ROLE OF MICROGLIA DURING MOTONEURON REGENERATION AND DEGENERATION: RELEVANCE FOR PATHOGENESIS AND TREATMENT OF AMYOTROPHIC LATERAL SCLEROSIS

By

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This dissertation is dedicated to my family and friends for their support and encouragement throughout graduate school.

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Recently, microglial activation has been identified as a contributing factor in a number of neurodegenerative diseases and been targeted for therapeutic treatment. However, this view fails to consider the neuroprotective role of microglia observed in injury models where microglial activation accompanies neuronal regeneration. The main goal of this study was to investigate the role of microglia activation in an injury model as well as the transgenic SOD1 rat model and characterize the accompanying neuronal responses. In order to determine if microglial activation in the facial nerve paradigm is beneficial, minocycline, a drug shown to inhibit microglial activation, was utilized allowing neuronal regeneration to be assessed in the absence of microglial activation. Microglial activation was assessed in the SOD1 transgenic rat model to characterize the role of microglial activation in a neurodegenerative disease.

In this study we investigated the effect of minocycline specifically on microglial mitotic activity and neuronal regeneration within the facial nucleus following a nerve

crush injury. No significant difference was found between minocycline treated and control rats when comparing the ^{3}H-thymidine labeled microglial cells or fluorogold labeled neurons at all post-injury time points investigated.

To assess microglial activation in the ALS rat model, microglial morphology and activation were assessed in various brain regions at three stages of the disease, asymptomatic, onset of symptoms and end stage. Microglia were found to have a normal resting morphology in the motor cortex at all time points assessed. In the ventral spinal cord and brainstem there were signs of intense microglial activation. In addition, microglia fusions and multinucleated giant cells were seen dispersed throughout the brainstem and ventral horn of the lumbar spinal cord. To further assess microglial response and function in the transgenic model a facial nerve axotomy was performed and apoptotic microglial were quantified. Transgenic animals were found to have significantly reduced numbers of apoptotic microglial when compared to the age-matched controls 14 days post axotomy. The findings in the current study suggest that microglia may undergo both functional and morphological changes as a result of mutant SOD1 contributing to the disease.

CHAPTER 1
INTRODUCTION AND LITERATURE REVIEW

Microglia: An Overview

Introduction

Microglia, the resident central nervous system (CNS) macrophage, represent about 10% of the adult brain cell population (Lawson et al., 1990). Historically, microglia research focused on the ontogeny of microglia with two conflicting hypotheses. One hypothesis states microglia precursors are cells of neuroectodermal origin (Kitamura et al., 1984; Fedoroff and Hao, 1991; Hao et al., 1991; Fedoroff et al., 1997); the other proposes they proceed from mesodermal cells and originate outside of the developing CNS (Perry and Gordon, 1991; Ling and Wong, 1993; Cuadros and Navascues, 1998). The latter view is currently the most accepted by those in the field who believe that microglia derive either from monocytes that leave the blood stream and populate the brain parenchyma or from primitive hemopoietic cells that differentiate as microglial cells within the CNS. The presence of primitive microglia in the developing CNS can be detected prior to the appearance of monocytes causing speculation on the theory that microglia precursors are monocytes (Hurley and Streit, 1996; Alliot et al., 1999). An alternative precursor for microglia is primitive hemopoietic cells, also called fetal macrophages (Streit, 2001). Fetal macrophages form in the blood islands of the yolk sac, enter the CNS via the meninges by transversing the pial surface (Navascues et al., 2000) and expand until assuming the fully differentiated, ramified adult microglial cells.

In the healthy adult CNS, microglia constitute a stable cell population, which maintains itself by proliferation of resident microglia or recruitment of bone-derived cells (Barron, 1995; Kreutzberg, 1996; Simard and Rivest, 2004; Bechmann et al., 2005). Resident microglia in the healthy brain are termed "resting" however this is far from their actual dynamic and motile functions performed. Resident microglia undergo constant structural changes allowing microglia to effectively survey and maintain the CNS environment by sampling the environment with highly motile protrusions (Nimmerjahn et al., 2005). Upon injury, microglia undergo morphological and phenotypic changes specifically cells undergo hypertrophy, proliferate and up regulate surface antigens and various cytokines to transform into an activated state (Graeber et al., 1988a; Streit et al., 1989b, 1989a).

Microglia Are Neurosupportive

Many studies have demonstrated the ability of microglia to produce growth factors (transforming growth factor ß1 (TGF-ß1), IL-ß, and nerve growth factor) (Giulian et al., 1986; Kreutzberg, 1996; Nakajima et al., 2001) that aid in neuronal survival during development and following injury. Further evidence for a neurosupportive role of microglia is seen *in vitro* studies in which cultured neocortical and mesencephalic neurons show enhanced survival and neurite outgrowth following treatment with conditioned microglial medium (Nagata et al., 1993). *In vivo*, microglial activation in the facial nerve paradigm accompanies neuronal regeneration following injury whereas a central axotomy, such as a transaction of the rubrospinal tract in the cervical spinal cord, does not result in regeneration and elicits only a minimal microglial response (Barron et al., 1990; Tseng et al., 1996; Streit et al., 2000). These *in vivo* observations clearly support that microglial activation is required to facilitate regeneration. More direct

evidence is seen in transplantation studies that show cultured microglial cells engrafted into the injured spinal cord promote neurite outgrowth (Rabchevsky and Streit, 1997).

Another function of microglia is to maintain the environment in the CNS by removing cellular debris and dysfunctional cells. In the presence of degenerating neurons microglia transform into phagocytic cells that remove damaged cells eliminating the potential for toxic products to be released into the CNS environment.

Facial Nerve Axotomy

The facial nucleus is the largest brainstem motor nucleus in the rat with approximately 3000-5000 motoneurons innervating muscles controlling facial movement, including whisker movement. The motoneurons are organized into several muscle and region specific nuclei located in the ipsilateral brainstem.

Axotomy of the facial nerve is a well-established paradigm for the study of microglial activation in response to injury. Peripheral injury of cranial nerve VII generates a response in the CNS while maintaining the integrity of the blood brain barrier restricting leukocyte infiltration. In addition, this model has proven useful in microglia research because the injury is reproducible and can be done in the absence of direct brain manipulation. The microglial response is well-documented following facial nerve injury. Early activation is seen within 24 hours of injury and is characterized by an increase in molecules with an immune function as well as up regulation of OX-42 immunoreactivity (Graeber et al., 1988a; Kreutzberg et al., 1989). The next stage of microglial activation occurs 2-4 days post injury when microglia proliferate and begin to home and adhere to the axotomized motoneurons allowing microglial processes to strip away afferent axon terminals (Blinzinger and Kreutzberg, 1968). Following nerve reinnervation which occurs 2 weeks post injury in the rat, microglia decline in number and return to a resting

state. It has been proposed that microglia undergo cell death following recovery of axotomized neurons, possibly through apoptosis (Gehrmann and Banati, 1995; Jones et al., 1997).

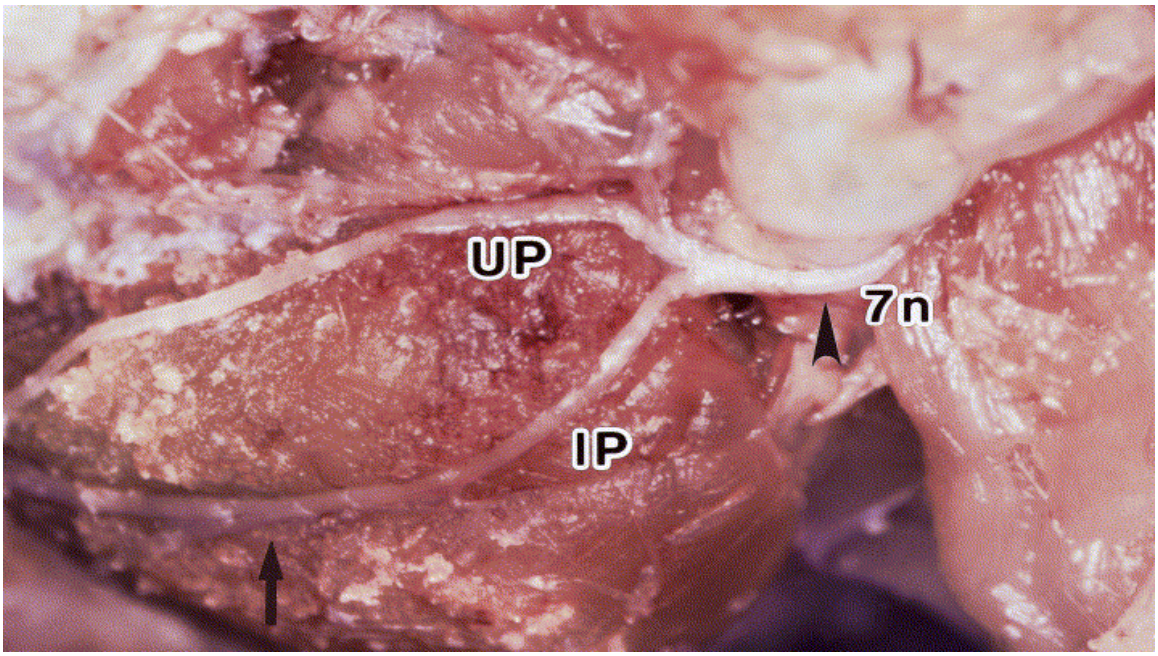


Figure 1-1. Lateral view of the facial nerve (7N) with its upper (UP) and lower (IP) peripheral branches that innervate the vibrissae follicular muscles. Arrowhead identifies the location of crush site. (Kamijo et al., 2003)

Amyotrophic Lateral Sclerosis

Amyotrophic Lateral Sclerosis (ALS) is one of the most common adult-onset neurodegenerative disease affecting ~5 per 100,000 individuals. First described by Charcot in 1869, ALS is characterized by the selective loss of upper and lower motor neurons invariably progressing to paralysis and death over a 1-5 year time course. Its etiology is still poorly understood; however a major breakthrough in the field occurred with the discovery that mutations in the Cu/Zn superoxide dismutase 1 (SOD1) gene affect approximately 20% of patients with familial ALS (Rosen, 1993; Siddique and Deng, 1996). This discovery allowed for generation of transgenic animal models which

closely resemble the motor weakness and degeneration seen in human disease (Gurney, 1994; Wong and Borchelt, 1995; Bruijn et al., 1997; Nagai et al., 2001; Howland et al., 2002). Prior to animal models, it was proposed the mutations in SOD1 reduced enzyme activity causing decreased free radical scavenging activity and an increase in oxidative stress. Contradicting evidence emerged from transgenic SOD1 mice that developed progressive motor neuron disease despite possessing two normal mouse SOD1 alleles (Bruijn et al., 1998; Jaarsma et al., 2001). In addition, SOD1 knockout mice live until adulthood and do not develop motor neuron disease (Reaume et al., 1996) indicating that the mutation in SOD1 causes a toxic gain of function rather then a loss of dismutase activity. The mutant SOD1 may catalyze aberrant biochemical reactions which result in production of damaging reactive oxygen species (ROS) such as the superoxide anion, the hydroxyl radical, hydrogen peroxide and peroxynitrite (Cluskey and Ramsden, 2001). Misfolding of the mutant protein causes copper at the active site to be less tightly bound increasing the release of copper. The unoccupied active site is more accessible to abnormal substrates such as peroxynitrite which leads to nitration of tyrosine residues (Beckman et al., 1993) and hydrogen peroxide which generates hydroxyl radicals that can damage cellular targets (Wiedau-Pazos et al., 1996). In addition, to aberrant chemical reactions occurring at the active site, the SOD1 toxic gain of function may be due to its participation in formation of protein aggregates. Protein aggregates or inclusion bodies intensely immunoreactive for SOD1 are found in motor neurons of the mouse model of ALS (Bruijn et al., 1997). Aggregates may be toxic due to additional proteins associating with them thereby depleting protein functions that may be essential for neuronal survival. Another hypothesis is that by repetitively misfolding, mutant proteins

are reducing availability of chaperones for proteins required for normal cell function. A final hypothesis relating to aggregates and their role in ALS is that SOD1 mutants reduce proteasome activity needed for normal protein turnover. SOD1 aggregates are very stable and even with treatment using strong detergents and reducing agents the aggregates are not easily dissociated. Formation of SOD1 aggregates disrupts normal balance of protein synthesis and degradation interrupting normal degradation of misfolded proteins critical to cell survival.

While familial ALS has been attributed to mutations located within the SOD1 gene, mechanisms underlying onset and progression of sporadic ALS, which accounts for the largest percentage of cases, are still largely unknown. It has been hypothesized that ALS is due to excitotoxcity, protein aggregation, mitochondrial dysfunction, and recently it has been proposed microglial activation is a contributing factor in the disease process.

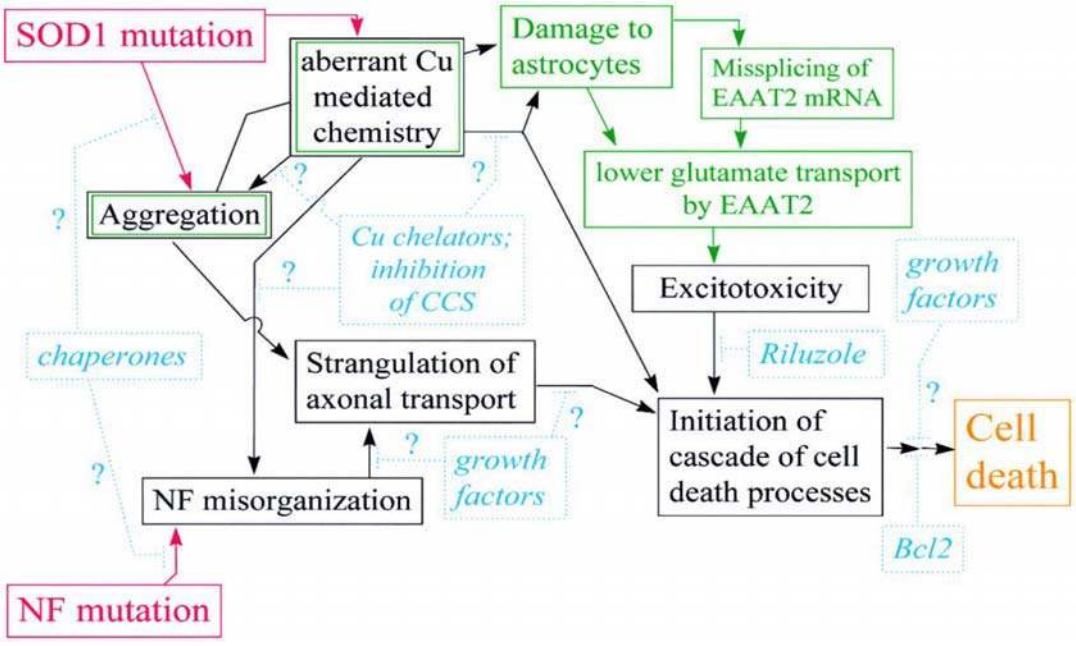


Figure 1-2. Proposed mechanisms of ALS. (Cleveland, 1999)

Microglia in ALS

Glial cell involvement in ALS pathology is unknown; however recent studies have presented strong evidence for non-neuronal cell involvement in ALS. A series of experiments revealed that SOD1 mutations are not directly toxic to motor neurons, but rather exert their neurotoxic effects in a non-cell autonomous fashion. Initial experiments showed expression of mutant SOD1 in neurons (Pramatarova et al., 2001; Lino et al., 2002) or astrocytes (Gong et al., 2000) alone failed to induce motor neuron degeneration. Clements et al., further implicated glial cells in ALS pathology by showing neuronal degeneration is dependent not on the type of cell carrying the mutant SOD1 gene, but rather the number of cells. This conclusion was reached from an experiment in which mutant SOD1 expression in individual neurons surrounded by wild type glial cells allowed neuronal populations to remain healthy whereas when the reverse occurred with wild type neurons surrounded by mutant SOD1 glial cells the result was neuronal degeneration (Clement et al., 2003). The role of glial cells in ALS is clearly demonstrated in experiments manipulating expression of mutant SOD1 in various cell types however further experimentation has specifically shown a role for microglia in ALS pathology. Cell-specific knock down of mutant SOD1 in microglia and macrophages in transgenic mice cause increased life span (Cleveland, 2004). Further evidence is seen in a study conducted by Weydt where microglia isolated from transgenic mice showed significantly higher levels of tumor necrosis factor alpha (TNF-α) when compared to age-matched controls (Weydt et al., 2004). Cellular evidence of microglia involvement can be seen in histological studies of both human and animal tissue where microglial activation and proliferation is seen in regions of motor neuron loss such as the spinal cord, brainstem and primary motor cortex (Kawamata et al., 1992; Hall et al., 1998;

Almer et al., 1999; Alexianu et al., 2001; Henkel et al., 2004). Microglial activation temporally corresponds to onset of motor weakness and neuronal loss. Accordingly, increased microglia production of TNF-α, IL-1ß, iNOS, and COX-2 all pro-inflammatory mediators are present in ALS patients suggesting a likely role of neuroinflammation in ALS etiology (Poloni et al., 2000; Elliott, 2001; Nguyen et al., 2001; Olsen et al., 2001; Hensley et al., 2002; Yoshihara et al., 2002). In addition to the products listed above, there are a number of microglial activating factors found to be elevated in ALS patients providing a plausible source for persistent microglial activation. It is unknown if microglial activation is triggered as a response to neuronal death or some other manner however it appears that once activated they are able to self-activate furthering an inflammatory environment. It should be noted that unlike other neurodegenerative diseases, influx of peripheral immune cells is a rare occurrence only seen at the end stage of ALS (Kawamata et al., 1992; Bruijn et al., 2004). Therefore, in ALS inflammatory reactions are generated and sustained only by CNS cells most likely microglia since they are the major immunocompetent cells of the CNS. Evidence to date strongly supports active participation of microglial cells in ALS pathogenesis however, it still remains unknown in what manner microglia exert their detrimental effects and if microglial activation is a secondary effect of the disease process or an initial contributing factor.

Neuroinflammation

Classically, inflammation is a complex response which aims to repair tissue damage and is accompanied by the cardinal points described by Celsus: pain, tumor, rubor and heat. Under normal conditions inflammation is a tightly controlled process maintained until the initial stimulus is repaired or eliminated however the inability of the immune system to clear the foreign target or repair the existing stimuli results in chronic

stimulation of immune cells thereby resulting in damage to tissue (Nathan, 2002). Chronic inflammation in the CNS, often referred to as neuroinflammation, is defined by the presence of activated microglia, reactive astrocytes and inflammatory mediators with activated microglia being the central component. Recently, neuroinflammation has been implicated in a number of neurodegenerative diseases such as Parkinson's, Alzheimer's and ALS. Microglial activation and its role in neurodegenerative diseases is a highly debated topic in that it still remains unclear whether microglial activation is beneficial or detrimental. In the non-diseased brain microglia are found in a resting ramified state where upon acute injury microglia proliferate and undergo morphological changes to attain a state of activation to aid in repair of damaged tissue. Following recovery microglia return to a resting state however in neurodegenerative diseases microglia are thought to maintain a persistent state of activation. It is proposed that neuroinflammation may drive a self-propagating toxic cycle of microglia in which several factors of disease such as protein aggregates, injured neurons, and AB plaques activate microglia exacerbating neuronal death through production of pro-inflammatory products which in turn further increases levels of microglial activation. Evidence supporting pro-inflammatory properties of microglia is extensive in neurodegenerative diseases however whether the microglial reaction is a secondary response to neuronal death or a causative factor still remains unresolved. Nonetheless, therapeutic treatments targeting inflammation in a number of neurodegenerative diseases are currently underway with variable results. COX-2 inhibitor, rofecoxib, has been used for treatment of Alzheimer's and Parkinson's however clinical trials failed to demonstrate a beneficial effect (Aisen et al., 2003; Przybylkowski et al., 2004; Reines et al., 2004). These findings suggest that

inflammation may be a secondary cause of neurodegenerative diseases due to the failure of treatment to halt disease progression.

Microglia Dysfunction

An alternative to neuroinflammation is that microglial cells undergo functional changes in the diseased brain where they acquire toxic functions or become incapable of performing normal functions in the CNS. The inability of microglia to perform their normal function may have detrimental consequences for neurons thereby propagating neuronal degeneration. Microglia dysfunction has been observed in HIV patients, the normal aging brain and neurodegenerative diseases.

HIV-1 associated dementia (HAD) is a syndrome of motor and cognitive dysfunction in 10% of patients infected with HIV-1 with acquired immune deficiency syndrome (McArthur et al., 1993; Sacktor et al., 2001). Neurons are not productively infected with the virus suggesting infected cells in the CNS, in particular microglia, release signals that lead to secondary neuronal injury. Microglia can be activated by HIV infection itself, by interaction with viral proteins or by immune stimulation in response to factors released from other infected cells (Lipton and Gendelman, 1995). In response to activation, microglia have increased production and release of neurotoxic immunomodulatory factors such as pro-inflammatory cytokines, free radicals and neurotoxic amines (Genis et al., 1992; Achim et al., 1993; Bukrinsky et al., 1995; Giulian et al., 1996; Zhao et al., 2001). Further evidence of abnormal microglia is the presence of multinucleated giant cells which are found in close proximity to apoptotic neurons.

Phenotypic changes have been shown in microglia with aging such as up regulated expression of MHC II (Perry et al., 1993; Ogura et al., 1994; DiPatre and Gelman, 1997; Streit and Sparks, 1997; Morgan et al., 1999), greater exhibition of phagocytic

morphology and IL-1α immunoreactivity (Sheng et al., 1998). In addition, several studies have reported significant changes in microglia morphology, including cytosolic inclusions (Peinado et al., 1998), higher incidence of clumping in and around white matter (Perry et al., 1993) and structural changes. Morphological abnormalities in microglia have been identified in the Huntington's mouse model (Ma et al., 2003) and human Alzheimer's tissue. Structural changes included bulbous swellings, long stringy processes, cytoplasmic fragmentation and deramified processes.

Corresponding to phenotypic changes microglia appear to undergo functional changes due to aging. In culture studies and in the facial nerve paradigm aged microglia display an increased proliferative response (Rozovsky et al., 1998; Conde and Streit, 2005). *In vitro*, microglial proliferation progressively increases with donor age up to 400% greater at 24 months vs. 3 months (Rozovsky et al., 1998). Following a facial nerve axotomy microglial proliferation in aged rats is significantly higher 4 days following axotomy (Conde and Streit, 2005). The enhanced proliferation of aged microglia may be due to a loss of response to regulatory mechanisms which is supported by culture experiments where aged microglia did not respond to TGF-ß1 in contrast to young microglia that showed an inhibition of proliferation following TGF- ß1 treatment (Rozovsky et al., 1998). This loss of sensitivity to TGF- ß1 with increased age was also demonstrated in the regulation of prolactin in rat anterior pituitary cells (Tan et al., 1997). The desensitization to anti-proliferative properties of TGFß -1 provides a plausible cause for the increased proliferative response of aged microglia. Further impairment of TGFß-1 regulatory mechanisms is seen in cultured microglia. Lipopolysaccharide (LPS) treatment induces NO production in all donor age cultures however in young donor

cultures NO production is inhibited following TGFß-1 but TGFß-1 has no effect on NO production in aged donor cultures (Rozovsky et al., 1998). Aging-related changes in microglia appear to affect the regulatory mechanism of microglial activation causing the homeostasis of the local CNS environment to be disrupted.

Minocycline

Minocycline is a second generation tetracycline with antibiotic activity against a broad-spectrum of bacterial types including both Gram-positive and Gram-negative bacteria. Completely separate and distinct from its antimicrobial activity, minocycline exhibits anti-inflammatory effects that are proven to be neuroprotective in a number of neurodegenerative diseases and brain ischemia (Yrjanheikki et al., 1998; Yrjanheikki et al., 1999; Du et al., 2001; Kriz et al., 2002; Wu et al., 2002). Although other tetracyclines can diffuse across the blood-brain barrier into the CNS in small amounts, the lipophilicity of minocycline allows it to attain significantly higher levels in the CNS furthering its therapeutic potential in neurodegenerative diseases (Barza et al., 1975). One proposed mechanism of minocycline is a direct neuroprotective action in which caspases are inhibited by preventing release of mitochondrial cytochrome c (Zhu et al., 2002; Teng et al., 2004) . Another proposed action of the drug is deactivation of microglial cells indirectly accounting for the observed neuroprotection (Yrjanheikki et al., 1999; Du et al., 2001; He et al., 2001; Kriz et al., 2002; Wu et al., 2002). Microglia deactivation occurs through inhibition of p38 MAPK which is thought to mediate the inflammatory process within microglia by inducing transcription factors that positively regulate inflammatory genes (Tikka et al., 2001; Koistinaho and Koistinaho, 2002).

Minocycline treatment administered to SOD1 mice delays onset of motor neuron degeneration and increases longevity of SOD1 mice lifespan (Kriz et al., 2002; Van Den

Bosch et al., 2002; Zhu et al., 2002). Currently, clinical trials are underway to determine the benefits of minocycline treatment in human ALS patients.

Minocycline appeared to hold great therapeutic treatment for neurodegenerative disease however recent findings showed a deleterious effect in Parkinson's (PD), Huntington's (HD) and hypoxic-ischemia (HI) animal models (Smith et al., 2003; Yang et al., 2003; Tsuji et al., 2004). In a PD model minocycline increased 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) toxicity and showed no effect in the transgenic mouse model of HD (Smith et al., 2003; Yang et al., 2003). It was also reported that in two chronic models: the MPTP-intoxicated non-human primate model of PD and the 3-nitropropionic acid (3-NP) intoxicated model of HD minocycline treatment resulted in earlier clinical motor symptoms during toxic treatment, decreased motor performance and greater neuronal loss when compared to controls (Diguet et al., 2003; Diguet et al., 2004). In addition, minocycline is proposed to exacerbate hypoxic-ischemia brain injury in the immature mouse cortex, thalamus and striatum while neuroprotective in the immature rat brain (Arvin et al., 2002; Tsuji et al., 2004). In models of HI, deleterious effects of minocycline may be due to the reduction in compensatory angiogenesis after HI by inhibiting endothelial proliferation.

CHAPTER 2
MINOCYCLINE DOES NOT INHIBIT MICROGLIA PROLIFERATION OR NEURONAL REGENERATION IN THE FACIAL NUCLEUS FOLLOWING A FACIAL NERVE CRUSH

Introduction

Minocycline is a second-generation tetracycline reported to have an anti-inflammatory activity independent of its antimicrobial function (Amin et al., 1996). Recently, minocycline has been shown to inhibit microglial activation and promote neuronal survival in animal models of neurodegenerative disease and stroke (Yrjanheikki et al., 1999; Du et al., 2001; He et al., 2001; Kriz et al., 2002; Wu et al., 2002). It has been hypothesized that in neurodegenerative disease microglia undergo detrimental activation characterized by increased production and release of neurotoxins that contribute to neuronal cell death. Accordingly, minocycline has been proclaimed as a potential treatment for neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS) and Parkinson's disease which are thought to have a neuroinflammatory component in their pathogenesis (Du et al., 2001; Kriz et al., 2002; Wu et al., 2002). On the other hand, experimental studies after acute brain injury show that microglial activation is a consequence of neuronal injury rather than the cause of it. In particular, experimental paradigms involving neuron regeneration such as motoneuron axotomy, show that microglial activation precedes successful regeneration of severed axons suggesting that activated microglia are neuroprotective and support motoneuron regeneration (Streit, 1993, 2002, 2005). Following axotomy, greater numbers of microglia are generated through local proliferation (Graeber et al., 1988b) and these cells

encircle the injured neurons in a manner that suggests neuroprotection through displacement of afferent synapses and close glial-neuronal apposition which may allow for targeted delivery of microglia-derived growth factors, such as TGF-beta (Mallat et al., 1989; Martinou et al., 1990; Araujo and Cotman, 1992; Elkabes et al., 1996; Lehrmann et al., 1998). Furthermore, there is little if any neuronal cell death within the facial nucleus of the rat following axotomy (Johnson and Duberley, 1998) suggesting that microglia are aiding with recovery of damaged neurons rather than harming them (Lieberman, 1971; Streit and Kreutzberg, 1988; Streit, 1993; Kuzis et al., 1999).

To directly test the hypothesis that minocycline inhibits microglia activation *in vivo*, we have quantified microglial proliferation in the axotomized facial nucleus. To determine if there are functional consequences for neurons of this potential microglial inhibition, we have also quantified and compared numbers of regenerating motoneurons between minocycline treated and non-treated animals.

Materials and Methods

Animals and Diet

Animal use protocols were approved by the University of Florida Institutional Use and Care of Animals Committee (IUCAC). Young adult male Sprague Dawley rats were divided into two groups, one receiving a standard rodent diet while the second group was fed a diet enriched with minocycline (1gram/kilogram) obtained from Harlan Tekland (Madison, WI). The diets were implemented one week prior to surgery and continued throughout the remainder of the experiment. Levels of food intake were recorded for each cage of animals. Both treatment groups underwent a unilateral facial nerve axotomy.

Table 2-1. Average intake of minocycline during experiments.

Survival Time Point for Proliferation Studies	**Number of Days on Diet**	**Average Drug Intake/Animal (grams)**
2 days post-axotomy	9	0.19286
3 days post-axotomy	10	0.24166
4 days post-axotomy	11	0.27190
Survival Time point for Flurogold Studies		
7 days post-axotomy	14	0.30158
14 days post-axotomy	21	0.50119
21 days post-axotomy	28	0.63218

Facial Nerve Axotomy

Animals were anesthetized with isoflurane using a precision vapor machine with gas scavenging system attached. Animals were placed in an inducing box where isoflurane was administered until the pedal and palpebral reflexes were absent. Animals were transferred to a nose cone where isoflurane was administered to the animal for the remainder of surgery. Upon full sedation, a small incision was made directly behind the right ear. Using a pair of angled scissors, the superficial levels of the muscle tissue were cut until the facial nerve was exposed. Both branches of the facial nerve were separated from the surrounding tissue and crushed with a pair of hemostats for 10 sec. The incision was closed with a surgical staple and animals were removed from isoflurane and closely monitored until fully recovered. The absence of whisker movement was assessed to confirm that both nerve branches were completely crushed.

^{3}H-Thymidine Injections and Radioactive Perfusions

Microglial proliferation was assessed at 2, 3 and 4 days after facial nerve crush since it is known that the burst of mitotic activity occurs during this time period. Each time point included ten animals, five in each treatment group. Animals were weighed and given an intraperitoneal (i.p) injection of 3 μCi per gram body weight of [methyl-^{3}H]

thymidine (Amersham Pharmacia Biotech) two hours prior to perfusion. Animals were caged in radioactive labeled cages until perfusion.

Two hours following the ^{3}H-Thymidine injection each animal was given a lethal dose of sodium pentobarbital (150 mg/kg; i.p.). In the absence of pedal and palpebral reflexes animals were transcardially perfused with phosphate buffered saline (PBS) followed by 4% paraformaldehyde (in PBS). Following perfusion brains were dissected out and placed in 4% paraformaldehyde overnight at room temperature. Each animal was assigned a random number to maintain objectivity in quantitative analysis. All liquid, dry and animal waste were labeled as radioactive and disposed according to the University of Florida waste management protocol.

Tissue Processing for Audoradiography

Following overnight fixation, a coronal section of the brainstem containing the facial nucleus was dissected out and rinsed in PBS. Each section was processed for paraffin embedding by slowly dehydrating through an ascending series of alcohol beginning with 70% ethanol for 2h, 45 min for 70%, 90%, 95%, 100% ethanol then 100% ethanol overnight. Following overnight incubation, brain sections were placed in two changes of xylenes for 2h and transferred to paraffin cassette holders. Cassette holders were immersed in 2 changes of Surgipath Formula R paraffin (Surgipath, Richmond, IL) at 60°C for 2h each. Lastly, the tissue was removed from cassette holders and embedded in Surgipath Tissue Embedding Medium paraffin (Surgipath, Richmond, IL) and allowed to cool. The facial nucleus was serially cut (7µm sections) from rostral to caudal on a microtome and collected on Superfrost Plus slides.

Audoradiography

Immediately before beginning the developing process, slides were deparaffinized and hydrated through a series of descending alcohols then rinsed in PBS for 5min. The sections were dipped in a 50% solution of NTB-2 emulsion (Eastman Kodak) and allowed to air dry in a darkroom with a safelight with Kodak #2 filter. Slides were exposed in light- protected slide boxes with desiccant at 4°C for 5 weeks and developed with 50% Dektol developer for 2.5min (Eastman Kodak), rinsed in ddH_2O for 10dips, fixed in Kodak fixer for 5min, washed, counterstained with 0.5% cresyl violet, dehydrated through alcohols and xylenes and coverslipped using Permount mounting medium (Fisher Scientific).

Quantitative Analysis for ^{3}H-thymidine Labeled Microglia

For quantitative analysis of proliferating microglia every fourth section of the facial nucleus was counted for ^{3}H-thymidine labeled microglial cells. The sections were viewed using a Zeiss Axiophot microscope with a Sony DXC970 camera attached. In the selected sections the facial nucleus was outlined and the area measured using MCID 6 software (Imaging Research, St. Catherine's, Ontario). The labeled cells in the outlined area were manually counted under 40X magnification. Labeled cells were pooled together for each animal and divided by the pooled area measured for each animal to determine a population density of proliferating microglia in the facial nucleus. Results are represented as mean values ± SEM. The density of dividing microglia was compared among minocycline treated and non-treated animals at each time point with a t test using GraphPad Prism software (GraphPad Software, San Diego, CA). A significance level of $p<0.05$ was used.

Fluorogold Labeling

Regeneration of motor neurons following minocycline treatment was investigated at 7, 14, and 21 days after facial nerve crush. Each time point included ten animals, five in each treatment group. Three days before perfusion, animals were given two 10 μL injections of 4% fluorogold (in saline): one into the whisker pad and one directly underneath the eye.

Perfusion and Tissue Processing for Fluorogold Labeling

The transcardial perfusion was performed as detailed in the previous radioactive perfusion section, however in the absence of radioactive precautions. Following the perfusion brains were dissected out and placed in 4% paraformaldehyde until sectioning. The brainstem containing the facial nucleus was dissected out and mounted onto a cutting block. The facial nucleus was sectioned caudal to rostral on a vibratome in 50 μm sections and collected on SuperFrost slides. Following collection the slides were allowed to air dry for one hour, dehydrated through a series of ascending alcohols and xylenes and coverslipped with Permount mounting medium (Fischer Scientific).

Quantitative Analysis of Fluorogold Labeling

For quantitative analysis of regenerating neurons the entire facial nucleus was counted for fluorogold labeled cells. Results are represented as mean values ± SEM. The number of fluorogold labeled cells were compared among minocycline treated and non-treated animals at each time point with a t test. A significance level of $p<0.05$ was used.

Results

Microglia proliferation is not inhibited in minocycline treated animals at 2, 3, and 4 days post facial nerve axotomy. Our objective was to determine *in vivo* if

minocycline could attenuate microglial activation, and to this end we decided to measure cell proliferation, which is a reliable, quantifiable parameter of microglial activation. Consistent with prior reports, we found ^{3}H-thymidine-labeled cells at all three time points examined. However no statistically significant difference between the number of ^{3}H-thymidine cells was found in the injured facial nucleus at any time point when comparing the control rats and the rats fed a diet enriched with minocycline in the injured facial nucleus at all three time points. No cells labeled with ^{3}H-thymidine were observed on the uninjured side of the facial nucleus within either treatment group.

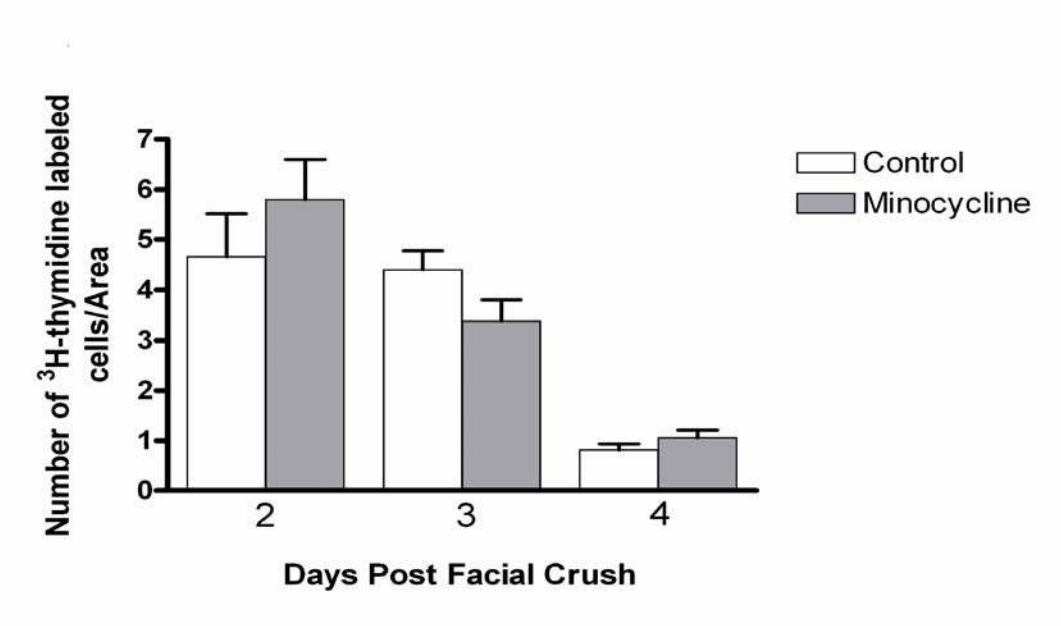


Figure 2-1. Microglial proliferation in the facial nucleus. There is no statistically significant difference in the cell proliferation between the control animals and the animals fed a diet enriched with minocycline at any post-axotomy time point. (Difference at day two is 1.14 ± 1.171 ($p = 0.3587$), at day three is 1.12 ± 0.5775 ($p = 0.1207$), and difference at day four is 0.29 ± 0.1922 ($p = 0.2364$)).

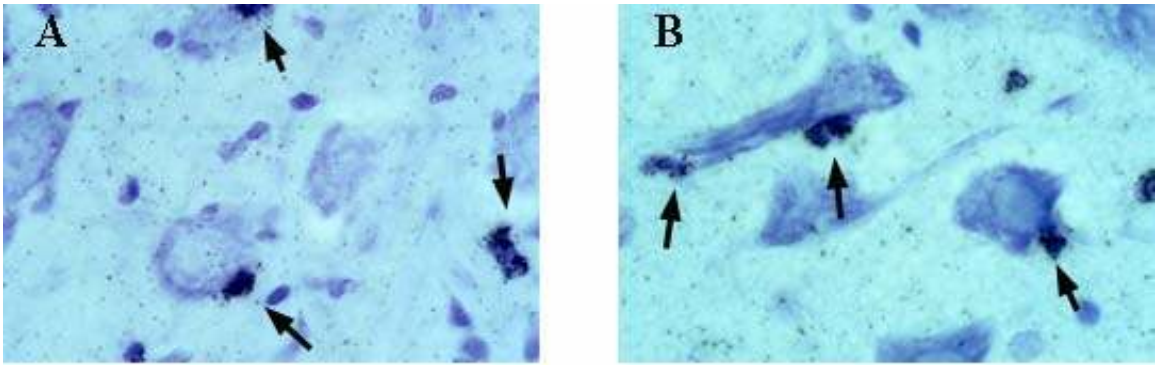


Figure 2-2. Photomicrographs of ^{3}H-thymidine labeled cells in the injured facial nucleus. A) Control rat 3 days post-injury. B) Minocycline treated rat 3 days post-injury. Note that ^{3}H-thymidine labeled microglia are in close proximity to the regenerating neurons. Magnification 250x.

Figure 2-3. Motor neuron regeneration in the facial nucleus. There is no statistically significant difference in the number of fluorogold labeled neurons between the control animals and the animals fed a diet enriched with minocycline at 14 and 21 days after nerve crush. Differences between treatment groups at day 14 is 6 ± 8.051 ($p = 0.4774$) and at day 21 is 10.8 ± 32.12 ($p = 0.7454$)

Neuronal regeneration is not inhibited by minocycline treatment. Since any attenuation of microglial activation by minocycline could have an effect on neuronal

regenerative ability after nerve crush we performed fluorogold injections 7, 14 and 21 days following a unilateral facial nerve crush to determine the number of neurons that underwent successful reinnervation. No fluorogold labeled neurons were found on day 7 after nerve crush; they first became apparent at 14 days and became more numerous by day 21. The results show that successful reinnervation of facial muscles takes place between 2 and 3 weeks (Soreide, 1981; Fawcett and Keynes, 1990). The counts of regenerating neurons throughout the facial nucleus show that minocycline did not influence regeneration of neurons between the control and minocycline treated groups.

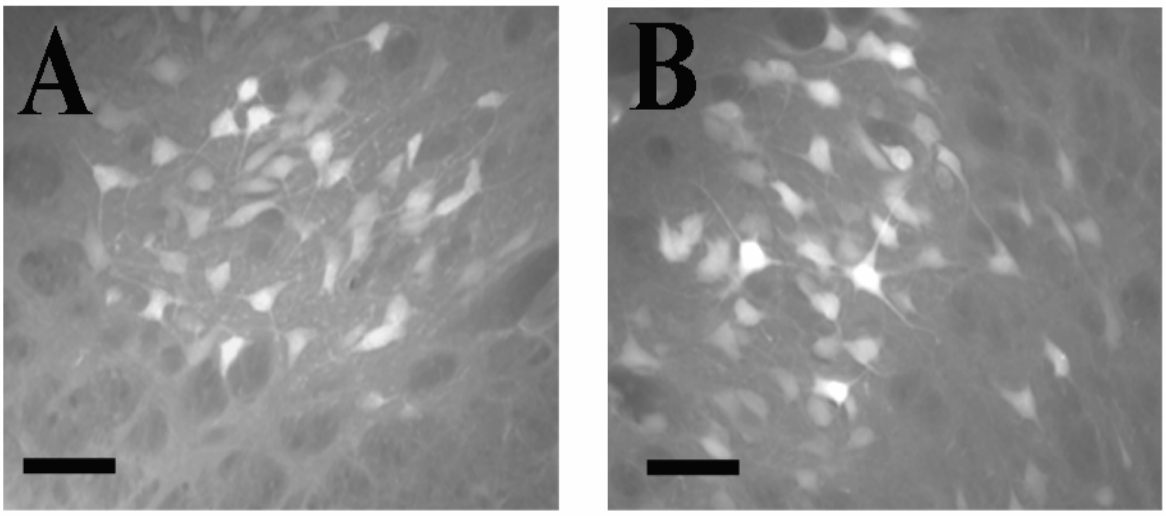


Figure 2-4. Fluorogold labled neurons within the lateral and ventral intermediate sections of the injured facial nucleus. A) Control rat 21 days post-facial nerve injury. B) Minocycline treated rat 21 days post-facial nerve injury. Bar = 100μm.

Discussion

In this study we report that microglial proliferation *in vivo* is not inhibited by minocycline within the facial nucleus following a nerve crush. These results differ from those reported in previous studies, which found that minocycline significantly inhibits

microglial proliferation *in vitro* (Tikka et al., 2001; Tikka and Koistinaho, 2001). To reconcile this apparent contradiction in experimental findings, one needs to consider the fact that there are fundamental differences between microglia *in vitro* and *in vivo* (Streit, 2005). Specifically, with regard to cell division, it is important to note that microglial cells in culture undergo mitosis constitutively and spontaneously, because they exist in a permanent state of activation *in vitro*. This differs from resting microglia in the CNS, which only become activated and proliferate in the presence of a stimulus, such as acute CNS injury. This mismatch of activation states between cultured microglia and microglia in the brain likely accounts for the differences in proliferation dynamics observed after minocycline treatment *in vitro* and *in vivo*. In addition, previous studies conducted in the SOD1 and Parkinson's mice (LPS and 6-hydroxydopamine induced) classified activated microglia by the presence of cell surface markers such as OX-6, OX-42 and Mac-2 (He et al., 2001; Kriz et al., 2002; Tomas-Camardiel et al., 2004). In these studies a decrease in expression of OX-6, OX-42 or Mac-2 was found following minocycline treatment which, in turn, led to the conclusion that by decreasing microglial activation life span in the SOD1 mouse is increased and dopaminergic cells become partially protected in the Parkinson's mouse. However, the exclusive use of cell surface markers is not a reliable method for quantification of activated microglia. OX-6 labeling of MHC II expression is not always indicative of microglial activation considering that not all activated microglia are MHC II positive and MHC II expression can be found on resting microglial cells in the normal rodent brain, often in the white matter (Streit et al., 1989a). The decrease in expression of OX-42 and Mac-2 following minocycline treatment could be a result of a direct neuroprotective effect of the drug which may allow greater neuronal survival

thereby decreasing the intensity of the reactive microgliosis. It has been shown *in vitro* and *in vivo* that minocycline is able to inhibit cyctochrome c release as well as activation of downstream caspase dependent and independent cell death pathways (Zhu et al., 2002; Wang et al., 2003). Presently, clinical trials are underway to test the effectiveness of minocycline vs. placebo in the treatment of ALS patients. These trials resulted after minocycline was found to increase life span in animal models representing familial ALS. It is hypothesized that minocycline increased life span in SOD1 mutant mice by inhibiting microglial activation through the p38 MAPK pathway. However, as we show in this study minocycline fails to inhibit microglial proliferation, a key characteristic of microglial activation. Perhaps, minocycline is effective in the SOD1 mouse by being directly neuroprotective. In the facial nucleus following injury the number of regenerating neurons was unchanged between the minocycline treated animals and the control group as was the number of proliferating microglial. This finding offers further support for the idea that microglial activation after CNS injury is a neuroprotective mechanism.

CHAPTER 3
DETERMINATION OF MINOCYCLINE CONCENTRATION IN THE BRAIN AFTER DRUG ADMINISTRATION IN DIET

Introduction

The semisynthetic second-generation minocycline has neuroprotective effects in models of cerebral ischemia (Yrjanheikki et al., 1998; Yrjanheikki et al., 1999; Tikka et al., 2001). Minocycline has also been shown to prevent nigrostriatal dopaminergic neurodengeneration in mouse models of Parkinson's disease (Du et al., 2001; Wu et al., 2002), delay disease progression in a transgenic model of Huntington disease (Chen et al., 2000) and increase life span in a mouse model of ALS (Kriz et al., 2002; Van Den Bosch et al., 2002; Zhu et al., 2002). While all these studies suggest that minocycline might offer a useful pharmological approach for treatment of numerous neurodegenerative disease it is still unknown on the concentrations reached in the brain and how they relate to blood concentrations in animals. Analyses based on simple protein precipitation (Birminham et al., 1995), liquid-liquid (Mascher, 1998; Araujo et al., 2001) or solid-phase extraction procedures (Wrightson et al., 1998; Orti et al., 2000) followed by high-performance liquid chromatography (HPLC) with ultraviolet detection have been reported for the determination of minocycline in biological samples.

The current study will investigate mincoycline concentrations in the brain following drug treatment administered through diet. The absorption of minocycline is believed to be affected by simultaneous administration with food (Leyden, 1985; Meyer, 1996) therefore, to confirm that the lack of inhibition of microglia proliferation in chapter

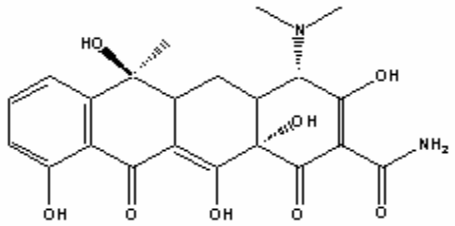
2 was not a result of insufficient levels of minocycline in the brain, HPLC MS/MS will be utilized to measure minocycline concentrations in the brain following drug intake through diet.

Materials and Methods

Reagents

Minocycline hydrochloride and tetracycline hydrochloride were obtained from Sigma-Aldrich. Stock solutions were prepared by dissolving tetracyclines in methanol at a concentration of 1 mg/mL. Working standard solutions were prepared from stock solutions by dilution with methanol. To prepare standards for the concentration curve, three concentrations of the analyte were prepared in methanol; 0.05 μg/mL, 0.5 μg/mL and 2.5 μg/mL.

Tetracycline



Minocycline

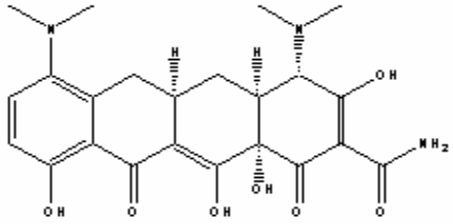


Figure 3-1. Chemical structures of internal standard and minocycline.

Animals

Two male Sprague Dawley rats weighing 200-250 g were used, one was fed a diet enriched with minocycline (1 gram/kilogram) for one week prior to analysis while the other rat was fed a standard rodent diet. Animals were killed by decapitation under deep anesthesia, brains were rapidly removed and blotted to remove excess surface blood and stored at -20ºC until analysis. Water and food were freely available throughout the study.

Extraction

A 200 mg section of the brainstem containing the facial nucleus was removed from the whole brain and homogenized in 1 mL of 0.01M phosphate buffer pH 7.4, on ice. To 1 mL of the resulting homogenate 25µL of the internal standard tetracycline (10 µg/mL) and 20 µL of H_3PO_4 were added, vortex mixed and centrifuged at 2000rpm at 4ºC for 30min. The precipitate was re-dissolved in 1 mL of 0.01M phosphate buffer, pH 7.4 and centrifuged. Following centrifugation, the supernatants were combined.

To clean up supernatants 1ml/30mg Oasis HLB extraction cartridges (Waters) were used. Prior to applying the homogenized tissue sample cartridges were preconditioned with 1 mL of methanol followed by 1 mL of distilled water. Samples were loaded into columns, the columns were rinsed with 1 mL of 5% methanol solution, and dried for 10 min through suction. The samples were eluted with 1 mL of methanol. A blank sample was processed in tandem with brain samples to eliminate concern of contamination during extraction.

Equipment

The apparatus used for High Performance Liquid Chromatography was an Agilent 1100 series binary pump (Palo Alto, CA) equipped with an Agilent 1100 UV/V detector

set at 353 nm. Separation was carried out on a Pehnomenex Synergi 4u Hydro-RP 80A (Torrace, CA) (2 x 150 mm, 4 um) plus C18 guard column (2 mm x 4 mm). Mass spectrometric detection was performed using a TehermoFinnigan LCQ (San Jose, CA) with electrospray ionization (ESI).

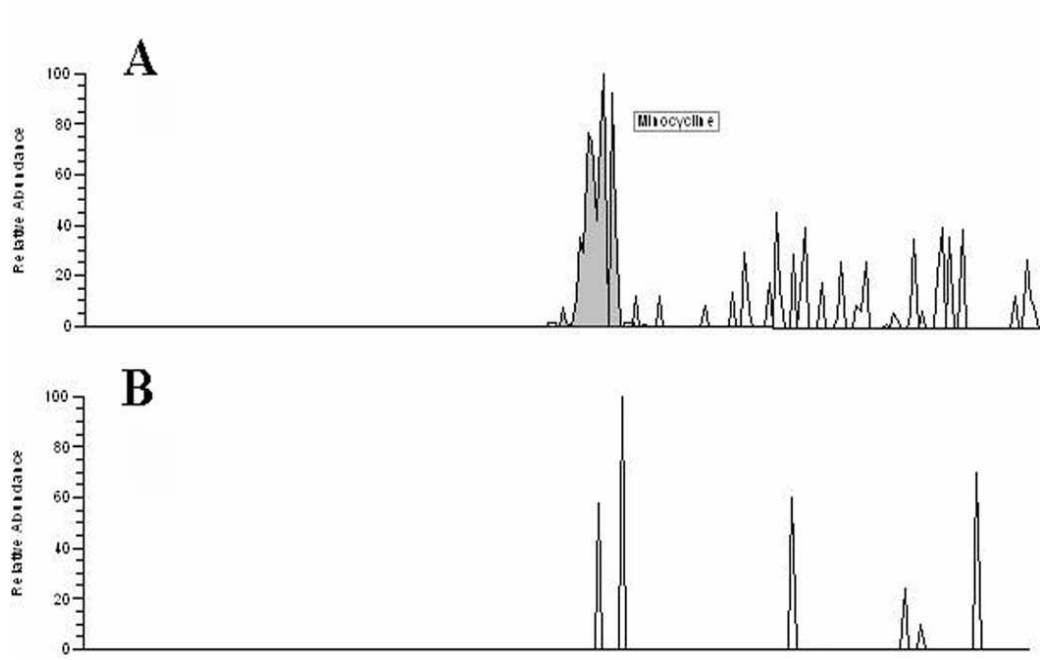


Figure 3.2. Minocycline concentrations found through HPLC/MS/MS analysis. A) In the minocycline treated animal there was a peak area showing a presence of minocycline at a concentration of 0.4968 µg/mL. B) In the control sample there was no peak area corresponding to minocycline.

Results

Minocycline was found at a significant concentration in the treated animal. In order to confirm that minocycline had crossed the blood brain barrier, HPLC MS/MS was performed on brainstem tissue from a control animal and from an animal fed minocycline. This showed a peak area for the minocycline compound in the treated animal but not in the control animal. The extract of the minocycline-treated animal was analyzed twice. From the concentration in the methanol extract and knowing the extraction procedure, the concentration of minocycline was determined and was found at

a concentration of 0.4968 μg/mL in the treated animal. Using the area of the standard tetracycline a calibration curve was created. A linear regression yielded the equation of the line: $y = 9.338x - 0.0314$ where y = the area ratio Minocycline/Tetracycline and x is the [Minocycline] in μg/mL. Rearrangement of this equation yielded the equation to determine the [M] in the unknown: $[M] = \{(M/T) + 0.0314\} /9.3338$.

Discussion

The current study demonstrates that significant levels of minocycline were detected in the brain following drug administration in rat rodent diet. A study conducted by Du and colleagues found that minocycline orally administered at a concentration of 120mg/kg in the 1-methyl-4-phenyl-1,2,3,6-tetraydropyridine (MPTP) Parkinson's model provided neuroprotection to midbrain dopamine neurons from the toxic effects of MPTP. Minocycline levels were assessed using liquid chromatography and mass spectral detection and found minocycline concentration in the midbrain at 0.32 μg/g compared to the 2.484 μg/g found in the rat brains assessed in this study. Accordingly, it appears the lack of inhibition of microglia proliferation in the study described in chapter 2 is not a result of insignificant levels in the brain but rather an actual lack of effect of minocycline on microglial activation.

A number of studies using intraperitoneal (i.p.) injection for drug delivery have claimed minocycline as a neuroprotective agent in neurodegenerative diseases (Van Den Bosch et al., 2002; Zhu et al., 2002; Wang et al., 2003). It is difficult to determine the levels of minocycline in the brain using this route of administration due to the lack of studies investigating minocycline concentrations in brain tissue following an i.p. injection. The majority of HPLC studies concentrate on the levels of minocycline in the plasma rather than actual brain tissue.

It can be concluded, that oral administration of minocycline is an effective method for drug delivery and that failure of minocycline to inhibit microglial activation in the facial nucleus is not a result of low levels of minocycline in the brain.

CHAPTER 4
TIMELINE OF MICROGLIA PROGRAMMED CELL DEATH IN THE FACIAL NUCLEUS FOLLOWING INJURY

Introduction

Activation and proliferation of microglia is one of the earliest and most common glial reactions in the injured brain. Upon injury in the CNS, specifically in the facial nucleus, microglial cells dramatically increase in cell number (Cammermeyer, 1965; Graeber et al., 1988b; Raivich et al., 1994) and are recruited to perineuronal sites where it is suggested they displace afferent synaptic terminals (Blinzinger and Kreutzberg, 1968). Following the initial microglial response and regeneration of motoneurons, activated microglia migrate into the nearby parenchyma (Angelov et al., 1995) and decline in number often reaching baseline levels several weeks following injury (Streit et al., 1988; Raivich et al., 1993). Initially, the mechanism used to maintain homeostasis of microglial cell numbers was attributed to migration of activated microglia to blood vessels where microglia exit through the walls (Del Rio-Hortega, 1932; Cammermeyer, 1965) however, recent studies suggest that microglia regulation is controlled by a form of programmed cell death (Gehrmann and Banati, 1995; Jones et al., 1997).

Programmed cell death is an important mechanism used to control cell population during development, growth and in regulation of the immune response (Allen et al., 1993; Bortner et al., 1995; Majno and Joris, 1995). Apoptosis is the most common type of programmed cell death investigated in the literature and is characterized by chromatin condensation, DNA fragmentation, membrane blebbing and caspase induced (Gavrieli et

al., 1992; Bohm and Schild, 2003; Jaattela and Tschopp, 2003). Apoptotic cells are quickly phagocyctosed without induction of an inflammatory response allowing homeostatic regulation of the CNS. More recently, a number of reports have described a caspase-independent form of programmed cell death that display activation of other proteases and changes in morphology not consistent with classical apoptosis (Jaattela and Tschopp, 2003; Nagy and Mooney, 2003; Lockshin and Zakeri, 2004).

The main objective of the study in the current chapter is to compare the post-mitotic turnover of microglia at different post-injury time points using the ApopTag assay, a kit variation of the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling method. The ApopTag kit labels DNA fragmentation, a key component of apopotsis, by detecting DNA strand breaks by enzymatically labeling the free 3'-OH termini with modified nucleotides. The findings from this study will be used for further experimentation in ALS animal models to determine key differences in post-mitotic microglia turnover in diseased and non-diseased animals.

Materials and Methods

Animals and Tissue Processing

Male Sprague Dawley rats weighing 200-250 grams were used. Animals underwent a unilateral facial nerve crush as described in chapter 2. Four animals per survival time were euthanized at 10, 14, 17 and 21 days post-injury by a transcardial perfusion as described in chapter 2 without radioactive precautions. Briefly, animals were given an overdose of sodium pentobarbital and transcardially perfused with 0.1 M PBS pH 7.4 followed by 4% paraformaldehyde. Immediately following perfusion, brains were quickly removed and stored in 4% paraformaldehyde for 2h and then transferred to

PBS. Brainstem containing the facial nucleus was paraffin processed as described in chapter 2 and 7 μm sections were cut on a microtome.

TUNEL and DAPI Staining

To assess apoptotic cells, TUNEL labeling was performed on the processed tissue. Sections were deparaffinized through xylenes (2 changes for 15 min each) and descending alcohols for 2 min each (100%, 100%, 95%, 90%, 70%, 70%) and rinsed in PBS for 5 min. The ApopTag Red *In Situ* detection kit (Serologicals Corporation, Norcross, GA) was used as described in the manufacturer's protocol however the pretreatment step was omitted. Negative controls omitted the terminal deoxynucleotidyl transferase (TdT) enzyme.

Following TUNEL labeling, slides were counterstained with DAPI. Slides were incubated with DAPI at a concentration of 1:333 for 5 min in a light protected box, rinsed in PBS and coverslipped.

Quantitative Analysis

Eight sections containing the facial nucleus were used for TUNEL quantification. Sections were imaged using a Spot RT digital camera (Diagnostic Instruments, Sterling Heights, MI) attached to a Zeiss Axioskop microscope. All TUNEL positive cells located in the facial nucleus were counted and pooled together per time point and divided by the total number of sections counted to give an average. Results are represented as mean values ± SEM.

Results

Differences in density of TUNEL positive cells in the injured facial nucleus were seen at different post-axotomy time points. TUNEL positive cells were found at

all time-points investigated in the injured facial nucleus. There were differences seen in the number of TUNEL labeled cells at 10, 14, 17 and 21 days (22 ± 1.194, 51 ± 3.061, 10 ± 0.064, and 13 ± 0.832) with the greatest density of TUNEL positive cells present at 14 days post-axotomy (Fig. 4-1). No TUNEL labeled cells were found in the contralateral, unoperated facial nucleus (Fig. 4-2d).

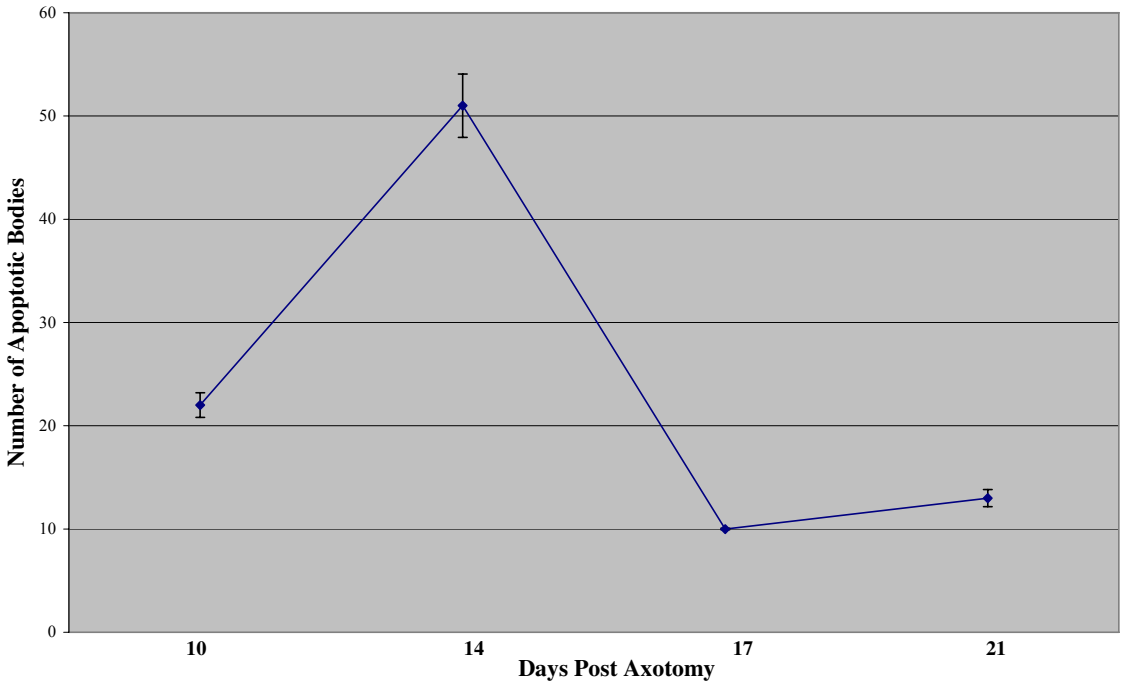


Figure 4-1. A time line of TUNEL positive microglia in the facial nucleus following injury. Results are represented as mean values ± SEM. (Day 10, 22 ± 1.194, Day 14, 51 ± 3.061, Day 17, 10 ± 0.064, and Day 21,13 ± 0.832)

TUNEL labeled microglia display abnormal cytoplasmic staining at all time points post-axotomy. The majority of TUNEL positive microglia showed staining diffusely dispersed throughout the cytoplasm however, lacked intense nuclear staining commonly found with classic apoptosis. The cytoplasmic staining revealed ramified processes and a perineuronal location of the TUNEL positive cells similar to that seen of microglial cells stained with microglia cell surface markers (Fig. 4-2c). A small

population of TUNEL cells displayed both cytoplasmic and nuclear staining revealing an overlap between TUNEL and DAPI staining (Fig. 4-2a,b). Cytoplasmic staining did not appear to be contained in lytic vesicles characteristic of phagocytosed debris making it unlikely to be degraded DNA from adjacent dying cells.

While no co-labeling was performed to identify the TUNEL labeled cells, previous studies have identified microglia as the only cell to die during the regenerative process in the facial nucleus (Gehrmann and Banati, 1995). In addition, the morphology of the TUNEL cells is identical to microglia morphology.

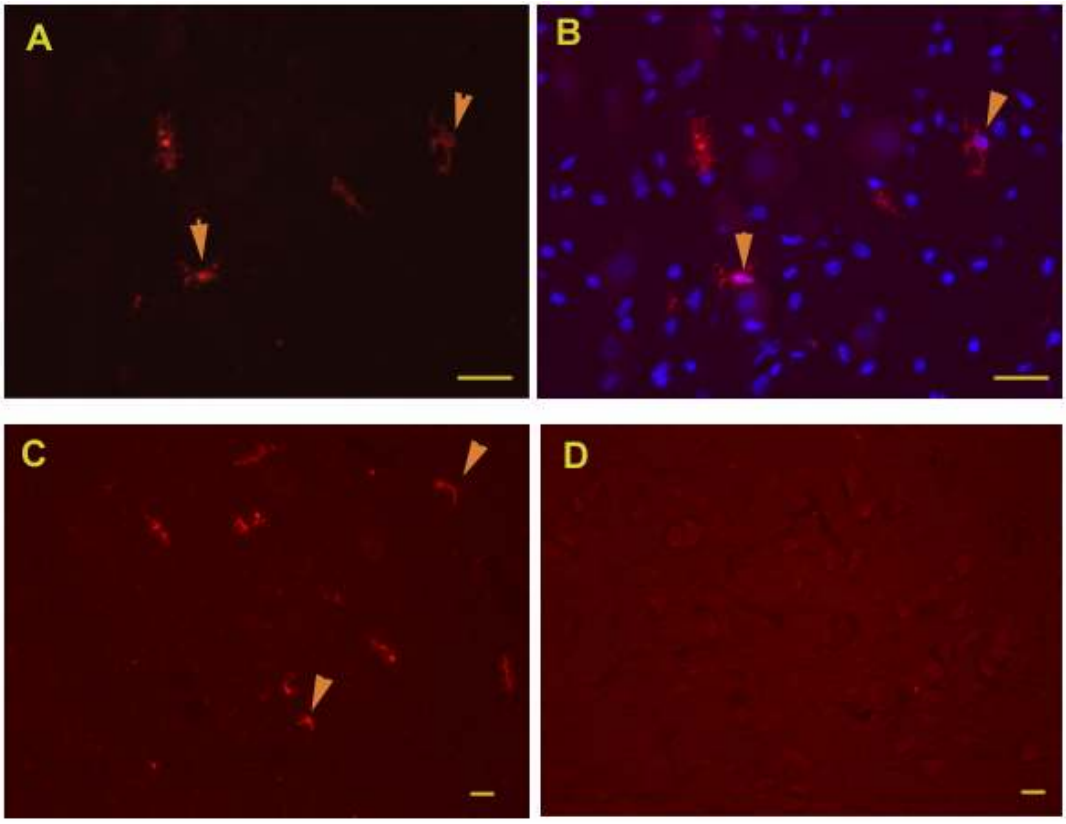


Figure 4-2. Non-classical TUNEL positive cells were found throughout the neuropil of the injured facial nucleus 14 days post-injury. A) TUNEL labeling in the facial nucleus displaying cytoplasmic staining representative of microglial cells. B) Section of double labeled TUNEL/DAPI cells. The arrows identify nuclear and cytoplasmic staining of microglia. C) Facial nucleus 14 days post-axotomy with arrows identifying the perineuronal position of microglia. D) Contralateral, unoperated facial nucleus. Bar = 80μm

Discussion

Axotomy of the rat facial nucleus leads to mitotic division of microglial cells leading to an increase in cell number (Graeber et al., 1988b; Gehrmann and Banati, 1995). Upon recovery of motoneurons, microglial are thought to undergo programmed cell death to return to normal homeostatic levels (Gehrmann and Banati, 1995; Jones et al., 1997). The current study investigated the time course of microglial turnover in the facial nucleus following a crush injury. The greatest density of TUNEL positive microglia was seen at 14 days post axotomy where successful reinnervation of the facial muscles has occurred (Kamijo et al., 2003). The correlation between regeneration and microglia turnover further supports the essential role of programmed cell death in regulating the immune response in the CNS following an injury in order to maintain the homeostatic CNS environment (Fig 4-3).

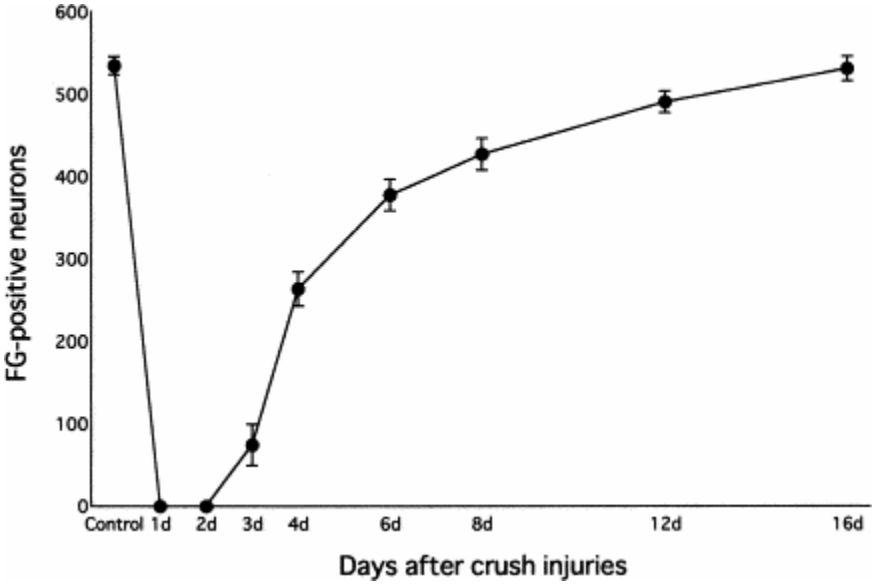


Figure 4-3. Flourogold labeled neurons at different survival times following a crush injury in the rat facial nucleus. The regenerative patterns of neurons shows a positive correlation with microglia turnover (Kamijo et al., 2003).

TUNEL labeling in the facial nucleus demonstrated diffuse cytoplasmic and nuclear staining of microglial cells, similar to previous studies that found non-typical cytoplasmic staining of microglial cells following facial nerve injury (Jones et al., 1997). To dismiss handling artifact, the study conducted by Jones and collages examined different fixations, pretreatment and staining methods where it was found cytoplasmic staining was present under all experimental conditions. The authors concluded that the non-typical TUNEL staining suggested microglial turnover occurred through a non-classical form of programmed cell death.

As the only cell in the CNS capable of transforming into phagocytic cells, microglia can engulf extracellular and neuronal debris which may contain degraded DNA. *In vivo* it is known that microglia are recruited to neuronal debris and responsible for their removal (Moller et al., 1996) however, this is an unlikely explanation for the diffuse cytoplasmic staining seen since there was no evidence of phagosomal structures. Furthermore, facial nerve injury in the rat does not induce neuronal death eliminating the phagocytic properties of microglia (Moran and Graeber, 2004). However, ricin/axotomy model leads to extensive neuronal degeneration followed by microglial phagocytosis but does not show a significant increase in the number of TUNEL labeled cells (Jones et al., 1997).

CHAPTER 5
MICROGLIA UNDERGO MORPHOLOGICAL AND FUNCTIONAL ABNORMALITIES IN THE SUPEROXIDE DISMUTASE 1 RAT

Introduction

Amyotrophic lateral sclerosis (ALS) is an adult onset neurodegenerative disease characterized by selective loss of upper and lower motor neurons. Loss of motor neurons results in muscle paralysis and ultimately death due to respiratory failure. 5-10% of ALS cases are familial, inherited in an autosomal dominant pattern (Mulder et al., 1986; Deng et al., 1993; Rosen et al., 1993; Siddique and Deng, 1996) Of familial ALS cases, 20% have been linked to mutations located in the Cu/Zn superoxide dismutase 1 (SOD1) gene (Rosen et al., 1993; Siddique and Deng, 1996). The identification of SOD1 gene mutations has provided insight into understanding the molecular pathology of ALS. Specifically, transgenic rodent models expressing SOD1 mutant G93A have provided a model that closely resembles the human form of the disease, however to date there is no single mechanism that can be identified in the etiology of ALS. Recent literature has focused on non-neuronal cells in the propagation of ALS. Several studies have shown expression of mutant SOD1 limited to motor neurons is insufficient to cause motor neuron degeneration supporting glial cell involvement in ALS (Pramatarova et al., 2001; Lino et al., 2002; Clement et al., 2003). *In vitro* studies found differences in TNF-α levels secreted following stimulation with LPS when comparing microglia isolated from transgenic and wild type mice at day 60 (Weydt et al., 2004). Transgenic microglia produced higher levels of TNF-α. In addition, increased levels of microglial activation

are readily discernible in affected areas in both human and animal models of the disease (Kawamata et al., 1992; Hall et al., 1998; Alexianu et al., 2001). Thus, from current evidence it may be proposed that mutant SOD1 may cause abnormalities in microglial cells in ALS that alter normal cell function. The abnormal microglia could undergo functional changes that result in increased levels of cytotoxicity further propagating disease. Alternatively, microglial may become senescent or dysfunctional due to the SOD1 mutation thereby reducing the number of functional microglia able to provide trophic support to motor neurons furthering neuronal cell death.

The aim of the present study is to characterize microglia in the mutant G93A SOD1 transgenic rat, specifically the morphological changes that occur throughout the brain and spinal cord at specific stages of the disease. In addition, this study will investigate microglia turnover in the spinal cord and following a facial nerve injury to determine if mutant SOD1 expression in microglia causes the cells to be more susceptible to apoptosis causing fewer microglial to be readily available in maintaining a healthy environment for neurons.

Materials and Methods

Animals and Surgery

Animal use protocols were approved by the University of Florida Institutional Use and Care of Animals Committee (IUCAC). All transgenic animals used in this study were male Sprague Dawley NTac:SD-TgN(SOD1G93A)L26H rats obtained from Taconic Farms. Animals were monitored daily to assess muscle weakness and to record disease progression.

To examine microglial morphology OX-42, OX-6 and TUNEL staining were used at three stages of the disease: asymptomatic where animals had no visual muscle

weakness, onset of symptoms where animals first showed evidence of weakness in the hind limb and end stage where animals were no longer able to right themselves after 30 s. Animals were sacrificed by transcardial perfusion as detailed in Chapter 2 at the specified stage of the disease. Immediately following perfusion brains and spinal cords were removed and fixed in 4% paraformaldhyde for 2 h. For OX-42, OX-6 and TUNEL labeling the tissue was placed in 30% sucrose until the tissue was saturated. All time points included 4 transgenic animals and 4 age-matched wild type controls.

To assess microglial turnover in the facial nucleus, 6 non-symptomatic SOD1 transgenic and 6 age-matched control animals underwent a facial nerve axotomy where under isoflurane anesthesia, the right facial nucleus was exposed at the exit from the stylomastoid foramen and crushed with a hemostat for 10 s. At 14 days post-axotomy animals were sacrificed by transcardial perfusion as described in Chapter 2. The brains were immediately removed and placed in 4% fixative overnight. Tissue sections containing the facial nucleus were paraffin embedded as detailed in Chapter 2, cut ona microtome at 7 μm, and mounted on SuperFrost Plus slides. A number of slides from these animals were labeled with lectin as described in the lectin histochemistry section found in a later paragraph in the materials and methods section.

Table 5-1. Age and corresponding disease stage for animals used in experiments.

	Age of Asymptomatic Animals	**Age of Onset Animals**	**Age of Endstage Animals**
OX-42/OX-6 Labeling	74-84 days	113-117 days	135-140 days
TUNEL/Lectin Labeling	107-114	N/A	N/A

OX-42 and OX-6 Immunohistochemistry

Lumbar spinal cord, cortical, and brainstem sections were serially cut at 20 μm on a cryostat, mounted on Superfrost Plus slides and air dried for one hour. Sections were pretreated in 0.5% PBS-Triton for 15 min, blocked in 10% normal goat serum for 30 min and incubated overnight at room temperature in the primary antibody diluted in buffer. The primary antibodies included MRC OX-42 (Serotec, Cambridge, UK) and MRC OX-6 (Serotec, Cambridge, UK) at 1:500. The slides were rinsed in PBS and incu bated in secondary antibody (1:500) for 1h. Following incubation, slides were rinsed for 9 min and Horseradish Peroxidase Avidin D was applied (1:500) (Vector, Burlingame,CA) and incubated for 30 min. Slides were washed and immunoreactivity was visualized with 3,3'-diaminobenzidine (DAB)-H_2O_2 substrate. After a brief rinse, slides were dehydrated in increasing concentrations of ethanols, cleared in xylene, and coverslipped using Permount mounting medium (Fisher Scientific).

Quantification of Immunohistochemistry Labeling in the Ventral Spinal Cord

OX-42 and OX-6 expression in the ventral spinal cord was quantified using Image Pro Plus software. The area occupied by labeled cells was highlighted and measured for each section of spinal cord (6 sections per animal) then expressed as a percentage of total area of ventral spinal cord. Using GraphPad Prism software (San Diego, CA) a t-test was performed to determine statistical significance between transgenic SOD 1 and control animals at each time point. A one-way ANOVA was performed to compare differences among the transgenic animals followed by a Tukey multiple comparison test. A significance level of $p<0.05$ was used.

TUNEL labeling and cell identification in the spinal cord

Lumbar spinal cord sections were serially cut at 20 μm on a cryostat, mounted on Superfrost Plus slides and air dried for one hour. The ApopTag Red *In Situ* Apoptosis Detection Kit (Serologicals Corporation, Norcross, GA) was used as described in the manufacturer's protocol omitting the pretreatment step. To identify the cell type of TUNEL positive cells RIP1 (oligodendrocytes), GFAP (astrocytes), OX-42 and OX-6 (microglia) were used. Following the TUNEL procedure, sections were pretreated in 0.5% PBS-Triton for 15 min, blocked in 10% normal goat serum for 30 min and incubated overnight at room temperature in the primary antibody diluted 1:500 in buffer. The slides were rinsed in PBS and incubated in secondary antibody (1:500) for 1h. Following incubation, slides were rinsed for 9 min and FITC-Avidin D was applied (1:500) (Vector, Burlingame,CA) and incubated for 30 min. Slides were rinsed briefly and coverslipped.

Lectin histochemistry

Prior to lectin staining, sections were deparaffinized through xylenes, graded alcohols and rinsed in PBS. Next, the slides were trypsin treated (0.1% trypsin, 0.1% $CaCl_2$) for 12 min at 37°C. Following a 10 min wash the slides were incubated overnight at 4°C in lectin GSA I-HRP (Sigma Chemical Co.) diluted 1:10 in PBS containing cations (0.1 mm of CaCl2, MgCl2 and MnCl2) and 0.1% Triton-X100. After overnight incubation slides were briefly rinsed in PBS and visualized with 3,3'-diabimobenzidine (DAB)- H_2O_2 substrate. Sections were counterstained with cresyl violet, dehydrated through ascending alcohols, cleared in xylenes and coverslipped with Permount.

TUNEL and Lectin Double Labeling

Microglial cell death was visualized in the facial nucleus using TUNEL on tissue sections from animals 14 days post-facial nerve axotomy. Sections were deparaffinized, washed in PBS, trypsin treated (0.1% trypsin, 0.1% $CaCl_2$) for 12 min at 37°C and rinsed two times in PBS for 5 min each. The ApopTag Red *In Situ* Apoptosis Detection Kit (Serologicals Corporation, Norcross, GA) was then used as described in the manufacturer's protocol omitting the pretreatment step. Following TUNEL, sections were stained using GSA I-FITC (Sigma, St.Louis, MO) according to protocol described in the previous lectin section. Six sections containing the facial nucleus per animal were manually counted for TUNEL labeled cells and statistically evaluated using an unpaired t test.

Brown and Brenn Gram Stain

A gram stain was performed on tissue containing the facial nucleus from transgenic SOD1 animals 14 days post-axotomy. The tissue was handled and processed following the same protocol detailed for the TUNEL labeling in the facial nucleus. Slides were deparaffinized and hydrated to distilled water. Next 1 mL of crystal violet solution and 5 drops of 5% sodium bicarbonate solution were added to sections and allowed to sit for one minute then rinsed in tap water. The slides were decolorized with acetone, rinsed in water, flooded with basic fuchsin working solution for 1 min, rinsed again and placed in water. Each slide was individually dipped in acetone to start reaction and immediately differentiated with picric acid-acetone solution until turning a yellowish pink. Finally slides were quickly rinsed in acetone, then in acetone-xylene solution, cleared in 2 changes of xylene and mounted with resinous medium.

Results

No change in microglia morphology or activation was seen in the cortex of SOD1 animals. To investigate changes in microglia morphology in the transgenic SOD1 rat, a number of microglia markers were used for staining at various time points throughout disease progression. Numerous CNS regions were investigated to determine if microglia change was limited to areas where extensive neuronal degeneration occurred or rather was a widespread effect.

The cortex revealed OX-42 positive microglia with a normal resting morphology distributed evenly throughout the cortex (Fig. 5-1a). OX-6 staining labeled a small number of microglial cells in the gray matter indicating an absence of MHC II positive microglia in the cortex of ALS rats at all stages of the disease (Fig. 5-1b).

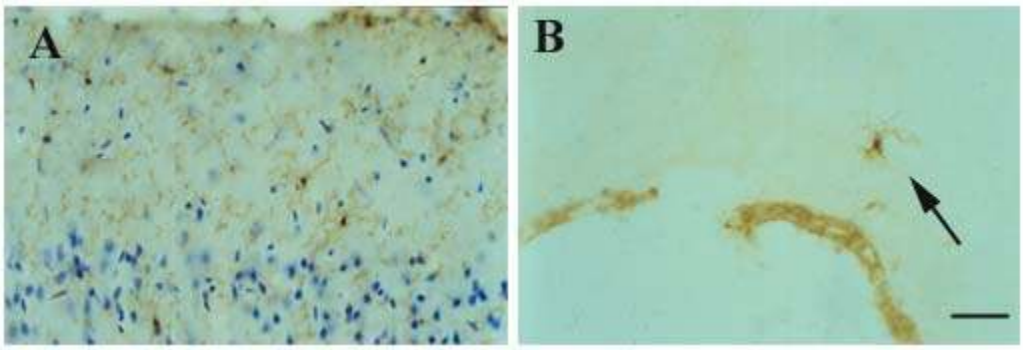


Figure 5-1. Photomicrographs of microglia labeling in the cortex of SOD1 rats. A) OX-42 labeled cells in animals with early onset of symptoms displaying a ramified morphology representative of a resting state. B) Cortex of end stage animal labeled with OX-6 showing that there is a lack of MHCII expression in SOD1 animals. The arrow identifies a labeled microglia confirming that the OX-6 stain worked in the cortex. Bar = 40μm

Abnormal microglial fusion and activation was present at the level of the red nucleus. The brainstem portrayed drastically different characteristics of microglia throughout disease progression. Morphological changes of microglial cells were seen prior to onset of symptoms at the level of the red nucleus (Fig. 5-2a,d) and persisted until

end stage. OX-42 staining revealed intense microglial activation within the red nucleus at all 3 stages of the disease. A distinguishable border was present between the red nucleus and the surrounding tissue clearly demonstrating that intense microglial activation was contained to the red nucleus (Fig. 5-2e). It appears that activation occurred prior to neuronal degeneration since neuronal populations of the red nucleus were similar between control and transgenic animals in asymptomatic animals. Further evidence of microglial changes at the level of the red nucleus were seen with the presence of microglial fusions (Fig. 5-2f). The majority of fusions were located within the nucleus and contained a large number of microglial cells clumped together. Upon close observation, it is evident that the fusions were microglia because the cells were OX-42 positive and displayed a morphology representative of microglia. With further disease progression the fusions were distributed throughout the entire level of the red nucleus.

In the same sections containing the red nucleus, the oculomotor nucleus and substania nigra were investigated and found to have no microglial fusions and a population of evenly dispersed ramified microglial cells which remained throughout disease progression (Fig. 5-2b,c).

SOD1 animals present abnormal microglia fusions at the level of the facial nucleus. When looking at the level of the facial nucleus similar changes were seen as in the red nucleus. Asymptomatic animals showed a small number of fused microglia however no other abnormalities or activation were seen. With the appearance of muscle weakness through the end stage of the disease, increasing numbers of microglial fusions were present with the majority of fusions distributed outside the facial nucleus. The fusions varied in appearance with some displaying a long string of microglial cells

whereas others were rounded fusions of microglial cells representing multinucleated giant cells (Fig. 5-3a,b,c). The giant cells had microglial nuclei orientated in a circle and were intensely stained with both OX-42 and lectin (Fig. 5-3d). Unlike the red nucleus, the facial nucleus did not have increased levels of microglial activation throughout the disease process. The microglial population in the nucleus was found to have a ramified morphology indicative of a resting state. An unexpected finding was seen in one animal where a nidus of bacillus bacteria was detected within the tissue section (Fig. 5-4d). However, when trying to confirm the presence bacteria using a gram stain there was no evidence of bacteria.

In addition to microglial abnormalities there was evidence of pathological changes of neurons. It appears that neuronal bodies are undergoing degenerative changes as seen by the separation of dendrites from their neuronal bodies (Fig. 5-4a,b). The majority of fragmented dendrites were located in vacuoles that become increasingly apparent in the diseased brainstem (Fig. 5-4c).

Microglial activation and abnormalities were evident in the ventral spinal cord prior to onset of muscle weakness. Microglial response to disease progression in the ventral spinal cord was assessed using OX-42 and OX-6 markers. OX-6 labeling revealed an increase in density of MHC II positive labeling with progression of the disease and was significantly higher in transgenic animals when compared to age-matched controls after the occurrence of symptoms (Figs.5-5, 5-6). OX-42 density in the ventral spinal cord initially increased with the occurrence of symptoms however decreased in number at the end stage of the disease. Elevated levels of OX-42 expression

were found in the transgenic SOD1 animals at all time points when compared to age-matched controls (Figs.5-7, 5-8).

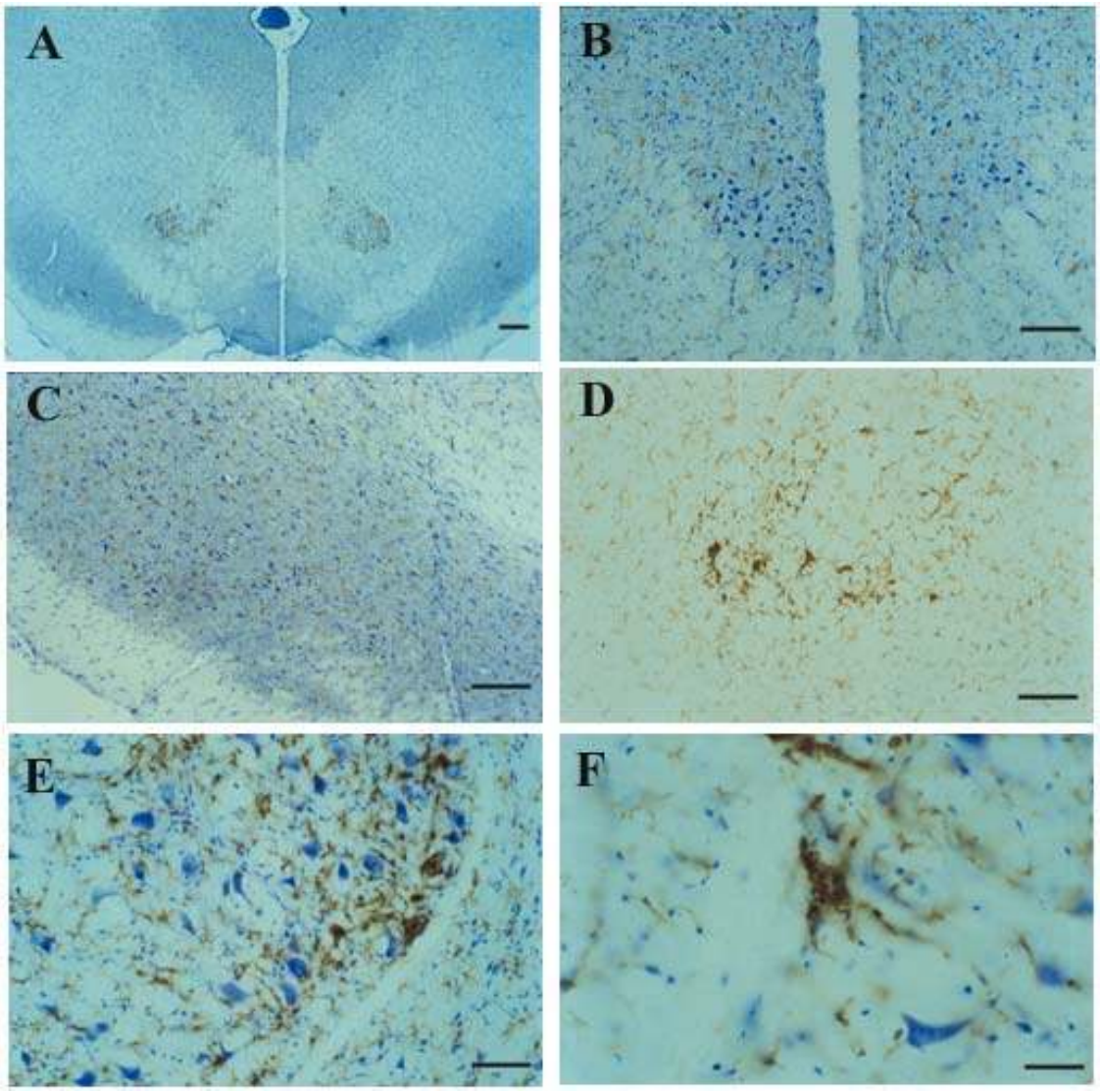


Figure 5-2. Photomicrographs representing aberrant microglial activation at the level of the red nucleus in early onset SOD1 animals. All sections were stained with OX-42. A) Midbrain section displaying intense microglial activation located in the red nucleus. B) Oculomotor nucleus showing a lack of aberrant microglial activation. C) Subtania nigra displaying microglia with a ramified resting morphology. D) Higher magnification of the microglial response in the red nucleus. (A-D) Bar = 160μm E) The border separating the microglial activation in the red nucleus from the surrounding tissue. Bar = 80μm F) Presence of fused microglia located in the red nucleus intensely stained with OX-42. Bar = 40 μm

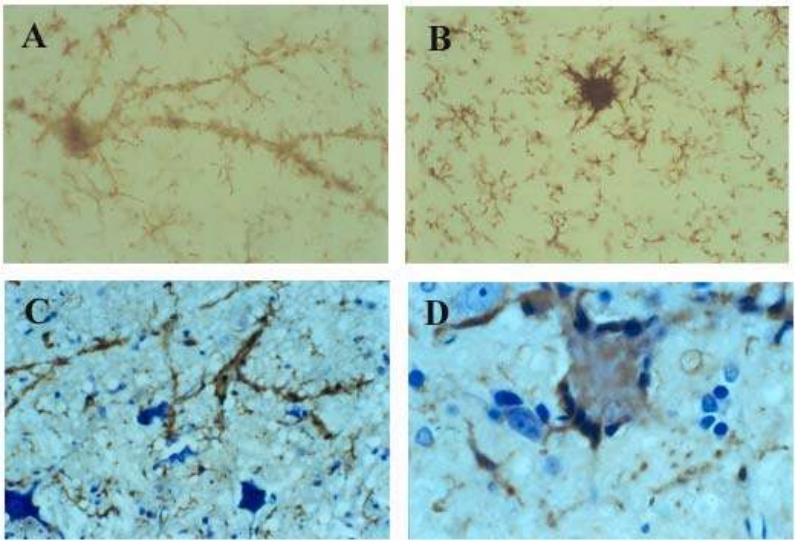


Figure 5-3. Abnormal morphological changes seen at the level of the facial nucleus in SOD1 rats. A) Microglial fusion displaying a long rod-like structure in asymptomatic animals (day 74) labeled with OX-42. Note the individual microglial cells and the ability to observe the processes of microglia. B) Microglial fusion displaying a rounded structure in asymptomatic animals labeled with OX-42. C) Rod-like microglial fusions (lectin and creysl violet labeled) in day 107 animals displaying hind limb weakness. (A-C) Magnification 250x. D) Multinucleated giant cell of the Langhans type (lectin labeled) in animals with hind limb weakness. Magnification 630x.

Microglial fusions as well as multinucleated giant cells were present in the gray matter of the spinal cord with the majority distributed in the ventral horn (Fig.5-9b). The fusions were identical to those seen in the brainstem and were in addition to phagocytic clusters that were present as a direct result of neuronal degeneration (Fig. 5-9a). The two were distinguishable because the clusters were limited to areas surrounding ventral horn neurons whereas the fusions were not always in the vicinity of neurons. Only one fusion was located in the white matter, with the majority of the microglial population appearing normal. In the end stage animals, OX-42 staining revealed swollen, fragmented, non-ramified microglial cells dispersed throughout the ventral horn suggesting that cell integrity was impaired (Fig. 5-9c). OX-6 labeled microglial were often associated with

multinucleated giant cells at the end stage, however also revealed a population of fragmented swollen microglial cells (Fig. 5-9d).

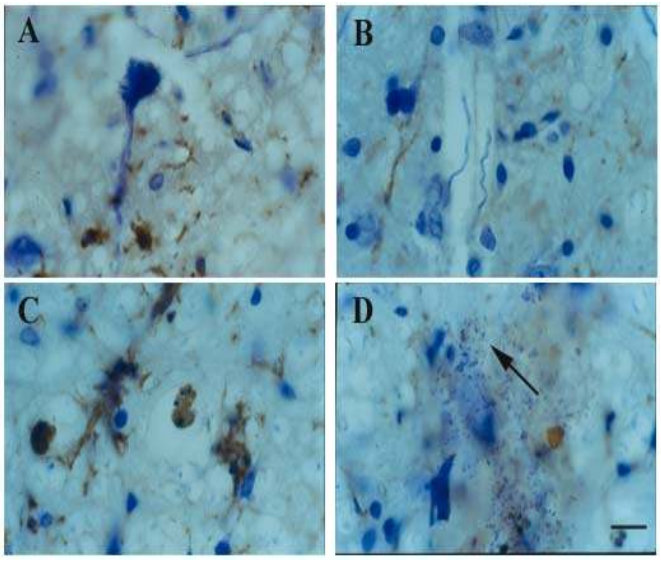


Figure 5-4. Pathological changes occurring in early SOD1 symptomatic animals. A) Neuronal fragmentation due to vacuolization. B) Dendrites separated from neuronal bodies located in vacuoles. C) Vacuole containing a lectin labeled cell body. D) Evidence of bacillus bacteria suggesting an impaired immune response. Bar = 16μm

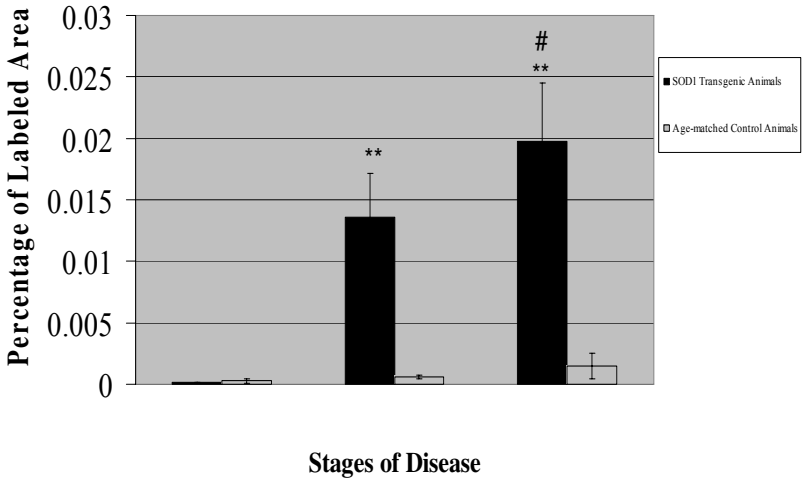


Figure 5-5. Percentage of area covered by OX-6 immunoreactive cells in the ventral horn of lumbar spinal cord of SOD1 transgenic rats and age-matched control rats from 74 days to156 days. Columns represent mean ± S.E.M of 4 animals for each time point. **P<.001 with respect to the corresponding age-matched control rats and #P<.05 with respect to presymptomatic transgenic rats.

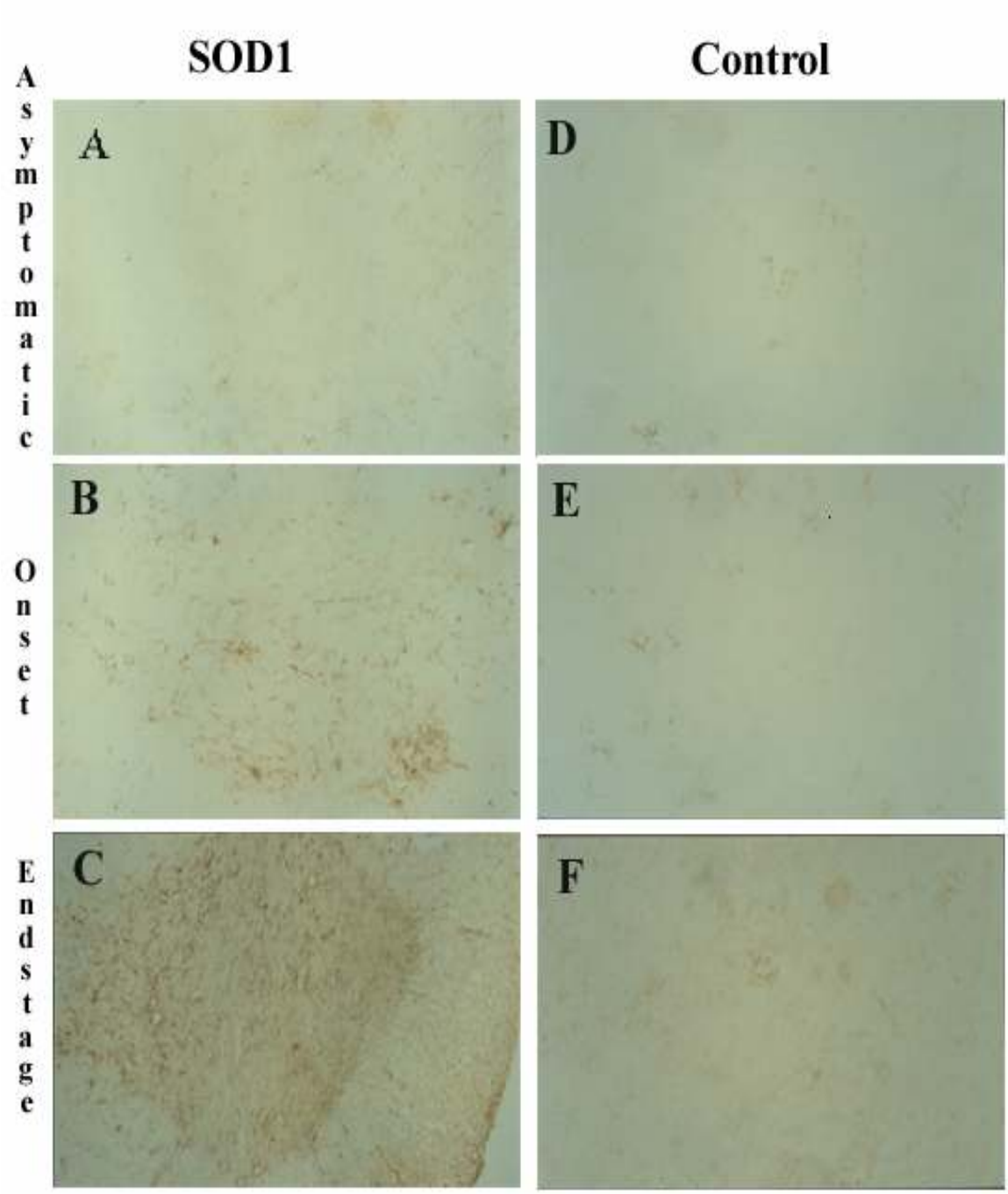


Figure 5-6. Photomicrographs demonstrating change in OX-6 expression with age. A-C) OX-6 labeled microglia in the ventral spinal cord of SOD1 animals throughout the disease progression. OX-6 density increased throughout the disease progression. Magnification 60x. D-F) OX-6 labeled microglia in the ventral spinal cord in age-matched control animals. Magnification 125x.

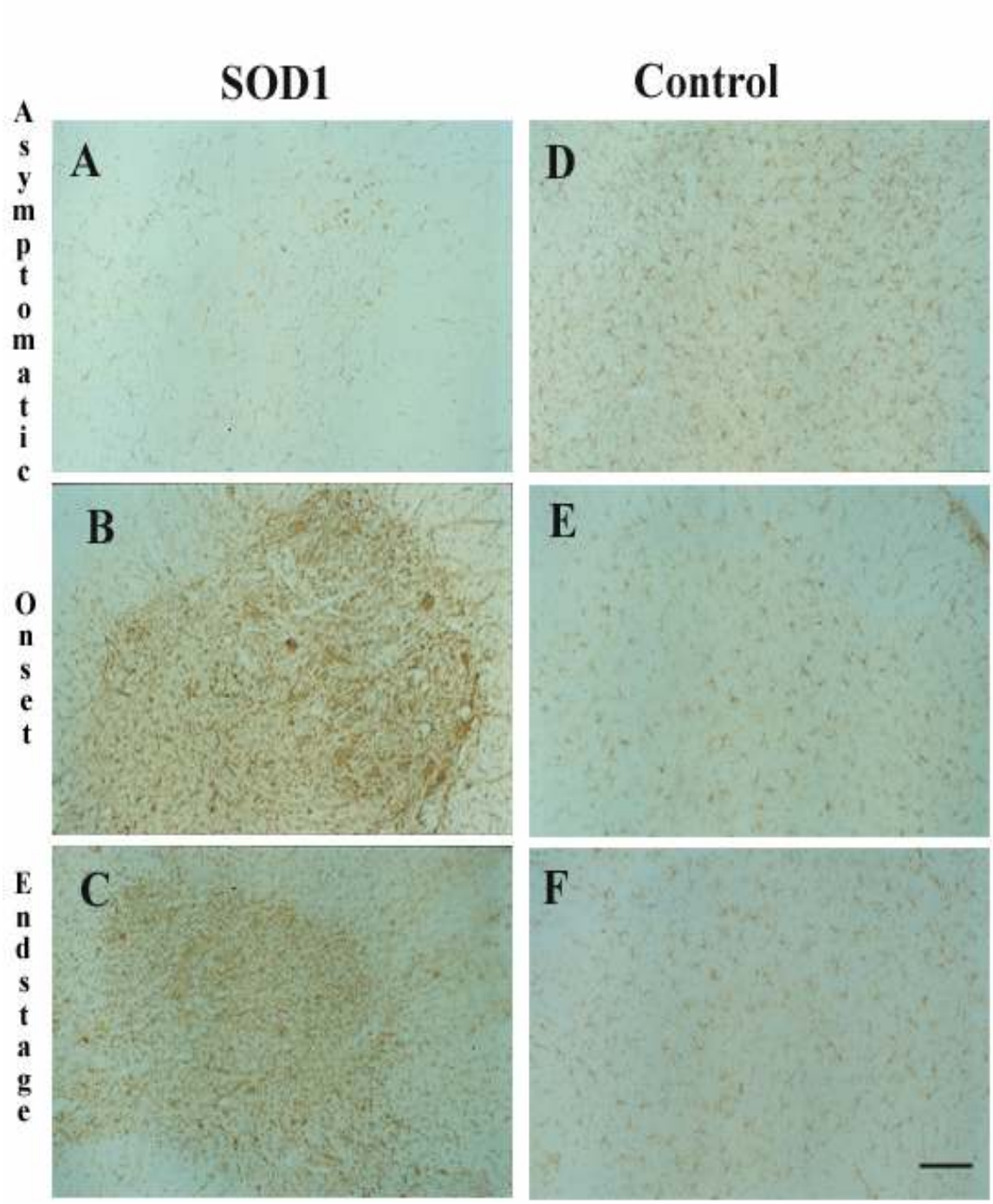


Figure 5-7. Photomicrographs demonstrating change in OX-42 expression with age. A-C) OX-42 labeled microglia in the ventral spinal cord of SOD1 animals throughout the disease progression. An initial increase of OX-42 density is seen in with the onset of symptoms that declines in end stage animals. D-F) OX-42 labeled microglia in the ventral spinal cord in age-matched control animals. Bar = 160μm

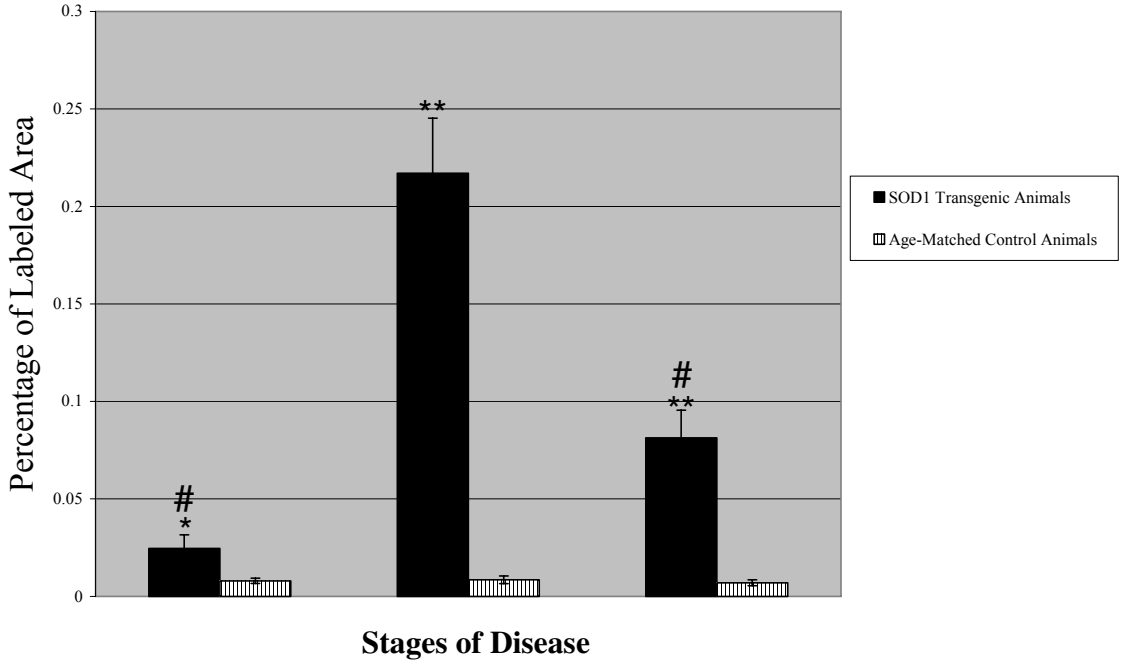


Figure 5-8. Percentage of area covered by OX-42 immunoreactive cells in the ventral horn of the lumbar spinal cord of SOD1 transgenic rats and age-matched control rats from 74 days to156 days. Columns represent mean ± S.E.M of 4 animals for each time point. *P<0.05 and **P<0.001 with respect to corresponding age-matched control rats and #P<0.05 with respect to transgenic onset of symptom rats.

TUNEL positive cells were dispersed throughout the lumbar ventral spinal cord in ALS animals. TUNEL labeling was performed on spinal cord sections to determine if microglial cells were undergoing apoptosis due to cellular senescence. TUNEL positive cells were found at all stages of the disease with the majority located in the ventral area of the spinal cord displaying classic nuclear staining. A few isolated TUNEL positive cells in asymptomatic and early symptomatic animals portrayed nuclear and diffuse cytoplasmic staining similar to the TUNEL positive cells that were identified as microglial cells in the facial nucleus (Fig. 5-10a). Control spinal cord sections had similar occurrence of cytoplasmic/nuclear stained TUNEL positive cells suggesting that microglial turnover in the spinal cord regulated by apoptosis is unchanged in transgenic

animals (Fig. 5-10c). End stage animals displayed nuclear staining orientated in a string of labeled cells not representative of microglial cells (Fig 5-10b). Co-labeling with markers for each CNS cell type revealed no overlap between cell-specific markers and TUNEL labeled cells not allowing conclusive identification of apoptotic cells.

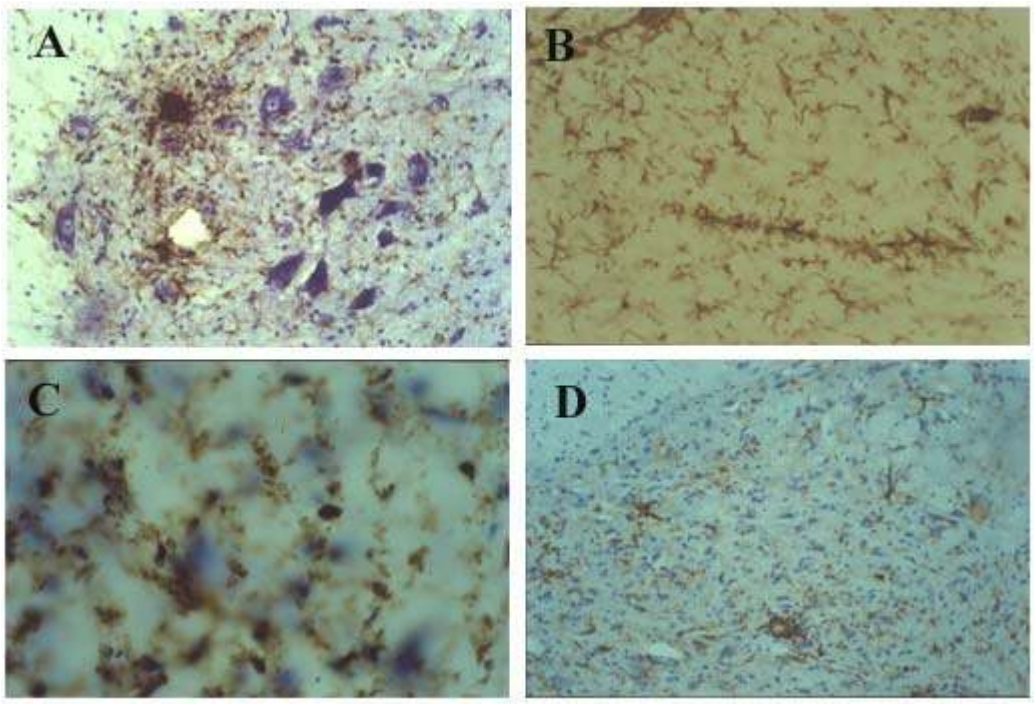


Figure 5-9. Microglial response and changes in SOD1 animals in the ventral spinal cord. A) Microglial activation seen in close proximity to motor neurons located in the ventral horn of asymptomatic animals (OX-42 labeled). B) Rod-like microglial fusions stained with OX-42 in asymptomatic animals. The fusions are similar to those seen in the brainstem in SOD1 animals. C) OX-42 labeled microglia in end stage animals portraying an abnormal swollen morphology representative of degenerative changes. D) OX-6 labeling of giant cells in end stage animals suggesting a relationship between giant cells and MHCII expression. Magnification A,B,D 125x and C 630x.

TUNEL positive cells were found at significantly lower numbers in the SOD1 facial nucleus 14 days post-axotomy. TUNEL-positive cells in the SOD1 transgenic and wild type axotomized facial nucleus lacked the classic nuclear staining instead having cytoplasmic staining similar to previous studies of apoptotic microglia (Gehrmann and Banati, 1995; Jones et al., 1997). In addition, double-labeling revealed an overlap

between lectin and TUNEL staining further identifying TUNEL-labeled cells as microglia (Fig. 5-12). TUNEL/lectin positive cells distribution was limited to the facial nucleus.

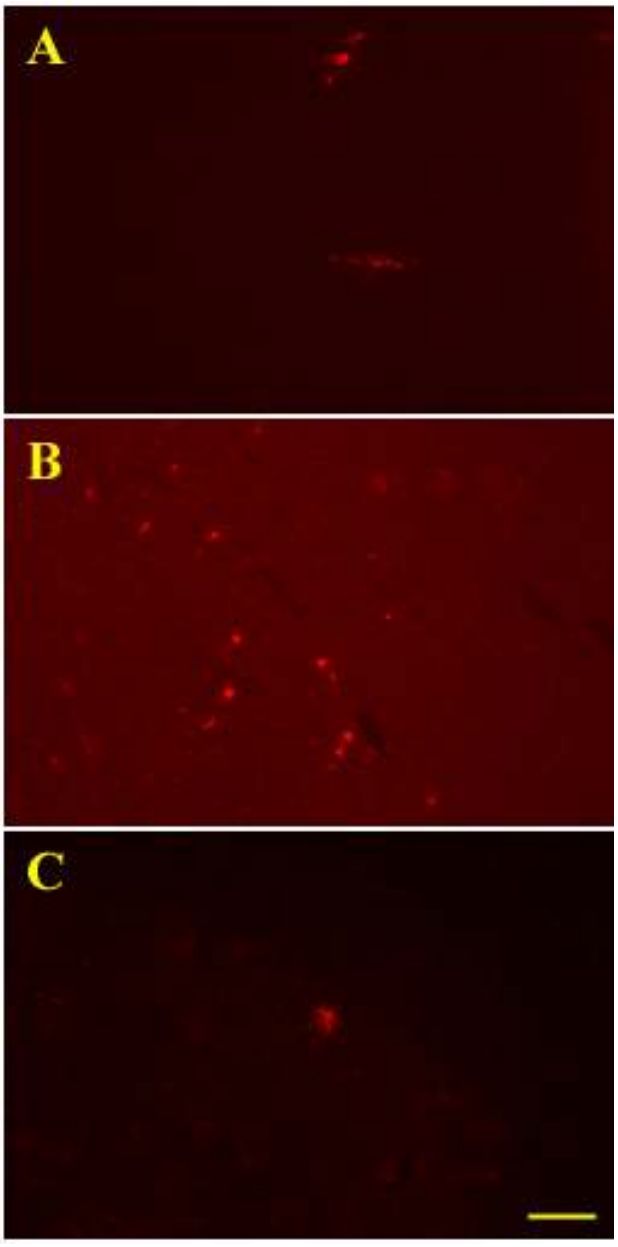


Figure 5-10. TUNEL positive cells in the ventral lumbar spinal cord in ALS animals. A) Diffuse cytoplasmic and nuclear staining in asymptomatic animals (Day 74). B) End stage TUNEL labeling with exclusive nuclear staining orientated in a string of positive cells. C) Control TUNEL labeling revealing cytoplasmic and nuclear staining similar to TUNEL positive cells seen in asymptomatic and early symptomatic animals. Bar = 80μm.

The number of TUNEL labeled cells in the injured facial nucleus was significantly lower ($p<0.001$) in the transgenic animals when compared to age matched controls ((Fig. 5-11) SOD1 transgenic animals 0.5278 ± 0.1847, control animals 5.357 ± 0.5058)). No TUNEL-positive cells were found in the unoperated facial nucleus in both animal groups. Qualitative analysis of a nissil stain revealed similar numbers of nuclei in the transgenic and control animals. In addition, the microglial response 14-days post axotomy appeared to be similar between control and transgenic animals both displaying perineruonal microglia and qualitatively revealing a similar density of lectin labeling within the nucleus.

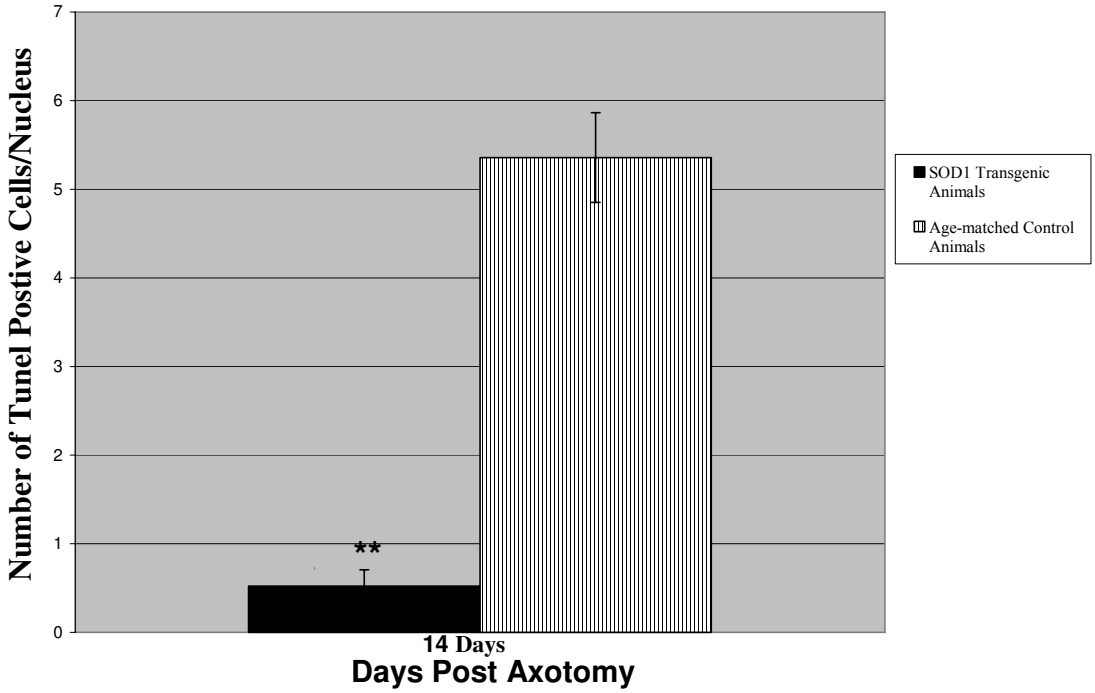


Figure 5-11. Number of TUNEL positive cells in facial nucleus 14 days post axotomy in non-symptomatic transgenic and age-matched control animals. Columns represent mean ± S.E.M of 6 animals for each time point (**P<0.001).

Discussion

The current study demonstrated for the first time a presence of microglial fusions and Langhans giant cells in the ALS rat model. Additional pathological changes were observed at the levels of the red and facial nucleus in the brainstem where aberrant microglial activation and neuronal fragmentation was seen. Evidence of abnormal microglial function was found in the TUNEL studies where SOD1 animals had significantly reduced levels of TUNEL positive cells in the facial nucleus compared to age matched controls 14 days post-axotomy. The current findings demonstrate that microglial cells undergo morphological and functional changes during the disease progression in ALS animals.

The presence of multinucelated giant cells was an unexpected finding since these cells are often only associated with viral and bacterial infections, which to date have not been reported in ALS animal models. The current study demonstrated a large number of multinucleated giant cells of the Langhans type throughout the CNS of the rat transgenic model. Langhans giant cells are characterized by nuclei orientated around the periphery of the cell and most often associated with granulomatous reactions, specifically *Mycobacterium tuberculosis*. The presence of indigestible particles of an organism causes macrophages to aggregate at the site and engulf the foreign particle. During the ensuing days, a cell-mediated immunity to the bacterium develops leading to T lymphocyte recruitment and release of cytokines some of which cause further recruitment of macrophages and cell fusion through induction of cell surface adhesion molecules (Lee et al., 1993). *In vitro* studies have found that interleukin-3 and interferon-γ induced Langhans multinucleated giant cell formation in the presence of granulocyte macrophage-colony stimulating factor (GM-CSF) (McNally and Anderson, 1995). The characteristics

of Langhans giant cell formation suggests the presence of bacteria in the ALS rat model which was supported by the observed nidus of bacillus bacteria in a tissue section of a transgenic animal. The fact that we detected bacilli and giant cells in animals that develop neurodegenerative disease leads us to conclude that a common link for the development of brain infection and neurodegeneration may be found in dysfunction of microglia that results in impaired immunological defense mechanisms and diminished neuroprotection. Further evidence of this novel theory is found in human immunodeficiency virus encephalitis (HIVE) cases where viral infected microglial cells portray altered cytokine production and formation of mulitnucleated giant cells that accompany neurodengerative changes (Nottet et al., 1997; Zheng and Gendelman, 1997; Kaul et al., 2001). In ALS, microglial dysfunction may be a direct result of the SOD1 mutation whereas in HIV encephalopathy microglial dysfunction may be a result of viral infection both rendering detrimental consequences for the neuronal cell population. An additional link between ALS and HIV is seen in a number of HIV patients that were clinically diagnosed with ALS suggesting a related mechanism of disease propagation (Verma et al., 1990; Huang et al., 1993; Casado et al., 1997; Galassi et al., 1998; MacGowan et al., 2001; Moulignier et al., 2001; Zoccolella et al., 2002).

In addition to the presence of giant cells, intense microglial activation was seen throughout the brainstem and in the ventral horn of the lumbar spinal cord. Microglial activation was seen in the spinal cord corresponding to neuronal degeneration, however in the brainstem the activation is not a direct result of neuronal degeneration but may be due to elevated levels of microglial-activating factors identified in cerebral spinal fluid and serum of ALS patients.

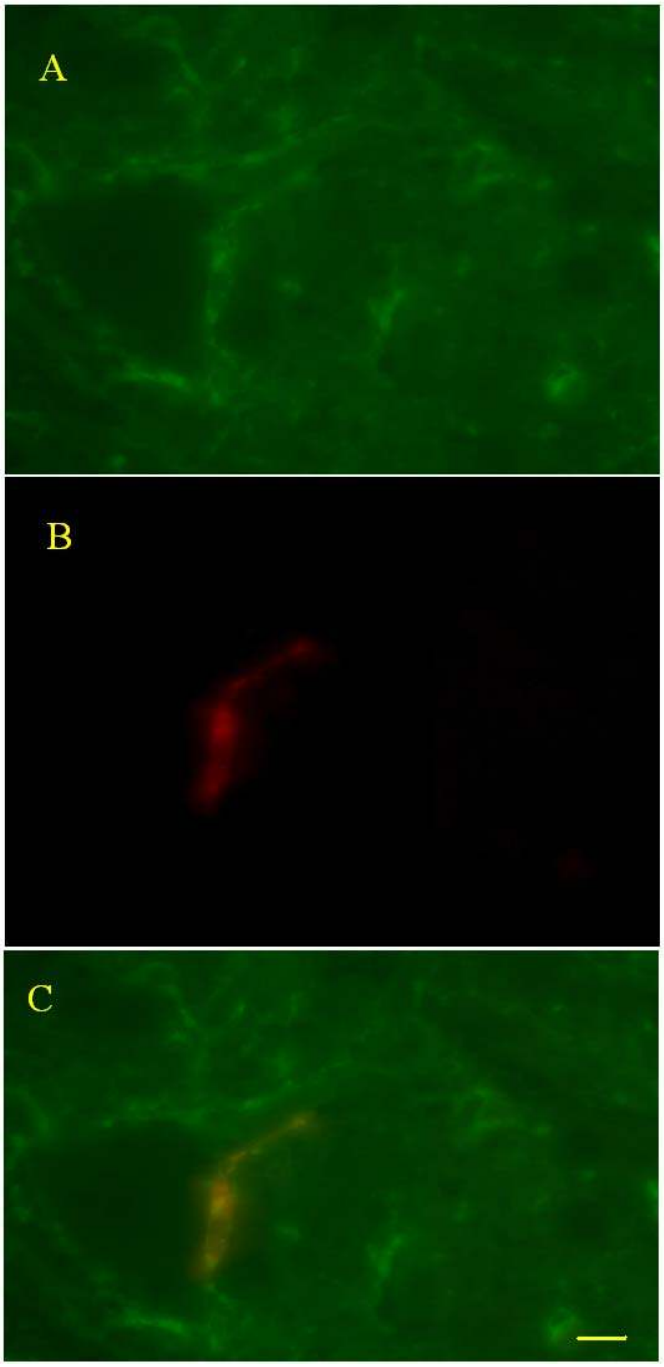


Figure 5-12. Apoptotic microglial cells in the facial nucleus following injury. A) Lectin labeled perineuronal cell. B) TUNEL labeled cell. C) Merged images confirming TUNEL labeled cells are microglial cells. Bar = 40μm.

Macrophage colony stimulating factor (M-CSF), monocyte chemoattract protein 1 (MCP-1), tumor necrosis factor alpha (TNF-α), and transforming growth factor beta 1 (TGF-ß1) have all been found at elevated levels in ALS when compared to controls (Poloni et al., 2000; Elliott, 2001; Hensley et al., 2002; Ilzecka et al., 2002; Yoshihara et al., 2002; Hensley et al., 2003; Wilms et al., 2003; Henkel et al., 2004). The mechanism in which microglia activating factors are produced is unclear, but may be released by glial cells themselves. The glial cells may be affected by the mutant SOD1 thereby becoming neurotoxic and increasing production of pro-inflammatory molecules. In addition, to activating microglia the elevated levels of inflammatory molecules may further affect the function of microglia propagating the disease. Chronic expression of MCP-1 in the central nervous system causes impairment of microglia function in mice, specifically the ability of microglia to respond to environmental stimuli (Huang et al., 2005).

It is unclear the cause for isolated microglia activation in the red nucleus, but absent in other motor nuclei such as the oculomotor and facial nucleus. The aberrant microglia activation may be dependent on the projection of cells in the specific nuclei. Cells in the red nucleus are the only cells that directly project to spinal cord levels whereas those in the oculomotor, facial and substania nigra have no direct projections to the spinal cord.

Initial observations in the spinal cord confirmed prior reports of prominent microglial activation in areas of motor neuron degeneration. Microglial activation was assessed in the ventral spinal cord using OX-42 and OX-6. A variation in expression between OX-42 and OX-6 was observed. OX-6 expression transiently increased throughout the disease whereas OX-42 levels were increased in asymptomatic animals,

continued to increase with the onset of symptoms followed by a decline in density in end stage animals. OX-6 is often used as a marker of microglial activation which suggests that microglial activation occurs in response to neuronal degeneration in the spinal cord. However, MHC II is not always indicative of activation since non-activated ramified microglia present in young and non-diseased human subjects are positively stained with MHC II (Streit and Sparks, 1997; Streit et al., 2004). Alternatively, MHC II may be a marker of microglia maturation and an early stage of cellular senescence which is supported by the increasing number of MHC II positive microglial cells from infancy to old age (Streit and Sparks, 1997). Therefore, the progressive increase in OX-6 in SOD1 animals may be a result of the SOD1 mutation negatively effecting microglial cells causing increased cellular dysfunction and senescence. The conflicting decline of OX-42 density in end stage animals may be attributed to differential regulation of cell surface markers dependent on the life stage of microglia. OX-6 labeling remains elevated in end stage animals because the remaining microglial cells are senescent whereas OX-42 density declines as a result of microglial degeneration causing a decrease in the overall microglia population. Evidence of abnormal microglia structure as seen with fragmented, swollen OX-42 positive cells in end stage animals is suggestive of microglial degeneration however, the lack of increased levels of apoptotic microglia observed in the transgenic spinal cord suggests that if microglial are under going cell death it is through an alternative pathway.

To investigate microglial function in the SOD1 transgenic animal, this study utilized the facial nerve injury model. Peripheral nerve lesions like the facial nerve axotomy maintain the integrity of the blood brain barrier thus allowing resident microglia

to be studied in the absence of infiltrating blood-derived cells. In non-diseased animals microglia proliferate in response to facial nerve injury (Kreutzberg, 1968; Graeber et al., 1988; Gehrmann and Banati, 1995) reaching a peak at 3 days and gradually decrease in number (Streit et al., 1988; Raivich et al., 1993). The decline in microglia has been attributed to non-classical programmed cell death, a possible mechanism used to regulate post-mitotic microglia populations through elimination of activated microglia (Gehrmann and Banati, 1995; Jones et al., 1997). The lack of TUNEL-positive microglia in transgenic animals may be a mark of abnormalities in microglial regulatory mechanisms. If microglia fail to undergo programmed cell death the levels of microglia will remain elevated and may maintain an activated state causing a cytotoxic environment. Evidence for dysfunctional microglial function in ALS has been seen in the study by Weydt et al. where microglia isolated from the transgenic SOD1 mouse produced higher levels of TNF-α. Another explanation for the lack of TUNEL positive cells in the transgenic animals is that post-mitotic turnover of microglia is controlled by an alternative type of programmed cell death.

Neuronal abnormalities must also be addressed as a factor in the reduction of microglia turnover in transgenic animals. The motoneuron population in the facial nucleus may be affected by the disease process resulting in reduced numbers of motoneurons thereby upon injury a diminished neuronal response is present to induce microglia activation requiring less cell turnover in order to maintain a resting CNS environment. However, qualitative analysis of nissl staining of motoneurons in the facial nucleus revealed similar numbers between transgenic and control animals, which is further supported by magnetic imaging and histochemical studies that establish

motoneuron populations in the facial nucleus remain unaffected in mice prior to onset of symptoms (Nimchinsky et al., 2000; Haenggeli and Kato, 2002; Angenstein et al., 2004). Although, neuronal numbers remain unchanged, previous findings have shown an impaired neuronal response to peripheral injury in the transgenic SOD1 mouse (Mariotti et al., 2002). Following a facial nerve axotomy, levels of NOS immunoreactivity was significantly reduced in the axotomized facial nucleus of transgenic animals. The precise function of NOS induction in the facial nucleus is debated however in cranial motoneurons it has been identified as an indicator of the cell body response to both lethal (Wu, 2000) and non-lethal (Mariotti et al., 2001) peripheral injuries. The lack of NOS induction in the transgenic animals may impair microglia-neuronal signaling thereby reducing the microglia response following injury diminishing microglial turnover.

Contradicting findings on levels of TUNEL labeled cells were seen in the spinal cord and facial nucleus. The facial nucleus showed decreased levels of TUNEL labeled microglia whereas the spinal cord had similar levels of TUNEL labeled microglia as the controls. The discrepancy may be due to regional differences as well as activation levels of the microglial population. The spinal cord presents intense neuronal degeneration accompanied by an intense microglial response whereas the facial nucleus had no evidence of neuronal death.

The current findings suggest an aberrant microglial response in the SOD1 transgenic animal which may be a direct result of the SOD1 mutant resulting in impaired function of microglial cells. Previous studies have found that exclusive expression of SOD1 mutant in neurons (Pramatarova et al., 2001; Lino et al., 2002) or astrocytes (Gong

et al., 2000) is insufficient to cause neuronal degeneration indicating that the mutation must be affecting both the neuronal and glial population.

CHAPTER 6
CONCLUSION

In spite of the large body of evidence indicating that microglial activation might influence the pathogenesis of degenerative diseases, there is considerable debate regarding whether microglial activation is beneficial or harmful. To address this fundamental question we decided to examine the effect of minocycline on microglial activation and neuronal regeneration. Initially, it was thought that minocycline would inhibit microglial activation, as seen in a number of previous studies, allowing neuronal regeneration to be investigated in the absence of a robust microglial response providing evidence for or against a pro-regenerative role of microglial activation. However, there was no difference seen in proliferating microglia in the facial nucleus 2, 3, and 4 days post-axotomy between minocycline treated and control animals. The unexpected effect of minocycline did not allow the role of microglial activation in neuronal regeneration to be addressed. Nonetheless, the results provided important insight to the mechanism in which minocycline provides neuroprotection in a number of neurodegenerative and injury models. From this study it appears that minocycline does not provide neuroprotection as a result of microglial deactivation. Further experiments in a lethal motor neuron injury model would address if minocycline functions by providing direct neuroprotection to injured neurons. The conflicting findings of minocycline on microglial activation may be attributed to the model used as well as the marker used to assess microglial activation. The facial nerve model may elicit a different microglial response than that in a neurodegenerative model since motor neurons in the facial regenerate and fully recover

whereas the neurons in a neurodegenerative model degenerate providing a chronic source for microglial activation.

Future studies need to address microglia cytokine production following minocycline treatment to assess if microglial activation is truly inhibited, specifically, pro-inflammatory molecules that have been suggested to be detrimental in the CNS. In addition, a different route of drug administration should be tested to determine if minocycline protection is dependent on the route of drug administration. An alternative to oral drug administration would be an i.p. injection of a corresponding dose of drug and then assessment of microglial proliferation in the facial nucleus following axotomy. An i.p. injection would determine if the facial nerve paradigm portrays a different response than that seen in neurodegenerative models following an i.p. injection of minocycline.

To confirm that the lack of effect of minocycline on microglial activation was not a result of insufficient drug levels in the brain, we performed HPLC/MS/MS to determine levels of minocycline in the brain following drug administration through oral diet. HPLC/MS/MS revealed minocycline levels similar to those found in studies where microglial activation was significantly inhibited following minocycline treatment.

The second aim of the current study investigated changes in microglia morphology and activation in the rat transgenic model of ALS to assess the role of microglia in disease progression. Morphological changes of microglia were observed in the brainstem and spinal cord of ALS transgenic animals at all stages of the disease. Included in the morphological changes was the formation of giant cells and microglial clusters. The giant cells portrayed the classic characteristics of Langhans giant cells suggesting a bacterial infection accompanying neurodegeneration in the ALS animal

model. This finding was further supported by the presence of bacilli bacteria seen in a symptomatic animal. However, a gram stain failed to identify bacteria in brain tissues from ALS animals. The failure of the gram stain to identify bacteria may be due to the short time frame in which the bacteria are present prior to their clearance or the bacteria present in the brain are acid-fast thereby causing them to be gram resistant. To further assess the presence of bacterial infections in ALS transgenic animals, future studies should directly culture brains to determine the presence of bacteria as well as performance of a broader range of bacterial stains to include all types of bacteria.

Further aberrant microglial activation was present in the red nucleus where intense activation was seen at all stages of the disease. However, in the same animals the oculomotor nucleus remained unaffected. The discrepancy between microglial activation in the two motor nuclei may be a direct result of the nucleuses cell projections. Cells in the red nucleus directly project into cervical, thoracic and lumbar levels whereas the oculomotor has no direct pathways to the spinal cord. Degenerative changes in the spinal cord affecting the rubrospinal tract may cause neuronal changes in the red nucleus eliciting a microglial response.

Levels of microglial activation in the ventral spinal cord were investigated using OX-42 and OX-6 to determine a time line of microglial activation in the spinal cord in response to disease progression. OX-6 expression was found at increased levels in the transgenic animal when compared to the age-matched controls and showed a continuous increase in density that corresponded with disease progression. OX-42 density initially increased with the onset of hind limb weakness however declined in end stage animals. The differences in OX-42 and OX-6 expression may be due to the heterogeneity of

microglia cells found in the CNS and the differential regulation of cell surface markers during microglia development. OX-6 positive microglial cells may be in early stages of cellular senescence as a direct result of the SOD1 mutation thereby increasing in number during the disease process. OX-42 labels the majority of microglial cells representing the overall response of microglial cells which declines through microglia degeneration in end stage animals. Therefore, MHC II positive cells are indicative of late stages of microglial life cycle thereby remaining elevated in end stage animals whereas the majority of OX-42 cells have undergone structural changes indicating degenerative changes as a result of cellular senescence therefore, no longer staining with MHC II.

To determine if microglial cells in the ALS model were more susceptible to apoptosis due to cellular senescence TUNEL labeling was utilized to assess apoptotic microglia. In the spinal cord TUNEL labeled cells had a morphology that was representative of microglia however failed to co-label with any of the microglial makers used. The failure to co-label may be due to differential expression of cell surface markers on cells undergoing programmed cell death. Future experiments need to further investigate the identity of the apoptotic cells with of a wider range of microglial markers. Rather than using a surface antigen, a nuclear stain allowing identification of fragmented cells should be tested.

In the facial nucleus, TUNEL labeled microglial were significantly fewer in number in the transgenic animals 14 days post-axotomy. The findings seem to contradict the hypothesis of cellular senescence and favor dysfunction of transgenic microglia. In chapter 5, a number of theories were postulated to explain the findings of fewer apoptotic microglia in the transgenic animals however until the initial microglial response is further

investigated little speculation can be made on the cause behind the lack of microglial turnover. In order to investigate the initial microglial response, the microglial response at 4 days post-axotomy in the facial nucleus should be compared between asymptomatic ALS animals and age-matched controls. This experiment will determine if the lack of turnover is due to a diminished microglial response or is in fact dysregulation of deactivating mechanisms in microglia. A follow-up to the above experiment would be to measure microglia-neuronal signals to determine if the lack of response is due to neuronal dysfunctions or microglia dysfunctions. The cytokine interleukin-6 (IL-6) has been suggested to be a potential signaling molecule between neurons and microglia and fractalkine is a chemokine that is found to be constitutively expressed on CNS neurons while the corresponding receptor CX3CR1 is found on microglial cells another potential signal between microglia and neurons (Harrison et al., 1998; Nishiyori et al., 1998). Both of the potential microglia-neuron signals would be key candidates to investigate microglia-neuron signaling in the facial nucleus.

In conclusion, the microglial cells in the ALS rat appear to be abnormal and undergoing functional changes due to the SOD1 mutation. It is still unclear in what manner the mutation renders the microglia dysfunctional, but may be attributed to oxidative stress. Oxidative stress has been shown to be a key contributing factor in familial ALS where the mutation catalyzes aberrant chemical reactions that initiate a cascade of oxidatively damaged products. Experiments addressing oxidative damage directly effecting the microglial cell population would provide great insight to the functional and morphological changes of microglia in the ALS rat. Furthermore, the dysfunction of microglia appears to compromise the integrity of the immune system in

the CNS allowing for a bacterial infection to occur and may contribute to neurodegeneration due to diminished neuroprotective properties.

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BIOGRAPHICAL SKETCH

Sarah Emily Fendrick was born in Mansfield, Ohio and remained in Ohio until her sophomore year in high school. She then relocated to Minnesota with her family where she graduated from high school in 1998. For her undergraduate education she attended the University of Wisconsin-Madison where she wanted to pursue a career in journalism. However, when attending orientation she made a last minute decision to follow a path that would allow her to attend medical school. Her mind changed a number of times until she finally decided that she would like to pursue her degree in genetics.

While in college, her first experience in the field of research was through employment in a stem cell lab. Her time spent in the stem cell lab was short due to her dislike of working in close proximity to monkeys. She then obtained employment in a functional genomics lab where she spent the two years working on a project that did functional genomics within the E. coli genome. The summer of her junior year Sarah was selected for an internship funded through the National Science Foundation at the Whitney Lab in St. Augustine, Florida. During her internship, Sarah's fondness for the warm weather and sunny days persuaded her to continue her education in Florida.

In 2002, she began her graduate education at the University of Florida in the Interdisiplinary Program in Biomeidcal Reserach. Entering into graduate school, her interest was in neuroimmunlogy which caused her to immediately pursue Jake Streit to be her mentor. Upon active persuasion, Jake accepted Sarah into his lab where her research focused on the role of microglial activation in ALS. After completing her Ph.D. Sarah

would like to pursue a career in patent law related to biomedical research and plans to begin law school in the Fall of 2006 at The Ohio State University.