EFFECTS OF AGING ON MICROGLIAL ACTIVATION IN RESPONSE TO NEURONAL INJURY

By

JESSICA RENEE CONDE

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

2005

This dissertation is dedicated to my husband Dan for all of his love and encouragement, and to my daughters Haley and Athena for being my sources of inspiration.

ACKNOWLEDGMENTS

First, I would like to thank my mentor, Dr. Wolfgang "Jake" Streit, for his guidance, for enduring my endless questions, and most of all for teaching me to appreciate the beauty of microglia. 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 that I needed to complete this dissertation. Dr. Harrison was especially helpful in providing advice and allowing me the use of his lab and equipment.

I am indebted to all the current and former members of the Streit lab that I have worked with though the years. In particular, I would like to thank Kelly Miller for her multitude of assistance, and Amanda Kuhns, my Photoshop savior, who also kept the lab running smoothly and provided other assistance when needed. I also appreciate Dr. Tanya Ferguson for being a wonderful teacher, Sarah Fendrick and Barry Flanary for their technical advice, Dr. Chris Mariani for his compassionate assistance with my animals both inside and outside of the lab (and for sharing intelligent political discourse and humor on the "evening shift"), and Dr. Parker Mickle for his infectious optimism and support.

Dr. Defang Luo of the Harrison lab deserves much credit for her patience while teaching me the ways of *in situ* hybridization. I would also like to thank former members of the Harrison lab, Drs. Violetta Zujovic and Shuzhen Chen, for their technical advice and assistance.

I thank Dr. Kevin Anderson for taking the time to teach me how to emulsion dip my slides and allowing me to use some of his darkroom equipment when I first got started.

Tim Vaught of the MBI Microscopy core is appreciated for his assistance in getting me started with image analysis and stepping in when the computer just did not want to behave.

Neuroscience office staff members B.J. Streetman, John Neely, and Diane Sherley are deeply appreciated for making sure that I was correctly registered, paid, funded, and guided through the bureaucracy. I would also like to thank Jon Akers for enduring my frequent pestering regarding computer problems.

I owe a special acknowledgement to Dr. Robert Schmidt, my undergraduate research mentor, for starting me on this path and continuing to provide moral support for all of my endeavors.

Finally, and most of all, I would like to thank my friends and family for always being supportive, especially when things were going rough and I needed all the encouragement that I could find. My husband Dan deserves special accolades for keeping it together at home during my long evenings in the lab. I could not have done this without him. And I owe all of my inspiration to the brightest lights in my life, my daughters Haley and Athena.

TABLE OF CONTENTS

page

LIST OF TABLES

LIST OF FIGURES

Abstract of Dissertation Presented to the Graduate School
of the University of Florida in Partial Fulfillment of the
Requirements for the Degree of Doctor of Philosophy

EFFECTS OF AGING ON MICROGLIAL ACTIVATION IN RESPONSE TO NEURONAL INJURY

By

Jessica Renee Conde

May, 2005

Chair: Wolfgang J. Streit
Major Department: Neuroscience

Microglia have been studied extensively in aging-related neurodegenerative diseases, but to a lesser extent in the normally aged central nervous system (CNS), and little is known about how aging affects the ability of microglia to respond to neuronal injury. Microglial activation in response to facial nerve injury is distinguished by proliferation in conjunction with morphological and immunophenotypic changes, followed by turnover to return the microglial cell population to homeostatic levels as the acute phase of the injury subsides. The main goal of this study was to elucidate what changes, if any, occur in the microglial activation program with aging.

We found that proliferation of microglia in old (30 m.o.) rats remained significantly higher than in young (3 m.o.) rats 4 days after axotomy, demonstrating that the downregulation of microglial activation is attenuated with aging. We found no aging-related change in microglial cell death as part of post-mitotic turnover of microglia to balance the prolonged period of proliferation. Because evidence suggests that neuronal-

microglial signaling is fundamental in modulating the extent and course of microglial activation, including the downregulation of microglial activation after the acute phase of the injury, we investigated the effect of aging on the putative neuronal-microglial regulatory signal fractalkine as a possible source of the aging-related decline in regulation of microglial proliferation. There was no evidence of an aging-related change in the mRNA expression of fractalkine and its microglial receptor CX3CR1; however, this does not dismiss possible aging-related changes at other steps in the signaling pathway. We found that microglia in old animals exhibited hypertrophy of their somata with dense perinuclear lectin staining, indicative of an accumulation of heterogeneous material, and upon activation the dense perinuclear lectin staining was accompanied by fragmented staining of their somewhat truncated processes. Taken together, the changes in regulation of microglial activation and morphology may be indicative of aging-related changes in neuronal signaling and/or microglial senescence; such changes have the potential to disrupt CNS homeostasis and may play a role in the aging-associated susceptibility to neurodegenerative diseases. These studies provide an impetus for further investigation into aging-related changes in neuronal-microglial communication and microglial senescence.

CHAPTER 1
INTRODUCTION AND LITERATURE REVIEW

Microglia: An Overview

Introduction

Microglia are unique glial cells of the central nervous system (CNS) parenchyma capable of both neurosupportive and immunocompetent functions. Microglia are one of 3 major types of glial cells in the CNS, the others being astrocytes and oligodendrocytes, and represent approximately 5-20% of the total glial cell population (Lawson et al., 1990; Streit, 1995). Microglia exhibit great morphological and immunophenotypic plasticity in accordance with their variety of supportive and protective functions.

The embryonic tissue origin of microglia was debated for many years, with some proposing a neuroectodermal origin (Fedoroff and Hao, 1991; Fedoroff et al., 1997; Hao et al., 1991; Kitamura et al., 1984; Miyake et al., 1984; Oehmichen et al., 1979), and others a mesodermal origin in common with blood monocytes (Cuadros and Navascues, 1998; Ling and Wong, 1993; Perry and Gordon, 1991; Streit, 2001; Streit et al., 1988). Accumulating evidence, particularly the expression of cell surface markers also found on monocytes, has now made it clear that microglia originate from the embryonic mesoderm and colonize the CNS early in development (Ling and Wong, 1993). However, the fact that primitive, ameboid microglia are detectable in the developing CNS earlier than the origin of blood monocytes (Alliot et al., 1999; Hurley and Streit, 1996) suggests that the ameboid microglia are not derived directly from monocytes, but instead from a common progenitor cell, the fetal macrophage (Streit, 2001). Microglial precursors primarily enter

the developing CNS via the meninges by transversing the pial surface (Navascues et al., 2000), then cluster in the white matter tracks of the supraventricular corpus callosum before migrating to other areas of the CNS and differentiating into adult, ramified microglia (Del Rio-Hortega, 1932; Streit, 2001). During the course of differentiation, the morphological change is accompanied by a change in immunophenotype, so that the resting, ramified microglia of the normal rat brain are no longer expressing major histocompatibility complex (MHC) or ED1 antigens by postnatal day 21 (Domaradzka-Pytel et al., 1999). Down-regulation of their immunophenotype under physiological conditions is probably important to their adaptation to the specialized microenvironment of the CNS (Kreutzberg, 1996).

The adult parenchymal microglia population undergoes steady-state turnover, but there has been debate as to how the population is renewed. The process of renewal of adult microglia is presumably different from the establishment of the microglia population during development (Navascues et al., 2000), and understanding the mechanisms of renewal may be important in understanding how aging affects microglia. Traditionally, it was held that renewal of the population was exclusively via proliferation of resident microglial cells (Barron, 1995; Kreutzberg, 1996) and that monocytes do not differentiate into microglia in the mature CNS (Graeber et al., 1988; Schelper and Adrian, 1986; Streit et al., 1988). However, Lawson and colleagues (1992) provided evidence that the steady-state turnover of parenchymal microglia occurred by both local division and recruitment from the circulating monocyte pool. It has more recently been demonstrated using bone marrow chimeras that bone marrow-derived precursors do have a low but noteworthy ability to infiltrate the adult CNS and become part of the highly

differentiated parenchymal microglia population after a simple, non-blood-brain barrier disrupting facial nerve lesion, and this infiltration is increased in animals concurrently undergoing experimental autoimmune encephalitis (Flugel et al., 2001). Differentiated parenchymal microglia were also shown to arise from bone marrow stem cells in irradiated mice transplanted with GFP-expressing stem cells (Bechmann et al., 2005; Simard and Rivest, 2004), and exogenous microglia were purported to have greater antigen presenting potential than resident microglia (Simard and Rivest, 2004). The transformation of bone marrow progenitors into parenchymal microglia lend further support to a mesodermal origin of microglia. Furthermore, if exogenously recruited microglia in the adult CNS do indeed have a somewhat more immunogenic phenotype than resident microglia, this could have serious implications in situations where the infiltration of exogenous precursors is increased.

The Functional Plasticity of Microglia

The CNS differs from other systems in that the presence of the blood-brain barrier (BBB) largely excludes the peripheral immune system from exerting its influence in the delicate CNS environment. In the absence of peripheral leukocytes, microglia fulfill the protective role in the CNS by acting as the resident immunocompetent cells (Streit, 2002; Streit et al., 1999). However, microglia are much more than CNS versions of their monocyte cousins. In addition to immunosurveillance and mediation, microglia maintain overall tissue homeostasis in the CNS by removing potentially harmful debris, protecting neurons from excitotoxicity, and secreting growth factors to provide neuronal support (Kreutzberg, 1996). In the adult CNS, microglia exhibit diverse morphologies which are associated with their functional state within the local microenvironment (Streit et al., 1988; Streit et al., 1999). Microglia have a ubiquitous but heterogenous distribution

throughout the CNS, with widely varying densities and morphologies consistent with architecturally and functionally distinct areas of the CNS (Lawson et al., 1990).

Microglial surveillance. In the normal adult CNS, microglia are in a highly ramified "resting state," but it should be emphasized that "resting" microglia are not dormant, but are actively and sensitively surveying their environment for signs of even the most subtle of changes (Kreutzberg, 1996). Microglia are able to detect and respond to disturbances in CNS homeostasis, and they display plasticity in their response depending on the nature of the disturbance. Microglial morphology ranges from cells with long, ramified processes associated with the "resting state" (a structure conducive to surveillance of the microenvironment), to cells with shortened, hypertrophied processes, to cells with an amoeboid form (conducive to phagocytosis). Microglial immunophenotype ranges from low constitutive expression of molecules such as complement receptor-3 (CR3) and cluster of differentiation-4 (CD4) to activation-induced upregulated expression of their constitutive markers along with expression of immunogenic markers such as MHC antigens and ED1 macrophage marker (Kreutzberg et al., 1989; Kreutzberg, 1990; Streit, 1995).

Microglia respond to pathogenic invaders or neuronal injury by rapidly initiating their activation program, including proliferation, motility, and upregulation of the immunophenotypic repertoire that they share in common with their monocyte relatives (Streit, 2002). Activated microglia have been shown to express toll-like receptors (Bsibsi et al., 2002; Nguyen et al., 2002), CD14 (Peterson et al., 1995), and mannose receptors (Marzolo et al., 1999), which are associated with recognition of pathogen-associated molecular patterns (Rock et al., 2004). In line with their change in immunophenotype,

activated microglia are also capable of acting as antigen presenting cells (APCs) (Frei et al., 1987; Hetier et al., 1988; Hickey and Kimura, 1988), although they are not as strong APCs as other cells with this function in peripheral organs (Carson et al., 1998; Flugel et al., 1999; Ford et al., 1995). This limited immune competence of microglia is nature's way of providing some protection to the CNS against disease while protecting it from the ravages of a full-fledged immune response (Streit, 2002).

Microglia are neurosupportive. Though all types of neuronal injury elicit microglial activation, the degree and nature of activation differ depending on whether the neuron has the potential for recovery or is destined for degeneration (Kreutzberg, 1990). Many studies have demonstrated that microglia are capable of secreting growth factors that may aid in neuronal development, support, and survival, including transforming growth factor beta (TGF-β) (Kreutzberg, 1996), interleukin-1 beta (IL-1β) (Giulian et al., 1986), basic fibroblast growth factor (bFGF) (Shimojo et al.1991), brain-derived neurotrophic factor (BDNF) and nerve growth factor (Nakajima et al., 2001a), and hepatocyte growth factor (Hamanoue et al., 1996). *In vitro* studies demonstrate that neuronal survival and proliferation are enhanced when neurons are cultured with microglial-conditioned medium (Morgan et al., 2004; Nagata et al., 1993; Polazzi et al., 2001; Watanabe et al., 2000) or in mixed cultures with microglia (Zhang and Fedoroff, 1996; Zietlow et al., 1999). Microglia have been shown to express neurotrophic molecules such as BDNF and glial cell line-derived neurotrophic factor (GDNF) *in vivo* in response to striatal injury (Batchelor et al., 1999), and grafting cultured microglia into the CNS enhances neuroregeneration (Prewitt et al., 1997; Rabchevsky and Streit, 1997).

Microglia are neuroprotective. Another function of microglia is the protection of neurons as they undergo the retrograde changes that are part of their regeneration program. It has been shown in mice deficient in colony stimulating factor (CSF)-1 that a decreased microglial response results in neuronal vulnerability to ischemia (Berezovskaya et al., 1995). Microglia have been shown to have glutamate scavenging capability (Nakajima et al., 2001b), which may protect neurons from oxidative glutamate toxicity. After initiation of activation and proliferation, perinuclear microglia participate in the detachment of afferent synaptic terminals from the surface of regenerating neurons, a process known as synaptic stripping (Blinzinger and Kreutzberg, 1968). There has been some controversy as to whether microglial participation in synaptic stripping is an important part of the regeneration process. One study showed that synaptic stripping did not occur in the injured facial nucleus when microglial proliferation was inhibited by adriamycin (Graeber et al., 1989). However, in another study using macrophage colony stimulating factor (MCSF, a microglial mitogen) deficient mice, deficiencies in microglial proliferation and expression of early markers of activation (i.e., thrombospondin, MCSF receptor, aMb2- and a5b1-integrins) did not affect neuronal survival, and the speed of axon recovery was also unaffected (Kalla et al., 2001). Additionally, when microglial proliferation after hypoglossal injury was blocked with ARA-C, synaptic stripping and axon regeneration still occurred (Svensson and Aldskogius, 1993a; Svensson and Aldskogius, 1993b). Some authors have implied that because neuron regeneration occurs in the absence of perineuronal microglia, microglia do not live up to the regeneration potential demonstrated *in vitro* and are not involved in supporting neuronal survival, regeneration, or plasticity (Aldskogius, 2001). Considering

the conservative nature of biological systems, however, the tightly orchestrated, specific response of microglia to neuronal injury is not likely in vain, and the increased vulnerability to ischemic injury in CSF-1 deficient mice discussed above provides evidence to the contrary. A possible explanation for normal regeneration of peripheral axons in the absence of microglial activation is that normal microglial functions may be backed up by compensatory mechanisms; for example, astrocytes may fulfill the roles normally occupied by microglia in the injury response program. There is evidence of communication between microglia and astrocytes (Giulian and Baker, 1985; Rezaie et al., 2002; Verderio and Matteoli, 2001); thus, it is feasible that astrocytes may be able to sense and compensate for deficient microglial activation in certain circumstances.

Another way in which microglia protect healthy neurons is by the maintenance of CNS homeostasis. In the presence of degenerating neurons, microglia transform into brain macrophages and phagocytose neuronal debris (Kreutzberg, 1990). Microglia are also able to detect, kill, and phagocytose dysfunctional neurons, such as those with altered lysosomal storage phenotype (Nakanishi, 2003). By removing dysfunctional and dying/dead cells, microglia protect other cells in the local microenvironment from potentially toxic products released by or spilled from these cells. Internalization of beta amyloid aggregates by microglia has been a hot topic of Alzheimer's disease research, as will be discussed later.

Neuronal-Microglial Communication

The dynamic ability of microglia to change phenotype and the existence of microglial subpopulations demonstrate the immense impact of the local CNS microenvironment on the regulation of these multi-potential cells. However, it is not entirely clear which components of this microenvironment determine the phenotypic

specialization of microglia (Perry et al., 1993). A complex variety of factors have been suggested as molecular regulators and mediators of microglial interactions with neurons (Harrison et al., 1998; Neumann et al., 1998; Streit et al., 2001; Streit et al., 2000), as will be discussed below.

Evidence of Communication between Neurons and Microglia

Given the rapid response of microglia to neuronal injury, there is little doubt that neurons are capable of rapid and direct communication with microglia. The transient and graded upregulation of microglial antigens and inflammatory mediators after injury suggests that the response is tightly regulated (Streit et al., 1999). Additional credence to the concept of neuronal-microglial communication is provided by the demonstration that cultured microglia change their morphology and survive longer when in contact with neurons (Zhang and Fedoroff, 1996). However, the neuronal signals regulating microglial phenotype and function are not well characterized.

There are at least two potential types of signals involved in neuronal-microglial communication: stimulatory and attenuating. Stimulatory signals may be released from degenerating synapses or from the neuronal perikarya itself, and expression of such signals would presumably be upregulated or activated in the event of neuronal distress. There is also evidence that neuronal-microglial signaling occurs in the absence of neuronal distress. It has been shown that microglia co-cultured with neurons have a neuron survival-promoting phenotype in contrast to microglia in isolated cultures, which release factors neurotoxic to dopaminergic neurons (Zeitlow et al., 1999). Thus, there is evidence that a neuronal signal (or combination of signals) is needed for the necessary tight control over microglial phenotype. Neuronal regulation of the microglial activation program makes sense considering neurons' limited capacity for repair. It has been

suggested that increased microglial activation during aging may be due to disinhibition after loss of an attenuating neuronal signal. This theory is supported by evidence that activation of cultured microglia by LPS is attenuated when they are co-cultured with neurons (Chang et al., 2001). However, pinpointing the putative attenuating neuronal-microglial signal has been difficult. A neuronal regulatory signal would ideally be constitutively and exclusively expressed by neurons (with a constitutive and exclusive receptor on microglia), demonstrate a change in expression in the event of neuronal distress that allows for the rapid response of microglia, and also allow for the graded activation of microglia depending on the nature of the injury.

Candidate Signaling Molecules

Many ligand-receptor pairs have been investigated as potentially having a primary role in neuronal-microglial signaling, including many cytokine/cytokine receptors (Hanisch, 2002). Interleukin-1β (IL-1β), interferon-γ (IFN-γ) and tumor necrosis factor (TNF) have been shown to affect proliferation and immunophenotype in cultured microglia (Gehrmann, 1995; Loughlin et al., 1992; Suzumura et al., 1990). While TNF expression is rapidly induced in neurons in response to a variety of stimuli (Liu et al., 1994; Minami et al., 1991; Taupin et al., 1993; Tchelingerian et al., 1993), expression of TNF was also detected in microglia and macrophages surrounding damaged tissue 2-5 days after neuronal injury (Liu et al., 1994), demonstrating that TNF is not exclusive to neuronal-microglial signaling. The cytokine interleukin-6 (IL-6) has been shown to have the potential for being a signal between neurons and microglia. In *in vivo* studies, IL-6 was found primarily in neurons in both the CNS and the peripheral nervous system (Arruda et al., 1998; Lemke et al., 1998; Marz et al., 1998; Murphy et al., 1995). Studies

in our laboratory found that cultured microglia express IL-6 receptor and respond to IL-6 stimulation in a dose-dependent manner. Additionally, robust microglial activation occurs *in vivo* in response to injury of motoneurons expressing IL-6, which is in starke contrast to the lack of microglial activation after injury of rubrospinal neurons, which do not exhibit expression of IL-6 (Streit et al., 2000).

The phospholipid platelet activation factor (PAF) is another possible mediator of neuronal-microglial interactions. Aihara and colleagues (2000) found PAF was synthesized in neurons but not glia in response to glutamic acid stimulation. The receptor for PAF (PAFR) was previously shown to be predominantly expressed in microglia (Mori et al., 1996). PAF-induced chemotaxis was demonstrated in cultured microglia, but did not occur with microglia cultured from PAFR-deficient mice (Aihara et al., 2000), suggesting that PAFR is the only receptor for PAF in microglia and PAF is a mediator of neuronal-microglial interactions. Stem cell factor (SCF) is another molecule implicated in neuronal-microglial signaling. SCF is expressed primarily in neurons in normal, intact brain (Hirota et al., 1992; Manova et al., 1992; Motro et al., 1991; Zhang and Fedoroff, 1997), though some SCF mRNA has also been detected in astrocytes and microglia adjacent to a CNS wound site (Zhang and Fedoroff, 1999). The receptor for SCF, c-kitR, is expressed by both microglia and astrocytes in culture (Zhang and Fedoroff, 1997), but c-kitR immunoreactivity was shown to primarily localize to microglia in the vicinity of a cortical stab injury (Zhang and Fedoroff, 1999), suggesting that microglia are the primary brain cell type responding to SCF *in vivo*. SCF has been shown to maintain cultured microglia in a process-bearing phenotype, inhibit microglial proliferation in response to colony stimulating factor-1, stimulate microglial expression of neurotrophic factors, and

down-regulate microglial expression of inflammatory cytokines (Zhang and Fedoroff, 1998). Thus, SCF is a potential attenuating signal, though its non-exclusive cell type expression does not make it the ideal putative neuronal-microglial regulatory signal.

The mRNA expression of secondary lymphoid-tissue chemokine (SLC) has been localized to neurons in ischemic areas of the mouse cortex (Biber et al., 2001). This study also showed that treatment with SLC induced a transient intracellular calcium increase and chemotaxis in cultured microglia, and the CXCR3 but not the CCR7 receptor for SLC was found on microglia. Thus, SLC has the potential for involvement in neuronal-microglial signaling, but the promiscuity of SLC receptor binding makes it an unlikely candidate as a major regulator of microglial activation. Another chemokine, fractalkine, has been suggested to be involved in neuronal-microglial signaling. Two independent studies found that, *in vivo*, fractalkine is constitutively expressed in the brain only on neurons, while its receptor, CX3CR1, is found only on microglia (Harrison et al., 1998; Nishiyori et al., 1998). The fractalkine/CX3CR1 pair is one of only two chemokine ligand-receptor pairs known to be constitutively expressed in the CNS (SDF-1/CXCR4 is the other pair), and is the only one with exclusive cell expression *in vivo*. In addition, studies in which the murine CX3CR1 gene was replaced with GFP indicate that CX3CR1 is the only murine fractalkine receptor (Jung et al., 2000). Because of the exclusivity of the fractalkine ligand-receptor pair, it is an excellent candidate for a neuronal-microglial regulatory signal.

The Aging CNS

Overview

Aging is generally characterized by changes in the biochemical composition of tissues, a progressive decrease in physiological capacity, a reduction in the adaptive response to

environmental stimuli, increased mortality, and an increase in susceptibility and vulnerability to disease (Troen 2003). The CNS not only demonstrates all of these characteristics of aging, but their manifestation in the CNS is particularly concerning to our species that relies so much on cognitive functioning. Identifying the mechanisms and symptoms of aging in the CNS, and translating that information into preventatives or treatments for aging-related cognitive or functional decline, is a major focus of current neurological research.

Theories of aging. Multiple theories of aging have emerged from different lines of study of the molecular and cellular changes that occur during the aging process (Troen, 2003). One line of thinking that primarily focuses on genetic factors is the evolutionary biologic theory of aging. The major premise behind this theory is that senescence is a constellation of phenotypes that have escaped the evolutionary pressure of natural selection. In his review of the theory, Martin proposed six classes of gene action without selective pressure that potentially modulate senescent phenotypes, all falling under the umbrella of the evolutionary biologic theory of aging (Martin, 2002). One of these classes is the premise of the classic "mutation accumulation" theory of aging, in which the phenotypic threshold of expression of deleterious inborn mutations is not surpassed until after reproductive senescence, thereby escaping natural selection (Medawar, 1957a; Medawar, 1957b). Huntington's disease and "early onset" familial Alzheimer's disease are readily explainable by this theory (Haldane, 1942; Martin, 2002), but whether this theory can explain all of the changes of normal aging is not clear. The "antagonistic pleiotroic" theory proposes another genetic mechanism of aging. This theory surmises that some genetic alleles that confer evolutionary advantageous phenotypes early in life

have detrimental effects later in life (Williams, 1957). Another genetic action that may explain senescence with aging is down regulation of "good genes," such as those involved in protein synthesis, after the cessation of somatic growth in favor of other genes involved in reproduction (Kirkwood, 1988). Martin suggests that once these genes are switched off there is no evolutionary pressure for them to switch back on at the end of the prime reproductive period, explaining findings of decreased protein synthesis with aging (Kelly et al., 2000; Martin, 2002). A decrease in protein synthesis in combination with a decrease in the rate of protein turnover may lead to an increase in post-translationally altered proteins (Rattan, 1996), which is a proposed factor in age-related physiological decline (Kohn and Hamlin, 1978; Troen, 2003). An expansion of Martin's (2002) concept of the down-regulation of "good genes" with aging is the "gene silencing" theory, which holds that aging results from reduced expression/silencing of important genes, including tumor suppressors and genes involved in the control of cell cycle, apoptosis, detoxification, and cholesterol metabolism (Burzynski, 2005). Alternatively, it has been proposed that senescence may occur because of inappropriate upregulation of certain genes (loss of gene silencing) later in life (Burzynski, 2005; Guarente, 2000). It should be noted that changes in gene silencing in aged animals may potentially be affected by environmental influences, or they may be related to other stochastic mechanisms of senescence, such as somatic mutation. Somatic mutations or epimutations are random genetic or epigenetic changes that may accumulate over a lifetime and lead to a senescent phenotype after the threshold of natural selection has been surpassed (Failla, 1958; Martin, 2002). An additional theory of aging with a genetic basis is cellular senescence due to telomere shortening. Telomeres at the end of

chromosomes progressively shorten with proliferation in non-immortalized cultured cells (Flanary and Streit, 2004; Harley et al., 1990; Reaper et al., 2004) and with aging in animals (Flanary and Streit, 2003; Kajstura et al., 2000; von Zglinicki et al., 2000) until they are reduced to a threshold length that results in replicative senescence of the cell (Harley, 1991; Harley et al., 1992; Reaper et al., 2004). Supporters of the telomere theory of aging postulate that cellular senescence can lead to aging of the whole organism (Von Zglinicki, 2003). Evidence suggests that this may be an antagonistically pleiotropic function that protects from tumorigenesis but contributes to overall senescence (Troen, 2003).

There is much evidence that somatic mutations or epimutations are not entirely stochastic, but are significantly increased by environmental factors. Likewise, changes in gene silencing and post-translational modification of proteins may be affected by environmental factors. The free radical/oxidative stress theory of aging attempts to explain how environmental factors may influence genetic actions and lead to a senescent phenotype. This theory holds that reactive oxygen species generated by aerobic metabolism influence gene expression, cell division, and most importantly play a role in mitochondrial DNA damage (Barja, 1998; Barja, 2004b; Bossy-Wetzel et al., 2003; Droge, 2003; Shigenaga et al., 1994). This concept explains why lifespan is increased with caloric restriction (Barja, 2004a; Mattson et al., 2002). Lifespan is limited by free radical generation during the minimal metabolic processes required for the sustenance of life, and environmental variables such as diet influence the metabolic rate and ultimately further influence lifespan. Free radicals are also released during tissue injury (Khaldi et al., 2002; Lewen et al., 2000), and microglia have the potential to release free radicals

(Colton and Gilbert, 1987; Hu et al., 1996; Hu et al., 1995; Peterson et al., 1995). Other environmental influences may have an effect on aging through the introduction of somatic mutation or protein modification.

It is likely that both genetic and environmental influences play a role in the development of the senescent phenotype, with genetic factors determining the maximum potential lifespan of the organism, and environmental influences affecting the ability to live up to that potential. Additionally, it is not likely that only one mechanism of gene action influences the development of senescence, and the contribution of each mechanism may vary within different cells, tissues, and organisms (Troen, 2003).

Aging-Related Neurodegenerative Disease

The primary focus of aging-related changes in the CNS has centered around neurodegenerative diseases, particularly Alzheimer's disease (AD). This focus on neurodegenerative diseases stems from the fact that the incidence of such diseases increases substantially with aging (Perls, 2004). It has been shown that elements of neurodegenerative diseases of humans can be induced in animal models, including chronic cerebral hypoperfusion (Farkas et al., 2004) and transgenic expression of human amyloid precursor protein (APP) and tau (Higgins and Jacobsen, 2003). Thus, data gathered from animal models provide additional insight into the changes that occur in the aging human brain.

The role of microglia in neurodegenerative diseases of aging. Microglia have a leading role in many of the hypotheses regarding the etiology of neurodegenerative diseases of aging. The role of microglia in neurodegenerative diseases has been the subject of so many studies that a considerable number of reviews cover the topic (e.g., (Akiyama et al., 2000; Rogers et al., 2002; Streit, 2004; Wyss-Coray and Mucke, 2002).

From the large number of studies and reviews have risen what initially appear to be paradoxical hypotheses about what role microglia play in the process of neurodegeneration, particularly within the AD literature. For at least a decade the prevailing view was that microglial-mediated inflammation in AD was solely pathogenic and detrimental (the "Inflammatory Hypothesis"), and this was the basis of the promotion of non-steroidal anti-inflammatory drugs (NSAIDs) for delaying the onset or progression of AD. Indeed, microglia exposed to amyloid β (Aβ) peptide or clustered around amyloid plaques have increased expression of MHC class II (Haga et al., 1989; Rogers et al., 1988) and have been shown to produce a number of inflammatory mediators and potentially cytotoxic products, including superoxide free radicals, chemokines and chemokine receptors, IL-1, IL-6 and TNF-α (Combs et al., 2000; Cotman et al., 1996; Dickson et al., 1993; Giulian et al., 1996; Goodwin et al., 1995; Griffin et al., 1995; Klegeris and McGeer, 1997; Lue et al., 2001; McDonald et al., 1997; Szczepanik et al., 2001; Xia et al., 1998). On the other hand, microglia have been shown to phagocytose Aβ (Ard et al., 1996; Bard et al., 2000; Frautschy et al., 1992; Rogers et al., 2002; Weldon et al., 1998), and there is evidence that immunization with antibodies against Aβ peptide stimulates microglia to clear plaques through phagocytosis (Bard et al., 2000; Games et al., 2000; Rogers et al., 2002; Schenk et al., 1999), demonstrating that microglial activation is potentially beneficial in removing plaque pathology. Thus, the more recent view of microglial activation in AD (and possibly other neurodegenerative diseases of aging) is as a "double-edged sword" (Wyss-Coray and Mucke, 2002).

An unanswered question regarding AD pathology is why, if microglia have the potential to clear Aβ deposits, Aβ continues to accumulate in the presence of microglial

activation. Aβ fibrils and fragments have been shown within microglia in the AD brain (Akiyama et al., 1996; Frackowiak et al., 1992), although the transgenic AD mouse Aβ immunization studies suggest that microglial phagocytosis of Aβ in AD brain is not at maximum efficiency. It has both been suggested that Aβ deposits in AD human brain may be difficult for microglia to remove (Rogers et al., 2002) and the ability of microglia to effectively phagocytose and/or clear amyloid may be impaired (Streit, 2004), leading to the accumulation of Aβ, and perhaps a viscious cycle of further microglial activation. The concept of microglial impairment leads to more questions regarding the etiology of this impairment. Microglia may undergo aging-related senescence (Streit et al., 2004) and/or gradually become dysfunctional as a result of chronic stimulation, such as that which occurs with insoluble protein deposition (including Aβ) and after CNS injury. Another unanswered question is whether or not the production of the potentially neurotoxic molecules by activated microglia localized to Aβ plaques is actually responsible for the neurofibrillary degeneration found in AD brains (the "by-stander damage" hypothesis). There is no direct *in vivo* evidence showing microglial activation as the cause of neurofibrillary tangle formation, though this should not be interpreted to mean that the potential for neurotoxicity is not there. Clearly, however, there is a disruption of homeostasis, and potential therapies will need to promote microglial Aβ clearance while maintaining balance in the local microenvironment. Further studies in the normal aging brain may provide insight as to which factors change with aging to tip the balance and increase the incidence of neurodegenerative diseases.

The Normal Aging Brain.

When referring to the normal aging brain, "normal" refers to universal, unavoidable physiological changes that occur with aging. For example, gradual loss of white matter

volume, nerve fibers, and nerve fiber length are phenomena of normal aging (Albert, 1993; Sandell and Peters, 2002; Tang et al., 1997), but the formation of amyloid plaques in the neocortex is considered pathological.

Neuronal loss occurs in the aging substantia nigra (McGeer et al., 1977) and hippocampus (Ishimaru et al., 1991), but in most of the normal aging cerebral cortex, structural changes are primarily a product of changes in neuronal or myelin sheath structure rather than a decrease in cell number. For example, a thinning of layer 1 was found in some cortical areas of the aging rhesus monkey brain, but the number of neurons and glia did not decrease significantly with age (Peters and Sethares, 2002). Other studies of the rhesus monkey brain have provided an explanation for the cortical layer thinning without cell loss, including a loss of synapses and dendritic branches from the apical tufts of pyramidal neurons in layer 1 (Peters et al., 1998). Additionally, aging-related changes in the ultrastructure of myelin have been found throughout the CNS (Peters, 2002; Peters et al., 1996; Sandell and Peters, 2002). Activated calpain-1 levels have been shown to be increased in the white matter of aged rhesus monkeys, and it has been postulated that this enzyme may play a role in the aging-related increase in myelin protein degradation (Hinman et al., 2004; Sloane et al., 2003). Neuronal atrophy also occurs, with regional variation, during normal aging (Finch, 1993; Haug, 1997).

Changes in the nature of the blood-brain barrier (BBB) are another significant finding in the normally aging brain. The BBB changes tend to be regionally and species specific, but common changes include thickening of the basal lamina (Alba et al., 2004), elongation and loss of capillary endothelial cells, decreases in transport function (e.g., glucose, choline, triiodothyronine, hexose, and butyrate) (Mooradian, 1988; Shah and

Mooradian, 1997), alterations in protein composition and decreased reactivity of the cerebral microvasculature, and arteriovenous shunting (Mooradian, 1994; Mooradian and McCuskey, 1992). Permeability to water soluble molecules and high molecular weight solutes does not appear to be affected by normal aging, though such changes have been found in neurodegenerative brains (Mooradian, 1988). Endothelial cells, pericytes and vessel-associated (end-feet) astrocytes contain inclusions and vacuoles with aging, which may contribute to the aging-associated thickening of the vascular wall (Alba et al., 2004; Peinado et al., 1998).

Aging-related changes in glial cells have the potential for significant affects on the maintenance of CNS homeostasis and neuron function. Of the three major glial cell types, microglia probably show the most significant aging-related changes, and such changes will be discussed thoroughly in the next section. Oligodendrocytes, glial cells responsible for myelin production and maintenance, have been shown to aggregate and develop inclusions in the aging brain (Peters, 1996; Peters et al., 1991). These changes in oligodendrocytes may be related to aging-related myelin abnormalities, and ultimately influence the rate of conduction along affected nerve fibers. Astrocytes are also affected by aging, demonstrating hypertrophy, inclusions and increased production and degradation of GFAP (Linnemann and Skarsfelt, 1994; Peters et al., 1991; Sloane et al., 2000). There are conflicting reports as to whether or not the number of astrocytes increases with aging (Diamond et al., 1977; Peinado et al., 1998).

A multitude of molecular changes have been found to occur with aging in the CNS, with a general trend towards an upregulation in the expression of cytokines, such as IL-6 (Ye and Johnson, 2001), IL-1β, TNF (Bodles and Barger, 2004), and chemokines

macrophage inflammatory protein (MIP)-1a, MIP-1b, and RANTES (Felzien et al., 2001). A general trend towards changes characteristic of inflammation, oxidative stress and reduced neurotrophic support has been revealed through gene expression profiling of the aging mouse brain (Lee et al., 2000).

Microglia in the Normal Aging Brain.

As discussed above, normal aging is associated with a variety of molecular, cellular and microenvironment changes within the CNS, and given that microglia are sensitive surveyors of their environment, it is logical to expect to find aging-related changes in their phenotype. The vast majority of studies of aging-related phenotypic changes in microglia have demonstrated a steady increase in the expression of markers generally associated with microglial activation. Increases in MHC class II expression have been reported in humans (DiPatre and Gelman, 1997; Streit and Sparks, 1997), monkeys (Sheffield and Berman, 1998; Sloane et al., 1999), and rats (Morgan et al., 1999; Ogura et al., 1994; Perry et al., 1993). There is no evidence, however, of microglial proliferation above the low basal rate of young animals (Long et al., 1998) (however, see Vaughan and Peters (1974)); thus, increases in MHC II expression represent immunophenotypic changes in the existing microglial population. Increased numbers of ED1 macrophage marker, leukocyte common antigen (LCA), and CD4 positive microglia have been found in the rat CNS with aging (Kullberg et al., 2001; Perry et al., 1993), and microglial hypertrophy has been reported in the aging rat retina (Kim et al., 2004). An aging-related increase in the number of IL-1α+ microglia was found in the temporal lobe of humans (Sheng et al., 1998). Studies of aging-related expression of complement receptor 3 (CR3) have had more varied results. CR3 is constitutively expressed on microglia, but its cell surface expression is upregulated with microglial activation (DiPatre and Gelman, 1997).

Increases in the number of CR3 expressing microglia were reported in the aging rat spinal cord white matter (Kullberg et al., 2001; Stuesse et al., 2000) and brain (Kullberg et al., 2001; Morgan et al., 1999), but other studies found no difference in the number or density of CR3+ microglia in the mouse hippocampus (Long et al., 1998) or rat brain (Ogura et al., 1994; Perry et al., 1993). Because CR3 is constitutively expressed on microglia, the conflicting reports may be a reflection of the species and regional heterogeneity of the microglial population density, as well as differences in tissue processing and/or data collection.

In studies that have compared grey and white matter, the aging-related increase in MHC II expression was much more significant in white matter (Ogura et al., 1994; Perry et al., 1993; Sheffield and Berman, 1998; Sloane et al., 1999), suggesting a correlation between aging-related myelin breakdown and microglial activation. It is known that microglia have the ability to phagocytose myelin (Mosley and Cuzner, 1996; Smith, 1993) and become activated by myelin *in vitro* (Williams et al., 1994). A recent study also demonstrated that activated microglia in the white matter of rhesus monkeys had an aging-related increase in the expression of active calpain-1 (Hinman et al., 2004), a proteolytic enzyme that was previously proposed to have a role in aging-related myelin protein degradation (Sloane et al., 2003); thus, age-dependent increases in calpain-1 expression may be a reflection of increased degradation of phagocytosed myelin fragments by microglia. Aging-related increases in the breakdown of myelin and other cellular components, and the subsequent phagocytosis of these substances by microglia, may account for the substantial increase in inclusions within microglia reported in old

monkeys (Peters et al., 1991; Peters and Sethares, 2002; Sandell and Peters, 2002) and rats (Peinado et al., 1998).

In addition to aging-related increases in the expression of markers of microglial activation, several studies have reported significant changes in microglial morphology, including the presence of cytosolic inclusions mentioned above. These membrane bound inclusions have been described in monkeys (Peters et al., 1991; Peters and Sethares, 2002; Sandell and Peters, 2002) and rats (Peinado et al., 1998; Vaughan and Peters, 1974) as heterogeneous, foamy or dense, generally filling the microglial perikarya and often pushing the nucleus off to the side. Lamellar inclusions filled with phagocytosed myelin sheaths have been described in microglia of the monkey optic nerve (Sandell and Peters, 2002). Vaughan and Peters (1974) also demonstrated extension of these heterogeneous inclusions into the microglial processes of older rats, and they suggested other possible sources of the aging-related accumulation of material, including phagocytosis of degenerating axon terminals and dendritic spines, and pinocytosis of plama proteins, metabolic waste or other material. Perry and colleagues (1993) reported vacuolated processes in OX-6+ microglia in aging rats, and though they found no aging-related difference in the density of OX-42+ microglia, they described OX-42+ microglia in old rats as having abnormal morphology and a higher incidence of clumping, particularly in and around white matter. Microglial structural changes, including bulbous swellings, long stringy processes, and cytoplasmic fragmentation, found in a mouse model of Huntington's disease were mirrored with aging in wild type mice (Ma et al., 2003). Cytoplasmic vacuoles were also noted in old mouse microglia visualized with GSA I-B4 lectin (Fotheringham et al., 2000). The most striking changes in microglial morphology

have been described in the human cerebral cortex in a recent study by our laboratory. Microglia from old humans had a significant number of deramified processes, occasionally displaying beading or spheroid formation, and were classified as "dystrophic microglia". Other dystrophic changes found included shortened, tortuous processes and, most notably, cytoplasmic fragmentation (Streit et al., 2004).

Table 1-1. Summary of reported microglial changes in the normal aging brain.

Microglial marker	Observed change with aging?	Species	References
Ultrastructural phenotype	↑ heterogeneous inclusions	monkey	Peters et al., 1991; Peters and Sethares, 2002; Sandell and Peters, 2002
		rat	Peinado et al., 1998; Vaughan and Peters, 1974
	↑ number/density	monkey	Peters et al., 1991; Sandell and Peters, 2002
		rat	Vaughan and Peters, 1974
MHC class II	↑ number/density/area	human	DiPatre and Gelman, 1997; Streit and Sparks, 1997
		monkey	Sheffield and Berman, 1998; Sloane et al., 1999
		rat	Morgan et al., 1999; Ogura et al., 1994; Perry et al., 1993
	morphological changes	human	Streit et al., 2004
		rat	Perry et al., 1993
Complement receptor 3 (CR3)	↑ number/area	rat	Morgan et al., 1999; Stuesse et al., 2000
	phenotypic changes	rat	Kullberg et al., 2001; Perry et al., 1993
	no change	rat	Ogura et al., 1994
		mouse	Long et al., 1998
ED1 macrophage marker	↑ number	rat	Kullberg et al., 2001; Perry et al., 1993
GSA I-B4 lectin	phenotypic changes	rat	Kim et al., 2004
	↑ density/intensity, cytoplasmic vacuoles	mouse	Fotheringham et al., 2000

Inhibition of microglial proliferation by TGF-β1 was impaired in cultures from old donors, suggesting that the regulation of microglia may be impaired with aging (Rozovsky et al., 1998). It has previously been shown that TGB-β1 modulates fractalkine-stimulated signaling through microglial CX3CR1, a system believed to be

involved in the regulation of microglial activation (Chen et al., 2002). Thus, it is attractive to speculate that the aging-related impairment of TGF-β1's inhibitory effect may involve the fractalkine-CX3CR1 ligand-receptor pair. In the rat brain, TGF-β1 mRNA expression is increased in microglia with normal aging, and evidence suggested that this increase is due to age-related changes in glucocorticoid regulation (Nichols, 1999).

Aging and CNS Injury

While neurons that die within the CNS generally can not be replaced, axon sprouting and remodeling may occur to reestablish circuits disrupted by neuronal death. There is evidence, though, that these compensatory responses are impaired in the aged CNS. After a central lesion of the entorhinal cortex, the response of septohippocampal and commissural-associational fibers have been shown to decrease progressively in both rate and magnitude with increasing age (Scheff et al., 1980). Along with a decreased sprouting response of hippocampal neurons to deafferentation, lack of induction of GAP-43 mRNA has been found in old rats (Schauwecker et al., 1995). When the nigro-striatal pathway is lesioned, 18-month-old rats demonstrate a progressive delay in lesion-induced degeneration, and consequent delay in the beginning of regeneration, compared to 2-month-old rats (Calderini et al., 1987). Aging is also associated with more severe swelling, increased neurological deficits and diminished functional recovery after intracerebral hemorrhage and stroke in rats in old rats (Badan et al., 2003a; Gong et al., 2004). In humans, there is a correlation between advancing age and poor recovery from traumatic brain injury (Cifu et al., 1996; Hukkelhoven et al., 2003; Kilaru et al., 1996; Mosenthal et al., 2004; Susman et al., 2002).

Aging also affects the regeneration of axons with peripheral targets. For example, aging affects recovery from facial nerve injury (Streppel et al., 1998); reestablishment of functional facial motoneuron connections has been shown to be delayed by 4 days and accompanied by less robust increase in nucleolar size in 15-month-old rats compared to 3-month-olds (Vaughan, 1990; Vaughan, 1992). Numerous other studies have demonstrated delayed and/or aberrant peripheral nerve regeneration with aging in various models (Kerezoudi and Thomas, 1999). However, nearly all of these studies focused on factors affecting the axon rather than examining aging-related effects at the level of the neuronal cell body or the surrounding microenvironment. For example, Tanaka and Webster (1991) found that in aging mice, delayed sciatic nerve regeneration is associated with delayed Schwann cell ensheathment of regenerating axons. While peripheral factors are almost certainly involved in impaired peripheral nerve regeneration in aging, there are compelling reasons to investigate whether or not the microglial response to neuronal injury is impaired with aging. Specifically, aging-related impairment of peripheral factors can not explain the decline in CNS plasticity with aging, phenotypic changes in microglia with normal aging have been demonstrated (discussed above), and microglia are clearly involved in the response to facial nerve injury in young adults.

Effects of Aging on the Microglial Response to Injury.

While most studies have found changes in the basal expression of immunophenotypic markers on microglia in the normal aging brain, whether or not aging affects the response of microglia to neuronal injury is not entirely clear from the limited number of studies examining this question. For example, old rats demonstrate greater microglial lectin reactivity in the retina, and transient retinal ischemia increases this lectin reactivity in old rats more than in young rats (Kim et al., 2004). Similarly, 1-methyl-4-

phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced dopaminergic neurodegeneration has been shown to increase microglial CR3 expression more in old compared to young mice (Sugama et al., 2003). In contrast, although baseline microglial expression of CR3 was higher with aging in the primary afferent terminations and motoneuron columns of the rat sciatic nerve, sciatic nerve injury did not increase CR3 expression as much in old animals as in young animals (Stuesse et al., 2000). CR3 expression has been found to be greater in old versus young rats 3 days after intracerebral hemorrhage (Gong et al., 2004), but the authors did not state if the net increase over control was different between age groups. Likewise, ED-1 macrophage marker immunoreactivity was found to be greater with aging after cortical stab injury (Kyrkanides et al., 2001) and stroke (Badan et al., 2003a; Badan et al., 2003b). Although these studies also did not indicate the ratio of lesioned to control immunoreactivity, it is clear that the time course of ED-1 reactivity changes with aging, with ED-1 reactivity peaking earlier in old rats. Hurley and Coleman (2003) did not find aging-related differences in microglial CR3 or MHC II expression in either the control or injured facial nucleus, though they did note that basal CR3 expression was increased in other areas of the aging brainstem. There are several possible reasons for the conflicting results of these studies. Different injury models, including both neurodegenerative and regenerative, species, anatomical regions, microglial markers, and methods of analysis were employed in the various studies. For example, the study examining aging-related differences in CR3 expression after sciatic nerve injury, a model of regeneration, in the rat (Stuesse et al., 2000) had different results than the study using MPTP-induced dopaminergic neurodegeneration in the mouse (Sugama et al., 2003). It should also be taken into consideration the possibility of infiltrating peripheral

macrophages, particularly with the use of blood-brain barrier disrupting models or ED-1 macrophage marker.

In addition to the injury models, several studies have examined how aging affects the response of microglia to viral or bacterial stimuli. Miyazaki and colleagues (2002) found basal levels of MT-III were increased with aging in the rat brain, but LPS administration did not induce a further increase in MT-III expression in microglia and oligodendrocytes of old rats as it did in young rats. Because MT-III has neuroprotective antioxidant properties, the increased basal levels and lack of induction with oxidative stress in old rats suggest that microglia in old animals have already reached saturating levels of oxidative stress and MT-III production. This supposition is supported by an earlier study in which chronic LPS infusions did not result in as significant an increase in the number of activated, MHC II expressing microglia or decrease memory performance in old rats compared to young rats (Hauss-Wegrzyniak et al., 1999). Additionally, although microglia cultured from aged hippocampus and cortex showed increased LPS-induction of IL-1 and IL-6, inducibility of nitric oxide and subsequent neurotoxicity were decreased in microglia cultured from aged versus young cortex (Xie et al., 2003). In the mouse brain, no aging-related difference was found in the response of microglia to intracerebroventricular injection of LPS (Kalehua et al., 2000) or striatal injection of an adenoviral vector (Davies et al., 2004).

In reviewing the studies on aging-related changes in the microglial reponse to neuronal injury listed in Table 1-2, it becomes obvious that there is still much unknown about how aging affects the microglial activation program. The published studies have not examined if changes in the microglial activation program other than

immunophenotypic changes occur with aging, such as the location, time course and degree of microglial proliferation, the subsequent turnover of microglia after the acute phase of the injury, or regulatory signaling from neurons. Analysis of the effects of aging on these aspects of microglial activation was the overall aim of this dissertation, as will be discussed in subsequent chapters.

Table 1-2. Aging-related differences in the response of microglia to injury: varying results based on injury model, marker, and method of analysis.

Injury paradigm, species	Microglial marker, data compared	Aging related difference after injury?	Aging-related difference in ratio of lesioned to control	Reference(s)
Intracerebral hemorrhage, rat	CR3, cell density	Old ↑	Not stated	Gong et al., 2004
Stroke, rat	ED-1, qualitative	Old ↑	Not stated	Badan et al., 2003a; Badan et al., 2003b
Transient retinal ischemia, rat	GSA I-B4 lectin, qualitative	Old ↑	Old ↑	Kim et al., 2004
Cortical stab injury, rat	ED1, qualitative	Old ↑	Not stated	Kyrkanides et al., 2001
MPTP-induced neurotoxicity, mouse	CR3, cell counts	Old ↑	Old ↑	Sugama et al., 2003
Facial nerve axotomy, rat	MHC II, CR3, area ratio	No	No	Hurley and Coleman, 2003
Sciatic nerve constriction, rat	CR3, area ratio	Old ↑	Old ↓	Stuesse et al., 2000

The Facial Nerve Axotomy Model

All studies of aging-related changes in the course of microglial activation in this manuscript were conducted using the facial nerve axotomy model. The facial nerve (cranial nerve VII) in the rat splits into several branches that innervate the muscles involved in facial movement, including those controlling whisker movement (Fig. 1-1A). The facial nerve cell bodies are located in the ipsilateral facial motor nucleus in the

brainstem (Fig. 1-1B), and are arranged in a strict somatotopic organization of subnuclei. Transection (axotomy) of the facial nerve results in loss of whisker movement with complex cellular changes and somatotopic reorganization in the nucleus of the ipsilateral side (Moran and Graeber, 2004; Thomander, 1984).

The facial nerve axotomy model is ideal for the study of the effects of aging on microglial activation because: (1) the response of microglia and the time course of regeneration of facial motoneuron axons are already well established in young adult animals; (2) the procedure is simple and highly reproducible; (3) the blood-brain barrier remains intact, (4) the uninjured neurons in the contralateral nucleus are visible in the same section as the experimental nucleus in coronally sectioned brainstem, and thus the contralateral facial nucleus serves as a convenient internal control, and (5) a remote lesion allows assessment of the microglial response without direct brain manipulation. In the facial nerve axotomy model, microglia are not responding to direct injury of the tissue itself, only to signals from neurons who have had a remote injury of their axons (Moran and Graeber, 2004). Thus, the model is an ideal system for examining neuronal-microglial signaling.

In young adult rats, there is a well-established microglial response following axotomy of the facial nerve: by 24h after injury there is evidence of microglial activation with hypertrophy and increased OX-42 immunoreactivity (Graeber et al., 1988; Kreutzberg et al., 1989); 2-4 days after axotomy there is a peak in microglial proliferation, and microglial envelopment of neuronal perikarya with accompanying

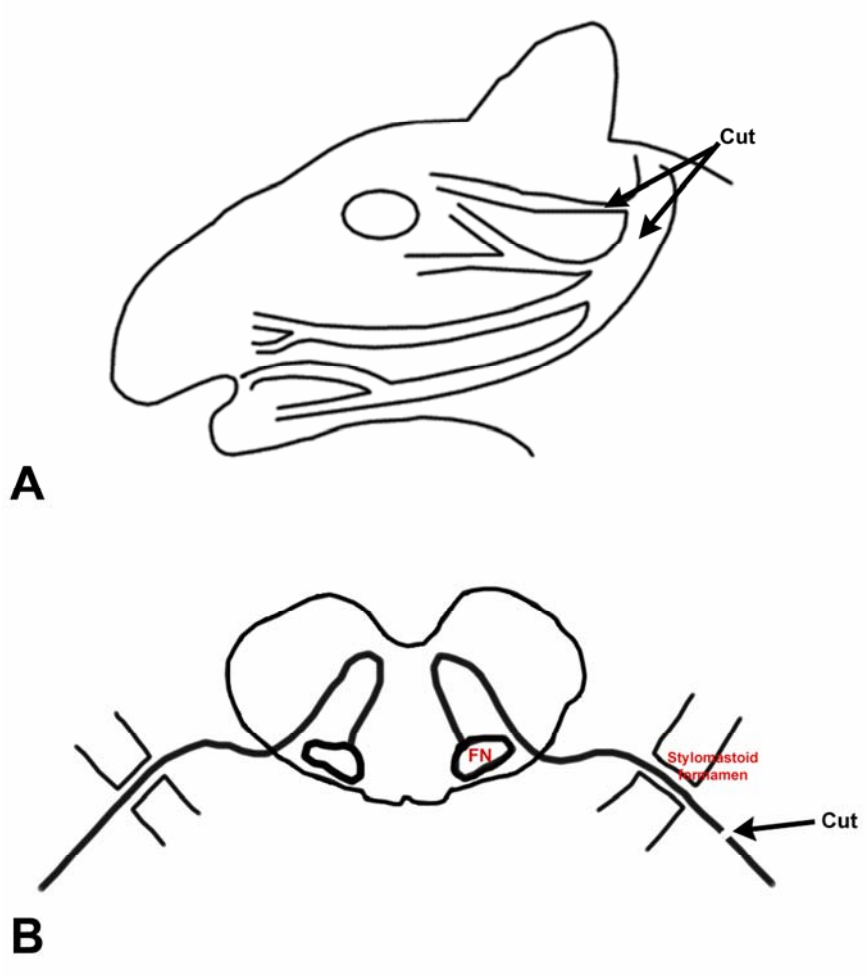

Figure 1-1. The facial nerve axotomy model. (A) The facial nerve exits the skull at the stylomastoid foramen, branching to innervate the facial musculature, including those muscles involved in whisker function. The first primary branching from the main trunk occurs just after the nerve exits the skull, and both the main trunk and temporal branch are transected during the axotomy procedure (arrows). (B) The facial nuclei containing the motoneuron cell bodies are located on either side of the ventral region of the brainstem. The motoneuron axons loop around the abducens nucleus in the dorsal portion of the brainstem (genu), and then project from the ventral surface of the brainstem as the facial nerve before exiting the skull at the stylomastoid foramen.

synaptic displacement begins (Blinzinger and Kreutzberg, 1968); neuronal ensheathment and synaptic displacement continue for about 2 weeks, and are accompanied by neuronal chromatolysis (retrograde change) in the facial nucleus and extensive sprouting near the site of injury. While disruption of the somatotopic organization of the facial nucleus

continues indefinitely after axotomy in the rat (Choi and Raisman, 2002; Thomander, 1984), the majority of facial motoneurons survive in the rat, with reinnervation first observed approximately 2 weeks after axotomy, reaching the maximum observed reinnervation of 80% around 5 weeks post-axotomy (Moran and Graeber, 2004). Thus, the facial nerve axotomy is a model of regeneration.

CHAPTER 2
THE EFFECT OF AGING ON THE PROLIFERATIVE RESPONSE OF MICROGLIA TO FACIAL NERVE INJURY

Introduction

In the intact CNS, the number of microglia is roughly equal to the number of neurons, although the ratio has regional variation (Lawson et al., 1990; Streit, 1995). As with the heterogeneity of their morphology, the regional variation in microglial density is likely a reflection of their function in the local microenvironment, with the microglial population adjusting in size to fulfill their duties of surveillance, protection and support of neurons. It has been shown that facial nerve axotomy specifically triggers a wave of microglial proliferation in the adult rat (Cammermeyer, 1965; Gehrmann and Banati, 1995; Graeber et al., 1988; Kreutzberg, 1966; Kreutzberg, 1968), and evidence suggests that the activation of microglia is a crucial component of the regeneration program (Streit, 2002). The few studies that have examined aging-related changes in the microglial reaction to neuronal injury have only measured upregulation of microglial immunophenotypic markers (Badan et al., 2003a; Badan et al., 2003b; Gong et al., 2004; Hurley and Coleman, 2003; Kim et al., 2004; Kyrkanides et al., 2001; Stuesse et al., 2000; Sugama et al., 2003). This is the first aging study to employ a more quantifiable means of labeling dividing microglia with tritiated (^{3}H)-thymidine. Immunohistochemical methods do not distinguish between microglial hyperplasia (mitosis) and phenotypic changes such as microglial hypertrophy and upregulation of surface antigens, while cells that have incorporated ^{3}H-thymidine into their DNA are explicitly identifiable as proliferating cells. Because previous studies have shown delayed recovery from cranial nerve

injury in old animals (Streppel et al., 1998; Vaughan, 1990; Vaughan, 1992), we hypothesized that microglial proliferation in response to neuronal injury would be decreased or delayed in old animals. However, the results demonstrate that rather than an attenuated or delayed response, it is the downregulation of microglial proliferation after the peak response that is attenuated with aging.

Materials and Methods

Animals

Animal use protocols were approved by the University of Florida Institutional Use and Care of Animals Committee (IUCAC). The animals used for this study were 3 month old (young), 15 month old (middle-aged) and 30 month old (old) virgin male Fisher 344-Brown Norway (F344BN) F1 hybrid rats obtained from the National Institute on Aging in Bethesda, MD. All animals arrived at their appropriate ages immediately (1-3 days) before use in these experiments. After arrival at our facility, animals were housed in the specific pathogen-free, climate-controlled animal care facility within the McKnight Brain Institute of the University of Florida. Animals were fed sterile rat chow and water without restriction. Equal numbers of animals from each age group were ordered and operated at the same time for each experiment.

Facial Nerve Axotomy

Animals were anesthetized with isoflurane using a precision vaporizer machine with gas scavenging system attached. Anesthesia was induced with an isoflurane mixture of 5% in the inducing box, and then maintained during surgery with an isoflurane mixture of 1-3% using a nose cone. The level of sedation was monitored by assessing for absence of pedal (toe pinch) and palpebral reflexes. In addition, respiratory rate, mucous membrane color and skin warmth were monitored during the procedure. Once the animal

was fully sedated, a small patch of skin behind the right ear was shaved and prepped with Betadine skin cleanser. A hemostat was used to fold the ear towards the face, and a small (~1 cm) opening at the base of the dorsal aspect of the ear (near the exit of the stylomastoid foramen) was cut using sterile scissors. The primary and temporal branches of the facial nerve were visible under the superficial muscle layer as white fiber-like tissue. Using sterile forceps, both the primary and temporal branches of the nerve were gently lifted and cut using surgical scissors until two separate stumps were visible for each branch. Care was taken to completely cut both branches in every animal to reduce inter-animal variation in the degree of microglial activation, as each branch is associated with distinct topographical subnuclei within the facial nucleus (Moran and Graeber, 2004). The incision was closed using one surgical staple. The anesthesia apparatus was removed and the animal monitored until fully recovered. Once the animal recovered from anesthesia, absence of whisker movement on the side of the injury was assessed to insure that the injury was complete. Animals were monitored for 1-2 hours before returning to the animal care facility, and monitored daily thereafter.

^{3}H-Thymidine Injection

For the labeling of microglial proliferation following facial nerve axotomy, animals were weighed and given intraperitoneal (i.p.) injections of 3 μCi per gram body weight [methyl-^{3}H]Thymidine (Amersham Pharmacia Biotech) at 47, 71, or 95 hours post transection of the facial nerve. Tritiated-thymidine is incorporated into DNA during the pre-mitotic S-phase (Kreutzberg, 1968). All cells demonstrating ^{3}H-thymidine incorporation will be referred to in this manuscript as "^{3}H-thymidine labeled cells". After injection, the animals were kept in cages with radioactive caution labels for 2 hours until euthanasia via transcardial perfusion.

Radioactive Perfusion

Animal euthanasia was performed using a transcardial perfusion procedure with modifications to prevent contact or environmental contamination with radioactive blood or tissue. All permanent surfaces that might possibly come into contact with blood or tissue were covered with absorbent pads, and the perfusions were performed in a hood over a collection box designated for collection of radioactive blood. Gloves, masks, protective eyewear, gowns, and shoe covers were worn. All surgical instruments were designated for use with radioactive animals only, and were cleaned thoroughly with surface radioactivity decontaminant after use. All blood, unused tissue, and disposable materials were collected for pick up by the office of Radiation Control. The procedure areas were thoroughly cleaned and surveyed for contamination after use.

Two hours after injection with ^{3}H-thymidine, each animal was given a lethal dose of the anesthetic sodium pentobarbital (150 mg/kg; i.p.; Schering Plough Animal Health). The perfusion procedure was started after the animal was fully anesthetized but before respirations ceased. The level of anesthesia was monitored by assessing for absence of pedal (toe pinch) and palpebral reflexes. Once the animal was fully anesthetized, the animal was transcardially perfused with phosphate buffered saline (PBS, pH 7.2) followed by 4% paraformaldehyde (in PBS) according to our laboratory's University of Florida IUCAC standard operating procedure #0026. At the end of the perfusion, the brain of the animal was dissected from the cranial vault and transferred to a vial containing 4% paraformaldehyde for post-fixation. At this point a random and unique identification number was assigned to each animal by a laboratory member not involved in the quantitative analysis. The number was recorded on the surgical record and the tissue vial. All subsequent identification of each animal (including on slides) was via this

number until the identity of each animal was revealed at the completion of data collection.

Tissue Processing

Brain tissue was post-fixed overnight in 4% paraformaldehyde. After rinsing the tissue in PBS, an approximately 5 mm thick coronal brainstem tissue slice containing the facial nuclei was dissected from each brain. Each brainstem slice was processed for paraffin embedding by slowly dehydrating through 70% ethanol for 2h, 45 min. each of 70%, 90%, 95%, and 100% ethanol, 100% ethanol overnight, 100% ethanol for 30 min., and then cleared in 2 changes of xylenes for 1.5h each. Each slice of tissue was transferred into its own paraffin cassette (with animal identification number), and infiltrated in 2 changes of Surgipath Formula R paraffin (Surgipath, Richmond, IL) at 60°C for 2h each. Using a TissueTek paraffin embedding console and metal molds, the tissues were embedded in Surgipath Tissue Embedding Medium paraffin at 60°C and allowed to cool. A 26 gauge needle was inserted through the dorsal portion of the brainstem of the unoperated side as a mark for future orientation.

Using a Reichert Autocut microtome, 7 μm paraffin brainstem sections were collected from the rostral to the caudal end of the facial nuclei of each animal. All sections were collected in a warm water bath and transferred to Superfrost Plus (Fisher Scientific) slides in a systematic pattern so that every third section was placed on a different slide. Each slide was labeled with the sequential order number of each section on the slide, and with the animal's unique identification number.

Autoradiography

Immediately before autoradiography, paraffin sections were deparaffinized through xylenes (2 changes of 15 min. each) and descending alcohols (3 min. each of 100%,

100%, 95%, 90%, 75%, and 75% ethanol), and then rinsed in PBS. Equipment (safety light (15 watt) with Kodak #2 filter, water bath, thermometer, beakers, conical tube with 20-30 ml distilled, deionized water (ddH_2O), glass single slide dipping vessel, paper towels, slide box with desiccant packet, aluminum foil, gloves) was set up in a darkroom. The water bath was set at 43-45°C, and Kodak NTB2 emulsion was placed in the water bath and allowed to melt for approximately 20-30 min. Once the emulsion was melted, under darkroom conditions the emulsion container was opened, and an equal volume of emulsion was poured into the conical tube containing water and gently mixed. The diluted emulsion mixture was poured into the glass slide dipping vessel and kept in the water bath. Each slide was gently dipped into the emulsion mixture, the back of the slide was wiped, and the slide was allowed to air dry on paper towels. After all the slides were dipped and allowed to dry, the slides were transferred to a slide box containing a packet of desiccant (sheet of filter paper shaped into a packet around a small amount of desiccant). The slide box was covered in aluminum foil before turning room lights back on. The emulsion dipped slides were kept at 4°C for 5 weeks.

After 5 weeks, slides were developed under dark room conditions using a safety light with Kodak #2 filter. Slides were transferred to slide racks and processed through 50% diluted Dektol (Eastman Kodak) developer for 2.5 min., ddH_2O for 10 dips, Kodak fixer for 5 min., and ddH_2O for 5 min. Slides were immediately counterstained with 0.5% cresyl violet acetate (Sigma Chemical Co.), quickly dehydrated through ascending alcohols, cleared in 2 changes of xylenes for 2 min. each, and coverslipped using Permount mounting medium (Fisher Scientific).

Quantitative Analysis of the Number of Cells with ^{3}H-Thymidine Incorporation

For quantitative analysis of the density of ^{3}H-thymidine labeled cells in the facial nucleus, sections were selected systematically throughout each facial nucleus. In each selected section, the facial nucleus was outlined, the area within the outline was measured, and ^{3}H-thymidine labeled cells within the outline were counted using MCID 6 software (Imaging Research, St. Catharines, Ontario) and a Sony DXC970 camera attached to a Zeiss Axiophot microscope. This method is illustrated in Figure 2-1. The total number of ^{3}H-thymidine labeled cells counted for each animal was pooled, and this number was divided by the total area measured to determine the population density (labeled cells per unit area) of proliferating microglia within the facial nucleus of each animal.

Results are represented as mean values ± SEM. The density of dividing microglia in the lesioned facial nucleus were compared between age-groups at each time point with a one-way ANOVA using GraphPad Prism software (GraphPad Software, San Diego, CA). Tukey's multiple comparison test was used for post hoc comparisons. The distribution of dividing cells for each age group and time point was analyzed with a two-way ANOVA in GraphPad Prism. A significance level of $p \leq 0.05$ was used.

A preliminary study (N=2) was performed to compare variability in the measurement of ^{3}H-thymidine-labeled cell population density when using approximately every 10th section (approximately 70 μm apart) versus every 20th section (approximately 140 μm apart). No difference was found in the population density measured using either distance between sections (Fig. 2-2). Therefore, data collection for the entire study was conducted using approximately every 20th section (6-9 sections/animal, N=5-6).

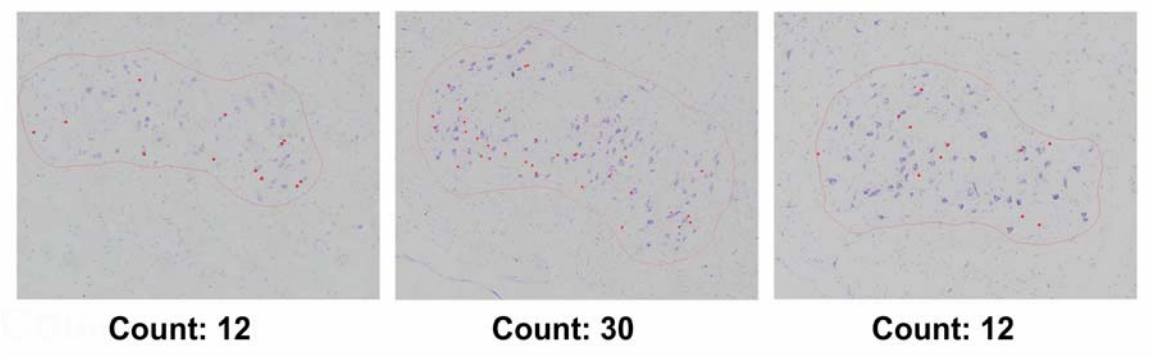

Figure 2-1. Method of quantifying the number of ^{3}H-thymidine labeled microglia per unit area. Systematic random sections (~140 μm apart) were selected throughout each facial nucleus (6-9 sections/animal). The facial nucleus was outlined, the area within the outline was measured, and ^{3}H-thymidine labeled cells were counted (red circles) using MCID 6 software (Imaging Research) and a Sony DXC970 camera attached to a Zeiss Axiophot microscope. The total count was divided by the total area to determine the population density of mitotic microglia within the facial nucleus of each animal.

Co-labeling of ^{3}H-Thymidine Incorporated Cells with Lectin

To confirm that proliferating cells in the vicinity of the injured facial nucleus are microglia, sections were labeled with a histochemical marker of microglia before emulsion dipping. Sections from the ^{3}H-thymidine injected animals were deparaffinized as described above, but were left in the last change of 70% ethanol overnight. After rinsing in PBS, sections were encircled with a grease pen. The microglia-specific Griffonia simplicifolia B4 isolectin conjugated to horseradish peroxidase (GSA I-B4-HRP, Sigma Chemical Co.) diluted 1:100 in PBS containing cations (0.1mm of CaCl2, MgCl2, and MnCl2) and 0.1% Triton-X100 was applied to each section overnight at 4°C in a moist chamber. After incubation, the slides were washed briefly in PBS 3 times and incubated with 3,3'-diaminobenzidine (DAB)-H2O2 substrate until a brown color reaction was visible (approximately 5 min.). Lectin-stained sections were immediately taken for autoradiography as described above. After development, sections co-labeled with lectin and 3H-thymidine incorporation (silver grains) were counterstained with 0.5%

cresyl violet acetate, dehydrated through ascending alcohols and coverslipped. Images of the co-labeled sections were taken using a Spot RT color camera attached to a Zeiss Axioskop 2 microscope.

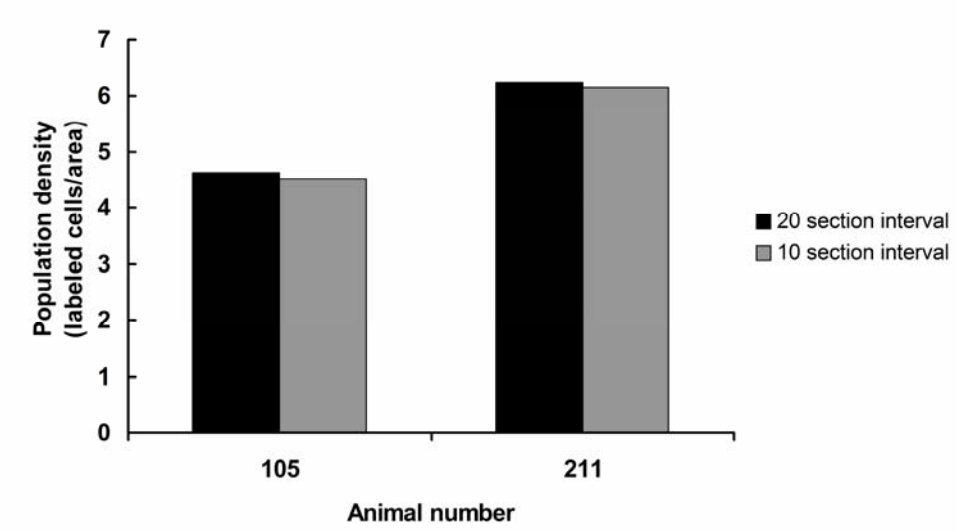

Figure 2-2. The quantitative analysis of ^{3}H-thymidine labeling does not significantly differ when using 10 (70 μm) versus 20 (140 μm) section intervals. Labeled cell nuclei in the lesioned facial nucleus of 2 animals were counted using both intervals between sections. Based on these preliminary results, the quantitative analysis of all animals was conducted using the larger (20) section interval.

Technical Considerations

Tritiated-thymidine incorporation into the DNA of pre-mitotic cells is visualized in autoradiography sections as clusters of silver grains over the cell nuclei (Fig. 2-3). It should be noted that equal numbers (N=6) of animals from each age/time point group were injected with ^{3}H-thymidine, but most of the 9 different age/time point groups had 1 animal excluded because the animal failed to demonstrate any clusters of silver grains in the brainstem after autoradiography. Because the animals who did demonstrate labeling had clearly distinguishable labeled cells, and the differences among the standard deviations within each group were not significant (Bartlett's test), it appears that those

few animals without labeling did not take up the radioactive label due to a technical error in the injection technique. It appears that the dose of ^{3}H-thymidine was strong enough to label all dividing microglia as long as some of it infiltrated into the brain. In the future, it may be beneficial to reserve tissue with high mitotic activity, such as the testes, to confirm lack of systemic distribution of the ^{3}H-thymidine tag when no labeling is found in the injured facial nucleus.

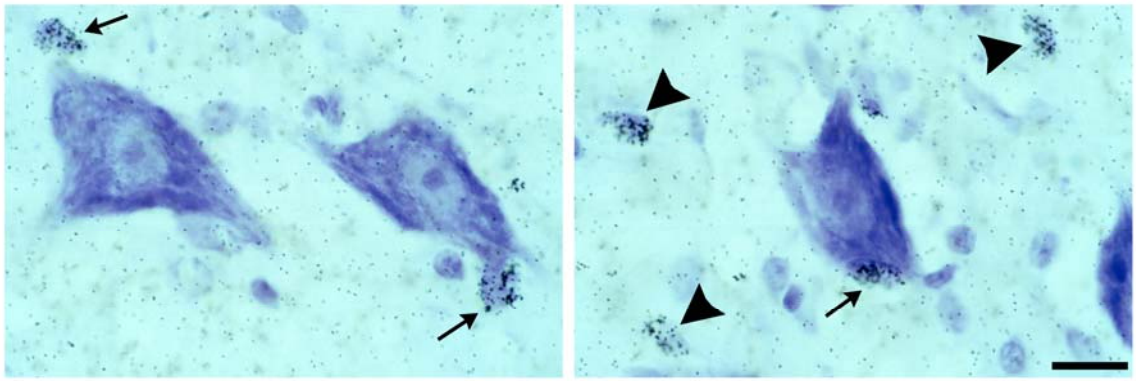

Figure 2-3. Tritiated-thymidine labeled cells in the lesioned facial nucleus. Labeled cell nuclei are found adjacent to neurons (arrows) and within the neuropil (arrowheads). Bar= 20 μm.

Results

Dividing microglia are present in the injured facial nucleus of young, middle-aged and old animals 2 days following axotomy. Originally it was hypothesized that microglial activation, including the proliferative response, might be delayed in old animals based on reports that the regeneration of peripheral axons after facial nerve injury is delayed in old rats (Streppel et al., 1998; Vaughan, 1990; Vaughan, 1992). Two days following facial nerve axotomy, clearly identifiable labeled cell nuclei were visible in the vicinity of the lesioned facial nucleus in most ^{3}H-thymidine injected animals (Fig. 2-5D, E, F), with no detectable difference in the density of labeled cells between the three

age groups (Fig. 2-4). Therefore, there appears to be no aging-related difference in the initiation of the proliferative response of microglia to motoneuron injury.

Microglial proliferation after facial nerve axotomy peaks at 3 days in all age groups. Three days following axotomy, dividing microglia were at their highest density within the lesioned facial nuclei of all animals. Additionally, the population density during this peak did not appear to differ between age groups (Fig. 2-4). Thus, there is no discernable aging-related difference in the robustness and time course of the initial proliferative response of microglia to facial nerve injury. On day 3, dividing microglia were frequently found in perineuronal positions (Figs. 2-3), although many were scattered throughout the neuropil within and immediately adjacent to the injured facial nucleus (Figs. 2-5G, H, I; 2-7C).

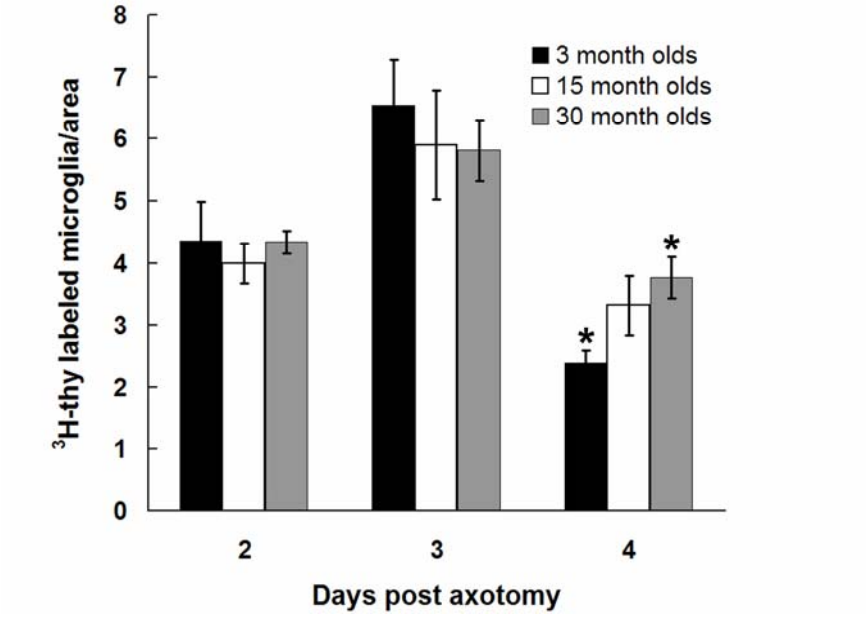

Figure 2-4. Aging attenuates downregulation of the microglial proliferative response to facial nerve axotomy. The density of dividing cells was significantly greater in old (30 m.o.) animals compared to young (3 m.o.) animals 4 days after injury (*$p < 0.05$), but no aging-related difference in microglial proliferation was detected on post-axotomy days 2 or 3.

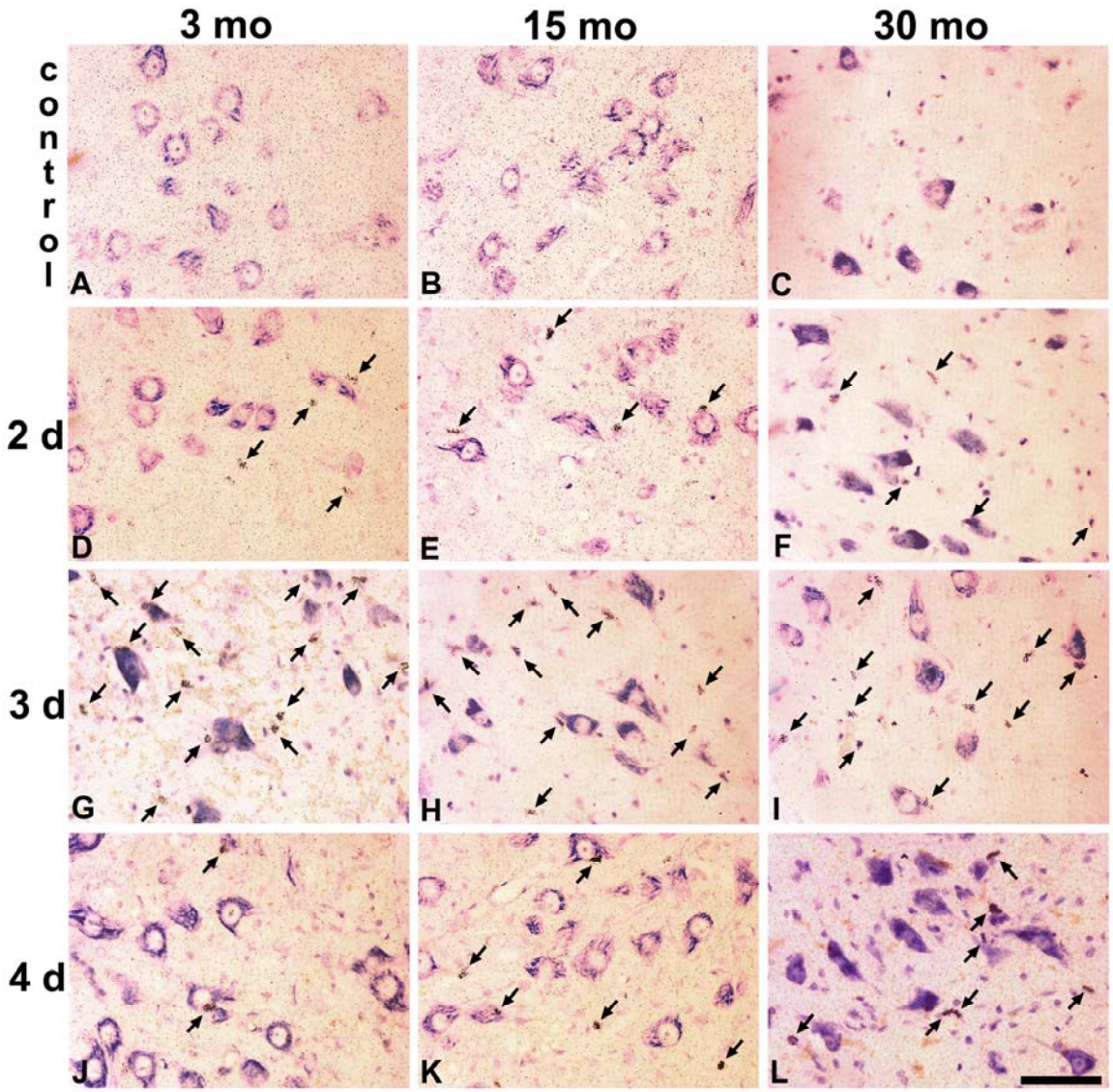

Figure 2-5. Photomicrographs of the time course of ^{3}H-thymidine labeling. (A-C) Generally, no labeled cells are found in the unlesioned (control) facial nuclei. (D-L) ^{3}H-thymidine labeled cell nuclei (arrows) in the lesioned facial nucleus demonstrating the time course of microglial proliferation at 2 (D-E), 3 (G-I) and 4 (J-L) days following axotomy in young (3 mo), middle-aged (15 mo) and old (30 mo) rats. On post-axotomy day 4 more labeled cells are found in old animals (L) compared to young animals (J). Bar= 100 μm

Aging attenuates the downregulation of the microglial proliferative response to facial nerve axotomy. By day 4, proliferation had sharply declined in the young animals, but the decline was not as sharp in the old age group, with the number of dividing microglia remaining significantly higher ($p<0.05$) in 30 month old versus 3

month old animals (Figs. 2-4, 2-5J, L). The population density of dividing microglia in the lesioned facial nucleus of 15 month old animals fell in between that of young and old animals and was not significantly different from either group (Fig 2-4, 2-5K). Thus, there appears to be an age-related trend in the degree of microglial proliferation continuing to occur 4 days following axotomy.

No significant microglial proliferation occurs in the non-lesioned facial nucleus. In all animals, very few (a maximum of 4 in all examined sections of each animal) ^{3}H-thymidine labeled cell nuclei were found in the unoperated facial nucleus (Fig. 2-5A, B, C), and the same applied to the rest of the brainstem outside of the vicinity of the lesioned facial nucleus. Thus, the basal level of microglial proliferation does not appear to increase with aging in the rat brainstem.

All cells that proliferate in response to facial nerve axotomy are microglia. Tritiated-thymidine labeled cells all co-localized with lectin labeling (Fig. 2-6). This is the first histochemical co-labeling confirmation that microglia are the only cells that divide after facial nerve axotomy. Previous studies demonstrated that microglia are the only glia to undergo cell division in response to a peripheral nerve injury using ultrastructural morphology (Graeber et al., 1988; Svensson et al., 1994).

The distribution of dividing microglia in and around the lesioned facial nucleus changes between post-axotomy days 2 and 3 in all age groups. During quantitative analysis of the ^{3}H-thymidine labeled microglia, it was noted that in some animals many of the labeled dividing cells were found in the white matter along the ventral border between the lesioned facial nucleus and the section edge (Fig. 2-7B). Because the counts were performed without knowledge of the age or time point of the

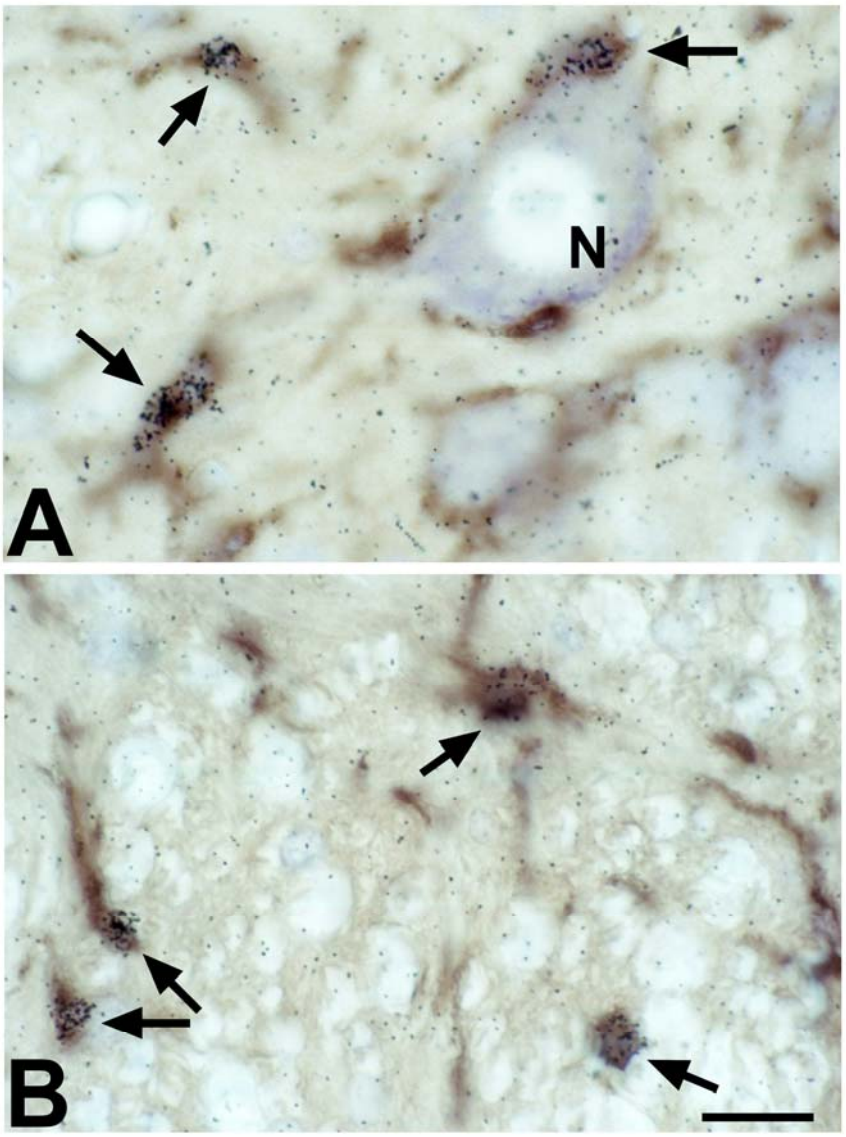

Figure 2-6. All cells that proliferate in response to facial nerve axotomy are microglia. ^{3}H-thymidine labeled nuclei co-localize with lectin histochemistry for microglia in the perikarya of the lesioned facial nucleus (A) and in the white matter immediately adjacent to the ventral border of the lesioned facial nucleus (B). N, neuron with a perineuronal labeled microglia. Bar= 20 μm.

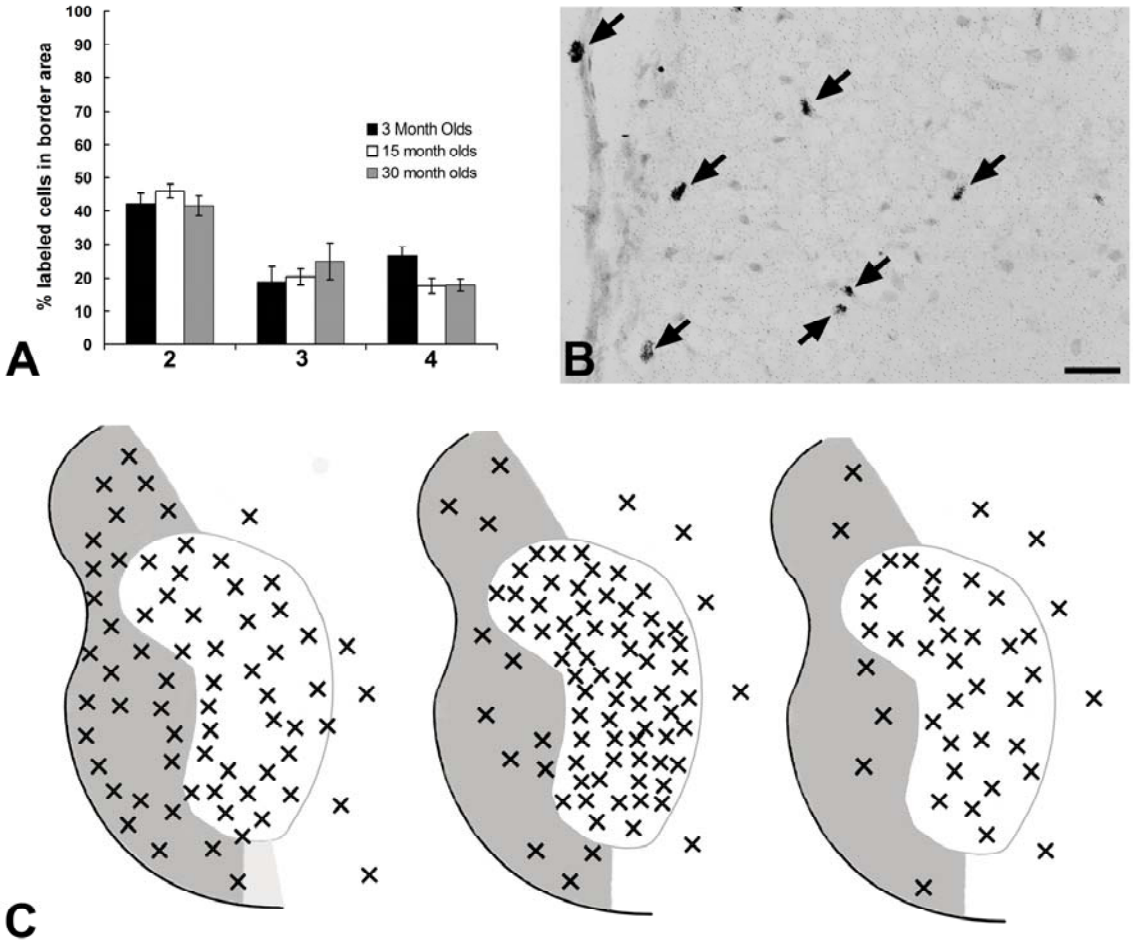

Figure 2-7. Distribution of dividing microglia in the vicinity of the lesioned facial nucleus 2, 3 and 4 days following axotomy. (A) Graph showing the percentage of ^{3}H-thymidine labeled cells found in the white matter of the ventral border region at each time point. The percent of labeled cells in this border region on post-axotomy day 2 was significantly higher than on days 3 and 4 in all age groups ($p<0.0001$ for all age groups). No difference in the distribution was detected between age groups. (B) Two days after axotomy many of the dividing cells (arrows) are found in the white matter region between the ventral border of the facial nucleus and the edge of the section. (C) Diagrams of the distribution of ^{3}H-thymidine labeled cells at 2, 3 and 4 days following axotomy. The facial nucleus is outlined in gray, and the ventral border region is shaded in gray. Bar= 35 μm

animal, initially it was not known if there was a pattern to this distribution. During data collection, the ratio of labeled cells inside the outlined border of the facial nucleus and between the ventral border and section edge was noted for each section. After decoding

the identity of the animals, we examined whether a correlation existed between the distribution of labeled dividing microglia and time point and/or age. On day 2, many of the dividing cells were detected in the white matter along the ventral border of the injured facial nucleus, but by day 3 the distribution of ^{3}H-thymidine labeled cells was centered within the injured facial nucleus; thus there was a correlation between distribution of labeled cells and post-injury time point ($p<0.0001$) (Fig. 2-7C). However, the distribution of dividing microglia within the injured facial nucleus and along the ventral border did not appear to differ between age groups at any of the time points examined (Fig. 2-7A).

Discussion

In young rodents, microglia normally respond to neuronal injury by increasing in number, with microglial mitosis serving as a major contributor to this population expansion (Gehrmann and Banati, 1995; Graeber et al., 1988; Kreutzberg, 1966; Kreutzberg, 1968). Microglia ensheath motoneurons while displacing afferent synapses, presumably a neuroprotective function (Blinzinger and Kreutzberg, 1968; Raivich, 2002; Streit, 2002). In contrast, the lack of regeneration after a central axotomy is associated with poor induction of microglial activation (Barron et al., 1990; Streit et al., 2000; Tseng et al., 1996). Based on this knowledge and reports that cranial nerve regeneration is delayed in old rodents (Vaughan, 1990; Vaughan, 1992), we initially hypothesized that the microglial response to motoneuron injury would be weak or delayed in old rats. However, our ^{3}H-thymidine data did not show a significant aging-related difference in microglial proliferation at 2 and 3 days following axotomy of the facial nerve, and proliferation peaked at 3 days in all three age groups. Therefore, the initial microglial proliferative response to peripheral axotomy appears to be just as robust in old animals as

in young animals. Microglial mitosis attenuated substantially by post-injury day 4 in the young animals, but surprisingly in the old animals the number of dividing microglia remained significantly higher. Attenuation of the proliferative response is likely an important factor in maintaining the proper balance of microglial cells needed for success of the motoneuron regeneration program, but the downregulation of this response is changed with aging.

The initial distribution of many of the dividing microglia outside of the injured facial nucleus demonstrates that initiation of microglial mitosis does not primarily occur in perineuronal locations, as previously thought (Graeber et al., 1988; Kreutzberg, 1968). Other studies have demonstrated microglial recruitment and migration from adjacent areas to sites of injury (Dong et al., 2004; Gehrmann et al., 1991; Hailer et al., 1997; Hailer et al., 1996; Jensen et al., 1997; Jensen et al., 1994). The time course of the distribution of dividing microglia found in this study shows a pattern of migration from the white matter towards the injured motoneurons between 2 and 3 days following axotomy (Fig. 2-7C). Neuronal injury results in a much greater need for the protective and supportive functions of microglia, and the resting state population density of microglia within the facial nucleus becomes inadequate. Thus, it is not surprising to find proliferation of microglia in the white matter area adjacent to the facial nucleus. An aging-related difference in the distribution pattern of dividing microglia was not found. Although there may be aging-related changes in the densities of different immunophenotype sub-populations of microglia (e.g., those expressing MHC class II) (DiPatre and Gelman, 1997), the lack of an aging-related difference in the basal rate of microglial proliferation or the distribution of proliferating cells after injury suggests that

the ratio of gray and white matter total microglial population density in the intact brainstem does not change with aging.

The location of many dividing microglia outside of the facial nucleus also suggests that microglia must receive some sort of diffusible mitosis-inducing signal from injured neurons. Delayed axonal regeneration (Vaughan, 1992) may sustain the putative mitosis-stimulating signaling pathway, prolonging microglial proliferation. Alternatively, an aging-related change in the ability to modulate the diffusible signal as the acute phase of neuronal injury subsides may result in impaired regulation of microglial mitosis in old animals. This notion is supported by an *in vitro* study showing that the ability of transforming growth factor-beta1 (TGF-β1) to inhibit proliferation of microglia was impaired in cultures from aging donors compared to young donors (Rozovsky et al., 1998). TGF-β1 has been shown to modulate the putative neuronal-microglial signaling function of the chemokine fractalkine (CX3C1) and its receptor, CX3CR1 (Chen et al., 2002). The evidence from this study indicating an aging-related attenuation of the regulation of the microglial proliferative response to neuronal injury prompted further investigation into aging-related changes in neuronal-microglial signaling via fractalkine and CX3CR1, which is the focus of Chapter 5 of this work.

Another potential explanation for the extended microglial proliferative response in old animals is the possible presence of inherent effects of aging within microglia themselves. The high mitotic potential of microglia may make them particularly vulnerable to the effects of aging. Human microglia demonstrate significant dystrophic changes with aging (Streit et al., 2004), and the telomeres of cultured microglia shorten until they undergo replicative senescence (Flanary and Streit, 2004). It is plausible that

microglia, particularly those which have undergone repeated rounds of replication (such as in the case of head injury), may reach a state of dysfunction with aging that makes them unable to attenuate their proliferative response in spite of neuronal regulatory signals. Furthermore, senescent changes within microglia may make them less sensitive and responsive to modulators of neuronal-microglial signaling pathways (i.e., TGF-β1). Whether it is a product of changes in neuronal signals, microglial dysfunction, or both, a change in the regulation of microglial activation with aging may have significant implications for older individuals with brain, cranial nerve or spinal cord injuries, and may play a role in the aging-related increase in susceptibility to neurodegenerative diseases.

CHAPTER 3
THE EFFECT OF AGING ON MICROGLIAL PROGRAMMED CELL DEATH FOLLOWING THE PROLIFERATIVE RESPONSE TO NEURONAL INJURY

Introduction

Microglia activated in response to facial nerve axotomy proliferate and migrate towards injured motoneurons (Moran and Graeber, 2004), significantly increasing the microglial population density within the lesioned facial nucleus. Following their proliferative burst in response to neuronal injury, microglia appear to gradually decrease in number (Raivich et al., 1993; Streit et al., 1988). Previously it was thought that homeostatic regulation of the post-mitotic microglia population occurred by the exit of activated microglia through blood vessel walls (Cammermeyer, 1965; Del Rio-Hortega, 1932), but more recent evidence shows that a form of programmed cell death may play a role in the return of microglia to their pre-injury numbers (Gehrmann and Banati, 1995; Jones et al., 1997).

The terms apoptosis and programmed cell death are routinely used interchangeably to describe the process by which cells die under tight regulation by signaling pathways in a manner which does not disrupt the surrounding environment or invoke an inflammatory response. The classical morphological characteristics of apoptosis are cell shrinkage, chromatin condensation, cell membrane blebbing (with retention of membrane integrity), and the formation of apoptotic bodies, and the process is mediated by caspases (Bohm and Schild, 2003; Gavrieli et al., 1992; Jaattela and Tschopp, 2003). Recently, however, alternative mechanisms of programmed cell death (as opposed to "accidental" cell death

associated with necrosis) other than classical apoptosis are being acknowledged by a growing number of researchers (Graeber and Moran, 2002; Jaattela and Tschopp, 2003; Lockshin and Zakeri, 2004; Nagy and Mooney, 2003). Like classical apoptosis, alternative mechanisms of cell death are highly regulated, but they may or may not have the classical morphological characteristics of apoptosis (e.g., chromatin margination), and are often caspase-independent (Lockshin and Zakeri, 2004). Jones and colleagues (1997) provided evidence that the programmed cell death of microglia occurs via a non-classical mechanism.

The study of aging-related changes in the proliferative response of microglia described in Chapter 2 of this dissertation indicated that the downregulation of microglial proliferation is attenuated in old animals. This suggests that, unless the prolonged period of proliferation is coupled with a higher rate of microglial turnover, there may be a transient period of higher numbers of activated microglia in the lesioned facial nucleus of old animals. The main objective of the study in the current chapter was to compare the post-mitotic turnover of activated microglia via programmed cell death in the lesioned facial nucleus of young and old animals using the ApopTag assay, a kit variation of the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling (TUNEL) method. TUNEL is a method for visualizing the DNA fragmentation that typically accompanies apoptosis by enzymatically labeling the free 3'-OH ends of DNA fragments with modified nucleotides (Gavrieli et al., 1992). In the immune system, programmed cell death of the expanded lymphocyte population is an essential component of returning the system to homeostasis at the decline of an immune response (Jaattela and Tschopp, 2003), and it is reasonable to assume that a balance between proliferation and

turnover of microglia at the decline of the injury response is essential to the homeostasis of the CNS microenvironment. While we suspected that there might be an increase in microglial turnover to balance the prolonged period of microglial proliferation in the old animals, studies of physiological apoptosis in other systems have found aging-related declines in the potential for apoptosis (Warner, 1997). However, we found no evidence of either an increase or decrease in the number of dying microglia with aging, nor a change in the time course of microglial turnover.

Materials and Methods

Animals, Surgery and Tissue Processing

Male young (3 month old) and old (30 month old) F344BN hybrid rats were obtained from the National Institute on Aging. Animals were subjected to a right facial nerve axotomy as described in Chapter 2. Animals were euthanized at 7, 14 or 21 days following facial nerve axotomy using transcardial perfusion. The transcardial perfusion procedure was described in detail in Chapter 2. The perfusion procedure for TUNEL analysis was performed in the same manner, except that radioactive precautions were not needed and the procedure was performed directly in the perfusion hood rather than over a collection container. Briefly, animals were anesthetized with an overdose of sodium pentobarbital (150 mg/kg) and transcardially perfused with 0.1 M PBS (pH 7.2) followed by 4% paraformaldehyde in PBS. Brain tissue was removed and post-fixed in the same fixative overnight. Brainstem tissue containing the facial nuclei was paraffin processed and sectioned on a microtome at 7 μm as described in Chapter 2.

TUNEL

For the assessment of microglial programmed cell death using TUNEL, immediately before performing the TUNEL procedure 7 μm paraffin sections were

deparaffinized through xylenes (2 changes of 15 min. each) and descending alcohols (3 min. each of 100%, 100%, 95%, 90%, 75%, and 75% ethanol), and then washed in PBS. Deparaffinized sections were pre-treated with 0.5% Triton-X100 in PBS for 20 min. The ApopTag Peroxidase *in situ* Apoptosis Detection Kit (Serologicals Corporation, Norcross, GA) was then used as described by the manufacturer. Negative controls included omission of the terminal deoxynucleotidyl transferase (TdT) or the anti-digoxigenin-peroxidase conjugate. Positive controls included sections pretreated with DNAse I, sections of post-natal day 5 rat cortex, and sections of Bouin's fixed brain tissue. Bouin's fixative contains picric acid that nicks DNA and results in false-positive labeling similar to that of DNAse I treated tissue. Cells were counterstained with 0.1% methyl green, rinsed in deionized water, dehydrated in 100% N-butanol, cleared in xylenes, and cover-slipped using Permount.

Quantitative Analysis

For quantitative analysis of TUNEL positive microglia in the facial nucleus, sections were imaged using a color Spot RT digital camera (Diagnostic Instruments, Sterling Heights, MI) attached to a Zeiss Axioskop 2 microscope and Spot Advanced (version 3.4.5) software running on a Dell PC. Labeled cells within a counting frame placed over the axotomized or control facial nuclei were counted (5-8 sections/animal) using Image Pro Plus software (version 4.5.1, Media Cybernetics, Carlsbad, CA). The total number of labeled cells counted per total area measured was calculated. Results are represented as mean values ± SEM. Significant differences were determined with t-tests comparing young and old at each time point using GraphPad Prism software (GraphPad Software, San Diego, CA). A significance level of $p \leq 0.05$ was used.

Results

Aging-related differences in the density or distribution of TUNEL-positive cells in the lesioned facial nucleus were not detected. TUNEL-positive cells were distributed throughout the neuropil of the regenerating facial nucleus (Fig. 3-1A), and the distribution of TUNEL-positive cells did not appear to differ with aging. The greatest population density of TUNEL-positive cells within the facial nucleus was found at 14 days post injury in all animals. There was no evidence of a difference in the density of TUNEL-positive cells in the lesioned facial nucleus at 7, 14 or 21 days following axotomy between young (1.35 ± 0.08, 2.07 ± 0.19, and 1.14 ± 0.06 cells/unit area, respectively) and old (0.78 ± 0.13, 2.71 ± 0.20, and 0.84 ± 0.08 cells/unit area, respectively) animals (Fig. 3-1D). No TUNEL-positive cells were detected in the unoperated facial nucleus of any of the animals (Fig. 3-1B).

Microglia demonstrate an unusual cytoplasmic distribution of TUNEL in both young and old animals. TUNEL-positive microglia in the regenerating facial nucleus were found to have the non-typical cytoplasmic labeling described previously (Jones et al., 1997) (Fig. 3-2). The labeling was frequently distributed throughout the cytoplasm, revealing the morphological characteristics of microglia (e.g., ramified processes, apposition of the soma and processes around the neuronal perikarya in perineuronal cells), and there did not appear to be distinct nuclear labeling in such cells. The cytoplasmic labeling did not appear to be isolated in phagocytic vacuoles, thus making it unlikely that the microglia became labeled due to ingestion of fragmented DNA from adjacent dying cells. A few cells had a ring of labeling around the nucleus that did not extend into the processes (Fig. 3-2D). While we did not employ co-labeling with a specific microglial marker, microglia have previously been shown to be the only cell type

dying in this injury model during this time frame of the regeneration program (Gehrmann and Banati, 1995), and the majority of TUNEL-positive cells demonstrated the morphological characteristics of microglial. There appeared to be no aging-related difference in the degree of cytoplasmic TUNEL labeling between young and old animals.

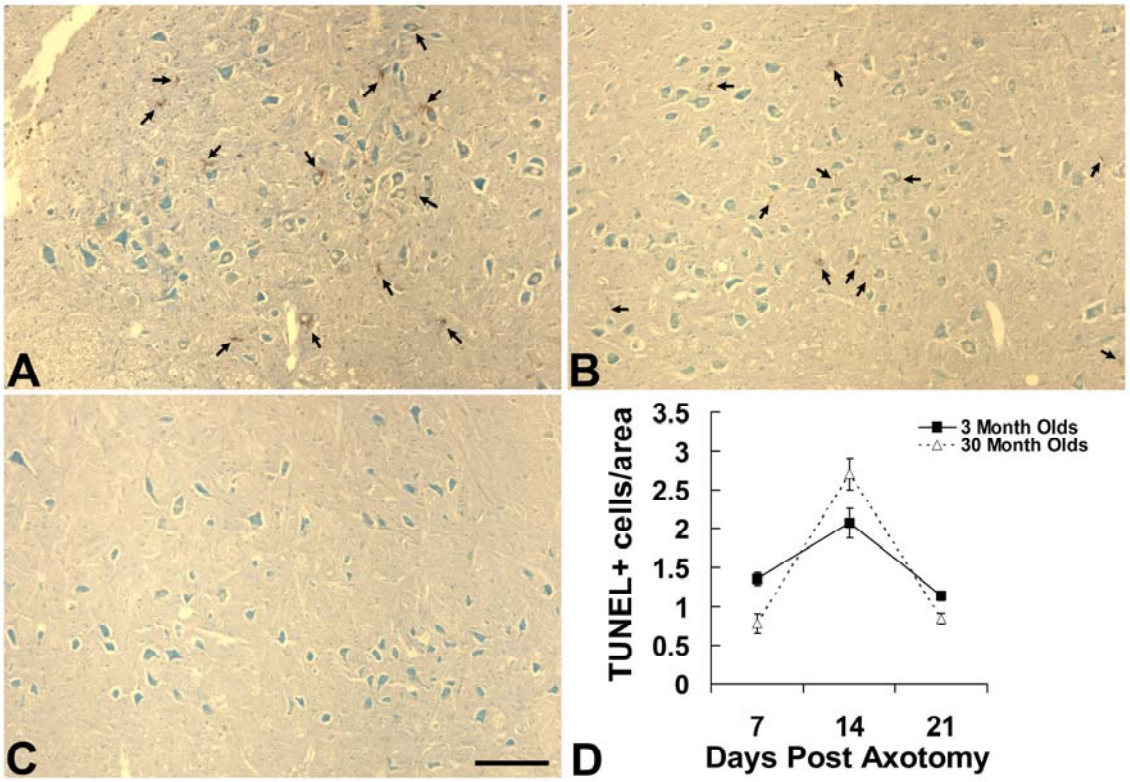

Figure 3-1. Aging does not affect the density or distribution of TUNEL-positive microglia in the lesioned facial nucleus. TUNEL-positive cells (arrows, brown color) are distributed throughout the parenchyma of the lesioned facial nucleus in both young (A) and old (B) animals 14 days following axotomy. No TUNEL-positive cells are detected in the unoperated facial nucleus (C). Graph of the time-course of the change in density of TUNEL-positive cells in the lesioned facial nucleus demonstrates no evidence of a significant difference between young and old (D). Bar= 200 μm.

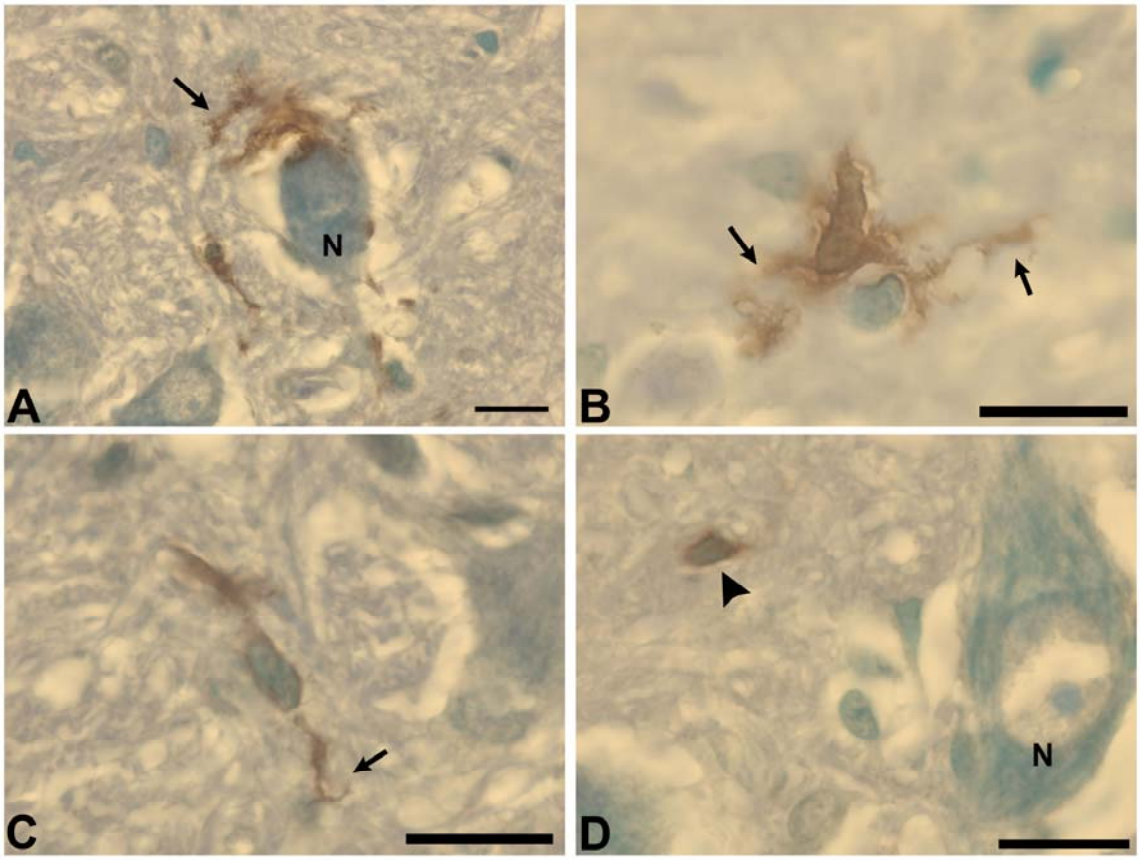

Figure 3-2. The cytoplasmic distribution of TUNEL staining of microglia is found in both young (A and C) and old (B and D) animals. The cytoplasmic labeling is often diffusely distributed throughout the cytoplasm in a manner which reveals the morphological shape of microglia (A-C), including ramified processes (arrows). Occasionally, only perinuclear labeling is present (arrow head) (D). Note the TUNEL positive microglia in (A) directly in contact with a non-apoptotic neuron; N. Bar= 25 μm.

Discussion

Injury to the CNS induces a rapid expansion of microglial cell number (Gehrmann and Banati, 1995; Graeber et al.,1988; Kreutzberg, 1966; Kreutzberg, 1968), and it is known that as the injured neurons recover, microglial cell numbers return to normal homeostatic levels. Current evidence points to programmed cell death as the primary means by which microglia return to their homeostatic numbers (Gehrmann and Banati, 1995; Jones et al., 1997), and we employed TUNEL to examine the regulated death of

microglia in the regenerating facial nucleus. Given that microglial proliferation remained elevated 4 days after injury in old animals, we expected to find an aging-related difference in the number of TUNEL-positive cells between 1 and 3 weeks post-injury, but there was no evidence of a difference between old and young animals. The higher rate of proliferation at day 4 with no difference in the number of dying microglia at later time points in old animals suggests that the number of microglia should be greater in old versus young animals at later time points. This supposition was investigated further as part of the study described in Chapter 4. Additionally, if microglial proliferation is sustained longer in old animals because of delayed neuronal regeneration, we would except to have found a corresponding shift in the time course of microglial turnover, but we did not find evidence of such a shift.

TUNEL labeling demonstrated an unusual cytoplasmic staining in microglia of the injured facial motor nucleus more frequently than the typical specific nuclear labeling of classically apoptotic cells. The phenomenon of TUNEL labeling of microglial cytoplasm has previously been investigated in a study by Jones and colleagues (1997). In that study the possibility of artifact was dismissed, and it was shown that at the ultrastructural level very little of the microglial nucleus was TUNEL positive, while labeling was found on the outer nuclear membrane and often diffusely distributed throughout the cytoplasm (rather than compartmentalized into phagocytic vacuoles). Because TUNEL-positive microglia generally do not exhibit the classical apoptotic characteristics of membrane blebbing, chromatin condensation and apoptotic bodies, the authors concluded that microglia may undergo a non-classical form of programmed cell death. Another study found microglia with cytoplasmic TUNEL labeling in AD brains (Li et al., 2004). This

study also demonstrated that cultured microglia internalized fragmented DNA through scavenger receptors, resulting in cytoplasmic TUNEL labeling, and thus they concluded that the cytoplasmic TUNEL labeling of microglia found in AD brains was due to uptake of fragmented DNA from nearby apoptotic neurons. Given this finding, the possibility of microglial internalization of fragmented DNA should not be dismissed. However, TUNEL positive microglia in the AD brains were usually found adjacent to apoptotic neurons or Aβ plaques, but in the facial nerve axotomy model used in our study the majority of neurons survive (Moran and Graeber, 2004), and TUNEL positive microglia were frequently directly adjacent to non-apoptotic neurons (Fig 3-2A).

A review of the literature revealed no other descriptions of diffuse cytoplasmic TUNEL labeling aside from that which has been reported for microglia. The literature on programmed cell death of microglia and mechanisms of microglial turnover is almost non-existent, and clearly such research should be the focus of future studies. The cytoplasmic TUNEL labeling was found in both young and old animals, and taken together with the finding of no significant differences in the temporospatial distribution of TUNEL-positive microglia, aging does not appear to affect the mechanism of microglial programmed cell death.

CHAPTER 4
A HISTOCHEMICAL ASSESSMENT OF AGING-RELATED DIFFERENCES IN MICROGLIAL ACTIVATION AND MORPHOLOGY

Introduction

An aging-related attenuation of the downregulation of microglial proliferation after the peak response to facial nerve axotomy was observed in the study described in Chapter 2. Assuming that the number of microglia within the intact facial nucleus does not differ between age groups, the finding of prolonged microglial proliferation following axotomy in the old animals suggests that the number of microglia should be higher in the aged facial nucleus from post-axotomy day 4 unless there is also an aging-related increase in microglial post-mitotic turnover. However, in the study described in Chapter 3, we found no such increase in microglial turnover via programmed cell death with aging, although the possibility of aging-related changes in other means of population regulation, such as exit of activated microglia through blood vessel walls (Cammermeyer, 1965; Del Rio-Hortega, 1932), has not been dismissed. The main objective of the study described in the current chapter was to quantitatively compare axotomy-induced microglial activation during the normal time course of proliferation and turnover (from 2 to 21 days) between young and old using a histochemical marker to identify microglia. The hypothesis was that the increase in the area occupied by microglia would be greater in old animals between 4 and 21 days following axotomy. Aging related differences in the morphology of both resting and activated microglia were also assessed.

Qualitative and quantitative aging-related histological differences were investigated using the microglia-specific *Griffonia simplicifolia* B_4 isolectin conjugated to horseradish peroxidase (GSA I-B_4-HRP). Lectins are proteins with strong affinities to the oligosaccharide chains of glycoproteins, primarily membrane-associated glycoproteins in the CNS (Streit et al., 1985). The lectin conjugate GSA I-B_4 selectively binds to terminal α-D-galactose residues on the membranes of microglia. These residues are constitutively expressed on microglia, but lectin reactivity increases with microglial activation (Streit and Kreutzberg, 1987). We found evidence that the increase in lectin-reactive area associated with activation does not significantly differ with aging, but visualization of microglia with lectin revealed significant age-related differences in the morphology of individual microglia.

Materials and Methods

Animal Tissue

Tissue sections processed for lectin histochemistry in this study were remaining brainstem sections from young (3 month old) and old (30 month old) F344BN hybrid rats obtained during the studies described in Chapters 2 and 3 of this dissertation. All tissue was paraffin processed and sectioned in the same manner.

Lectin Histochemistry

For histochemistry with microglia-specific GSA I-B_4-HRP, 2, 3, 4, 7, 14, and 21-day post-facial nerve axotomy brainstem sections of young and old animals were used. Sections were deparaffinized through xylenes (2 changes of 15 min. each) and descending alcohols (3 min. each of 100%, 100%, 95%, 90%, 75%, and 75% ethanol), and then were allowed to incubate in 70% ethanol overnight. The next day the sections were washed in PBS (pH 7.2) and incubated in PBS containing cations (0.1mm of $CaCl_2$,

$MgCl_2$, and $MnCl_2$) and 0.1% Triton-X100 for 10 min. Lectin GSA I-B4-HRP (Sigma Chemical Co.) was diluted 1:100 in PBS containing cations and 0.1% Triton-X100. A grease pen was used to encircle the sections before aliquoting the lectin solution, and the slides were incubated overnight at 4ºC in a moist chamber. After incubation, the slides were washed briefly in PBS 3 times, and then sites containing bound lectin-HRP conjugates were visualized with 3,3'-diaminobenzidine (DAB)-H_2O_2 substrate. Sections were counterstained for with cresyl violet, dehydrated through ascending alcohols, cleared in xylenes, and coverslipped with Permount mounting medium (Fisher Scientific).

Quantitative Analysis

Lectin histochemistry was quantified using a Dell computer running Image Pro Plus software (version 4.5.1, Media Cybernetics, Carlsbad, CA) and a Spot RT color camera (Diagnostic Instruments, Sterling Heights, MI) attached to a Zeiss Axioskop 2 microscope. Obtaining accurate measurements of total microglial numbers is difficult using histochemical means during the peak period of microglial activation; therefore, measurements of the relative lectin reactive area were used for quantitative analysis. The area occupied by lectin-positive cells was highlighted and measured within an area of interest (AOI) box of known area (Fig. 4-1). The data obtained from all sections examined in each facial nucleus was pooled, and the total lectin-positive area measured for each facial nucleus was expressed as a percentage of the total area of the AOI boxes (6-10 sections per animal were used). The fold increase in area after axotomy was determined by subtracting the unoperated facial nucleus percent area from the lesioned facial nucleus percent area, and then dividing by the unoperated nucleus percent area.

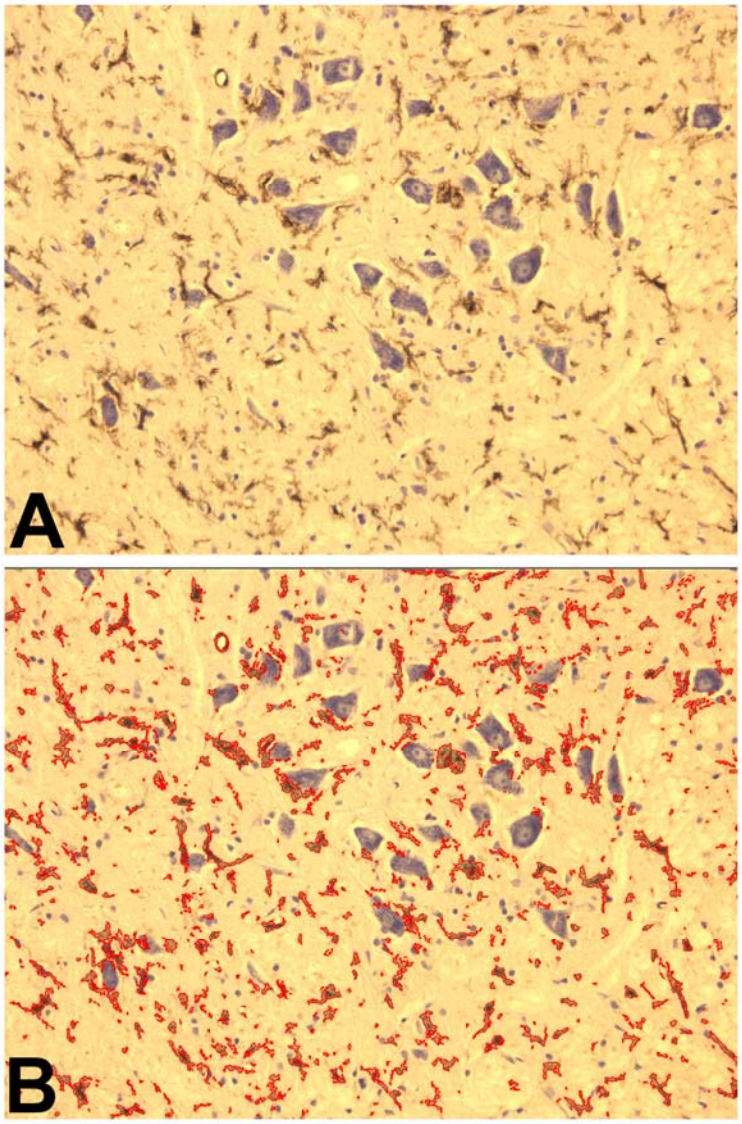

Figure 4-1. Method of quantifying the percent area occupied by lectin-positive microglia. Images of each lectin-stained facial nucleus were taken using a color Spot RT digital camera attached to a Zeiss Axioskop 2 microscope with a 20X objective. (A) The image was opened in Image Pro Plus software, and an AOI box of known area was laid over the image. (B) The measurement threshold was set so that only the brown colored areas of lectin positive cells were highlighted (red outlines), and then the area of the highlighted cells within the border of the AOI box was measured. The measured area of lectin positive cells was divided by the total area of the AOI boxes to determine the percent area occupied by lectin-positive microglia.

Results are represented as mean values ± SEM. Lectin-reactive area was compared between age groups and lesioned/unoperated with a two-way ANOVA using GraphPad Prism software (San Diego, CA). The fold increase in lectin-reactive area over control was analyzed with t-tests comparing young and old at each time point. A significance level of $p \leq 0.05$ was used.

Qualitative Analysis

For qualitative assessment of microglia morphology, photomicrographs of lectin stained sections were taken using a Zeiss MC100 camera attached to a Zeiss Axioplan microscope using Fujichrome iso100 daylight 35mm color slide film. Developed slides were scanned using a Hewlett Packard scanner. Images were processed for the figure panels using Adobe PHOTOSHOP 6.0.

Results

Aging affects the total lectin reactive area in the lesioned facial nucleus, but not the fold increase in lectin reactive area over control. Lectin histochemistry was quantified by determining the percent area occupied by lectin-positive microglia within the area of interest (Fig. 4-1). In all animals the area occupied by lectin-positive microglia was significantly higher ($p<0.01$) in the axotomized facial nuclei compared to the contralateral unoperated nuclei (Figs. 4-2A, 4-3). The percent area occupied by lectin-positive microglia at 7, 14, and 21 days post-axotomy (Fig. 4-2A) was significantly higher ($p< 0.05$) in old animals (14.13 ±1.25%, 15.77 ± 0.16%, 12.82 ± 2.00%, respectively) versus young animals (9.22 ± 0.88%, 10.43 ± 0.85%, 7.50 ± 0.57%, respectively). However, the fold increase in lectin-positive area over control was not significantly different between age groups (Fig. 4-2B).

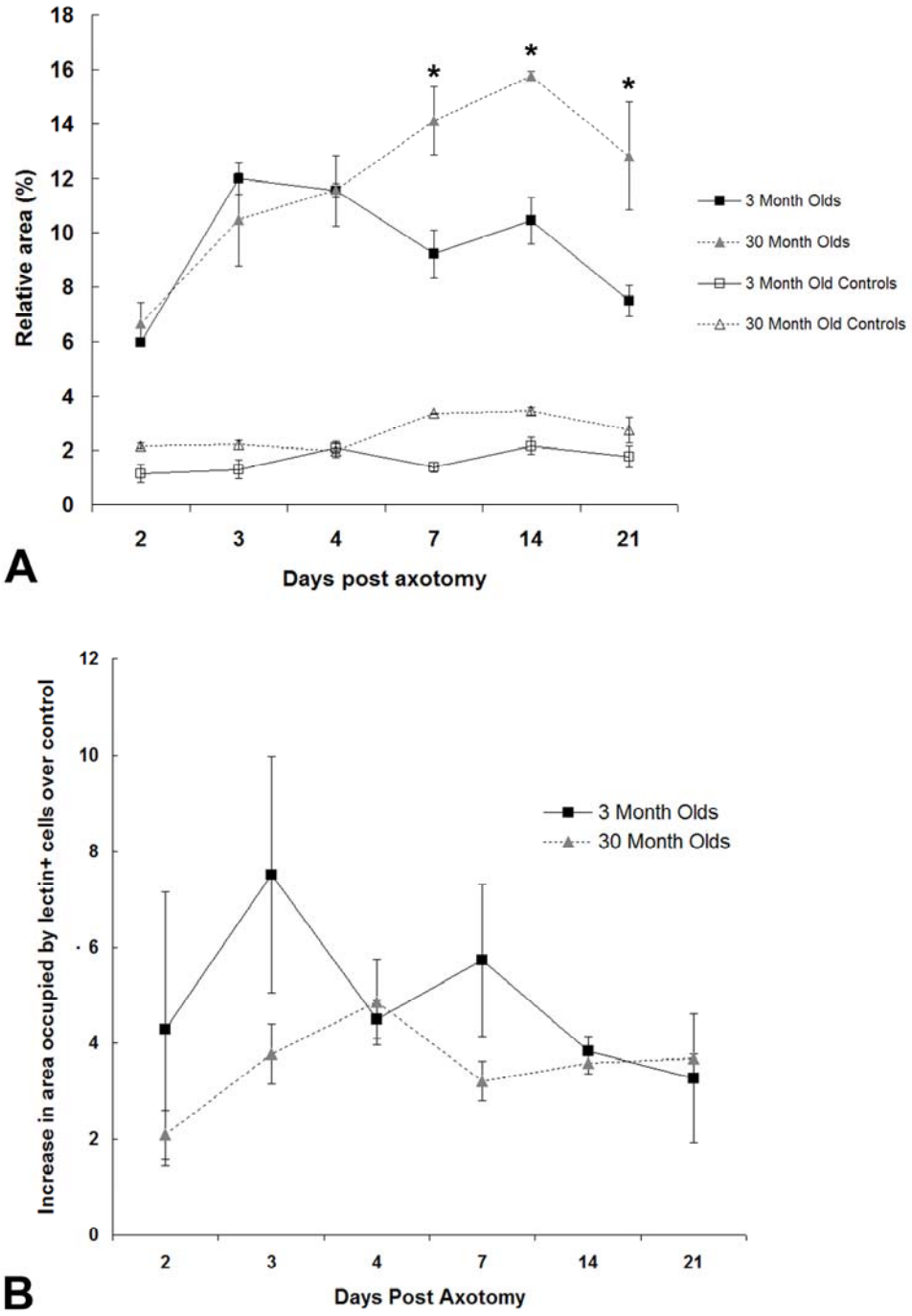

Figure 4-2. Comparison of the relative area occupied by lectin-reactive young and old microglia in the lesioned and control facial nuclei. There appears to be an aging-related increase (*$p<0.05$ between age groups) in lectin-reactive area in the lesioned facial nucleus between days 7-21 (A), but an aging-related difference is not detected when the lectin-reactive area of the lesioned side is expressed as the fold increase over control levels (B).

Aging affects the morphology of non-activated microglia. Conspicuous qualitative morphological differences between the aged and young microglia were evident in the unoperated facial nucleus. In fact, a blinded observer was able to correctly distinguish between young and aged brainstem sections in 11 out of 12 cases by observing only lectin-stained microglial morphology in the unoperated facial nucleus. Control side microglia of young rats exhibited finer, more tenuous processes than their older counterparts. The non-activated young microglia were typically lightly stained with lectin (Fig. 4-3C). They had elongated or oval nuclei surrounded by a thin rim of cytoplasm, and finely ramified processes protruding from the somata (Fig. 4-4A). In contrast, microglia in the uninjured facial nucleus of old animals were more intensely lectin-stained (Fig. 4-3D), displaying a morphology similar to that of activated microglia. Frequently, the old microglia had swollen somata with densely stained perinuclear cytoplasm, and a rounded nucleus pushed to one side. Microglial processes of old animals generally remained ramified, though the proximal portions showed darker lectin staining with some hypertrophy (Fig. 4-4B). The distinct microglial morphology in the aged rats was not limited to the unoperated facial nucleus, but was present throughout most of the brainstem.

Aging affects the morphology of activated microglial. Morphological differences between young and old activated microglia within the injured facial nuclei were more subtle, but at high magnification it was noted that many activated microglia in the old animals retained the dense perinuclear staining (Fig. 4-4D); the nucleus and somata were elongated as found in the young animals. Often, it was difficult to visualize the nucleus in activated cells that exhibited dense perinuclear lectin-reactivity.

Activation resulted in hypertrophy of microglial processes in both young and old animals, but there was a fragmented, somewhat shorter appearance to the lectin stained processes of old activated microglia (Fig 4-4D). Because their somata were hypertrophied before activation, microglia in old animals did not appear to have as significant of an increase in overall size upon axotomy-induced activation as did microglia in young animals.

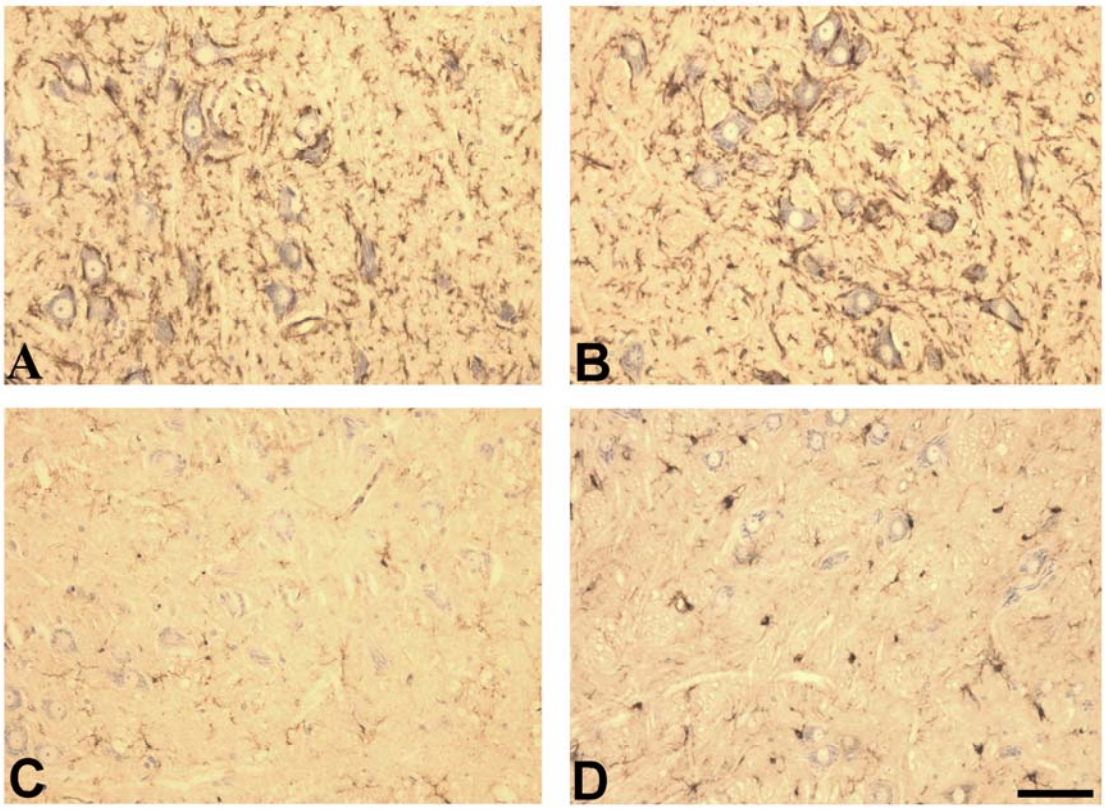

Figure 4-3. Comparison of lectin staining in the facial nucleus before and after facial nerve axotomy between young and old. Lectin reactivity in the young (A and C) and old (B and D) lesioned (A and B) and unoperated (C and D) facial nuclei 3 days following axotomy. While aging-related qualitative differences in microglia of the lesioned nucleus are not readily apparent at this low magnification, morphological differences between young (C) and old (D) are notable even at low magnification in the unoperated facial nucleus. Bar= 100 μm.

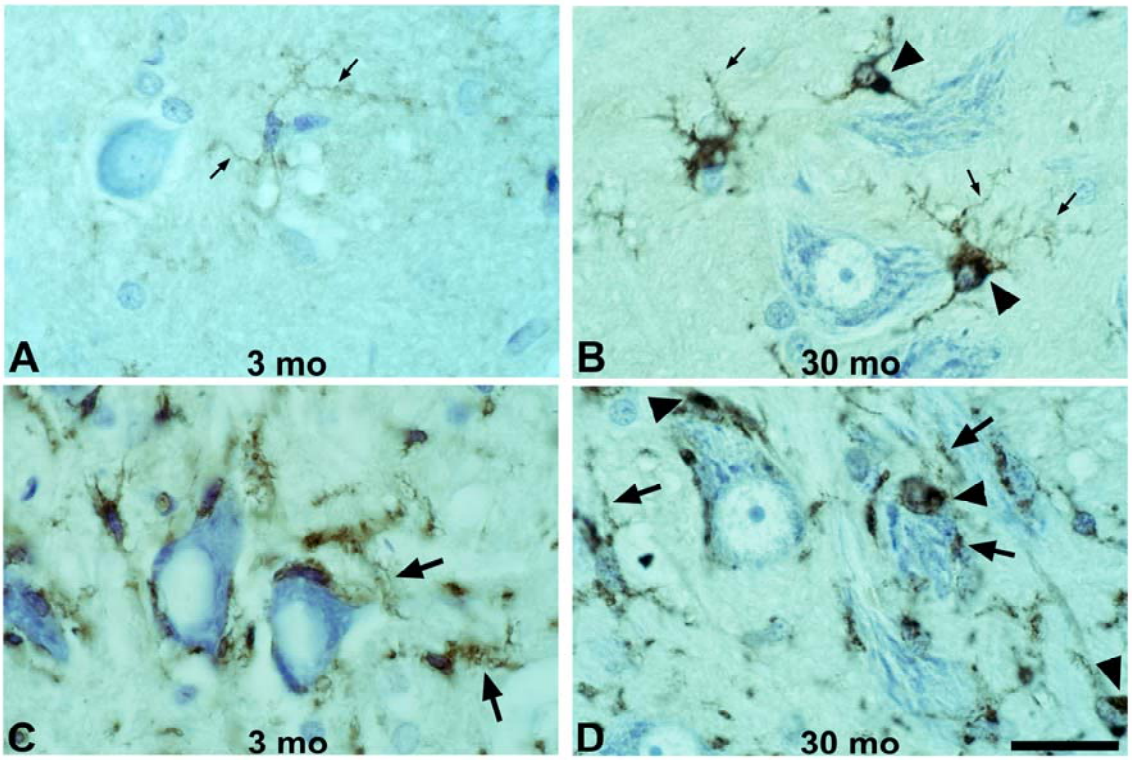

Figure 4-4. Higher magnification images of lectin-reactivity in young and old animals. (A) Microglia in the unoperated facial nucleus of young animals have elongated nuclei surrounded by a thin rim of cytoplasm, extending into thin, ramified processes from the cell body (small arrows). In contrast, microglia in the unoperated facial nucleus of old animals (B) typically have rounder nuclei, with a cluster of densely stained cytoplasm to one side of the nucleus (arrow heads). Despite the increased lectin reactivity, their processes remain ramified (small arrows). (C) Three days post-axotomy, microglia of young animals increase in lectin reactivity and develop hypertrophied processes (arrows). (D) Microglial activation in old animals resembles that of young animals, including perineuronal positioning and retracted, hypertrophied processes, but many old activated microglia retain the densely stained perinuclear cytoplasm (arrowheads), and there is a fragmented appearance to the lectin staining of the hypertrophied processes (arrows). Bar= 25 μm.

Discussion

Because the microglia of old animals demonstrated a higher rate of microglial proliferation at day 4 (Chapter 2, this dissertation) with no difference in the number of dying microglia at later time points (Chapter 3, this dissertation), we hypothesized that the number of microglia should be greater in old versus young animals at later time

points. While there was a higher density of lectin reactive area in the old animals at the later time points of 7, 14 and 21 days, the fold increase in lectin reactive area over baseline levels did not differ with aging. This finding is not surprising because of our finding of non-activated microglia with hypertrophied cell bodies in old animals, which may not change as much in area upon activation compared to those of young animals. The increase in lectin reactive area after axotomy is normally due to a combination of microglial hypertrophy and proliferation, but in old animals proliferation may account for more of the increase.

The morphological differences between purportedly non-activated microglia of young and old animals were readily discernable in lectin-stained brainstem sections. Such a distinction was an unexpected finding because, while studies have demonstrated aging-related differences in microglial morphology in other areas of the CNS (Kim et al., 2004; Morgan et al., 1999; Ogura et al., 1994; Peinado et al., 1998; Perry et al., 1993; Vaughan and Peters, 1974), previously it was shown that in the facial nucleus there was not a difference between young and old microglia visualized using OX-42 or OX-6 immunohistochemistry (Hurley and Coleman, 2003). Using lectin histochemistry, we found that the majority of microglia in the unoperated facial nucleus of old animals exhibited swelling and dense lectin reactivity of the perinuclear cytoplasm, and this microglial phenotype was also exhibited in other areas of the brainstem sections. One may conclude that the hypertrophied cell bodies of the microglia in the unoperated facial nucleus of old animals indicate that these microglia are already in a state of mild activation. However, careful examination reveals that the hypertrophied microglia of the aged brainstem do not completely resemble the activated microglia of the young injured

facial nucleus (compare Fig. 4-4B to 4-4C). The activated microglia of the young animals do indeed show hypertrophy and increased lectin reactivity, but they do not have the dense perinuclear cytoplasmic staining or rounded nuclei of the old microglia. While it is difficult to determine the localization of the dense lectin binding using light microscopy, it is known through ultrastructural cytochemistry that lectin conjugates can bind to intracytoplamic membranes (Streit and Kreutzberg, 1987). Microglia are known to have small, membrane-bound vesicles throughout their cytoplasm and proximal portions of their processes (Vaughan and Peters, 1974), and aging-related increases in membrane-bound, heterogeneous inclusions have been found in ultrastructural studies of microglia in the rhesus monkey brain (Peters and Sethares, 2002) and rat auditory (Vaughan and Peters, 1974) and parietal (Peinado et al., 1998) cortices in a pattern similar to the perinuclear density and nuclear displacement we detected in this study. Thus, the hypertrophy and dense lectin reactivity is indicative of an accumulation of heterogeneous material in microglia of the aging CNS, possibly through phagocytosis or pinocytosis (Vaughan and Peters, 1974). In contrast, the injury-induced hypertrophy of microglia in the young rodent facial nucleus is not due to a significant increase in accumulated cytoplasmic material. A recent study by our laboratory demonstrated that purportedly "activated" microglia in the aged human brain have different immunophenotypic features than microglia activated by CNS injury, and these microglia of the aged human CNS may be more accurately described as dystrophic (Streit et al., 2004). Upon injury-induced activation, microglia of old animals assumed a morphology similar to the microglia of young animals, but the dense perinuclear lectin reactivity was still a prominent feature. Additionally, many activated microglia of old animals appeared

to have shorter processes than their young counterparts, and there was a fragmented appearance to the lectin staining of the processes. Vacuolated processes have previously been described in microglia of the aging rat (Perry et al., 1993). Microglia in the aged rodent brain do not possess all of the features of dystrophy described in microglia of the aged human brain, but it is plausible to consider that the presence of morphological changes in conjunction with a less regulated mitotic response in the aged facial nucleus may be a reflection of microglial dysfunction related to senescence. Given their high mitotic and phagocytic potential, microglia may be particularly vulnerable to the affects of aging.

CHAPTER 5
THE EFFECT OF AGING ON NEURONAL-MICROGLIAL SIGNALING

Introduction

Microglia demonstrate an ability to dynamically change phenotype and function in response to even minor changes in their microenvironment (Kreutzberg, 1996). The conserved course of microglial activation in response to facial nerve axotomy, and neuronal regulation of microglial phenotype *in vitro* (Zhang and Fedoroff, 1996; Zietlow et al., 1999), provide convincing evidence for regulatory signaling between neurons and microglia. A multitude of molecules released by neurons have been shown to activate microglia, particularly in cultured cells (Bruce-Keller, 1999). Regulation of microglial activation is a very stringent process, however, and activation of microglia does not automatically qualify a molecule as a candidate for "the" putative neuronal-microglial regulatory signal. Ideally, such a regulatory signal would be constitutively and exclusively expressed by neurons (with a constitutive and exclusive receptor on microglia), and would change expression during the course of microglial activation. The chemokine fractalkine (CX3CL1) and its receptor CX3CR1 are the best known candidates for such a signal ligand-receptor pair. In the study described in Chapter 2, we found that the downregulation of microglial mitosis is attenuated with aging. This finding prompted us to examine the expression of fractalkine and CX3CR1 mRNA in young and old animals to determine if aging-related changes in these transcripts may account for aging-related changes in microglial regulation.

Fractalkine in neuronal-microglial signaling. Fractalkine, the only known member of the chemokine CX3C subfamily, is unique among chemokines in that it can exist in both membrane-bound and secreted forms, and is expressed constitutively in many non-hematopoietic tissues, including the brain. Fractalkine expression in the CNS has been shown to be localized to neurons *in vivo* (Harrison et al., 1998; Nishiyori et al., 1998; Tarozzo et al., 2002). The membrane-bound form functions as an adhesive molecule, while the soluble form has been shown to induce chemotaxis in receptive cells (Imai et al., 1997). CX3CR1, a seven-transmembrane G-protein-coupled receptor, is the exclusive receptor for fractalkine, and in the intact CNS it is constitutively and exclusively expressed on microglia (Harrison et al., 1998; Tarozzo et al., 2002). In response to facial nerve axotomy, transient increases in CX3CR1 mRNA expression in microglia parallel microglial proliferation and perineuronal positioning, while expression of the soluble (chemotaxic) form of fractalkine increases (Harrison et al., 1998). Fractalkine has been shown to inhibit Fas-ligand mediated cell death of microglia *in vitro* (Boehme et al., 2000), thus demonstrating that neurons may influence microglial survival. Additionally, neuronal mediation of microglial phenotype through fractalkine is evident from studies showing fractalkine modulation of LPS-induced production of TNF-α and neurotoxicity by microglia (Zujovic et al., 2000; Zujovic et al., 2001). There is some confusion about the functional role of the fractalkine ligand-receptor pair as a neuronal-microglial signal *in vivo* because targeted deletion of CX3CR1 does not result in changes in microglial proliferation and perineuronal positioning in response to facial nerve axotomy (Jung et al., 2000). However, the constitutive expression of both molecules in the CNS along with their pattern of expression in the course of the injury

response make it unlikely that there is not biological significance to signaling through this unique ligand-receptor pair; rather, compensatory mechanisms are likely enacted in the absence of fractalkine signaling.

TGF-β1 modulation of fractalkine signaling. TGF-β1 is a multifunctional cytokine that is associated with the regulation of cell growth and differentiation (Massague, 1996). TGF-β1 has both pro- and anti-apoptotic and pro- and anti-inflammatory effects depending upon the cell type, environment, and duration/amount of TGF-β1 production. In the CNS, very low basal TGF-β1 expression is increased after injury and in neurodegenerative diseases, and this increase appears to have a neuroprotective role based on studies of TGF-β1 deficient/knockout (Brionne et al., 2003) and over-expressing (Wyss-Coray et al., 1995) transgenic mice. Injury-induced TGF-β1 mRNA is primarily localized to microglia, and appears to follow the same time-course as microglial activation and CX3CR1 upregulation (Harrison et al., 1998; Kiefer et al., 1995).

Recently, TGF-β1 was shown to modulate fractalkine signaling by increasing transcription of CX3CR1 in microglia and attenuating fractalkine-induced ERK1/2 phosphorylation (Chen et al., 2002). The attenuation of ERK1/2 phosphorylation occurs by TGF-β1-induced upregulation of RGS (regulator of G-protein signaling)-2 and RGS10, which inhibit activation of the MAPK pathway and may shift signaling to other pathways (Chen, 2004). Because increased TGF-β1 expression follows the same time course as microglial activation and increased CX3CR1 expression after facial nerve axotomy, we postulated that TGF-β1 may regulate microglial proliferation by modulating fractalkine signaling through CX3CR1, and that a disruption in this regulation may

explain the increased microglial proliferation in the lesioned facial nucleus of old animals on post-injury day 4. Thus, we compared the course of the facial nerve axotomy-induced increase in TGF-β1 mRNA in the facial nuclei of young and old animals.

HN1 as a marker of neuronal injury. Hematopoietic and neurologic-expressed sequence-1 (HN1) is so named because it was first characterized by its expression primarily in hemopoietic cells and neurons (Tang et al., 1997). HN1 expression is high during CNS development, and remains constitutively expressed in some neuronal populations of the adult CNS. Significant upregulation of HN1 mRNA transcripts occurs after injury in regenerating neurons (e.g., axotomized adult facial motoneurons), but not in degenerating neurons (e.g., axotomized neonatal facial motoneurons or rubrospinal neurons) (unpublished data from the laboratory of Jeffrey Harrison, PhD, University of Florida). Thus, HN1 is a putative marker of neuronal regeneration, and because its expression correlates well with the course of axotomy-induced microglial activation and upregulation of CX3CR1 in the regenerating facial nucleus, we compared its expression in young and old animals at the peak of CX3CR1 expression (4 days following injury) as a potential indicator of aging-related changes in neuronal regeneration.

Materials and Methods

Surgery

Young (3 m.o.) and old (30 m.o.) male F344BN hybrid rats from the NIA were subjected to facial nerve axotomy. Animals were sacrificed at 2, 3, 4, 7, 14, and 21 days (N=2 per time point/age group) using transcardial perfusion as described in Chapter 2, with modifications. Briefly, each animal was transcardial perfused with PBS followed by 4% paraformaldehyde. Brains were post-fixed at 4°C for 1h in 4% paraformaldehyde, then cryoprotected in sterile 30% sucrose/PBS solution at 4°C until each brain sunk to the

bottom of the tube (~2d). Sucrose-protected brains were snap frozen in liquid nitrogen-cooled isopentane, then quickly transferred to a pre-cooled, pre-labeled storage container and placed in the -80°C freezer.

Tissue Sectioning for *In Situ* Hybridization

Frozen brains were allowed to equilibriate in the cryostat chamber at -20°C for 30 min. before sectioning. A block of the brainstem containing the facial nuclei was dissected from each brain, and then embedded in OCT and mounted onto a cryostat chuck. Twenty-micron coronal cryostat sections were mounted onto Superfrost Plus slides in a pattern that allowed 3-4 sections from different caudal-rostral regions of the facial nucleus to be mounted on each slide. 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 fractalkine, CX3CR1, TGF-β1, and HN1was carried out on 20 μm cryostat brainstem sections from both young and old animals. Plasmids containing inserts of rat fractalkine, CX3CR1 and HN1 cDNA were obtained from the laboratory of Dr. Jeffrey Harrison in order to generate riboprobes for ISH (Table 5-1). However, the cDNA used to create the riboprobe for TGF-β1 was generated by RT-PCR from total RNA extracts of rat cortex.

RT-PCR and cloning of TGF-β1 cDNA. Fresh frozen rat cortex was homogenized in TRIZOL reagent (Invitrogen, Carlsbad, CA) using a glass homogenizer, and total RNA was extracted following the manufacturer's instructions. Reverse transcription (RT) of 1 μg total extracted RNA was carried out, followed by polymerase chain reaction (PCR) with oligonucleotide primers flanking sequences within the rat TGB-β1 gene (forward 5' CTA CTG CTT CAG CTC CAC AGA G 3'; reverse 5' ACC

TTG GGC TTG CGA CC 3'). A 281 base-pair cDNA corresponding to nucleotides 1261-1541 of the full rat TGF-β1 mRNA sequence (GenBank accession number X52498) was generated. Purified TGF-β1 cDNA was blunt-end ligated into the *Sma*I site of plasmid pGEM7 (Promega, Madison, WI), and JM109 competent cells were transfected with the plasmid. Restriction digestion with *Hin*dIII and *Xba*I was used to verify the presence of the insert in miniprep clone DNA, the correct sequence of the insert was verified by the University of Florida Interdisciplinary Center for Biotechnology Research (ICBR) DNA sequencing lab, the plasmid was amplified in maxiprep cultures, and purified TGF-β1-pGEM7 was extracted using the Qiagen Plasmid Maxi Kit (Valencia, CA).

Table 5-1. pGEM7 cDNA inserts and respective linearization restriction enzymes and RNA polymerases for *in situ* hybridization.

cDNA (species)	pGEM7 insertion site	Insert size	Anti-sense riboprobes		Sense riboprobes	
			Restriction enzyme	RNA polymerase	Restriction enzyme	RNA polymerase
CX3CR1 (rat)	*Kpn*I	450bp	*Hin*dIII	T7	*Eco*RI	Sp6
Fractalkine (rat)	*Nco*I	400bp	*Eco*RI	Sp6	*Bam*HI	T7
TGF-β1 (rat)	*Sma*I	281bp	*Xba*I	Sp6	*Hin*dIII	T7
HN1 (murine)	*Bam*HI/ *Hin*dIII	490bp	*Bam*HI	T7	*Hin*dIII	Sp6

ISH. Sense and antisense riboprobes for ISH were generated by linearizing the plasmids containing the respective inserts and then transcribing the cRNA using either T7 or SP6 RNA polymerase (see Table 5-1) 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 and subsequently dipped in Kodak NTB2 emulsion and exposed in

light-tight boxes at 4°C for 1-8 weeks. After development, sections that were to be used for quantitative analysis of the hybridization signal were not counterstained. Sections probed for fractalkine mRNA were counterstained with hematoxylin and eosin.

Quantitative Analysis

Quantitative analysis of the intensity of the hybridization signal was performed on non-counterstained emulsion dipped sections hybridized with CX3CR1 and HN1 riboprobes. Sections were imaged at 10X magnification using a color Spot RT digital camera (Diagnostic Instruments, Sterling Heights, MI) attached to a Zeiss Axioskop 2 microscope and Spot Advanced (version 3.4.5) software running on a Dell PC. The intensity of the hybridization signal within the facial nucleus was measured using the thick line profile feature in the measurements menu of Image Pro Plus software (version 4.5.1, Media Cybernetics, Carlsbad, CA). The intensity of the hybridization signal in the lesioned facial nucleus was expressed as the percent increase over the control side (unoperated) facial nucleus signal in the same section ([(exp.-con.)/con.] x 100) in order to account for possible processing-introduced variations in inter-brainstem section hybridization signal intensity. Intensity was measured in 4-7 sections from each animal. Results are represented as mean values ± SEM. Significant differences in CX3CR1 and HN1 hybridization signal were determined with t-tests at the respective time points examined using GraphPad Prism software (GraphPad Software, San Diego, CA). A significance level of $p \leq 0.05$ was used.

Results

Aging does not affect the time course of CX3CR1 mRNA expression in the lesioned facial nucleus. The axotomy-induced increase in CX3CR1 mRNA hybridization signal was compared in the lesioned facial nucleus of young and old rats to

determine whether aging-induced changes in the transcription of CX3CR1 account for the attenuated downregulation of microglial proliferation in old animals. Analysis of the intensity of the *in situ* hybridization signal demonstrated that the transient in increase in CX3CR1 mRNA followed the same general time-course in both age groups as previously reported (Harrison et al., 1998), peaking at roughly 4-7 days post-axotomy (Fig. 5-1). No hybridization signal was observed with the corresponding sense riboprobe (not shown).

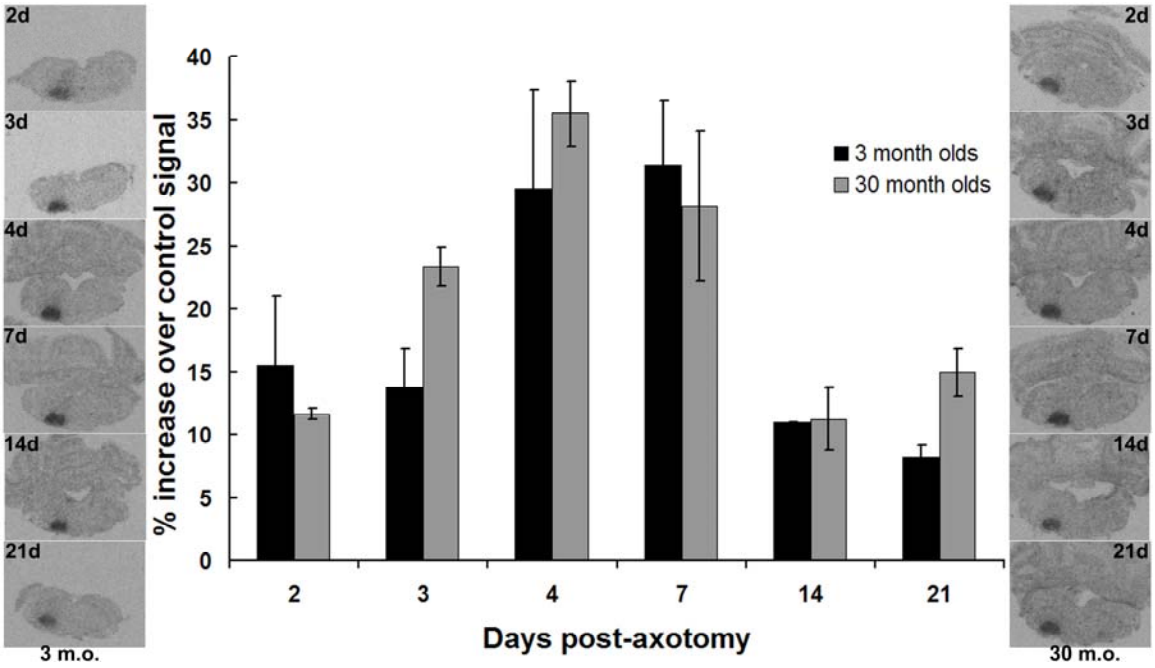

Figure 5-1. Aging does not affect the time course of CX3CR1 mRNA expression in the lesioned facial nucleus. CX3CR1 mRNA expression in the rat facial nucleus after facial nerve axotomy was analyzed by measuring the intensity of the *in situ* hybridization signal in both the lesioned and control facial nuclei, and the results are expressed as the percent increase in lesion signal over control. CX3CR1 expression appears to peak at 4 days in old (30 m.o.) and 7 days in young (3 m.o.) animals, but the difference is not significant.

The pattern of silver grain distribution in CX3CR1 ISH sections was similar in young and old animals in both the lesioned and unoperated facial nucleus (Fig. 5-2). Hybridized sections from both age groups show that the pattern of CX3CR1 expression parallels the pattern of microglial distribution.

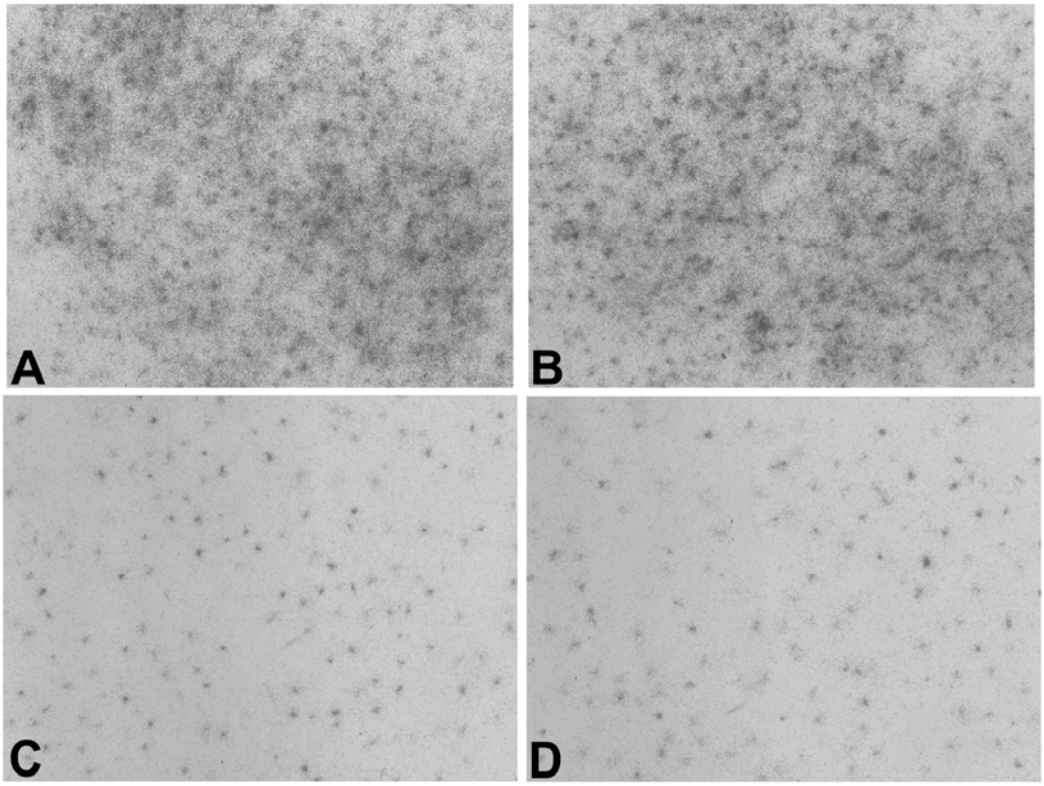

Figure 5-2. Photomicrographs of CX3CR1 mRNA expression in the lesioned (A and B) and control (C and D) facial nucleus of young (A and C) and old (B and D) rats 4 days following facial nerve axotomy. The pattern of silver grain clustering is similar to the shape and distribution of microglia in the facial nucleus. There is no discernable aging-related change in either the lesion-induced or basal level of CX3CR1 mRNA.

Aging does not affect fractalkine mRNA expression in the lesioned and non-lesioned facial nucleus. We examined the expression of fractalkine mRNA in the brainstem of young and old rats at several time points after facial nerve axotomy. The hybridization signal in the facial nucleus was too faint to do accurate quantitative analysis, as motoneurons of the facial nucleus have a relatively low hybridization signal for fractalkine compared to neurons in other areas of the brainstem, and the signal appears to slightly decrease after axotomy. Nonetheless, no evidence of an aging-related change in the fractalkine hybridization signal was observed in either the lesioned facial nucleus (Fig. 5-3C, D), control side (unoperated) facial nucleus (Fig. 5-3A, B), or in other

areas of the brainstem (Fig. 5-3E, F). No hybridization signal was observed in sections probed with the sense fractalkine riboprobe (data not shown).

Aging does not affect TGF-β1 mRNA expression in the lesioned facial nucleus. Because TGF-β1 has been shown to attenuate microglial proliferation, increase the transcription of CX3CR1, and modulate fractalkine-induced signaling pathways in cultured microglia, we investigated the expression of TGF-β1 mRNA after facial nerve axotomy in young and old rats to determine if aging-related changes in TGF-β1 mRNA account for the attenuated downregulation of microglial proliferation in old animals. A 281bp rat TGF-β1 cDNA fragment was generated via RT-PCR for use in creating a TGFβ1 ISH ^{33}P-riboprobe. The hybridization signal from this probe was weak and required prolonged exposure to film (2 weeks) and of emulsion-dipped slides (7-8 weeks), which may be due to a combination of relatively low abundance of TGF-β1 transcripts and low target hybridization of the relatively short riboprobe. Ultimately, an axotomy-induced increase in hybridization signal over control was detected only at the previously reported (Kiefer et al., 1995) peak of TGF-β1 expression, 4-7 days post-axotomy. This increase was detected in sections from both young and old animals at these time points (Fig. 5-4). It should be noted that even at the peak time points, the signal was not detected in all sections examined, but was generally detectable in sections from the central rostral-caudal region of each lesioned facial nucleus in both age groups.

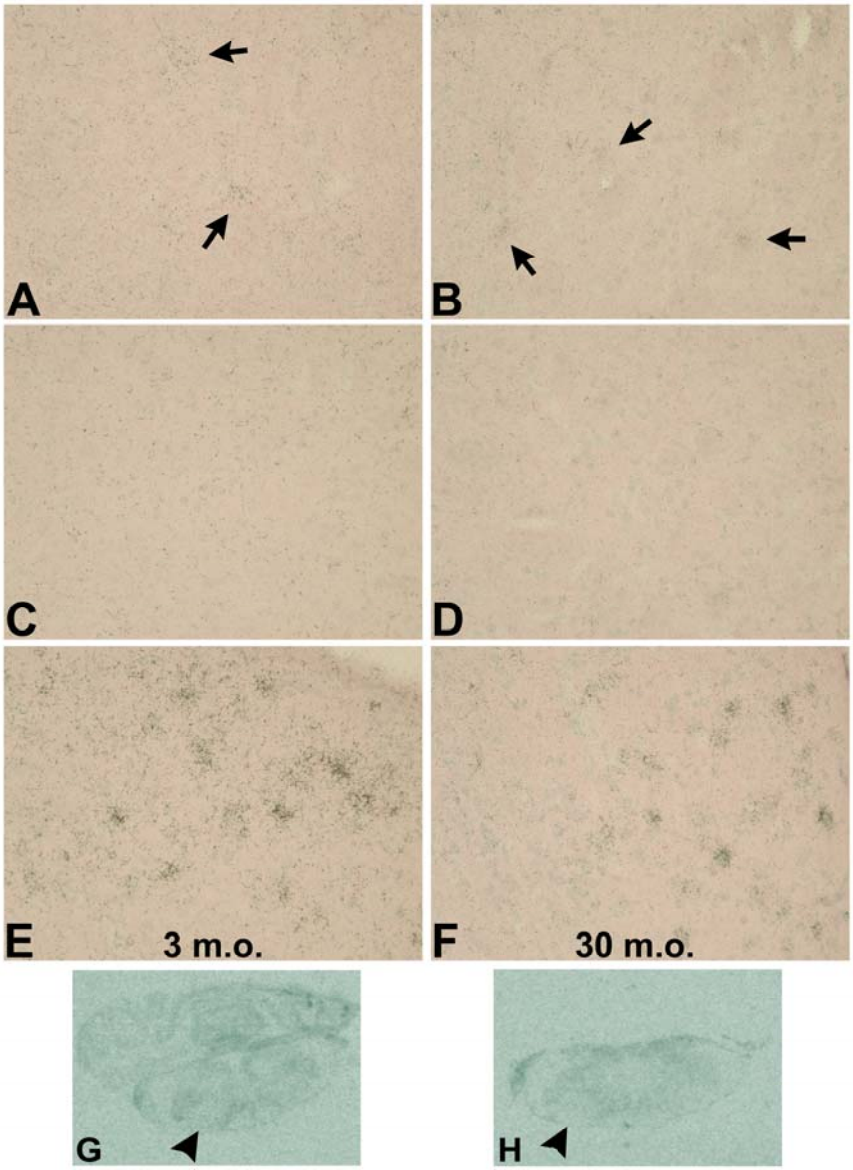

Figure 5-3. Fractalkine mRNA expression in the lesioned and unoperated facial nuclei does not change with aging. A faint fractalkine hybridization signal (clustered silver grains, arrows) was detected over facial motoneurons in the unoperated facial nucleus of both young (A) and old (B) animals, and the signal decreased in the lesioned facial nucleus of both age groups (C and D). Some groups of neurons outside the facial nucleus exhibited a stronger fractalkine hybridization signal in both age groups (E and F). In film images of whole brainstem sections, less signal is evident over the lesioned facial nucleus (arrowheads) in both young (G) and old (H) animals.

There was a low TGF-β1 hybridization signal throughout the rest of the brainstem and cerebellum (including the unoperated facial nucleus) compared to the lack of signal from sections incubated with the sense probe (Fig. 5-4E).

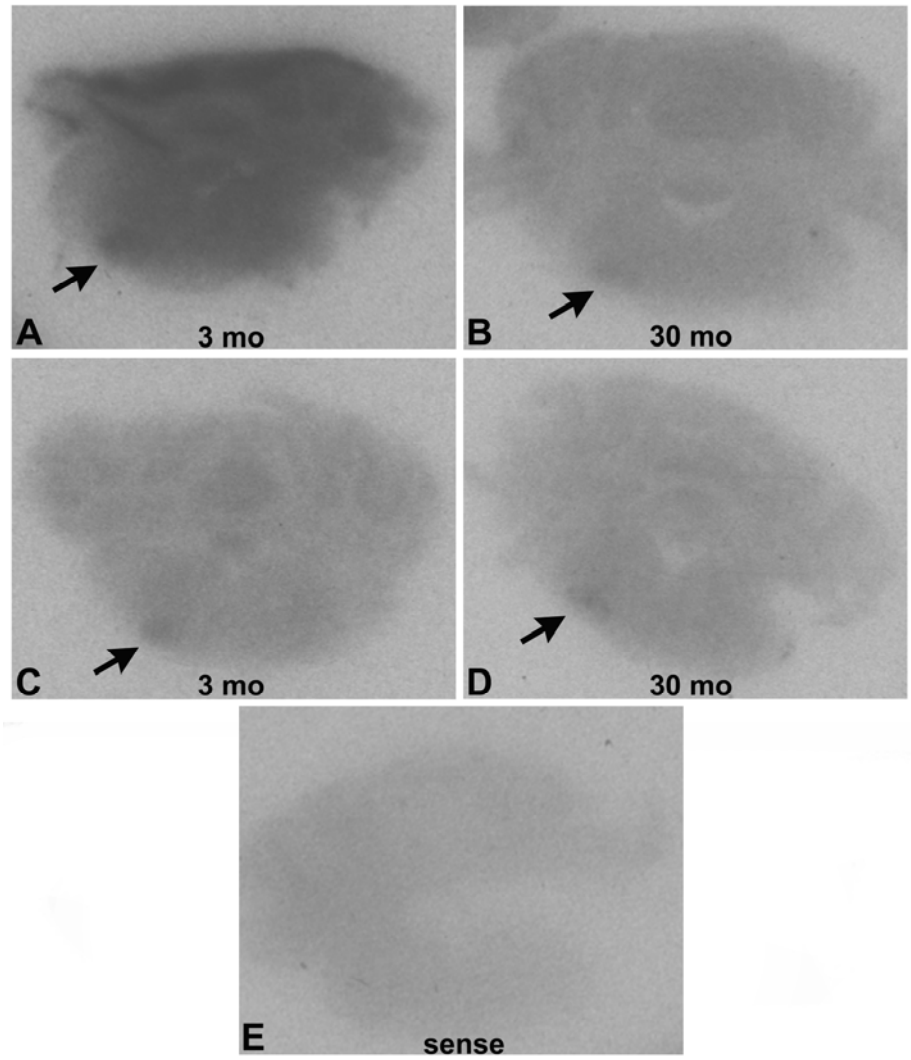

Figure 5-4. Aging does not affect the axotomy-induced increase in TGF-β1 mRNA in the lesioned facial nucleus. TGF-β1 mRNA expression is increased at 4 (A and B) and 7 (C and D) days following axotomy in the lesioned facial nucleus (arrows) of both young (A and C) and old (B and D) rats. Note the presence of a low hybridization signal throughout the brainstem and cerebellum in all sections incubated with the anti-sense TGF-β1 riboprobe (A-D) compared to the sense riboprobe (E).

There is no evidence of an aging-related change in the basal or axtomy-induced expression of HN1 in facial motoneurons. HN1 is a putative marker of neuronal regeneration, and the time course its increased expression in axotomized facial motoneurons correlates with the time course of the increased expression CX3CR1 and microglial activation following facial nerve axotomy. The purpose of examining HN1 expression was to assess whether there are aging-related changes in neuronal regeneration that may in turn explain why microglia in old animals maintain a high rate of proliferation 4 days post-axotomy. However, HN1 mRNA expression did not appear to differ between young and old animals 4 days following axotomy (Fig. 5-5A, B). If HN1 is assumed to be a reliable marker of the state of neuronal regeneration, then aging does not appear to affect the neuronal regeneration program on post-axotomy day 4, and thus the higher level of microglial proliferation on day 4 in old animals is not because neuronal regeneration is lagging behind in those animals. However, given previous observations of delayed neuronal regeneration with aging (Streppel et al., 1998; Vaughan 1990; Vaughan 1992) and the lack of knowledge about the sensitivity of HN1 as a marker of regeneration, it is not possible to rule out delayed neuronal regeneration as the stimulus of prolonged microglial proliferation in old animals.

Discussion

Our finding suggesting that the downregulation of microglial proliferation is attenuated with aging led us to examine the possibility of aging-related changes in molecules involved in neuronal-microglial signaling. We focused on the mRNA expression of the chemokine fractalkine and its receptor, CX3CR1, as well as the mRNA expression of TGF-β1, which has previously been shown to modulate fractalkine-CX3CR1 signaling. We found no evidence of aging-related changes in mRNA

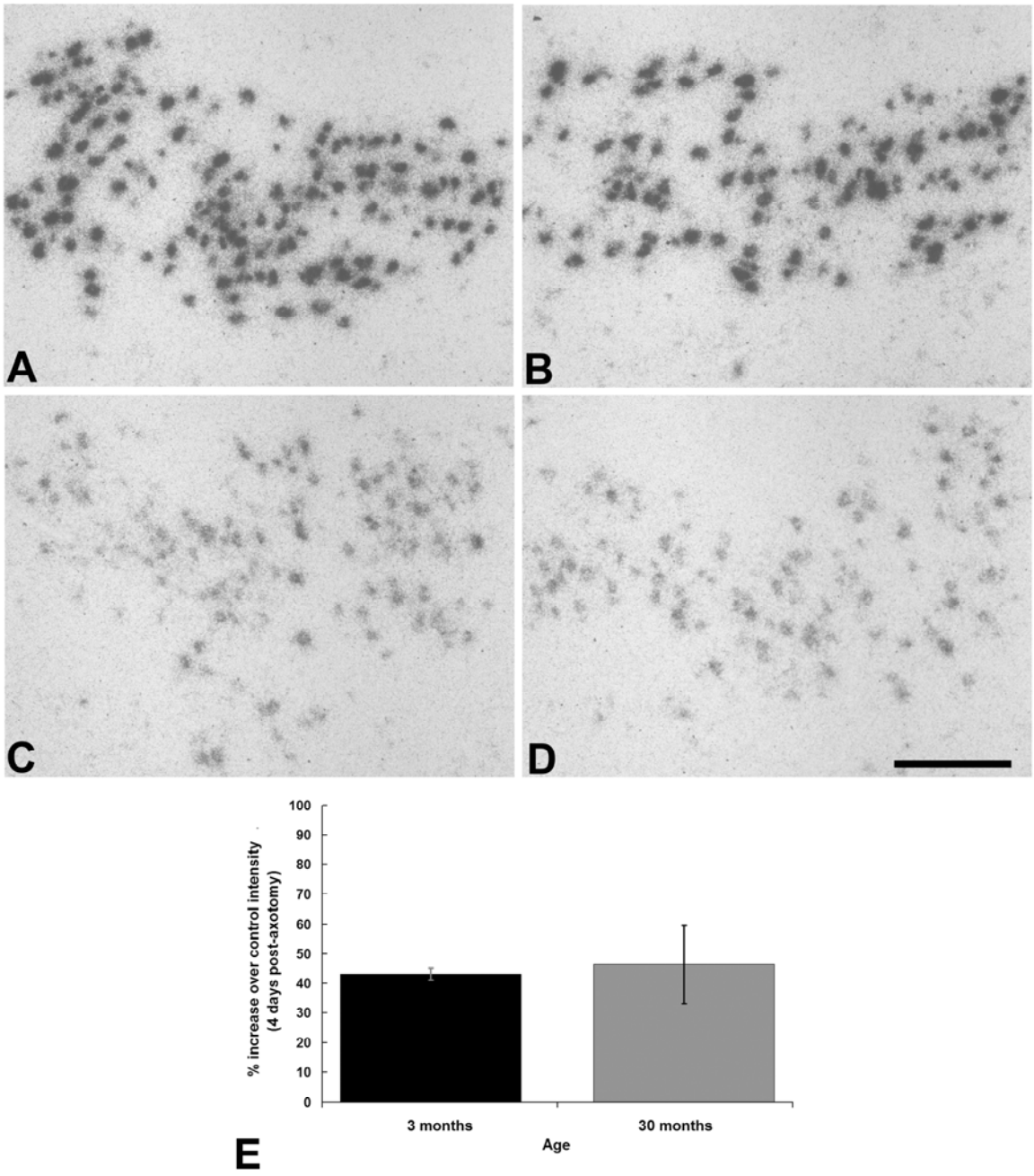

Figure 5-5. Levels of basal and axotomy-induced HN-1 mRNA in the facial nucleus do not change with aging. The expression of HN1 mRNA, a putative marker of neuronal regeneration, was assessed via ISH in the 4-days post-axotomy (A and B) and unoperated (C and D) facial nuclei of young (A and C) and old (B and D) rats. HN1 hybridization signal in intact facial motoneurons does not discernibly differ between young (C) and old (D) animals. The percent increase in intensity of the hybridization signal over control (unoperated facial nucleus) was quantified 4 days following axotomy, and did not significantly differ between age groups (E). Bar= 200 μm.

transcripts of these molecules. There are at least two possible explanations for this finding: either there is no aging-related change in fractalkine-CX3CR1 signaling, or changes occur at the level of protein expression. Fractalkine has the unique property of existing in both a membrane-tethered and a soluble form. The form of fractalkine expressed likely depends on environmental cues, and cleavage of the membrane bound form into the soluble form has been shown to be moderated by proteases such as ADAM17 (TNF-α-converting enzyme) (Garton et al., 2001; Tsou et al., 2001). The membrane-bound form of fractalkine functions in adhesion of CX3CR1-expressing cells, while the soluble form is associated with chemoattraction of CX3CR1-expressing cells (Imai et al., 1997). Additionally, the soluble form of fractalkine has been shown to inhibit Fas-mediated cell death of cultured microglia (Boehme et al., 2000), and to arbitrate microglial phenotype (Zujovic et al., 2000). Despite the different properties of the two forms, few studies have examined how CNS injury affects membrane-bound versus soluble fractalkine expression *in vivo*. Western blot analysis of fractalkine expression in the lesioned facial nucleus has demonstrated a transient increase in smaller molecular weight soluble fractalkine after axotomy (Harrison et al., 1998). It is quite possible that rather than affecting transcription of fractalkine, aging may affect the mechanism of cleavage into the soluble form of the protein. A better understanding of the role of membrane-bound versus soluble forms of fractalkine in the progression of CNS injury and recovery will be necessary in order to understand what changes may occur with aging.

Further complicating the picture are variable data on whether or not fractalkine mRNA expression changes after CNS injury. The results of the current study indicate

that facial motoneuron basal expression of fractalkine mRNA is low compared to neurons in other areas of the brain, and facial nerve axotomy slightly decreases the hybridization signal. No indication of an aging-related change in basal or axotomy-induced expression of fractalkine in the brainstem was found. Other studies have shown an increase in neuronal fractalkine mRNA after kainic-acid induced hippocampal seizure (Suzuki, 2000), decreased expression in the ischemic striatal core accompanied by increased expression in intact neurons of the perifocal region after transient occlusion of the middle cerebral artery (Tarozzo et al., 2002), and a decrease in the expression of fractalkine mRNA in the lesioned facial nucleus (Harrison et al., 1998). Thus, analysis of fractalkine mRNA expression by itself does not give a clear picture of changes in neuronal-microglial signaling, and future studies might be improved by assessing aging-induced changes in the ratio of membrane-bound to soluble fractalkine *in vivo*.

The axotomy-induced increase in the hybridization signal of CX3CR1 mRNA in the lesioned facial nucleus appeared to follow the same time course in both young and old animals, peaking at approximately 4-7 days following axotomy. Likewise, the hybridization signal of TGF-β1 mRNA appeared to peak at the same time points in both young and old animals. We chose to examine TGF-β1 mRNA expression because this cytokine has been previously shown to regulate the transcription of CX3CR1 (Chen et al., 2002). Because we found no evidence of an aging-related difference in the transcription of CX3CR1, further investigation of the expression of TGF-β1 at the protein level is not likely to demonstrate aging-related differences relevant to regulation of CX3CR1 expression. However, it should be noted that TGF-β1 also attenuates fractalkine-CX3CR1 binding-induced ERK1/2 phosphorylation (possibly shifting fractalkine-

dependent signaling towards alternate pathways) by inducing expression of RGS2 and RGS10 (Chen, 2004; Chen et al., 2002). Thus, there exists the possibility that there may be aging-related changes in TGF-β1-induced expression of RGS2 or RGS10 without changes in TGF-β1 regulation of CX3CR1 transcription. TGF-β1 has been shown to inhibit GM-CSF-induced microglial proliferation *in vitro* (Suzumura et al., 1993), and this inhibitory function of TGF-β1 is impaired when applied to microglia cultured from old donors (Rozovsky et al., 1998). Aging-related attenuation of TGF-β1's inhibitory effect on microglial proliferation may be independent of its regulation of CX3CR1 expression, and may even be a sign of senescent changes in microglia. Thus, further studies of aging-related changes in TGF-β receptor signaling and modulation of fractalkine signaling pathways are warranted.

CHAPTER 6
REPEAT FACIAL NERVE INJURY AS A MODEL OF MICROGLIAL AGING

Introduction

In Chapter 2, we reported that the downregulation of microglial proliferation in response to facial nerve axotomy is attenuated in old rats, and we proposed two hypothetical explanations for this change. The first hypothesis, that aging disrupts neuronal-microglial regulatory signals, was examined in Chapter 5. The second hypothesis stated that the aging-related attenuation of the regulation of microglial activation is due to microglial senescence- cellular dysfunction as a product of the aging process. Senescence may be the result of an accumulation of genomic DNA, mtDNA, and protein changes with aging that ultimately interrupt the proper functioning of the cell (Martin, 2002; Troen, 2003). Cell division and exposure to reactive oxygen species increase the incidence of DNA and protein changes. Because microglia have a high proliferative capacity and increased exposure to reactive oxygen species as part of their activation program, microglia may be particularly vulnerable to senescence. Exposure to reactive oxygen species is also an unavoidable consequence of normal cellular metabolism (Barja, 2004; Droge, 2003; Shigenaga et al., 1994), and minor brain insults throughout the lifespan are probably an unavoidable factor of living for nearly every animal. Thus, it is not surprising to find aging-related morphological changes (such as those described in Chapter 4) in microglia of otherwise normally aging, intact areas of the brain. Previous brain injury has been associated with increased incidence of neurodegenerative disease with aging (Guo et al., 2000; Lye and Shores, 2000; Plassman

et al., 2000), and it is plausible that injury-accelerated senescence of microglia may contribute to an earlier onset of aging-related changes in the brain and lower the threshold for onset of neurodegenerative disease. To investigate whether a previous insult can induce aging-like changes in microglia, we employed a repeat facial nerve injury model. We hypothesized that an earlier round of microglial proliferation induced by facial nerve crush would result in attenuated downregulation of microglial proliferation after a second facial nerve injury via axotomy, mimicking the effects found in old animals.

Materials and Methods

Experimental Design

All animals used in this experiment were male F344BN hybrid rats obtained from the National Institute on Aging. Animals receiving double facial nerve lesions were ordered at 2 months of age, subjected to facial nerve crush within 2 days of arrival, and housed in the University of Florida McKnight Brain Institute Animal Care Services facility between surgeries. Animals receiving single lesions were ordered at 3 months of age in order to be the same approximate age as double injury animals at the time of axotomy.

Repeat (double) injury animals were initially subjected to crush of the right facial nerve. The crush surgery is similar to the axotomy surgery described in Chapter 2, with a minor modification. Briefly, once the nerve was exposed, the nerve was gently lifted and crushed with sterile forceps for 10 seconds (rather than axotomized into 2 separate branches). The incision was closed with a surgical staple, and once the animal recovered from anesthesia lack of whisker movement on the right side was verified. Although facial nerve crush generates microglial activation in the same manner as axotomy, reinnervation proceeds much more quickly in the crush model, with recovery of whisker

function as early as 2-3 weeks after injury (Moran and Graeber, 2004). From post-injury day 7, animals were monitored daily for recovery of whisker function, which began to appear by day 13-14 in all animals. Approximately 5 weeks after the initial crush surgery, repeat injury animals were subjected to facial nerve axotomy of the ipsilateral nerve. Animals were given ^{3}H-thymidine injections and sacrificed (with age-matched single axotomy animals) at 2, 3 and 4 days post-axotomy as described in Chapter 2. Paraffin processing, sectioning, autoradiography, and development were also carried out as described in Chapter 2.

Quantitative Analysis

Quantitative analysis of single (N= 3-4 per time point) and repeat injury animals (N= 3-4 per time point) was carried out by counting the number of ^{3}H-thymidine labeled cell nuclei within the outlined area of the lesioned facial nucleus and calculating the population density, as described in Chapter 2. Results are represented as mean values ± SEM. Data were analyzed with t-tests at each time point using GraphPad Prism software (GraphPad Software, San Diego, CA). A significance level of $p \leq 0.05$ was used.

Results

A previous facial nerve injury does not change the time course or extent of the microglial proliferative response after facial nerve axotomy. The hypothesis behind this experiment was that previous episodes of microglial proliferation (i.e., from a previous injury) lead to the phenotype of microglial senescence (i.e., attenuated ability to downregulate the proliferative response) associated with aging. However, we found no evidence that one previous episode of microglial proliferation after a facial nerve crush leads to a senescent phenotype after a second facial nerve injury. Although there appeared to be a somewhat more robust initiation of the proliferative response on post-

injury day 2 in double injury animals, the difference was not statistically significant (Fig 6-1). Microglial proliferation peaked at 3 days following facial nerve axotomy in both groups. The density of ^{3}H-thymidine labeled dividing microglia within the lesioned facial nucleus declined significantly by 4 days post-axotomy in both single and repeat injury animals.

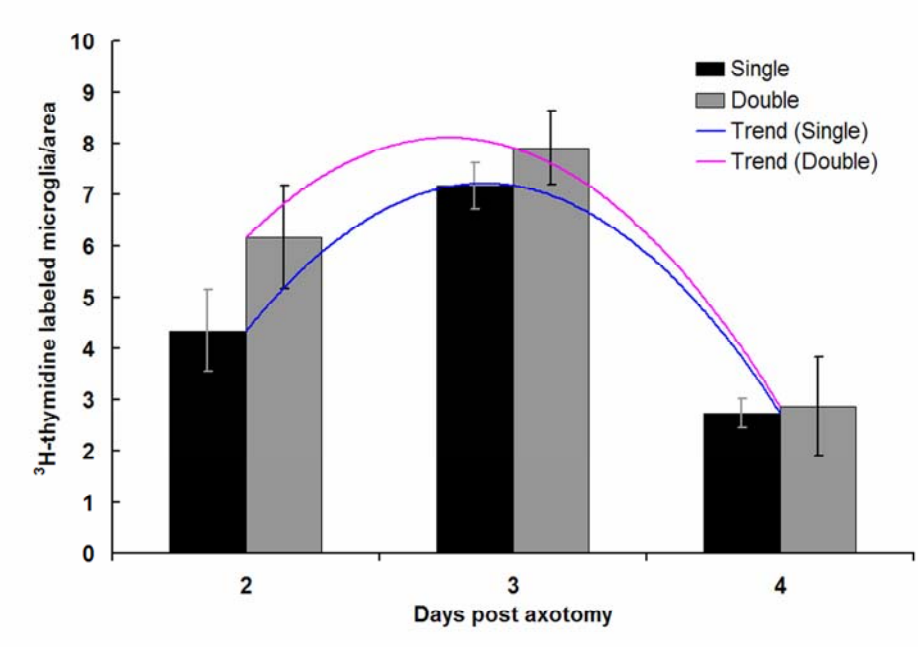

Figure 6-1. The time course of microglial proliferation in single vs. repeat (double) facial nerve injury animals. An increase in the robustness of proliferative response was noted on post-axotomy day 2 in animals receiving a previous facial nerve injury, but the increase was not statistically significant. Microglial proliferation peaked at 3 days and declined sharply by 4 days post-axotomy in both single and repeat injury animals.

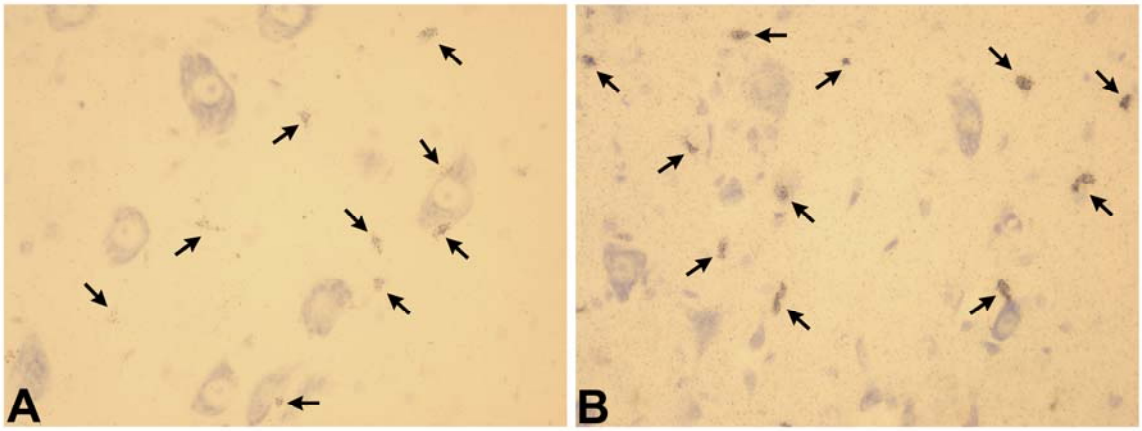

Figure 6-2. Photomicrographs of ^{3}H-thymidine labeled dividing microglia (silver grains, arrows) in the lesioned facial nucleus 2 days after facial nerve axotomy in single (A) and repeat (B) injury animals. At this time point, there was a trend towards more labeled microglia in repeat injury animals.

Discussion

We hypothesized that aging-related changes in microglial activation may be a sign of microglial senescence, and that microglial senescence may be accelerated by previous stimulation of microglial mitosis. Thus, we employed a model of repeat facial nerve injury to determine if previously activated microglia would mimic the senescent (i.e., attenuated downregulation of proliferation) phenotype of old microglia. Repeat injury rats were subjected to a facial nerve crush, followed 5 weeks later by an axotomy of the same nerve. Using ^{3}H-thymidine labeling, the density of dividing microglia in the lesioned facial nucleus of these animals was compared at 2, 3 and 4 days following axotomy to the density in animals sacrificed at the same time points after receiving only a single axotomy.

On the second day following axotomy, the repeat injury animals appeared to have a more robust proliferative response of microglia than single injury animals. This may be due to "priming" of the microglia by the first injury. However, the general time course of proliferation did not differ significantly between the two groups, and proliferation was

significantly downregulated in both groups by the fourth day following axotomy. Thus, microglia in repeat injury animals did not mimic the attenuated downregulation of proliferation phenotype of old microglia. However, this does not dismiss the hypothesis that microglial senescence may be accelerated by microglial activation and proliferation. The facial nerve crush and axotomy models are models of neuroregeneration and do not elicit the full extent of the microglial activation program compared to degenerative conditions (e.g., traumatic brain injury). Thus, a single previous stimulus of microglial proliferation by facial nerve crush is probably not enough to generate the cellular changes that result in microglial senescence. In fact, our finding is not surprising in light of recent data from our laboratory demonstrating that telomerase activity is increased and telomeres lengthened in microglia stimulated by a single facial nerve axotomy (data submitted for publication by Flanary and Streit). Increased microglial telomerase activity after a short burst of proliferation in young animals may be a mechanism to protect microglia from senescence, as telomerase activity initially increases in stimulated microglia *in vitro*, but chronic stimulation of cultured microglia gradually results in decreased telomerase activity, shortened telomeres and, ultimately, in cellular senescence (Flanary and Streit, 2004). It is reasonable to postulate that chronic stimulation of microglial proliferation *in vivo* may also eventually result in microglial senescence. A more extensive exploration of this hypothesis could be accomplished through future studies in which the facial nerve is crushed at regular intervals throughout the lifespan, and axotomy-induced microglial proliferation assessed at various ages in these animals to determine if the putative threshold of microglial senescence is reached earlier in these animals compared to single axotomy controls.

CHAPTER 7
CONCLUSION

Summary of Findings

Because little is known about how aging affects microglia beyond changes in cell surface marker expression, we decided to examine the effects of aging on facial nerve axotomy-induced microglial activation, proliferation, and turnover. We found no discernable difference in the robustness of the initiation of the microglial proliferative response to motoneuron axotomy, and proliferation peaked at 3 days in both age groups. However, while microglial proliferation was strongly downregulated by day 4 in young animals, a significantly higher number of dividing microglia remained in the lesioned facial nucleus of old animals at this time point. We speculated that the prolonged period of proliferation might be balanced by a subsequent higher rate of microglial turnover via programmed cell death in old animals, but we did not detect an aging-related difference in the number of TUNEL-positive microglia at 7, 14 or 21 days following axotomy. Furthermore, the unusual cytoplasmic labeling of fragmented DNA in TUNEL-positive microglia was found in both young and old animals, and the distribution of labeled cells in the lesioned facial nucleus did not differ between age groups at any of the time points examined. Using lectin histochemistry to label microglia, we examined if there was an aging-related change in the axotomy-induced increase in lectin-positive area in the lesioned facial nucleus during the first 21 days following axotomy. We hypothesized that there may be a greater axotomy-induced increase in lectin-positive area within the lesioned facial nucleus of old animals at 7, 14 and/or 21 days given the prolonged period

of microglial proliferation without a corresponding increase in microglial turnover. However, there was no significant aging-related difference in the fold increase in lectin-positive area at any of the time points analyzed. Upon light microscopic examination of lectin-positive microglia, we found conspicuous aging-related morphological changes, including perinuclear hypertrophy and dense lectin reactivity, in microglia of the unoperated facial nucleus. Furthermore, because they already exhibited perinuclear hypertrophy in the unoperated facial nucleus, the axotomy-induced increase in microglial cell size did not appear to be as significant in old animals compared to young animals. Thus, the lack of a detectable aging-related difference in axotomy-induced increase in lectin-positive area may be due to less of an axotomy-induced increase in cell size (and lectin reactivity) in old animals.

The finding of an attenuated down regulation of axotomy-induced microglial proliferation with aging led us to two possible hypotheses: either aging affects neuronal-microglial regulatory signals (for example, delayed regeneration may prolong the upregulation of a signal stimulating microglial activation or the downregulation of an inhibitory signal), or microglia become inherently senescent with aging and are unable to downregulate their proliferative response in spite of regulatory signals from neurons. To explore the neuronal-microglial signaling hypothesis, we compared the axotomy-induced change in mRNA expression of fractalkine, its G-protein coupled receptor (CX3CR1), and the regulatory growth factor TGF-β1 in young and old animals. Aging did not appear to affect expression of fractalkine or CX3CR1 mRNA in the lesioned facial nucleus, but possible aging-related changes in fractalkine or CX3CR1 protein expression have not yet been examined. Additionally, aging did not appear to affect the axotomy-

induced transient increase in TGF-β1 mRNA or TGF-β1 regulation of CX3CR1 transcription. Previous studies have shown that TGF-β1 has an inhibitory affect on microglial proliferation *in vitro* (Suzumura et al., 1993) that is impaired in cultures from old donors (Rozovsky et al., 1998), and we initially hypothesized that the aging-related impairment of TGF-β1's inhibitory effect may involve disregulation of CX3CR1 transcription. The lack of a detectable aging-related change in CX3CR1 mRNA expression suggests that this hypothesis is wrong. However, the inhibitory effect of TGF-β1 on microglial proliferation may be independent of its regulation of CX3CR1 expression, and it is possible that the aging-related attenuation of the regulation of microglial proliferation may be due to changes in TGF-β receptor signaling and TGF-β1 modulation of fractalkine signaling pathways.

Such changes in signaling pathways within microglia would also lend support to the alternative hypothesis of microglial senescence. Microglia have high mitotic and phagocytic potential that may make them particularly vulnerable to aging-related mutations and oxidative damage. We employed a repeat facial nerve injury model to examine whether previous stimulation of microglial proliferation via facial nerve crush would cause changes that mimic aging when microglia are later subjected to a second proliferative stimulus via facial nerve axotomy. We were unable to detect changes in the microglial proliferative response (such as those which occur with aging) between single and double-injury animals, but it must be acknowledged that a single previous stimulus is not likely enough to mimic the effects of aging. Stimulation of chronic microglial proliferation would be a more suitable model for examining the hypothesis of microglial senescence in future studies, as described in Chapter 6.

We have shown that aging does affect regulation of microglial proliferation, but we have only begun to examine the many potential factors that may change with aging to result in the deregulation of the microglial proliferative response. For example, while we did not find changes in the mRNA expression of fractalkine and its receptor, CX3CR1, transcription of these molecules are only two potential points at which aging may affect a complex signaling process (e.g., neuronal expression of soluble vs. membrane-bound fractalkine protein). Aging-related changes in the microenvironment, such as the presence of aberrant inhibitory molecules in the extracellular space or changes in the expression of moderating molecules, may also disrupt the neuronal-microglial signal. Whether it is a product of changes in neuronal signals or microglial dysfunction, deregulation of microglial activation with aging may have significant implications for older individuals with brain, cranial nerve or spinal cord injuries, and may play a role in the aging-related increase in susceptibility to neurodegenerative diseases.

Future Studies

Beyond the Facial Nerve Axotomy Model

The choice of the facial nerve axotomy model for use in the studies conducted in this dissertation was explained in Chapter 1. The model is advantageous because of its well established time course of microglial activation and neuronal regeneration, and because it involves a remote lesion that ensures that microglial activation is due to changes in neuronal signals rather than from direct tissue injury. However, information gained from this model, however insightful, does not necessarily apply to other regions of the CNS. The microglial population is heterogeneous in phenotype and density throughout the CNS (Lawson et al., 1990; Streit et al., 1988; Streit et al., 1999), and the microglial reaction to neuronal injury also varies with region of the CNS (Gehrmann et

al., 1991; Streit et al., 2000). Comparing microglial proliferation, turnover, morphology, and neuronal-microglial signaling between age groups after lesion to another region of the brain may provide additional insight into the effects of aging on the microglial activation response. A lesion of the entorhinal cortex, which leads to anterograde axonal degeneration in the molecular layer of the dentate gyrus, would be a good choice for future studies because microglia are known to proliferate in response to the injury (Fagan and Gage, 1994; Gehrmann et al., 1991); and any detected aging-related changes in microglia of the hippocampus would have more relevance to aging-related memory deficits.

Aging and Neuronal-Microglial Signaling

As discussed above, changes in neuronal-microglial signaling are one possible cause of aging-related changes in the regulation of the microglial activation program. Fractalkine was considered the best candidate for the putative regulatory neuronal-microglial signal for multiple reasons as discussed in Chapters 1 and 5 of this dissertation. Although we did not find aging-related changes in the axotomy-induced change in mRNA expression of fractalkine or its receptor, the next logical step would be to examine their expression at the protein level. It has previously been shown that fractalkine protein expression in the intact facial nucleus is primarily the larger molecular mass (65 kDa) membrane-bound form, but after axotomy includes expression of smaller molecular mass soluble forms, with a peak in the expression of the 50 kDa form on post-axotomy day 4 (Harrison et al., 1998). The peak in expression of the soluble form coincides with the peak expression of CX3CR1, and there is much evidence that the two different forms of fractalkine have different functions. Thus, any change in the time course of axotomy-induced soluble fractalkine expression would likely affect activation

of CX3CR1-expressing microglia. A Western blot comparative analysis of fractalkine protein expression in the facial nucleus of young and old animals at various times after axotomy would be useful in determining if there is an aging-related change in the induction of soluble fractalkine expression.

Another aspect of the fractalkine-CX3CR1 signaling mechanism that may be affected by aging is the TGF-β1-induced increase in microglial RGS10 expression, which attenuates G-protein coupled receptor (i.e., CX3CR1) signaling and may shift fractalkine-triggered signaling away from the MAPK pathway to other signaling pathways (Chen, 2004). Future studies may examine the effects of aging on RGS10 expression in the lesioned facial nucleus. Aging related changes in microglial signaling pathways, including changes in RGS10, may also be a reflection of microglial senescence.

Neuronal expression of the cytokine IL-6 has previously been shown to induce robust activation of microglia in the regenerating facial nucleus (Streit et al., 2000), and because, among many of its pleiotropic functions, IL-6 is known to induce cellular proliferation (Kishimoto et al., 1995), IL-6 is a potential stimulator of microglial proliferation. Thus, any aging-related changes in the time course of expression of IL-6 and IL-6 receptor in the lesioned facial nucleus would possibly provide an explanation for the prolonged period of microglial proliferation with aging, and should be investigated in future studies.

Ultrastructural Analysis of Aging-Related Morphological Changes in Activated Microglia

An ultrastructural comparison of microglia activated by neuronal injury in young and old animals has not yet been performed. In the study described in Chapter 4, dense perikaryal lectin reactivity (suggestive of membrane-bound inclusions) was observed in

both resting and activated microglia of old animals, and old activated microglia had shorter processes with a fragmented appearance to the lectin staining compared to their younger counterparts. Comparing young and old activated microglia at the ultrastructural level using electron microscopy may provide further insight into the nature of the membrane bound inclusions and other aging-related effects on the structure of activated microglia. Microglial morphology is an indication of the functional state of microglia, and significant aging-related changes in the ability of microglia to morph their cytoarchitecture during activation may affect their ability to provide protection and support to regenerating neurons (e.g., perineuronal microglia with shorter processes may be less able to envelope the motoneuron soma). Additionally, a comparison of cytoskeletal protein (i.e., actin, beta-actin and tubulin) expression in young and old activated microglia may provide further insight into aging-related morphological changes.

Potential Causes of Aging-Related Microglial Dysfunction/Senescence

Microglia exhibit clearly discernable morphological changes with aging, including the observation of somatal hypertrophy and dense perinuclear lectin reactivity described in Chapter 4 and previous reports of dystrophic changes (Streit et al., 2004) and cytosolic inclusions (Peinado et al., 1998; Peters et al., 1991; Sandell and Peters, 2002; Vaughan and Peters, 1974), but it remains unclear if such morphological changes are signs, or possibly even the cause (in the case of inclusions), of microglial senescent dysfunction. The potential causes of aging-related microglial dysfunction run the gamut of all the theories of aging discussed in Chapter 1. For example, the high mitotic potential of microglia throughout the lifespan of the organism may make them vulnerable telomere shortening. Obviously, the extended proliferative response observed in the aging animals

demonstrates that these microglia have not yet reached replicative senescence (the point where the shortest telomere has shortened to the critical length such that mitosis is stopped), but there are aberrant effects on genes near the telomeres as telomere shortening progresses, referred to as the telomere position effect (Wright and Shay, 1992). It is possible that telomere position effect may result in microglial dysfunction. Microglial dysfunction as a result of telomere shortening is consistent with the observation that previous traumatic insults to the brain, which would initiate microglial proliferation, are a risk factor for early onset of aging-related brain changes. Previous studies in our lab have demonstrated that telomere shortening occurs over time in cultured microglia (Flanary and Streit, 2004) and with aging in the rat cerebellum and cortex (Flanary and Streit, 2003), but a study of whether microglia isolated from old donors have shorter telomeres than those from young donors has not been conducted yet. Additionally, expansion of the repeat facial nerve injury model (as discussed in Chapter 6) to include multiple injuries throughout the lifespan may provide further insight as to whether chronic microglial activation hastens the appearance of putative characteristics of microglial senescence, including telomere shortening and impaired regulation of activation/proliferation.

Aging-related microglial dysfunction may also be a product of oxidative damage. Microglia exhibit risk factors for vulnerability to oxidative damage, including their release of reactive oxygen species as part of their defense function (Colton and Gilbert, 1987; Hu et al., 1996), and along with oligodendrocytes, a decreased ability to repair the effects of oxidative damage to mtDNA than astrocytes (Hollensworth et al., 2000;

Ledoux et al., 1998). Future studies of aging-related changes in microglial mtDNA may provide insight into the putative phenomenon of microglial senescence.

Aging and Exogenously Derived Microglia

In the ^{3}H-thymidine labeling experiments it was noted that early in the response to facial nerve axotomy a large percentage of proliferating microglia were found outside of the injured facial nucleus (i.e., in the white matter between the ventral border of the facial nucleus and edge of the brainstem), which suggested that injured neurons release some sort of diffusible signal that is capable of recruiting and inducing mitosis in microglia from adjacent areas. In light of evidence showing that blood-derived monocytes or bone marrow precursors are capable of infiltrating the adult CNS and differentiating into the ramified phenotype of endogeneous parenchymal microglia (Bechmann et al., 2005; Beck et al., 2003; Kennedy and Abkowitz, 1997; Krall et al., 1994; Lawson et al., 1992; McMahon et al., 2002; Priller et al., 2001; Schilling et al., 2003; Simard and Rivest, 2004; Vallieres and Sawchenko, 2003), the possibility that some of the recruited ^{3}H-thymidine labeled cells observed in this study were exogeneously derived should be considered. One study of exogeneously derived microglia found evidence that such cells have higher antigen presenting potential than resident microglia (Simard and Rivest, 2004). Future studies might investigate if there are aging-related differences in the degree of exogenous infilitration after injury. If there are a greater percentage of exogenously derived microglia with higher antigen presenting potential in old animals, this could have implications in the manifestation of neurodegenerative diseases of aging.

Aging and Basal Levels of Microglial Proliferation

Low levels of microglial proliferation occur in the normal, intact brain and spinal cord, but no previous study has examined if the basal rate of microglial proliferation

changes with aging. As discussed in Chapter 1, several studies have found an aging-related increase in the density and/or total number of microglial immunophenotypic sub-populations, including those expressing MHC class II (DiPatre and Gelman, 1997; Morgan et al., 1999; Perry et al., 1993; Sheffield and Berman, 1998), ED1 macrophage marker (Kullberg et al., 2001; Perry et al., 1993) and IL-1α (Sheng et al., 1998). However, there are conflicting reports as to whether the total microglial population density changes with aging (Long et al., 1998; Ma et al., 2003), probably because of variances in the method of analysis, as well as species and regional heterogeneity of the microglia population. As part of the ^{3}H-thymidine labeling experiment in Chapter 2, aging-related differences in proliferation of microglia in the unoperated facial nucleus were examined. Very few proliferating microglia were found in the unoperated facial nucleus, or anywhere else in the brainstem outside the immediate vicinity of the injured facial nucleus, in all of the animals examined. Thus, there is no aging-related difference in the basal level of microglial proliferation in the brainstem. It is not known, however, if the observations in the brainstem reflect the rate of basal microglial proliferation in other areas of the brain. Because changes in the basal rate of microglial proliferation in other intact areas of the aging brain may result in, or be a reflection of, a disruption of CNS homeostasis, a comparison of microglial proliferation in various regions of the intact brain between young and old animals should be a subject of future studies.

LIST OF REFERENCES

Aihara M, Ishii S, Kume K, Shimizu T. 2000. Interaction between neurone and microglia mediated by platelet-activating factor. Genes Cells **5**(5): 397-406.

Akiyama H, Barger S, Barnum S, Bradt B, Bauer J, et al. 2000. Inflammation and Alzheimer's disease. Neurobiol Aging **21**(3): 383-421.

Akiyama H, Schwab C, Kondo H, Mori H, Kametani F, et al. 1996. Granules in glial cells of patients with Alzheimer's disease are immunopositive for C-terminal sequences of beta-amyloid protein. Neurosci Lett **206**(2-3): 169-72.

Alba C, Vidal L, Diaz F, Villena A, de Vargas IP. 2004. Ultrastructural and quantitative age-related changes in capillaries of the dorsal lateral geniculate nucleus. Brain Res Bull **64**(2): 145-53.

Albert M. 1993. Neuropsychological and neurophysiological changes in healthy adult humans across the age range. Neurobiol Aging **14**(6): 623-5.

Aldskogius H. 2001. Microglia in neuroregeneration. Microsc Res Tech **54**(1): 40-6.

Alliot F, Godin I, Pessac B. 1999. Microglia derive from progenitors, originating from the yolk sac, and which proliferate in the brain. Brain Res Dev Brain Res **117**(2): 145-52.

Ard MD, Cole GM, Wei J, Mehrle AP, Fratkin JD. 1996. Scavenging of Alzheimer's amyloid beta-protein by microglia in culture. J Neurosci Res **43**(2): 190-202.

Arruda JL, Colburn RW, Rickman AJ, Rutkowski MD, DeLeo JA. 1998. Increase of interleukin-6 mRNA in the spinal cord following peripheral nerve injury in the rat: potential role of IL-6 in neuropathic pain. Brain Res Mol Brain Res **62**(2): 228-35.

Badan I, Buchhold B, Hamm A, Gratz M, Walker LC, et al. 2003a. Accelerated glial reactivity to stroke in aged rats correlates with reduced functional recovery. J Cereb Blood Flow Metab **23**(7): 845-54.

Badan I, Platt D, Kessler C, Popa-Wagner A. 2003b. Temporal dynamics of degenerative and regenerative events associated with cerebral ischemia in aged rats. Gerontology **49**(6): 356-65.

Bard F, Cannon C, Barbour R, Burke RL, Games D, et al. 2000. Peripherally administered antibodies against amyloid beta-peptide enter the central nervous system and reduce pathology in a mouse model of Alzheimer disease. Nat Med **6**(8): 916-9.

Barja G. 1998. Mitochondrial free radical production and aging in mammals and birds. Ann N Y Acad Sci **854**: 224-38.

Barja G. 2004a. Aging in vertebrates, and the effect of caloric restriction: a mitochondrial free radical production-DNA damage mechanism? Biol Rev Camb Philos Soc **79**(2): 235-51.

Barja G. 2004b. Free radicals and aging. Trends Neurosci **27**(10): 595-600.

Barron KD. 1995. The microglial cell. A historical review. J Neurol Sci **134 Suppl**: 57-68.

Barron KD, Marciano FF, Amundson R, Mankes R. 1990. Perineuronal glial responses after axotomy of central and peripheral axons. A comparison. Brain Res **523**(2): 219-29.

Batchelor PE, Liberatore GT, Wong JY, Porritt MJ, Frerichs F, et al. 1999. Activated macrophages and microglia induce dopaminergic sprouting in the injured striatum and express brain-derived neurotrophic factor and glial cell line-derived neurotrophic factor. J Neurosci **19**(5): 1708-16.

Bechmann I, Goldmann J, Kovac AD, Kwidzinski E, Simburger E, et al. 2005. Circulating monocytic cells infiltrate layers of anterograde axonal degeneration where they transform into microglia. Faseb J. published online Jan 25, 2005, doi:10.1096/fj.04-2599fje.

Beck H, Voswinckel R, Wagner S, Ziegelhoeffer T, Heil M, et al. 2003. Participation of bone marrow-derived cells in long-term repair processes after experimental stroke. J Cereb Blood Flow Metab **23**(6): 709-17.

Berezovskaya O, Maysinger D, Fedoroff S. 1995. The hematopoietic cytokine, colony-stimulating factor 1, is also a growth factor in the CNS: congenital absence of CSF-1 in mice results in abnormal microglial response and increased neuron vulnerability to injury. Int J Dev Neurosci **13**(3-4): 285-99.

Biber K, Sauter A, Brouwer N, Copray SC, Boddeke HW. 2001. Ischemia-induced neuronal expression of the microglia attracting chemokine Secondary Lymphoid-tissue Chemokine (SLC). Glia **34**(2): 121-33.

Blinzinger K, Kreutzberg G. 1968. Displacement of synaptic terminals from regenerating motoneurons by microglial cells. Z Zellforsch Mikrosk Anat **85**(2): 145-57.

Bodles AM, Barger SW. 2004. Cytokines and the aging brain - what we don't know might help us. Trends Neurosci **27**(10): 621-6.

Boehme SA, Lio FM, Maciejewski-Lenoir D, Bacon KB, Conlon PJ. 2000. The chemokine fractalkine inhibits Fas-mediated cell death of brain microglia. J Immunol **165**(1): 397-403.

Bohm I, Schild H. 2003. Apoptosis: the complex scenario for a silent cell death. Mol Imaging Biol **5**(1): 2-14.

Bossy-Wetzel E, Barsoum MJ, Godzik A, Schwarzenbacher R, Lipton SA. 2003. Mitochondrial fission in apoptosis, neurodegeneration and aging. Curr Opin Cell Biol **15**(6): 706-16.

Brionne TC, Tesseur I, Masliah E, Wyss-Coray T. 2003. Loss of TGF-beta 1 leads to increased neuronal cell death and microgliosis in mouse brain. Neuron **40**(6): 1133-45.

Bruce-Keller AJ. 1999. Microglial-neuronal interactions in synaptic damage and recovery. J Neurosci Res **58**(1): 191-201.

Bsibsi M, Ravid R, Gveric D, van Noort JM. 2002. Broad expression of Toll-like receptors in the human central nervous system. J Neuropathol Exp Neurol **61**(11): 1013-21.

Burzynski SR. 2005. Aging: gene silencing or gene activation? Med Hypotheses **64**(1): 201-8.

Calderini G, Bellini F, Consolazione A, Dal Toso R, Milan F, et al. 1987. Reparative processes in aged brain. Gerontology **33**(3-4): 227-33.

Cammermeyer J. 1965. Juxtavascular karyokinesis and microglia cell proliferation during retrograde reaction in the mouse facial nucleus. Ergeb Anat Entwicklungsgesch **38**: 1-22.

Carson MJ, Reilly CR, Sutcliffe JG, Lo D. 1998. Mature microglia resemble immature antigen-presenting cells. Glia **22**(1): 72-85.

Chang RC, Chen W, Hudson P, Wilson B, Han DS, et al. 2001. Neurons reduce glial responses to lipopolysaccharide (LPS) and prevent injury of microglial cells from over-activation by LPS. J Neurochem **76**(4): 1042-9.

Chen S. 2004. TGF-beta1 regulation of chemokine receptors in rat microglia and human macrophages. Thesis, University of Florida**:** 24-63.

Chen S, Luo D, Streit WJ, Harrison JK. 2002. TGF-beta1 upregulates CX3CR1 expression and inhibits fractalkine-stimulated signaling in rat microglia. J Neuroimmunol **133**(1-2): 46-55.

Choi D, Raisman G. 2002. Somatotopic organization of the facial nucleus is disrupted after lesioning and regeneration of the facial nerve: the histological representation of synkinesis. Neurosurgery **50**(2): 355-62; discussion 362-3.

Cifu DX, Kreutzer JS, Marwitz JH, Rosenthal M, Englander J, et al. 1996. Functional outcomes of older adults with traumatic brain injury: a prospective, multicenter analysis. Arch Phys Med Rehabil **77**(9): 883-8.

Colton CA, Gilbert DL. 1987. Production of superoxide anions by a CNS macrophage, the microglia. FEBS Lett **223**(2): 284-8.

Combs CK, Johnson DE, Karlo JC, Cannady SB, Landreth GE. 2000. Inflammatory mechanisms in Alzheimer's disease: inhibition of beta-amyloid-stimulated proinflammatory responses and neurotoxicity by PPARgamma agonists. J Neurosci **20**(2): 558-67.

Cotman CW, Tenner AJ, Cummings BJ. 1996. beta-Amyloid converts an acute phase injury response to chronic injury responses. Neurobiol Aging **17**(5): 723-31.

Cuadros MA, Navascues J. 1998. The origin and differentiation of microglial cells during development. Prog Neurobiol **56**(2): 173-89.

Davies CA, Gollins H, Stevens N, Fotheringham AP, Davies I. 2004. The glial cell response to a viral vector in the aged brain. Neuropathol Appl Neurobiol **30**(1): 30-8.

Del Rio-Hortega P. 1932. Microglia. In: Penfield W. Cytology and Cellular Pathology of the Nervous System. New York: PB Hoeber**:** 489-534.

Diamond MC, Johnson RE, Gold MW. 1977. Changes in neuron number and size and glia number in the young, adult, and aging rat medial occipital cortex. Behav Biol **20**(4): 409-18.

Dickson DW, Lee SC, Mattiace LA, Yen SH, Brosnan C. 1993. Microglia and cytokines in neurological disease, with special reference to AIDS and Alzheimer's disease. Glia **7**(1): 75-83.

DiPatre PL, Gelman BB. 1997. Microglial cell activation in aging and Alzheimer disease: partial linkage with neurofibrillary tangle burden in the hippocampus. J Neuropathol Exp Neurol **56**(2): 143-9.

Domaradzka-Pytel B, Ludkiewicz B, Morys J, Wisniewski HM. 1999. Expression and distribution of various antigens of developing microglial cells in the rat telencephalon. J Hirnforsch **39**(3): 283-91.

Dong JH, Ying GX, Zhou CF. 2004. Entorhinal deafferentation induces the expression of profilin mRNA in the reactive microglial cells in the hippocampus. Glia **47**(1): 102-8.

Droge W. 2003. Oxidative stress and aging. Adv Exp Med Biol **543**: 191-200.

Fagan AM, Gage FH. 1994. Mechanisms of sprouting in the adult central nervous system: cellular responses in areas of terminal degeneration and reinnervation in the rat hippocampus. Neuroscience **58**(4): 705-25.

Failla G. 1958. The aging process and cancerogenesis. Ann N Y Acad Sci **71**(6): 1124-40.

Farkas E, Donka G, de Vos RA, Mihaly A, Bari F, et al. 2004. Experimental cerebral hypoperfusion induces white matter injury and microglial activation in the rat brain. Acta Neuropathol (Berl) **108**(1): 57-64.

Fedoroff S, Hao C. 1991. Origin of microglia and their regulation by astroglia. Adv Exp Med Biol **296**: 135-42.

Fedoroff S, Zhai R, Novak JP. 1997. Microglia and astroglia have a common progenitor cell. J Neurosci Res **50**(3): 477-86.

Felzien LK, McDonald JT, Gleason SM, Berman NE, Klein RM. 2001. Increased chemokine gene expression during aging in the murine brain. Brain Res **890**(1): 137-46.

Finch CE. 1993. Neuron atrophy during aging: programmed or sporadic? Trends Neurosci **16**(3): 104-10.

Flanary BE, Streit WJ. 2003. Telomeres shorten with age in rat cerebellum and cortex in vivo. J Anti Aging Med **6**(4): 299-308.

Flanary BE, Streit WJ. 2004. Progressive telomere shortening occurs in cultured rat microglia, but not astrocytes. Glia **45**(1): 75-88.

Flugel A, Bradl M, Kreutzberg GW, Graeber MB. 2001. Transformation of donor-derived bone marrow precursors into host microglia during autoimmune CNS inflammation and during the retrograde response to axotomy. J Neurosci Res **66**(1): 74-82.

Flugel A, Labeur MS, Grasbon-Frodl EM, Kreutzberg GW, Graeber MB. 1999. Microglia only weakly present glioma antigen to cytotoxic T cells. Int J Dev Neurosci **17**(5-6): 547-56.

Ford AL, Goodsall AL, Hickey WF, Sedgwick JD. 1995. Normal adult ramified microglia separated from other central nervous system macrophages by flow cytometric

sorting. Phenotypic differences defined and direct ex vivo antigen presentation to myelin basic protein-reactive CD4+ T cells compared. J Immunol **154**(9): 4309-21.

Fotheringham AP, Davies CA, Davies I. 2000. Oedema and glial cell involvement in the aged mouse brain after permanent focal ischaemia. Neuropathol Appl Neurobiol **26**(5): 412-23.

Frackowiak J, Wisniewski HM, Wegiel J, Merz GS, Iqbal K, et al. 1992. Ultrastructure of the microglia that phagocytose amyloid and the microglia that produce beta-amyloid fibrils. Acta Neuropathol (Berl) **84**(3): 225-33.

Frautschy SA, Cole GM, Baird A. 1992. Phagocytosis and deposition of vascular beta-amyloid in rat brains injected with Alzheimer beta-amyloid. Am J Pathol **140**(6): 1389-99.

Frei K, Siepl C, Groscurth P, Bodmer S, Schwerdel C, et al. 1987. Antigen presentation and tumor cytotoxicity by interferon-gamma-treated microglial cells. Eur J Immunol **17**(9): 1271-8.

Games D, Bard F, Grajeda H, Guido T, Khan K, et al. 2000. Prevention and reduction of AD-type pathology in PDAPP mice immunized with A beta 1-42. Ann N Y Acad Sci **920**: 274-84.

Garton KJ, Gough PJ, Blobel CP, Murphy G, Greaves DR, et al. 2001. Tumor necrosis factor-alpha-converting enzyme (ADAM17) mediates the cleavage and shedding of fractalkine (CX3CL1). J Biol Chem **276**(41): 37993-8001.

Gavrieli Y, Sherman Y, Ben-Sasson SA. 1992. Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. J Cell Biol **119**(3): 493-501.

Gehrmann J. 1995. Colony-stimulating factors regulate programmed cell death of rat microglia/brain macrophages in vitro. J Neuroimmunol **63**(1): 55-61.

Gehrmann J, Banati RB. 1995. Microglial turnover in the injured CNS: activated microglia undergo delayed DNA fragmentation following peripheral nerve injury. J Neuropathol Exp Neurol **54**(5): 680-8.

Gehrmann J, Schoen SW, Kreutzberg GW. 1991. Lesion of the rat entorhinal cortex leads to a rapid microglial reaction in the dentate gyrus. A light and electron microscopical study. Acta Neuropathol (Berl) **82**(6): 442-55.

Giulian D, Baker TJ. 1985. Peptides released by ameboid microglia regulate astroglial proliferation. J Cell Biol **101**(6): 2411-5.

Giulian D, Baker TJ, Shih LC, Lachman LB. 1986. Interleukin 1 of the central nervous system is produced by ameboid microglia. J Exp Med **164**(2): 594-604.

Giulian D, Haverkamp LJ, Yu JH, Karshin W, Tom D, et al. 1996. Specific domains of beta-amyloid from Alzheimer plaque elicit neuron killing in human microglia. J Neurosci **16**(19): 6021-37.

Gong Y, Hua Y, Keep RF, Hoff JT, Xi G. 2004. Intracerebral hemorrhage: effects of aging on brain edema and neurological deficits. Stroke **35**(11): 2571-5.

Goodwin JL, Uemura E, Cunnick JE. 1995. Microglial release of nitric oxide by the synergistic action of beta-amyloid and IFN-gamma. Brain Res **692**(1-2): 207-14.

Graeber MB, Moran LB. 2002. Mechanisms of cell death in neurodegenerative diseases: fashion, fiction, and facts. Brain Pathol **12**(3): 385-90.

Graeber MB, Streit WJ, Kreutzberg GW. 1989. Formation of microglia-derived brain macrophages is blocked by adriamycin. Acta Neuropathol (Berl) **78**(4): 348-58.

Graeber MB, Tetzlaff W, Streit WJ, Kreutzberg GW. 1988. Microglial cells but not astrocytes undergo mitosis following rat facial nerve axotomy. Neurosci Lett **85**(3): 317-21.

Griffin WS, Sheng JG, Roberts GW, Mrak RE. 1995. Interleukin-1 expression in different plaque types in Alzheimer's disease: significance in plaque evolution. J Neuropathol Exp Neurol **54**(2): 276-81.

Guarente L. 2000. Sir2 links chromatin silencing, metabolism, and aging. Genes Dev **14**(9): 1021-6.

Guo Z, Cupples LA, Kurz A, Auerbach SH, Volicer L, et al. 2000. Head injury and the risk of AD in the MIRAGE study. Neurology **54**(6): 1316-23.

Haga S, Akai K, Ishii T. 1989. Demonstration of microglial cells in and around senile (neuritic) plaques in the Alzheimer brain. An immunohistochemical study using a novel monoclonal antibody. Acta Neuropathol (Berl) **77**(6): 569-75.

Hailer NP, Heppner FL, Haas D, Nitsch R. 1997. Fluorescent dye prelabelled microglial cells migrate into organotypic hippocampal slice cultures and ramify. Eur J Neurosci **9**(4): 863-6.

Hailer NP, Jarhult JD, Nitsch R. 1996. Resting microglial cells in vitro: analysis of morphology and adhesion molecule expression in organotypic hippocampal slice cultures. Glia **18**(4): 319-31.

Haldane J. 1942. New paths in genetics. New York: Harper Brothers.

Hamanoue M, Takemoto N, Matsumoto K, Nakamura T, Nakajima K, et al. 1996. Neurotrophic effect of hepatocyte growth factor on central nervous system neurons in vitro. J Neurosci Res **43**(5): 554-64.

Hanisch UK. 2002. Microglia as a source and target of cytokines. Glia **40**(2): 140-55.

Hao C, Richardson A, Fedoroff S. 1991. Macrophage-like cells originate from neuroepithelium in culture: characterization and properties of the macrophage-like cells. Int J Dev Neurosci **9**(1): 1-14.

Harley CB. 1991. Telomere loss: mitotic clock or genetic time bomb? Mutat Res **256**(2-6): 271-82.

Harley CB, Futcher AB, Greider CW. 1990. Telomeres shorten during ageing of human fibroblasts. Nature **345**(6274): 458-60.

Harley CB, Vaziri H, Counter CM, Allsopp RC. 1992. The telomere hypothesis of cellular aging. Exp Gerontol **27**(4): 375-82.

Harrison JK, Jiang Y, Chen S, Xia Y, Maciejewski D, et al. 1998. Role for neuronally derived fractalkine in mediating interactions between neurons and CX3CR1-expressing microglia. Proc Natl Acad Sci U S A **95**(18): 10896-901.

Harrison JK, Luo D, Streit WJ. 2003. In situ hybridization analysis of chemokines and chemokine receptors in the central nervous system. Methods **29**(4): 312-8.

Haug H. 1997. The aging human cerebral cortex: Morphometry of areal differences and their functional meaning. In: Dani SU, Hori, A., Walter, G.F. Principles of neuronal aging. Amsterdam: Elsevier**:** 247-261.

Hauss-Wegrzyniak B, Vraniak P, Wenk GL. 1999. The effects of a novel NSAID on chronic neuroinflammation are age dependent. Neurobiol Aging **20**(3): 305-13.

Hetier E, Ayala J, Denefle P, Bousseau A, Rouget P, et al. 1988. Brain macrophages synthesize interleukin-1 and interleukin-1 mRNAs in vitro. J Neurosci Res **21**(2-4): 391-7.

Hickey WF, Kimura H. 1988. Perivascular microglial cells of the CNS are bone marrow-derived and present antigen in vivo. Science **239**(4837): 290-2.

Higgins GA, Jacobsen H. 2003. Transgenic mouse models of Alzheimer's disease: phenotype and application. Behav Pharmacol **14**(5-6): 419-38.

Hinman JD, Duce JA, Siman RA, Hollander W, Abraham CR. 2004. Activation of calpain-1 in myelin and microglia in the white matter of the aged rhesus monkey. J Neurochem **89**(2): 430-41.

Hirota S, Ito A, Morii E, Wanaka A, Tohyama M, et al. 1992. Localization of mRNA for c-kit receptor and its ligand in the brain of adult rats: an analysis using in situ hybridization histochemistry. Brain Res Mol Brain Res **15**(1-2): 47-54.

Hollensworth SB, Shen C, Sim JE, Spitz DR, Wilson GL, et al. 2000. Glial cell type-specific responses to menadione-induced oxidative stress. Free Radic Biol Med **28**(8): 1161-74.

Hu S, Chao CC, Khanna KV, Gekker G, Peterson PK, et al. 1996. Cytokine and free radical production by porcine microglia. Clin Immunol Immunopathol **78**(1): 93-6.

Hu S, Sheng WS, Peterson PK, Chao CC. 1995. Cytokine modulation of murine microglial cell superoxide production. Glia **13**(1): 45-50.

Hukkelhoven CW, Steyerberg EW, Rampen AJ, Farace E, Habbema JD, et al. 2003. Patient age and outcome following severe traumatic brain injury: an analysis of 5600 patients. J Neurosurg **99**(4): 666-73.

Hurley SD, Coleman PD. 2003. Facial nerve axotomy in aged and young adult rats: analysis of the glial response. Neurobiol Aging **24**(3): 511-8.

Hurley SD, Streit WJ. 1996. Microglia and the mononuclear phagocyte system. In: Ling EA TC, Tan CBC. Topical issues of microglial research. Singapore Neuroscience Association.

Imai T, Hieshima K, Haskell C, Baba M, Nagira M, et al. 1997. Identification and molecular characterization of fractalkine receptor CX3CR1, which mediates both leukocyte migration and adhesion. Cell **91**(4): 521-30.

Ishimaru H, Ogawa S, Fuji K, Fukuta T, Kameyama T, et al. 1991. Aged-related changes in learning and memory, choline acetyltransferase activity and number of neuronal cells in rats. J Pharmacobiodyn **14**(6): 321-5.

Jaattela M, Tschopp J. 2003. Caspase-independent cell death in T lymphocytes. Nat Immunol **4**(5): 416-23.

Jensen MB, Finsen B, Zimmer J. 1997. Morphological and immunophenotypic microglial changes in the denervated fascia dentata of adult rats: correlation with blood-brain barrier damage and astroglial reactions. Exp Neurol **143**(1): 103-16.

Jensen MB, Gonzalez B, Castellano B, Zimmer J. 1994. Microglial and astroglial reactions to anterograde axonal degeneration: a histochemical and immunocytochemical study of the adult rat fascia dentata after entorhinal perforant path lesions. Exp Brain Res **98**(2): 245-60.

Jones LL, Banati RB, Graeber MB, Bonfanti L, Raivich G, et al. 1997. Population control of microglia: does apoptosis play a role? J Neurocytol **26**(11): 755-70.

Jung S, Aliberti J, Graemmel P, Sunshine MJ, Kreutzberg GW, et al. 2000. Analysis of fractalkine receptor CX(3)CR1 function by targeted deletion and green fluorescent protein reporter gene insertion. Mol Cell Biol **20**(11): 4106-14.

Kajstura J, Pertoldi B, Leri A, Beltrami CA, Deptala A, et al. 2000. Telomere shortening is an in vivo marker of myocyte replication and aging. Am J Pathol **156**(3): 813-9.

Kalehua AN, Taub DD, Baskar PV, Hengemihle J, Munoz J, et al. 2000. Aged mice exhibit greater mortality concomitant to increased brain and plasma TNF-alpha levels following intracerebroventricular injection of lipopolysaccharide. Gerontology **46**(3): 115-28.

Kalla R, Liu Z, Xu S, Koppius A, Imai Y, et al. 2001. Microglia and the early phase of immune surveillance in the axotomized facial motor nucleus: impaired microglial activation and lymphocyte recruitment but no effect on neuronal survival or axonal regeneration in macrophage-colony stimulating factor-deficient mice. J Comp Neurol **436**(2): 182-201.

Kelly A, Mullany PM, Lynch MA. 2000. Protein synthesis in entorhinal cortex and long-term potentiation in dentate gyrus. Hippocampus **10**(4): 431-7.

Kennedy DW, Abkowitz JL. 1997. Kinetics of central nervous system microglial and macrophage engraftment: analysis using a transgenic bone marrow transplantation model. Blood **90**(3): 986-93.

Kerezoudi E, Thomas PK. 1999. Influence of age on regeneration in the peripheral nervous system. Gerontology **45**(6): 301-6.

Khaldi A, Chiueh CC, Bullock MR, Woodward JJ. 2002. The significance of nitric oxide production in the brain after injury. Ann N Y Acad Sci **962**: 53-9.

Kiefer R, Streit WJ, Toyka KV, Kreutzberg GW, Hartung HP. 1995. Transforming growth factor-beta 1: a lesion-associated cytokine of the nervous system. Int J Dev Neurosci **13**(3-4): 331-9.

Kilaru S, Garb J, Emhoff T, Fiallo V, Simon B, et al. 1996. Long-term functional status and mortality of elderly patients with severe closed head injuries. J Trauma **41**(6): 957-63.

Kim KY, Ju WK, Neufeld AH. 2004. Neuronal susceptibility to damage: comparison of the retinas of young, old and old/caloric restricted rats before and after transient ischemia. Neurobiol Aging **25**(4): 491-500.

Kirkwood TB. 1988. The nature and causes of ageing. Ciba Found Symp **134**: 193-207.

Kishimoto T, Akira S, Narazaki M, Taga T. 1995. Interleukin-6 family of cytokines and gp130. Blood **86**(4): 1243-54.

Kitamura T, Miyake T, Fujita S. 1984. Genesis of resting microglia in the gray matter of mouse hippocampus. J Comp Neurol **226**(3): 421-33.

Klegeris A, McGeer PL. 1997. beta-amyloid protein enhances macrophage production of oxygen free radicals and glutamate. J Neurosci Res **49**(2): 229-35.

Kohn RR, Hamlin CR. 1978. Genetic effects on aging of collagen, with special reference to diabetes mellitus. Birth Defects Orig Artic Ser **14**(1): 387-401.

Krall WJ, Challita PM, Perlmutter LS, Skelton DC, Kohn DB. 1994. Cells expressing human glucocerebrosidase from a retroviral vector repopulate macrophages and central nervous system microglia after murine bone marrow transplantation. Blood **83**(9): 2737-48.

Kreutzberg G. 1966. Autoradiographische Untersuchung uber die Beteiligung von Gliazellen an der axonalen Reaktion im Facialiskern der Ratte. Acta Neuropathol (Berl) **7**: 149-161.

Kreutzberg GW. 1968. [Autoradiographic studies on perineuronal microgliocytes]. Acta Neuropathol (Berl): Suppl 4:141-5.

Kreutzberg GW. 1996. Microglia: a sensor for pathological events in the CNS. Trends Neurosci **19**(8): 312-8.

Kreutzberg GW, Graeber MB, Streit WJ. 1989. Neuron-glial relationship during regeneration of motorneurons. Metab Brain Dis **4**(1): 81-5.

Kreutzberg GW, Graeber, M.B., Raivich, G., Streit, W.J. 1990. Neuron-glial relationship during regeneration of motoneurons. In: Regulation of gene expression in the nervous system. Wiley-Liss**:** 333-341.

Kullberg S, Aldskogius H, Ulfhake B. 2001. Microglial activation, emergence of ED1-expressing cells and clusterin upregulation in the aging rat CNS, with special reference to the spinal cord. Brain Res **899**(1-2): 169-86.

Kyrkanides S, O'Banion MK, Whiteley PE, Daeschner JC, Olschowka JA. 2001. Enhanced glial activation and expression of specific CNS inflammation-related molecules in aged versus young rats following cortical stab injury. J Neuroimmunol **119**(2): 269-77.

Lawson LJ, Perry VH, Dri P, Gordon S. 1990. Heterogeneity in the distribution and morphology of microglia in the normal adult mouse brain. Neuroscience **39**(1): 151-70.

Lawson LJ, Perry VH, Gordon S. 1992. Turnover of resident microglia in the normal adult mouse brain. Neuroscience **48**(2): 405-15.

Ledoux SP, Shen CC, Grishko VI, Fields PA, Gard AL, et al. 1998. Glial cell-specific differences in response to alkylation damage. Glia **24**(3): 304-12.

Lee CK, Weindruch R, Prolla TA. 2000. Gene-expression profile of the ageing brain in mice. Nat Genet **25**(3): 294-7.

Lemke R, Hartig W, Rossner S, Bigl V, Schliebs R. 1998. Interleukin-6 is not expressed in activated microglia and in reactive astrocytes in response to lesion of rat basal forebrain cholinergic system as demonstrated by combined in situ hybridization and immunocytochemistry. J Neurosci Res **51**(2): 223-36.

Lewen A, Matz P, Chan PH. 2000. Free radical pathways in CNS injury. J Neurotrauma **17**(10): 871-90.

Li Y, Liu L, Liu D, Woodward S, Barger SW, et al. 2004. Microglial activation by uptake of fDNA via a scavenger receptor. J Neuroimmunol **147**(1-2): 50-5.

Ling EA, Wong WC. 1993. The origin and nature of ramified and amoeboid microglia: a historical review and current concepts. Glia **7**(1): 9-18.

Linnemann D, Skarsfelt T. 1994. Regional changes in expression of NCAM, GFAP, and S100 in aging rat brain. Neurobiol Aging **15**(5): 651-5.

Liu T, Clark RK, McDonnell PC, Young PR, White RF, et al. 1994. Tumor necrosis factor-alpha expression in ischemic neurons. Stroke **25**(7): 1481-8.

Lockshin RA, Zakeri Z. 2004. Caspase-independent cell death? Oncogene **23**(16): 2766-73.

Long JM, Kalehua AN, Muth NJ, Calhoun ME, Jucker M, et al. 1998. Stereological analysis of astrocyte and microglia in aging mouse hippocampus. Neurobiol Aging **19**(5): 497-503.

Loughlin AJ, Woodroofe MN, Cuzner ML. 1992. Regulation of Fc receptor and major histocompatibility complex antigen expression on isolated rat microglia by tumour necrosis factor, interleukin-1 and lipopolysaccharide: effects on interferon-gamma induced activation. Immunology **75**(1): 170-5.

Lue LF, Rydel R, Brigham EF, Yang LB, Hampel H, et al. 2001. Inflammatory repertoire of Alzheimer's disease and nondemented elderly microglia in vitro. Glia **35**(1): 72-9.

Lye TC, Shores EA. 2000. Traumatic brain injury as a risk factor for Alzheimer's disease: a review. Neuropsychol Rev **10**(2): 115-29.

Ma L, Morton AJ, Nicholson LF. 2003. Microglia density decreases with age in a mouse model of Huntington's disease. Glia **43**(3): 274-80.

Manova K, Bachvarova RF, Huang EJ, Sanchez S, Pronovost SM, et al. 1992. c-kit receptor and ligand expression in postnatal development of the mouse cerebellum suggests a function for c-kit in inhibitory interneurons. J Neurosci **12**(12): 4663-76.

Martin GM. 2002. Gene action in the aging brain: an evolutionary biological perspective. Neurobiol Aging **23**(5): 647-54.

Marz P, Cheng JG, Gadient RA, Patterson PH, Stoyan T, et al. 1998. Sympathetic neurons can produce and respond to interleukin 6. Proc Natl Acad Sci U S A **95**(6): 3251-6.

Marzolo MP, von Bernhardi R, Inestrosa NC. 1999. Mannose receptor is present in a functional state in rat microglial cells. J Neurosci Res **58**(3): 387-95.

Massague J. 1996. TGFbeta signaling: receptors, transducers, and Mad proteins. Cell **85**(7): 947-50.

Mattson MP, Duan W, Chan SL, Cheng A, Haughey N, et al. 2002. Neuroprotective and neurorestorative signal transduction mechanisms in brain aging: modification by genes, diet and behavior. Neurobiol Aging **23**(5): 695-705.

McDonald DR, Brunden KR, Landreth GE. 1997. Amyloid fibrils activate tyrosine kinase-dependent signaling and superoxide production in microglia. J Neurosci **17**(7): 2284-94.

McGeer PL, McGeer EG, Suzuki JS. 1977. Aging and extrapyramidal function. Arch Neurol **34**(1): 33-5.

McMahon EJ, Suzuki K, Matsushima GK. 2002. Peripheral macrophage recruitment in cuprizone-induced CNS demyelination despite an intact blood-brain barrier. J Neuroimmunol **130**(1-2): 32-45.

Medawar P. 1957a. Old-age, and natural death; uniqueness of the individual. London: Methuen Co. Ltd.

Medawar P. 1957b. An unsolved problem of biology. In: Medawar P. The uniqueness of the individual. London: Methuen Co. Ltd.**:** 44-70.

Minami M, Kuraishi Y, Satoh M. 1991. Effects of kainic acid on messenger RNA levels of IL-1 beta, IL-6, TNF alpha and LIF in the rat brain. Biochem Biophys Res Commun **176**(2): 593-8.

Miyake T, Tsuchihashi Y, Kitamura T, Fujita S. 1984. Immunohistochemical studies of blood monocytes infiltrating into the neonatal rat brain. Acta Neuropathol (Berl) **62**(4): 291-7.

Miyazaki I, Asanuma M, Higashi Y, Sogawa CA, Tanaka K, et al. 2002. Age-related changes in expression of metallothionein-III in rat brain. Neurosci Res **43**(4): 323-33.

Mooradian AD. 1988. Effect of aging on the blood-brain barrier. Neurobiol Aging **9**(1): 31-9.

Mooradian AD. 1994. Potential mechanisms of the age-related changes in the blood-brain barrier. Neurobiol Aging **15**(6): 751-5; discussion 761-2, 767.

Mooradian AD, McCuskey RS. 1992. In vivo microscopic studies of age-related changes in the structure and the reactivity of cerebral microvessels. Mech Ageing Dev **64**(3): 247-54.

Moran LB, Graeber MB. 2004. The facial nerve axotomy model. Brain Res Brain Res Rev **44**(2-3): 154-78.

Morgan SC, Taylor DL, Pocock JM. 2004. Microglia release activators of neuronal proliferation mediated by activation of mitogen-activated protein kinase, phosphatidylinositol-3-kinase/Akt and delta-Notch signalling cascades. J Neurochem **90**(1): 89-101.

Morgan TE, Xie Z, Goldsmith S, Yoshida T, Lanzrein AS, et al. 1999. The mosaic of brain glial hyperactivity during normal ageing and its attenuation by food restriction. Neuroscience **89**(3): 687-99.

Mori M, Aihara M, Kume K, Hamanoue M, Kohsaka S, et al. 1996. Predominant expression of platelet-activating factor receptor in the rat brain microglia. J Neurosci **16**(11): 3590-600.

Mosenthal AC, Livingston DH, Lavery RF, Knudson MM, Lee S, et al. 2004. The effect of age on functional outcome in mild traumatic brain injury: 6-month report of a prospective multicenter trial. J Trauma **56**(5): 1042-8.

Mosley K, Cuzner ML. 1996. Receptor-mediated phagocytosis of myelin by macrophages and microglia: effect of opsonization and receptor blocking agents. Neurochem Res **21**(4): 481-7.

Motro B, van der Kooy D, Rossant J, Reith A, Bernstein A. 1991. Contiguous patterns of c-kit and steel expression: analysis of mutations at the W and Sl loci. Development **113**(4): 1207-21.

Murphy PG, Grondin J, Altares M, Richardson PM. 1995. Induction of interleukin-6 in axotomized sensory neurons. J Neurosci **15**(7 Pt 2): 5130-8.

Nagata K, Takei N, Nakajima K, Saito H, Kohsaka S. 1993. Microglial conditioned medium promotes survival and development of cultured mesencephalic neurons from embryonic rat brain. J Neurosci Res **34**(3): 357-63.

Nagy ZA, Mooney NA. 2003. A novel, alternative pathway of apoptosis triggered through class II major histocompatibility complex molecules. J Mol Med **81**(12): 757-65.

Nakajima K, Honda S, Tohyama Y, Imai Y, Kohsaka S, et al. 2001a. Neurotrophin secretion from cultured microglia. J Neurosci Res **65**(4): 322-31.

Nakajima K, Tohyama Y, Kohsaka S, Kurihara T. 2001b. Ability of rat microglia to uptake extracellular glutamate. Neurosci Lett **307**(3): 171-4.

Nakanishi H. 2003. Neuronal and microglial cathepsins in aging and age-related diseases. Ageing Res Rev **2**(4): 367-81.

Navascues J, Calvente R, Marin-Teva JL, Cuadros MA. 2000. Entry, dispersion and differentiation of microglia in the developing central nervous system. An Acad Bras Cienc **72**(1): 91-102.

Neumann H, Misgeld T, Matsumuro K, Wekerle H. 1998. Neurotrophins inhibit major histocompatibility class II inducibility of microglia: involvement of the p75 neurotrophin receptor. Proc Natl Acad Sci U S A **95**(10): 5779-84.

Nguyen MD, Julien JP, Rivest S. 2002. Innate immunity: the missing link in neuroprotection and neurodegeneration? Nat Rev Neurosci **3**(3): 216-27.

Nichols NR. 1999. Glial responses to steroids as markers of brain aging. J Neurobiol **40**(4): 585-601.

Nishiyori A, Minami M, Ohtani Y, Takami S, Yamamoto J, et al. 1998. Localization of fractalkine and CX3CR1 mRNAs in rat brain: does fractalkine play a role in signaling from neuron to microglia? FEBS Lett **429**(2): 167-72.

Oehmichen M, Wietholter H, Greaves MF. 1979. Immunological analysis of human microglia: lack of monocytic and lymphoid membrane differentiation antigens. J Neuropathol Exp Neurol **38**(2): 99-103.

Ogura K, Ogawa M, Yoshida M. 1994. Effects of ageing on microglia in the normal rat brain: immunohistochemical observations. Neuroreport **5**(10): 1224-6.

Peinado MA, Quesada A, Pedrosa JA, Torres MI, Martinez M, et al. 1998. Quantitative and ultrastructural changes in glia and pericytes in the parietal cortex of the aging rat. Microsc Res Tech **43**(1): 34-42.

Perls T. 2004. Dementia-free centenarians. Exp Gerontol **39**(11-12): 1587-93.

Perry VH, Gordon S. 1991. Macrophages and the nervous system. Int Rev Cytol **125**: 203-44.

Perry VH, Matyszak MK, Fearn S. 1993. Altered antigen expression of microglia in the aged rodent CNS. Glia **7**(1): 60-7.

Peters A. 1996. Age-related changes in oligodendrocytes in monkey cerebral cortex. J Comp Neurol **371**(1): 153-63.

Peters A. 2002. Structural changes in the normally aging cerebral cortex of primates. Prog Brain Res **136**: 455-65.

Peters A, Josephson K, Vincent SL. 1991. Effects of aging on the neuroglial cells and pericytes within area 17 of the rhesus monkey cerebral cortex. Anat Rec **229**(3): 384-98.

Peters A, Rosene DL, Moss MB, Kemper TL, Abraham CR, et al. 1996. Neurobiological bases of age-related cognitive decline in the rhesus monkey. J Neuropathol Exp Neurol **55**(8): 861-74.

Peters A, Sethares C. 2002. The effects of age on the cells in layer 1 of primate cerebral cortex. Cereb Cortex **12**(1): 27-36.

Peters A, Sethares C, Moss MB. 1998. The effects of aging on layer 1 in area 46 of prefrontal cortex in the rhesus monkey. Cereb Cortex **8**(8): 671-84.

Peterson PK, Gekker G, Hu S, Sheng WS, Anderson WR, et al. 1995. CD14 receptor-mediated uptake of nonopsonized Mycobacterium tuberculosis by human microglia. Infect Immun **63**(4): 1598-602.

Plassman BL, Havlik RJ, Steffens DC, Helms MJ, Newman TN, et al. 2000. Documented head injury in early adulthood and risk of Alzheimer's disease and other dementias. Neurology **55**(8): 1158-66.

Polazzi E, Gianni T, Contestabile A. 2001. Microglial cells protect cerebellar granule neurons from apoptosis: evidence for reciprocal signaling. Glia **36**(3): 271-80.

Prewitt CM, Niesman IR, Kane CJ, Houle JD. 1997. Activated macrophage/microglial cells can promote the regeneration of sensory axons into the injured spinal cord. Exp Neurol **148**(2): 433-43.

Priller J, Flugel A, Wehner T, Boentert M, Haas CA, et al. 2001. Targeting gene-modified hematopoietic cells to the central nervous system: use of green fluorescent protein uncovers microglial engraftment. Nat Med **7**(12): 1356-61.

Rabchevsky AG, Streit WJ. 1997. Grafting of cultured microglial cells into the lesioned spinal cord of adult rats enhances neurite outgrowth. J Neurosci Res **47**(1): 34-48.

Raivich G, Gehrmann J, Graeber MB, Kreutzberg GW. 1993. Quantitative immunohistochemistry in the rat facial nucleus with [125I]-iodinated secondary antibodies and in situ autoradiography: non-linear binding characteristics of primary monoclonal and polyclonal antibodies. J Histochem Cytochem **41**(4): 579-92.

Rattan SI. 1996. Synthesis, modifications, and turnover of proteins during aging. Exp Gerontol **31**(1-2): 33-47.

Reaper PM, di Fagagna F, Jackson SP. 2004. Activation of the DNA damage response by telomere attrition: a passage to cellular senescence. Cell Cycle **3**(5): 543-6.

Rezaie P, Trillo-Pazos G, Greenwood J, Everall IP, Male DK. 2002. Motility and ramification of human fetal microglia in culture: an investigation using time-lapse video microscopy and image analysis. Exp Cell Res **274**(1): 68-82.

Rock RB, Gekker G, Hu S, Sheng WS, Cheeran M, et al. 2004. Role of microglia in central nervous system infections. Clin Microbiol Rev **17**(4): 942-64, table of contents.

Rogers J, Luber-Narod J, Styren SD, Civin WH. 1988. Expression of immune system-associated antigens by cells of the human central nervous system: relationship to the pathology of Alzheimer's disease. Neurobiol Aging **9**(4): 339-49.

Rogers J, Strohmeyer R, Kovelowski CJ, Li R. 2002. Microglia and inflammatory mechanisms in the clearance of amyloid beta peptide. Glia **40**(2): 260-9.

Rozovsky I, Finch CE, Morgan TE. 1998. Age-related activation of microglia and astrocytes: in vitro studies show persistent phenotypes of aging, increased proliferation, and resistance to down-regulation. Neurobiol Aging **19**(1): 97-103.

Sandell JH, Peters A. 2002. Effects of age on the glial cells in the rhesus monkey optic nerve. J Comp Neurol **445**(1): 13-28.

Schauwecker PE, Cheng HW, Serquinia RM, Mori N, McNeill TH. 1995. Lesion-induced sprouting of commissural/associational axons and induction of GAP-43 mRNA

in hilar and CA3 pyramidal neurons in the hippocampus are diminished in aged rats. J Neurosci **15**(3 Pt 2): 2462-70.

Scheff SW, Benardo LS, Cotman CW. 1980. Decline in reactive fiber growth in the dentate gyrus of aged rats compared to young adult rats following entorhinal cortex removal. Brain Res **199**(1): 21-38.

Schelper RL, Adrian EK, Jr. 1986. Monocytes become macrophages; they do not become microglia: a light and electron microscopic autoradiographic study using 125-iododeoxyuridine. J Neuropathol Exp Neurol **45**(1): 1-19.

Schenk D, Barbour R, Dunn W, Gordon G, Grajeda H, et al. 1999. Immunization with amyloid-beta attenuates Alzheimer-disease-like pathology in the PDAPP mouse. Nature **400**(6740): 173-7.

Schilling M, Besselmann M, Leonhard C, Mueller M, Ringelstein EB, et al. 2003. Microglial activation precedes and predominates over macrophage infiltration in transient focal cerebral ischemia: a study in green fluorescent protein transgenic bone marrow chimeric mice. Exp Neurol **183**(1): 25-33.

Shah GN, Mooradian AD. 1997. Age-related changes in the blood-brain barrier. Exp Gerontol **32**(4-5): 501-19.

Sheffield LG, Berman NE. 1998. Microglial expression of MHC class II increases in normal aging of nonhuman primates. Neurobiol Aging **19**(1): 47-55.

Sheng JG, Mrak RE, Griffin WS. 1998. Enlarged and phagocytic, but not primed, interleukin-1 alpha-immunoreactive microglia increase with age in normal human brain. Acta Neuropathol (Berl) **95**(3): 229-34.

Shigenaga MK, Hagen TM, Ames BN. 1994. Oxidative damage and mitochondrial decay in aging. Proc Natl Acad Sci U S A **91**(23): 10771-8.

Shimojo M, Nakajima K, Takei N, Hamanoue M, Kohsaka S. 1991. Production of basic fibroblast growth factor in cultured rat brain microglia. Neurosci Lett **123**(2): 229-31.

Simard AR, Rivest S. 2004. Bone marrow stem cells have the ability to populate the entire central nervous system into fully differentiated parenchymal microglia. Faseb J **18**(9): 998-1000.

Sloane JA, Hinman JD, Lubonia M, Hollander W, Abraham CR. 2003. Age-dependent myelin degeneration and proteolysis of oligodendrocyte proteins is associated with the activation of calpain-1 in the rhesus monkey. J Neurochem **84**(1): 157-68.

Sloane JA, Hollander W, Moss MB, Rosene DL, Abraham CR. 1999. Increased microglial activation and protein nitration in white matter of the aging monkey. Neurobiol Aging **20**(4): 395-405.

Sloane JA, Hollander W, Rosene DL, Moss MB, Kemper T, et al. 2000. Astrocytic hypertrophy and altered GFAP degradation with age in subcortical white matter of the rhesus monkey. Brain Res **862**(1-2): 1-10.

Smith ME. 1993. Phagocytosis of myelin by microglia in vitro. J Neurosci Res **35**(5): 480-7.

Streit WJ. 1995. Microglial Cells. In: Kettenmann HaR, BR. Neuroglia. Oxford University Press.

Streit WJ. 2001. Microglia and macrophages in the developing CNS. Neurotoxicology **22**(5): 619-24.

Streit WJ. 2002. Microglia as neuroprotective, immunocompetent cells of the CNS. Glia **40**(2): 133-9.

Streit WJ. 2004. Microglia and Alzheimer's disease pathogenesis. J Neurosci Res **77**(1): 1-8.

Streit WJ, Conde JR, Harrison JK. 2001. Chemokines and Alzheimer's disease. Neurobiol Aging **22**(6): 909-13.

Streit WJ, Graeber MB, Kreutzberg GW. 1988. Functional plasticity of microglia: a review. Glia **1**(5): 301-7.

Streit WJ, Hurley SD, McGraw TS, Semple-Rowland SL. 2000. Comparative evaluation of cytokine profiles and reactive gliosis supports a critical role for interleukin-6 in neuron-glia signaling during regeneration. J Neurosci Res **61**(1): 10-20.

Streit WJ, Kreutzberg GW. 1987. Lectin binding by resting and reactive microglia. J Neurocytol **16**(2): 249-60.

Streit WJ, Sammons NW, Kuhns AJ, Sparks DL. 2004. Dystrophic microglia in the aging human brain. Glia **45**(2): 208-12.

Streit WJ, Schulte BA, Balentine DJ, Spicer SS. 1985. Histochemical localization of galactose-containing glycoconjugates in sensory neurons and their processes in the central and peripheral nervous system of the rat. J Histochem Cytochem **33**(10): 1042-52.

Streit WJ, Sparks DL. 1997. Activation of microglia in the brains of humans with heart disease and hypercholesterolemic rabbits. J Mol Med **75**(2): 130-8.

Streit WJ, Walter SA, Pennell NA. 1999. Reactive microgliosis. Prog Neurobiol **57**(6): 563-81.

Streppel M, Angelov DN, Guntinas-Lichius O, Hilgers RD, Rosenblatt JD, et al. 1998. Slow axonal regrowth but extreme hyperinnervation of target muscle after suture of the facial nerve in aged rats. Neurobiol Aging **19**(1): 83-8.

Stuesse SL, Cruce WL, Lovell JA, McBurney DL, Crisp T. 2000. Microglial proliferation in the spinal cord of aged rats with a sciatic nerve injury. Neurosci Lett **287**(2): 121-4.

Sugama S, Yang L, Cho BP, DeGiorgio LA, Lorenzl S, et al. 2003. Age-related microglial activation in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced dopaminergic neurodegeneration in C57BL/6 mice. Brain Res **964**(2): 288-94.

Susman M, DiRusso SM, Sullivan T, Risucci D, Nealon P, et al. 2002. Traumatic brain injury in the elderly: increased mortality and worse functional outcome at discharge despite lower injury severity. J Trauma **53**(2): 219-23; discussion 223-4.

Suzuki Y, Onodera H, Nagata T, Yoshie O, Itoyama Y. 2000. Enhanced expression of chemokines and their receptors in the rat hippocampus after kainic-acid induced seizure. Soc Neurosci Abstr (26): 1945.

Suzumura A, Sawada M, Yamamoto H, Marunouchi T. 1990. Effects of colony stimulating factors on isolated microglia in vitro. J Neuroimmunol **30**(2-3): 111-20.

Suzumura A, Sawada M, Yamamoto H, Marunouchi T. 1993. Transforming growth factor-beta suppresses activation and proliferation of microglia in vitro. J Immunol **151**(4): 2150-8.

Svensson M, Aldskogius H. 1993a. Regeneration of hypoglossal nerve axons following blockade of the axotomy-induced microglial cell reaction in the rat. Eur J Neurosci **5**(1): 85-94.

Svensson M, Aldskogius H. 1993b. Synaptic density of axotomized hypoglossal motorneurons following pharmacological blockade of the microglial cell proliferation. Exp Neurol **120**(1): 123-31.

Svensson M, Mattsson P, Aldskogius H. 1994. A bromodeoxyuridine labelling study of proliferating cells in the brainstem following hypoglossal nerve transection. J Anat **185 (Pt 3)**: 537-42.

Szczepanik AM, Funes S, Petko W, Ringheim GE. 2001. IL-4, IL-10 and IL-13 modulate A beta(1--42)-induced cytokine and chemokine production in primary murine microglia and a human monocyte cell line. J Neuroimmunol **113**(1): 49-62.

Tanaka K, Webster HD. 1991. Myelinated fiber regeneration after crush injury is retarded in sciatic nerves of aging mice. J Comp Neurol **308**(2): 180-7.

Tang W, Lai YH, Han XD, Wong PM, Peters LL, et al. 1997a. Murine Hn1 on chromosome 11 is expressed in hemopoietic and brain tissues. Mamm Genome **8**(9): 695-6.

Tang Y, Nyengaard JR, Pakkenberg B, Gundersen HJ. 1997b. Age-induced white matter changes in the human brain: a stereological investigation. Neurobiol Aging **18**(6): 609-15.

Tarozzo G, Campanella M, Ghiani M, Bulfone A, Beltramo M. 2002. Expression of fractalkine and its receptor, CX3CR1, in response to ischaemia-reperfusion brain injury in the rat. Eur J Neurosci **15**(10): 1663-8.

Taupin V, Toulmond S, Serrano A, Benavides J, Zavala F. 1993. Increase in IL-6, IL-1 and TNF levels in rat brain following traumatic lesion. Influence of pre- and post-traumatic treatment with Ro5 4864, a peripheral-type (p site) benzodiazepine ligand. J Neuroimmunol **42**(2): 177-85.

Tchelingerian JL, Quinonero J, Booss J, Jacque C. 1993. Localization of TNF alpha and IL-1 alpha immunoreactivities in striatal neurons after surgical injury to the hippocampus. Neuron **10**(2): 213-24.

Thomander L. 1984. Reorganization of the facial motor nucleus after peripheral nerve regeneration. An HRP study in the rat. Acta Otolaryngol **97**(5-6): 619-26.

Troen BR. 2003. The biology of aging. Mt Sinai J Med **70**(1): 3-22.

Tseng GF, Wang YJ, Lai QC. 1996. Perineuronal microglial reactivity following proximal and distal axotomy of rat rubrospinal neurons. Brain Res **715**(1-2): 32-43.

Tsou CL, Haskell CA, Charo IF. 2001. Tumor necrosis factor-alpha-converting enzyme mediates the inducible cleavage of fractalkine. J Biol Chem **276**(48): 44622-6.

Vallieres L, Sawchenko PE. 2003. Bone marrow-derived cells that populate the adult mouse brain preserve their hematopoietic identity. J Neurosci **23**(12): 5197-207.

Vaughan DW. 1990. Effects of advancing age on the central response of rat facial neurons to axotomy: light microscope morphometry. Anat Rec **228**(2): 211-9.

Vaughan DW. 1992. Effects of advancing age on peripheral nerve regeneration. J Comp Neurol **323**(2): 219-37.

Vaughan DW, Peters A. 1974. Neuroglial cells in the cerebral cortex of rats from young adulthood to old age: an electron microscope study. J Neurocytol **3**(4): 405-29.

Verderio C, Matteoli M. 2001. ATP mediates calcium signaling between astrocytes and microglial cells: modulation by IFN-gamma. J Immunol **166**(10): 6383-91.

Von Zglinicki T. 2003. Replicative senescence and the art of counting. Exp Gerontol **38**(11-12): 1259-64.

von Zglinicki T, Serra V, Lorenz M, Saretzki G, Lenzen-Grossimlighaus R, et al. 2000. Short telomeres in patients with vascular dementia: an indicator of low antioxidative capacity and a possible risk factor? Lab Invest **80**(11): 1739-47.

Warner HR. 1997. Aging and regulation of apoptosis. Curr Top Cell Regul **35**: 107-21.

Watanabe H, Abe H, Takeuchi S, Tanaka R. 2000. Protective effect of microglial conditioning medium on neuronal damage induced by glutamate. Neurosci Lett **289**(1): 53-6.

Weldon DT, Rogers SD, Ghilardi JR, Finke MP, Cleary JP, et al. 1998. Fibrillar beta-amyloid induces microglial phagocytosis, expression of inducible nitric oxide synthase, and loss of a select population of neurons in the rat CNS in vivo. J Neurosci **18**(6): 2161-73.

Williams G. 1957. Pleiotrophy, natural selection, and the evolution of senescence. Evolution **11**: 398-411.

Williams K, Ulvestad E, Waage A, Antel JP, McLaurin J. 1994. Activation of adult human derived microglia by myelin phagocytosis in vitro. J Neurosci Res **38**(4): 433-43.

Wright WE, Shay JW. 1992. Telomere positional effects and the regulation of cellular senescence. Trends Genet **8**(6): 193-7.

Wyss-Coray T, Feng L, Masliah E, Ruppe MD, Lee HS, et al. 1995. Increased central nervous system production of extracellular matrix components and development of hydrocephalus in transgenic mice overexpressing transforming growth factor-beta 1. Am J Pathol **147**(1): 53-67.

Wyss-Coray T, Mucke L. 2002. Inflammation in neurodegenerative disease--a double-edged sword. Neuron **35**(3): 419-32.

Xia MQ, Qin SX, Wu LJ, Mackay CR, Hyman BT. 1998. Immunohistochemical study of the beta-chemokine receptors CCR3 and CCR5 and their ligands in normal and Alzheimer's disease brains. Am J Pathol **153**(1): 31-7.

Xie Z, Morgan TE, Rozovsky I, Finch CE. 2003. Aging and glial responses to lipopolysaccharide in vitro: greater induction of IL-1 and IL-6, but smaller induction of neurotoxicity. Exp Neurol **182**(1): 135-41.

Ye SM, Johnson RW. 2001. An age-related decline in interleukin-10 may contribute to the increased expression of interleukin-6 in brain of aged mice. Neuroimmunomodulation **9**(4): 183-92.

Zhang SC, Fedoroff S. 1996. Neuron-microglia interactions in vitro. Acta Neuropathol (Berl) **91**(4): 385-95.

Zhang SC, Fedoroff S. 1997. Cellular localization of stem cell factor and c-kit receptor in the mouse nervous system. J Neurosci Res **47**(1): 1-15.

Zhang SC, Fedoroff S. 1998. Modulation of microglia by stem cell factor. J Neurosci Res **53**(1): 29-37.

Zhang SC, Fedoroff S. 1999. Expression of stem cell factor and c-kit receptor in neural cells after brain injury. Acta Neuropathol (Berl) **97**(4): 393-8.

Zietlow R, Dunnett SB, Fawcett JW. 1999. The effect of microglia on embryonic dopaminergic neuronal survival in vitro: diffusible signals from neurons and glia change microglia from neurotoxic to neuroprotective. Eur J Neurosci **11**(5): 1657-67.

Zujovic V, Benavides J, Vige X, Carter C, Taupin V. 2000. Fractalkine modulates TNF-alpha secretion and neurotoxicity induced by microglial activation. Glia **29**(4): 305-15.

Zujovic V, Schussler N, Jourdain D, Duverger D, Taupin V. 2001. In vivo neutralization of endogenous brain fractalkine increases hippocampal TNFalpha and 8-isoprostane production induced by intracerebroventricular injection of LPS. J Neuroimmunol **115**(1-2): 135-43.

BIOGRAPHICAL SKETCH

Jessica Renee Locke was born in Orlando, Florida in 1974, but grew up in Miami, Florida. She graduated from G. Holmes Braddock Senior High School with honors in 1992. Her original plans to attend college in Miami were serendipitously changed with the arrival of her beautiful daughter Haley in the summer of 1992. That same summer Jessica married Haley's father, her high school sweetheart Dan Conde, and they have been married ever since. With a family to support, Jessica decided to enter nursing school, graduating and obtaining licensure as a Licensed Practical Nurse in 1994.

Even as she began her career as a nurse, Jessica knew that she wanted to further her education. In the spring of 1995, Jessica easily convinced Dan to move to Gainesville so that they could both attend school at the University of Florida. During her undergraduate tenure, Jessica began her science career by working in the plant biochemistry laboratory of Dr. Robert Schmidt. At the same time, Jessica worked full time as a nurse, first with geriatric patients in a sub-acute rehabilitative center, and then in the homes of children with neurological disorders and injuries. It soon became clear to her that she wanted to continue research at the graduate level, but in a field that might provide the most benefit to her patients: neuroscience.

After graduating with a B.S. in microbiology and cell science, with honors, in the spring of 1999, Jessica entered the University of Florida College of Medicine's Interdisciplinary Program in Biomedical Sciences that Fall. Inspired by her patients, her initial intent was to pursue spinal cord injury research. She learned that one research

focus of Dr. Wolfgang “Jake” Streit’s laboratory was spinal cord injury, and given her secondary interest in immunology she was especially intrigued and asked to do a rotation in the Streit laboratory. It did not take much effort for her to be lured in by the beauty of the microglial cell, and she officially joined the Streit lab in May of 2000. Jessica initially planned a project examining communication between neurons and microglia in spinal cord injury, but lack of funding led her to change her plans. As with all unexpected changes in her life, the switch to a project involving microglial activation in the aging brain turned out to be serendipitous.

During her graduate career, Jessica and Dan welcomed their second daughter, Athena. Being in the medical environment during prenatal and pediatric visits made her realize how much she misses the health care field, and she has now decided to enter the Nurse Practitioner program at UF upon completion of her dissertation. Ultimately, Jessica hopes to combine a clinical career with research.