MICROGLIAL FUNCTION IN THE AGED AND INJURED RODENT BRAIN

By

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A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

2009

To the memory of my brother, father and stepfather.

ACKNOWLEDGMENTS

I thank my mentor Dr. Wolfgang Streit for his guidance, patience and willingness to teach me. I also thank the other members of my committee, Dr. Jeffrey Harrison, Dr. John Petitto and Dr. William Millard for providing the guidance and support needed to complete this dissertation. Dr. Harrison was especially helpful in providing advice and allowing me the use of his lab and equipment. Finally, I would like to extend thanks to Dr. Edwin Meyer for his initial guidance and service on my committee.

A special thank you is extended to current and former members of the Streit lab. In particular, I would like to thank Jessica Conde for providing training in my early days in the lab and paving the way for my dissertation project, Sarah Fendrick and Kryslaine Lopes for their constant support and encouragement both in and out of the lab, and Chris Mariani for being a supportive lab member and friend. Finally, I extend my sincere gratitude to Dr. Defang Luo of the Harrison lab for her invaluable assistance with my *in situ* hybridization studies and Dr. Qing-Shan Xue of the Streit lab for technical assistance and imaging help.

I thank Dr. James Resnick for allowing me the use of his lab and equipment and for making me feel like one of the family. Emily Smith of the Resnick lab also deserves many thanks for her technical assistance and for her friendship that I sincerely appreciate. I am also very grateful to Michael Poulos of the Swanson lab for generously lending his time and expertise.

Finally, and most of all, I thank my friends and family for providing support and encouragement. I am especially grateful to my mother for providing me with the love and support I needed to succeed both in and out of school.

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of the University of Florida in Partial Fulfillment of the
Requirements for the Degree of Doctor of Philosophy

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August 2009

Chair: Wolfgang J. Streit
Major: Medical Sciences-- Neuroscience

Neuroinflammation resulting from chronic reactive microgliosis is thought to contribute to age-related neurodegeneration, as well as age-related neurodegenerative diseases, specifically Alzheimer's disease (AD). Support of this theory comes from studies reporting a progressive, age-associated increase in microglia with an activated phenotype. While the underlying cause of this microglial reactivity is idiopathic, a popular therapeutic strategy for the treatment of AD is inhibition of microglial activation through the use of anti-inflammatory agents. While the effectiveness of anti-inflammatory treatment for AD remains equivocal, microglial inhibition is being tested as a potential treatment for additional neurodegenerative disorders including amyotrophic lateral sclerosis (ALS) and Parkinson's disease (PD). Given the important and necessary functions of microglia, careful evaluation of microglial function in the aged and injured brain is a necessary first step in targeting better treatment strategies.

Recent evidence suggests that microglia may undergo cellular senescence in response to normal aging and other exogenous insults. To further investigate this possibility we evaluated

the replicative potential of microglia following repeated peripheral nerve injury. We found that repeated challenge to the same pool of microglial cells, in the form of facial nerve crush injury, results in decreased cell proliferation in rats 3, 4 or 5 days after axotomy. Concomitant with decreased mitotic potential, microglia challenged by multiple nerve injuries exhibited an altered immunophenotype characterized by increased expression of macrosialin, a macrophage marker known to be upregulated in the aged brain. To investigate normal macrosialin expression in the facial motor nucleus, we analyzed protein immunoreactivity in aged and young rats following a single facial nerve injury. We determined that macrosialin is not expressed by activated microglial cells in the facial nucleus of young rats after a single nerve injury, but rather is upregulated in response to aging in both injured and uninjured nuclei. These results suggest that macrosialin is a marker of aged microglial cells. Taken together, the diminished proliferative response concomitant with macrosialin expression seen in response to repeated injury may be indicative of microglial senescence. Changes in microglial function resulting from a senescent cellular phenotype may be detrimental to brain homeostasis and act to exacerbate aging-related neurodegenerative disease or play a role in increasing the susceptibility to such degenerative conditions. These studies provide an impetus for further investigation into the causes and effects of microglial senescence.

CHAPTER 1
INTRODUCTION AND LITERATURE REVIEW

Microglia: An Overview

Introduction

Once thought to be immune-privileged, it is now known that the brain contains immunocompetent microglial cells. Highly adaptable in structure and function, microglia are prepared to respond to changes in the microenvironment in an attempt to maintain brain homeostasis. Microglia in the quiescent brain are described as "resting" based primarily on morphological characteristics. Contrary to what this classification may suggest, in vivo experiments have shown that resting microglia exhibit extremely motile processes and protrusions (Nimmerjahn *et al*., 2005). Distributed along their processes and on the cell body are copious surface receptors that allow microglia to detect and respond to many types of incoming signals. In response to insult or injury to the brain, microglia become "activated" undergoing phenotypical and physiological changes, including the elaboration of neurotrophic and/or neurotoxic cytokines and growth factors. Severe injury resulting in neuronal degeneration renders microglia phagocytic and they take on a rounded, amoeboid morphology characteristic of peripheral macrophages. While the primary role of microglia is believed to be one of neuroprotection, these cells are also thought to contribute to the onset of neuronal degeneration in many neurodegenerative disorders. Currently, the functional capacity of microglia in the aged brain is poorly understood, and it remains unclear whether microglial reactivity is a cause of neurodegeneration or merely a secondary reaction to insult in the brain.

What is microglial activation?

Microglia function not only as phagocytes, but are highly dynamic cells that display exceptional morphological and functional plasticity. In the quiescent brain, microglia are

described as “resting”, based primarily on their morphological characteristics. Contrary to what this classification may suggest, it has long been the working assumption that resting microglia are in fact busy monitoring their microenvironment in an attempt to maintain homeostasis within the central nervous system (CNS). This assumption was confirmed recently through *in vivo* experiments revealing extremely motile processes and protrusions on resting microglia in the living neocortex of mice (Nimmerjahn *et al*., 2005). Distributed along their processes and on the cell body are a plethora of surface receptors and ion channels, allowing microglia to detect and respond to myriad signals, such as neurotransmitters, neuropeptides, cytokines, chemokines, ions, growth factors and serum derived components like immunoglobulins, thrombin and complement. Upon insult or injury to the brain, microglia become “activated” undergoing phenotypical changes that include hypertrophy, mitosis, as well as changes in immunophenotype and in cytokine/growth factor production.

Morphology

In accordance with their highly adaptable nature, microglial morphology varies in correlation with unique functional states. Microglia in the normal, healthy brain are approximately 30-40 μm in diameter, smaller than both astrocytes and oligodendrocytes (Raivich *et al.*, 1999). Resting microglia exhibit a stellate morphology in gray matter, while in the white matter they lie in parallel to nerve fibers. Electron microscopy performed in organotypic hippocampal slice culture reveals microglia with oval or elongated nuclei, dense cytoplasm, dense laminar bodies, homogenous droplets, lysosomes, lipofuscin and a granular endoplasmic reticulum (Skibo *et al.*, 2000). Because of their long, highly branched processes, resting microglia are often referred to as “ramified.” Primary branches on resting, ramified microglia may extend more than 50 μm in length, with thin finger-like protrusions extending outward sometimes forming bulbous endings (Nimmerjahn *et al.*, 2005; Stence *et al.*, 2001).

Furthermore, both Nimmerjahn *et al.*, (2005) and Stence *et al.*, (2001) provide evidence that microglial processes undergo cycles of formation and withdrawal that occur within minutes, thereby resulting in extensive morphological changes within an hour's time.

Upon insult or injury to the brain, microglia undergo a stereotypical, graduated response commensurate with the severity of brain damage incurred. Prior to becoming fully activated, or in the event of a mild perturbation, microglia may take on a hyper-ramified form (Streit *et al.*, 1999). Fully reactive microglia retract their processes and develop an enlarged cell body. Shortened processes exhibit increased thickness proximally and deramification of distal branches. Additionally, experiments performed using the electron microscope describe reactive microglia as having enlarged nuclei and perikaryon, increased size and number of lysosomes and the appearance of phagosomes (Blinzinger & Hager, 1962). Ultimately, activated microglia responding to injury that does not involve frank neuronal degeneration will decrease in number and return to a resting state (Graeber *et al.*, 1989). When brain damage leads to neuronal degeneration, microglia undergo further transformation from an activated phenotype to that of a phagocyte (Streit & Kreutzberg, 1988). In cases of neuronal cell death, microglia with a macrophage appearance can be detected as early as one to four hours post-injury (Kaur & You, 2000; Skibo *et al.*, 2000). Microglial-derived macrophages take on a rounded, amoeboid shape similar to that of peripheral macrophages . When examined under the electron microscope, phagocytic microglia showed abundant lysosomes and phagosomes as well as copious lipid droplets and lipofuscin material (Kaur & You, 2000; Sobaniec-Lotowska, 2005). Further examination revealed oval or round nuclei with dense heterochromatin accumulated under the nuclear envelope and sparse euchromatin. Finally, microglia-derived macrophages customarily revert to a resting phenotype within a few days to weeks, but active macrophages have been

found in white matter tracts up to ten years following middle cerebral artery occlusion (Kosel *et al.*, 1997).

Proliferation

The mitotic potential of microglia in the adult brain was discovered when autoradiographic studies employing [^{3}H] thymidine incorporation showed that microglia undergo mitosis following brain injury (Friede & Johnstone, 1967; Kreutzberg, 1966; Sjostrand, 1971) Microglial proliferation occurred to a much lesser degree in the absence of an injury, likely reflecting normal cell turnover (Dalton *et al.*, 1968; Lawson *et al.*, 1992; Tonchev *et al.*, 2003). Later experiments confirmed that microglia are the only glial cell type to undergo mitosis after facial nerve axotomy in the rat (Graeber *et al.*, 1988b). However, astrocytic proliferation has been reported in other models (Cao *et al.*, 2003; du Bois *et al.*, 1985; Li *et al.*, 2005; McGinn *et al.*, 2004). Despite the variations in experimental injury models, species and strain differences, as well as methods of detection used to assess glial proliferation, mitosis proves to be a prominent and consistent component of the microglial response to injury.

Microglial proliferation has been studied most exhaustively in the facial nerve axotomy model (Cammermeyer, 1965; Fendrick *et al.*, 2005; Graeber *et al.*, 1988b; Kreutzberg, 1966; Streit & Kreutzberg, 1988). This well established injury paradigm is advantageous in the study of microglial activation primarily because there is no direct trauma to the CNS and the blood brain barrier remains intact, providing an opportunity to study purely endogenous glial responses. An additional advantage is that the injury is well tolerated and highly reproducible from animal to animal. Insights gained from studies employing the facial nerve axotomy and other regenerating nerve models, as well as from acute and chronic neural injury models reveal that microglial proliferation begins as early as 12 hours post-lesion (Ziaja & Janeczko, 1999), peaks at approximately three to four days after insult (Kreutzberg, 1966; Ladeby *et al.*, 2005; Sjostrand,

1971; Streit & Kreutzberg, 1988; Stuesse *et al.*, 2000) and declines thereafter. In contrast to the prevailing mitotic response described above, Tonchev et al. (2003) have shown that there is a differential proliferative response exhibited by microglia after ischemic insult in the macaque monkey. As expected, their study showed that microglial proliferation peaks four days after ischemia in the hippocampus, but surprisingly, mitotic activity in the superior temporal gyrus was delayed until 15 days post-injury. There was no significant increase in microglial proliferation in the parahippocampal region or olfactory bulb. This study highlights the fact that microglial responses are highly specialized and context-specific. Furthermore, there is evidence to show that after microglia have proliferated, population control is implemented by apoptosis. In models of facial (Jones *et al.*, 1997), as well as hypoglossal and sciatic nerve injuries (Gehrmann & Banati, 1995), apoptosis of microglia was measured using terminal transferase mediated d-UTP nick end labeling (TUNEL) and in situ end labeling (ISEL) and found to occur beginning four to six days after injury and continuing for up to 21 days.

While the proliferative response of microglia is well-documented and characterized, little is known about mechanisms underlying its regulation. The literature abounds with reports of pharmacological agents and/or endogenous chemicals that stimulate or inhibit microglial proliferation *in vitro*, which is not unexpected given the high sensitivity of microglia to their surrounding milieu. However, these findings are difficult to extrapolate to the *in vivo* situation. Upon careful review of current data, a picture emerges of likely common mechanisms governing microglial mitosis *in vivo*. Specifically, it seems that inducers of microglial proliferation could include interleukin-6 (IL-6) (Streit *et al.*, 2000), the neurotrophin NT-3 (Elkabes *et al.*, 1996) and macrophage colony-stimulating factor (M-CSF) (Kloss *et al.*, 1997). Insights into the molecular mechanisms by which these microglial mitogens exert their effects has been gained in the last

few years. *In vitro* experiments have shown that GM-CSF activates Hck tyrosine kinase, which in turn activates the phosphatidylinositol 3-kinase/Akt (PI3K/Akt) pathway (Ito *et al.*, 2005; Suh *et al.*, 2005). Additionally, studies have shown that microglial mitosis induced by GM-CSF administration (Koguchi *et al.*, 2003) and cerebral ischemia (Kato *et al.*, 2003) lead to expression of the cell cycle-associated proteins cyclin D1, E, A and cyclin-dependent kinase inhibitor p21 as well as cyclin D1 and cyclin-dependent kinase-4, respectively. Given the fact that Akt is known to activate cyclins (Mirza *et al.*, 2004) these data collectively provide a highly plausible mechanism for GM-CSF induced microglial mitosis.

The macrophage colony-stimulating factors have received considerable attention for their abilities as microglial mitogens, but IL-6 has also come to be thought of as an inducer of glial proliferation. This is not completely surprising given that IL-6 was previously known as "B cell growth factor" for its stimulation of proliferation in B lymphocytes. Studies strongly suggest that IL-6 released from injured neurons serves as a signal for microglial proliferation and activation in general (Kiefer *et al.*, 1993; Streit *et al.*, 2000; Streit *et al.*, 1998). It was shown that there is an early and robust upregulation of IL-6 mRNA following facial nerve injury that precedes the onset of microglial mitosis (Streit *et al.*, 2000). Concordantly, low levels of IL-6 expression were seen in the red nucleus following rubrospinal tractotomy, as well as in the facial nucleus of neonates post-axotomy, both situations wherein microglial proliferation does not occur. Furthermore, experiments performed on IL-6 deficient mice show significantly delayed microglial responses. Specifically, impairment of microglial proliferation was reported in IL-6 -/- mice following facial nerve axotomy (Galiano *et al.*, 2001; Klein *et al.*, 1997) and in the substantia nigra pars compacta (SNpc) after MPTP lesions (Cardenas & Bolin, 2003). Thus, when examining the beneficial proliferative response of microglia to IL-6, we are again

reminded of the highly specialized and context-specific reaction of microglia to a molecule that is known to have multiple effects, both pro- and anti-inflammatory.

Cytokine/growth factor production

Microglial production of cytokines and growth factors is complex and occurs in a heterogeneous and escalating manner. Certain cytokines known to be constitutively expressed by microglia are thought to act in an autocrine fashion, specifically, transforming growth factor β (TGFβ) (Kiefer *et al.*, 1993; Lehrmann *et al.*, 1998), a pleiotropic growth factor. TGFβ has been shown to exert inhibitory effects on microglial phagocytosis (Stoll *et al.*, 2004) and proliferation (Jones *et al.*, 1998), as well as prevent the induction of microglial genes involved in chemotaxis and cell migration, among others (Paglinawan *et al.*, 2003). The list of cytokines, chemokines and growth factors produced by microglia upon activation is extensive (Hanisch, 2002); however, it is important to note that many cytokines exert both positive and negative effects on the CNS and that it is the degree of microglial activation, or severity of neuronal damage, that determines the ensuing cytokine expression patterns. For example, microglia rapidly upregulate IL-1β, IL-6, TNF-α mRNAs following traumatic spinal cord damage (Bartholdi & Schwab, 1997; Streit *et al.*, 1998; Yang *et al.*, 2005), whereas in the regenerating facial nerve injury paradigm mRNAs of TNF-α and IL-1β, both prototypic proinflammatory cytokines, are only minimally elevated and there is no change in M-CSF mRNA (Raivich *et al.*, 1999; Streit *et al.*, 1998). Interleukin-6, which shows prolonged expression after facial axotomy, is rapidly downregulated after spiking initially in spinal cord injury (Streit *et al.*, 1998). Finally, microglial activation resulting from infection, such as viral meningitis or bacteria-induced encephalitis, leads to production of not only those cytokines listed above, but also interferon-γ (IFN-γ) (Frei *et al.*, 1988; Suzuki *et al.*, 2005). IFN-γ acts to promote upregulation of surface molecules like major histocompatibility complex (MHC) class I and II molecules, complement receptors, Fc

receptors and CD14, as well as induce the release of cytokines, complement and nitric oxide (NO) (Hanisch, 2002). In addition, IFN-γ acting synergistically with beta-amyloid (Aβ) peptide has been shown experimentally to induce microglial production of the chemokine monocyte chemotactic protein (MCP-1) (Meda *et al.*, 1996). Microglia are capable of producing many additional cytokines, chemokines and neurotrophins not discussed herein and for additional information the interested reader is referred elsewhere (Hanisch, 2002; van Rossum & Hanisch, 2004).

Immunophenotype

As with all other aspects of microglial biology, surface molecule expression is highly dynamic and exhibits changes in association with various states of microglial activation. Resting microglia constitutively express type three complement receptors (Graeber *et al.*, 1988a), and Fc and macrophage-specific antigen (Perry *et al.*, 1985), as well as CD4 (Perry & Gordon, 1987). However, when microglia become activated, there are changes in surface marker expression that suggest changes in cell function. Within 24 hours of activation, microglia express many molecules important for interactions between lymphocytes and antigen-presenting cells. Specifically, they exhibit an upregulation of CR3 (OX-42) expression (Graeber *et al.*, 1988a) accompanied by an increase in IgG-immunoreactivity, thrombospondin, and intercellular adhesion molecule 1 (Kloss *et al.*, 1999; Moller *et al.*, 1996; Raivich *et al.*, 1999). Peak expression of integrin subunits α5 and α 6 occurs at day four post-injury and the αM-subunit at day 1 and again at days 14-42. Furthermore, within three days of CNS injury, proliferating microglia have been shown to express the stem cell antigen CD34 (Ladeby *et al.*, 2005). Consistent with a role as antigen-presenting cells, reactive microglia show enhanced major histocompatibility complex type I and II (MHC I and II) expression during the first week after injury (Streit *et al.*, 1989a, 1989b). Upregulation of MHC I can be detected in all activated

microglia, while MHC II expression is restricted primarily to microglia in degenerating white matter tracts (Streit *et al.*, 1989b; Watanabe *et al.*, 1999). Phagocytic microglia are known to display all of the surface molecules previously discussed, as well as the macrophage surface antigens ED1 and ED3 (Graeber *et al.*, 1990). All in all, there is great heterogeneity in microglial immunophenotypes, which can vary with the type and severity of a lesion, the location within the parenchyma (white *versus* grey matter), and perhaps also with the cells' age.

Cellular Senescence

Throughout much of the early 20th century it was believed that cells in culture were immortal and had the capacity to divide indefinitely. This perception was due primarily to work conducted by Carrell (Carrell, 1912) using cultured chick cells. It wasn't until the 1960's that Hayflick and Moorehead (Hayflick, 1965; Hayflick and Moorehead, 1961) disproved this theory by demonstrating that human fetal lung fibroblasts in culture display a limited proliferative lifespan that culminates in a state of irreversible cell-cycle arrest termed replicative senescence. Further work has since demonstrated the presence of senescent cells in numerous renewable tissues of a variety of organisms, including, but not limited to the haematopoietic system, epithelium and the vasculature (Dimri et al., 1995; Campisi, 2005; Jeyapalan et al., 2007; Rossi et al., 2007). Cellular senescence results from varied causes including telomere attrition, non-telomeric DNA damage, chromatin perturbations and strong mitogenic signals; while the senescent phenotype includes apoptosis resistance, an inability to proliferate and altered gene expression (Campisi and d'Adda di Fagagna, 2007).

Causes of Cellular Senescence

Telomere-dependent senescence

Telomeres are structures composed of repeating TTAGGG DNA sequences and binding proteins located at the ends of linear chromosomes. This nucleoprotein complex forms a

specialized structure responsible for protecting against chromosome fusions, translocations and non-dysjunctions (Blackburn, 2000). Because DNA polymerase requires a template for semi-conservative DNA replication, the telomeric end on the lagging DNA strand is progressively shortened with each round of DNA replication, resulting in what is referred to as the "end replication problem" (Levy et al., 1992). When telomeres reach a critical length, cells can no longer divide and enter a state of growth arrest termed replicative senescence. In some cell types, the end replication problem can be circumvented by the enzyme telomerase. Telomerase is a ribonucleoprotein enzyme that catalyzes the synthesis and extension of telomeric DNA repeats (Cech, 2004). While most cells do not express telomerase, exceptions include germ cells (Campisi, 1997; Zalenskaya and Zalensky, 2002), stem cells (Mason, 2003; Deville et al., 2009), tumor cells (Shay et al., 1996; Belgiovine et al., 2008; Straat et al., 2009) and transiently-expressing dividing cells (Buchkovich and Greider, 1996; Flanary and Streit, 2005). Importantly, although telomerase activity can impart heightened proliferative capacity, its expression is not always sufficient to confer unlimited proliferation (Kiyono et al., 1998; Dickson et al., 2000) and some cells undergo senescence despite telomerase activity or telomere maintenance (Kang et al., 2003; Flanary and Streit, 2004; Itahana et al., 2004; Kang et al., 2004).

Stress-induced senescence

While numerous studies demonstrate that progressive telomere shortening leads to cell senescence and that telomerase activity can prevent entrance into replicative senescence, additional evidence proves that a variety of physiological stressors can lead to rapid cellular senescence independent of telomere dynamics. These senescence-inducing factors include DNA damage, oxidative stress, oncogene expression and damage to chromatin structure (Serrano and Blasco, 2001; Ben-Porath and Weinberg, 2004; Kujoth et al., 2005; Campisi and d'Adda di Fagagna, 2007; Rossi et al., 2007). While each cause can occur distinctly, common molecular

pathways underlie the ultimate induction of senescence for all antecedents, including telomere-attrition.

Severe DNA damage at any location within the genome, including mitochondrial DNA (Kujoth et al., 2005; Ma et al., 2009), can lead to cellular senescence. Double strand breaks (DSBs) or exposure of single-stranded DNA serve to initiate a DNA damage response (DDR) (Gire et al., 2004; Rossi et al., 2007; Ohtani et al., 2009). When DNA damage is detected, specialized protein kinases, ataxia telangiectasia and Rad3-related (ATR) or ataxia telangiectasia mutated (ATM) are recruited to the lesion. Other important participants in the DDR include p53-binding protein 1 (p53BP1) and mediator of DNA-damage checkpoint 1 (MDC1) that act to recruit ATM to histones where histone phosphorylation takes place. Furthermore, ATM or ATR phosphorylation leads to activation of checkpoint kinase CHK2 or CHK1, respectively (Buscemi et al., 2004). These kinases diffuse throughout the nucleus phosphorylating their substrates thereby propagating the DDR. Finally, DDR pathways converge upon either p53, p16 (Shapiro et al., 2000; Beausejour et al., 2003) or cell-division cycle 25 phosphatases known to be essential for cell proliferation (Mailand et al., 2000). p53 serves to activate p21 which then inhibits retinoblastoma protein (Rb) and subsequently E2F transcription factors resulting in stable cell cycle arrest (Di Leonardo et al., 1994; Shapiro et al., 2000; Herbig et al., 2004; Hinkal et al., 2009). In addition to DSBs and single-strand exposure, telomere attrition also activates DDR. Shortening of telomeres results in a loss of telomere-bound inhibitors, ATM and ATR, subsequently resulting in the initiation of a DDR (Takai et al., 2003). The processes that differentiate between a DDR that results in DNA repair versus a response that culminates in permanent and irreversible cell cycle arrest, or senescence, are unknown but usually involve large or protracted DNA damage foci.

Early *in vitro* experiments revealed that human fibroblasts undergo cellular senescence when cultured at high ambient oxygen levels; while their viability is preserved at lower, more physiologically relevant levels (Packer and Smith, 1977). More recently, Parrinello et al. (2003) demonstrated that premature senescence of mouse embryonic fibroblasts *in vitro* is a direct result of oxidative stress. Furthermore, these cells exhibit high levels of DNA damage (Parrinello et al., 2003). Oxidative stress occurs when ROS in a cell overwhelm the capacity of antioxidant defenses. Moreover, oxidation can occur to numerous cellular components including DNA, lipids and proteins (Sitte et al., 2000). Reactive oxygen species (ROS) are a byproduct of normal cellular metabolism and primary sources include mitochondrial respiration, peroxisomes, antimicrobial oxidative bursts of phagocytic cells and cytochrome p450 enzymes ((Finkel and Holbrook, 2000; Weinert and Timiras, 2003). Further work has provided insight into the mechanisms underlying oxidative stress-induced senescence revealing that oxidative stress operates along the same transduction pathways described above, including p53 signaling to p21 and Rb (Itahana et al., 2003). Finally, elevated levels of oxidant-damaged DNA and proteins and products of oxidatively-damaged DNA (8-oxoguanine) are known to accumulate with age coincident with age-related increases in senescent cells. This suggests that oxidative damage is an important inducer of cell senescence (Beckman and Ames, 1998; Hamilton et al., 2001; Shringarpure and Davies, 2002).

Oncogenes are mutated versions of normal genes that have the potential to induce malignant transformation. When expressed in normal cells, oncogenes such as RAS, BRAF MOS, MEK, MYC, RAF and E2F induce cellular senescence (Serrano et al., 1997; Pearson et al., 2000; Lazzerini Denchi et al., 2005; Michaloglou et al., 2005). It is thought that oncogene-induced senescence (OIS) serves as a tumor suppressor mechanism by inhibiting uncontrolled

cellular proliferation (Ohtani et al., 2001; Chen et al., 2005; Lazzerini Denchi et al., 2005; Ohtani et al., 2009). This theory is supported by the fact that many oncogenes are positive regulators of cell cycle progression. Furthermore, it has been shown that when cells are cultured under conditions that reduce mitogenic stimulation, namely in serum-free medium, they are able to bypass premature senescence and exhibit extended survival time (Mathon et al., 2001). Interestingly, like telomere-induced and oxidative stress-induced senescence, OIS also initiates a DDR (Bartkova et al., 2006). However, it is rather the ATR to CDC25 and/or the p16 to Rb pathways that predominately modulate this form of cellular senescence, although not exclusively (Serrano et al., 1997; Ohtani et al., 2001) .

The Senescent Phenotype

Over the last 50 years our understanding of cellular senescence has expanded beyond the sole inclusion of replicative arrest to include a much broader phenotype which is characterized by several distinct anatomical and physiological changes. These alterations most commonly include morphological distortions, growth arrest, functional dysregulation, altered gene expression and resistance to apoptosis (Campisi, 1997; Campisi and Fagagna, 2007; Cristofalo et al., 2004). The most notable and easily identifiable characteristic of senescent cells is their inability to respond to mitogenic stimuli. This occurs in metabolically active cells despite appropriate growth conditions and functional signal transduction capacity (Rittling et al., 1986; Seshadri and Campisi, 1990; Di Leonardo et al., 1994). Growth arrest of senescent cells occurs due to overexpression of inhibitors of cell cycle progression and repression of positively-acting growth regulators (Seshadri and Campisi, 1990; Hara et al., 1994; Alcorta et al., 1996). Changes in genes encoding cell cycle regulators account for a large proportion of genetic alterations seen in senescent cells, but there are also reports of overexpression of genes encoding secreted proteins that could potentially affect surrounding cells and ultimately compromise tissue

homeostasis (Shelton et al., 1999; Mason et al., 2004; Santra et al., 2009). While senescent cells exhibit growth arrest and dysregulation of genes regulating cell cycle progression, they remain distinct from dying cells in that they exhibit a resistance to apoptosis. This may explain why senescent cells accumulate with age (Jeyapalan et al., 2007). The phenomenon of apoptosis resistance is variable among different cell types and apoptotic-inducing stimuli making it difficult to identify the factors responsible for this resistance (Chen et al., 2000; Hampel et al., 2004). Studies suggest that the intensity of stressors and the regulation of pro and anti-apoptotic genes may play an important role (Chen et al., 2000; Marcotte et al., 2004). Finally, morphological and immunophenotypical changes are also a prominent characteristic of senescent cells. Morphological alterations are cell-type specific, but include such things as cellular and nuclear hypertrophy (Cristofalo and Kritchevsky, 1969; Greenberg et al., 1977; Conde and Streit, 2006), the presence of vacuolizations, increased microfilaments, lipofuscin, neuromelanin and ceroid (Giannessi et al., 2005; Sulzer et al., 2008) and the propensity for formation of multinucleated giant cells (Matsumura, 1980; Fendrick et al., 2007).

Microglial Senescence

Microglial Telomere Maintenance

Microglia are derived from haematopoietic precursor cells that populate the rodent brain in late embryonic development (corresponding to the second trimester in humans). Parenchymal microglia have a slow rate of turnover, and studies have revealed that while a small degree of repopulation is achieved through infiltration of bone marrow- derived cells, cell maintenance occurs primarily through mitosis of resident microglia (Streit *et al.*, 1989; Lawson *et al.*, 1992; Priller *et al.*, 2001; Ladeby *et al.*, 2005). As the only mature cell type in the brain exhibiting significant mitotic activity, microglia may be subject to replicative senescence. As discussed

above, cellular senescence results from varied causes including both telomere attrition and non-telomeric DNA damage (Campisi and d'Adda di Fagagna, 2007).

Telomere attrition and telomerase activity have been investigated in numerous cell types (see above), but little is known about telomere dynamics in microglial cells. However, recent studies have provided some clues for understanding microglial telomere maintenance *in vitro*, as well as in the aged and AD brain. Using primary microglial cell cultures derived from newborn rats, experiments were conducted to assess the potential that microglial telomeres shorten when the cells are stimulated to proliferate (Flanary and Streit, 2004). It was found that telomere attrition occurs over time in both stimulated and non-stimulated cells. Cells that were not exposed to the microglial mitogen granulocyte macrophage colony stimulating factor (GM-CSF) underwent proliferative arrest when telomeres reached a critical length somewhere below 14 kb. Interestingly, stimulated cells, which were found to express higher telomerase activity than controls, did not enter replicative senescence when their telomeres reached critical length (i.e. below 14 kb). Alternatively, stimulated microglia underwent senescence, as demonstrated by reduced viability and mitotic activity, after several weeks in culture and in the absence of telomerase repression. This data shows that microglia *in vitro* are subject to both telomere-dependent and independent mechanisms of cellular senescence. To determine if this phenomenon also occurs *in vivo*, telomere length and telomerase activity were measured in rat cerebellar and cortical tissue taken from 21 day-old and 5 month old animals (Flanary and Streit, 2003). In agreement with reports from other researchers (Prowse and Greider, 1995; Coviello-McLaughlin and Prowse, 1997), this study showed that telomere length declines in association with increasing age in the rat brain.

Microglial Morphology in the Aging and AD Brain

Resting, non-reactive microglia exhibit a highly ramified morphology *in vivo*. Alternatively, injury-induced microglial activation involves a retraction of cellular processes and an enlargement of the cell body. In contrast, microglia from aged and AD human brains have been shown to display many of the features of degenerating cells (Streit, 2004; Streit *et al.*, 2004). These cells exhibit aberrant changes in cellular structure including deramification, shortening, twisting and fragmentation of cellular processes. Additionally, studies have identified microglia in rat brains that exhibit age-related, progressive hypertrophy of the perinuclear cytoplasm (Conde and Streit, 2006). This data is in agreement with reports from Peters *et al.* (1991), Peinado *et al. (1998)* and Peters and Sethares (2002) who found microglia in aged monkeys and rats that contain abnormal membrane-bound, cytosolic inclusions that occupy the perikarya resulting in displacement of the nucleus. Moreover, these atypical morphological alterations are accompanied by immunophenotypical changes including increased MHC II expression (Perry *et al.*, 1993; Sheffield and Berman, 1998; Sloane *et al.*, 1999), increases in ED1 macrophage antigen and an upregulated expression of leukocyte common antigen (LCA) (Perry *et al.*, 1993; Kullberg *et al.*, 2001) . While these changes have been assumed to be an indication of activation, they are often found occurring in the absence of any observable pathology. Taken together, these morphological and immunophenotypical anomalies suggest that dystrophic microglia may be in a state of cellular senescence.

Dissertation Project

Microglia comprise a mitotically active cell population located within the brain parenchyma. It has been demonstrated that microglial replenishment from peripherally-derived precursor cells is a minor source of cellular renewal. Rather, microglial cell population is

maintained primarily through mitosis of resident parenchymal microglia. As such, microglial cells may likely be subject to replicative senescence. Furthermore, microglia in aged and diseased human brains are now known to display abnormal morphological and phenotypical characteristics typical of other types of senescent cells, as discussed above. This suggests that potential age-related increases in oxidative stress, accumulation of DNA mutations and damage and unidentified pathological processes associated with neurodegenerative diseases may all contribute to microglial senescence. The aim of this dissertation is to assess microglial senescence *in vivo* in response to advanced age and an exogenous stressor, namely repeated nerve injury.

CHAPTER 2
EFFECTS OF REPEATED PERIPHERAL NERVE INJURY ON MICROGLIAL PROLIFERATION

Introduction

Microglia serve as immunocompetent cells in the central nervous system. It is in this capacity that microglia play a prominent role in neuroinflammation following brain injury. It is hypothesized that brain inflammation acts to exacerbate neurodegeneration and for this reason microglia have been implicated in the pathogenesis of neurodegenerative diseases, including Alzheimer's disease (AD). There have been numerous reports of changes in microglial cells in the aged brain (Vaughan and Peters, 1974; Peters *et al.*, 1991; Perry *et al.*, 1993; DiPatre and Gelman, 1997; Sheffield and Berman, 1998; Sheng *et al.*, 1998; Sloane *et al.*, 1999; Kullberg *et al.*, 2001; Peters and Sethares, 2002), often considered to be indicative of microglial activation. These findings have led to the idea that alterations in the aged brain induce chronic microgliosis, which leads to the production of pro-inflammatory and neurotoxic mediators that induce neuronal degeneration and further microglial reactivity, thereby creating a pathological cycle of events. While many of the criteria used to characterize microglial activation in the above referenced reports are well accepted measures of microglial reactivity, careful examination of aged and AD brains suggests that many of these ostensibly activated cells may instead be undergoing senescent changes. A distinction between healthy, activated microglia and dysfunctional, senescent cells is critical when considering future approaches to maintain brain health and treat neurodegenerative disease. The present study examines the potential for microglia *in vivo* to undergo senescence in response to repeated peripheral nerve injury. Because microglia are known to be subject to telomere attrition (Flanary and Streit, 2003, , 2004; Flanary et al., 2007) and because strong mitogenic stimulation is an established inducer of cell senescence (Mathon et al., 2001; Tang et al., 2001) independent of telomere function, it is

hypothesized that repeated nerve injury will act to exhaust the replicative potential microglia in the affected pool of cells through the induction of cellular senescence.

Materials and Methods

Animals and surgery

Animal use protocols were approved by the University of Florida Institutional Use and Care of Animals Committee (IUCAC). All animals used in this study were male Sprague-Dawley rats (Harlan, Indianapolis, IN) aged 3 (multiple injury (experimental) groups), 9 (single injury (control) group; 4 and 5 day-post axotomy time points) or 12 months (4 injuries (control) group; 3 day time point) at the time of initial or sole injury, respectively (Figure 2-1). Animals were housed under standard SPF conditions in the McKnight Brain Institute animal facility. Under isoflurane anesthesia, the right facial nerve was exposed near its exit from the stylomastoid foramen. The nerve was crushed once with a pair of fine forceps for 10 sec, approximately 2 mm from the stylomastoid foramen (~12–14 mm from the facial nucleus in the brainstem). In multiple injury animals, nerve crush was performed as close as possible to the site of primary injury, moving proximal to the brain as necessary. Lack of whisker movement on the right side was verified after the animals recovered from anesthesia. Any animals that retained whisker movement after surgery were excluded from the study. The contralateral (unoperated) facial nucleus served as an internal control in all experiments. Three, 4 or 5 days after the final injury (4^{th} axotomy in 3 day experimental group; 3^{rd} axotomy in 4 and 5 day experimental groups; 1^{st} axotomy for all control groups) all animals received a single intraperitoneal injection of bromodeoxyuridine (BrdU; Sigma) at a dose of 100 mg/kg body weight. Animals were sacrificed 2 hours after BrdU administration. Subjects were anesthetized with an overdose of sodium pentobarbital and transcardially perfused with 0.1M phosphate-buffered saline (PBS, pH 7.2) followed by 4% paraformaldehyde in PBS. Brain tissue was removed and post-fixed at 4° C

in the same fixative overnight. Subsequently, brains were placed in 30% sucrose at 4° C until tissue processing.

Alternatively, tissue used for microglial Iba1 immunohistochemistry was collected and processed separately. A total of 1 or 3 surgeries were carried out as described above and animals were sacrificed 5 days post-injury. Subjects were anesthetized with an overdose of sodium pentobarbital and transcardially perfused with 0.1M phosphate-buffered saline (PBS, pH 7.2) followed by 4% paraformaldehyde in PBS. After brains were collected, they were post-fixed for two hours in 4% paraformaldehyde and subsequently frozen in methyl butane cooled in liquid nitrogen and stored at -80° C until tissue processing.

Tissue Processing

Following fixation, all brains used for this experiment, except those collected for Iba1 immunohistochemistry, were frozen at -25° C and 20 µm cryosections were collected encompassing the entire span of the facial motor nucleus in the brainstem. A puncture mark was made on the contralateral side of the brain to distinguish the injured and non-injured nuclei. Sections were stored at -20° C in cryoprotectant solution until immunohistochemistry was performed. Brain tissue collected for Iba1 histochemistry was removed from storage at -80° C and equilibriated to -25° C in the cryostat. Next, 20 µm cryosections were collected encompassing the entire span of the facial motor nucleus in the brainstem. A puncture mark was made on the contralateral side of the brain to distinguish the injured and non-injured nuclei. Sections were mounted on glass slides and stored in slide boxes at -80° C until used.

Imunohistochemistry

All tissue was rinsed in PBS for 10 minutes prior to staining to remove cryoprotectant. Approximately every third section spanning the facial motor nucleus was examined for BrdU immunohistochemistry. Tissue was incubated in a 1:1 solution of 2X SDS and formamide for 2

hours at 65° C. Next, the tissue was rinsed in 2X SDS for 10 minutes. The samples were then incubated in 2N HCl for 30 minutes at 37° C. Following HCl treatment, the sections were rinsed in 0.1M borate buffer for 10 minutes. Next, a blocking solution containing PBS with 0.1% Triton-X100 and 10% NGS was applied for 1 hour. Rat anti-BrdU antibody (Abcam) was applied at a concentration of 1:100 in PBS with 0.1% Triton- X100 and 10% NGS and incubated overnight at 4° C. Antibody binding sites were visualized using fluorescent goat anti-rat secondary antibody (Alexafluor 568, Molecular Probes) diluted to a concentration of 1:1500 in PBS with 0.1% Triton-X100 and 10% NGS. Lastly, sections were mounted onto glass slides and coverslipped with gel-mount mounting media.

A random sampling of sections from each subject was chosen for double-immunohistochemistry. For these samples, BrdU histochemistry was performed as described above, and subsequently the microglia-specific *Griffonia simplicifolia* B_4 isolectin (biotinylated lectin GSA I-B_4, Sigma) was applied at a concentration of 1:100 in PBS containing cations (0.1mm of CaCl2, MgCl2, and MnCl2) and 0.1% Triton-X100 and incubated overnight at 4° C. The sites containing bound lectin-biotin conjugates were visualized using fluorescent Avidin substrate at a concentration of 1:1000 (Alexafluor 468 Avidin, Molecular Probes) in PBS with 0.1% Triton-X100. These sections were then mounted onto slides and coverslipped.

Tissue collected 3 days after a sole or 4th injury was used to visualize motor neurons in the facial nucleus. Approximately 10-15 sections per brain were stained with cresyl violet, dehydrated through ascending alcohols, cleared in xylenes and coverslipped with Permount mounting medium (Fisher Scientific).

Finally, immunohistochemistry was performed to identify microglia within the injured facial nucleus. Slides were removed from storage at -80° C and acclimated at -20° C for 15-30

minutes. Slides were then warmed and allowed to dry at room temperature for 30 minutes. All tissue was rinsed in PBS for 10 minutes prior to staining. A blocking solution containing PBS with 0.1% Triton-X100 and 10% NGS was applied for 1 hour at 37° C. Rabbit anti-Iba1 antibody (Wako Chemicals USA, Inc., Richmond, VA) was applied at a concentration of 1:500 in PBS with 0.1% Triton- X100 and 5% NGS and incubated overnight at 4° C. Antibody binding sites were visualized using fluorescent goat anti-rabbit secondary antibody (Alexafluor 568, Molecular Probes) diluted to a concentration of 1:300 in PBS with 0.1% Triton-X100 and 5% NGS incubated at room temperature. Slides were rinsed in PBS and coverslipped with gel-mount mounting media.

Quantitative Analysis

For the quantitative analysis of BrdU-positive cells we were interested in determining if the average number of labeled cells in the injured facial motor nucleus changes following multiple nerve injuries. Therefore, we measured and compared the mean number of labeled cell profiles per unit area. An important consideration in using this technique is to sample from all areas of the facial nucleus to account for uneven spatial distribution of microglia in different areas of the facial nucleus (rostral to caudal- most regions). Therefore, approximately every 3rd section through the facial nucleus was analyzed. An assumption of this method is that any bias in counting profiles is the same for all groups. The injured facial nucleus on each section was imaged and photographed using a Spot RT digital camera (Diagnostic Instruments, Sterling Heights, MI) attached to a Zeiss Axioskop 2 microscope. BrdU-labeled cell profiles within a counting frame placed over the facial nuclei were counted (approximately 15 sections per animal) using Image Pro Plus software (version 4.5.1, Media Cybernetics, Carlsbad, CA). The total number of labeled cells was divided by the total area measured to estimate the mean number of BrdU-positive cells within the injured facial nucleus of each animal. Significant differences

were determined by Two-way ANOVA followed by Bonferroni posttests using GraphPad Prism software (GraphPad Software, San Diego, CA). Results are represented as mean values ± SEM. A significance level of $p < 0.05$ was used.

Neuronal quantification was achieved by comparing the ratio of neurons in the control nuclei versus the injured nuclei for both single and repeat (4 facial nerve crush (FNCx4)) injury groups. This was done by counting the total number of cresyl violet-stained motor neurons in both the control and injured facial nuclei of each animal (approximately 10 sections per animal). The mean number of neurons in both contralateral and ipsilateral nuclei for both single and repeat injury groups was calculated. Then, the ratio of neurons in the uninjured nuclei vs. injured nuclei was established for each animal and ratios were compared between groups. Significant differences were determined by *t*-test using GraphPad Prism software. Results are represented as mean values ± SEM. A significance level of $p < 0.05$ was used.

Quantification of Iba1 positive microglia in the injured facial nucleus was achieved by calculating the percent of total area occupied by Iba1 positive cell bodies. The area occupied by Iba1 cells was ascertained using Image Pro Plus software (version 6.2, Media Cybernetics, Carlsbad, CA) and divided by the total area measured. Approximately every 4^{th} slide (containing 2 sections each) spanning the facial motor nucleus was examined for Iba1 immunohistochemistry. This equates to approximately 6 slides/12 sections per brain. Significant differences were determined by t-test using GraphPad Prism software. Results are represented as mean values ± SEM. A significance level of $p < 0.05$ was used.

Technical Considerations

The facial nerve injury model has been successfully used for decades to study nerve degeneration and regeneration as well as microglial function and physiology (Moran and Graeber, 2004). Advantages of the facial nerve paradigm include the fact that facial nerve

transection and crush are believed to be mild injuries that are generally well-tolerated by rats, which is important for our study in light of the fact that the animals will have long post-injury survival times (up to 9 nine months) and will endure the procedure several times. The repeated injury model is a novel variation on the classical model that has not been previously tested. It was not anticipated that multiple injuries may lead to health problems, excessive pain, discomfort or sickness. In the first trial of this experiment 30 animals were used to assess proliferation after a total of 4 facial nerve injuries each (n =10 for each time point, 3, 4 or 5 days post-injury). Ultimately, 13% of the animals used in the study had to be sacrificed prior to the conclusion of the experiment due to illness (Figure 2-2). Noted maladies included swollen hind limbs, tumors of variable localization across animals and idiopathic weight loss. An additional 3% of subjects died from unknown causes, with notable weight loss. All animals that became sick or died did so toward the end of the study, usually after a total of 3 injuries at 9-12 months of age. It is not suspected that weight loss was due to impaired mastication or overall decrease in consumption because the remaining animals in the study were not underweight. In fact, animals that underwent repeated injury weighed slightly more on average than those that had only 1 injury at the same age (Figure 2-3). Alternatively, it is thought that the animals experienced excessive levels of stress and possibly chronic, excessive or prolonged pain resulting from their injuries. It is not recommended that this injury model be used in the future due to animal welfare concerns.

In addition to animal welfare issues, the repeat injury model proved problematic because of excess scar tissue formation. Following the fourth and final injury, 44% percent of the remaining animals in the study retained whisker movement after surgery (Figure 2-2). A high

degree of scarring and disfigurement of the facial nerve made localization and identification of the nerve problematic.

To resolve the animal welfare and technical issues raised above, the repeat injury paradigm was modified to include a total of only 3 injuries (Figure 2-1). Eliminating the fourth surgery resulted in a better surgery success rate and improved the survival rate of the animals (Figure 2-4). Importantly, despite the reduction in the number of injuries, 4% of the subject group still died or had to be euthanized due to similar problems encountered in the 4 injury design. While this modification allowed for successful completion of the study, we maintain the position that this model (including either 3 or 4 surgeries) should not be repeated due to animal welfare concerns.

Finally, the assessment of whisker recovery was performed 14 days post- injury. This time point was chosen because of literature reports purporting that functional recovery occurs between 14 and 21 days post crush injury. Animals regained function of their whiskers by 14 days after 1 or 2 injuries. Because it was not anticipated that regeneration would occur faster with subsequent injuries, the first assessment after a 3rd injury was also carried out 14 days post-injury. No animal had regained function at this time and a second evaluation was done 10 days later. It was not expected that microglial dysfunction would significantly inhibit axonal regeneration, but because we noticed a delay in functional recovery beyond 14 days, this assumption may have been incorrect. Future studies should include a careful evaluation of recovery of whisker movement post-injury.

Results

Dividing Cells are Present in the Injured Facial Motor Nucleus Following Repeat Injury

Following a single facial nerve injury, microglia in the injured facial motor nucleus undergo a proliferative burst that begins 2 days after injury, peaks at 3 days post-injury and

declines thereafter (Kreutzberg, 1968; Streit et al., 1999; Moran and Graeber, 2004). In agreement with the established time course of microglial proliferation post-axotomy, clearly identifiable BrdU+ cells were visible at 3, 4 and 5 days post-injury in the lesioned facial nucleus of singly-injured control rats as well as experimental animals that underwent 3 (4 and 5 days post-injury) or 4 (3 days post-injury) nerve crush injuries. (Figure 2-5). There were no BrdU+ cells in the uninjured nuclei of any animals in the control (FNCx1) or experimental (FNCx3 or x4) groups.

Repeated Facial Nerve Injury Results In a Significant Reduction in Cell Proliferation in the Lesioned Facial Nucleus

It was hypothesized that repeated facial nerve injury would induce replicative senescence of the pool of microglia in the lesioned nucleus. Consistent with this hypothesis, there was a significant reduction ($p<0.05$) in the number of proliferating cells in the injured facial motor nucleus 3 days post-injury in animals that had a total of 4 nerve injuries (Figure 2-6). Similarly, there was an appreciable, but not significant, reduction in BrdU+ cells at 4 and 5 days post-injury after a total of 3 injuries (Figure 2-6).

All Proliferating Cells in the Singly-Injured or Repeatedly Injured Facial Nucleus are Microglia

All BrdU positive cells colocalized with lectin positive microglia after 1, 3 or 4 facial nerve crush injuries at all time points examined (Figure 2-7). BrdU labeled microglia were located perineuronally as well as in the perikarya of the lesioned facial nucleus.

Repeated Facial Nerve Injury Does Not Lead to Significant Neuronal Loss

Numerous studies demonstrate that facial nerve axotomy in adult rats does not lead to marked neuronal degeneration (Streit and Kreutzberg, 1988; Mattsson et al., 1999; Moran and Graeber, 2004). Accordant with neuronal survival patterns after a single nerve injury, there was no significant change in the number of neurons in the injured facial nucleus 3 days after a fourth

nerve crush, as identified by Nissl staining (Figure 2-8, 2-9). Similarly, few, if any, phagocytic microglial clusters were visible within the injured nuclei (Figure 2-10). Taken together, this suggests that there is little, if any neuronal degeneration taking place in the repeatedly injured facial nucleus.

There is No Change in the Overall Number of Microglia in the Facial Nucleus After Repeat Nerve Injury

To determine if there was an overall change in the total number of microglia in the facial motor nucleus after repeated injuries, we calculated the percent of total area in the facial nucleus that was occupied by Iba1-positive microglia 5 days post-injury after a single or 3 nerve crush injuries (Figure 2-11). There was no significant difference in the density of microglia in the facial nucleus after multiple injuries compared to single injuries (Figure 2-12). Thus, it can be concluded that decreased overall numbers of microglial cells is not the cause of reduced numbers of proliferating cells seen after multiple nerve injuries.

There is a Delay in Functional Recovery Following Multiple Nerve Facial Nerve Injuries

Facial motor neurons innervate the facial musculature of the rat that control whisker movement (Moran and Graeber, 2004). Functional control of whiskers is temporarily lost after nerve injury and is regained only after nerve regeneration. Reports show that functional recovery of whisker movement occurs as early as 14 days after nerve crush injury. We evaluated whisker movement in all rats at 14 days after 1 or 2 injuries. All animals had regained whisker movement at this time point. Following a 3rd nerve injury, whisker movement was assessed at 14 days and no animal had yet recovered function. The next analysis was at 24 days, by which time all animals had regained whisker control to the same level exhibited 14 days post-lesion 2.

Discussion

The facial nerve injury paradigm has been well established as an ideal model for studying microglial activation (Moran and Graeber, 2004). This model is advantageous primarily because there is no direct trauma to the CNS and the blood brain barrier remains intact, providing an opportunity to study purely endogenous glial responses. Insights gained from studies employing the facial nerve axotomy reveal that microglial proliferation begins as early as 12 hours post-lesion, peaks at approximately 3 to 4 days after insult and declines thereafter. To expand our understanding of the replicative potential of microglia and gain better insight into the potential effects of exogenous stressors, such as strong, repeated mitogenic stimulation and prolonged high metabolic demand on microglial function, we conducted the present study examining the cells' proliferative ability following repeated facial nerve injury. In contrast to the more heterogeneous effect of aging in the brain, we hypothesized that challenging the same pool of microglia multiple times would result in replicative senescence independent of aging effects. To test this theory, rats were subjected to either one or a series of 3 or 4 facial nerve crush injuries and euthanized by 9 or 12 months of age, respectively. To illustrate that this model reflects a repeat-injury model as opposed to a model of chronic nerve injury, we assessed whisker function in all animals and found that functional recovery had occurred in both control and experimental animals by 2 weeks-post-axotomy following 1 or 2 injuries and by 24 days post-injury after a third nerve crush. We then counted the number of proliferating microglia in the facial nucleus after multiple injuries and contrasted that to the number of dividing microglia in the nuclei of animals that received only one nerve injury. Consistent with the established microglial response to nerve injury, we found numerous proliferating cells in the injured facial nucleus of both groups at 3, 4 and 5 days post-injury. We performed double-immunohistochemistry with

microglia-specific *Griffonia simplicifolia* B_4 isolectin and verified that the dividing cells were microglia.

In agreement with our hypothesis that repeated injury would induce replicative senescence, we found a statistically significant decrease in the number of BrdU-positive microglia in the facial motor nucleus after repeated nerve crush injury. After multiple injuries there was an approximate 50% reduction in microglial proliferation in the injured facial nucleus at 3 days post-crush (Figure 2-6). Similarly, there was an obvious trend toward reduced proliferation in repeatedly injured animals at 4 and 5 days, but this difference was not significant. The induction of cellular senescence in this model is supported by numerous studies describing the effects of strong or persistent mitogenic stimulation on other cell types (Pearson et al., 2000; Mathon et al., 2001; Michaloglou et al., 2005; Bartkova et al., 2006). These aforementioned reports provide evidence that excessive mitogenic stimulation serves to activate signaling pathways that lead to proliferative arrest and senescence. "Mitogen-induced" senescence occurs in response to cell-intrinsic alterations in genes encoding cell-cycle regulators, but has been shown to be influenced by extrinsic factors. For example, it has been shown that culturing cells in serum free medium, thereby eliminating mitogenic stimulation, prevents or delays cellular senescence (Mathon et al., 2001; Bartkova et al., 2006). The induction of senescence and growth arrest in response to oncogene expression or immoderate mitogenic stimulation is believed to represent a tumor-suppressive mechanism. As such, it is likely that this mechanism of senescence induction is highly conserved and active in microglial cells in the same manner as other cell types. Furthermore, it has been shown that microglia maintained in culture with mitogenic medium are subject to telomere shortening and replicative arrest. Moreover, it has been found that telomere attrition occurs in association with aging and AD *in vivo* in both rodent and human brains

(Flanary and Streit, 2003, , 2004; Flanary et al., 2007). It is possible that repeated rounds of proliferation induced by multiple injuries may have led to critical telomere shortening and the induction of replicative senescence in our model. Complicating this argument is an experiment that reports telomerase upregulation and an increase in telomere length following a single facial nerve injury (Flanary and Streit, 2005), suggesting that microglia upregulate telomerase in response to mitogenic stimulation in an attempt to bypass senescence. However, careful review of the literature reveals that this phenomenon also occurs in cultured microglia that ultimately undergo senescence via telomere-independent mechanisms despite telomerase upregulation (Flanary and Streit, 2004).

In contrast to the significant decline in proliferating cells 3 days post-injury in repeat injury animals, the number of proliferating cells was not significantly different between single or multiple injury animals at 4 and 5 days post-crush, although there were notably fewer dividing cells at both of these time points. There is one potentially meaningful difference between animals sacrificed 3 days post-injury versus those sacrificed 4 or 5 days post-injury that may explain the smaller decline in microglial proliferation seen in the latter groups. Namely, the three groups sustained different numbers of injuries. Animals included in the 3 day post-injury time point group underwent a total of 4 nerve crushes, while animals included in the 4 and 5 day time-point groups only received 3 injuries (due to modifications in experimental design intended to minimize animal pain and suffering). If microglia in the injured facial nucleus are undergoing senescence in response to repeated injury, it makes sense that the number of senescing cells would increase with time and increasing numbers of injuries. In fact, it is known that cells within a population undergo senescence at varying times and rates so that a given population of cells at any one time is heterogeneous in regards to senescent versus viable cells (Cristofalo and

Sharf, 1973). This may explain why some cells in the lesioned facial mucleus still proliferate after repeated injury.

Considering the regulatory role of neuronal signaling in microglial activation and the possibility that neurons may undergo degeneration after repeated axonal injury, we evaluated neuronal survival 3 days after either a single facial nerve crush or 4 such injuries. Nissl staining revealed that there was a slight, but not significant, decrease in motor neuron number in repeatedly injured nuclei compared to the uninjured internal control nuclei and singly-injured animals (Figure 2-9). Additionally, we observed no changes in neuronal morphology or phagocytic microglial clusters, suggesting that the cells remained viable. Due to the inappreciable difference between groups, we do not attribute the decline in microglial proliferation to a lack of mitogenic signaling from injured facial motor neurons.

Following the proliferative burst that microglia undergo in response to facial nerve injury, the cells migrate away from the facial motor nucleus or undergo apoptosis in order to maintain homeostatic population levels (Gehrmann and Banati, 1995; Jones et al., 1997). Another possible explanation for the decreased number of proliferating cells in the repeatedly injured facial nucleus is that the overall number of microglia in the injured nuclei declined owing to increased microglial death following multiple injury. In order to investigate this possibility, we used microglial-specific Iba1 immunohistochemistry to assess the number of microglia present in the facial nucleus of repeat injury animals compared to singly-injured animals. We evaluated the percent area of the lesioned nucleus that was occupied by Iba1-positive cell bodies, and found no difference between groups. Therefore, it can be concluded that although the same number of microglia are present, only a subpopulation of cells proliferated in response to nerve injury.

The data presented in this chapter show that microglia experience a decline in mitotic activity in response to repeated crush injury of cranial nerve VII. Importantly, this change in proliferative activity occurs in the absence of neuronal degeneration and therefore any suspected changes in neuronal signaling. Furthermore, there is no difference in the population size of microglia in the repeatedly injured nuclei as compared to singly injured nuclei. This suggests that although the same number of cells are present, not all are responsive to mitogenic signals. Collectively, this data supports the hypothesis that microglia are subject to cellular senescence and growth arrest in response to repeated injury. Like most senescent cells in the body, senescent microglia likely exhibit a deterioration of cell function that is detrimental to tissue homeostasis. The evaluation of one such critical aspect of microglial function in response to repeated injury, namely the production of the neuroprotective cytokine TGFβ, is discussed in Chapter 3.

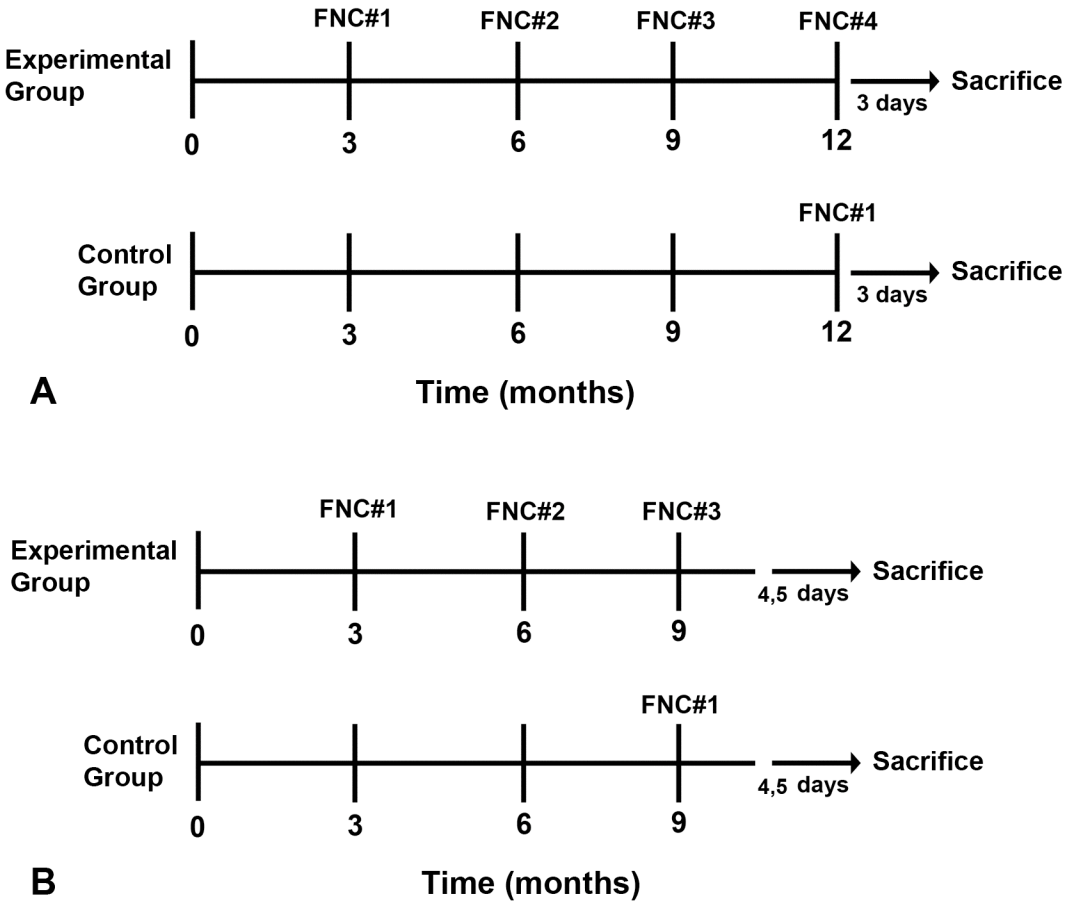

Figure 2-1. The repeat facial nerve injury model. Experimental animals received a total of A) 4 or B) three crush injuries to the right facial nerve over the course of A) 1 year or B) nine months. The first nerve injury was performed at 3 months of age, with subsequent injuries at 3-month intervals. Animals were sacrificed 3 (A), 4 or 5 (B) days after the final injury. Single nerve crush controls received 1 right facial nerve crush at 12 (A) or 9 (B) months of age.

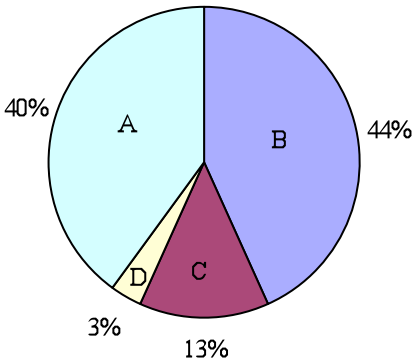

Figure 2-2. Repeat injury study subject data. Facial nerve crush x 4. A) % animals that survived and were included in study. B) % animals that retained whisker movement post-operatively. C) % animals that were sacrificed due to illness. D) % animals that died during study. Percentages are calculated from a total n =30.

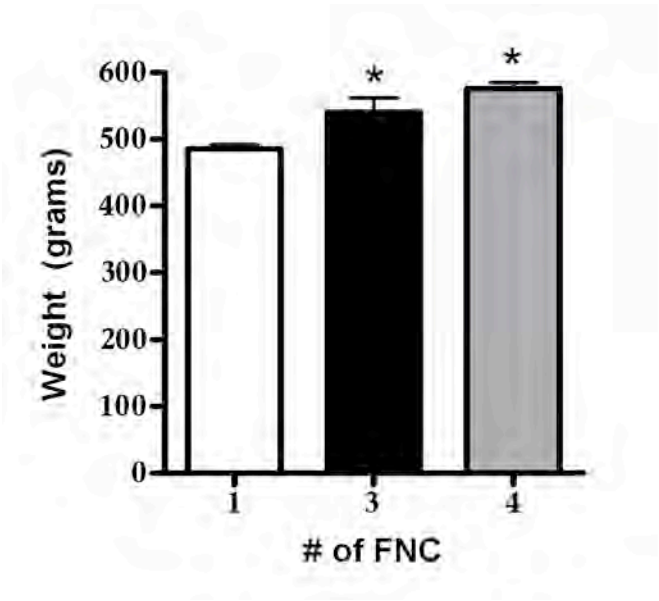

Figure 2-3. Animal Weight. Repeat nerve injury did not result in impairment of mastication or appetite. Animals were weighed at the time of sacrifice. All rats were aged 9-12 months at weighing. N = 7 for FNCx4; n = 26 for FNCx3; n = 24 for FNCx1. * $p < 0.001$.

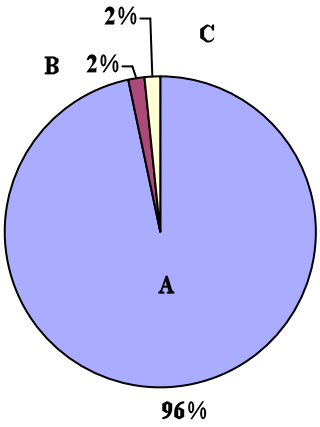

Figure 2-4. Repeat injury study subject data. Facial nerve crush x 3. A) % animals that survived and were included in study. B) % animals that were sacrificed due to illness. C) % animals that died during study. Percentages are calculated from a total n = 60.

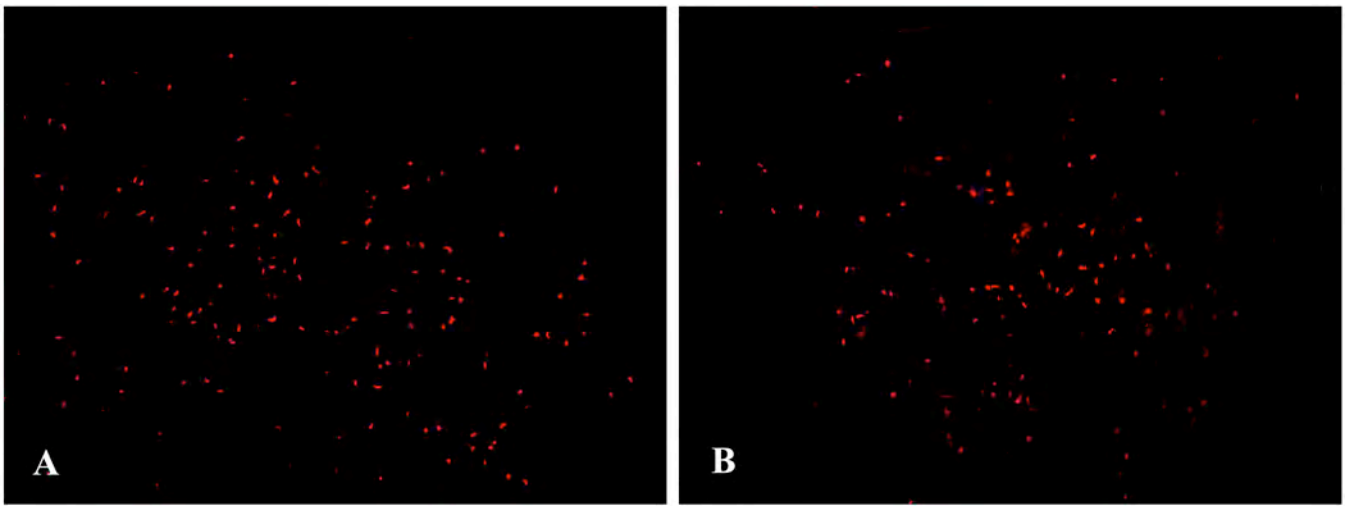

Figure 2-5. Proliferating cells in the injured facial motor nucleus. A, B) BrdU-positive cell nuclei are distributed throughout the facial nucleus after one (A) or four (B) nerve crush injuries in animals aged 12 months. Images taken at 10x magnification.

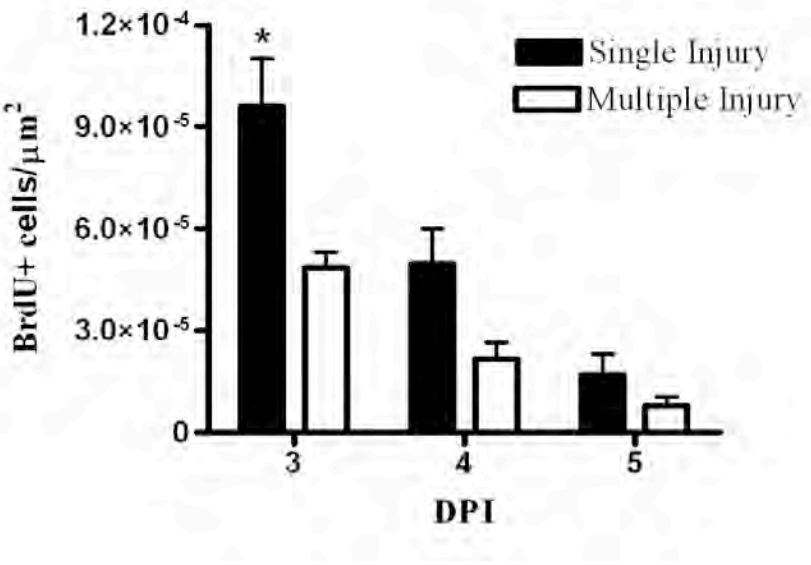

Figure 2-6. Cell proliferation in the injured facial motor nucleus. There were significantly fewer proliferating cells in the lesioned facial nucleus 3 days post-injury in animals that received multiple (4) nerve injuries. There was also a trend toward reduced numbers of proliferating cells at 4 and 5 days post-injury in repeatedly (3) injured animals. N = 5 per group. Data are represented as mean number of BrdU + cells $\pm$ SEM per μm^2. * $p < 0.05$. DPI = days post injury.

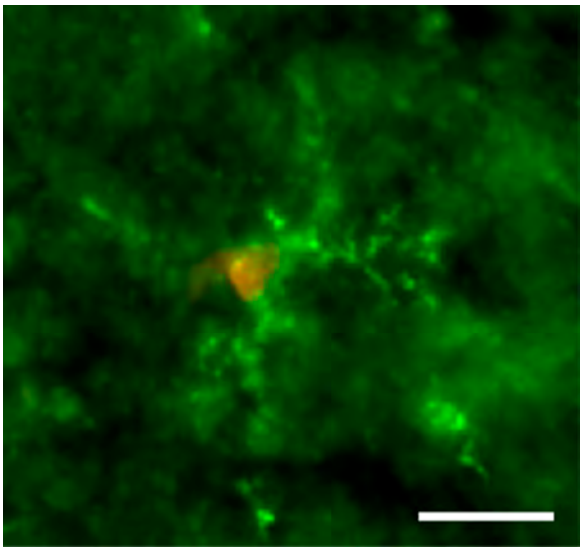

Figure 2-7. Proliferating cells in the injured facial motor nucleus are microglia. Shown is co-labeling of microglia-specific *G. simplicifolia* B_4 isolectin (green) and anti-BrdU antibody (red). Scale bar, 10 μm.

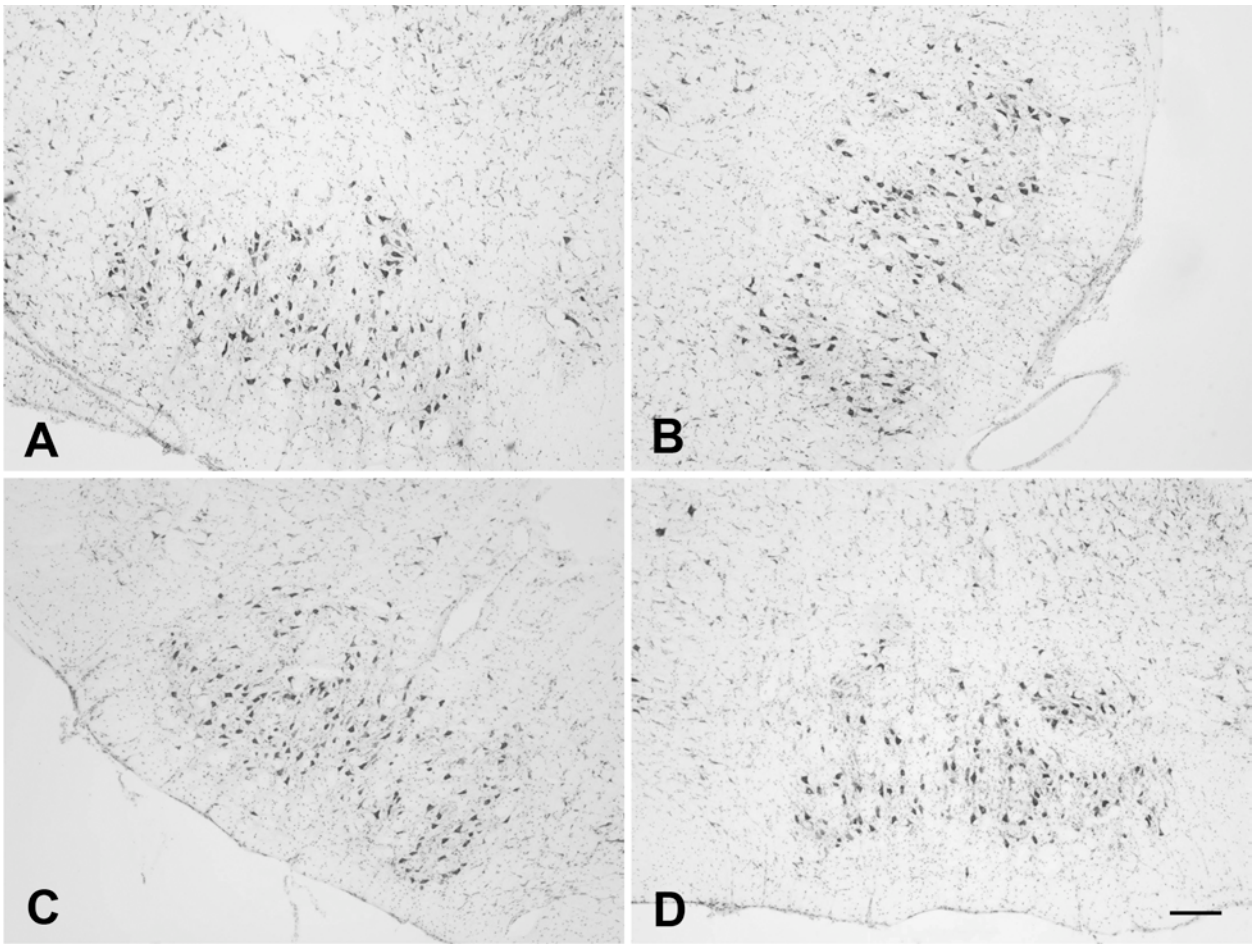

Figure 2-8. Repeated facial nerve injury and neuronal survival. Repeated injury did not result in a prominent reduction in the number of motor neurons in the facial nucleus 3 days after injury. A) The unoperated (contralateral) facial nucleus of an animal that received a single nerve crush. B) There is no appreciable difference in the number of neurons in the injured (ipsilateral) nucleus of the same animal. C, D) There is a similar pattern of neuronal survival in animals that received four nerve crush injuries. Repeated injury did not result in a pronounced difference in the number of neurons in the unoperated (C) or injured (D) facial nuclei. Cells were visualized with cresyl-violet staining. Scale bar, 200 μm.

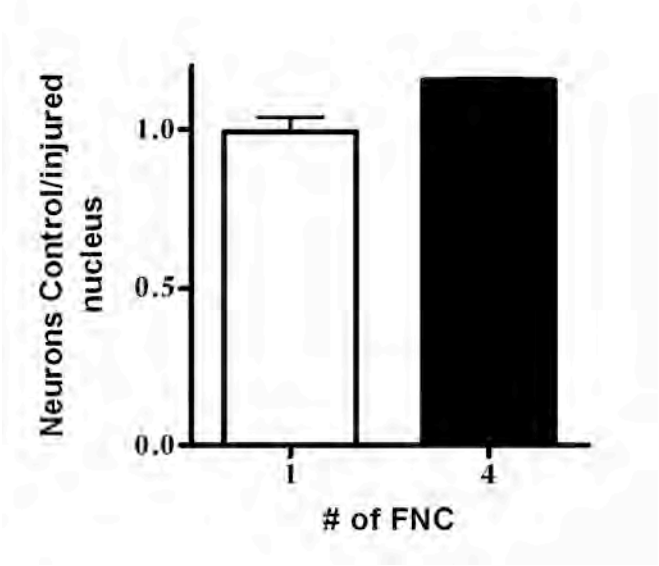

Figure 2-9. Neuronal survival in the injured facial motor nucleus. The ratio of neurons in the unoperated (control) nuclei and the lesioned nuclei of animals that received one or four facial nerve crush injuries is similar. There is only a slight decline in neuronal survival following repeated nerve injury. Data is represented as the mean $\pm$ SEM ratio of cresyl-violet stained neurons present. N = 4-5 per group.

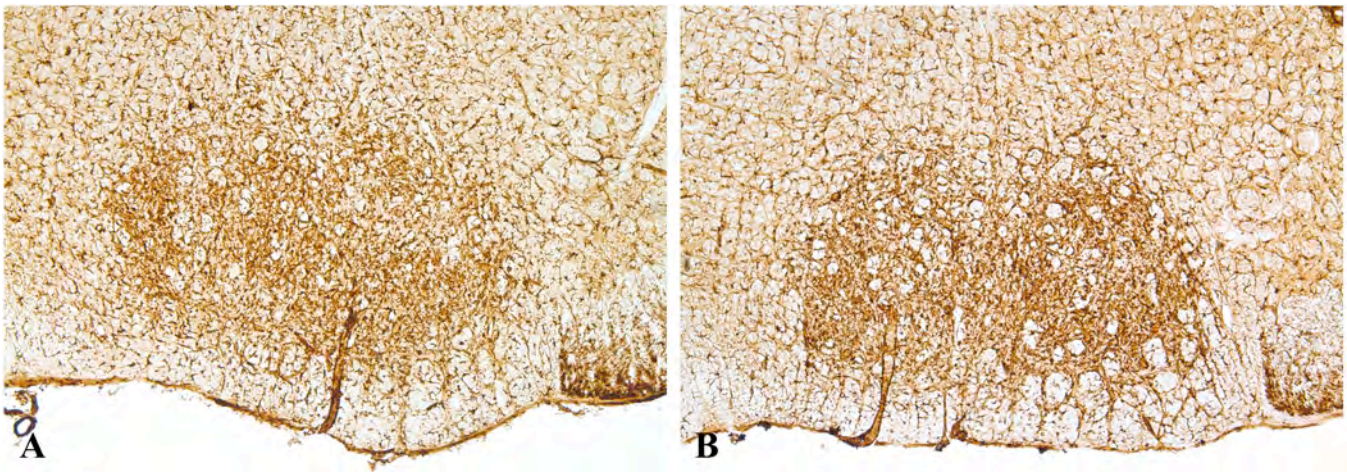

Figure 2-10. Microglial-specific lectin. Lectin staining in the injured facial motor nucleus after one (A) or four (B) nerve crush injuries. Reactive microgliosis occurs in the absence of microglial cluster formation after 1 or multiple injuries. Images taken at 5x magnification.

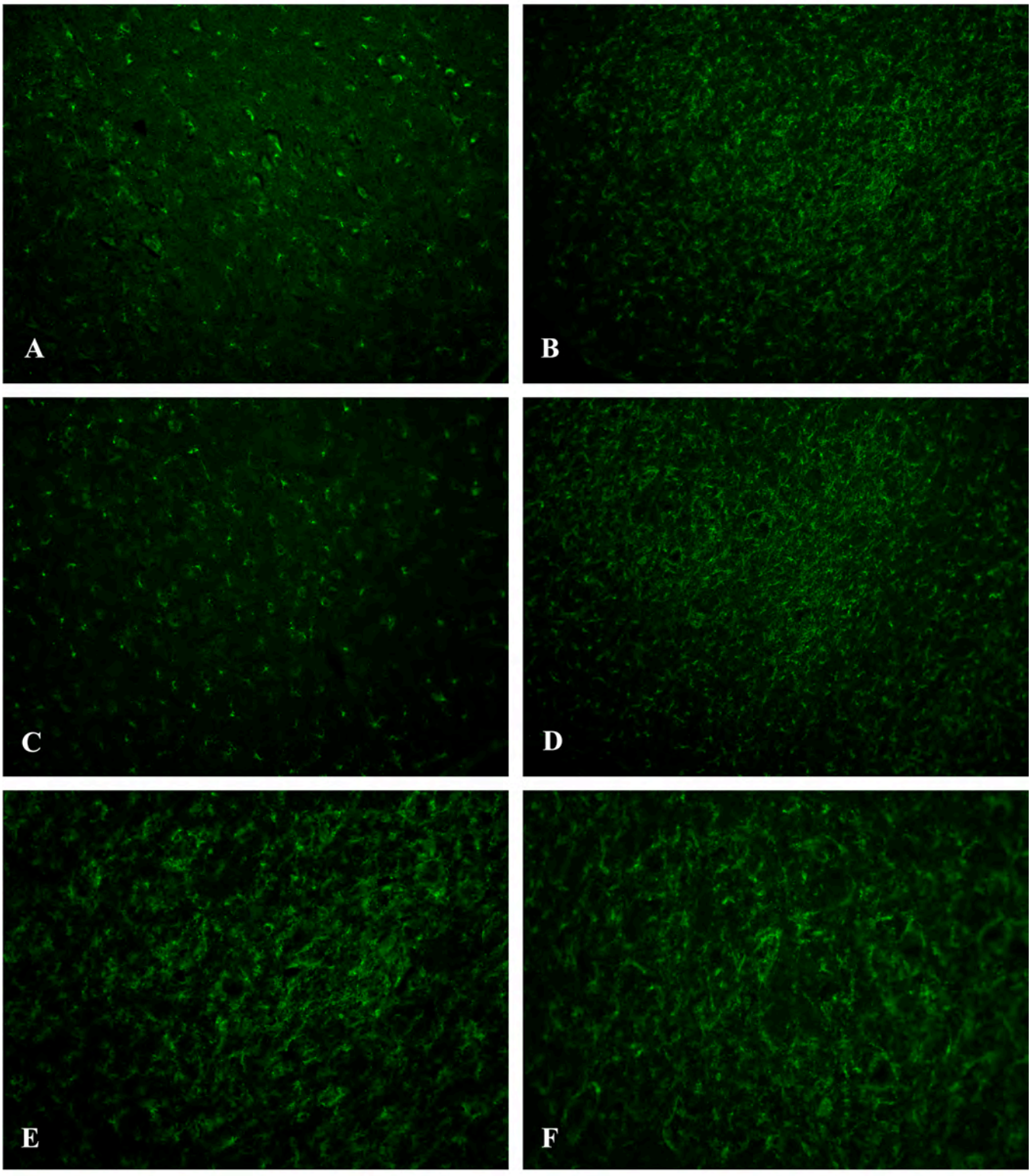

Figure 2-11. Microglial-specific Iba1.

Figure 2-11 continued. Microglial-specific Iba1. Iba1 staining in the control (A, C) and injured (B, D, E, F) facial motor nuclei. The distribution and number of microglial in the facial nucleus does not change after repeated nerve injury. A, B) Noninjured and injured facial nuclei of animal that received 1 nerve crush injury. C, D) Noninjured and injured facial nuclei of animal that received 3 nerve crush injuries. E, F) High magnification images of the injured facial motor nucleus after 1 (E) or 3 (F) nerve crush injuries. All images taken 5 days post-injury. A-D taken at 10x magnification; E and F taken at 20x magnification.

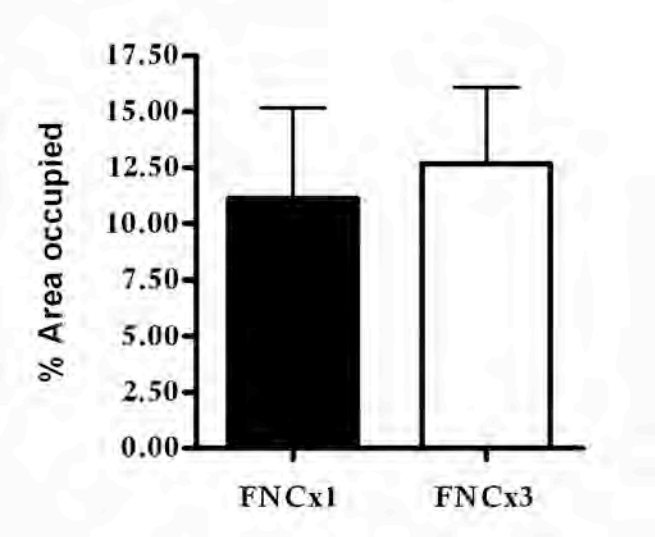

Figure 2-12. Microglia in the injured facial motor nucleus. There is no significant decrease in the number of microglia in the facial nucleus in response to repeated injury. Data represent the % area of the facial nucleus occupied by Iba1+ microglia 5 days after 1 or 3 nerve crush injuries. N = 4 per group. FNC = facial nerve crush.

CHAPTER 3
THE EFFECT OF REPEAT NERVE INJURY ON MICROGLIAL-DERIVED TRANSFORMING GROWTH FACTOR BETA PRODUCTION

Introduction

Transforming growth factor- betas (TGFβs) comprise a family of multifunctional growth factors. Five isoforms of TGFβs have been identified and named TGFβ1-5. Isoforms β1, β2 and β3 are expressed in mammals (Ohta et al., 1987; Madisen et al., 1988), while β5 and β6 represent TGFβ1 homologues in the chicken and Xenopus, repsectively (Burt and Law, 1994). There are three TGFβ receptors that have been identified and designated types I, II and III (Massague et al., 1990; Derynck, 1994). Receptor types I and II belong to a class of serine/threonine kinase receptors and act to mediate signal transduction through interaction of both receptors, probably as a tetramer structure composed of two type I and two type II molecules (Wrana et al., 1992; Massague and Weis-Garcia, 1996). Type III TGFβ receptors are proteoglycans thought to mediate ligand binding to receptor II (Massague et al., 1994). TGFβ is produced as a preproprotein typical of secreted proteins (Lindholm et al., 1992; Flanders et al., 1998; Bottner et al., 2000). The TGFβ precursor molecule consists of an N-terminal signaling peptide that targets the molecule to the secretory pathway, a pro-domain responsible for protein folding and a C-terminal fragment that is released upon cleavage to generate a bioactive molecule (Sporn and Roberts, 1990; Kingsley, 1994). Although the TGFβs share more than 95% identity between mature isoform sequences (Flanders et al., 1998), each isoform has distinct expression patterns and functional repertoires.

The spectrum of functions attributed to the TGFβs includes cell proliferation and differentiation, extracellular matrix production, chemotaxis, angiogenesis, immunosuppression and regulation of apoptosis ((Roberts et al., 1990). TGFβ2 and 3 are widely expressed throughout the nervous system in neurons, astrocytes and Schwann cells (Flanders et al., 1991;

Pelton et al., 1991; Unsicker et al., 1991; Unsicker et al., 1996; Bottner et al., 2000). On the other hand, TGFβ1 is nearly undetectable in the absence of injury or disease (Wilcox and Derynck, 1988; Flanders et al., 1991; Pelton et al., 1991; Unsicker et al., 1991; Unsicker et al., 1996; Bottner et al., 2000). However, TGFβ1 is consistently upregulated in response to a variety of insults including hypoxic-ischemic events, stab wounds, electrolytic entorhinal cortex lesion (ECL), kainic acid exposure, cranial nerve axotomy, HIV infection, Alzheimer's disease and Down syndrome (Nichols et al., 1991; Wahl et al., 1991; Klempt et al., 1992; Lindholm et al., 1992; Morgan et al., 1993; Colosetti et al., 1995; Streit et al., 1998; Chen et al., 2002). In the periphery, TGFβ1 participates in wound healing resulting in improved tissue repair (Roberts and Sporn, 1996). The combined understanding of TGFβ1 function in the periphery and its established upregulation in the injured nervous system suggests a neuroprotective role for TGFβ1 in the CNS.

While TGFβ2 and 3 isoforms are expressed primarily in neurons and astrocytes of the CNS, TGFβ1 has been localized to microglial cells following cranial nerve axotomy, cortical stab wound, transient global ischemia and hippocampal lesion (Lindholm et al., 1992; Kiefer et al., 1993b; Morgan et al., 1993; Lehrmann et al., 1995). Levels of TGFβ1 begin increasing as early as 2 days post-injury (Kiefer et al., 1993b), reaching peak levels between 4 and 7 days (Morgan et al., 1993; Streit et al., 1998). After facial nerve axotomy, a second peak is reported at about 21 days post-lesion, corresponding to the approximate time of nerve regeneration in this model (Kiefer et al., 1993a). Although the exact role of TGFβ in the facial nerve injury model is unknown, evidence suggests at a neurosupportive function. For example, TGF has been proven to promote survival of spinal cord neurons, cultured rat and chick embryonic motorneurons and midbrain dopaminergic neurons (Martinou et al., 1990; Krieglstein et al., 1995; Gouin et al.,

1996). However, the co-activation of TGFβ with cytokines, such as glial-derived neurotrophic factor (GDNF), ciliary neurotrophic factor (CNTF) or nerve growth factor (NGF) is sometimes required for neurotrophic actions (Martinou et al., 1990; Lindholm et al., 1992; Krieglstein et al., 1995; Gouin et al., 1996; Unsicker and Strelau, 2000).

We have described in Chapter 2 of this report that microglia exhibit decreased proliferation following repeated facial nerve injury. It is hypothesized that this change in mitotic potential is a result of cellular senescence. In addition to growth arrest, senescent cells often exhibit an overall dysregulation of coordinated processes (see Chapter 1). Because TGFβ1 is one of the most robustly upregulated cytokines following facial nerve injury and has been shown to produced by microglial cells, we analyzed TGFβ1 expression by *in situ* hybridization in animals that underwent repeated facial nerve injury and in aged rodents. It was suspected that aging-related or injury-induced cellular senescence may result in altered cytokine production.

Materials and Methods

Animals and Surgery

Repeat injury experiment

Male Sprague-Dawley rats (Harlan, Indianapolis, IN) aged 3 (multiple injury (experimental) groups) or 9 (single injury (control) group) months at the time of initial or sole injury, respectively, were used (Figure 2-1B). Animals were housed under standard SPF conditions in the McKnight Brain Institute animal facility. Under isoflurane anesthesia, the right facial nerve was exposed near its exit from the stylomastoid foramen. The nerve was crushed once with a pair of fine forceps for 10 sec, approximately 2 mm from the stylomastoid foramen (~12–14 mm from the facial nucleus in the brainstem). In multiple injury animals, nerve crush was performed as close as possible to the site of primary injury, moving proximal to the brain as necessary. Lack of whisker movement on the right side was verified after the animals recovered

from anesthesia. Any animals that retained whisker movement after surgery were excluded from the study. The contralateral (unoperated) facial nucleus served as an internal control in all experiments. Animals were sacrificed 3, 4 or 5 days after the final injury (3^{rd} axotomy in experimental groups; 1^{st} axotomy for all control groups). Subjects were anesthetized with an overdose of sodium pentobarbital and transcardially perfused with 0.1M phosphate-buffered saline (PBS, pH 7.2) followed by 4% paraformaldehyde in PBS. Brain tissue was removed and post-fixed for two hours in 4% paraformaldehyde and subsequently frozen in methyl butane cooled in liquid nitrogen and stored at -80° C until tissue processing.

Aging experiment

Young (3 months) and old (30 months) male Fisher 344 Brown Norway hybrid rats (National Institute of Aging) were subjected to facial nerve crush. Animals were sacrificed at 3, 5 or 7 days post-injury using transcardial perfusion as described above. Following 2 hours of post-fixation in 4% paraformaldehyde, brains were frozen in methyl butane cooled in liquid nitrogen and stored at -80° C until tissue processing.

Tissue Processing

Frozen brains were allowed to equilibriate in the cryostat chamber at -20°C for 30 min. before sectioning. Twenty micrometer coronal sections were cut on the croyostat and mounted onto Superfrost Plus slides in a pattern that allowed 2 sections from different caudal-rostral regions of the facial nucleus to be mounted on each slide. A puncture mark was made on the contralateral side of the brain to distinguish the injured and non-injured nuclei. Slides were immediately stored at -80°C until use for *in situ* hybridization.

***In Situ* Hybridization**

In situ hybridization (ISH) to analyze the mRNA expression of TGF-β1 was carried out on brainstem sections containing the facial motor nucleus. Sections were collected from repeat or

single injury animals as well as young and old animals. Linearized TGFβ cDNA 281 base-pairs long, corresponding to nucleotides 1261-1541 of the full rat TGF-β1 mRNA sequence was obtained from the lab of Dr. Jeffery Harrison. Sense and antisense riboprobes for ISH were generated by transcribing the cRNA using either T7 or SP6 RNA polymerase, respectively, in the presence of ^{33}P-UTP. Hybridization of ^{33}P-riboprobes to rat brainstem sections was carried out according to the Harrison laboratory published protocol (Harrison et al., 2003). After ISH, radiolabeled sections were exposed to film for 8 days and subsequently dipped in Kodak NTB2 emulsion and exposed in light-tight boxes at 4°C for 4 weeks. After slides were developed, they were counterstained with hematoxylin and eosin.

Quantitative Analysis

Quantitative analysis of the TGFβ hybridization signal was conducted by densitometric analysis of the autoradiograohic films using MCID software (InterFocus Imaging, Cambridge, UK). The intensity of the hybridization signal in the facial nucleus was determined by measuring the relative optical density (ROD) of the signal. Intensity was measured in 4-6 sections from each animal. Results are represented as mean values $\pm$ SEM. Significant differences were determined by One-way ANOVA with nonparametric Kruskal-Wallis test using GraphPad Prism software (GraphPad Software, San Diego, CA). A significance level of $p \leq 0.05$ was used.

Results

There is No Age-Related Change in TGFβ1 mRNA Expression in Response to Facial Nerve Injury

TGFβ1 mRNA levels were analyzed by ISH to determine if there is an age-related change in microglial signaling in the injured facial nucleus. Young (3 months) and aged (30 months) rats were subjected to a single nerve crush and brain sections containing the facial motor nucleus

were probed with sense and antisense TGFβ1 riboprobes at 3, 5 and 7 days post-injury. Autoradiographs showed clearly that there is an upregulation of TGFβ mRNA expression after nerve crush at all time points examined (Figure 3-1). However, densitometric analysis of hybridization signals in the facial nucleus revealed no difference in TGFβ1 mRNA levels between young and aged animals (Figure 3-2). Sense probes showed no hybridization signal.

There is No Change in TGFβ1 mRNA levels in the Facial Motor Nucleus in Response to Repeated Nerve Injury

In an attempt to determine if changes in proliferative changes in the repeat facial nerve injury model are accompanied changes in the mRNA expression levels of microglial-derived TGFβ1, we performed ISH using tissue collected from rats 3, 4 or 5 days after a 1st or 3rd facial nerve crush injury. Autoradiographs showed clearly that there is an upregulation of TGFβ mRNA expression after nerve crush at all time points examined (Figure 3-3). However, densitometric analysis of hybridization signals in the facial nucleus revealed no difference in TGFβ1 mRNA expression levels between single or repeat injury groups (Figure 3-4). Sense probes showed no hybridization signal. Emulsion dipping revealed silver grains localized to microglial cells located both perineuronally and throughout the perikarya of the facial nucleus (Figure 3-5).

Discussion

TGFβ1 expression following facial nerve injury is a well-established microglial response to nerve lesion (Kiefer et al., 1993a; Streit et al., 1998). TGFβ1 upregulation is reported as early as 2-4 days post-injury and reaches peak levels 7 days after axotomy. We reported in Chapter 2 that repeated injury to the facial nerve results in a decline in microglial mitosis. It is hypothesized that this effect is a result of cellular senescence. In order to evaluate if other changes in microglial function occur as a result of repeated injury, we evaluated mRNA

expression levels of the microglial-derived cytokine TGFβ1. It was hypothesized that senescent microglia may exhibit changes in the levels of TGFβ1 produced after multiple injuries. In consideration of data showing that TGFβ inhibits microglial proliferation *in vitro* (Suzumura et al., 1993), increased levels of TGFβ could explain the decline seen in mitotically active microglia after repeated nerve lesion. We also assessed TGFβ1 expression in aged rats to determine if normal aging leads to alterations in the microglial response to injury. We sought to compare potential age-related changes in cytokine production to those anticipated after repeated injury. Such a result could support the hypothesis that microglia in the latter case are exhibiting senescence-associated changes.

ISH revealed that normal increases in TGFβ1 mRNA are seen at all time points analyzed in aged rats after nerve crush injury. These results are consistent with another ISH study conducted on aged animals after facial nerve axotomy that also reported no age-related changes in TGFβ1 mRNA levels (J. Conde, unpublished data). When proliferation was analyzed in aged rats in the same study, slight, albeit statistically significant, alterations in microglial mitosis were only seen at only one time point (4 days post-injury) (Conde and Streit, 2005). It was proposed that these changes were a result of aging-related functional dysregulation, but additional experiments may be needed to verify these results. One explanation for the modest changes in proliferation seen in aged rats and the lack of differences in TGFβ mRNA demonstrated in this and Dr. Conde's study is that age-induced alterations in cytokine production may be modest and undetectable by the methods employed in these studies. Another possibility is that viable microglia in the facial nucleus are compensating for decreased cytokine production by senescent cells. Finally, it is possible that aged microglia do not undergo changes in the normal pattern of TGFβ mRNA expression.

As seen in aged rats subjected to facial nerve axotomy, there is no change in TGFβ1 mRNA expression levels in the facial nucleus of animals subjected to repeated nerve injury. Furthermore, TGFβ mRNA was localized to microglia as expected. This result must be considered in combination with the data presented in Chapter 2. We showed that repeated injury results in significantly reduced microglial proliferation. It was hypothesized that proliferative changes occurring in response to multiple injuries are a result of cellular senescence and as such, mitotic alterations might be expected to be accompanied by other functional changes in microglia, such as cytokine production. This theory is supported by experiments demonstrating that aged peripheral macrophages, close relatives of microglia, produce altered levels of pro- and anti-inflammatory cytokines, including TGFβ (Ashcroft et al., 1997; Swift et al., 1999). Although this was not the case, the data reported herein do not rule out the possibility that repeated nerve injury results in cell senescence. It is possible, and most likely, that microglia in the repeatedly injured facial motor nucleus enter senescence without undergoing changes in all functions. Furthermore, as discussed in regards to results seen in aged rats, senescent-associated changes in cytokine expression levels are likely to be modest and may require more sensitive methods of quantization. Moreover, compensatory mechanisms may act to maintain necessary levels of growth factor in order to protect injured neurons. Finally, it is also possible that while mRNA levels remain normal, protein levels are altered. Support of this idea is demonstrated by evidence showing that TGFβ bioactivity is largely reliant upon protein processing, and changes in physiological activity levels may not be accompanied by changes in mRNA expression (Assoian et al., 1987).

TGFβ has a proven neuroprotective effect and likely serves to improve neuronal survival after injury. Consistent with this role and with the lack of change seen in TGFβ1 mRNA

expression levels after repeated nerve injury, no significant neuronal degeneration was seen following multiple injuries (see Chapter 2). Finally, although TGFβ can inhibit microglial and astrocyte proliferation (Lindholm et al., 1992; Suzumura et al., 1993), this effect has not been demonstrated in the facial motor nucleus. Furthermore, studies demonstrate region-specific differences in such effects (Johns et al., 1992). Thus, decreased levels of microglial division need not be accompanied by increased levels of TGFβ mRNA.

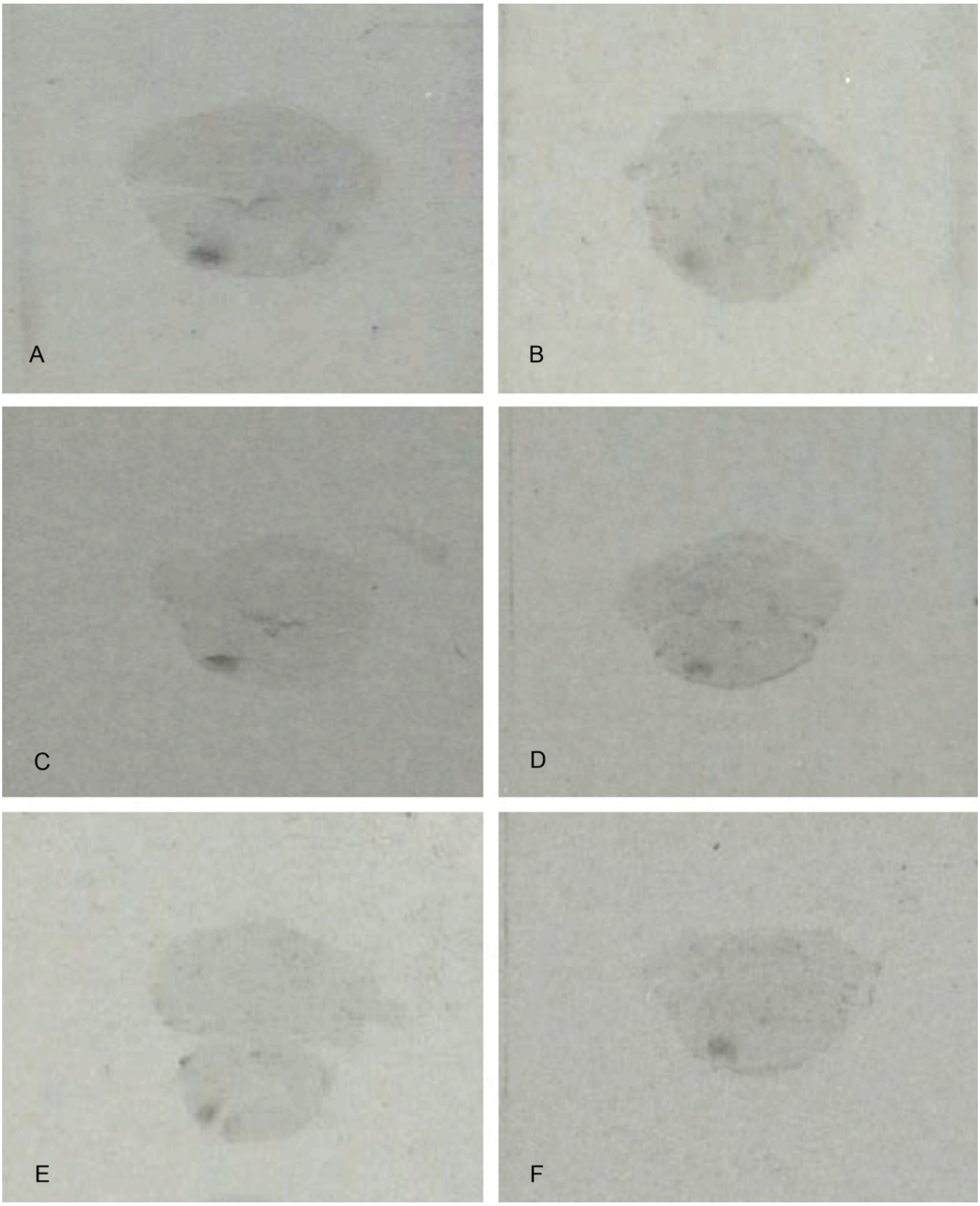

Figure 3-1. TGFβ1 mRNA in the aged brain.

Figure 3-1 continued. TGFβ1 mRNA in the aged brain. TGFβ1 mRNA hybridization signal in the injured facial nuclei of young (3 months) (A, C, E) or aged (30 months) (B, D, E) rats 3 (A, B), 5 (C, D) or 7 (E, F) days post-injury.

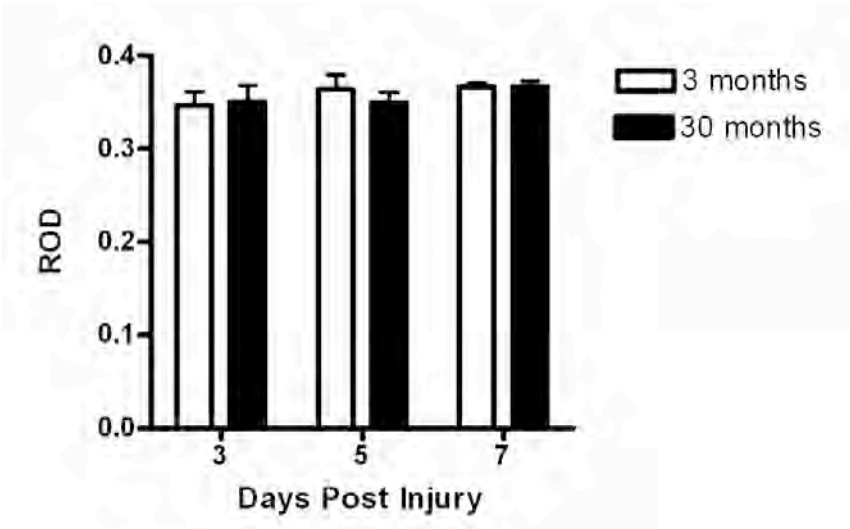

Figure 3-2. TGFβ1 mRNA expression in the aged brain. Relative optical density (ROD) of hybridization signals in the injured facial motor nucleus 3, 5 or 7 days-post injury. Data are represented as means ± SEM. N= 4 aged, n= 2 young.

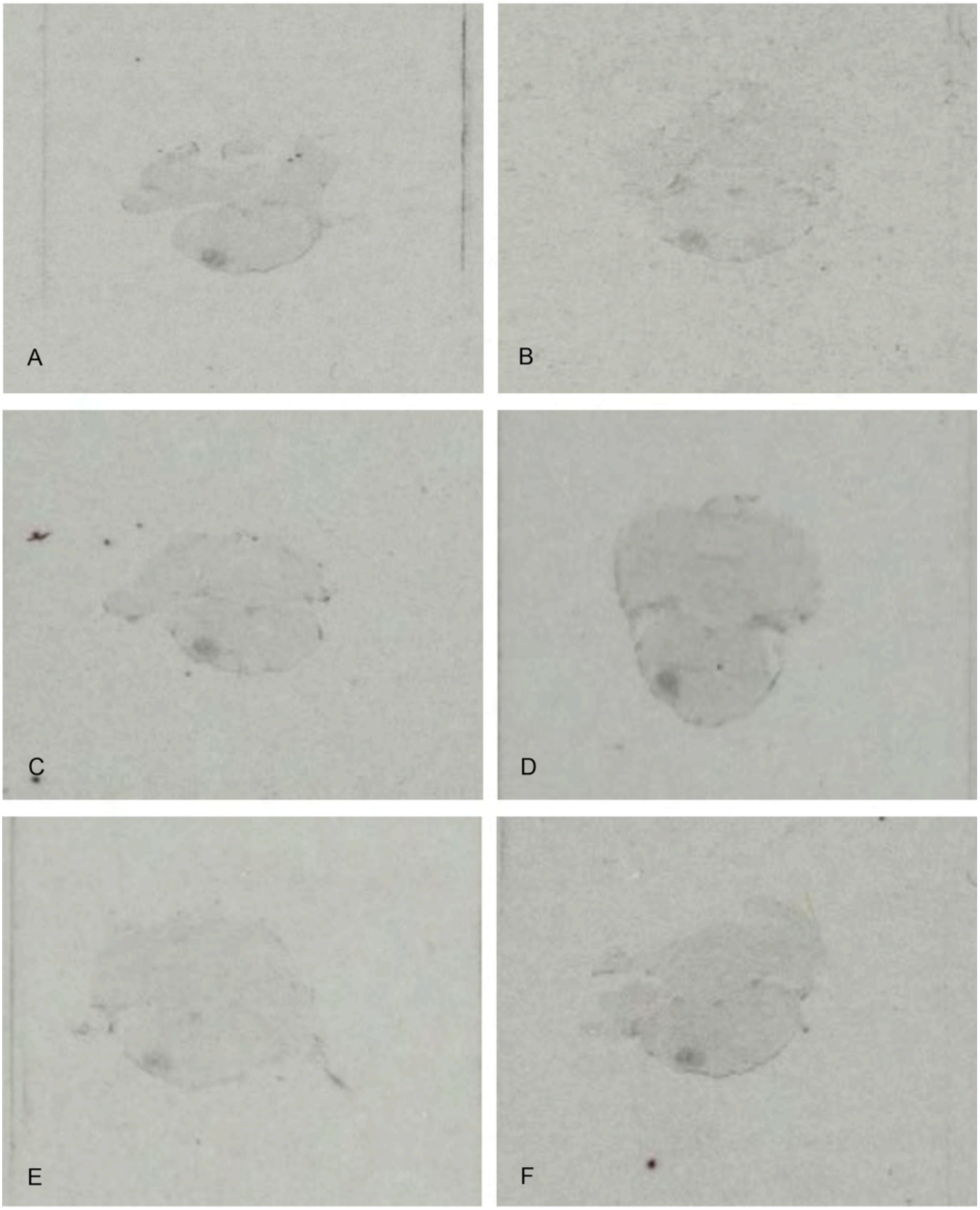

Figure 3-3. TGFβ1 mRNA after repeat nerve injury.

Figure 3-3 continued. TGFβ1 mRNA after repeat nerve injury. TGFβ1 mRNA hybridization signal in the injured facial nuclei after 1 (A, C, E) or 3 (B, D, E) facial anerve crush injuries 3 (A, B), 4 (C, D) or 5 (E, F) days post-injury.

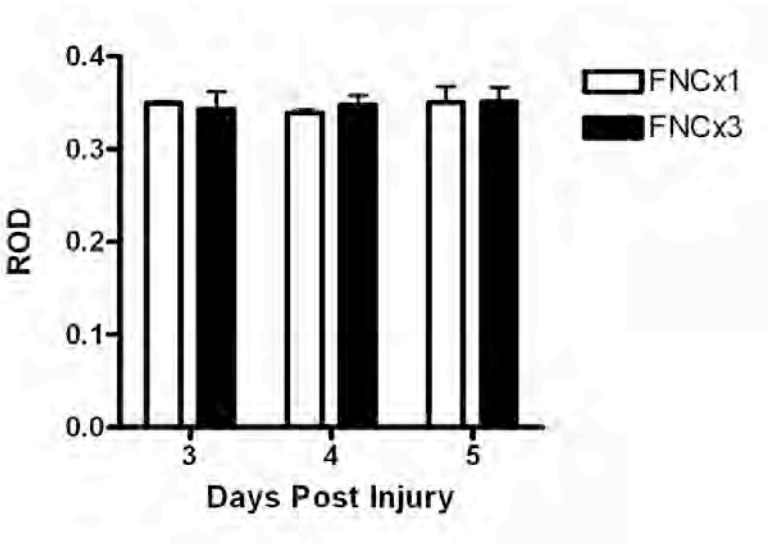

Figure 3-4. TGFβ1 mRNA expression in response to repeated facial nerve injury. Relative optical density (ROD) of hybridization signals in the injured facial motor nucleus 3, 4 or 5 days-post injury. Data are represented as means ± SEM. FNC = facial nerve crush. N= 2 FNC x 1, n= 4 FNC x 3.

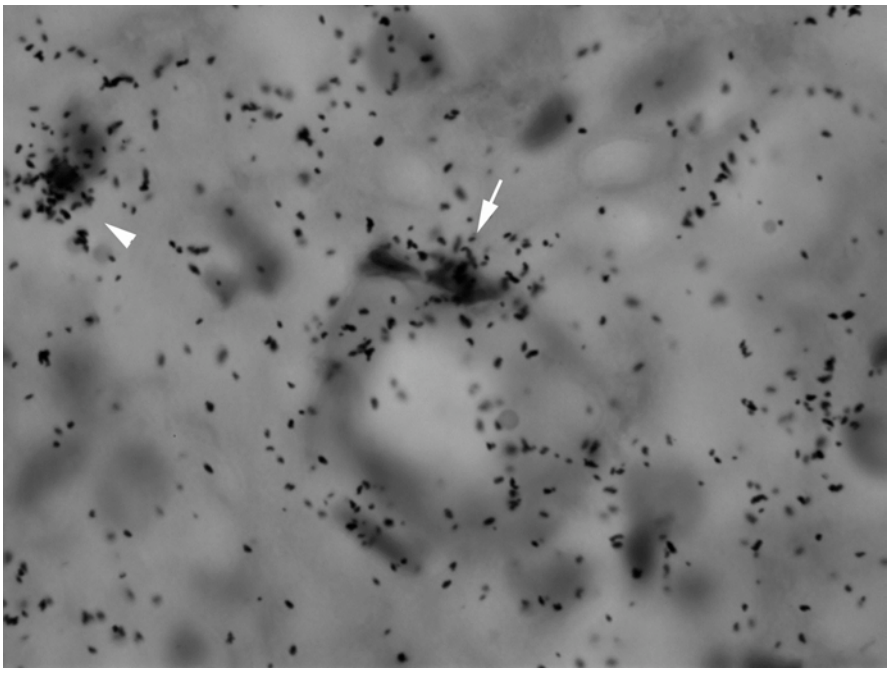

Figure 3-5. TGFβ1 mRNA in the facial nucleus. Silver grains localize to perineuronal microglial (arrow) and microglial scattered throughout the perikarya (arrowhead) of the injured facial motor nucleus. 100x magnification.

CHAPTER FOUR
IMMUNOHISTOCHEMICAL ANALYSIS IN THE REPEATED FACIAL NERVE INJURY MODEL

Introduction

A reduction in microglial proliferation in response to repeated nerve injury was described in Chapter 2. Signals from injured motor neurons are thought to induce microglial activation and the accompanying proliferative response and changes in immunophenotype and cytokine production. There was no reduction in neuronal number observed after repeated injury, suggesting that neuronal signaling remains intact. Furthermore, there was no decrease in the overall number of microglia present in the repeatedly injured facial nucleus. Taken together, this data points to an intrinsic change in microglial physiology as the cause of the altered proliferative response seen in the repeat injury model. In this chapter we analyze numerous components of the facial motor nucleus system immunohistochemically in order to explore potential injury-induced changes in the facial nucleus that may be indicative of degenerative or senescent changes in neurons or microglia that could explain the differences in proliferation observed in the repeat injury model. Specifically, we performed histochemical analyses of ferritin, macrosialin, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling (TUNEL), CD34, alpha-synuclein, neurofilament heavy chain (NFH), leukocyte common antigen (LCA), CD6 and glial fibrillary acidic protein (GFAP).

Two molecules expressed by microglial cells, macrosialin and ferritin, were chosen for investigation because of previously identified age-related changes in expression levels and association with dystrophic microglia, respectively. Macrosialin, or ED1 as it is also referred, is homologous to human CD68 (Holness and Simmons, 1993; Wong et al., 2005). Peripheral expression of macrosialin protein is nearly exclusive to macrophages and to a lesser extent dendritic cells (Dijkstra et al., 1985; Holness et al., 1993; Damoiseaux et al., 1994; Wong et al.,

2005). This molecule is mainly expressed cytoplasmically, specifically localized in endosomes or lysosomes, with a small amount present on the cell surface (Saito et al., 1991; Rabinowitz et al., 1992). The endosomal/lysosomal localization of macrosialin and its expression in phagocytic macrophages suggests a role in phagocytosis (Holness and Simmons, 1993). In fact, expression levels have been correlated to phagocytic activity, but macrosialin antibodies are not able to block phagocytosis (Damoiseaux et al., 1994). In the normal brain, ED1 is expressed by some perivascular cells and by phagocytic microglia in areas of neuronal degeneration, but not resting or activated parenchymal microglia (Kullberg et al., 2001; McKay et al., 2007; Soulas et al., 2009). It has been established that microglia experience age-related increases in basal expression levels of macrophage markers, such as MHC II and CR3 (see Chapter 1). Recently, it has also been demonstrated that microglial macrosialin expression is increased with age in the absence of injury or disease (Kullberg et al., 2001; Wong et al., 2005). Furthermore, caloric restriction, which is known to attenuate oxidative damage and inflammation associated with aging, was shown to reduce age-related macrosialin upregulation (Wong et al., 2005). Based on this data, we investigated macrosialin expression after a single or multiple facial nerve injuries. We also analyzed macrosialin expression in the brainstem of aged rodents after a single nerve crush to verify that reported ED1 upregulation is not restricted to regions of the brain excluding the facial nucleus. As a marker of aged microglia, macrosialin expression in response to repeated nerve injury would indicate that there is ongoing cellular degeneration and phagocytosis in the facial nucleus or that ED1 expressing cells are exhibiting senescence-associated changes. Additionally, studies conducted in aged and AD human brains revealed that a subpopulation of the microglial pool that express the iron storage molecule L-ferritin display a propensity for

morphological dystrophy. As another potential marker of senescent microglia, we analyzed expression of L-ferritin in the repeat injury model.

Another line of inquiry included histochemical evaluation of markers that provide information about microglial population dynamics, namely TUNEL and CD34. Because there are fewer proliferating cells in the repeatedly lesioned nuclei, yet the same overall number of microglia present, there must be changes in other means of population regulation besides mitosis. One possibility is that there is more microglial turnover occurring via programmed cell death after a single injury than following multiple lesions. To explore this possibility, we performed TUNEL labeling to visualize apoptotic cells. An alternative mechanism for population maintenance in the repeatedly injured facial nucleus might involve the infiltration of peripherally-derived microglial precursor cells. Studies have shown that CD34+ cells infiltrate the CNS after injury (Asheuer et al., 2004; Davoust et al., 2006) and this occurrence could contribute to microglial cell numbers in repeatedly injured nuclei. In consideration of this information, we performed histochemical analyses of CD34 expression after repeat nerve injury. Lastly, we investigated the presence of additional peripherally-derived cells, such as T-cells, using antibodies directed against CD6 and LCA.

Finally, to ensure that there were no unusual changes in neurons or astrocytes in response to repeated injury, we performed immunohistochemical analyses of the astrocyte marker, GFAP, as well as NFH and alpha-synuclein. Importantly, alpha-synuclein is expressed *de novo* by facial motorneurons after transection or crush injury (Moran et al., 2001). It is reported that expression levels of alpha-synuclein correspond to the severity of injury, with greater upregulation occurring after nerve axotomy compared to crush. Moreover, it was found that alpha-synuclein expression in the facial nerve model was associated with a non-apoptotic, slow form of

neurodegeneration (Moran et al., 2001). Therefore, knowledge of alpha-synuclein expression after repeated nerve crush injury could provide further confirmation that neurons remain viable after multiple injuries, as concluded in Chapter 2.

Materials and Methods

Animals and Surgery

Male Sprague-Dawley rats (Harlan, Indianapolis, IN) aged 3 (multiple injury (experimental) groups) or 9 (single injury (control) group) months at the time of initial or sole injury, respectively, were used (Figure 2-1B). Animals were housed under standard SPF conditions in the McKnight Brain Institute animal facility. Under isoflurane anesthesia, the right facial nerve was exposed near its exit from the stylomastoid foramen. The nerve was crushed once with a pair of fine forceps for 10 sec, approximately 2 mm from the stylomastoid foramen (~12–14 mm from the facial nucleus in the brainstem). In multiple injury animals, nerve crush was performed as close as possible to the site of primary injury, moving proximal to the brain as necessary. Lack of whisker movement on the right side was verified after the animals recovered from anesthesia. Any animals that retained whisker movement after surgery were excluded from the study. The contralateral (unoperated) facial nucleus served as an internal control in all experiments. Animals were sacrificed 3, 4 or 5 days after the final injury (3rd axotomy in experimental groups; 1st axotomy for all control groups). Subjects were anesthetized with an overdose of sodium pentobarbital and transcardially perfused with 0.1M phosphate-buffered saline (PBS, pH 7.2) followed by 4% paraformaldehyde in PBS. Brain tissue was removed and post-fixed for two hours in 4% paraformaldehyde and subsequently frozen in methyl butane cooled in liquid nitrogen and stored at -80° C until tissue processing.

To analyze ED1 expression in the aged brain, young (3 months) and old (30 months) male Fisher 344 Brown Norway hybrid rats (National Institute of Aging) were subjected to facial

nerve crush as described above. Animals used for ED1 analysis were sacrificed at 5 days post-injury (N = 4 or 2 per group for aged and young animals, respectively) using transcardial perfusion as described above. Following 2 hours of post-fixation in 4% paraformaldehyde, brains were frozen in methyl butane cooled in liquid nitrogen and stored at -80° C until tissue processing.

Tissue Processing

Frozen brains were allowed to equilibriate in the cryostat chamber at -20°C for 30 min. before sectioning. Twenty micrometer coronal sections were cut on the croyostat and mounted onto Superfrost Plus slides in a pattern that allowed 2 sections from different caudal-rostral regions of the facial nucleus to be mounted on each slide. A puncture mark was made on the contralateral side of the brain to distinguish the injured and non-injured nuclei. Slides were stored at -80°C until use.

Immunohistochemistry

TUNEL

TUNEL was used to assess programmed cell death. Frozen sections were removed from storage at -80°C and equilibriated at -20°C for 15-30 minutes before drying at room temperature. The ApopTag Red *in situ* Apoptosis Detection Kit (Chemicon International, Temecula, CA) was then used as described by the manufacturer. Negative controls included omission of the terminal deoxynucleotidyl transferase (TdT) or the anti-digoxigenin-Rhodamine conjugate. Positive controls included spinal cord sections taken from SOD1 transgenic animals at late stage of disease (courtesy of Dr. Celeste Karch). Slides were cover slipped using gel mount mounting medium.

Ferritin, CD34, alpha-synuclein, NFH, CD6, LCA, GFAP, Iba1 and ED1

Slides were removed from storage at -80° C and acclimated at -20° C for 15-30 minutes. Slides were then warmed and allowed to dry at room temperature for 30 minutes. All tissue was rinsed in PBS for 10 minutes prior to staining. A blocking solution containing PBS with 0.1% Triton-X100 and 10% NGS was applied for 1 hour at 37° C. Primary antibodies were used as follows: rabbit anti-horse spleen L-ferritin (Sigma, St. Louis, MO; 1:1000), rabbit anti-Iba1 antibody (Wako Chemicals USA, Inc., Richmond, VA; 1:500), anti-rat CD34 (R&D Systems; 1:100), mouse anti-alpha synuclein (Gift of Dr. Gerard Shaw; 1:100), mouse anti-NFH (Gift of Dr. Gerard Shaw; 1:200), mouse anti-rat CD6 (SeroTec; 1:500), mouse anti-rat CD45 (BD Biosciences; 1:200), rabbit anti-GFAP (Gift of Dr. Gerard Shaw; 1:200) and mouse anti-ED1 (Chemicon; 1:300) were applied at given concentrations diluted in PBS with 0.1% Triton- X100 and 5% NGS and incubated overnight at 4° C. Antibody binding sites were visualized using corresponding fluorescent secondary antibody (Alexafluor 568 or 488, Molecular Probes) diluted to a concentration of 1:300 in PBS with 0.1% Triton-X100 and 5% NGS incubated at room temperature. Slides were rinsed in PBS and coverslipped with gel-mount mounting media. Double staining of Iba1/ED1 was accomplished by co-incubation of the primary antibodies under conditions described above.

Quantitative Analysis

For the quantitative analysis of ED1-positive cells we were interested in determining the average number of labeled cells in both the control (uninjured) and injured facial motor nuclei following multiple nerve injuries. Therefore, we measured and compared the mean number of labeled cell profiles per unit area. An important consideration in using this technique is to sample from all areas of the facial nucleus to account for uneven spatial distribution of microglia in different areas of the facial nucleus (rostral to caudal- most regions). An assumption of this

method is that any bias in counting profiles is the same for all groups. The control and injured facial nuclei on each section was imaged at 10x magnification and photographed using a Spot RT digital camera a (Diagnostic Instruments, Sterling Heights, MI) attached to a Zeiss Axioskop 2 microscope. ED1-labeled cell profiles within a counting frame placed over the facial nuclei were counted (approximately 10-15 sections per animal) using Image Pro Plus software (version 6.2, Media Cybernetics, Carlsbad, CA). The total number of labeled cells was divided by the total area measured to estimate the mean number of ED1-positive cells within the facial nuclei of each animal. Significant differences were determined by Two-way ANOVA followed by Bonferroni posttests using GraphPad Prism software (GraphPad Software, San Diego, CA). Results are represented as mean values $\pm$ SEM. A significance level of $p < 0.05$ was used.

Qualitative Analysis

TUNEL analysis was carried out as a pilot study using 6 brain sections from each of 2 animals per group sacrificed 3, 4 or 5 days post-injury. Staining was visualized using a Zeiss Axioskop 2 microscope. Sections were examined at low (10x) and high (40x) magnification.

Histochemical preparations of CD34, ferritin, GFAP, NFH, alpha-synuclein, LCA and CD6 in the facial nucleus were visualized using a Zeiss Axioskop 2 microscope. Four to 6 sections were analyzed from each of at least 2 brains/ group/ time point. Sections were examined at low and high magnification for the presence of positive immunohistochemical labeling.

Results

There were no TUNEL, CD34, Ferritin, LCA or CD6 Positive Cell Bodies in the Repeatedly Injured Facial Motor Nucleus

TUNEL labeling revealed no positive cell bodies on any section of any experimental animal analyzed. Positive control tissue from SOD1 transgenic animals processed in parallel

with experimental tissue revealed modest staining in lumbar spinal cord sections. Similarly, little ferrtin labeling was seen in the injured or control facial nuclei of repeat or single injury animals. CD34-positive labeling was restricted to endothelial cells, while no LCA or CD6-positive cells were seen in any sections analyzed (Table 4-1).

GFAP and NFH Immunoreactivity Are Normally Expressed Following Repeat Facial Nerve Injury

Analysis of the astrocyte marker GFAP revealed an injury induced upregulation following facial nerve crush injury. However, this response did not differ after 1 versus 3 nerve injuries. Similarly, high levels of NFH were expressed in the facial nuclei, but no detectable difference was seen in animals that received multiple injuries as compared to only 1.

There is an Age-Related Increase in ED1 Expression in the Brainstem in Response to Facial Nerve Injury

ED1 immunoreactivity was analyzed in the brainstem of young (3 months) and aged (30 months) rats 5 days after facial nerve crush injury. As expected, there was no ED1 labeling in control or injured facial nuclei of young rats (Figure 4-1C, D). Further, there was no ED1 labeling throughout the rest of the brainstem at the level of cranial nerve VII. Alternatively, there were numerous ED1-positive cells present in aged animals in both uninjured and injured facial nuclei, as well as throughout the rest of the brainstem at the level of the facial nerve (Figure 4-1A, B, E, F). There was no significant difference between the numbers of ED1+ cells in the uninjured versus injured facial nuclei of aged rats (Figure 4-2).

There is a Significant Increase in ED1 Expression in the Facial Motor Nucleus in Response to Repeat Nerve Injury

Immunohistochemical analysis of ED1-positive cells was carried out in uninjured and injured facial nuclei of adult rats after 1 or 3 nerve crushes. Animals were 9 months old at the time of sacrifice. There were numerous ED1-labeled cells visible in both the control and injured

facial nuclei of rats that received 1 or 3 facial nerve injuries (Figure 4-3). While there were ED1-positive cells present in animals that received only 1 injury, there was no significant difference in the number of immunoreactive cells in control versus injured nuclei. However, repeat nerve injury induced a significant increase in ED1 expression in injured nuclei compared to uninjured control nuclei ($p \leq 0.05$) (Figure 4-4). There were few ED1 positive cells outside of the facial motor nucleus in 9 month old rats after single or repeat facial nerve injury.

ED1-positive Cells Are Microglia

The identity of ED1 positive cells in the facial motor nuclei and brainstem at the level of the facial nerve was investigated by colabeling with the microglial-specific marker Iba1 (Figure 4-1, 4-3). ED1 and Iba1 immunoreactive cells colocalized in all regions examined. ED1/Iba1 positive microglia were located perineuronally and in the perikarya of the facial nucleus (4-5). In aged animals, ED1/Iba1+ cells were also distributed ubiquitously throughout the brainstem at the level of cranial nerve VII.

Discussion

Microglia respond to repeat facial nerve injury with significantly reduced proliferative levels compared to the response typically seen after a single injury (Chapter 2). Because we have shown that there is no difference in the number of microglia present in the repeatedly injured facial nucleus compared to the singly injured nucleus, we must assume there is another, as yet unidentified change affecting population dynamics. For example, one mechanism that would allow for the maintenance of equal microglia numbers in single versus repeatedly injured nuclei is a difference in programmed cell death. In order to maintain homeostatic population numbers after a proliferative burst, microglia undergo apoptosis (Gehrmann and Banati, 1995; Jones et al., 1997; Moran and Graeber, 2004). We have demonstrated that there are more proliferating cells after a single nerve crush than after 3. Therefore, it is possible that more

apoptosis takes place in the singly injured brain. We performed TUNEL labeling after 1 or 3 nerve injuries to assess cell death in the injured facial nucleus. A pilot analysis of 2 brains per group collected 3, 4 or 5 days post-injury did not reveal any TUNEL labeling. While this tells us that there is no early cell death taking place, the possibility remains that there may be an increased level of apoptosis in the singly injured facial nucleus that occurs at a later time point. If this experiment were to be repeated, an important consideration should be the inclusion of subjects sacrificed at least 14 days post-injury allowing for a more thorough examination of mechanisms regulating population control after repeated injury.

Another potential means of maintaining equal cell numbers in the facial nucleus despite reduced proliferation involves infiltration of cells from outside of the facial nucleus into the repeatedly injured nucleus. These cells could consist of microglia from the nearby parenchyma or may originate in the periphery. One such peripherally-derived cell type reported to infiltrate the CNS after injury is CD34+ cells (Asheuer et al., 2004; Davoust et al., 2006). We analyzed CD34 immunoreactivity after 1 or 3 facial nerve crushes and saw no visible CD34 staining, with the exception of endothelial cells. These results indicate that CD34 progenitor cell infiltration can not explain the maintenance of the microglial population in the repeat injury model. Alternatively, if microglia from surrounding brain regions migrate into the repeatedly injured facial nucleus, this may be mediated by increased or prolonged expression of neuronally-derived chemoattractants. The analysis of such cytokines and chemokines is another avenue for investigation in the repeat facial nerve injury model.

A prominent difference was detected in ED1/macrosialin expression in the repeat versus singly injured facial nuclei, as well as in the aged brain compared to young. Macrosialin is not normally expressed in quiescent non-phagocytic microglia, such as those in the injured rat facial

motor nucleus (Graeber et al., 1990; Kullberg et al., 2001; McKay et al., 2007; Soulas et al., 2009). However, studies have shown an age-related increase in ED1 expression throughout the brain in the absence of injury or disease (Kullberg et al., 2001; Wong et al., 2005). Similarly, we show age-related increases in ED1-positive, Iba1-positive microglia throughout the brainstem including the injured and uninjured facial nucleus. Because no ED1 immunostaining is present in young rats even after injury, it is concluded that this aberrant expression results from aging-related changes indicative of cellular senescence.

When ED1/macrosialin protein expression was evaluated in the facial nucleus after repeat nerve injury, we found a significant increase in immunoreactivity restricted to the injured facial motor nucleus. Notably, this high level of ED1 immunoreactivity was seen in the absence of any detectable neuronal degeneration. Furthermore, there was little expression in the uninjured facial nucleus or throughout the brainstem. The moderate ED1 expression visible throughout the brainstem at the level of the facial nucleus and in the uninjured facial motor nucleus can be attributed to the age of the rats used in the repeat injury model. Because of the experimental design, animals were 9 months old at the time of sacrifice. On the other hand, the number of ED1 expressing microglia in the repeatedly lesioned facial nucleus is significantly greater than that seen in any other region examined in experimental animals. The expression of ED1 in the absence of cellular degeneration suggests that injury-induced ED1/macrosialin expression is not related to phagocytic activity. Taken together with the injury-induced reduction in mitotic activity reported in Chapter 2, ED1 expression in the repeatedly injured facial motor nucleus is suggestive of cellular senescence.

Molecular Marker/Antigen	Post-FNCx1	Post-FNCx3
Ferritin	–	–
GFAP	+	+
Alpha-synuclein	+	+
TUNEL	+/-	+/-
CD34	+ (endothelial cells)	+ (endothelial cells)
CD6 (OX-52)	–	–
LCA (OX-1)	–	–
ED1/CD68	–	+ + +
NFH	+ +	+ +

Table 4-1. Immunohistochemical analysis in the facial motor nucleus after injury. FNC = facial nerve crush. - = not detected; +/- = little to no immunoreactivity detected; + = some immunoreactivity detected; ++ = high level of immunoreactivity detected; +++ = very high level of immunoreactivity detected.

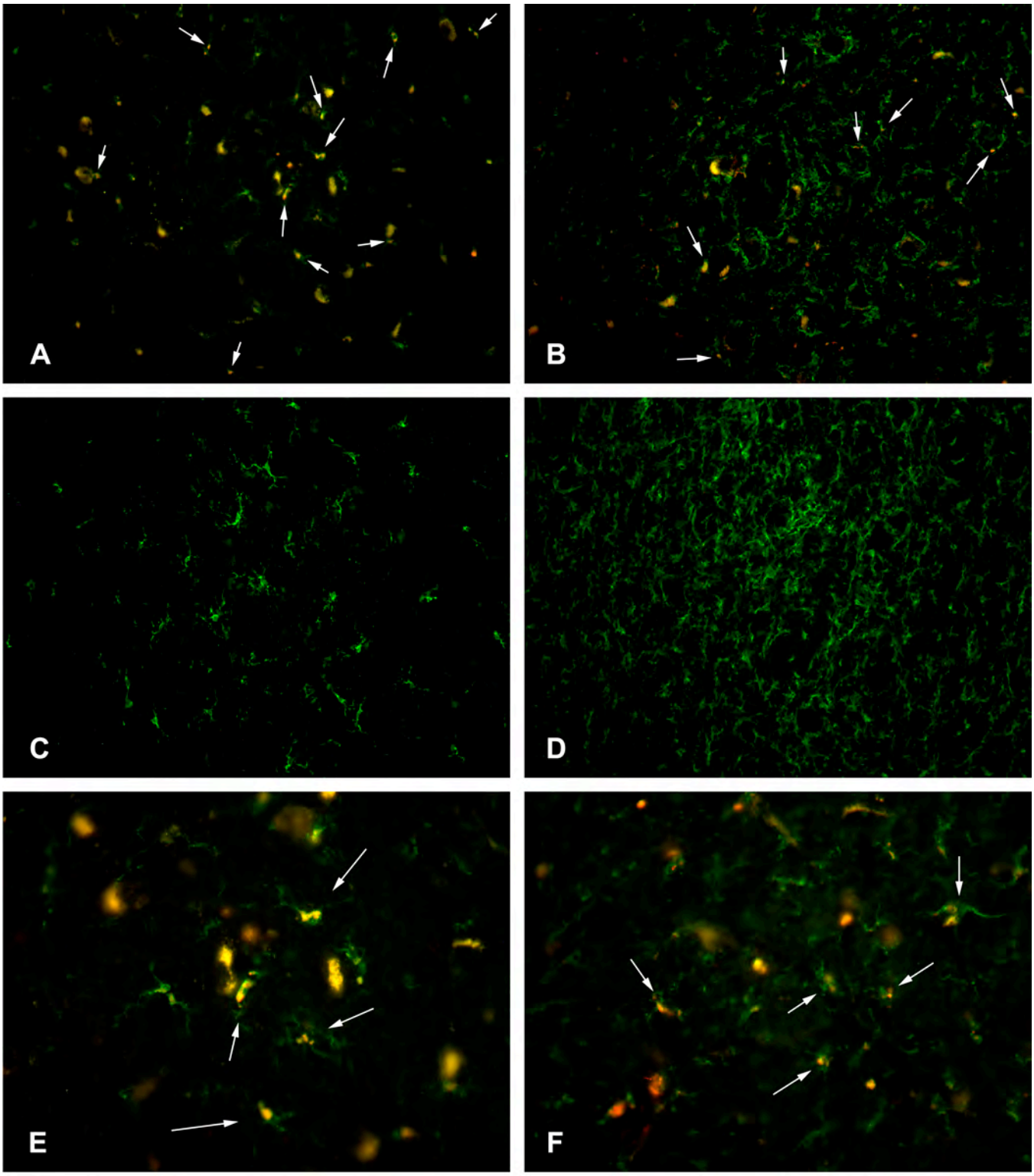

Figure 4-1. ED1 in the aged rat brain.

Figure 4-1 continued. ED1 in the aged rat brain. Numerous ED1 immunoreactive cells (arrows) are visible in the uninjured (A) and injured (B) facial nuclei of 30 month old rats after a single facial nerve crush injury. C, D) No ED1-poisitve cells are present in the uninjured (C) or injured (D) facial nuclei of 3 month old rats after a single nerve crush injury. ED1 immunoreactive cells in the uninjured (E) and injured (F) facial nuclei of aged rats are microglia. Green= Iba1, Red= ED1. 40x magnification.

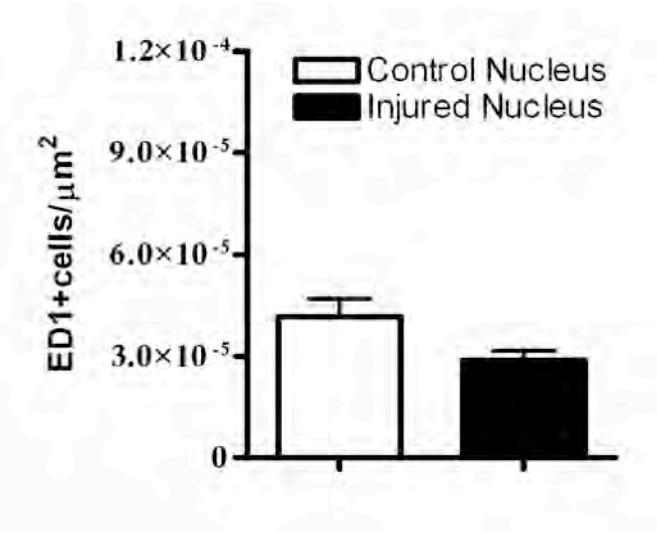

Figure 4-2. ED1 immunoreactivity in aged rats. ED1-positive cells were quantified in the control or injured facial nuclei of 30 month old rats. Data are presented as the number of ED1+ cells per square micrometer. Results are represented as means ± SEM.

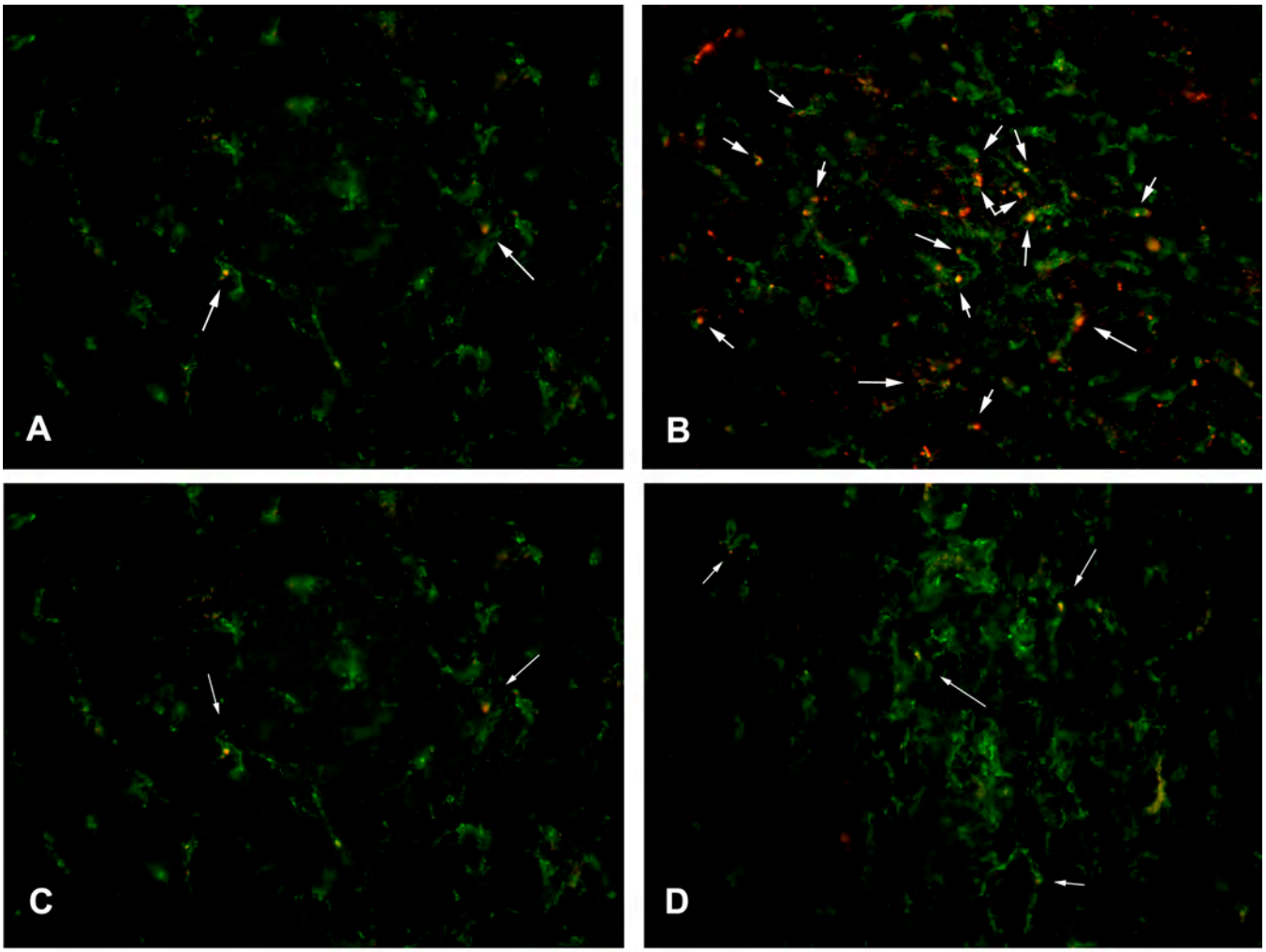

Figure 4-3. ED1 immunoreactivity in the facial nucleus in response to repeat nerve injury. A, C) There are few ED1-positive cells (arrows) in the uninjured, contralateral nucleus of 9 month old rats after 3 (A) or 1 (C) facial nerve crush injury. While there are few ED1+ cells in the injured facial nucleus after a single crush injury (D), there is a significant upregulation of ED1 immunoreactivity in response to repeat nerve injury (B). Green= Iba1, Red= ED1. 40x magnification.

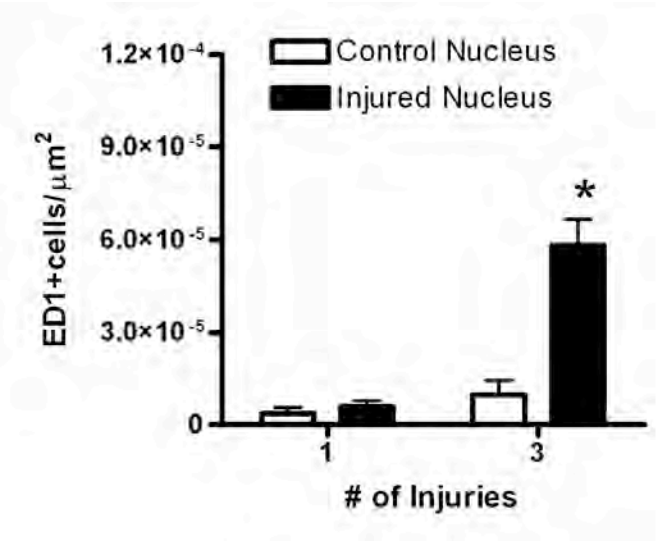

Figure 4-4. ED1 expression cells in response to repeat nerve injury. ED1 immunoreactive cells were quantified in the control (uninjured) or injured facial motor nucleus of rats after 1 or 3 injuries. There is no difference between the number of ED1+ cells in the control versus injured nuclei after 1 nerve crush. There is a significant increase in ED1 expression the injured facial nucleus after 3 nerve injuries. $P \leq 0.05$. 5 days post-injury. N= 4 per group. Data are presented as the number of ED1+ cells per square micrometer. Results are represented as means $\pm$ SEM.

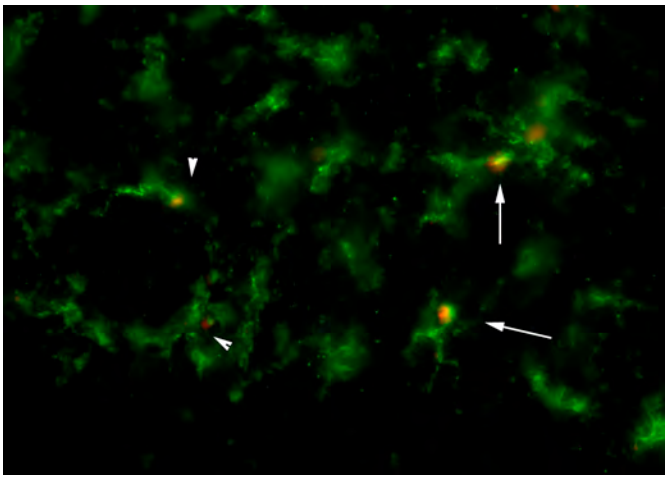

Figure 4-5. ED1 and Iba1 colocalization. ED1-positive cells colocalize with Iba1-positive microglia in the injured facial motor nucleus. Cells are found perineuronally (arrowhead) and throughout the perikarya (arrows) of the facial nucleus. Green= Iba1, Red= ED1. 40x magnification.

CHAPTER 5
CONCLUSION

Microglia are responsible for performing numerable essential functions in the brain. Due to their critical role in maintaining neuronal health and viability, the capacity of microglia to function properly and respond to neuronal injury throughout the lifespan is of considerable importance. Recent data has demonstrated a multitude of age and disease-related changes in microglial morphology, immunophenotype and function. However, limited studies on microglia in the aging, diseased and injured brain have left a void in our understanding of the kinds of effects that lifelong metabolic stress and disease have on microglial viability. Our goal is to understand how cellular function changes in relation to endogenous and exogenous stressors and to use this knowledge to better determine the role of microglia in neurodegenerative diseases, such as AD.

One prominent theory in AD research is that chronic microglial reactivity and production of neurotoxic mediators results in disease exacerbation and neuronal death. This idea has spurred approaches towards the treatment of AD and other neurodegenerative diseases designed to inhibit microglial activation. However, because microglia play an important role in brain homeostasis, it is critical to assess whether inhibition of overactive cells or support for dysfunctional, failing cells is the appropriate strategy.

Microglia participate in key actions such as phagocytosis, CNS surveillance, antigen presentation and the production and secretion of cytokines and neurotrophic factors. Compromise of any of these critical elements of microglial function could result in neurodegenerative changes. Studies have shown through telomere and telomerase analysis that microglia are subject to cellular senescence *in vivo* (Flanary and Streit, 2003; Flanary et al., 2007). In addition, previously identified changes in microglia in the aging rat brain include

altered injury-induced proliferative responses (Conde and Streit, 2005). In aged rats, microglial proliferation was upregulated 4 days after injury when mitosis normally begins to decline in younger animals. One explanation could be that microglia are responding to age-related changes in neuronal signaling. However, studies have shown morphological abnormalities in microglial cells in the healthy aged brain (Streit *et al.*, 2004), suggesting that the observed changes in those dystrophic cells are the result of a primary problem in the microglia. Similarly, if individual microglia lose their ability to properly support damaged neurons, increased proliferation may serve as a compensatory mechanism to ensure survival of the neuronal population.

To better understand the potential for microglia to undergo cellular senescence *in vivo*, we sought to exhaust the replicative potential of microglial cells through repeated facial nerve injury. We hypothesized that repeated challenge of the same pool of cells would deplete their capacity to proliferate properly. In support of our hypothesis, our results revealed a significant decline in the proliferative potential of microglia in the injured facial nucleus. These results are not interpreted as aging effects because the animals were only 9 or 12 months of age at the time of sacrifice. However, alternative explanations could not be ruled out without additional investigation. Therefore, to further substantiate the theory of repeat injury-induced senescence, we also evaluated mRNA expression of a microglial-derived cytokine, TGFβ, and conducted immunohistochemical analyses in the facial motor nucleus following multiple nerve injuries.

An obvious consideration when interpreting data showing decreased microglial proliferation was that of significant neuronal degeneration and death in response to repeated injury. Fewer neurons in the facial motor nucleus would result in decreased levels of microglial mitogens and diminished demand for neurotrophic support requiring less microglial population expansion. After quantifying the number of neurons in the repeatedly injured facial nucleus, we

found no significant decrease compared to contralateral control nuclei. Furthermore, microglial lectin staining did not reveal any phagocytic microglial clusters, which coincide with neuronal death. Finally, we evaluated alpha-synuclein immunoreactivity and found no difference between singly or repeatedly-injured nuclei. Based on a study that shows increased alpha-synuclein expression in degenerating motor neurons (Moran et al., 2001), this data further substantiates the conclusion that there is no significant neuronal degeneration in the repeat injury model. Therefore, we conclude that neuronal signaling to microglia is undiminished. This suggests that alterations in microglial mitosis result from primary changes in microglia.

Another simple explanation for reduced numbers of dividing microglia is that there are fewer total microglia present in the repeatedly injured facial nucleus. To address this question, we performed immunohistochemical analysis of Iba1-positive microglia. There was no difference in the overall number of microglia visible in the repeatedly injured facial nucleus compared to the singly-injured nucleus. This raises further questions about population dynamics in the repeat injury model. If there are more dividing cells in the facial nucleus after a single injury, there must either be more microglial cell death occurring to counteract this increase or there have to be more cells occupying the repeatedly injured nucleus that originate from extrinsic sources. In consideration of these points, we assessed both programmed cell death and the presence of peripherally-derived cells, specifically CD34+ microglia progenitors. There was no notable cell death occurring in the repeatedly injured facial nucleus up to 5 days post-injury. From this we can conclude that there is no early increase in cell death, but the possibility of altered levels of apoptosis occurring at later time points post-injury remains an attractive explanation.

Another explanation for the maintenance of cell numbers in the facial nucleus takes into account our assumption that a subpopulation of microglia in the repeatedly injured nucleus are senescent. A prominent characteristic of other types of senescent cells in the body is a resistance to apoptosis (Campisi and d'Adda di Fagagna, 2007). This is a prominent characteristic differentiating senescent from dying cells. If senescent microglia share this characteristic with other cell types, apoptotic resistant, senescent cells may accumulate in the facial nucleus after the injury-induced proliferative burst, thereby contributing to the overall number of cells in the nucleus.

A final possibility for the maintenance of equal microglial numbers in the repeatedly versus singly-injured facial nucleus involves an intriguing and as yet, unproven potential that microglia may possess immunological "memory". This characteristic, exhibited by some peripheral immune cells, would allow for a more efficient microglial response to injury. Such an improved response would necessitate fewer activated cells to produce an equivalent response to that seen after 1 injury. Therefore, fewer cells would have to divide in order to meet neuronal needs. Furthermore, if microglia were to respond to injury more quickly after previous exposure to such an insult, the peak level of proliferation seen at 3 days post-injury in the facial nerve injury model would shift to an earlier time point (for example, 2 days post-injury). If this were the case, the number of proliferating microglia may not differ after 1 or many injuries, but analysis conducted at 3 days after injury would provide misleading results. To rule out this possibility, proliferation should be analyzed at earlier and more numerous time points. A detailed analysis of the time course of microglial mitosis would provide a more complete understanding of the dynamics of the microglial response to repeated nerve injury. Although

more experiments are required to disprove or validate this idea, immunohistochemical studies described in Chapter 4 provide evidence that supports the alternate theory of cell senescence.

Following facial nerve injury, microglia upregulate mRNA for the neuroprotective cytokine TGFβ1. In situ hybridization studies revealed that there is no effect of repeated nerve injury on this microglial function. Numerous studies describe DNA modifications that occur in other types of senescent cells involving genes that encode secreted proteins capable of altering the microenvironment (Campisi and d'Adda di Fagagna, 2007). Based on this information, it could be expected that senescent microglia may produce altered levels of secreted factors, including TGFβ1, but this lack of change in TGF mRNA expression does not preclude the possibility of microglial senescence in the repeat injury model. It is possible that non-dividing, senescent microglia in the facial nucleus are producing diminished levels of TGFβ1, but viable cells are providing compensation. Furthermore, it is also possible that even in dysfunctional cells, alterations in the production of cytokines, such as TGF in this model, are minimal and require more sensitive methods of analysis. Finally, as discussed in Chapter 3, TGFβ bioactivity is largely reliant upon protein processing, and changes in physiological activity levels may not be accompanied by changes in mRNA expression (Assoian et al., 1987). Therefore, evaluation of TGFβ1 protein levels should also be assessed.

Lastly and of considerable importance in our analysis was the detection of significant numbers of ED1/macrosialin-expressing microglia in the aged brain and after repeated facial nerve crush injury. Our identification of ED1-positive cells in the aged brain is consistent with other reports (Kullberg et al., 2001; Wong et al., 2005). While not expressed in the young brain or in activated microglia in the absence of cellular degeneration and phagocytosis, ED1 serves as a marker of aging microglia. Consistent with this assumption, we found a significant expression

of macrosialin in the repeatedly injured facial nucleus, but not in the uninjured control nucleus or brainstem of 9 month old rats. We have also established that no significant cellular degeneration is taking place in this model and conclude that microglial ED1 expression in the repeatedly injured facial nucleus in not associated with phagocytosis. Taken together this data supports the conclusion that repeated facial nerve crush injury results in microglial senescence.

Senescence of microglia in the repeat facial nerve injury model could occur as a result of multiple factors such as telomere attrition or acquired DNA damage resulting from increased metabolic and catabolic demands. Determining the cause of replicative senescence as seen in this study is an important future direction because repeated injury may place similar demands on microglia as experienced over decades of life or in instances of chronic neurodegenerative disease. Additionally, while we know that microglia are losing the ability to proliferate after repeated axotomy, it would be enlightening to know what biochemical, physiological and genetic changes occur in these senescent cells. It should be assumed that injury-induced senescent microglia in the facial nucleus will exhibit many of the same changes as senescent microglia in any brain region, making any information about these cells useful in furthering our understanding of microglial senescence.

On the other hand, a study conducted using macrophage-colony stimulating factor – deficient mice demonstrated no effect on neuronal survival or axonal regeneration after facial nerve injury, despite a lack of early microglial activation (Kalla et al., 2001). This suggests that compensatory mechanisms in this model counteract the lack of microglial support. Importantly, this underscores the fact that while the facial nerve injury model can provide clues about injury-induced senescent microglia, it is not ideal for the investigation of the effects of senescent, dysfunctional microglia on neuronal survival and viability. Furthermore, we contend that the

repeat facial nerve injury model inflicts undue pain and suffering on the animals and should not be repeated. Although other injury models, such as entorhinal cortex lesion, may prove valuable for similar studies, it is likely that any repeated nervous system injury would be poorly tolerated by animals. Alternatively, analysis of microglia in the injured and diseased brain could be carried out using human brain tissue. Although this avenue of experimentation is limiting in some aspects, particularly experimental control, tissue availability and preservation, there are valuable insights to be gained. In addition to immunohistochemical analyses, laser capture microdissection allows for the isolation and subsequent characterization of individually chosen cells. This technique would be particularly useful because the microglial population is heterogeneous and any non-selective isolation of cells, from animals or human tissue, would require subsequent cell sorting. Such an endeavor would prove laborious and inefficient since markers specific for senescent cells are as yet unidentified.

Taken together, our data strongly support the theory that microglia are subject to injury-induced senescence. Just as peripheral immune function declines in aged populations and in the face of excessive physiological stress and disease rendering the body increasingly susceptible to injury and disease, we believe that central immune function also declines in response to aging, excessive or prolonged physiological stress, disease and environmental toxins. Microglial dysfunction is certain to compromise brain homeostasis rendering the CNS less able to repair itself after injury and more susceptible to age-related neurodegenerative disease. Understanding senescence-associated changes occurring in microglia may provide opportunities for early intervention and disease treatments that are aimed at revitalizing rather than suppressing the brain's failing immune system.

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BIOGRAPHICAL SKETCH

Kelly Renee Miller was born in Galion, Ohio, in 1979 and spent her early childhood in nearby Bucyrus. In 1985, she relocated with her family to Orange Park, Florida. Kelly graduated in 1996 from Orange Park High School. For her undergraduate education, Ms. Miller attended the University of Florida in Gainesville, Florida, studying behavioral neuroscience. During her time as an undergraduate student, Kelly began working in a neuroscience lab and decided she would attend graduate school to pursue a career in science.

After obtaining her bachelor's degree, Kelly entered the Interdisciplinary Biomedical Science Program at the University of Florida in 2004. Because of her interest in neuroimmunology and neurodegenerative disease, Kelly decided to carry out her dissertation work in the laboratory of Dr. Wolfgang Streit. Her research focused on the analysis of microglial cell function in the aging and injured brain. She received her Ph.D. from the University of Florida in the summer of 2009. Kelly will continue her research career as a post-doctoral scientist in the Department of Neuropathology at Charite´ Medical University Berlin, Germany.