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EFFECTS OF SHORT-TERM MINOCYCLINE TREATMENTS ON INFLAMMATORY CELL RESPONSE IN THE ACUTE STAGE FOLLOWING TBI

**A thesis submitted in partial fulfillment of the requirements for the degree of Master of
Science at Virginia Commonwealth University**

by

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LIST OF ABBREVIATIONS

ABC	Avidin-Biotin Complex
ARG1	Arginase 1
BBB	Blood-Brain Barrier
BrdU	5-bromo-2-deoxyuridine
CCI	Controlled Cortical Impact
CDC	Center for Disease Control and Prevention
CNS	Central Nervous System
DAB	5,5-diaminobenzidine
DG	Dentate Gyrus
DIA	Digital Image Analysis
i.p.	Intraperitoneal
Iba1	Ionized Calcium Binding Adaptor Molecule 1
IFN- γ	Interferon- γ
IL-1 β	Interleukin-1 β
IL-4	Interleukin-4
IL-10	Interleukin-10
IL-12	Interleukin-12
IL-13	Interleukin-13
IL-23	Interleukin-23
iNOS	Inducible Nitric Oxide Synthase
LPS	Lipopolysaccharide
MHC	Major Histocompatibility Complex
MMP	Matrix Metalloprotease
NO	Nitric Oxide
PBS	Phosphate Buffered Saline
PFA	Paraformaldehyde
ROI	Region of Interest
ROS	Reactive Oxygen Species

SEM	Standard Error of the Mean
SSC	Saline Sodium Citrate
TBI	Traumatic Brain Injury
TGF- β	Transforming Growth Factor β
TNF- α	Tumor Necrosis Factor α

ABSTRACT

EFFECTS OF SHORT-TERM MINOCYCLINE TREATMENTS ON INFLAMMATORY CELL RESPONSE IN THE ACUTE STAGE FOLLOWING TBI

Henna Ayub

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science
at Virginia Commonwealth University

Virginia Commonwealth University, 2013

Advisor: Dong Sun, MD, PhD., Department of Neurosurgery

Following traumatic brain injury (TBI), neuroinflammation contributes to the secondary injury. Microglia are the resident immune cells of the central nervous system (CNS), and when activated can exert either protective or detrimental effects on surrounding tissue. They are often segregated into subpopulations based on their type of activation, either pro-inflammatory (M1, classically activated), or anti-inflammatory (M2, alternatively activated). Minocycline, an anti-inflammatory drug, is known to be neuroprotective and to have effect on microglia. However, the effect of minocycline on subpopulations of inflammatory cells in the acute stage following injury is unclear. It is also unclear whether minocycline has a different treatment effect on injury-induced inflammatory responses in young and aged populations. In this study, we compared the effect of minocycline treatments on the microglial markers and the M2 subtype in both young (3-month-old) and aged (20-month-old) rats, sacrificed at three days after a moderate controlled cortical impact (CCI) injury. Minocycline treatments were either given beginning at 30 minutes post-injury or 4 hours post-injury for three days. Inflammatory cell markers ED1, OX6, and Iba1, as well as Arginase 1, a marker for alternatively

activated macrophages/microglia, were used to label inflammatory cells. Staining intensity of each marker was analyzed in both the peri-lesion cortical tissue and the ipsilateral hippocampus regions. Our findings have found that 3-day minocycline treatment significantly attenuated TBI-induced inflammatory cell response especially in the aged rats. Minocycline treatment did not show any significant changes in the prevalence of the M2 phenotype. Our findings suggest that minocycline may exert its anti-inflammatory effect mostly on inhibition of M1 phenotype rather than promoting M2 phenotype.

INTRODUCTION

Traumatic Brain Injury (TBI) is a worldwide health concern, affecting an estimated 10 million people yearly (Hyder et al. 2007). In the United States alone, the Centers for Disease Control and Prevention (CDC) reports that there are about 1.7 million cases annually, and that TBI contributes to one-third of all injury-related deaths. While TBI is the most common cause of death in young people (<40 years of age), it is also a major problem for the aged population, who are vulnerable to falls (Hyder et al. 2007). TBI involves not only the primary trauma, but also a multi-faceted secondary injury, characterized by several mechanisms including neuroinflammation, excitotoxicity, and the production of free radicals. Because of the extensive damage associated with these two phases, hospitalization and rehabilitation costs related to TBI can also be astounding, amounting to over \$48 billion in the U.S. each year (Huang 2013).

It is widely understood that after the primary injury, or the direct tissue loss, cell death, and disruption of the blood-brain barrier (BBB) caused by the biomechanical impact, a secondary injury extends the damage. This insult is caused in large part by neuroinflammation involving the activation of glial cells and the accumulation of leukocytes (Greve and Zink 2009). Microglia, the resident immune cells of the central nervous system (CNS), play a large part in this immune response. They not only phagocytize cellular debris (and possible pathogens), but also produce pro-inflammatory cytokines as well as growth-promoting factors. Microglia have several activation phenotypes, but are generally classified into the M1 and M2 subtypes. M1, also known as classically activated microglia/macrophages, are characterized by the production of high levels of pro-inflammatory cytokines and increased phagocytic activity. M2, or alternatively activated

microglia/macrophages, are distinguished by the production of high levels of anti-inflammatory cytokines and growth factors, as well as other neuroprotective properties (Fairweather et al. 2009). While the inflammation caused by TBI can persist for days to years, cytokine levels can increase in minutes following injury and some can peak within hours (Jin et al. 2012). Therefore, it is important to address the acute response in order to limit secondary injury.

After childhood, incidences of TBI peak in elderly populations, and past 65 years, the frequency of brain trauma doubles every 10 years (Hyder et al. 2007). In general, aged individuals have higher morbidity and mortality associated with TBI, as well as lower functional recovery (Livingston et al. 2005). In the aged brain there are increased levels of systemic inflammation and a prolonged inflammatory response (Stout and Suttles 2005), which may exacerbate the damage incurred during the secondary response, compared to young animals. There is also evidence suggesting that the M1 pro-inflammatory phenotype is increased in aged mice after injury, when compared to young animals (Kumar et al. 2013). These changes indicate that suppressing the inflammatory response in aged individuals will improve outcome.

Currently, different therapies to treat secondary injury are being explored. Minocycline is a tetracycline-derived antibiotic that has a wide range of anti-inflammatory effects. Minocycline is known to reduce microglial activation in the brain following TBI, and has been associated with improved functional outcome in the reported studies (Bye et al. 2007). It has also been found to reduce lesion size following injury (Sanchez Mejia et al. 2001; Bye et al. 2007). Although minocycline's anti-inflammatory effects have been well studied, its effects on the M1 and M2 phenotypes and the age-related effect following TBI

are not yet completely understood. It was therefore the goal of this study to investigate the effect of minocycline on the immune response and reduction of microglial activation in the brain, and whether M1/M2 cell type switching is involved. The study also sought to compare the differences in the inflammatory response between young and aged animals with minocycline treatment.

Based on prior research, we predicted that acute minocycline treatment would suppress the post-injury inflammatory response in both age groups, with a greater effect in aged animals. We also expected to see an increase in the prevalence of reparative M2 type cells and a reduction in M1 type cells in all animals given minocycline treatment.

TRAUMATIC BRAIN INJURY

Traumatic brain injury (TBI) is defined by many groups, including the Brain Injury Association of America, as “an alteration in brain function, or other evidence of brain pathology, caused by an external force” (Menon et al. 2010). In humans, an alteration in brain function is manifested in changes in level of consciousness, amnesia, neurologic deficits (weakness, paralysis, ataxia), or altered mental status (confusion). The key element in this classification is the term “external force,” which separates TBI from other types of brain injury, such as stroke. There are different types of external forces that can be applied to the brain to cause traumatic injury, and they are typically divided into two categories. One is a penetrating injury, in which an object, such as a bullet or a knife, enters the brain. The other is a non-penetrating, or closed head, injury. This type of injury can be caused by either contact forces (like an object striking, but not penetrating, the head), or by inertial

forces (like rapid acceleration or deceleration). Each type of injury can further be described as being either diffuse (involving large portions or the entire brain) or focal (involving only the site of impact) (Greve and Zink 2009).

EPIDEMIOLOGY OF TBI

Traumatic brain injury is both a national and an international public health issue. The mortality, long-term disability, and financial burden associated with this type of injury create a pressing problem that deserves to be addressed. In the United States, TBI results in over 1.36 million ER visits annually, of which about 35% are made by children (0 to 14 years). TBI also produces 275,000 hospitalizations and approximately 52,000 deaths each year, with the highest incidence rates falling on the elderly (age 75 and older) (Faul et al. 2010). Worldwide, it is estimated that TBI affects over 10 million people each year, although this is often considered a conservative assessment due to underreporting (Hyder et al. 2007).

In the United States, falls leads the list of causes of TBI, with young children and the elderly comprising the majority of this group (Faul et al. 2010). Internationally, however, motor vehicle or road traffic injuries are the leading cause of TBI (Hyder et al. 2007). In both cases, motor vehicle-related injury is the most frequent cause of TBI-related death.

TBI also places financial strain on healthcare systems and individuals due to the associated prolonged disability and time spent on rehabilitation of survivors. It was estimated that for cases of TBI occurring in the US in the year 2000, total lifetime costs, including productivity losses, amounted to \$60.4 billion. Per person costs were almost

\$45,000 (Corrigan et al. 2010). TBI is a problem that affects all ages and socioeconomic groups, making the need to explore new options in treatment all the more necessary.

EXPERIMENTAL TBI

Although larger mammals are more physiologically similar to humans, the low cost, small size, and standardized outcome measurements of laboratory rodents have made them the most commonly used animal models in TBI research (Xiong et al. 2013). To model differences in injury response between young and aged brains, two rat age groups are used in experimental studies. Three-month-old rats and 20-month-old rats are used to correspond with middle-aged and elderly human patients, respectively.

Several injury models for rodents have been developed, each with its own advantages. One of the most common is the controlled cortical impact (CCI) injury model, which uses a pneumatic piston to inflict mainly focal damage upon the intact dura via a unilateral craniotomy halfway between lambda and bregma. The consequences of this impact include cortical tissue loss and damage, as well as degeneration in the hippocampus and thalamus (Hall et al. 2005).

The CCI method allows the experimenter to control the velocity, length (time), and depth of impact, when other methods, such as lateral fluid percussion injury (LFPI), cannot. The CCI model is also considered to be histopathologically similar to contusion injuries in humans (Morales et al. 2005). While this model of injury is highly reproducible and incurs a

low mortality rate, there are sometimes incidences of hypoxia and hypotension that affect outcomes (Xiong et al. 2013).

PHASES OF INJURY

TBI may be divided into two phases, known as the primary injury and the secondary injury. The primary injury is the initial blow or trauma experienced, and is characterized by immediate tissue loss, cell death, and mechanical disruption of cells and cellular structures, including the blood-brain barrier (BBB). There is either focal or diffuse damage to blood vessels, axons, neurons, and glia (Finnie et al. 2013). Primary injury is usually characterized by contusions, lacerations, hemorrhage, axonal injury/transectioning, and hypoxia (Finnie et al. 2003; Das et al. 2012). Mechanoporation of the cell membrane leads to an influx of ions, including calcium. The disruption of the mitochondrial membranes halts the production of ATP, and releases certain apoptotic factors. Breakdown of the lysosomal membrane releases degradative enzymes throughout the cell. Some cells are able to recover from these insults and repair the membranes, whereas others are forced to die (Greve and Zink 2009). After the traumatic event has occurred, cells are left vulnerable to a secondary injury, which may begin within minutes to hours and persist for days to months (Das et al. 2012). While the primary injury is untreatable (although sometimes preventable), there is a window of opportunity for treatment during the secondary insult, to improve survival and outcome.

The secondary injury can be characterized by further breakdown of the BBB, ischemia, edema, hematomas, elevated intracranial pressure, and infection (in a penetrating injury). The secondary injury is multi-faceted, but mostly occurs in response to the metabolic disruption caused by the primary insult. It is now well-documented that trauma causes excitotoxicity in the brain, due to a large and uncontrolled release of neurotransmitters from cells, which contributes to the huge ionic fluxes seen after injury (Giza and Hovda 2001). The large influx of calcium observed is detrimental for several reasons, including its impairment of mitochondrial oxidative metabolism and involvement in cell death pathways (Giza and Hovda 2001). There is also a large efflux of potassium from cells, which causes the release of more excitatory amino acids, further contributing to the excitotoxicity. Ultimately, the dramatic changes in ion concentrations lead to depression of neuronal activity (Katayama 1990). There is also evidence that increased metabolism after injury enhances the production of oxygen radicals (Kontos and Povlishock, 1986). These free radicals then initiate cascades including lipid peroxidation, leading to loss of vascular integrity (disruption of the BBB) (Smith et al. 1994).

The damage caused by excitotoxicity after trauma and neuronal impairment is exacerbated when damaged cells also undergo a large influx of calcium and sodium, and an efflux of potassium. The increase in intracellular calcium then stimulates the release of internal stores of calcium (calcium-induced calcium-release), causing a further rise in calcium concentration inside the cell. This calcium is responsible for the activation of certain calcium-dependent proteases inside the cells, such as calpains and caspases. These proteases, once activated, lead to the break down of the cytoarchitecture of the cell, and thus contribute to cell death (Greve and Zink 2009; Raghupathi 2004).

A large contributor to the secondary injury is the inflammatory response, mediated principally by microglia in the brain. However, breakdown of the BBB following TBI causes increased permeability of the CNS to infiltrating inflammatory cells and signaling molecules (Das et al. 2012). Microglia, as well as infiltrating leukocytes, release pro-inflammatory cytokines like $\text{TNF-}\alpha$ and $\text{IL-1}\beta$. These cells also remove inhibitory tissue debris and secrete growth-promoting factors, involved in regeneration of cells and tissue.

The cytokine levels rise within minutes of injury and can peak within hours, so it is necessary to suppress the acute response in order to prevent or reduce secondary damage. The cytokine $\text{IL-1}\beta$, for example, reaches maximal levels in the hippocampus one to two hours after injury (Dalgard et al. 2012). Because the secondary injury may in many cases be preventable it is important to investigate possible treatments and therapies to minimize damage caused by this phase.

AGING AND TBI RESPONSE AND RECOVERY

While advanced age is known to correlate with poor outcome following TBI, the reasons for the phenomenon are still not completely understood. Often, the age of the patient determines survival after TBI, as well as the severity of the injury and degree of recovery. Possible causes for this strong association include an increased inflammatory response in aged individuals, more severe secondary injuries, and an inability to compensate for neurological deficits (e.g. decreased neuroplasticity). Generally, the inflammatory response is less coordinated and therefore not as efficient at responding to an insult as in a younger individual (Godbout et al. 2005). In aged animal models of TBI, we see

delayed recruitment of monocytes, less chemotactic activity, decreased ability to regulate microglial proliferation, decreased cytokine production, and reduced phagocytosis (Conde and Streit 2006; Stout and Suttles 2005). Also, there is a decrease in the production of certain growth factors, like FGF-2 and VEGF, leading to impaired recovery after injury (Stout and Suttles 2005).

The idea of “immunosenescence,” or changes to the immune system caused by aging, is continuing to be explored in research. Studies have shown that with age there is an increased presence of dystrophic microglia, or microglia with abnormal cytoplasmic structures and morphology, in the brain (Streit et al. 2004). This, along with the fact that microglial expression of MHC class II (a marker of activation) increases with age, seems to indicate that the normal aged brain is “primed” for an exaggerated response after injury (Godbout et al. 2005).

A comparison of healthy rat cortical tissue by age showed that systemic microglial activation increases with age, thought to be due to minor insults to the CNS incurred over time (Vaughan et al. 1974). In a recent TBI study in mice, it was found that aged subjects had a higher mortality rate and worse neurologic outcomes that did not correlate with histopathological damage (Timaru-Kast et al. 2012). Examination of certain inflammatory markers, including iNOS and IL1- β , revealed that aged mice already had higher basal levels of these cytokines in the CNS, which may have lead to an exaggerated inflammatory response after injury. In comparing the responses of young and aged mice, it was found that old animals exhibited early and high increases in the markers IL-1 β and TNF- α , while young animals had delayed increases (Timaru-Kast et al. 2012). This strong, early expression

in aged animals could exacerbate the secondary damage and cause increased neuronal cell death.

Other studies comparing aged and young rodents have found similar correlations. Increased age has often been associated with higher numbers of activated microglia in specific brain regions, including the hippocampus (Sandhir et al. 2008). The hippocampus, being one of the few brain regions that continue to produce new neurons even in adulthood, is particularly vulnerable to TBI damage. Increased glial activation may affect repair and recovery in these regions, and lead to worse outcomes in aged brains.

After young children, incidences of TBI peak again in the elderly, and adults age 75 and older have the highest rates of TBI-associated hospitalization and death (Faul et al. 2010). In order to address this significantly affected population, the differences in inflammatory responses between young and aged brains must be further investigated, particularly in regard to the presence of specific microglial subpopulations (discussed further in the following sections). Additionally, the impact of an anti-inflammatory drug, like minocycline, on aged animals following TBI has not been well examined and requires further exploration.

IMMUNE RESPONSE IN THE CNS

First described in detail by Del Rio-Hortega in 1932, microglia are now known to constitute about 10-20% of the total CNS cell population. In contrast to the other glial cells of the CNS (i.e. astrocytes and oligodendrocytes), which originate from the neuroectoderm, microglia have a monocyte/macrophage lineage and originate from the mesoderm.

Circulating monocytes invade the developing brain during early developmental stages and transform into microglial cells. Microglia are best known as the resident immune cells of the central nervous system (CNS), meaning that they are always present and monitoring the environment for pathogens via pattern recognition receptors (PRRs) (Block et al. 2007). In this way, they play an important role in maintaining normal tissue homeostasis. In addition, microglia are known to be involved in synaptic regulation, neuronal cell death, and neuronal differentiation (Loane and Byrnes 2010).

At rest, microglia have a “ramified” morphology as they send out long, branched processes that survey the immediate surroundings, reacting to chemical as well as physical signals. Resting microglia in this ramified state present a relatively continuous population in the adult brain, and exhibit a slow turnover (little proliferation or recruitment) (Ohsawa and Kohsaka 2009). Cells in this state are characterized by constitutive expression of cell surface markers like CD11b and Iba1, as well as a reduced level of major histocompatibility complex (MHC) markers and phagocytic markers (ED1) (Ohsawa and Kohsaka 2009).

When stimulated, microglia take on a more “amoeboid” shape and appear more hypertrophied with shorter processes. These cells up-regulate certain cell-surface molecules, including MHC molecules, CD14, Iba1 and chemokine receptors (Block et al. 2007). Causes of microglial activation are varied, but include environmental toxins as well as neuronal damage (Block et al. 2007). After TBI, there is often disruption of the BBB, leading to infiltration of monocytes and macrophages from the blood into the CNS. These cells are indistinguishable from resident microglia that have been activated after injury.

Microglia provide the first line of defense against potentially harmful microbes or molecules and thus comprise the innate immune system of the CNS. This innate response,

being immediate and non-specific, contrasts with the subsequent adaptive response, which is delayed but specific to the type of damage. Microglia phagocytize cellular debris as well as pathogens, and secrete cytokines that activate more cells and draw other types of immune cells to the area (Martinez et al. 2008). The PRRs on their surface, such as toll-like receptors (TLRs), interact with various pathogen-associated molecular patterns (PAMPs) or, as in the case of most TBIs, damage-associated molecular patterns (DAMPs) in the environment (Colton 2009). These interactions start a cascade of events within the cell, ultimately triggering the induction of certain genes that effect the expression of inflammatory cytokines, such as TNF- α and IL-1 β (Colton 2009).

It can also be harmful to the host when necrotic and apoptotic pathways are strongly expressed, or the reaction is prolonged. Chronic activation can be detrimental due to the continuous release of potentially cytotoxic molecules, such as pro-inflammatory cytokines, ROS and their intermediates, proteinases, and complement proteins. However, microglia are also involved in the wound healing process after injury, and release growth factors, such as TGF- β , necessary for neuron repair and vascular regeneration (Vereyken et al. 2011). In addition, studies showing the detrimental effects of the impaired function of microglia or depletion of the cell type indicate the necessity of the microglial response to recovery (Lalancette-Hebert et al. 2007; Loane and Byrnes 2010).

TYPES OF MICROGLIA/MACROPHAGES

This dual role of macrophages in the inflammatory response has led to the classification system used today to differentiate the two major phenotypes of microglial activation. The

first are known as “classically activated” microglia, or simply M1, meaning that the cell releases primarily pro-inflammatory cytokines. The second are termed “alternatively activated”, or M2, meaning that the cell secretes primarily anti-inflammatory cytokines. It has been determined that in the intact CNS, microglia exhibit an M2 phenotype (Jiang et al. 2012). In the case of injury, however, the specific lesion microenvironment determines the activation of the microglia (Vereyken et al. 2011). These M1/M2 designations have been further clarified through extensive studies, and a more detailed explanation of differences between the two groups follows.

M1 (pro-inflammatory) cells are inducible by the introduction of inflammatory cytokines, such as IFN- γ , as well as LPS (lipopolysaccharide). They are themselves characterized by high expression of inflammatory cytokines, including IL-12, IL-23, TNF- α , and IL1- β , and chemokines, like CCL15/Hcc-2. M1 cells exhibit enhanced endocytic functions, as well as an increased ability to destroy intracellular pathogens. They accomplish this via several different mechanisms, including restriction of nutrients (e.g. iron), acidification of phagosomes, and synthesis of reactive oxygen species (ROS). M1 macrophages also express inducible nitric oxide synthase (iNOS), which skews the metabolism of arginine toward production of nitric oxide (NO) (Martinez et al. 2008). These microglia/macrophages are necessary in protecting the host against exogenous pathogens and debris left by injury, but can also cause increased tissue damage by attacking healthy host cells. M1 cells also exhibit higher levels of phagocytic activity involving myelin and neuronal fragments (Vereyken et al. 2011).

M2 anti-inflammatory microglia may be induced by factors such as IL-4, IL-13, IL-10, and TGF- β (Stein et al. 1992;). These cells then express similar anti-inflammatory molecules, in

addition to extracellular matrix molecules (e.g. fibronectin), scavenger receptors (e.g. CD163), and glucocorticoids. Specifically, IL-10 is known to inhibit production of pro-inflammatory cytokines, TGF- β controls microglial activation, cytokine production, and chemotaxis. Glucocorticoids bind receptors in the nucleus and inhibit the production of pro-inflammatory cytokines, as well as the enzymes that produce/mediate them, and down-regulate the genes that are usually up-regulated by IFN- γ . They also enhance expression of anti-inflammatory cytokines (Martinez et al. 2008). M2 cells are mainly involved in tissue repair mechanisms and are believed to have a neuro-protective role (Zhang et al. 2012). M2 microglia/macrophages express high levels of arginase I (ARGI), which competes with iNOS for use of arginine as a substrate. Arginase I produces proline and polyamines that promote cell growth, collagen formation, and tissue repair (Martinez et al. 2008). M2 cells also promote angiogenesis by producing necessary growth factors, such as PDGF, VEGF, and FGF (Verheyken et al. 2011). M2 cells are also known to secrete matrix metalloprotease-1 (MMP-1) and MMP-12, which are involved in substrate remodeling, and downregulate MMP-9, which has been implicated in regeneration failure after CNS injury (Busch et al. 2011).

The term M2 is now regarded as a general name for a continuum of anti-inflammatory cell types, which are functionally and phenotypically related. Subsets have been named with various terminologies (e.g. M2a and M2b), which differentiate the cell types by assorted characteristics, such as how they are induced and whether they express certain enzymes.

In a study by Dheen and associates (2007), neurogenesis in the adult hippocampus was examined. The hippocampus is uniquely appropriate for this type of study due to its permanent population of neural stem cells, which allows it to continually produce new

neurons even in adulthood. In this study, neurogenesis, in vitro, was inhibited when the NSCs were exposed to activated microglia. This suggests that the pro-inflammatory factors released by M1 cells could contribute to blocking of neurogenesis. Accordingly, treatment with NSAIDs restored neurogenesis in the hippocampus.

Some research has been done to explore the relationship between the two types of macrophages. One such study saw that the introduction of Multipotent Adult Progenitor Cell (MAPC) -secreted factors induced a shift from M1 to M2, which enhanced regrowth after a spinal cord injury by minimizing axonal dieback at the injury site (Busch et al. 2011). Axonal dieback is thought to be mediated by MMP-9, a protease that is upregulated in M1 cells.

The idea of a dual role of microglia in injury and repair has led to an interest in experimenting with the balance between M1 and M2 cells. Specifically, whether suppressing the pro-inflammatory cells and activating the anti-inflammatory cells leads to better recovery after injury. In a study by Jiang and colleagues (2012), Substance P (SP) was used as an inducer of the M2 phenotype in macrophages after spinal cord injury. It was found that with SP treatment the area around the injury site was induced toward a more anti-inflammatory state, by way of up-regulating IL-10 and arginase-1 expression and down-regulating iNOS, IL-6, and TNF- α expression by the microglia in the CNS. These effects of the drug on M1/M2 phenotype modulation were noticed at one-day-post-injury. Not only was functional recovery greater in the SP treatment groups, as compared to controls, but neuronal processes and vasculature were seen in the lesion sites as well.

MINOCYCLINE AS A TREATMENT

Minocycline is a commonly used tetracycline-derived antibiotic. Already FDA-approved, it has been used for decades to effectively treat infections ranging from periodontitis to acne vulgaris. Side effects are usually mild, and the symptoms most commonly reported by people taking the drug have been gastrointestinal issues and dizziness (Elewa et al. 2006). In addition to its anti-microbial properties, minocycline also has also been shown to exert anti-inflammatory effects, making it a strong candidate for therapy during the secondary injury following TBI.

Minocycline is lipid-soluble and able to cross the blood-brain barrier (BBB), meaning it can be administered orally, intravenously (I.V.), or even intra-peritoneally (I.P.). Other advantages of minocycline treatment include its ability to exert its protective effect for a relatively long time (>3 hours after injection). It is also able to inhibit several mechanisms of secondary injury, as opposed to very specific treatments (calcium channel blockers, glutamate antagonists) that leave other lines of damage open (Wells et al. 2003).

It has been repeatedly demonstrated that minocycline has anti-inflammatory as well as neuroprotective properties, in a variety of experimental models. In a study by Ng and associates (2012), mice in a closed head injury model treated with minocycline had significantly reduced activation and recruitment of microglia/macrophages to both the hippocampus and injury site. This finding was also associated with better neurological outcome. Another murine study employing a CCI injury found that minocycline treatment resulted in decreased lesion volume, improved neurological function, and reduced production of an inflammatory cytokine, IL-1 β (Bye et al. 2007). The drug has also been

associated with suppression of microgliosis, apoptosis, free radical formation, and peripheral inflammation (Hewlett et al. 2006).

The anti-inflammatory effects of minocycline are numerous. In general, the drug inhibits migration and infiltration of inflammatory cells, as well as suppresses ROS formation. It blocks enzymes that contribute to inflammation, such as iNOS and caspase-1 (Sanchez Mejia et al. 2001). Caspase-1 has been implicated in cellular apoptotic mechanisms that are activated following TBI. It also inhibits the production of ROS and expression of pro-inflammatory cytokines, like IL1- β , by microglia (Dheen et al. 2007).

Minocycline also suppresses apoptosis by inhibiting the release of cytochrome c from mitochondria and inducing the up-regulation of Bcl-2 (Matsukawa et al. 2005). The drug, like all tetracyclines, also inhibits matrix metallo-proteases (MMPs), which are frequently associated with the degeneration of extracellular proteins. MMPs are enzymes that are key in the process of leukocyte infiltration because they cleave tissue components like fibronectin and collagen (Parks et al. 2004).

This research suggests that minocycline could suppress inflammation and promote repair in the injured brain by supporting a switch from the M1 to M2 microglial phenotype. Therefore, the current study will investigate the effect of minocycline treatments on the acute inflammatory response following CCI injury. The study will also compare drug's effect on young animals (3-month-old) to aged animals (20-month-old), which are known to have elevated microglial activation and pro-inflammatory cytokine production after injury.

METHODS

SUBJECTS

The experimental procedures used in this study followed all NIH guidelines and were approved by the Institutional Animal Care and Use Committee at Virginia Commonwealth University. Three-month-old (approximately 300g each) and 20-month-old (approximately 450g each) Fisher 344 rats were used in this study. All animals were housed under normal conditions (temperature of 20-22°C with a 12-hour light/dark cycle), and were provided food and water *ad libitum*. The young rats were housed in pairs, while the aged rats were housed individually. The current study used tissue obtained from 32 animals, consisting of 18 young rats and 14 aged rats, to examine the effects of minocycline treatment on the injury-induced inflammatory response. The animals were divided into sham, vehicle, and two minocycline treatment groups (Table 1).

TABLE 2-1: ANIMAL GROUPING

	Controlled Cortical Impact (CCI) Injury	
	Young (3M)	Aged (20M)
Sham	3	2
Vehicle	5	4
Minocycline Treatment Group 1	6	4
Minocycline Treatment Group 2	4	4

SURGICAL PREPARATION AND PROCEDURE

Fifteen of the young animals and twelve of the aged animals used in this study endured CCI injury. The rest of the animals were shams and underwent the same procedures without injury. All surgeries were carried out in an aseptic environment and used autoclave-sterilized equipment, following protocol guidelines. Anesthetization before surgery was achieved with 5% isoflurane delivered in a Plexiglas chamber. The subject was then removed from the chamber and kept on continuous 2.5% isoflurane/gas (30% oxygen and 70% nitrogen) ventilation via a nose cone. The surgical area, shaved of fur, was cleansed with Betadine, and Puralube ointment was placed around the eyes to prevent drying out. After arranging the animal on the stereotaxic frame, over a heating pad. A midline incision was made in the scalp and hemostats were used to pull away the surrounding skin and connective tissue to expose the skull. A trephine and a Dremel drill were used to make a 4.9 mm craniotomy, without piercing the dura, at a position on the left parietal bone midway between lambda and bregma. The craniotomy was carefully cleared of bone fragments before continuing.

CONTROLLED CORTICAL IMPACT (CCI) INJURY

The injury model used for this study was the controlled cortical impact (CCI) model. After the craniotomy, the rat was disconnected from anesthesia. The subject was then placed in the CCI injury device (Figure 1) and the device was calibrated by lowering the 3mm steel impactor to the surface of the brain exposed by the craniotomy. This step ensured that the correct depth would be achieved during injury and that the dura would not be pierced. The subject was kept in place until it exhibited a tail reflex, at which point the

injury was delivered. Injury consisted of a 2.3 mm depression of the dura by the steel impactor at a velocity of 4 m/s and a dwell time of 50 milliseconds (ms).

After injury, the animal was returned to the surgical bench and placed in a supine position with gauze covering the wound. Time to regain paw reflexes and righting time were recorded to ensure that injury levels remained consistent. The animal was then returned to anesthesia (2.5% isoflurane/gas mixture) via nose cone ventilation, and the wound was then cleaned and the incision sutured. The animal was removed from ventilation and, after normal breathing returned, it was placed in its cage.



Impactor Tip

FIGURE 2-1: A PHOTOGRAPH OF THE DEVICE USED TO DELIVER A CONTROLLED CORTICAL IMPACT (CCI) INJURY TO THE ANIMALS USED IN THE STUDY.

MINOCYCLINE AND BRDU INJECTIONS

In this study, minocycline (Sigma) was administered to two treatment groups in both the young and the aged rat groups. In the first treatment group, rats were given intraperitoneal (IP) injections at 30 minutes post injury, and again at 8 hours after TBI. In the second treatment group, animals were given injections first at 4 hours after injury and again at 8 hours. All doses were given at 45mg/kg. Both groups were also subsequently given twice-daily injections on the first and second days post injury in every 12 hours, at doses of 22.5 mg/kg. Animals in the vehicle group received sterile saline injections at the same time points. Minocycline solutions for injection were prepared by dissolving 15 mg/ml minocycline in sterile saline.

In rodents, minocycline reaches peak serum levels about 2.5 hours after injection (IP), and has an average half-life of 3 hours (Elewa et al. 2006). Several studies have demonstrated improved cognitive and functional recovery in animals given acute minocycline treatments after injury (Sanchez Mejia et al. 2001; Bye et al. 2007). In a rodent model of mild, blast-induced TBI, Kovesdi and colleagues (2012) saw that animals treated with minocycline beginning 4 hours after injury, for four days, performed comparably to shams in three different behavioral tests.

BrdU (Sigma) was also administered to each animal used in the study in order to label cell proliferation following TBI. BrdU (5-bromo-2'-deoxyuridine) is a thymidine analog, and is incorporated into newly replicating cells. At two days post injury, each animal underwent 4 i.p. injections of BrdU (at 50 mg/kg), 2 hours apart. All animals used in this study were sacrificed at 3 days post injury, corresponding to 24 hours after the last BrdU or minocycline administration.

TISSUE PROCESSING

Under deep anesthesia with isoflurane, the rats were transcardially perfused with 400 ml of phosphate buffered saline (PBS) followed by 400 ml of 4% paraformaldehyde (PFA) in PBS. The brains were then removed from the rats' skulls and post-fixed in the same fixative, at 4°C. Brains were then transferred to PBS before being prepared for slicing. Coronal sectioning was carried out at a thickness of 60µm, by Vibratome (Leica). The slices were gathered into four serial groups of 30 slices each, arranged in five 24-well plates. Sections were stored in PBS + 0.01% sodium azide, at 4°C.

IMMUNOHISTOCHEMISTRY

Sections from each brain were selected to stain for one of following microglia cell type markers used in the study: Iba1, ED1, Ox6, Arg1, iNOS, CD206, CD86. For each marker, we used two or three sections, spaced 720 µm apart, from each brain for staining.

Iba1 is the ionized calcium-binding adaptor molecule 1, an intracellular signaling protein expressed by myeloid cells. It is constitutively expressed, but up-regulated on activated cells (Donnelly et al. 2009). ED1 is a marker for activated microglia/macrophages. It stains specifically for CD68, a lysosomal membrane protein that is predominantly found in phagocytizing macrophages and reactive microglia (Zhang et al. 2012). Ox6 is an MHC Class II protein, and also a marker for activated microglia. Arginase is an enzyme involved in the metabolism of arginine, and a marker for M2 cells. BrdU, a thymidine analog, is a marker for newly proliferating cells.

We originally selected two markers for each microglia subtype, but a serial dilution performed for optimization revealed no staining for three of the four markers. Therefore,

the two M1 markers, CD86 and iNOS, were not used for this study (Table 2-2). CD86 is a cell-surface protein expressed on antigen-presenting cells and iNOS is the inducible nitric oxide synthase, an enzyme that also uses arginine as a substrate, driving the production of NO. Another M2 marker, for CD206 – the mannose receptor, was also excluded due to poor staining (Table 2-2).

TABLE 2-2. PRIMARY ANTIBODIES USED IN IMMUNOSTAINING AND IMMUNOFLUORESCENT STAINING PROCEDURES.

Antibody	Manufacturer	Dilution	
Iba1	Wako (anti-rabbit)	1:1000	General microglia/macrophage marker
ED1	Millipore (anti-mouse)	1:1000	General microglia/macrophage marker
Ox6	Serotec (anti-mouse)	1:1000	General microglia/macrophage marker
iNOS	abcam (anti-rabbit)		M1 cell type marker
CD86	Abnova (anti-rabbit)		M1 cell type marker
Arg1	Santa Cruz (anti-rabbit)	1:500	M2 cell type marker
CD206	abcam (anti-rabbit)		M2 cell type marker
BrdU	Dako (anti-mouse)	1:200	Newly proliferating cell marker
BrdU	Serotec (anti-rat)	1:200	Newly proliferating cell marker

IMMUNOSTAINING PROCEDURE

Free-floating sections were washed with phosphate buffered saline (PBS) for 5 minutes, twice, and then placed in 3% hydrogen peroxide for 1 hour on a shaker at room temperature. The sections were then washed with PBS + 0.3% Triton 100 for 5 minutes, three times. The sections were then blocked overnight at 4°C on a shaker in a blocking buffer of PBS + 0.3% Triton 100 and 5% normal horse serum or fetal bovine serum.

The next day, sections were brought to room temperature and another wash with PBS + 0.3% Triton 100 was performed for 10 minutes, 3 times. After the wash, the primary antibody was added (Figure 2). Arg1 (Santa Cruz; anti-rabbit) was added at a 1:500 dilution in blocking solution, and Iba1 (Wako; anti-rabbit), ED1 (Millipore; anti-mouse), and Ox6 (Serotec; anti-mouse) were added at a 1:1000 dilution. The sections were allowed to incubate at 4°C on a shaker for at least 48 hours.

Sections were then brought back to room temperature and rinsed in PBS + 0.3% Triton 100 for 10 minutes, three times. Tissue was blocked again for 3 hours at room temperature on a shaker, in the same blocking solution as previously described. After blocking, the appropriate secondary biotinylated antibody was added. For Arg1- and Iba1-stained sections, biotinylated anti-rabbit IgG (Dako) was added at a 1:200 dilution in blocking solution. For ED1- and Ox6-stained sections, biotinylated anti-mouse IgG (Dako) was added at a 1:200 dilution. Sections incubated with the secondary antibody overnight, at 4°C, on a shaker.

The next morning, the sections were brought back to room temperature and washed with PBS for 10 minutes, three times. A 1:200 solution of avidin-biotin substrate (ABC Kit, Vector Laboratories; made 30 minutes before use) in PBS was then added, and sections

were allowed to incubate at room temperature for 2 hours, on a shaker. After incubation with ABC, sections were washed three times with PBS, for ten minutes each. At this point, a DAB solution was added to the sections, one tray at a time. The liquid DAB (Vector Laboratories) was prepared by adding the following solutions to 5ml of nanopure water: 2 drops of the Buffer Stock Solution, 4 drops of the DAB Stock Solution, and 2 drops of the hydrogen peroxide solution, mixing well after each addition. The sections were monitored under a microscope while they incubated with the liquid DAB solution, and were quenched with PBS when they were sufficiently stained. The sections were washed with PBS for 5 minutes, three times, and stored at 4°C until ready for mounting. The sections were then rinsed in distilled water just before being mounted onto glass microscope slides, 2-3 sections per slide.

COUNTERSTAINING PROCEDURE

Slides with mounted sections were arranged in a rack and placed in distilled water for 2 minutes. Slides were then placed in a 0.1% Cresyl Violet solution for one minute. After a brief rinse under tap water (one minute) and, finally, distilled water (30 seconds), the slides were placed in two 75% baths and one 95% EtOH bath for one minute each. Sections were then differentiated in acid alcohol (95% EtOH + a few drops of HCl) to remove almost all of the background stain. The slides were then run through two baths of 100% EtOH for one minute each, and then placed in Citra Solv for 2.5 minutes and 5 minutes. Slides were then coverslipped using Permount (Fisher).

IMMUNOFLUORESCENT DOUBLE STAINING

Fluorescent double staining was carried out in order to assess whether the cell-type-specific markers co-localized with BrdU, a marker for newly proliferating cells. The sections were stained for BrdU and one other cell type marker (Iba1, ED1, or ARG1). One section from each selected brain was also doubled labeled with ARG1 and ED1 to assess co-localization of the M2 cell-type marker with a general microglia cell marker.

For the BrdU double labeling, the free floating sections were rinsed twice with PBS for 5 minutes, and then denatured in a 50% Formamide solution (in water and 20x saline sodium citrate (SSC)) for one hour in a 65°C oven. The sections were then removed from heat and rinsed twice with 2x SSC for 5 minutes before being placed in 2N HCl for 30 minutes in an oven set at 37°C.

The sections were removed from the oven, and all sections (including those not being labeled with BrdU) were rinsed with PBS for 5 minutes, twice. The sections were then quenched in 3% hydrogen peroxide, washed with PBS + 0.3% Triton 100, and blocked overnight as described in the previous immunostaining procedure.

The next day, the primary antibody solution was added, made in the same blocking buffer as described earlier. For BrdU double labeling with Arginase or Iba1, anti-mouse BrdU (Dako; 1:200) was used, and for double labeling with ED1, anti-rat BrdU (Serotec; 1:200) was used. All other cell type markers were used at the same dilutions as normal immunostaining. Sections were allowed to incubate with the primary antibody solutions for at least 48 hours, at 4°C on a shaker. After incubation, sections were rinsed with PBS + 0.3% Triton 100 for 10 minutes, three times. The sections were then allowed to block at room temperature on a shaker for 3 hours before adding the secondary antibody solutions. For

BrdU/Arginase, BrdU/Iba1, and Arginase/ED1 labeling, two Alexa fluorophore-conjugated secondary antibodies were used (anti-mouse IgG 488 and anti-rabbit IgG 568; 1:200; Molecular Probes) were used. For BrdU/ED1 double labeling, the Alexa fluorophore-conjugated anti-rat IgG 488 and anti-mouse IgG 568 (1:200; Molecular Probes) were used.

Sections were then washed with PBS for 10 minutes, three times, rinsed with distilled water twice, and then mounted on a glass microscope slide. Mounted sections were coverslipped using Vectashield mounting medium (Vector Laboratories) and sealed with clear nail polish, before being stored in a -20°C freezer.

Sections were examined by confocal microscopy in order to quantify the percentage of new, BrdU-labeled cells that had differentiated into a specific subtype. In the cortex, the peri-contusional area was assessed for BrdU+ cells and each cell was manually examined for co-labeling with another cell-type-specific marker. The percentage of double-labeled cells was calculated as the number of BrdU+ cells that also co-labeled with a cell-type-specific marker against the total number of BrdU+ cells in the same section. The percentages of double-labeled cells in vehicle cortical tissue were compared to double-labeled cells in minocycline-treated animals.

A section fluorescently labeled for both ARG1 and ED1 was also examined for co-labeling. The peri-contusional cortex was examined for co-localization of the two markers and only those cells with explicit staining for both markers were counted as double-labeled.

DENSITOMETRY ANALYSIS

For analysis of IHC, we used Digital Image Analysis (DIA), as described by Donnelly et al. (2009). In this comparative study, DIA was found to be consistently the most sensitive and least variable, as well as most time-efficient, when compared to standard profile counting (PC) and the unbiased sampling technique (UST).

Images were taken with the Olympus DP Controller program, employing an Olympus IX71 microscope using a 4x objective. Two cortical tissue images were taken when there was an injury that caused a concavity to form. One image was taken on either side of the concavity. For shams and those injured animals that suffered a mild injury and lacked an injury concavity, only one cortical tissue image was taken. For all sections, one image of the dentate gyrus of the hippocampus was taken. For each staining group, images were kept at a consistent exposure, and optimized for white balance and high contrast.

DIA was completed using the IP Lab program (Scanalytics). Images were converted to gray scale and the XY units were defined, using a predetermined pixel:μm ratio (1 pixel = 1.61 μm). A line was then drawn around the region of interest (ROI), which in cortical images included the visible ipsilateral cortical tissue, and in hippocampal images included the entire dentate gyrus.

The program was then used to determine a threshold value that included all positively stained cells in the densitometry measurements, with as little background as possible. For Iba1 staining, the “max” for this segmentation step was held constant at 190, and for all other staining the “max” was held constant at 155. After segmentation, the IP Lab program was used to analyze the ROI for the percent area occupied by the positively labeled cells. This data was then used for the statistical analyses.

STATISTICAL ANALYSIS

Data was preliminarily analyzed using post-hoc student two-tailed t-tests, assuming equal variances. T-test data was used to compare differences between the two age groups, within each injury group. Two-way ANOVA (analysis of variance) with LSD post-hoc tests were also performed, to analyze the differences in staining densities among the treatment groups, within an age group, for each marker. P-values less than 0.05 were considered statistically significant.

RESULTS

The inflammatory response is known to be involved in secondary damage following traumatic brain injury, contributing to reduced or prolonged regeneration and recovery. Studies have also shown that the aged brain has higher levels of systemic inflammation, which may make it poised to exhibit a robust and exaggerated inflammatory response after injury. In the current study, we sought to determine the effects of an anti-inflammatory drug, minocycline, on the inflammatory response in both young and aged rats in the acute stage following a controlled cortical impact (CCI) injury. We used two treatment groups, the first receiving the initial dose of minocycline at 30 minutes post injury, and the second receiving the initial dose at 4 hours post injury. The animals used were Fisher 344 rats, aged 3 months (corresponding to a young adult human) and 20 months (corresponding to a middle-aged human) (Hamm et al. 1991).

We examined the inflammatory cell response using the general inflammatory cell markers including Iba1, ED1, OX6. We also used M1 subtype markers iNOS and CD86, M2 subtype markers Arg1 and CD206. However, the antibodies we used for iNOS, CD86 and CD206 did not work immunohistochemically on rat brain sections. To determine whether minocycline treatment affects generation of the subtypes of microglial cells, double labeling of ED1, Iba1 with BrdU, as well as ED1 with Arg1 were performed.

In our assessment, we examined two regions of each brain sections: the peri-contusional cortical tissue and the ipsilateral dentate gyrus of the hippocampus. These areas were the focus because high level of inflammatory cell reaction around the lesion area after injury, and the hippocampus has a high number of resident microglia. The hippocampus is also particularly vulnerable to injury because it is a site of continuous

neuronal proliferation throughout the lifespan. Digital image analysis (DIA) was used to determine the proportional area of positive staining in each region, and the intensity/expression level of each marker was expressed as percentages.

	Peri-Injury Cortex				Ipsilateral Dentate Gyrus			
	Young (3M)		Aged (20M)		Young (3M)		Aged (20M)	
	Mino-30min	Mino-4hr	Mino-30min	Mino-4hr	Mino-30min	Mino-4hr	Mino-30min	Mino-4hr
General Microglial Marker								
Iba1	–	–	–	↓	–	–	–	↓
Activated Microglia Markers								
ED1	–	–	–	↓	–	–	–	–
OX6	↓	↓	↓	↓	–	–	–	–
M2 Marker								
ARG1	–	–	–	–	–	–	–	–

TABLE 3-1: Table shows the observed effect of minocycline treatments (30-minute and 4-hour) on cell-type-specific marker staining, compared to vehicle-treated animals. Arrows indicate either statistically significant increases (↑) or decreases (↓) in staining, and lines (–) indicate no significant changes.

EFFECT OF MINOCYCLINE ON MICROGLIA STAINING IN YOUNG AND AGED RATS FOLLOWING CCI – IBA1

Iba1 recognizes the ionized calcium-binding adaptor molecule 1, and detects both resting and activated microglia as well as different subpopulations of microglia (Ito et al. 1998). In previous studies, Iba1+ cells with a ramified morphology are normally observed throughout the parenchyma of the cerebrum and hippocampus of healthy brains (Ito et al. 1998). After injury, Iba1+ staining intensifies around the lesion site and the microglia take on a more amoeboid morphology on the ipsilateral side (Ito et al. 1998).

In this study, Iba1 staining was seen throughout the cortical tissue and hippocampus of all animals (Figure 3-1). In the cortex, staining was lightest in the sham and heavier in the three injury groups. The staining in the ipsilateral dentate gyrus was similarly heaviest in the injury groups and lightest in sham. Also in the dentate gyrus, we observed a change in staining pattern in the injured animals that was not seen in shams. Specifically, staining became denser in a band surrounding the Granular Cell Layer.

In the peri-lesion cortical tissue of young rats (3M), all three injury groups showed intense Iba1 staining in this region compared to sham (Figure 3-1). Densitometry analysis revealed that a significantly higher densities of Iba1+ staining in the vehicle-treated animals ($p=0.049$), animals treated with minocycline beginning at 30 minutes post-injury ($p=0.032$), and animals treated with minocycline beginning at 4 hours post-injury ($p=0.0009$), compared to sham (Figure 3-2). Similarly, in the ipsilateral dentate gyrus of young rats, all three injury groups had higher staining densities of Iba1+ cells, compared to shams (Figures 3-2 and 3-3).

In the aged group, in the cortical tissue, similar to young animals, all injured groups had increased Iba1 staining compared to sham (Figure 3-3). Densitometry analysis showed that compared to sham, the vehicle ($p=0.016$) and minocycline 30 minute ($p=0.031$) groups had statistically higher Iba1+ staining densities (Figure 3-4). The average staining density for the minocycline 4-hour group was higher than sham, but was not significant. Among injured groups, animals received minocycline treatment at 4 hours post-injury had significantly reduced Iba1+ staining density compared to the vehicle group ($p=0.002$) and the 30-min treatment group ($p=0.001$). In the ipsilateral hippocampal dentate gyrus, aged animals in injured groups had more intense Iba1 staining compared to sham (Figure 3-4). Statistically significant higher Iba1+ staining densities were only found in the vehicle group compared to shams ($p=0.017$) (Figure 3-4). Again, the 4-hour minocycline treatment group had significantly lower staining density compared to both the vehicle group ($p=0.003$) and the 30-minute minocycline treatment group ($p=0.005$).

We also examined differences in the proportional Iba1+ area, in the two regions of interest, between the young and aged animals in each treatment group. The aged animals generally had higher Iba1+ staining densities in shams, vehicles, and the 30-minute minocycline treatment group. Following minocycline treatment, the aged animals had reduced Iba1+ staining density in the 4-hour minocycline treatment group, compared to the same treatment regime in young animals, and was significant in the peri-lesion cortex ($p=0.0013$).

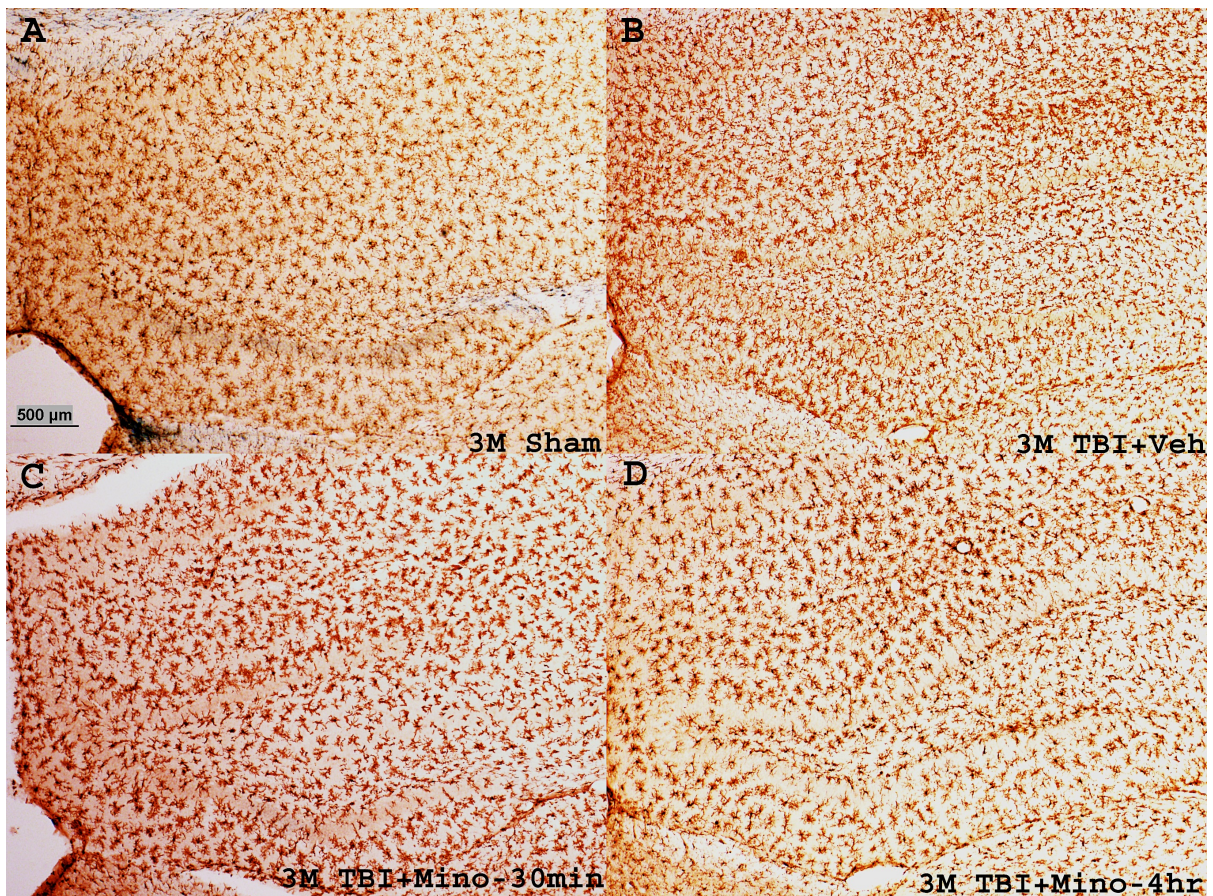
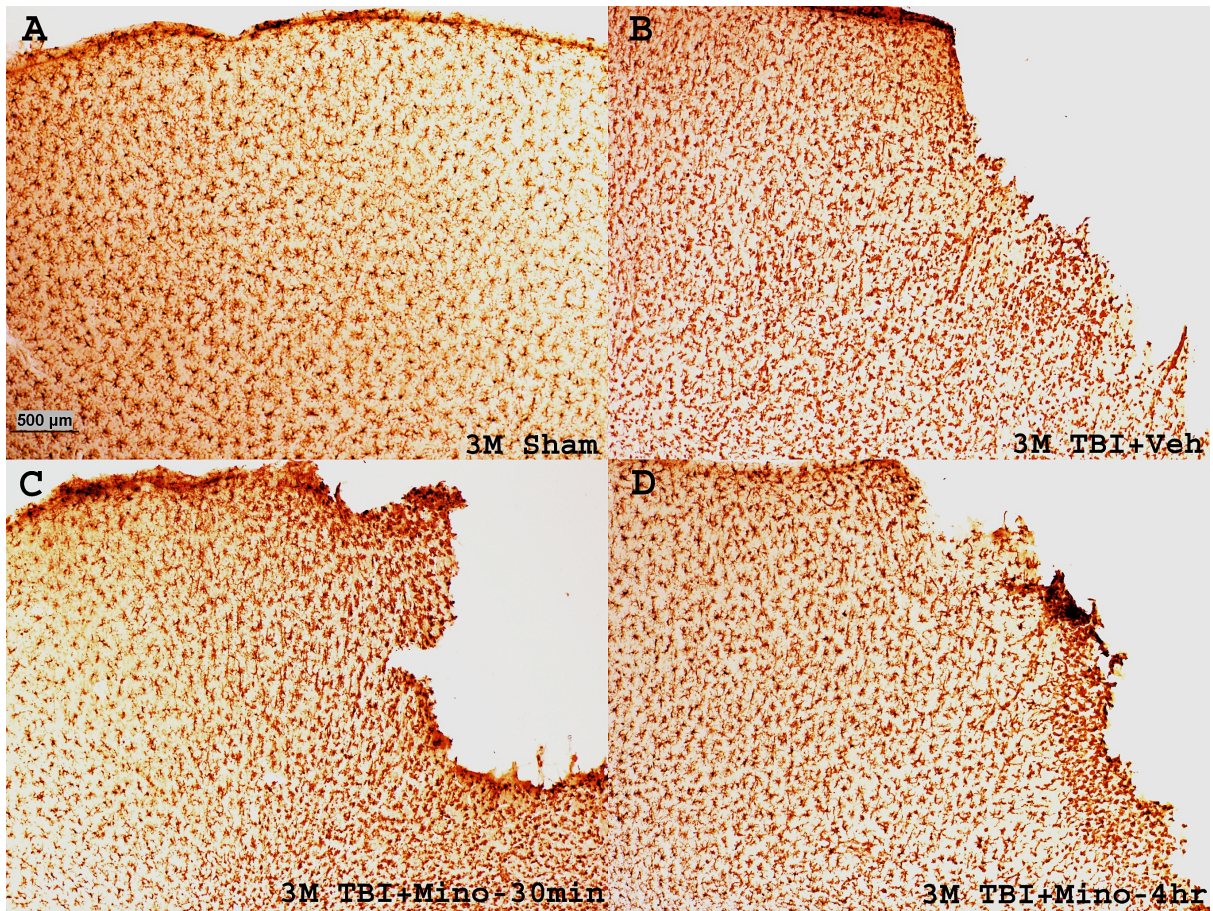
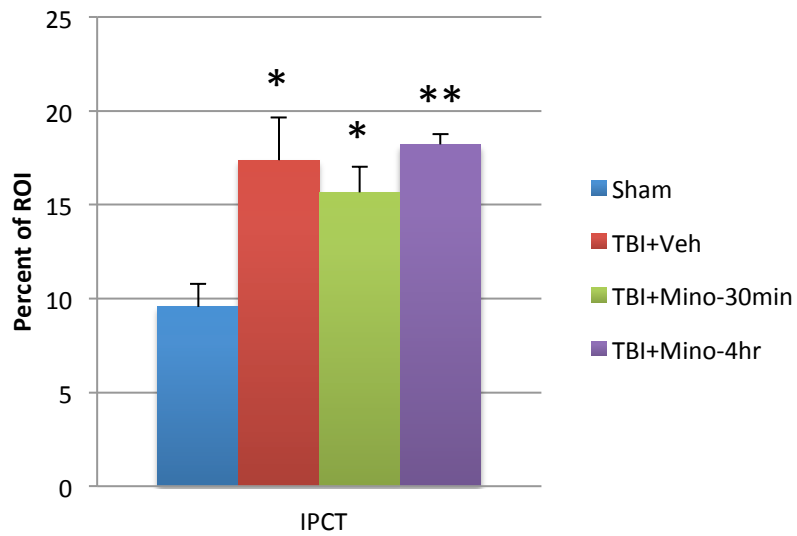


FIGURE 3-1: PATTERN OF IBA1+ STAINING IN THE PERI-LESION CORTEX AND IPSILATERAL DENTATE GYRUS (DG) IN YOUNG (3M) ANIMALS AT 3 DAYS POST-INJURY.

Representative micrograph images of Iba1 staining in the peri-lesion cortical region (upper panels) and the ipsilateral DG (lower panels) from shams (N=3), and injured animals treated with vehicle (N=5), minocycline beginning at 30 minutes after injury (N=5), and minocycline beginning at 4 hours after injury (N=4). For the sham animal, a representative cortical tissue area directly above the hippocampus was selected for comparison with injured animals. Strong Iba1+ staining was seen in all injured groups, compared to shams.

(A) **Percent of Cortical Tissue Stained with Iba1 in 3M Animals**



(B) **Percent of Ipsilateral Dentate Gyrus Stained with Iba1 in 3M Animals**

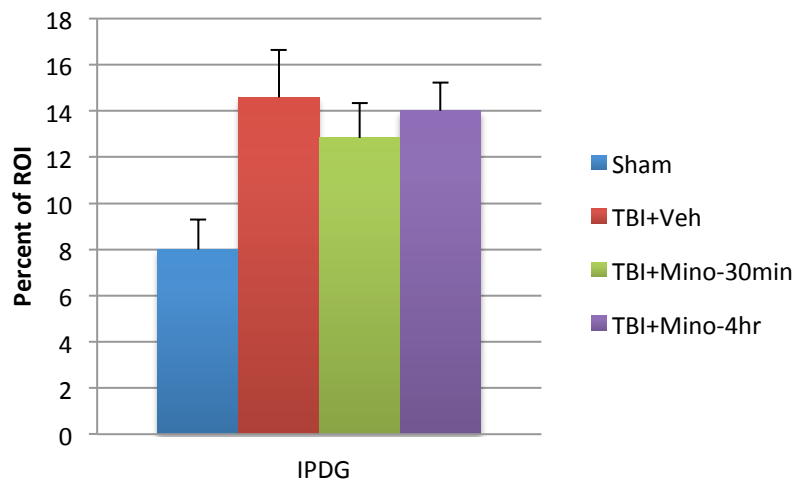


FIGURE 3-2: DENSITOMETRY ANALYSIS OF IBA1 STAINING INTENSITY IN THE IPSILATERAL PERI-LESION CORTEX AND IPSILATERAL DENTATE GYRUS (DG) OF YOUNG RATS (3 M).

Values are given as percent of region of interest (ROI) occupied by the positively labeled cells.

(A) In the peri-lesion cortex region of young animals, the vehicle-treated animals and animals in both minocycline treatment groups had significantly higher intensity of Iba+ cells staining compared to shams (* $p < 0.05$, ** $p < 0.01$). (B) In the hippocampus of young animals, the percentages of Iba staining intensity were higher in all injury groups compared to shams.

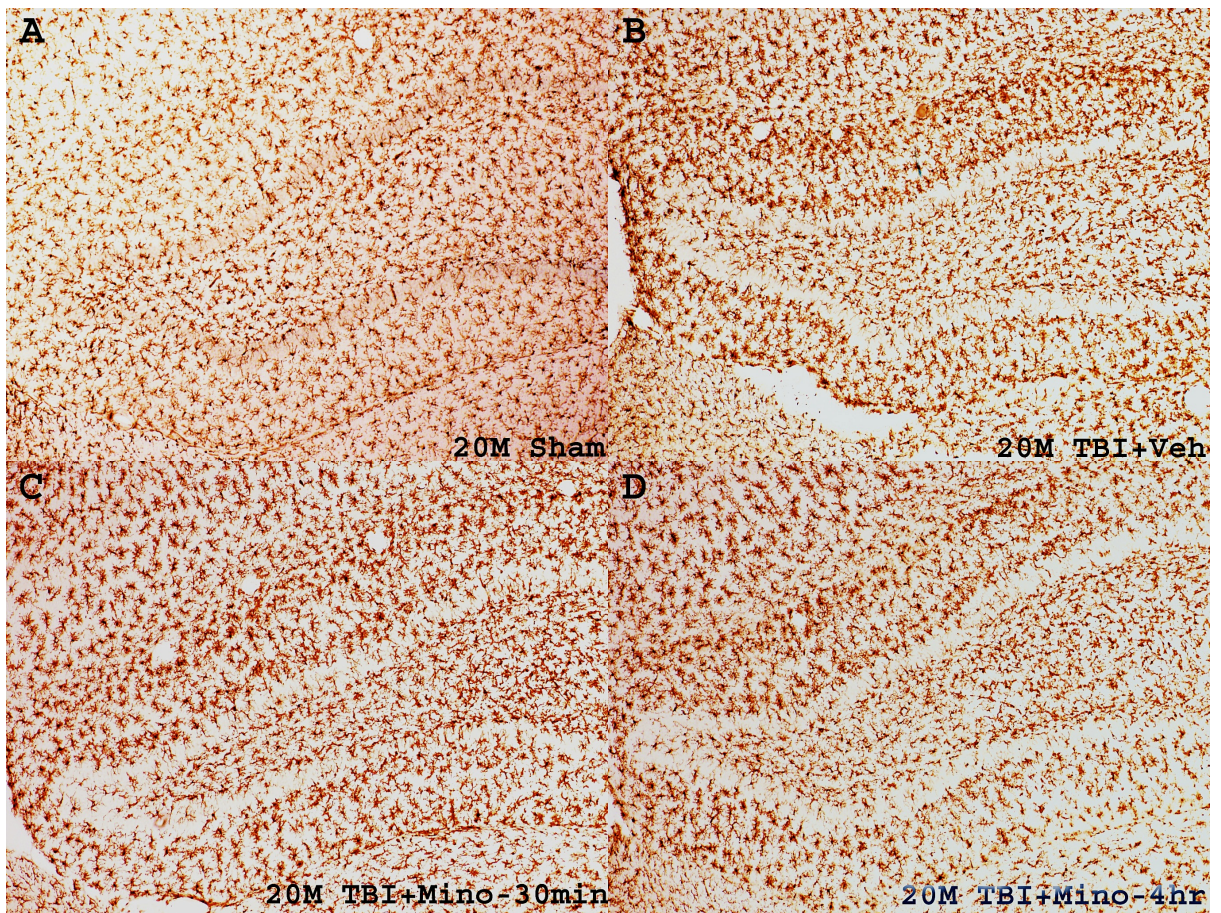
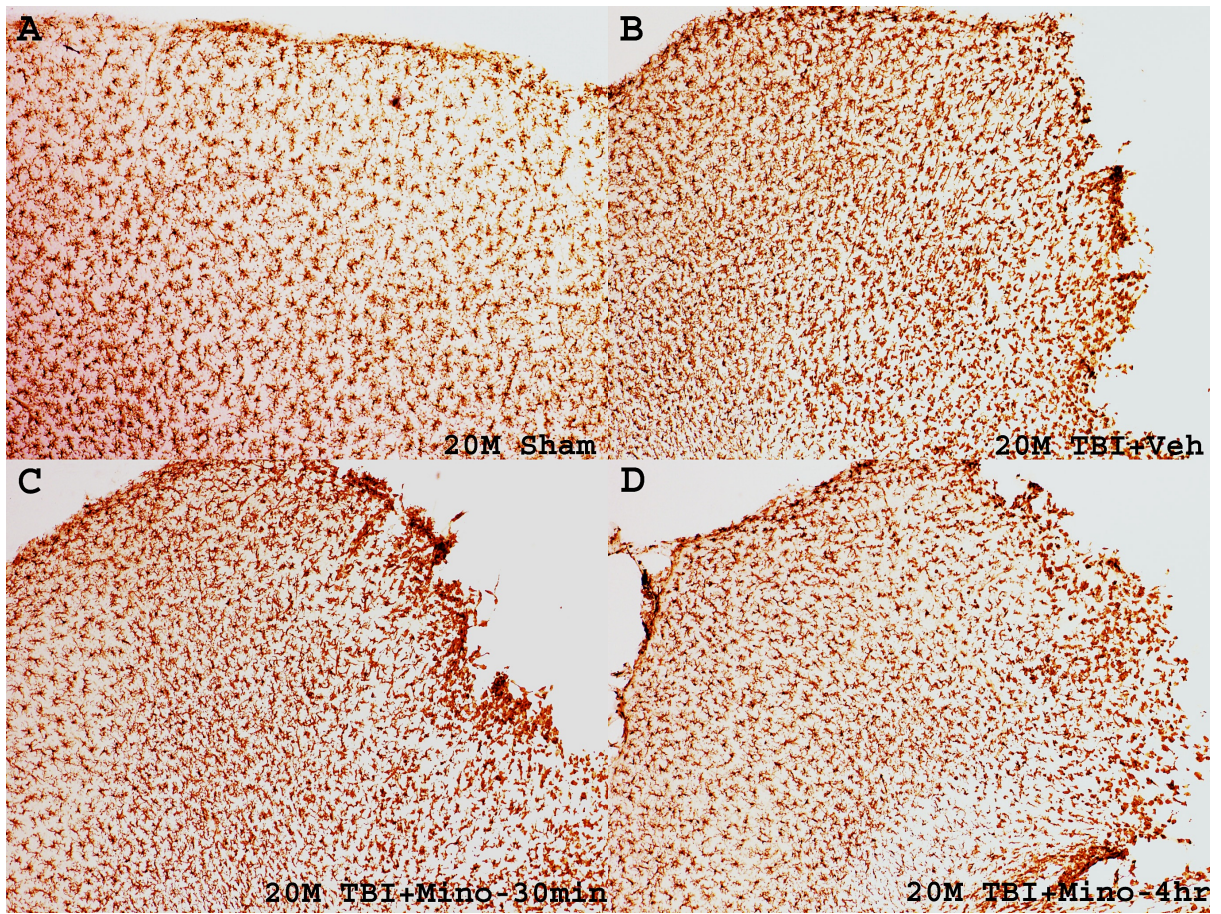
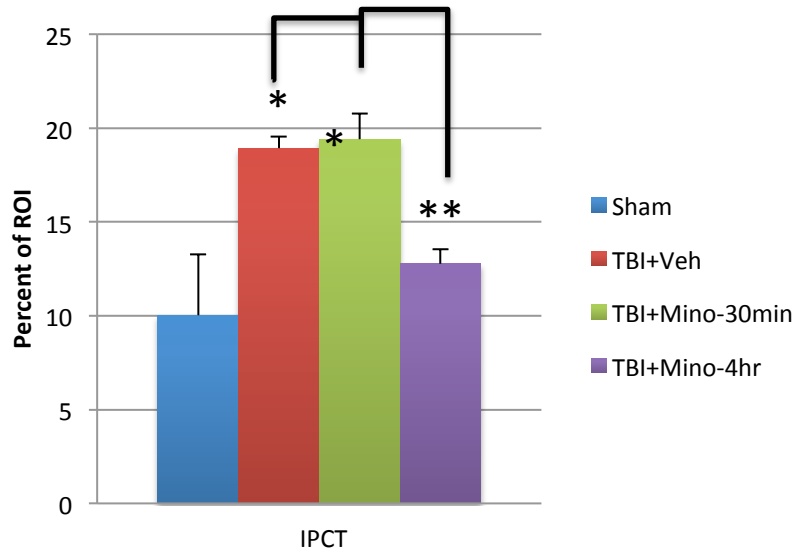


FIGURE 3-3: PATTERN OF IBA1+ STAINING IN THE PERI-LESION CORTEX AND IPSILATERAL DENTATE GYRUS (DG) IN AGED (20) ANIMALS AT 3 DAYS POST-INJURY.

Representative micrograph images of Iba1 staining in the peri-lesion cortical region (upper panels) and the ipsilateral DG (lower panels) from sham (N=2), injured animals treated with vehicle (N=4), minocycline beginning at 30 minutes after injury (N=4), and minocycline beginning at 4 hours after injury (N=4). For the sham animal, a representative cortical tissue area directly above the hippocampus was selected for comparison with injured animals. Strong Iba1+ staining was seen in all injured groups, compared to shams.

(A) Percent of Cortical Tissue Stained with Iba1 in 20M Animals



(B) Percent of Ipsilateral Dentate Gyrus Stained with Iba1 in 20M Animals

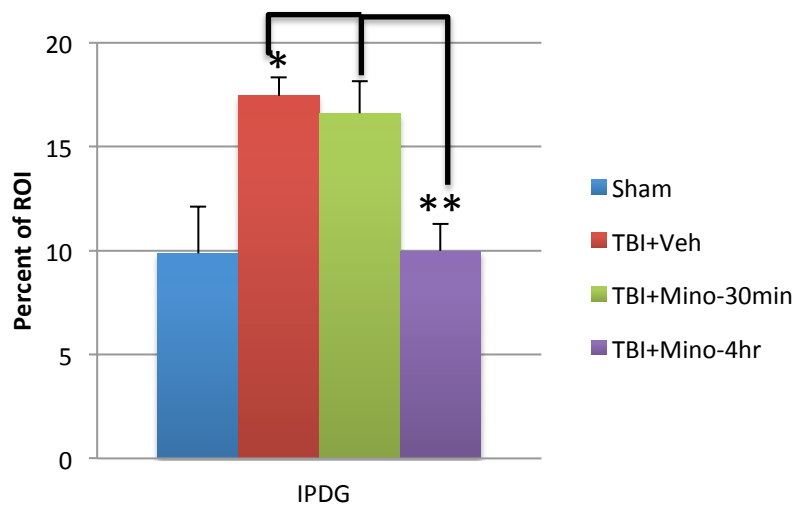


FIGURE 3-4: DENSITOMETRY ANALYSIS OF IBA1 LABELING INTENSITY IN THE IPSILATERAL CORTICAL TISSUE AND IPSILATERAL DENTATE GYRUS OF AGED RATS (20 MONTHS).

Values are given as percent of ROI area occupied by the positively labeled cells. (A) In the cortical tissue of aged animals, the vehicle-treated animals and TBI+Mino-30min groups had significantly higher densities of Iba1+ cells compared to shams (* $p < 0.05$). The density of Iba1 staining in the TBI+Mino-4hr group was also higher compared to shams, but it was not significant. Among three injury groups, the TBI+Mino-4hr group had a significantly lower density of Iba1+ cells compared to vehicles and TBI+Mino-30min group (** $p < 0.01$). (B) In the hippocampus of aged rats, in three injury groups, only the TBI-Vehicle group had a significantly higher intensity of Iba1+ staining compared to sham (* $p < 0.05$). In this region, among injured groups, the TBI+Mino-4hr group had a significantly lower density of Iba1+ cells compared to both vehicles and TBI+Mino-30min animals (** $p < 0.01$).

EFFECT OF MINOCYCLINE ON MICROGLIA STAINING IN YOUNG AND AGED RATS FOLLOWING CCI – ED1

ED1 is a general macrophage marker that labels infiltrating macrophages and activated microglia (Urrea et al. 2007). It recognizes CD68, a lysosomal membrane protein in macrophages. In previous studies, ED1+ staining has been restricted to the area just surrounding the contusion (Urrea et al. 2007). ED1+ microglia near the lesion have been observed to have an amoeboid morphology, whereas cells further away are smaller and are characterized by thick, elongated processes (Zhang et al. 2012).

In sections examined in sham animals, ED1+ staining was very low in both cortical and hippocampal regions (Figures 3-5 and 3-7). In the cortex, the positively labeled cells were concentrated at the injury site edge, with sparser staining moving away from the cavity (Figures 3-5 and 3-7). In both age groups, we see the heaviest staining seen in vehicle groups and lighter staining in the minocycline treatment groups. A similar pattern was seen among the groups when examining the dentate gyrus (Figures 3-5 and 3-7), although staining was not concentrated in any particular spot in the hippocampus. We therefore only compared the two minocycline treatment groups to the vehicle-treated group.

In three-month-old rats, in the injured cortical regions, there were no statistically significant differences among the three injury groups, due to high standard errors. Examining the young animal dentate gyri, we also found no significant differences among the vehicle-and minocycline-treated groups. For the 20-month-old animals in the two regions, a similar comparison was made only among the injured groups examined. In the cortex, among three injured groups, both minocycline treatment groups had lower level of ED1 staining compared to vehicle group, and the difference was statistically significant in

the 4-hour minocycline treatment group ($p=0.047$, Fig3-8A). There were no statistically significant differences between two minocycline treatment groups. In the DG, no significant differences were found among any of the injury groups (Fig.3-8B). Also, a comparison for each treatment group, between aged and young rats, revealed no significant differences in staining ED1+ staining density.

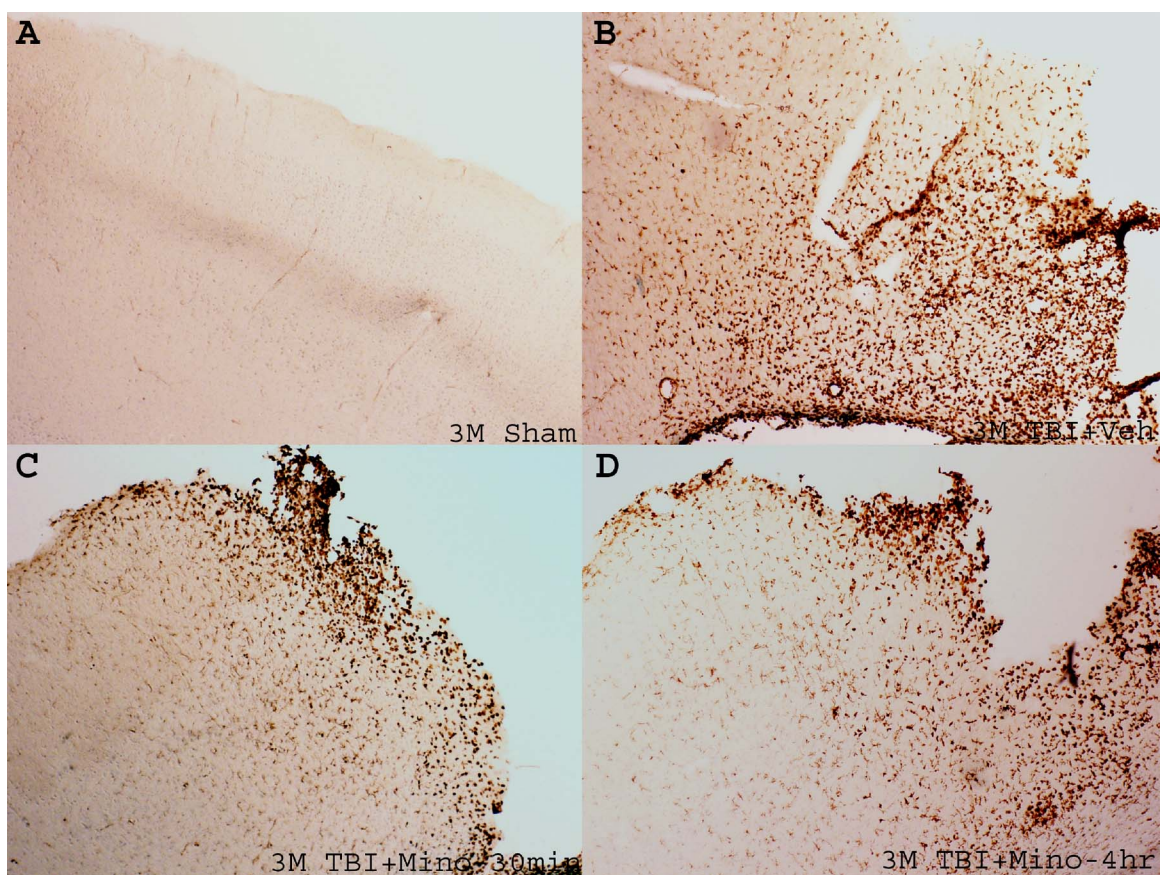
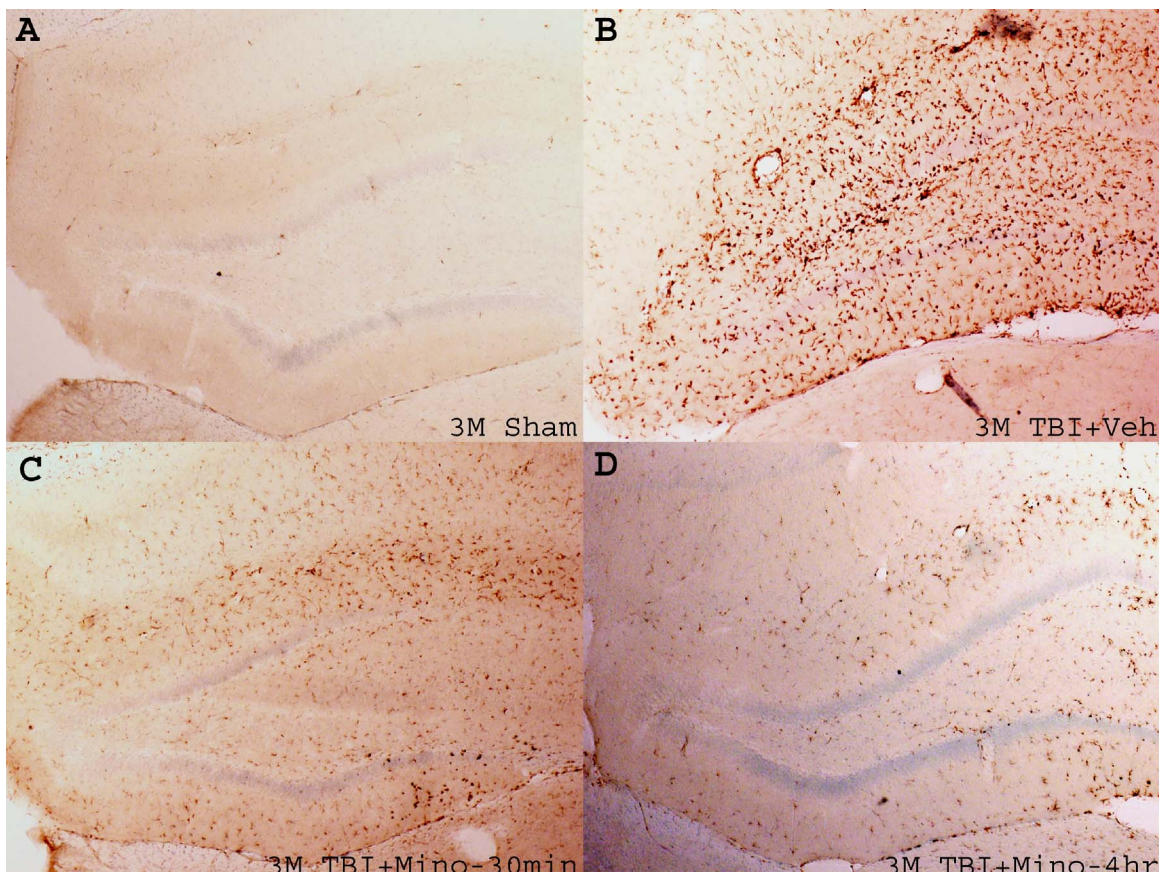
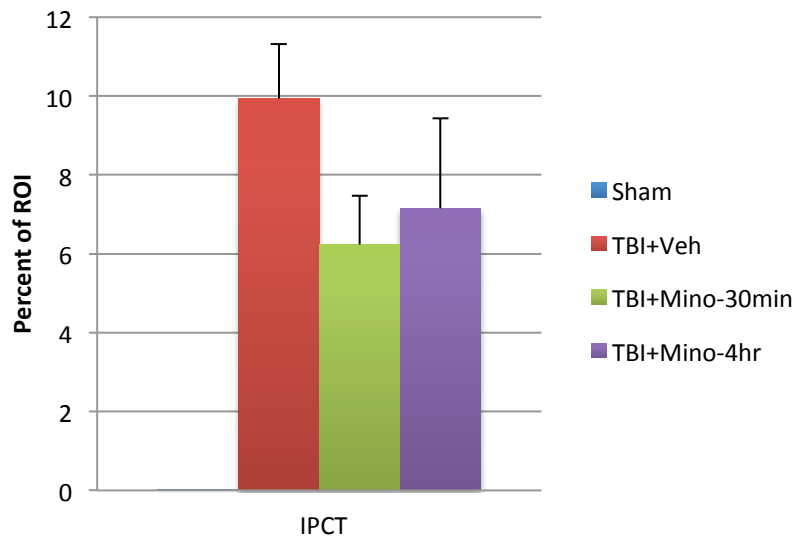


FIGURE 3-5: PATTERN OF ED1+ STAINING IN THE PERI-LESION CORTEX AND IPSILATERAL DENTATE GYRUS (DG) IN YOUNG (3M) ANIMALS AT 3 DAYS POST-INJURY.

Representative micrograph images of ED1 staining in the peri-lesion cortical region (upper panels) and the ipsilateral DG (lower panels) from shams (N=2), and injured animals treated with vehicle (N=4), minocycline beginning at 30 minutes after injury (N=5), and minocycline beginning at 4 hours after injury (N=3). For the sham animal, a representative cortical tissue area directly above the hippocampus was selected for comparison with injured animals. In both regions, strong ED1 staining was seen in all injured groups. ED1 staining was negligible in the shams.

(A) Percent of Cortical Tissue Stained with ED1 in 3M Animals



(B) Percent of Ipsilateral Dentate Gyrus Stained with ED1+ Cells in 3M Animals

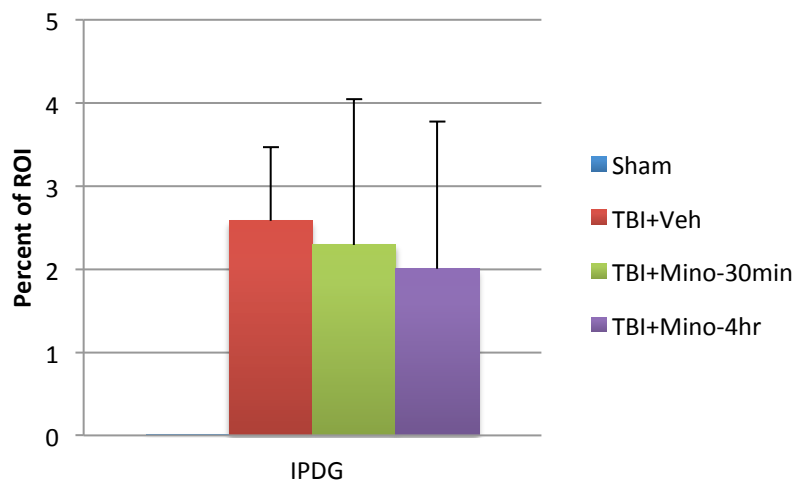


FIGURE 3-6: DENSITOMETRY ANALYSIS OF ED1 STAINING INTENSITY IN THE IPSILATERAL CORTICAL TISSUE AND IPSILATERAL DENTATE GYRUS OF YOUNG RATS (3 MONTHS).

Values are given as percent of ROI area occupied by the positively labeled cells. (A) In the cortical tissue of young rats, proportional areas of ED1+ cells were lower in both minocycline treatment groups, compared to vehicle, but did not reach significance. (B) In the dentate gyrus of young animals, all injury groups again had higher densities of ED1+ staining. Minocycline groups had slightly lower staining intensity than vehicle group but were not significant.

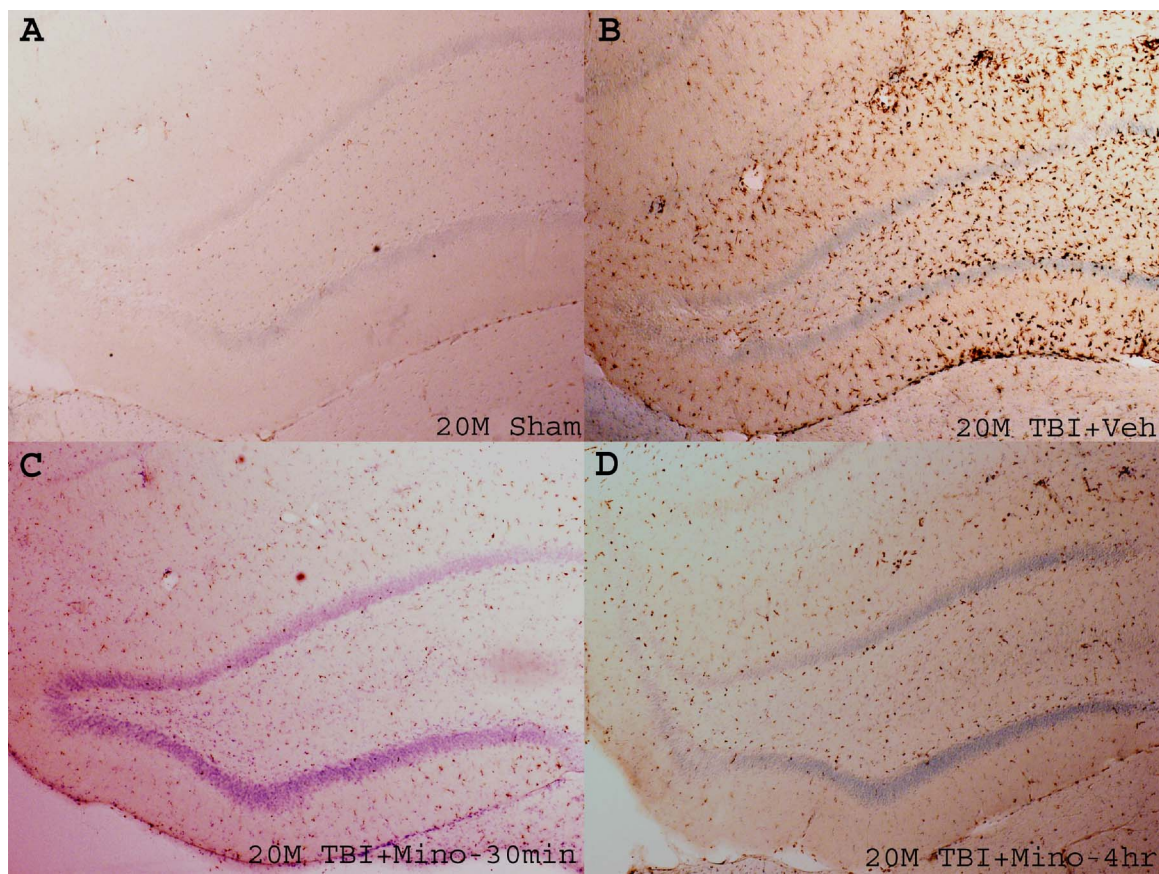
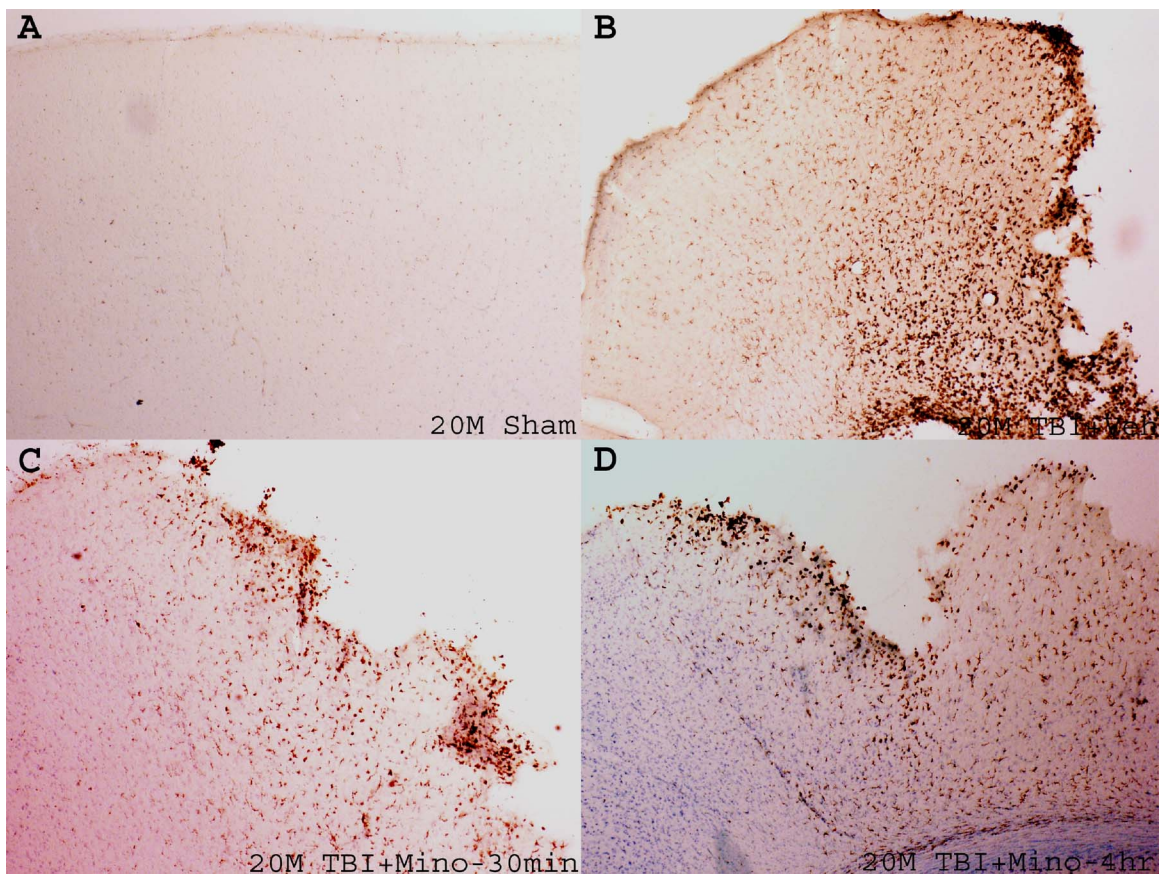
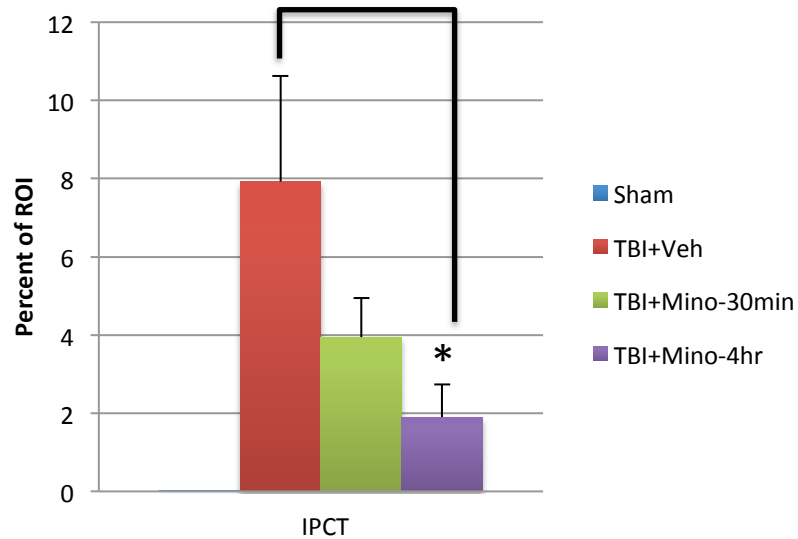


FIGURE 3-7: PATTERN OF ED1+ STAINING IN THE PERI-LESION CORTEX AND IPSILATERAL DENTATE GYRUS (DG) IN AGED (20M) ANIMALS AT 3 DAYS POST-INJURY.

Representative micrograph images of ED1 staining in the peri-lesion cortical region (upper panels) and the ipsilateral DG (lower panels) from shams (N=1), and injured animals treated with vehicle (N=4), minocycline beginning at 30 minutes after injury (N=4), and minocycline beginning at 4 hours after injury (N=4). For the sham animal, a representative cortical tissue area directly above the hippocampus was selected for comparison with injured animals. In both regions, strong ED1 staining was seen in all injured groups. ED1 staining was negligible in the shams.

(A) Percent of Cortical Tissue Stained with ED1 in 20M Animals



(B) Percent of Ipsilateral Dentate Gyrus Stained with ED1 in 20M Animals

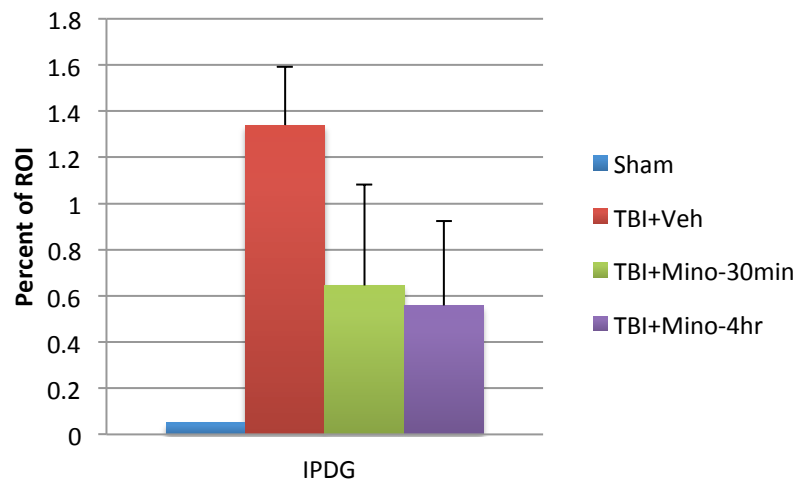


FIGURE 3-8: DENSITOMETRY ANALYSIS OF ED1 STAINING INTENSITY IN THE IPSILATERAL CORTICAL TISSUE AND IPSILATERAL DENTATE GYRUS OF AGED RATS (20 MONTHS).

Values are given as percent of ROI area occupied by the positively labeled cells. (A) In the aged rat cortical tissue, both minocycline treatment groups had lower intensity of ED1+ staining compared to vehicles and was significant in the 4-hr treatment group (* $p < 0.05$). Similarly, in the DG both minocycline groups had less ED1 staining compared to vehicle group, but did not reach statistical significance (B).

EFFECT OF MINOCYCLINE ON MICROGLIA STAINING IN YOUNG AND AGED RATS FOLLOWING CCI – OX6

OX6 is another general macrophage marker, which identifies an antigen on the major histocompatibility complex (MHC) class II that is present on antigen-presenting cells, including microglia and macrophages. In the sham animals in both young and aged groups, very little OX6+ staining was found (Figures 3-9 and 3-11). After injury, OX6+ cells have the morphology of activated microglia (i.e. amoeboid), and the density of OX6+ microglia is higher at the site of injury (Folkersma et al. 2011). In the cortex, the vehicles had the heaviest amount of staining compared to the minocycline treatment groups, in both age groups (Figures 3-9 and 3-11). In vehicles, the staining was mostly diffuse in the cortex, but in the minocycline treatment groups staining was primarily only seen at the cavity edge. In the dentate gyrus, staining appeared sparse and non-localized, in both age groups (Figures 3-9 and 3-11).

Densitometry analysis found that in young animals, the two minocycline treatment groups had significantly reduced OX6+ staining densities compared to the vehicles ($p=0.03$, for both, Figure 3-10). There was no difference in staining between the two minocycline treatment groups. In the dentate gyrus, the vehicle group had the highest density of OX6+ staining, and the two minocycline treatment groups both had lower densities (Figure 3-10).

In the cortex of aged animals, both of the minocycline treatment groups had significantly lower OX6+ cell densities compared to vehicles ($p=0.012$ for 30-minute group, $p=0.017$ for 4-hour group) (Figure 3-12). There were no significant differences between the two minocycline treatment groups. In the hippocampus of aged animals, we observed no significant differences in staining densities among any of the injury groups (Figure 3-12).

A comparison between the two age groups was also made using each treatment group in both ROIs. There were no statistically significant differences between the age groups in any of the animal groups.

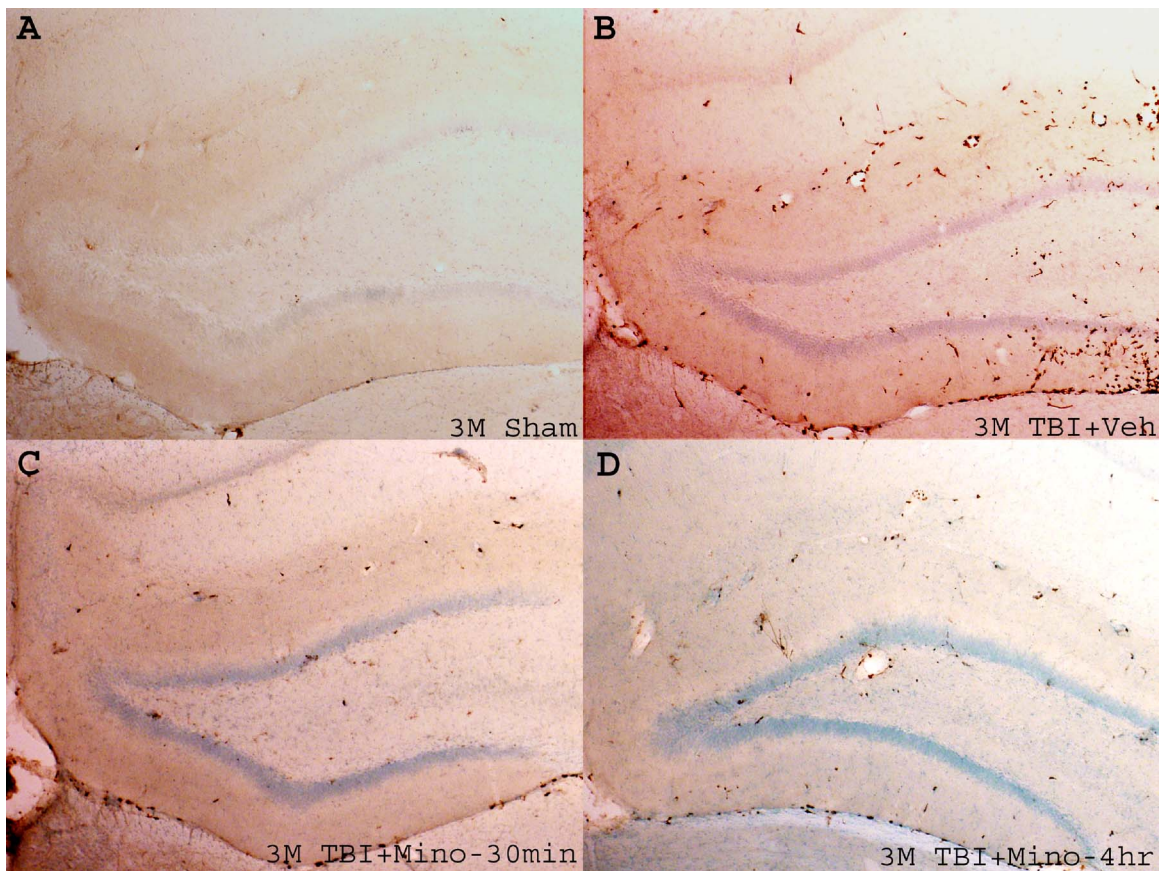
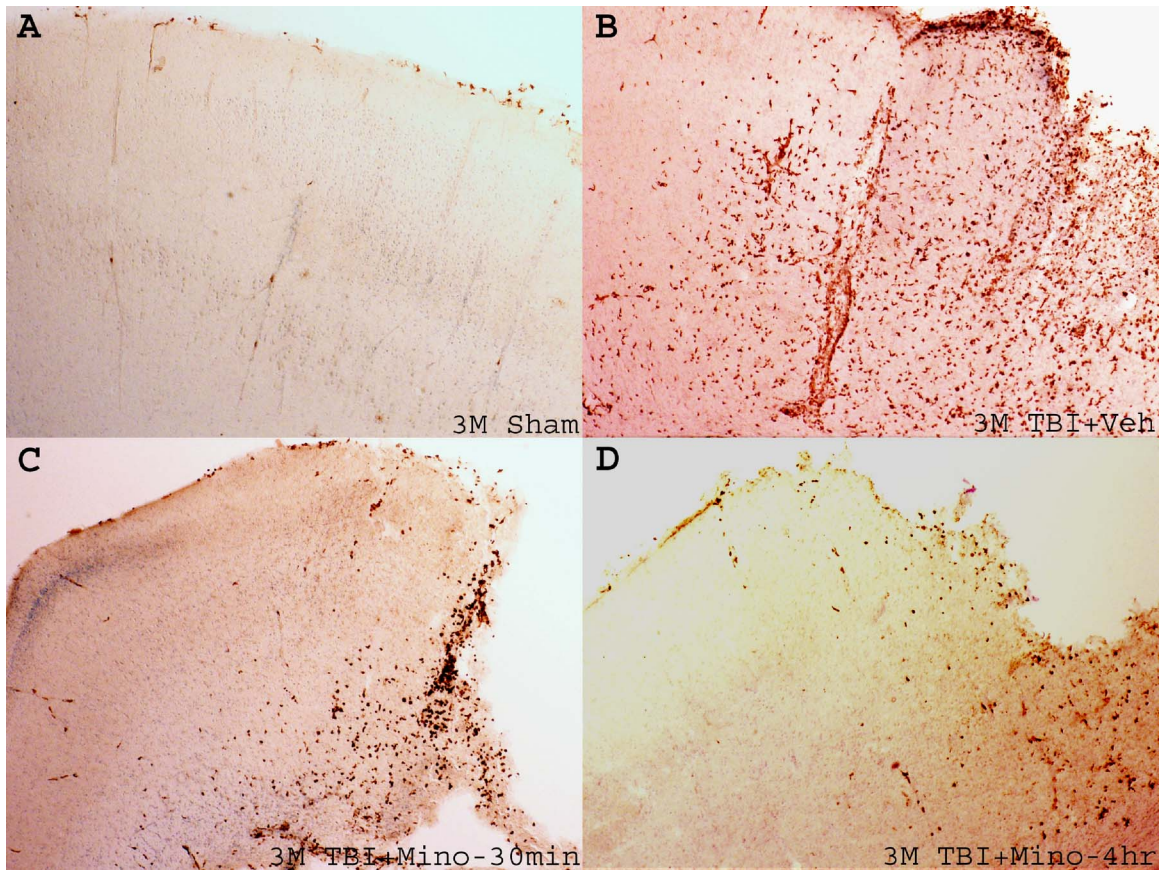
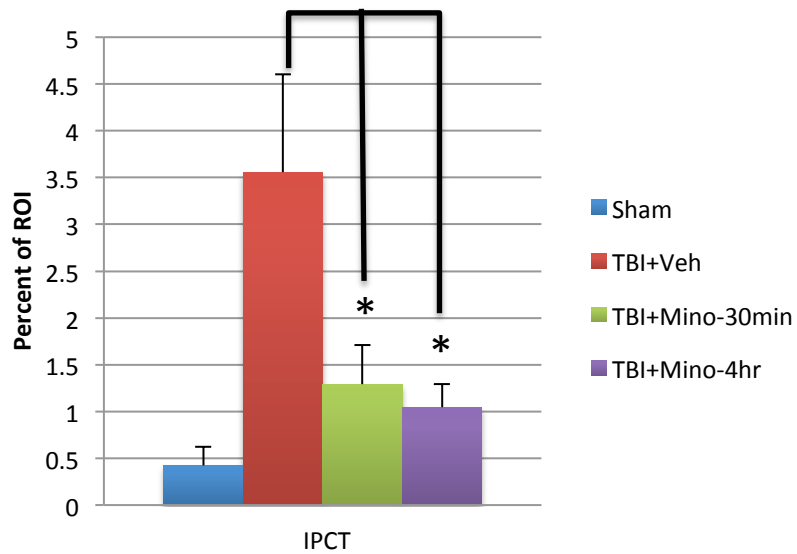


FIGURE 3-9: PATTERN OF OX6+ STAINING IN THE PERI-LESION CORTEX AND IPSILATERAL DENTATE GYRUS (DG) IN YOUNG (3M) ANIMALS AT 3 DAYS POST-INJURY.

Representative micrograph images of OX6 staining in the peri-lesion cortical region (upper panels) and the ipsilateral DG (lower panels) from shams (N=3), and injured animals treated with vehicle (N=5), minocycline beginning at 30 minutes after injury (N=6), and minocycline beginning at 4 hours after injury (N=4). For the sham animal, a representative cortical tissue area directly above the hippocampus was selected for comparison with injured animals. Strong OX6 staining was seen in all injured groups in the cortical region. In the DG, OX6 staining was found in the vehicle group. OX6 staining was negligible in the shams.

(A) Percent of Cortical Tissue Stained with OX6 in 3M Animals



(B) Percent of Ipsilateral Dentate Gyrus Stained with OX6 in 3M Animals

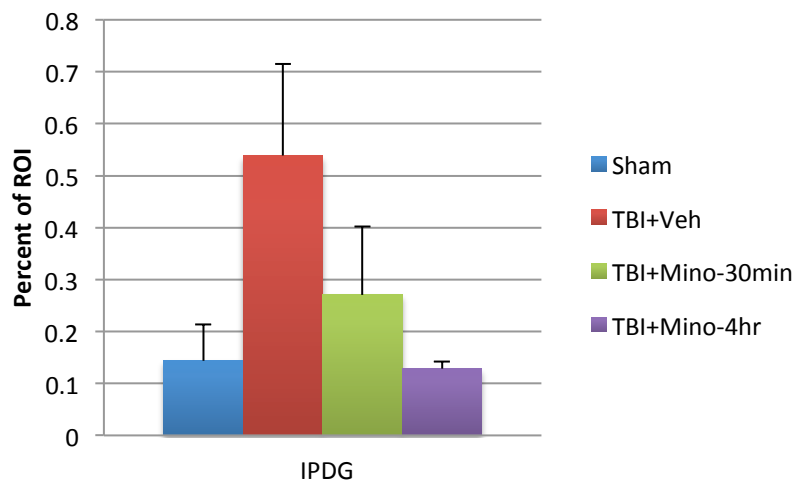


FIGURE 3-10: DENSITOMETRY ANALYSIS OF OX6 LABELING INTENSITY IN THE IPSILATERAL CORTICAL TISSUE AND IPSILATERAL DENTATE GYRUS OF YOUNG RATS (3 MONTHS).

Values are given as percent of ROI area occupied by the positively labeled cells. (A) In the cortical tissue of young animals, both minocycline treatment groups had significantly less OX6+ staining compared to vehicles (* $p < 0.05$). (B) In the hippocampus of young animals, there were also no significant differences in OX6+ cell area density among any of the treatment groups.

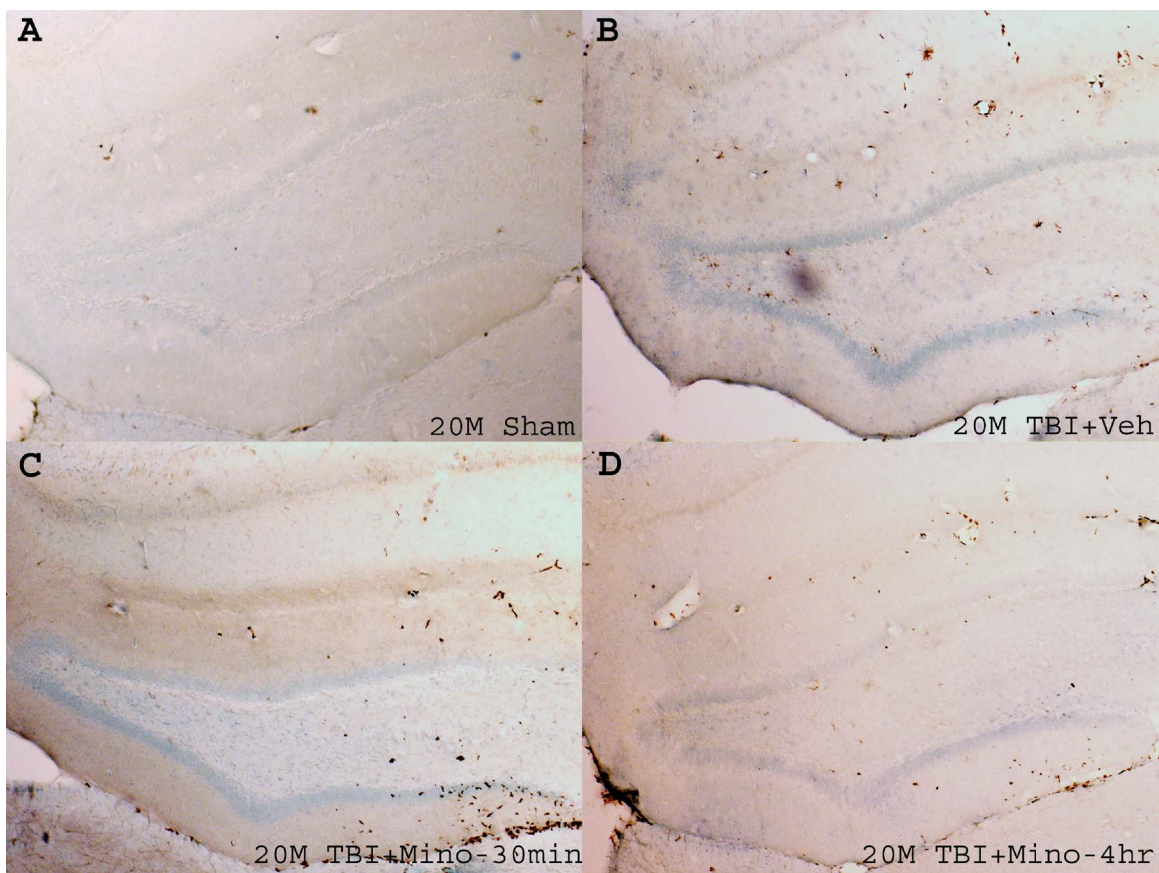
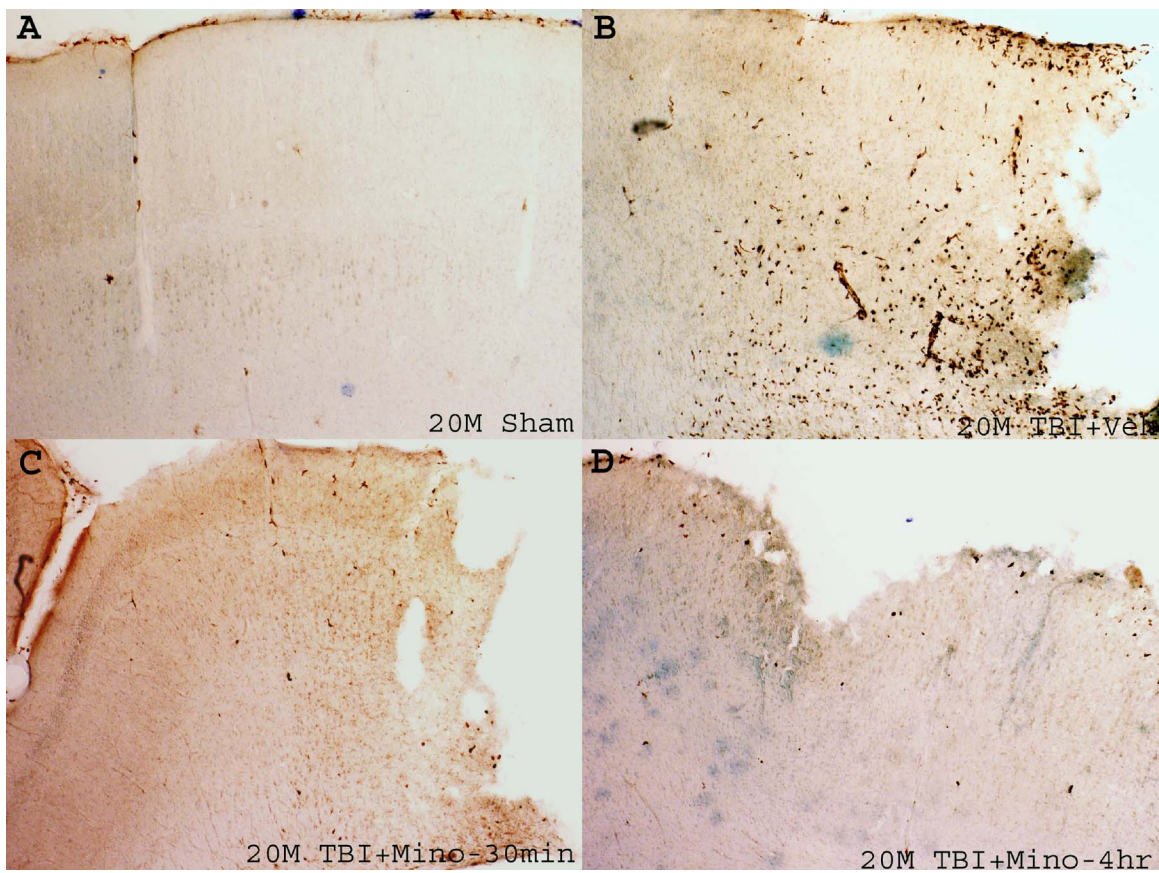
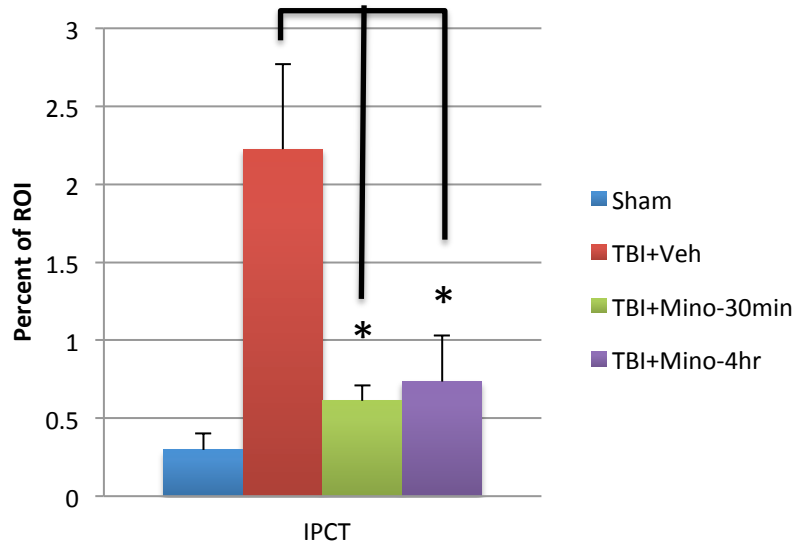


FIGURE 3-11: PATTERN OF OX6+ STAINING IN THE PERI-LESION CORTEX AND IPSILATERAL DENTATE GYRUS (DG) IN AGED (20M) ANIMALS AT 3 DAYS POST-INJURY.

Representative micrograph images of OX6 staining in the peri-lesion cortical region (upper panels) and the ipsilateral DG (lower panels) from shams (N=2), and injured animals treated with vehicle (N=4), minocycline beginning at 30 minutes after injury (N=4), and minocycline beginning at 4 hours after injury (N=4). For the sham animal, a representative cortical tissue area directly above the hippocampus was selected for comparison with injured animals. Strong OX6 staining was seen in injured vehicle group in the cortical region, both minocycline treated groups had a few scattered OX6+ cells. In the DG, OX6 stained cells were found scattered in the DG in all injury groups. OX6 staining was negligible in the shams.

(A) **Percent of Cortical Tissue Stained with OX6 in 20M Animals**



(B) **Percent of Ipsilateral Dentate Gyrus Stained with OX6 in 20M Animals**

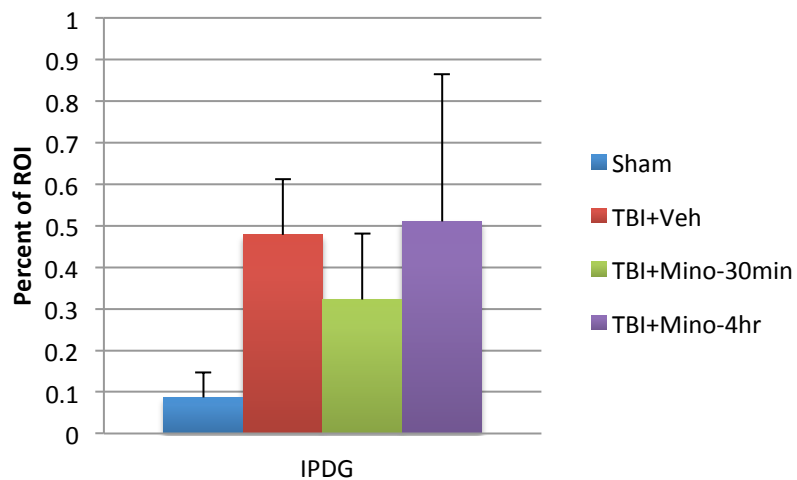


FIGURE 3-12: DENSITOMETRY ANALYSIS OF OX6 CELL LABELING IN THE IPSILATERAL CORTICAL TISSUE AND IPSILATERAL DENTATE GYRUS OF AGED RATS (20 MONTHS).

Values are given as percent of ROI area occupied by the positively labeled cells. (A) In the cortical tissue of aged animals, the OX6+ staining density was significantly lower ($*p<0.05$) in both minocycline treatment groups, compared to vehicle-treated animals. (B) In the dentate gyrus of aged animals, there were no significant differences in OX6+ cell area densities among any of the groups compared.

EFFECT OF MINOCYCLINE ON M2 PHENOTYPE IN YOUNG AND AGED RATS FOLLOWING CCI INJURY

To determine whether any changes in microglial phenotype occurred with minocycline treatment in both young and aged rats undergoing CCI injury, we stained sections for Arginase 1 expression. Arg1 antibody reacts with arginase, an enzyme involved in the metabolism of arginine, and is a specific marker for M2 cells. However, arginase 1 is also known to be weakly expressed in astrocytes (as well as some neurons) in the normal rat CNS (Ahn et al. 2012). In both young and aged animals, very little ARG1+ staining was found in the cortex and DG of shams (Figures 3-13 and 3-15). In the cortex in injury groups, Arg1+ cells were mostly localized around the injury cavity (Figures 3-13 and 3-15). In the dentate gyrus, staining is sparse and scattered across the region (Figures 3-13 and 3-15).

In the cortex of young animals, all injury groups were observed to have higher ARG1+ staining densities than sham (Figure 3-14). However, there were no statistically significant differences among any of the injury groups (vehicle, 30-minute minocycline, and 4-hour minocycline). In the hippocampus of young animals, there were also no significant differences among any of the injury groups, meaning there was no effect with minocycline treatment. In the aged animals, increased ARG1+ cell density was statistically significant in vehicles and the 30-minute minocycline group, when compared to shams. However, there were no significant differences among the injury groups. In the hippocampus of the aged animals, the ARG1+ staining densities of the injury groups were not significantly different from shams. Although, the ARG1+ cell density was significantly lower in the 4-hour minocycline treatment group when compared to vehicles ($p=0.014$). There were no differences in staining between the two minocycline treatment groups or between the 30-minute minocycline treatment group and vehicles.

When comparing the two age groups, we found no significant differences in Arg1 staining intensity between any of the animal groups in the cortical tissue. Examining the hippocampus, we found a significant difference between the two 4-hour minocycline treatment groups, with the aged group having lower staining density than the young.

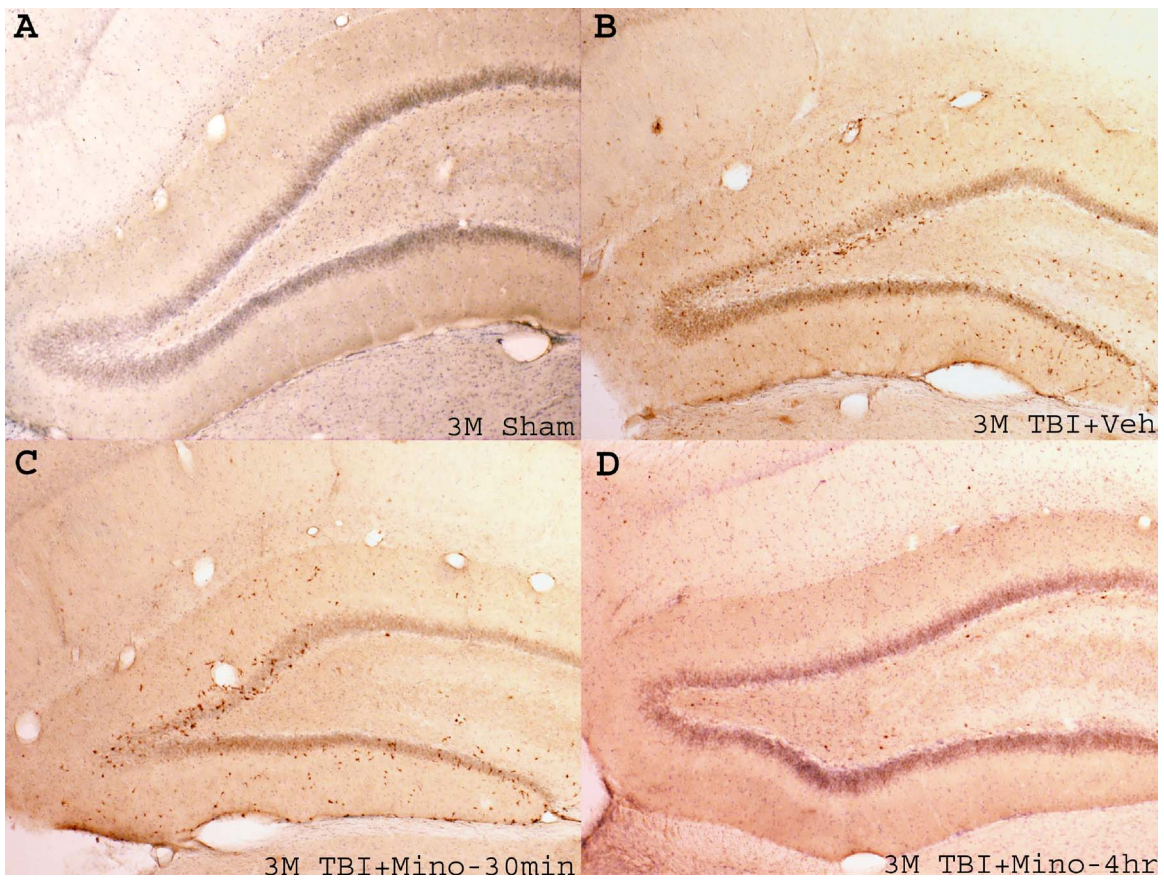
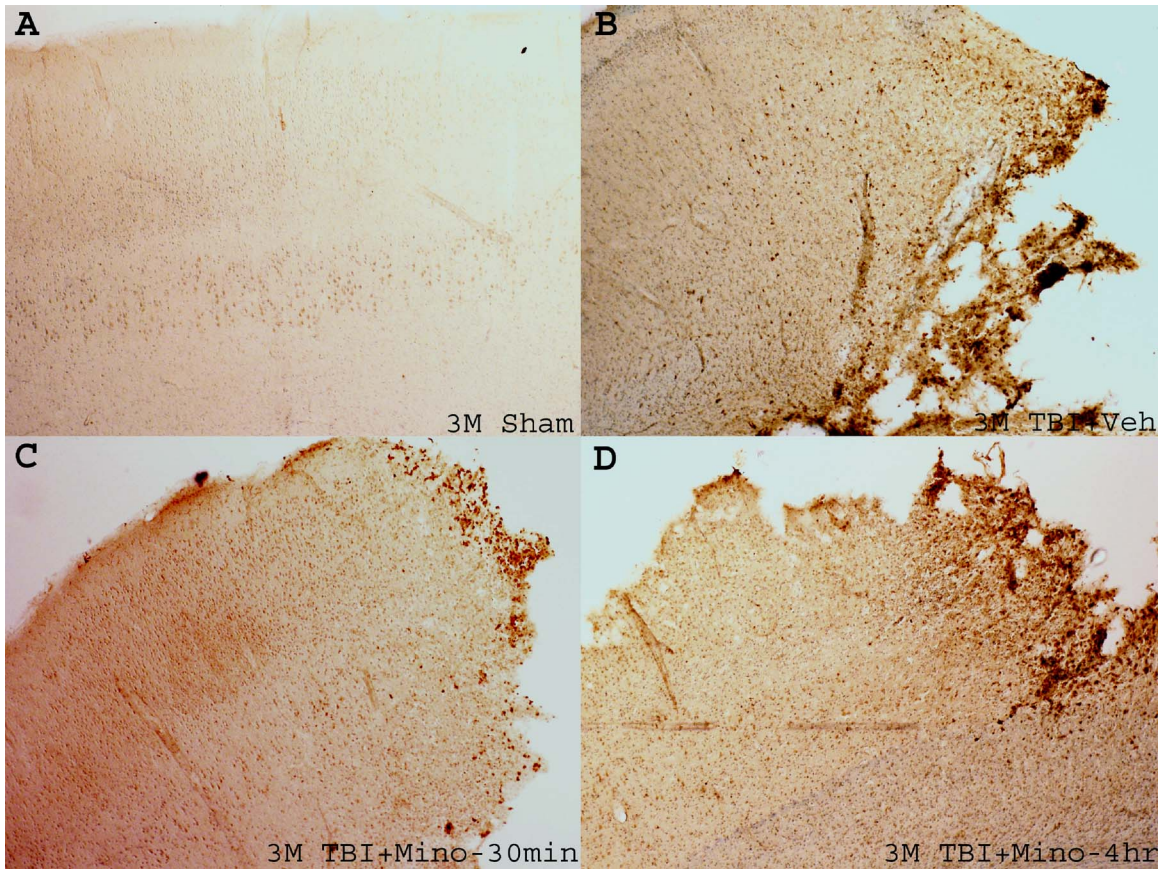
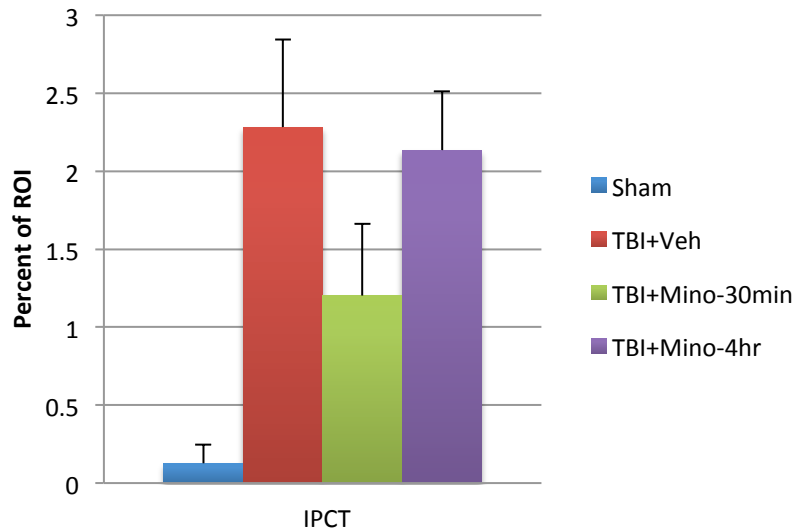


FIGURE 3-13: PATTERN OF M2 MARKER ARG1+ STAINING IN THE PERI-LESION CORTEX AND IPSILATERAL DENTATE GYRUS (DG) IN YOUNG (3M) ANIMALS AT 3 DAYS POST-INJURY.

Representative micrograph images of ARG1 staining in the peri-lesion cortical region (upper panels) and the ipsilateral DG (lower panels) from shams (N=2), and injured animals treated with vehicle (N=5), minocycline beginning at 30 minutes after injury (N=4), and minocycline beginning at 4 hours after injury (N=4). For the sham animal, a representative cortical tissue area directly above the hippocampus was selected for comparison with injured animals.

Strong ARG1 staining was seen in injured vehicle group in the cortical region, both minocycline treated groups had a few scattered ARG1+ cells. In the DG, ARG1 stained cells were found scattered in the DG in all injury groups. No ARG1+ staining was found in the shams.

(A) Percent of Cortical Tissue Stained with ARG1 in 3M Animals



(B) Percent of Ipsilateral Dentate Gyrus Stained with ARG1 in 3M Animals

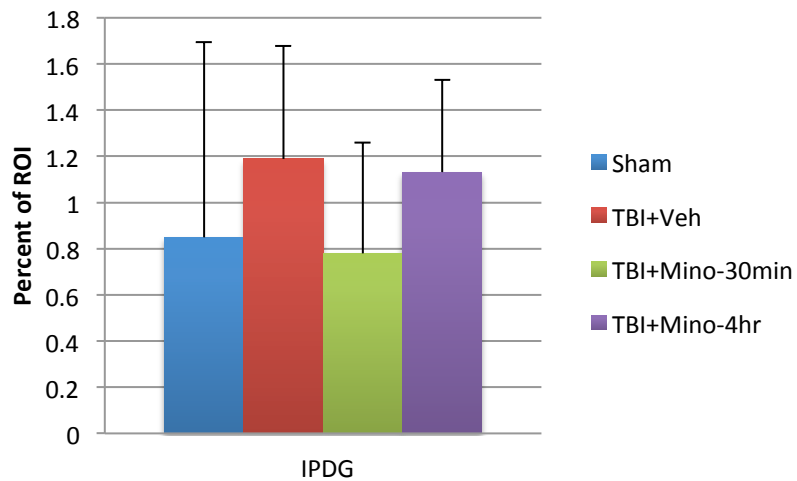


FIGURE 3-14: DENSITOMETRY ANALYSIS OF ARG1 LABELING IN THE IPSILATERAL CORTICAL TISSUE AND IPSILATERAL DENTATE GYRUS OF YOUNG RATS (3 MONTHS).

Values are given as percent of ROI area occupied by the positively labeled cells. (A) In the cortical tissue of young rats, there was negligible ARG1+ staining in shams. The mean ARG1+ staining density was higher in all three injury groups. The differences among the vehicle, Mino-30min, and Mino-4hr groups were not significant. (B) In the hippocampus of young rats, there were no significant differences in ARG1+ cell area densities among any of the injury groups.

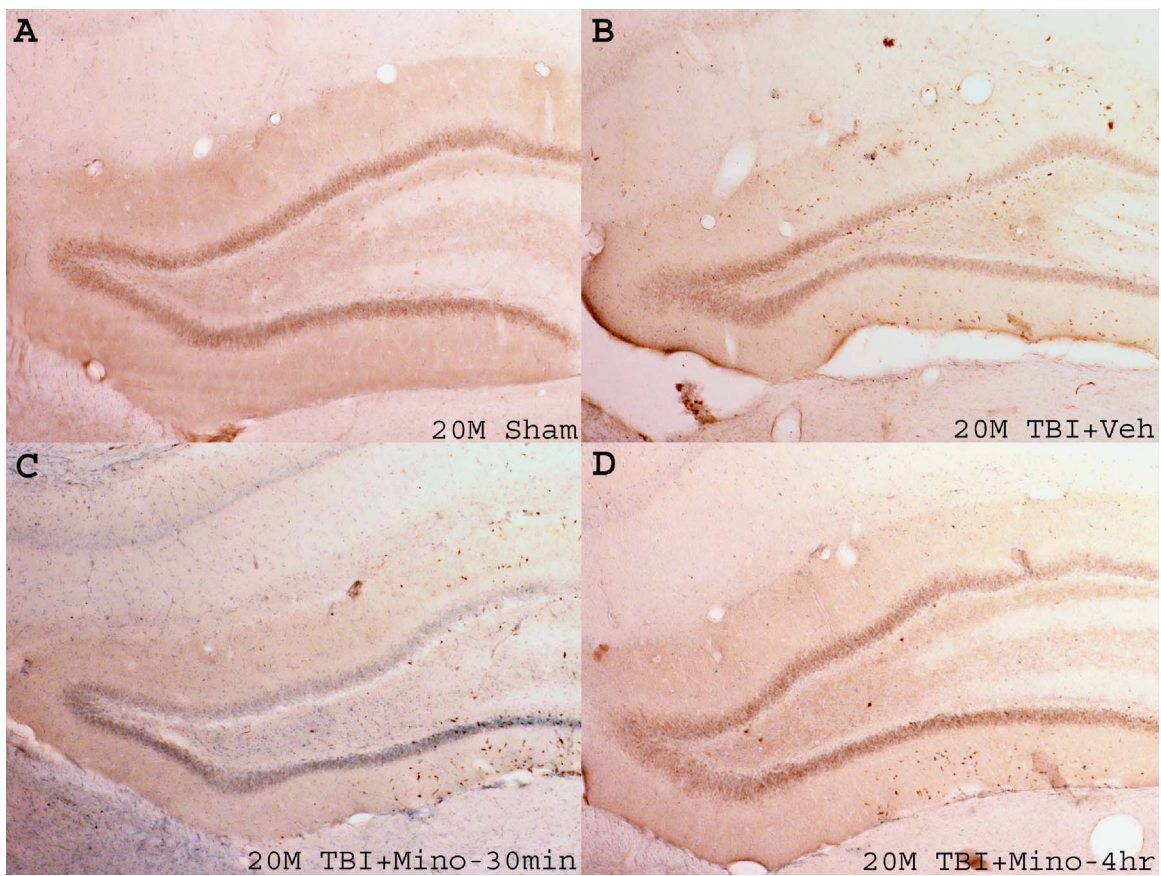
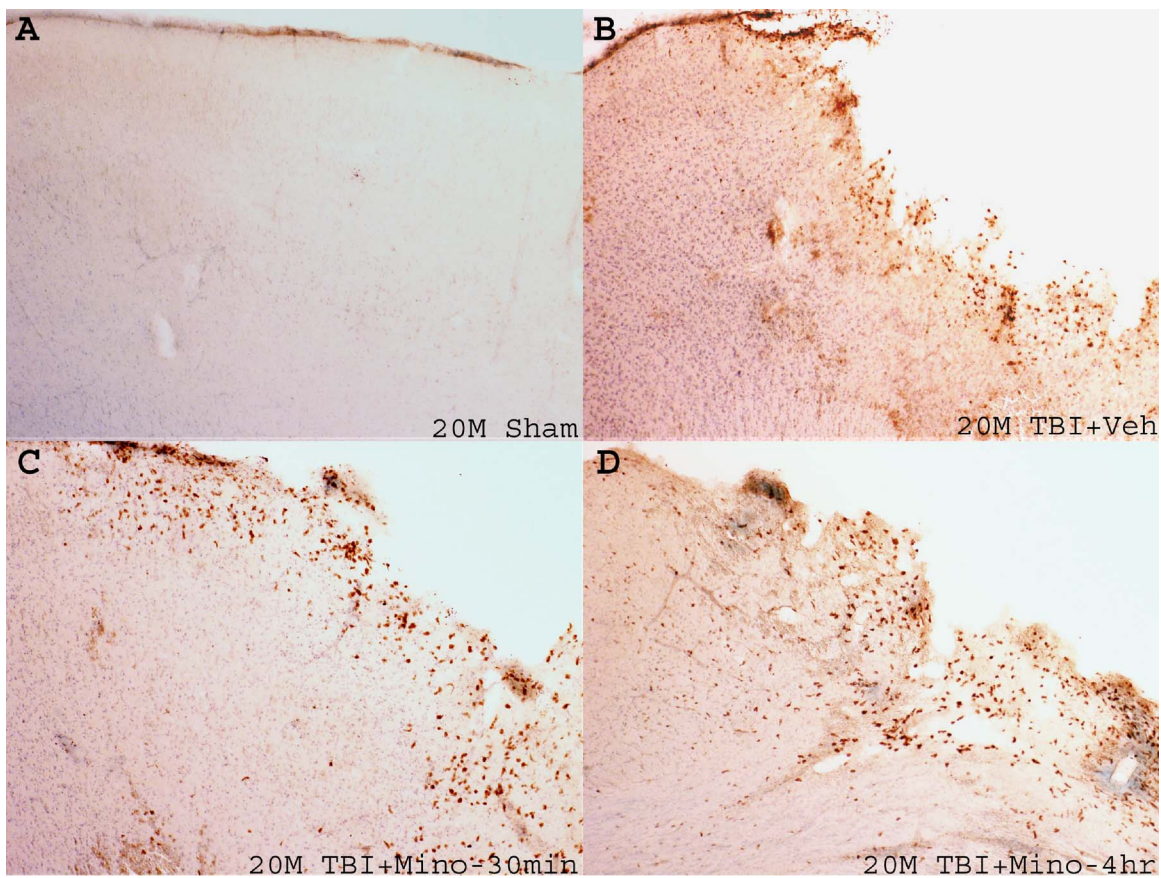
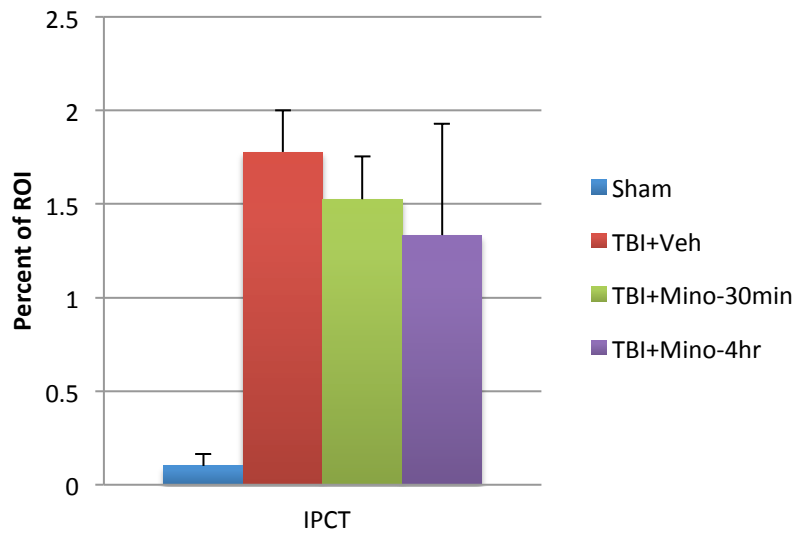


FIGURE 3-13: PATTERN OF M2 MARKER ARG1+ STAINING IN THE PERI-LESION CORTEX AND IPSILATERAL DENTATE GYRUS (DG) IN AGED (20M) ANIMALS AT 3 DAYS POST-INJURY.

Representative micrograph images of ARG1 staining in the peri-lesion cortical region (upper panels) and the ipsilateral DG (lower panels) from shams (N=2), and injured animals treated with vehicle (N=4), minocycline beginning at 30 minutes after injury (N=4), and minocycline beginning at 4 hours after injury (N=4). For the sham animal, a representative cortical tissue area directly above the hippocampus was selected for comparison with injured animals. Strong ARG1 staining was seen in injured vehicle group in the cortical region, both minocycline treated groups had a few scattered ARG1+ cells. In the DG, ARG1 stained cells were found scattered in the DG in all injury groups. No ARG1+ staining was found in the shams.

(A) Percent of Cortical Tissue Stained with ARG1 in 20M Animals



(B) Percent of Ipsilateral Dentate Gyrus Stained with ARG1 in 20M Animals

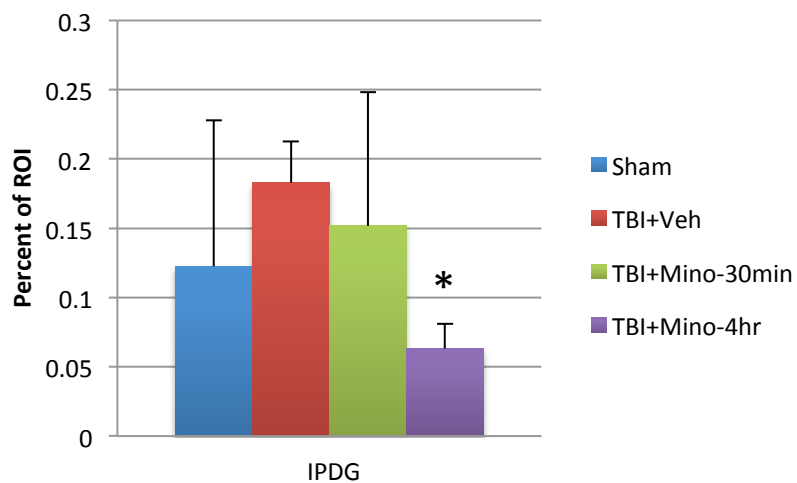


FIGURE 3-16: DENSITOMETRY ANALYSIS OF ARG1 LABELING IN THE IPSILATERAL CORTICAL TISSUE AND IPSILATERAL DENTATE GYRUS OF AGED RATS (20 MONTHS).

Values are given as percent of ROI area occupied by the positively labeled cells. (A) In the cortical tissue of aged rats, the ARG1+ staining in shams was also negligible. There were also no significant differences in ARG1+ cell area densities among the three injury groups. (B) In the hippocampus of aged rats, there were no significant differences between the injury groups and shams. However, the ARG1+ staining density in the Mino-4hr group was significantly lower than the vehicle-treated group (* $p < 0.05$).

CO-LOCALIZATION OF MICROGLIAL MARKERS

To determine whether the changes in microglial activation are due to phenotype switching or due to the generation of new cells, we double-labeled sections for BrdU and one of the aforementioned microglial cell markers. BrdU is a marker for newly proliferating cells, and co-localization with one of the other markers would tell us that the microglia/macrophage cell is newly formed. Double-labeling was performed on at least one section from each injury group (vehicle, 30-minute minocycline, and 4-hour minocycline) in both age groups, but damage to the sections and poor staining prevented some of them from being used. Because BrdU+ cells were mostly observed in the cortical regions around the injury, therefore, examination of double labeling was performed only in this region.

We found that 40% of the newly proliferating cells in the vehicle-treated animals also stained positively for Iba1, compared to 46% in the minocycline-treated group. We also observed that 10% of the BrdU+ cells in the vehicle animals were ED1+, compared to 15% in minocycline-treated animals. We saw that in minocycline-treated animals around 17% newly proliferating cells co-labeled with ARG1, however, we were not able to compare the result to vehicles due to poor morphology.

We evaluated ARG1/ED1 double-labeling to determine whether the M2 cell-type marker co-localized with the general microglia cell marker, and found that the two markers did have overlap. However, this labeling was only performed in a vehicle section, so no comparison with treatment could be made.

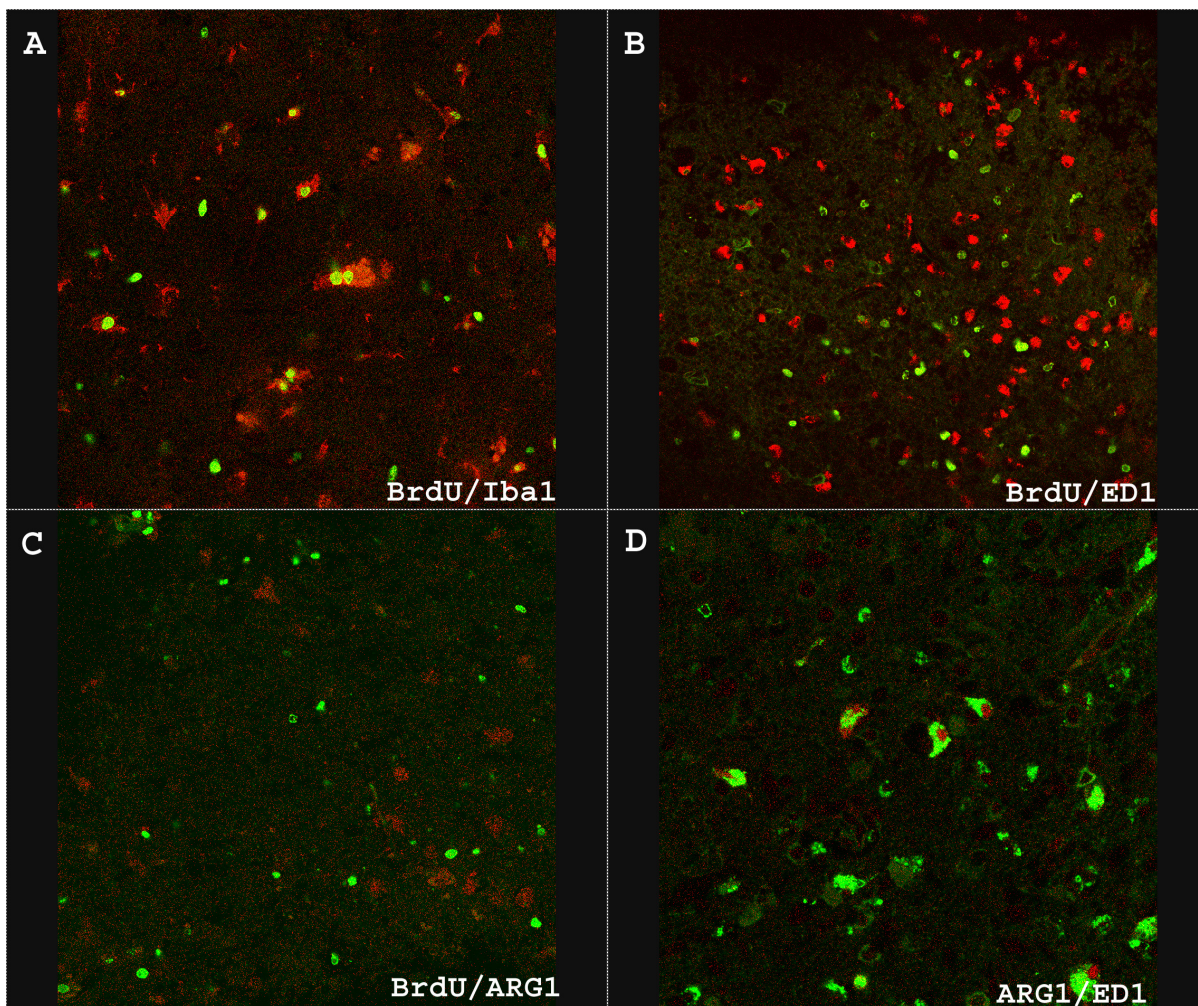


FIGURE 3-17: IMMUNOFLUORESCENT DOUBLE LABELING IN THE PERI-CONTUSIONAL CORTEX.

Representative confocal micrographs of double labeling examined. A) BrdU and Iba1; B) ED1 and BrdU; C) BrdU and ARG1; D) ED1 and ARG1.

DISCUSSION

SUMMARY OF RESULTS

This study examined the effects of short-term minocycline treatment on the inflammatory response in rats at the age of 3 months or 20 months following controlled cortical impact (CCI) injury. Two treatment groups were compared to controls, which included shams and vehicle-administered animals. Group one animals received the first dose of minocycline beginning at 30 minutes post-injury (30-minute minocycline group), while the other group of animals received the first dose at 4 hours post-injury (4-hour minocycline group). Both groups underwent the same subsequent minocycline injection schedule. All animals were sacrificed at 3 days post injury, so that the acute inflammatory response could be evaluated. In addition, two age groups were compared: young rats (3-month-old) and aged rats (20-month-old).

In order to assess the inflammatory response, three general microglia/macrophage markers were used: Iba1, ED1, and OX6. We also used the M2 cell type marker ARG1 to evaluate minocycline's effect on the phenotype of activated microglia after injury. We have also tested two M1 markers (iNOS and CD86) and another M2 marker, CD206. However, these antibodies did not work in immunohistochemistry on the rat brain. In this study, we focused on regions of the peri-lesion cortex and the dentate gyrus of the ipsilateral hippocampus. In young animals, minocycline treatment significantly reduced microglial staining intensity in the cortical tissue surrounding the injury site, as evidenced by OX6 staining. Changes in staining intensity in cortical tissue were not significant in ED1 or Iba1

staining. In the dentate gyrus of young animals, minocycline treatment did not significantly decrease microglial staining intensity with all markers.

In the aged animals, in the cortical tissue, minocycline treatment significantly reduced microglial staining intensity compared to vehicle-treated animals in all three pan-inflammatory markers. Between the two treatment regimes, the 4-hour minocycline treatment group had lower Iba1 staining compared to the 30-minute group. In the hippocampus of the aged animals, reduction of Iba1 and Ed1 staining was observed in minocycline treated groups particularly in the 4-hour treatment group. In both young and aged animals, M2 marker Arg1 expression was increased in all injured groups, but not significantly changed in both minocycline treatment regimes compared to vehicle treated group.

In comparing staining intensity between the two age groups, we found that minocycline treatment, particularly treatment beginning at 4hr post injury, had significant reduction in the expression of inflammatory cells markers in the aged animals compare to their younger counterparts.

CCI INJURY INDUCES AN INFLAMMATORY RESPONSE IN BOTH THE PERI-CONTUSIONAL CORTICAL TISSUE AND THE DENTATE GYRUS IN YOUNG AND AGED RATS

The presence of a robust secondary inflammatory response after TBI has been well described, especially in the acute stage. The inflammatory response is characterized by the activation of resident microglia and the infiltration of peripheral leukocytes through the now permeable blood-brain barrier (BBB). Increased staining in regions of the brain for

microglial markers, like Iba1, after injury suggests the presence of an inflammatory response and microglial activation (Sandhir et al. 2008). When activated in response to a mechanical or chemical stimulus, these immune cells secrete cytokines, and elevations in their concentrations are indicators of a response to injury. Levels of both pro- and anti-inflammatory cytokines, including IL-1 α , IL-1 β , IL-6, and TNF- α , can rise significantly within hours after injury, in the peri-contusional cortex (Mukherjee et al. 2011), as well as in the ipsilateral hippocampus (Harting et al. 2008).

In the current study, Iba1 staining revealed higher microglia cell densities in injured, vehicle-administered animals when compared to shams, which was statistically significant in young and aged cortical tissue, as well as in the aged hippocampus (Figures 3-2 and 3-4). Staining for ED1 and OX6 also demonstrated increased microglia cell densities in vehicles compared to shams. Consistent with published studies, our data indicates that the CCI injury initiated an inflammatory response in the peri-contusional cortex as well as in the hippocampus, as indicated by elevated densities of microglia/macrophages in these regions following injury in vehicle treated animals.

This study also compared the two age groups to each other. Aging has been associated with increased systemic inflammation, which is believed to contribute to an exaggerated inflammatory response after injury (Sandhir et al. 2008; Timaru-Kast et al. 2001). However, our comparison of cell densities in the sham and vehicle groups between the young and aged animals revealed no significant differences. Our findings did not indicate higher levels of inflammatory cell response in aged animals likely due to the quantification methods we used. More accurate assessment of protein expression, by a

method such as Western blotting, or more detailed analysis of morphological changes are needed for such comparison.

ACUTE MINOCYCLINE TREATMENT ATTENUATES THE INFLAMMATORY RESPONSE IN YOUNG AND AGED RATS FOLLOWING CCI INJURY

The anti-inflammatory properties of minocycline are well documented. The drug is known to attenuate production of reactive oxygen species (ROS), as well as secretion of pro-inflammatory cytokines from activated cells (Sanchez Mejia et al. 2001). Minocycline has also been shown to inhibit recruitment of microglia/macrophages to affected sites in the brain (Ng et al. 2012), thereby reducing the degree of the inflammatory response induced after injury. Minocycline's beneficial effects seem to be related to the attenuation of microglial activation (Fan et al. 2005) and proliferation (Tikka et al. 2001).

Consistent with these findings, the current study found that minocycline-treated animals had significantly reduced microglial staining intensity in both age groups, and was particularly significant in the aged group. These reductions in microglia/macrophage recruitment indicate a suppression of the secondary neuroinflammation following the CCI injury.

As we did not see any increase in the inflammatory response in aged animals compared to young, we cannot conclude that the significant reduction in staining with minocycline treatment is due to a suppression of elevated age-related systemic inflammation. However, using a different form of assessment, such as level of cytokine or cell-surface marker expression, may give a more accurate depiction of the cellular changes in the aging brain.

ACUTE MINOCYCLINE TREATMENT DOES NOT ENHANCE M2 PHENOTYPE IN YOUNG OR AGED RATS FOLLOWING CCI INJURY

During the TBI-induced secondary inflammatory response, microglia are the primary mediators. They play a defensive role, phagocytizing debris and pathogens and releasing signals to recruit and activate more microglia and other leukocytes, as well as a protective role, secreting anti-inflammatory cytokines and growth factors to enhance repair and neurogenesis. Microglia/macrophages exhibiting a defensive, pro-inflammatory phenotype are commonly referred to as M1 type cells, whereas those exhibiting protective, anti-inflammatory properties are known as M2 type cells. Recovery after injury requires a balance between these two phenotypes. Enhancement of the M1 phenotype and an impaired ability to promote the M2 phenotype have been suggested as reasons for poorer outcomes after injury in aged individuals compared to the young (Kumar et al. 2013).

Several studies have found that suppression of the inflammatory response, using a drug like minocycline, correlates with improved outcome and recovery and reduced lesion size (Ng et al. 2012; Bye et al. 2007). While much is known about the neuroprotective and anti-inflammatory effects of minocycline, little is understood regarding its effect on the phenotype of reactive microglia and macrophages. This study aimed to examine the effect of minocycline treatment on the density of M2 cells present in the peri-contusional cortex and the ipsilateral hippocampus, in the acute stage after CCI injury.

Arginase is an enzyme involved in the metabolism of arginine, driving the reaction toward production of proline and other molecules involved in cellular repair and

regeneration. As such, arginine expression is up-regulated in M2, alternatively activated, microglia/macrophages, making it a suitable marker for identifying the anti-inflammatory cell type.

While ARG1+ staining density was elevated in the cortex of all injury groups in both young and aged animals, our data showed no significant differences between vehicles and minocycline-treated animals (Figures 3-14 and 3-15). In the dentate gyrus, there was very little staining in all of the groups, and there were no significant differences among any of them (Figures 3-19 and 3-20). Our findings indicate that minocycline treatment had no effect on the recruitment of M2 type cells to the injury site. We therefore speculate that the neuro-protective effects of minocycline are exerted by suppressing microglial activation as a whole, and not by enhancing expression of the M2 phenotype.

Other studies have documented suppression of pro-inflammatory cytokine expression with minocycline treatment (Bye et al. 2007). In a recent model of amyotrophic lateral sclerosis (ALS) in mice, it was shown that minocycline treatment reduced expression of M1 markers, but had no effect on expression of the M2 phenotype (Kobayashi et al. 2013). Expanding this study to include a marker for the M1 cell type could further elucidate the effect of injury and minocycline treatment on the M1/M2 balance.

Our double-labeling study also found that not all cells labeled for ARG1 also labeled for ED1, indicating that ARG1 also picked up weak staining in other cells, likely astrocytes. This could be confirmed with further double-labeling of ARG1 with an astrocyte-specific cell marker, such as GFAP. The role of astrocytes in the inflammatory response cannot be ignored, as they are similarly activated after TBI and release many of the same cytokines and reparative factors that activated microglia do (Woodcock and Morganti-Kossmann

2013). Although microglial activation usually precedes astrocyte activation, both are noted within the first 24 hours after injury, and should therefore be considered when evaluating the acute response to injury (Sandhir et al. 2008). Further investigation examining the role and changes in astrocytes could also elucidate the effects of minocycline treatment on the inflammatory response following TBI.

MINOCYCLINE TREATMENT DOES NOT CHANGE THE PROLIFERATION RATE OF INFLAMMATORY CELLS

To examine whether minocycline affects proliferation of inflammatory cells, the animals in our study were given pulse injections of BrdU at the last day of minocycline treatment. We were therefore able to double-label our sections for BrdU and microglial cell-type-specific markers and evaluate changes in microglial proliferation.

Our study found a slightly higher rate of co-labeling of BrdU with Iba1+ or ED1+ in the injured cortex region in animals treated with minocycline as compared to vehicle-treated animals. As minocycline decreases microglia activation, this result could be due to reduction of both cell activation and cell proliferation. Our results suggest that the effect of minocycline used in our regime is more due to the reduction of cell activation. More precise analysis by counting the number of BrdU+ cells, and the number of activated microglial will provide more accurate conclusion. Other studies reported minocycline acts by attenuating microglial proliferation (Tikka et al. 2001), however, a proliferation marker was not used in this study therefore the term “proliferation” was not accurate. Consistent with other studies (Fan et al. 2005), our findings suggest that minocycline treatment in this case acted by largely by reducing microglial activation.

Double-labeling of BrdU and ARG1 also revealed that some of the newly proliferating microglia exhibited the M2 phenotype which may indicate generation of M2 cells, after minocycline treatment. However, we could not compare this result to vehicle-treated animals due to bad issue processing so the finding is inconclusive. Expanding this survey to include a marker for the M1 phenotype in the double-labeling would be able to tell us more about the M1/M2 cell type prevalence among newly proliferating cells.

CONCLUSION

This study, consistent with previous research, found that CCI injury induced a secondary inflammatory response in the acute stage. It also revealed that treatment with the anti-inflammatory drug minocycline attenuated the response, as evidenced by reduced densities of microglia in both the peri-contusional cortical tissue and the ipsilateral hippocampus. This response was especially pronounced in the aged group, compared to the young animals. However, analysis of changes to the density of the M2 phenotype with minocycline treatment revealed no differences when compared to vehicles. In this study, minocycline treatment had no effect on the recruitment of M2 anti-inflammatory cells to the injury areas. Our findings indicate a need for further study of the effects of minocycline on leukocyte subpopulations in order to fully understand its anti-inflammatory properties.

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