99, 183).

Recent work has begun to explore the intersection of insulin and NOX2 as a therapeutic target for both glucose metabolism and inflammation. Middle-aged and elderly mice have shown systemic increases in oxidative stress, high blood pressure, hyperglycaemia, and hyperinsulinaemia, and decreased insulin receptor expression and signaling (56). In transgenic NOX2 KO mice, both middle-aged and elderly mice demonstrate preserved glucose metabolism and decreased oxidative damage compared to young (56), clearly demonstrating an interaction between insulin signaling and NOX2 related oxidative stress. As NOX2 production is directly related to microglial activity, it can be suggested that this intersection of NOX2 with insulin would impact both glucose utilization and inflammation.

The intersection of these two systems has not previously been explored in animal models of SCI. However, age and SCI related alterations to inflammation are similar to that seen in neurodegenerative diseases. In fact, rodent models of neuroinflammation have been used as models of neurodegenerative diseases like AD (128). AD shows impaired insulin receptor signaling, associated with increased oxidative stress, altered glucose metabolism and energy consumption (14). In wildtype rats, inhibition of insulin receptor signaling mimics AD related impaired glucose metabolism and increased neuroinflammation (40, 79). In a rat AD model, longitudinal treatment of intranasal insulin showed attenuated microglial activation and improved neurogenesis (69). The modulation of

inflammation by insulin in an AD rat model and the improvement in glucose metabolism shown in aged NOX2 KO mice supports that both NOX2 and insulin have the potential to be used as therapeutics for both age-related alterations to inflammation and glucose uptake.

CONCLUSION

Characterization of basal microglial activation, inflammatory cytokine and NOX2 expression (Aim 1 – Chapter 2) demonstrates that by middle-age, inflammatory related activity in the spinal cord has begun to change from young adult, demonstrating a ‘primed’, pro-inflammatory environment. Age-related increases in DNA damage, oxidative stress and dysregulation of gene expression involved in stress response are most pronounced at elderly ages, but are seen beginning in middle-age (32, 108). We now demonstrate that changes by middle-age are prominent, including increased microglial activation, pro-inflammatory cytokines and partial ROS-producing NOX2 component expression, suggesting chronic levels of basal inflammation may lead to enhanced response to SCI.

We now show that chronic injury to the middle-aged spinal cord leads to a hyper-response of the ‘primed’ basal inflammatory environment, resulting in increased microglial activation, inflammatory cytokine and NOX2 expression, lesion size, and worsened functional recovery (Aim 2 – Chapter 2). It is important to note that age-related increased inflammatory cytokine expression is both pro and anti-inflammatory chronically after SCI, agreeing with recent work in a mouse model that has identified a novel ROS-producing M2 microglial phenotype

by middle-age (59). This implies that the presence of both anti-inflammatory and pro-inflammatory cytokines and related NOX2 ROS production may have a detrimental effect on recovery to SCI, and is important for therapeutic targets (like NOX2 inhibition) aimed at manipulating the inflammatory response to consider.

Finally, we now show that age alters glucose uptake patterns before and after injury, and correlates with our age-related increases in inflammation (Aim 3 – Chapter 3), demonstrating the interaction of age-related alterations on multiple systems in the CNS. Glucose metabolism and inflammation interact and influence functionality of each system, and dysregulation of those systems with age may lead to a compounded effect on the ability of the body to combat an insult (2, 87). Further, these findings help to elucidate a novel avenue in the glucose pathway for therapeutic targeting in aging and SCI.

The data presented here support our original hypothesis that middle-age related increases in pro-inflammatory microglial activation and oxidative stress lead to an exaggerated glial response, altered glucose utilization and diminished functional recovery to SCI. With the increasing age of onset of SCI, the findings in this dissertation contribute to the understanding of how age impacts recovery from an SCI, defining relevant and useful systems to target in the future development of therapeutic interventions.

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