The role of extracellular matrix and matrix-degrading proteases in neonatal hypoxic-ischemic injury

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The Role of Extracellular Matrix and Matrix-Degrading Proteases in Neonatal Hypoxic-Ischemic Injury

by

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A dissertation in partial fulfillment of the requirements for the degree of Doctor of Philosophy
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Note to the reader: The original of this document contains color that is necessary for understanding the data. The original dissertation is on file with the University of South Florida library in Tampa, Florida.
Dedication

This dissertation is dedicated to all of the individuals whom, through tireless efforts and extraordinary insights, have contributed to the progress of scientific discovery.
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ABSTRACT

Improvements in medical care over recent decades have increased the number of premature and low birth weight infants that survive hypoxic-ischemic (H-I) insults. Because there is a rising incidence in diseases associated with these events, it is critical to develop effective therapies to treat the various resulting neuropathies. Extracellular matrix constitutes the majority of brain parenchyma. Lecticans and matrix-degrading proteases including ADAMTSs (a disintegrin and metalloproteinase with thrombospondin repeats) and matrix metalloproteinases (MMPs) exert effects on cell viability and may be associated with either protective or destructive processes after H-I. Both ADAMTSs (Cross et al. 2006; Tian et al. 2007) and MMPs (del Zoppo et al. 2007; Gu et al. 2005; Rosenberg et al. 2001) have been associated with pathological states in brain, yet the relative contributions of lecticans, ADAMTSs and MMPs to inflammation and cell death remain unknown.

In the present study, the first series of experiments were conducted to characterize cellular damage and neuroinflammation in the postnatal day 7 rat after exposure to H-I, and to determine if cell death and inflammation were associated with alterations in lectican expression. Data showed that reduced brevican expression occurred 4 days after H-I in lesioned hippocampus. Additionally, reduced versican expression in white matter was concomitant with pre-OL cell death at this endpoint. In contrast, both lecticans were elevated at later endpoints (14, 21 days) that were associated with increased neuroinflammation and cavitary infarction. These data suggest that lectican loss is associated with cell death at the early endpoint, whereas increased lectican deposition over time likely leads to glial scar formation and a reduced capacity for neuroplasticity.

Two subsequent series of experiments were conducted to determine the relative contributions of matrix-degrading proteases to injury, and whether proteolytic activity was associated with neuroinflammatory events. The first objective was to determine whether...
treatment with AG3340, a selective inhibitor of gelatin-degrading MMPs, or the anti-inflammatory compound minocycline, could provide neuroprotection when administered at a delayed time point after insult, and to compare the efficacy of AG3340 with that of the well-known anti-inflammatory compound minocycline. Data showed that both compounds effectively dampened the recruitment of microglia/macrophages to the lesion site when administered 24 hrs after H-I. These effects were associated with reduced neurodegeneration, indicating that these compounds neuroprotect at a clinically relevant time point. The final series of experiments tested whether these compounds could neuroprotect in an ex vivo model of oxygen glucose deprivation (OGD) that lacks peripheral immune cell involvement, thus providing insight into the relative contributions of resident microglia and gelatinase activity to the inflammatory sequelae. Results showed that both compounds blocked the OGD-induced increase in gelatinase activity and were neuroprotective in the absence of peripheral immune cells. Taken together, these data indicate that resident microglia contribute to H-I injury through gelatinase activation. Thus, the present study demonstrates that gelatin-degrading MMPs are important targets to consider when developing therapies to combat neonatal H-I injury.
Background and Significance

Perinatal Hypoxic-Ischemic Brain Injury:

Clinical Pathology

Due to improvements in medical care over past decades, increasing numbers of premature and low birth weight infants survive the neonatal period. Not surprisingly, there has been a rise in the incidence of diseases associated with prematurity, including neuropathies. Advances in diagnostic methods for these neuropathies and general knowledge of the cellular and molecular consequences have provided insight into potential causes for potential candidate therapeutics. Hypoxia-ischemia (H-I) is thought to be a major cause of perinatal brain injury, producing lesions of variable severity including focal necrotic cell death, diffuse white-matter injury, and cystic or cavitary infarction. It is estimated that nearly 40% of premature infants may suffer from either intraventricular-periventricular hemorrhage or periventricular lesions. In both cases, age of prematurity and birth weight are associated with increased risk, with younger, lighter infants at highest risk (du Plessis and Volpe 2002).

The escalating incidence of these deficiencies and lack of effective therapies underscores the importance of research in this area. Perinatal brain injury has been attributed to impaired modulation of systemic blood pressure by an immature vascular system. The immature vasculature and low baseline blood flow render the periventricular brain highly vulnerable to even modest changes in perfusion pressure (Baier 2006). The resulting lesions range from dilated ventricles in mild cases to focal, cystic infarcts or cavitations in severe cases. This particular injury is associated with high risk for subsequent development of cerebral palsy (CP), a spastic motor condition that is often accompanied by learning deficits. Though less common in the term infant, perinatal brain injury is typical in preterm infants born with spastic diplegia, the most common form of CP (70 - 80% of patients) in which both legs are affected. An important clinical study revealed that oligodendrocyte precursor (pre-OL) proliferation and differentiation are
coincident with the developmental period (~23-32 gestational weeks) during which preterm infants are most vulnerable to H-I injury (Back et al. 2001). Since that time, a growing body of literature has emerged indicating that white matter pathology observed in response to H-I is directly related to the selective vulnerability of pre-OLs, resulting in lower numbers of mature OLs and compromised myelination in the adult (Back et al. 2002; Craig et al. 2003; Skoff et al. 2001). While white matter damage has clearly been shown to produce motor disturbances, other mechanisms account for distinct forms of pathology. Cognitive deficits associated with H-I injury also result from damage to fiber tracts in white and gray matter. This neurodegeneration occurs not only through disrupted myelination but also in response to deleterious biochemical cascades that are activated after the insult. For example, there is strong and convincing evidence supporting the roles of oxidative stress and free radicals (Back et al. 1998; Elibol et al. 2001; Ferriero et al. 1996; Jian Liu and Rosenberg 2005). These mechanisms contribute to cell death in both neurons and glia, demonstrating the complexity of this pathology. Indeed, most perinatal H-I injuries manifest later in development as motor disturbances accompanied by some degree of cognitive dysfunction that occurs as a result of neurodegeneration.

Rodent Model of Hypoxia-Ischemia

The most common experimental animal model currently used to recapitulate human perinatal H-I was originally developed by Levine (Levine 1960) and reprised by Vannucci and colleagues for use in the neonate (Rice et al. 1981; Vannucci et al. 1999). In this model, unilateral ligation of the common carotid artery is performed in the neonatal rat followed by transient (hours) exposure to hypoxia. The resulting pathology includes injury to cerebral white matter (Back et al. 2002; McQuillen and Ferriero 2004) and gray matter (Almli et al. 2000; Arteni et al. 2003; Rice et al. 1981; Towfighi et al. 1995; Vannucci et al. 1999), both of which are likely to result in loss of cognitive and motor function (Bona et al. 1997; Ikeda et al. 2001; Jansen and Low 1996; Young et al. 1986). Importantly, lesions mimic those observed in perinatal brain injury, including ventricular dilation, cerebral white matter infarctions and substantial neuronal cell death. A key feature that is relatively unique to this rodent model, however, is shrinkage or elimination of
hippocampal structure in the severely injured brain (Aya-ay et al. 2005). Although there is inherent variation in this model in terms of lesion severity, it has proven quite useful for investigations into the cellular and molecular consequences of H-I. Subsequent sections will primarily address knowledge accumulated from experimental rodent studies, as the Vanucci H-I model was used to collect the majority of the data contained within this dissertation.

**Behavioral Paradigms Used in Rodent Models**

A host of investigations have tested the efficacy of various therapeutic agents in improving neurohistological outcomes after H-I (du Plessis and Volpe 2002; McQuillen and Ferriero 2004; Vannucci et al. 1999). The ultimate proof of therapeutic efficacy, however, is improvement in sensorimotor function. An early study by Young and colleagues documented sensorimotor deficits in rat that resulted from H-I insult (Young et al. 1986), consistent with cognitive and motor deficits in the clinical population. Despite these data, little was accomplished to further assess behavioral deficits in the H-I model for quite some time. Recently, behavior has been revisited in efforts to bring external validity to animal models, demonstrating motor and cognitive deficits associated with H-I events (Arteni et al. 2003; Bona et al. 1997; Ikeda et al. 2001; Jansen and Low 1996). Furthermore, administration of brain-derived neurotrophic factor prior to (Almli et al. 2000) or insulin-like growth factor-1 shortly after (Guan et al. 2003) H-I have been shown to improve behavioral outcomes in rat. A subsequent study obtained similar results in mouse (Ten et al. 2003). In terms of the clinical condition associated with H-I, however, injury is generally not detected until much later when the behavioral manifestations appear. Therefore, although the data from animal studies were encouraging, the need for therapeutics that can be delivered at delayed time points is essential in treating patients affected by H-I.

Although the physical handling of neonates introduces a unique set of concerns regarding alterations of normal developmental processes, the data that can be obtained from behavioral batteries is essential to understanding the ultimate consequences of brain pathology. The fields of neurodevelopment and developmental biology have constructed useful tests of sensorimotor and cognitive functions that maintain reliability across species. Developmental
disabilities are assessed through observations of simple reflex behaviors, each of which represents a unique developmental milestone (Jansen and Low 1996). Neuromuscular deficits are often measured with the vertical pole test, one which exploits the requirements of grip strength and balance to orient the body and remain suspended in an inverted manner. Perhaps the most well-known behavioral assay is the open field test in which spontaneous locomotor activity is measured and recorded over some duration, typically after several habituation trials. The activity is then quantified either using the classical method of counting photocell beam breaks or with more recent technologies, such as video surveillance that sends a live digitized signal to a computer template with preset distance parameters and continuously records movement.

Selective Vulnerability of Pre-oligodendrocytes

Pre-OLs arise from cells in the subventricular zone of the lateral ventricles, where they proliferate, differentiate and migrate to regions composed of white and grey matter (Levison and Goldman 1993). In neural tracts, OLs are responsible for the myelination of axons. Myelination provides the framework for saltatory conduction, thus allowing for fast and efficient signal propagation throughout the CNS. In the perinate as well as in the adult, H-I insults produce white matter disturbances that can lead to cognitive and motor deficits. Several lines of evidence indicate that injury occurring after adult stroke, for example, is due to disruption of axoglial junctions. The adult brain exhibits complete myelination of neural tracts. The junctions formed between neuronal axons, astrocytes and the myelin sheaths of oligodendrocytes form an interface that aids in maintaining structural stability while allowing for the communication of signals obtained from sampling the extracellular space. Studies indicate that degradation of myelin proteins, such as myelin basic protein and myelin associated glycoprotein, compromises these junctions. After this occurs, neurons may not function at full capacity due to the loss of efficacy in signal propagation. Others have suggested that cleavage of myelin proteins leads to neuronal cell death via anoikis, the process whereby cells die due to the loss of critical signaling with the surrounding microenvironment (Grossmann et al. 2001). In contrast to the adult brain, the neonatal brain contains immature cerebral white matter. The active myelination period occurs
from ~postnatal day 7 (P7) to ~P21. White matter injury in the neonate less likely results from degradation of myelin proteins or disruption of axoglial junctions since these junctions are still being formed and myelination is at an early stage. In fact, to achieve effective myelination, OL progenitors must complete a maturational process. Throughout maturation, OLs differentiate into several distinct phenotypes prior to achieving the capacity to myelinate axons. The recognition that different OL phenotypes may predominate during specific stages of early development raised the intriguing possibility that the susceptibility of OLs to H-I injury may depend upon the specific maturational state of these cells.

As the specific stage of differentiation was potentially a crucial determinant of underlying susceptibility to H-I, a detailed study of OLs at each developmental stage was imperative. The ability to distinguish between distinct biological phenotypes within the OL lineage has been aided by the recognition that OLs express and cease to express specific antigens throughout differentiation. An elegant series of experiments by Reynolds and Hardy determined through combined data from OL cultures and whole tissue sections that the O4 antigen is a cell surface marker for both pre-OLs and mature OLs in rat, while galactocerebroside (GC) labels proliferating and myelinating OLs; O4+/GC+ cells are mature, non-mitotic and differentiated. In addition, the NG2 proteoglycan appears to be a reliable marker for pre-OLs (Reynolds and Hardy 1997). These data suggest that NG2+/O4+/GC- cells are likely to be either pre-OLs or immature OLs, a distinction that remains somewhat ambiguous and unresolved. Taken together, these data suggest that as pre-OLs begin to express the O1 antigen, they begin to differentiate into immature, non-mitotic cells and gain resistance to H-I. Further differentiation of immature OLs to mature, myelinating cells is accompanied by the surface expression of myelin markers. The characterized cell surface antigen profile for each biological phenotype is as follows; pre-OL (NG2+/O4+/O1-/GC-), pre-OL/immature OL (NG2+/O4+/O1+/GC-), mature OL (NG2-/O4+/GC+/MBP+).

Since that time, Stephen Back’s laboratory has completed an investigation of OL lineage progression in both rodent and in human in efforts to match the period of rodent white matter development with that of the human during the critical period of vulnerability. Data from these
experiments revealed that pre-OLs predominated at P2 in the rat corpus callosum, a white matter-rich region in brain. By P7, the majority of these cells had differentiated into immature OLs that expressed the O1 antigen. Therefore, it seemed likely that the pre-OLs present at P2 most closely resembled those that predominate during the period of vulnerability in human. Mature OLs, immature OLs and early progenitors were resistant to H-I (Back et al. 2001; Craig et al. 2003). Despite these data, other investigations into the mechanisms of neonatal H-I injury have been performed in the P7 rat. These studies have shown that developing OLs are susceptible to injury at this age (Aya-ay et al. 2005; Follett et al. 2000) and are similar to the phenotype that predominates in the human during the critical period of susceptibility, thus validating the window between P2 and P7 as a reasonable period to study pre-OL injury resulting from H-I. It is important to note that while OLs differentiate into the mature phenotype as they migrate to their targets, little is known regarding functional differences between the immature phenotypes.

Extracellular Matrix in the Developing Brain:
The Lectican Family of Chondroitin Sulfate Proteoglycans

Extracellular matrix (ECM) is widely distributed throughout the brain parenchyma. ECM proteoglycans subserve many functions that are imperative to cell viability. As such, ECM turnover in response to injury influences the potential for plasticity and repair. Matrix in brain is composed primarily of chondroitin sulfate proteoglycans (CSPGs) that aggregate with the linear polysaccharide hyaluronan to form structural lattices (Rauch 2004). Lecticans are a family of CSPGs consisting of aggrecan, neurocan, versican and brevican. Constituting the preponderance of ECM in the brain, lecticans provide structural support and modulate cell signaling, migration and neurite outgrowth (Yamaguchi 2000). These PGs are characterized by structurally conserved globular domains at the N and C termini, as well as central chondroitin sulfate glycosaminoglycan (CS-GAG) – binding domains. GAGs are repeating disaccharides composed of glucuronic acid and N-acetyl galactosamine. These disaccharides are sulfated in the Golgi apparatus by chondroitin sulfotransferases. The specific positions and patterns of sulfation influence GAG binding properties, and therefore largely determine lectican function. Lecticans are well known
inhibitory substrates to neuroplasticity and repair (Beggah et al. 2005; Jones et al. 2003a; Jones et al. 2003b). GAG chains link to serine residues within the central domain of the core protein via xylose, catalyzed by the enzyme xylotransferase, and increase the inhibitory properties of lecticans (Silver and Miller 2004). The central GAG-binding domains are structurally diverse throughout the family and contain varying numbers of GAG attachment sites (aggrecan: ~120; versican: ~20; neurocan: ~7; brevican: ~3) (Yamaguchi 2000). The N-terminal globular domain binds hyaluronan with high affinity. Hyaluronan serves as the backbone of the ECM, containing repeating disaccharide units that produce chains up to 25,000 disaccharides in length (Galtrey and Fawcett 2007). The C-terminal globular domain contains a C-type lectin-binding domain which is thought to associate with tenascin-C or tenascin-R, depending upon the specific developmental stage in brain and the particular lectican in question. The substrate specificities of these globular domains are crucial in permitting the formation of the ECM lattice (Rauch 2004).

The expression patterns of lecticans throughout development are temporally unique and tissue-specific. In general, brevican and versican are the most ubiquitous in brain. Neurocan and brevican appear to be specific to neural tissues, while aggrecan is expressed mainly in connective tissues. Brevican and aggrecan exhibit similar expression patterns in brain, steadily increasing from ~embryonic day 14 (E14) and reaching plateau at ~P150. Neurocan expression rises during the embryonic period, peaking shortly after birth and rapidly declining. To date, 4 splice variants have been identified for versican. Versican V1 expression is predominantly embryonic and is similar to that of neurocan. In contrast, versican V2 expression increases from ~P10 to P100, similar to that of brevican (Yamaguchi 2000).

Brevican and versican share common features in that they are both present in high abundance in brain and are secreted by glial cell types under developmental regulation. By virtue of their discreet, developmental expression and their key roles in neural plasticity, proteolytic degradation of brevican and versican V2 may contribute to neuronal cell death and white matter injury following ischemic insult. Previous studies have demonstrated elevated CSPG expression in the injured adult rodent brain (Beggah et al. 2005; Jones et al. 2003a; Jones et al. 2003b; Mayer et al. 2005; Vorisek et al. 2002). The capacity of lecticans to act as non-permissive
substrates to tissue remodeling has also been well-documented (Asher et al. 2000; Galtrey and Fawcett 2007; Jaworski et al. 1999). Despite these data, little is known regarding the relationship between lectican function and neonatal H-I injury.

**Lectican Expression in Developing White Matter**

The glia contained within neural tracts are surrounded by lecticans, particularly brevican, neurocan, and versican V2. In the rat hippocampal fimbria (FB) there is little brevican expression at the time of birth. During the first 2 postnatal weeks, brevican expression increases in OLs, peaking at approximately P14. Brevican expression is subsequently downregulated in OLs, as it is taken over by astrocytes by P21. Interestingly, the temporal pattern of brevican expression in OLs coincides with the active period of myelination. In adult FB, brevican expression is restricted almost entirely to astrocytes (Ogawa et al. 2001). The developmentally regulated expression of brevican, coupled with a late transition to expression in astrocytes, suggests that brevican plays an important role in the differentiation and proliferation of pre-OLs. Likewise, the rise in versican V2 expression that occurs throughout development may also aid in OL differentiation and subsequent myelination. Data from a previous study demonstrated reductions in brevican core protein, proteolytic fragments of cleaved brevican and NG2+ cells in FB just 4 days after H-I (Aya-ay et al. 2005), lending further support that brevican may provide survival signals to pre-OLs. It is also possible, however, that the reductions in brevican resulted from death of the cells from which it was produced. To date, this important question remains unanswered.

**Extracellular Matrix-Degrading Proteases**

ECM proteoglycans are important in providing signals to neighboring cells and structural integrity to the interstitial space in both normal and pathological states. ECM turnover in brain is achieved predominantly through proteolytic cleavage by two major families of matrix-degrading proteases; ADAMTSs (a disintegrin and metalloproteinase with thrombospondin motifs) and matrix metalloproteinases (MMPs). Both families of enzymes are metalloendopeptidases and are members of the metzincin superfamily of zinc-dependent proteases. The term metzincin reflects
the zinc ion that is present within the catalytic site, as well as the conserved methionine residue downstream of the catalytic site.

The ADAMTSs are a sub-family of the ADAM proteases. ADAMs are known to participate in ectodomain shedding and activation of various cell surface proteins (Seals and Courtneidge 2003). Though highly conserved structurally, the major difference between these 2 classes of molecules is that while ADAMs are primarily transmembrane proteins, ADAMTSs lack the transmembrane domain and are secreted into the ECM where they exert their effects. As such, these enzymes have an increased capacity to contribute to matrix remodeling and inflammatory responses in the injured brain. ADAMTSs are synthesized as inactive, pre-proenzymes. N-terminal processing of the signal peptide is required for trafficking to the plasma membrane, and subsequent cleavage of the pro-domain is necessary to render an activated enzyme. Processing of the prodomain occurs in the trans-Golgi network and is achieved most likely through cleavage of the furin recognition sequence by the proprotein convertase furin, though other evidence suggests the presence of multiple furin recognition sites in addition to a furin independent mechanism of activation (Wang et al. 2004). Substrate specificity is conferred via C-terminal truncation that occurs predominantly in the spacer regions of the ancillary domains (Apte 2004; Porter et al. 2005).

Substrates of ADAMTSs vary depending upon the specific physiological context and protease in question. The effects of substrate binding include growth factor sequestration and the proteolytic processing of various ECM proteoglycans (Flannery 2006; Tang 2001). For example, ADAMTS1 inhibits angiogenesis by binding to the heparin-binding isoform of vascular endothelial growth factor (VEGF-165), thus preventing its binding and subsequent phosphorylation of the endogenous receptor VEGFR2 (Luque et al. 2003). ADAMTS4 and ADAMTS5 proteolytically process lectican CSPGs including aggrecan (Nagase and Kashiwagi 2003), brevican (Matthews et al. 2000) and versican (Sandy et al. 2001). While the cleavage of aggrecan is associated with disease states such as rheumatoid and osteoarthritis, brevican and versican processing may have relevance to many physiological processes, as they are ubiquitous in brain and their expression is developmentally regulated (Yamagata and Sanes 2005; Yamaguchi 2000).
Some data exists regarding the potential roles of ADAMTSs in the injured rat brain. ADAMTS1 mRNA levels increased 24 hrs after permanent middle cerebral artery occlusion (MCAO) and remained elevated up to 21 days after the insult. In contrast, ADAMTS8 transcript decreased in the early phase of the injury (Tian et al. 2007). Other data showed alterations in ADAMTS message and protein levels after experimental autoimmune encephalomyelitis (Cross et al. 2006), a model producing aberrant myelination similar to that which is observed in multiple sclerosis. Despite these data, ADAMTSs are mainly known for their contributions to various cancers, skin disorders and arthritic conditions, and while several studies suggest the involvement of ADAMTSs in the neural response to injury, ADAMTS cleavage of lecticans did not appear to contribute to injury in a neonatal rodent model of H-I (unpublished observations; see “Discussion”).

While the role of ADAMTSs in ischemic injury is unclear, evidence indicates that other members of the metzincin superfamily may be important contributors to H-I injury. The MMPs are perhaps the most well-studied of the matrix-degrading proteases, yet it remains elusive exactly how these enzymes regulate most cellular processes during development and in pathological states. The MMPs can be divided into 7 sub-families based on structural homology (minimal domain: MMP-7, -26; collagenases, stromelysins and other MMPs: MMP-1, -3, -8, -10, -12, -13, -19, -20, -27; gelatinases: MMP-2, -9; furin-activated MMPs: MMP-11, -21, -28; membrane-type (MT) MMPs: MMP-14, -15, -16, -24; GPI-anchored MMPs: MMP-17, -25; type II transmembrane: MMP-23A, -23B). Collectively, these enzymes are capable of proteolytically cleaving all ECM proteins (Mott and Werb 2004). MMP and ADAMTS metalloendopeptidases share many structural and functional attributes. Like ADAMTSs, most MMPs (with the exception of membrane-anchored MMPs) are generally secreted from cells and exert their effects within the ECM. These enzymes are synthesized as latent zymogens. The N-terminus contains a signal peptide that is cleaved in the secretory pathway, thus targeting it for secretion, while the C-terminus (a hemopexin-like domain in the majority of MMPs) is most important in determining substrate specificity. In addition, cleavage of the propeptide domain is necessary to confer enzymatic activity. In contrast to the ADAMTS family, however, MMP activation is achieved via
sequential processing of the N-terminus that can occur by several distinct mechanisms, such as through the activity of serine proteases or other MMPs, the use of reagents such as sodium dodecyl sulfate or 4-aminophenylmercuric acetate, and alterations in temperature or pH. Ultimately, activation occurs via activation of a “cystein switch” mechanism, in which a covalent bond between an N-terminal cystein residue and the catalytic zinc site is disrupted upon cleavage of the cystein-containing prodomain. Furthermore, MMPs are capable of autocatalysis and therefore can modify their own proteolytic capabilities (Flannery 2006).

Several lines of evidence have demonstrated that MMP activity performs critical functions in the developing brain. Most importantly, the MMPs play a major role in developmental myelination. Data from rodent knockout studies showed that MMP-9 and MMP-12 null mice demonstrated reductions in myelination, while MMP-12 null OL progenitor cultures were also unable to differentiate into mature cells (Larsen et al. 2003; Larsen and Yong 2004). A series of experiments performed by Yong et al. investigated the effects of MMP-9 on OL process extension both in vitro and in vivo. Cultured OLs from human, bovine and mouse showed process extension concomitant with active MMP-9 expression. Furthermore, OL process extension was reduced after treatment with MMP inhibitors or an MMP-9 blocking antibody. Consistent with these data, cultures from MMP-9 knockout mice showed reduced process extension relative to non-transgenic controls (Oh et al. 1999). MMP-9 expression in the mouse corpus callosum, a white matter-rich region of brain, also correlated with the postnatal period during which myelination occurs (Uhm et al. 1998). A later study showed that MMP-9 knockout mice exposed to lysolecithin-induced demyelination showed a reduced capacity to recover (Larsen et al. 2003), demonstrating that MMP-9 may be beneficial in models that exhibit substantial white matter injury. These data clearly demonstrate the involvement of MMPs, and particularly MMP-9, in the ability of OLs to effectively differentiate and ultimately myelinate axonal tracts. Although MMP function regulates matrix turnover and is necessary during critical developmental stages, a multitude of studies have demonstrated that these enzymes contribute significantly to the neuropathies that result from ischemic insult. These data will be addressed in section IV.
Growth Factor Substrates for Matrix Metalloproteinases

Many additional MMP substrates have been identified in other experimental models. Data from these studies demonstrates the ability of MMPs to alter cell growth, signaling and migration (McCawley and Matrisian 2001). For example, MMPs have been shown to cleave ECM proteoglycans that are associated with growth factors, thus releasing these growth factors from sequestration. This has been demonstrated for fibroblast growth factor (FGF) in human endothelial cells after cleavage of the heparin sulfate proteoglycan perlecan (Whitelock et al. 1996), as well as for transforming growth factor beta via decorin proteolysis (Imai et al. 1997). Similarly, MMP cleavage of non-matrix proteins such as insulin-like growth factor binding protein 3 (IGFBP-3) can activate IGF in dermal fibroblast cultures (Fowlkes et al. 1994). Consistent with these data, blocking MMP activity with an endogenous inhibitor increased IGFBP-3 expression and reduced IGF-1 receptor signaling in a murine hepatic tumor model (Martin et al. 1999).

Other evidence suggests that gelatinase MMPs can act on growth factor receptors to alter receptor turnover. In experiments that used a cervical carcinoma cell/mixed lymphocyte co-culture system to study the effects of immunosuppression, MMP-9 was shown to cleave interleukin-2 receptor alpha on T-lymphocytes (Sheu et al. 2001). Additionally, MMP-2, but not MMP-9, cleaved FGFR1 from the cell surface, releasing a soluble form into the ECM that retained FGF-binding activity (Levi et al. 1996). Other known substrates for gelatinases include vascular endothelial growth factor and monocyte chemoattractant protein 3 (MCP-3), the cleavage of which could affect chemotaxis and thus alter the inflammatory response (Engsig et al. 2000; McQuibban et al. 2000).

Although there is a paucity of data from in vivo ischemic models linking MMP activity to growth factors, the aforementioned studies illustrate the distinct ways in which MMPs modulate numerous cellular processes and may, in fact, provide protection depending upon the temporal activation profile and the overall conditions of the microenvironment. Elucidating the mechanisms by which these proteases contribute to perinatal H-I injury and identifying the substrates for which MMPs demonstrate high affinity in vivo may serve as crucial information when developing alternative therapeutic approaches to treat H-I injury in the future.
Endogenous Inhibitors of Metalloproteinases

The primary endogenous inhibitor of MMPs in the systemic circulation is α2-macroglobulin. However, specialized tissue inhibitor of matrix metalloproteinase (TIMP) molecules regulate MMP activity in tissues and mediate ECM remodeling (Brew et al. 2000). As such, these proteins are important in regulating protease activity within the CNS. The TIMP family consists of 4 members. TIMP-1, -2 and -4 are soluble, secreted proteins. In contrast, TIMP-3 is a matrix-associated protein that is generally bound to ECM (Leco et al. 1994). Each TIMP contains an N-terminal and a C-terminal domain. The N-terminal domain confers inhibitory activity via a conserved cysteine residue that interacts with the zinc ion present within the MMP catalytic site. The C-terminal domain is variable among these proteins and therefore may reflect unique properties among the TIMPs. Inhibition results from a noncovalent bond that contacts both the catalytic and hemopexin domains of MMPs. The cell surface glycoprotein reversion-inducing cysteine-rich protein with kazal motifs (RECK) has also been shown to inhibit gelatinase MMPs (Oh et al. 2001; Takahashi et al. 1998). While RECK was shown to be involved in tumor metastasis, the role of RECK in the injured CNS is not clear. To date, many questions regarding the specific ways in which endogenous inhibitors regulate MMP function remain unanswered. Understanding the mechanisms in both the normal and injured brain may provide important insights into the molecular events leading to neural injury and potential therapeutic approaches to enhance neuroplasticity after injury.

Cellular Responses to Hypoxia-Ischemia:

The Early Response

In the developing brain, H-I results in a biphasic injury profile. Many of the initial events leading to progressive damage have been identified. Perinatal rodents are particularly ketogenic due to the high fat content in rodent milk. In fact, ketones are a major energy source of the perinatal brain (Nehlig and Pereira de Vasconcelos 1993). In response to H-I, the immature brain relies heavily on anaerobic glycolysis and glucose utilization increases markedly (Vannucci et al. 1994). The breakdown of glucose to lactic acid lowers pH, thus creating a more acidic...
microenvironment. Anaerobic glycolysis is energetically unfavorable and results in rapid depletion of ATP, among other energy stores. Energy failure initiates a series of deleterious biochemical cascades. Free oxygen radicals produced from xanthine byproducts (resulting from breakdown of ATP) and prostaglandin synthesis (resulting from breakdown of free fatty acids) attack polyunsaturated fatty acids of the plasma membrane, increasing membrane permeability and ultimately leading to necrotic cell death. Endothelial cell death compromises the blood brain barrier, resulting in vasogenic edema and hemorrhage (Wetzel 2005). Although extravasation into the neuropil increases levels of interstitial glucose, the developing brain cannot efficiently utilize this energy source due to low levels of glucose transporters (Vannucci and Hagberg 2004). The failure of energy-dependent ion pumps causes cellular depolarization and the subsequent release of glutamate into the extracellular space. Glutamate, in turn, activates NMDA receptors, resulting in calcium influx. Excessive levels of intracellular calcium contribute to the production of free oxygen radicals and nitric oxide (NO), a potent molecule that activates lipid peroxidation by combining with superoxide to form peroxynitrite (Shalak and Perlman 2004). There is also evidence that NO activates MMP-9 (Gu et al. 2002).

In addition to necrosis, apoptotic cell death occurs during the initial response to insult and may be the predominant mechanism accounting for cell death during the delayed response (Northington et al. 2001b). Mitochondrial dysfunction, in particular, is a major component of both the intrinsic and extrinsic apoptotic pathways. Either through death receptor activation (extrinsic) or some intracellular signal (intrinsic), the release of cytochrome c into the cytosol initiates the activation of caspases, proapoptotic cysteine proteases that induce programmed cell death upon activation. The perinatal brain expresses substantially high levels of several apoptotic proteins, including caspase-3 (Hu et al. 2000), APAF-1 (Ota et al. 2002), Bax (Vekrellis et al. 1997) and Bcl-2 (Merry et al. 1994). The expression of these proteins decreases throughout development, thus lending support to the notion that apoptosis may account for a significant proportion of cell death after ischemia. Indeed, previous studies have demonstrated increased activity of caspase-3 (Blomgren et al. 2001) and caspase-9 (Zhu et al. 2003) after H-I, suggesting that the intrinsic pathway was activated. Other data showed increased expression of the Fas death receptor ligand
(FasL) in conjunction with activation of caspase-8 and caspase-3, indicating that the extrinsic pathway is also activated (Northington et al. 2001a) in response to insult.

In the context of ECM interactions, these apoptotic pathways may be particularly important in initiating detachment-induced apoptotic cell death, termed “anoikis”. For example, pharmacological blockade of integrin signaling was shown to trigger anoikis, thereby reducing angiogenesis and inhibiting tumor progression (Brooks et al. 1994). Integrins are ECM components that mediate signaling in both the immature and the adult brain. As such, disruptions in integrin signaling after H-I may result in cell detachment from ECM and subsequent apoptotic cell death. To date, anoikis has not been adequately investigated in models of perinatal H-I. Despite this fact, several lines of evidence suggest that this mechanism may be largely responsible for cell death after ischemic insult. Anoikis has been documented after ischemia/reperfusion in the rat intestinal epithelium (Ikeda et al. 1998), indicating that ischemia does, in fact, trigger this mechanism. In experiments conducted with intestinal epithelial cells, the activation of several caspases that regulate both the intrinsic and extrinsic apoptotic pathways have been associated with anoikis (Grossmann et al. 2001) and mitochondrial dysfunction (Grossmann et al. 2001; Rytomaa et al. 2000). The increased expression of proapoptotic enzymes in the developing brain relative to the adult, coupled with the highly-regulated developmental expression profile of ECM lecticans and the high incidence of apoptotic cell death after H-I, demonstrate the need for future research into the potential involvement of ECM signaling in the context of apoptotic cell death after ischemia.

**Neuroinflammation: The Secondary Response**

The contributions of excitotoxicity and free radical production to the early ischemic response are clear. Despite this fact, selective targeting of these mechanisms has not led to significant functional improvements in clinical trials. Importantly, a second wave of cell death occurs in response to ischemic insult and is mediated by the neuroinflammatory response to injury. During this time, a myriad of proinflammatory molecules are released, initiating signaling cascades that ultimately exacerbate neural damage. Accumulating evidence suggests that
targeting neuroinflammatory mechanisms may be a promising avenue for therapeutic intervention.

The immune response in brain is complex and highly regulated by a host of different cell types and signaling pathways. Resident microglia are among the first to become activated (Lai and Todd 2006). These cells migrate to necrotic regions where they remove cellular debris from the interstitial space. Also during this time, activated astrocytes upregulate glial fibrillary acidic protein (GFAP) and migrate to the injured site, a phenomenon known as reactive astrogliosis. Cells in and around the lesion core upregulate the expression of lecticans and these proteoglycans are deposited into the extracellular space. Thus, a dense composition of proteoglycans, reactive astrocytes and microglia invade the injured site and form a tissue barrier that is referred to as a "glial scar" (Silver and Miller 2004). The formation and progression of glial scarring is thought to be an innate protective mechanism in brain to isolate the injured area from viable surrounding tissue. As time progresses, however, the glial scar prevents neuroplasticity and repair at the lesion site while activated astrocytes and microglia promote further injury by secreting proinflammatory cytokines and chemokines, thus attracting peripheral macrophages (Chew et al. 2006). In concert, these immune cells and proinflammatory molecules contribute to a feed-forward inflammatory response by further enhancing microglia and macrophage recruitment to the injured site (Alvarez-Diaz et al. 2007). The end result is a heightened state of inflammation in the brain that promotes apoptotic cell death.

Cytokines seated on cell surfaces are activated and subsequently released into the extracellular milieu, where they serve as important mediators of apoptotic cell death. Following ischemic insult, activated microglia upregulate the expression of various cytokines and chemokines (Fukui et al. 2006; Kim 1996; Van Lint and Libert 2007). In particular, tumor necrosis factor alpha (TNF-α) and interleukin-1 beta (IL-1β) have been shown to potentiate the neuroinflammatory response after H-I. There have been many investigations into the specific mechanisms by which cytokine activation enhances the inflammatory response in brain. Interleukin-1 type 1 receptor (IL-1R1) knockout mice showed significant reductions in the expression of macrophage inflammatory protein-1 alpha (MIP-1α), MIP-1β, MCP and RANTES
(regulated upon activation, normal T cell expressed and secreted) both 18 hrs and 72 hrs after H-I compared to non-transgenic controls. These effects were also associated with reduced leukocyte infiltration (Lazovic et al. 2005), suggesting that IL-1β mediates chemotaxis of peripheral immune cells to the injured site. Other data from acute rat hippocampal slices showed that activation of alpha chemokine receptor 4 (CXCR4) by the natural ligand stromal cell-derived factor-1 (SDF-1) caused glutamate to be released from astrocytes. Interestingly, this response was found to be dependent on the release of TNF-α following CXCR4 activation, and was amplified in the presence of lipopolysaccharide (LPS)-stimulated microglia (Bezzi et al. 2001). These results lend further support to the notion that microglia contribute to unfavorable conditions after insult, working in concert with cytokines and chemokine activation.

Matrix Metalloproteinases and Cerebral Ischemia:

Blood Brain Barrier Degradation

Following an ischemic event, activated glia contribute to the secondary inflammatory response by promoting glial scar formation and releasing proinflammatory molecules that further recruit immune cells to the injured site. Although the precise mechanisms by which these effects are achieved have yet to be determined, data from numerous studies suggests that MMPs are instrumental in the production and maintenance of a proinflammatory microenvironment (Manicone and McGuire, 2008). The release of proteases from activated microglia results in proteolytic degradation of basement membrane constituents (del Zoppo et al. 2007). This breakdown compromises the blood brain barrier, causing extravasation that permits entry of peripheral leukocytes and macrophages into the brain. Gelatinases, in particular, are major contributors to ischemic pathology (Wetzel 2005). In vivo experiments revealed MMP-9 expression that was localized to neutrophils and endothelial cells, and showed elevated MMP-2 expression in astrocytic endfeet (Rosenberg et al. 2001). The latter expression profile places MMP-2 in the ideal position to proteolytically process basement membrane proteins, as astrocytic endfeet play an important role in sealing the blood brain barrier. An elegant study later supported this mechanism by showing that laminin is a key substrate for MMPs after ischemia. Gelatinase
activity increased in cortex after MCA occlusion, and active MMP-9 was upregulated and proteolytically processed laminin when incubated with tissue homogenates from ischemic brain. These effects were attenuated after administration of a highly selective gelatinase inhibitor (Gu et al. 2005). There is now substantial data demonstrating the degradation of basement membrane proteins by several MMPs that are elevated after ischemia (Cunningham et al. 2005; Manicone and McGuire 2008; Rosenberg and Yang 2007).

**Sheddase Activity and Extracellular Matrix Remodeling**

There is accumulating evidence suggesting that gelatinases may contribute to the inflammatory response through “sheddase” activity, the process by which MMPs cleave proinflammatory cytokines attached to cell surfaces. This proteolytic processing, in turn, activates and releases these molecules into the extracellular milieu to exert a wide range of effects depending upon the specific state of the microenvironment. MMPs have previously been shown to process both TNF-α (Gearing et al. 1994) and IL-1β (Schonbeck et al. 1998) to their biologically active forms. In culture, MMP-2 – positive astrocytes produced MMP-9 when stimulated with either TNF-α or IL-1β (Gottschall and Yu 1995). In agreement with these data, mice lacking MMP-9 showed improved outcomes that were directly related to reduced microglial activation (Svedin et al. 2007), attenuated blood brain barrier degradation (Gidday et al. 2005) and limited white matter damage (Asahi et al. 2001). Other data showed that gelatinases cleaved SDF-1α and various MCP chemokines (Overall et al. 2002), and SDF-1 accelerated proteolytic processing of syndecans by MMP-9 (Brule et al. 2006). Cerebral blood vessels express high levels of syndecans, which modulate transendothelial migration of monocytes across the brain endothelium (Floris et al. 2003). Taken together, these data offer potential mechanisms by which gelatinase activity enhances neuroinflammation after H-I by facilitating immune cell chemotaxis.

In addition to their roles in activating cytokines and processing basement membrane proteins, MMPs are efficient at cleaving lecticans (Gottschall 2005). Lecticans are upregulated in glial scars, where their growth-inhibitory properties act as a barrier between injured and viable tissue (Sandvig et al. 2004; Silver and Miller 2004). Thus, lectican proteolysis by MMPs may have
profound effects on perinatal brain plasticity after injury. Both full-length brevican and the G1 proteolytic fragment were reduced 1 and 14 days after insult in hippocampi of neonatal rats that were exposed to H-I (Aya-ay et al. 2005). While the mechanisms of this loss were unclear, the temporal association of brevican loss with lesion progression suggests that brevican expression may be critical for cell viability in the developing brain. In this context, brevican loss resulting from either proteolytic cleavage or cellular injury may enhance neural cell death. Though MMPs are upregulated in glial scar after spinal cord transection (Duchossoy et al. 2001), very little is known regarding the specific effects of MMPs on glial scar formation and ECM proteolysis after neonatal H-I. Potential mechanisms may include the induction of cellular anoikis or the facilitation of microglia and macrophage migration via reduced steric hindrance resulting from increased ECM clearance.

Extracellular Matrix, Matrix Metalloproteinases and Hypoxic-Ischemic Injury: The Big Picture

To date, the majority of research into neonatal H-I has emphasized the contributions of glutamate excitotoxicity and free radical production to the resulting neuropathology (Shalak and Perlman 2004). Consistent with evidence supporting the roles of free radical production and oxidative stress (Back et al. 1998; Elibol et al. 2001; Ferriero et al. 1996; Jian Liu and Rosenberg 2005), therapeutic approaches targeting these mechanisms in rodents have been of some benefit (Arvin et al. 2002; Follett et al. 2000). Nonetheless, most studies showing improved outcomes have limited clinical relevance, as protective agents were administered prior to or shortly after insult. Thus, the search continues for more selective therapeutics that do not interfere with critical cellular functions (Hamrick and Ferriero 2003) and can be administered at clinically relevant time points. The intent of the current study was to characterize the injury profile in rat using the Vannuci model of neonatal H-I, to determine whether ECM lecticans and/or matrix-degrading proteases contribute to the maladaptive responses to insult, and to test whether the selective targeting of gelatin-degrading MMPs provides neuroprotection at a delayed time point.
Lecticans are ubiquitous ECM substrates that influence axonal targeting, providing signals to neighboring cells and structural stability throughout the extracellular milieu (Yamaguchi 2000). Lectican expression is highly regulated in developing rodents. Interestingly, increased expression is associated both temporally and spatially with OL differentiation from pre-OLs to mature, myelinating cells. Additionally, data from rodent models indicates that lecticans serve as inhibitory substrates to neuroplasticity in the injured brain (Asher et al. 2000; Jaworski et al. 1999). Despite these data, little is known regarding the mechanisms by which lecticans may contribute to white matter dysfunction or glial scar formation after H-I. Preliminary data from our laboratory showed that the lesion resulting from H-I is progressive in the rat neonate. It is also noteworthy that pre-OLs show selective vulnerability to H-I injury in the Vannucci model (Back et al. 2002; Craig et al. 2003; Skoff et al. 2001), similar to the clinical population. Upon considering the expression profile of lecticans in developing white matter, as well as their contribution to the formation and maintenance of the glial scar in other injury models, the first set of experiments were designed to test the following hypotheses: 1) the death of pre-OLs after H-I is associated with reduced lectican expression, and 2) increased lectican deposition is associated with lesion progression and glial scarring.

In addition to cleaving lecticans, matrix-degrading proteases (particularly MMPs) proteolytically process various ECM substrates and demonstrate “sheddase” activity on cell-surface proteins. MMP-9 knockout mice showed improved outcomes after cerebral ischemia induced using either the Vannucci model that mimics neonatal H-I (Lee et al. 2004; Svedin et al. 2007) or the MCAO model that mimics adult stroke (Asahi et al. 2000; Asahi et al. 2001; Gidday et al. 2005). While accumulating evidence indicates that gelatin-degrading MMPs mediate the neuroinflammatory response to injury, little is known regarding the specific mechanisms by which these proteases may contribute to H-I injury in the developing rat brain.

A couple of points are worthy of mention. Minocycline has been shown to exert neuroprotective effects in several rat injury models (Cai et al. 2006; Fan et al. 2006; Yao et al. 2001). This compound is a tetracycline derivative with anti-inflammatory properties that are achieved primarily by the chelation of calcium ions. However, minocycline chelates other divalent
cations as well and has previously been shown to exert MMP inhibition both in vitro and in vivo (Machado et al. 2006), most likely through chelation of the zinc ion that is present within the catalytic site of MMPs. In contrast, AG3340 (prinomostat), a small molecule hydroxamate-based inhibitor of MMPs, is a potent MMP inhibitor with high nanomolar affinity for gelatinases. Both minocycline and AG3340 are orally bioavailable and therefore are attractive therapeutic agents for clinical use. AG3340 has been tested in various experimental cancer models and has demonstrated efficacy in limiting tumor growth in rodents (Alves et al. 2001; Price et al. 1999). To date, the efficacy of AG3340 in reducing neural injury has not been tested in the neonatal H-I model. However, data from previous experiments lend support to the notion that selective targeting of MMP-2 and MMP-9 could improve the outcome. The effects of gelatinase inhibition were examined in a model of chronic cerebral hypoperfusion. Data revealed that AG3340 provided neuroprotection in adult rats and mice when administered just prior to insult. Importantly, reduced activation of astrocytes and microglia was associated with retained blood brain barrier integrity (Nakaji et al. 2006). In a mouse model of MCAO, the gelatinase-selective compound SB-3CT reduced infarct volume when administered either 2 or 6 hrs, but not 10 hrs, after insult (Gu et al. 2005).

Due to the distinct mechanisms of action between minocycline and AG3340, a direct comparison of the efficacy of these compounds within a single study would provide information as to the relative contributions of generalized anti-inflammatory actions and MMP inhibition, specifically. Furthermore, treatment at a delayed time point would extend the clinical relevance if neuroprotection is, in fact, achieved. Therefore, the second series of experiments were designed to test the following hypotheses: 1) Treatment with minocycline or the selective gelatinase inhibitor AG3340 will provide neuroprotection when administered at a delayed time point, 2) neuroprotection will be associated with reduced neuroinflammation, and 3) minocycline will demonstrate greater efficacy in providing neuroprotection and reducing neuroinflammation when compared to AG3340.

Resident microglia are among the first cells activated in response to cerebral ischemia (Lai and Todd 2006). In the developing brain, these immune cells mediate the neuroinflammatory
response through a variety of mechanisms (Chew et al. 2006). In the case of ischemia, activated microglia upregulate the expression of cytokines and chemokines, enhancing the inflammatory response (Fukui et al. 2006; Kim 1996; Van Lint and Libert 2007). Increased expression of MMPs, particularly gelatinases, occurs concomitantly. Another known mechanism by which gelatinase activity exacerbates injury is through proteolytic processing of blood brain barrier constituents (del Zoppo et al. 2007; Rosenberg et al. 2001). In this instance, extravasation resulting from blood brain barrier degradation permits entry of monocytes/macrophages into the brain, further enhancing the recruitment of immune cells to the lesion site (Alvarez-Diaz et al. 2007). Infiltrating leukocytes (Gidday et al. 2005) are also sources of MMPs, though there is some evidence that microglia are the primary macrophages that respond to neonatal ischemic injury (Denker et al. 2007). Nonetheless, the relative contributions of the resident and peripheral immune responses to neonatal H-I injury have not been elucidated.

To investigate this further, organotypic slice culture methodology would be advantageous in that it allows for mechanistic experimentation in the absence of peripheral immune cell effects, while more closely mimicking the microenvironment in brain when compared to other cell culture systems. Thus, slices could be incubated with known concentrations of the appropriate compounds that correspond to the in vivo concentrations achieved after dosing in the animal. Additionally, gelatinase activity could be measured in all treatment conditions to further assess the degree to which gelatinase activity was associated with cell death. Together, these data would provide insight into the relative contributions of anti-inflammatory and MMP-inhibitory effects on cell survival. Therefore, the final series of experiments were designed to test the following hypotheses: 1) Oxygen glucose deprivation will increase gelatinase activity and neurodegeneration, 2) treatment with either minocycline or AG3340 will reduce gelatinase activity and neurodegeneration in slices exposed to OGD, and 3) AG3340 and minocycline will demonstrate equal efficacy in providing neuroprotection in slices exposed to OGD.
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Chapter 1

Versican and Brevican are Expressed with Distinct Pathology of Neonatal Hypoxic-Ischemic Injury

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Abstract

The developing brain is uniquely susceptible to injury after exposure to hypoxia-ischemia (H-I). Lecticans are developmentally regulated in formative white matter and exert growth-inhibitory effects in several adult injury models, yet little is known regarding their role in neonatal H-I injury. The main objectives of this study were to examine the expression profiles of brevican and versican in rat using a standard H-I model, and to determine whether altered expression was associated with distinct components of white and gray matter pathology. The H-I procedure produced progressive injury limited to ipsilateral hemisphere. Cresyl violet staining revealed severe cavitory infarctions at 14 and 21 days that were absent at 4 days. Cellular damage, as measured by GFAP and fractin immunoreactivity, occurred in cortical and subcortical gray matter at all endpoints. O4 sulfatide immunoreactivity was reduced in white matter of external capsule, hippocampal fimbria and corpus striatum at 4 days relative to contralateral, suggesting the loss of pre-oligodendrocytes. Brevican expression was reduced in cortex and hippocampus at 4 days but was markedly elevated at later endpoints, localizing to regions of cellular damage both within and proximal to the lesion core. Versican deposition was increased most prominently at late endpoints in cortex of moderately lesioned tissue. In contrast, versican was reduced in external capsule 4 days after H-I, a reduction that was sustained at 21 days. These data demonstrate unique expression profiles for lecticans after neonatal H-I, suggesting brevican deposition is elevated in response to progressive gray matter injury while diminished versican expression may be associated with deep cerebral white matter injury.

Keywords: chondroitin sulfate proteoglycans; hypoxia-ischemia; oligodendrocytes; rat; developmental disorders
**Introduction**

The developing brain is uniquely susceptible to injury after exposure to a hypoxic-ischemic (H-I) environment (Volpe 2001). Such insults produce a neural sequelae characterized by cortical and periventricular white matter injury (PWMI) (Rezaie and Dean 2002). Current views regarding mechanisms of PWMI emphasize perturbations of normal developmental myelination, while gray matter damage is associated with a multitude of factors that aid in the formation and progression of a dense composition of proteoglycans, reactive astrocytes and glia that constitute a glial scar (Silver and Miller 2004). An important clinical study revealed that oligodendrocyte precursor (pre-OL) proliferation and differentiation are coincident with the developmental period (23-32 gestational weeks) during which pre-term infants are most vulnerable to H-I injury (Back et al. 2001). Since that time, a growing body of literature has emerged indicating that white matter pathology observed in response to H-I is directly related to the selective vulnerability of pre-OLs, resulting in lower numbers of mature OLs and compromised myelination in the adult (Back et al. 2002; Craig et al. 2003; Skoff et al. 2001).

To date, the majority of research has emphasized excitotoxicity, reactive oxygen species and inflammation as key contributors to both white and gray matter injury (Shalak and Perlman 2004). While there is strong and convincing evidence supporting the roles of oxidative stress and free radicals (Back et al. 1998; Elibol et al. 2001; Ferriero et al. 1996; Jian Liu and Rosenberg 2005), clearly other mechanisms are involved, and while therapeutic approaches targeting these cascades have been of some benefit in animal models (Arvin et al. 2002; Follett et al. 2000), the search continues for therapeutic interventions that do not interfere with normal maturational processes (Hamrick and Ferriero 2003). One potential avenue for novel therapeutic intervention may involve selective targeting of lecticans. Glia contained within developing white matter tracts are surrounded by lecticans, a sub-family of chondroitin sulfate-bearing proteoglycans (CSPGs) that include brevican, versican, aggrecan and neurocan. Lecticans constitute the preponderance of extracellular matrix (ECM) in the brain, exerting effects on cellular adhesion, signaling and neurite outgrowth (Yamaguchi 2000). Lectican expression is highly regulated in developing rodents. The PGs are deposited by pre-OLs during the transition period from immature to mature,
myelinating OLs, suggesting a role in this transition process, while deposition subsequent to the active myelination period occurs mainly via astrocytes (Asher et al. 2002; Ogawa et al. 2001). The capacity of lecticans to act as non-permissive substrates to tissue remodeling has also been documented in certain injury models (Asher et al. 2000; Jaworski et al. 1999). Despite these data, little is known regarding the mechanisms by which lecticans may contribute to white matter dysfunction or glial scar formation after H-I.

The most common experimental animal model currently used to recapitulate human neonatal H-I was originally developed by Levine (Levine 1960) and reprised by Vannucci and colleagues for use in the neonate (Rice et al. 1981). The pathology in this model mimics that observed in human neonatal brain injury, including ventricular dilation and deep cerebral white matter injury, while retaining the gray matter component that is observed in various animal models of injury. Lectican expression is developmentally regulated in white matter and up-regulated in glial scar tissue, suggesting that lecticans may play a supporting role in normal white matter development while impeding reparative processes in injury states. However, little is known regarding the pattern and location of lectican deposition in relation to the pre-OL. Therefore, the present study was conducted to investigate the spatio-temporal relationship of lectican deposition in the rat neonate after exposure to H-I, and to determine the association of brevican and versican with both white and gray matter pathology.

**Materials and Methods**

**Induction of Hypoxia-Ischemia**

All animal procedures were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Neonatal Sprague-Dawley rats were birthed from time-pregnant dams (Harlan Labs). Rats were maintained on a 12 hour light/dark cycle (7 am – 7 pm) and given access to food and water *ad libitum*. Two separate litters from 2 dams were culled to 10 pups per litter at P1 and cross-fostered randomly between dams prior to each experiment. Animals included in this study were pooled from 2 experiments. The H-I procedure was performed at P7 and consisted of permanent unilateral ligation of the common carotid artery followed by transient
exposure to hypoxia. All animals were placed on a heating pad (37°C) during surgery to minimize variation that results from differences in core body temperature. Animals were initially anesthetized with 2.5% isofluorane and subsequently maintained at 350 ml/ml of oxygen and 1.5% isofluorane with an interfaced scavenging system for the duration of the surgery. The right common carotid artery was isolated away from the vagus nerve with blunt dissection and permanent ischemia was achieved by ligating the carotid artery with 6 x 0 nylon suture. The musculature and skin were then sutured and animals were placed back with their corresponding dams for 2 h. After the recovery period, pups were exposed to 8% oxygen/N₂ balanced for 120 minutes. During hypoxia, pups were placed into custom-made chambers that maintained a temperature of 37°C while permitting a water-saturated oxygen mixture to be dispensed at a constant flow rate (Aya-ay et al. 2005). Since physiological parameters were not monitored during the hypoxic period, naïve (no ligation, no hypoxia) and sham (permanent ligation, no hypoxia) animals were included for direct comparisons with H-I littermates within each experiment. Pups were then returned to their respective dams and body temperature was maintained at 37°C for 2 h with a heating pad prior to the conclusion of the experiment.

**Perfusion and Tissue Preparation**

For immunohistochemistry, tissue was collected at 4 (P11), 14 (P21) and 21 days (P28) after H-I as previously described (Aya-ay et al. 2005), with minor adaptations. Briefly, animals were anesthetized with pentobarbital (60 mg/kg) and intracardially perfused with 60 ml phosphate-buffered saline (PBS, pH 7.4) followed by 60 ml 4% paraformaldehyde. Brains were removed and cryopreserved with increasing sucrose concentrations (15%, 30%), sectioned coronally on a cryostat at 30 µm thickness and thaw-mounted onto slides for cresyl violet staining or immunohistochemistry. Thaw-mounting was chosen over free-floating sections to achieve maximum preservation of morphology in tissue proximal to lesion. Every third section was collected from an area contained within the lesion that ranged from the most rostral aspect of the lateral ventricles to the initial emergence of brainstem.
Histology and Immunohistochemistry

Cresyl violet-stained brain sections were used for verification and quantification of gross lesion areas. Sections were rinsed briefly in PBS, dried overnight and baked for 60 minutes at 50°C. Sections were then rehydrated in decreasing concentrations of ethanol (100%, 95%, 70%), stained with cresyl violet solution (3%) for 3 minutes, dehydrated (70%, 95%, 100%), cleared with Histoclear and mounted in Permount. Immunohistochemistry was performed as described (Mayer et al. 2005) to assess cellular damage, lectican expression and relative abundance of pre-OLs. All data was obtained from 5 distinct regions in brain (figure 1) collected at P11 (N=6), P21 (N=5) and P28 (N=7). Briefly, slides were rinsed with PBS, permeabilized and blocked for 60 minutes (3% Triton-X, 3% 1M Lysine, 10% NGS in PBS) and incubated overnight with primary antibody. Sections were then rinsed, incubated with secondary antibody and coverslipped using Vectashield aqueous mounting media (Vector Labs, Burlingame, CA). Double-label immunohistochemistry was achieved by co-incubation with anti-mouse and anti-rabbit primary antibodies, and subsequent co-incubation with secondary antibodies conjugated to distinct fluorophores for each respective species of primary antibody.

Primary antibodies used in these studies were mouse anti-glial fibrillary acidic protein (GFAP, Boeringher-Manheim, 1:1000), rabbit anti-fractin (Chemicon, 1:1000), rabbit anti-brevican (BD Transduction Labs, 1:500), mouse anti-12C5 (Developmental Hybridoma Bank, 1:500), rabbit anti-NG2 (Chemicon, 1:1000) and mouse anti-O4 sulfatide (Chemicon, 1:1000). Primary antibodies were visualized using either Alexa Fluor 488 (green) or Alexa Fluor 594 (red) secondary antibodies (Molecular Probes, Eugene, OR).

O4 Sulfatide Quantification

O4 sulfatide was quantified using ImageJ software. All images were captured at the same exposure time on a single day to eliminate confounds of differential background intensity and diminished fluorescence intensity over time. Low power photomicrographs of adjacent tissue sections (2 sections per animal) from P11 rats (N=6) corresponding to approximately Bregma 0.70 mm (Paxinos and Watson) were imported into ImageJ and converted to 8 bit binary images.
External capsule (EC) was traced and background subtraction was achieved by adjusting contrast until background particles were eliminated from the selected regions. Analyses yielded 3 distinct measures that included total area of external capsule ("EC area"), total number of particles within the traced area ("O4 intensity") and the proportion derived from dividing the total particle area by the total area of the selected region ("area fraction").

**Statistical Analyses**

For quantification of external capsule white matter loss, data were expressed as X ± SEM of proportions derived from values obtained ipsilateral divided by contralateral within each animal. These data were subjected to one sample student’s t-tests using the null hypothesis that the mean of the ipsilateral to contralateral side proportion was equal to 1.0. When p< 0.02, deviation from the null hypothesis was considered significant. This "p value" was chosen, rather than p < 0.05, to account for potential type I errors that would occur due to conducting multiple t-tests.

Data from lesion area quantification were obtained as described above and converted to percentages, representing percent ipsilateral relative to contralateral for each animal. Group values were then subjected to a one-way ANOVA with "p value" set at 0.05. Pair-wise comparison of group means was made using a Tukey post-hoc test.

**Results**

**Progressive Lesion after H-I**

Neonates were subjected to H-I at P7 and sacrificed 4 (P11), 14 (P21) and 21 (P28) days after insult. Brain sections were collected and stained with cresyl violet to determine the extent of lesion. This model resulted in lesions of variable severity but demonstrated a clear profile whereby the extent of lesion increased as a function of time from P11 to the later endpoints (Figure 2). Data revealed significant increases in lesion area at P21 in all areas measured relative to P11 (p<0.05). While a clear trend for an increase was observed at P28, variability within the model (eg non-lesioned animals) prevented the mean from reaching statistical significance. However, when 3 animals from P28 whose values were similar to unlesioned animals (and
potentially never lesioned) were removed from the analysis, the P28 means reach significance in
each region measured. Animals sacrificed at P11 typically displayed ipsilateral hemispheric
shrinkage accompanied by ventricular dilation. In general, the total area encompassing each
region selected for analysis was slightly reduced. Cortical cavitations were present in some cases
but severe cavitations of hippocampus and cortex were rarely observed. In contrast, nearly all
lesioned animals sacrificed at later endpoints exhibited severe cavitory infarctions throughout the
cortex and complete or near complete elimination of hippocampal structure. This more severe
lesion profile was nearly identical at P21 and P28, indicating that the gray matter component is
progressive and probably reaches a maximum threshold, at least for grossly observable
infarction, 14 days or less after the insult. While elimination of white matter was observed in
regions of external capsule adjacent to severe cortical infarctions, sections from animals with only
mild to moderate cavitations largely retained the structure. Taken together, these data suggest
that the neurohistological outcome in this model is most closely associated with gray matter
injury.

**Reactive Gliosis and Apoptosis in Gray Matter**

Cresyl violet staining revealed a progressive lesion profile. Immunohistochemistry was
performed on H-I tissues to identify early and late cellular damage profiles (Figure 3). The glial
scar that is characteristic of gray matter injury is known to be composed largely of reactive
astrocytes and glia. Indeed, animals sacrificed at P11 demonstrated clear elevations in GFAP
immunoreactive positive cells in the ipsilateral hemisphere. The most prominent increases were
found to be localized to cortical, hippocampal and striatal regions, while only modest increases
were observed in white matter of external capsule and hippocampal fimbria. Intense GFAP
immunoreactivity was observed at P21 and P28 in cortex, exhibiting the ‘columnar’ pattern typical
of this model, in corpus striatum and remaining hippocampal structure. The extent and intensity of
labeling was clearly increased several-fold at later endpoints relative to P11. In addition to
reactive gliosis, we also sought to determine the degree to which apoptosis contributes to the
early phase of injury. Double-label immunohistochemistry was performed in the same P11
sections probing for the apoptotic marker fractin, a neoepitope of β-actin that is generated upon cleavage by caspases 3 and 5. Intense fractin immunoreactivity was observed in the ipsilateral cortex at P11 and was consistent with the anatomical location of severe infarctions that were observed at P21 and P28. Fractin labeling was also found in the CA3 region of hippocampus but was absent in deep cerebral white matter. These findings suggest that the gray matter lesions produced in this model occurred, at least in part, as a result of caspase-mediated apoptotic cell death. Interestingly, fractin labeling was conspicuously low or absent in white matter. Our inability to detect fractin in white matter may have been due to lack of sensitivity and/or differences in overall expression of β-actin throughout the brain.

Brevican Deposition is Associated with Lesion Progression in Gray Matter

Because glial scars have been previously shown to be rich in proteoglycans in several rodent injury models, we sought to characterize the expression of brevican and versican in this model of neonatal H-I. Sections obtained from P11, P21 and P28 animals that underwent H-I and demonstrated grossly observable infarctions and/or elevated cellular damage markers were first probed for brevican (Figure 4). Brevican immunoreactivity was reduced in the ipsilateral hemisphere 4 days after H-I. The greatest reductions were observed in hippocampus, where brevican was clearly reduced throughout the structure and most prominently in dentate gyrus. Cortical regions also exhibited reductions in brevican labeling overall, though we did detect sparsely distributed punctate staining in some sections. To determine whether the regions that exhibited reductions in brevican were also those regions affected by the insult, the same sections were probed for fractin. The results showed complete overlap between intense fractin immunoreactivity and the absence of brevican immunoreactivity, demonstrating that diminished brevican was coincident with elevated fractin and therefore suggesting that reduced brevican expression is associated with cellular apoptosis in the early phase of lesion progression.

Analyses of later endpoints revealed that the expression profile for brevican was the opposite of that observed 4 days after insult. Sections collected from animals at P21 and P28 demonstrated increased brevican immunoreactivity in cortical regions that were anatomically
consistent with gray matter damage. That is, brevican was elevated in those columnar regions where GFAP and fractin showed intense immunoreactivity (Figures 3 and 4). Because the lesions had progressed to severe cavitary infarctions by P21 and P28, very little hippocampus remained in the majority of tissues that were probed for brevican and therefore it was not possible to make direct comparisons relative to contralateral. We were occasionally able to visualize fragments of shrunken hippocampus, however, and in each of these cases brevican labeling was observed in the form of a thick border surrounding the structure. These results demonstrated an increase in brevican expression concomitant with a progression in lesion severity between 4 and 14 days after H-I. Upon comparison of P21 and P28 animals, we detected no differences in local expression or intensity of brevican labeling (not shown), indicating that the time course for peak brevican expression is similar to that of lesion progression from cellular damage, in the absence of cavitation, to severe cavitary infarction. Taken together, these data support the notion that increased brevican expression is associated with the progression of gray matter pathology.

**Versican is Differentially Expressed in Gray and White Matter**

Although versican has also been implicated in glial scar formation, little is known regarding the role of versican in neonatal H-I pathology. Therefore, we examined versican expression in sections from P11, P21 and P28 lesioned animals (figure 5). The expression pattern for versican was found to be similar, but not identical, to that of brevican. At P11, versican immunoreactivity was moderately reduced in ipsilateral hippocampus and striatum in the majority of sections analyzed, though these reductions were of a lesser magnitude when compared to P11 brevican. In contrast, no differences in cortical versican expression were detected at the early endpoint. In fact, cortical versican immunoreactivity was quite low 4 days after H-I and only in rare instances did we observe diffuse, punctate labeling. Analyses of later endpoints revealed elevations in cortical versican expression similar to those observed for brevican, with the exception that versican immunoreactivity, while elevated relative to control hemisphere at both late endpoints, was greatest in moderate lesions. We detected no differences in hippocampus at
P21 and P28. Although some remaining hippocampal structures exhibited the border-labeling similar to that observed with brevican, the intensity was low and inconsistent across sections.

The most interesting finding from these studies arose upon inspection of white matter. While we were unable to detect changes in white matter brevican expression, the developmental expression of versican clearly increased from P11 to P21 on the non-lesioned side. Versican immunoreactivity was detected in the contralateral external capsule and hippocampal fimbria of both hemispheres 4 days after H-I, but clear reductions were observed in ipsilateral external capsule (Figure 5). Closer inspection revealed that versican was diminished in lateral regions of external capsule that were seated ventral-medial from cortical regions of intense GFAP and fractin labeling. Smaller differences were found in medial regions of external capsule. Analyses of later endpoints revealed marked reductions in versican expression in ipsilateral external capsule. By P21, a developmental increase resulted in intense versican immunoreactivity in contralateral external capsule that was strikingly lower in the ipsilateral hemisphere (Figure 5). These data indicate that the basal increase in versican expression from P11 to P21 was diminished in the ipsilateral hemisphere following an H-I insult, suggesting that the perturbation of developing white matter that occurs after H-I may be linked to versican expression.

**White Matter Loss after Hypoxia-Ischemia**

White matter injury in response to H-I has been linked to selective vulnerability of pre-OLs. Animals were sacrificed 4 days after H-I (P11) and sections were probed for the putative pre-OL marker O4 sulfatide (Figure 6). Intense O4 labeling was detected in the contralateral hemisphere throughout external capsule, hippocampal fimbria and the white matter-containing fiber tracts of corpus striatum. Consistent reductions occurred ipsilateral in each selected region relative to the corresponding contralateral region. In general, the magnitude and intensity of O4 labeling was reduced in both corpus striatum and external capsule. While a reduction in the number of fiber bundles in the lateral portion of corpus striatum was evident, the relative proportions in the medial aspects of the structure were variable and inconsistent across animals. O4+ immunoreactivity was clearly reduced in external capsule after H-I and this effect, similar to
that observed in corpus striatum, was most prominent in lateral portions of the structure. To
determine the degree of deep cerebral white matter loss, O4+ immunoreactivity was quantified in
external capsule using 3 distinct measurements (Figure 7). The use of multiple measurements
provided us with internal validation of our method as well as additional descriptive data. The
results revealed significant reductions in all measures, including total area of external capsule
(total area = 37 +/- 8%), O4+ immunoreactivity count (O4 intensity = 25 +/- 8%) and relative
proportion of the total area that labeled O4+ (area Fraction = 31 +/- 7%).

**Discussion**

Current rodent H-I models that mimic human PWMI also display substantial cortical and
subcortical gray matter pathology (Towfighi et al. 1995; Vannucci et al. 1999). Several
mechanisms have been proposed to account for H-I injury, including excitotoxicity, free radical
production, inflammation and glial scar formation (Ashwal and Pearce 2001). The role of lecticans
as inhibitory substrates to neural plasticity and tissue remodeling is well documented both
throughout normal development and in several models of brain injury (for review see (Galtrey and
Fawcett 2007). By virtue of their discreet, developmental expression and their key role in neural
plasticity, lecticans are prime candidates for contributing to glial scar formation and white matter
injury. Brevican and versican are lecticans that share common features in that they are present in
high abundance in brain and are secreted by glial cell types under developmental regulation. The
role of these molecules in response to H-I, however, has not been extensively investigated. Our
objective was to characterize the expression of brevican and versican at selected endpoints that
included both early and late phases of injury, and to determine the association of these lecticans
with distinct H-I pathology. Data revealed a progressive lesion profile from the early to later
endpoints. Animals sacrificed 14 or 21 days after insult developed severe cortical and subcortical
cavitary infarctions that were less obvious 4 days after H-I. Ipsilateral elevations in fractin and
GFAP immunoreactivity support the notion that reactive astrogliosis and apoptosis may contribute
to neuronal cell death in cortex and hippocampus, in particular. A small proportion of animals
exposed to H-I did not develop lesions. Therefore, the assessments of cellular damage markers
served two purposes in that we were able to detect pathology at the cellular level, in the absence of grossly observable infarctions, as well as select only those tissues that exhibited H-I pathology for subsequent examination of lectican expression profiles and white matter injury.

Several studies have demonstrated elevations in CSPGs in response to injury (Beggah et al. 2005; Jones et al. 2003a; Jones et al. 2003b; Mayer et al. 2005; Vorisek et al. 2002). In general, these molecules are known to inhibit neurite outgrowth and therefore might be expected to act as non-permissive substrates to tissue repair in this model as well (Morgenstern et al. 2002). Here we report that two members of the lectican family of CSPGs respond differentially to H-I, and appear to be associated with distinct components of the resulting pathology. Increased brevican expression has been reported in cortex after a cortical stab wound (Jaworski et al. 1999), data consistent with our results. With the exception of the early endpoint where brevican was reduced in cortex, intense cortical brevican immunoreactivity was evident by P21. The reduction in the early phase of injury confirms data previously published by our laboratory (Aya-ay et al. 2005). Severe cavitary infarctions at later endpoints prevented any direct comparisons between contralateral and ipsilateral hippocampi. However, we were able to detect brevican that was deposited in thick borders surrounding the remaining ipsilateral hippocampal structures, indicating that brevican was being secreted at the perimeter of the infarctions and therefore was likely contributing to the production of an inhibitory microenvironment. The fact that brevican was reduced at P11 but elevated at later endpoints, a profile that occurred in conjunction with increased astrogliosis, provides evidence that increased expression and/or deposition of brevican may aid in glial scar formation resulting from extensive gray matter injury.

The pattern of versican expression after H-I was somewhat unpredicted, differed from that of brevican, and suggests a dual role for versican in H-I pathology. Only modest changes in expression were detected in gray matter at P11 and were limited to hippocampus. Versican was previously shown to be upregulated in cerebral cortex of adult rats 7 days after unilateral knife lesion (Asher et al. 2002). The fact that we observed no changes in cortical versican 4 days after H-I may be due to differences in the experimental models or detection methodologies employed. One likely possibility is that limitations in plasticity that are characteristic of adult rodents may
result in an accelerated response profile when compared to younger animals which display a greater potential for plasticity and repair.

Interestingly, our data suggest that versican may be more involved in the white matter component of H-I injury. Versican is secreted by OLs and its expression is developmentally regulated. Indeed, we observed reductions in the putative pre-OL marker O4 sulfatide in white matter of external capsule, corpus striatum and hippocampal fimbria at P11. The O4 antigen is a cell surface marker for both pre-OLs and adult progenitors in rat (Reynolds and Hardy 1997). Double-label immunohistochemistry revealed cells in the external capsule that co-expressed the O4 and NG2 antigens. While our quantification of O4 alone showing loss of immunoreactivity in white matter definitively demonstrates deep cerebral white matter loss, it is reasonable to suggest that at least a proportion of O4 immunoreactivity is specific to pre-OLs given the developmental stage and colocalization of these antigens. White matter versican expression was also reduced in ipsilateral external capsule at P11 and persisted to later endpoints, supporting the notion that versican may provide important signals to neighboring cells within developing white matter that are necessary to achieve adequate myelination. In the developing rodent, little brevican is expressed prior to P7. We were unable to detect changes in secreted brevican in white matter, possibly due to lack of sensitivity or low expression levels at this developmental stage. OLs are the major cell type responsible for the elevation in brevican expression that occurs from P7 to P14 (Ogawa et al. 2001) and therefore, while difficult to substantiate, it is tempting to speculate that the reductions in brevican after H-I observed at P11 were due, at least in part, to decreased deposition resulting from the early loss of pre-OLs.

Taken together, these data provide a framework for further investigations into the role of lecticans in the progression of H-I pathology. The observed expression profiles suggest that the family of lecticans may not collectively subserve the same general functions. Instead, each of these molecules may contribute to distinct components of injury. Future studies investigating the regulatory mechanisms of these molecules, as well as their specific contributions to unique aspects of injury, may lead to novel approaches with the potential for highly selective therapeutic interventions.
Acknowledgements

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**Figure 1 Legend**

*Rat brain regions selected for immunohistochemistry.*

Sections were collected 4 (P11), 14 (P21) and 21 (P28) days after H-I. Photomicrographs were obtained from regions of cortex (CTX), corpus striatum (STR) and external capsule (EC) from coronal sections corresponding to approximately 0.70 mm rostral from bregma (top), and from hippocampus (HC) and hippocampal fimbria (FB) from sections corresponding to approximately 2.56 mm caudal from bregma (bottom).
Figure 2
**Figure 2 Legend**

**P7 H-I produces progressive lesions with variable severity.** Cresyl violet stained coronal sections from animals subjected to H-I at P7. (A) Mild lesion 4 days after H-I displaying ventricular enlargement and slight hemispheric shrinkage. (B) Moderate lesion 14 days after H-I displaying ventricular enlargement, hemispheric shrinkage and cortical cavitary infarction. (C) Severe lesion 21 days after H-I displaying cortical and subcortical cavitary infarction with complete elimination of hippocampal structure. Quantification of lesion areas at 4 (P11), 14 (P21) and 21 (P28) days after H-I revealed a progressive injury profile represented as % decrease in total area of ipsilateral hemisphere (D), hippocampus (E), cortex (F) and corpus striatum (G) relative to contralateral control regions. Asterisks denote significant difference from P11 (p<0.05).
Figure 3
**Figure 3 Legend**

*Apoptosis and reactive gliosis after hypoxic-ischemic insult.* Photomicrographs demonstrating double-labeling of (A) fractin and (B) GFAP in the ipsilateral hemisphere 4 days after H-I. (A) Caspase-generated cleavage fragment of β-actin (fractin) appearing in columns of cortex and in hippocampus, but not in white matter (25x magnification). Inset shows specific immunoreactivity in layer CA3 of hippocampus (400x magnification). (B) GFAP immunoreactivity in somatosensory cortex of the same section (200x magnification). (C-F) At P21, GFAP immunoreactivity is markedly increased in ipsilateral cortex of an animal demonstrating severe hippocampal cavitory infarction (D) compared to respective contralateral hemisphere (C) (25x magnification). 100x magnification shows a robust increase in GFAP immunoreactivity (F) relative to both contralateral control (E) and P11 ipsilateral cortex (B). White letters = contralateral control; red letters = ipsilateral lesion.
Figure 4
Figure 4 Legend

*Brevican deposition is associated with lesion progression.* Photomicrographs demonstrating double-labeling of fractin (green) and brevican (red) after H-I. Brevican expression was reduced 4 days after H-I in hippocampus (B) and cortex (D) compared to respective contralateral regions (A) and (C) and was coincident with elevations in fractin labeling (F, H) compared to respective contralateral areas (E, G). At 14 days after H-I, brevican expression was elevated in cortex (J, K) compared to contralateral cortex (I) and elevated in the border of the remaining hippocampal structure (L). White letters = contralateral control; red letters = ipsilateral lesion; magnification: (A, B, E, F) = 200x, (C, D, G, H, I - L) = 100x.
Figure 5
Versican exhibits a unique expression profile with lesion progression. Photomicrographs demonstrating versican labeling after H-I. Versican expression was reduced 4 days after H-I in the dentate gyrus of the hippocampus (B), unchanged in cortex (D) and reduced in white matter of external capsule (F) compared to respective contralateral controls (A), (C) and (E). At 21 days after H-I, versican expression was unchanged in ipsilateral hippocampus (H), elevated in cortex (J) and markedly reduced in external capsule (L), compared to respective contralateral areas (G), (I), and (K). White letters = contralateral control; red letters = ipsilateral lesion; magnification = 200x.
**Figure 6 Legend**

*White matter loss after hypoxia-ischemia.*

(A) Confocal micrograph showing double-labeling of O4 sulfatide (green) and GFAP (red) in corpus striatum of contralateral control. (B) Putative pre-OL markers O4 and NG2 colocalize (white arrows) on cell surfaces in external capsule. (C - H) Photomicrographs demonstrating white matter abundance after H-I, as indicated by O4 sulfatide immunoreactivity. O4 sulfatide was reduced 4 days after H-I in corpus striatum (C), external capsule (D) and hippocampal fimbria (E) compared to respective contralateral control areas (F), (G) and (H). White letters = contralateral control; red letters = ipsilateral lesion. Confocal scale bar = 50µm; C - H magnification = 100x.
Figure 7

External Capsule White Matter Loss

Relative Ratio (ipsilateral/contralateral)

- EC Area
- O4 Intensity
- Area Fraction

* Indicates statistical significance.
Figure 7 Legend

Quantification of external capsule white matter loss.

Significant reductions occurred in all measures of O4+ immunoreactivity in external capsule including total area (EC Area), total intensity of labeling (O4 Intensity) and relative proportion of O4-labeled external capsule (Area Fraction). Asterisks denote significant difference from contralateral control ($p < 0.05$).
References


Chapter 2

Delayed Administration of a Small Molecule MMP Inhibitor Protects the Neonatal Rat Against Hypoxic-Ischemic Brain Injury

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Abstract

Hypoxia-ischemia (H-I) produces widespread neurodegeneration and deep cerebral white matter injury in the rat neonate. Resident immune cells may promote lesion progression by releasing proteases and other pro-inflammatory mediators. Gelatin-degrading matrix metalloproteinases (MMPs) participate in the loss of blood brain barrier integrity after injury, extracellular matrix (ECM) proteolysis and cytokine/chemokine activation. Altered ECM proteoglycan expression is associated with white matter injury. Thus, therapies targeting ECM degradation and progressive neuroinflammation may be beneficial in reducing H-I – induced neuropathy. Minocycline is neuroprotective in rat and has MMP-inhibitory properties. AG3340 is a small molecule MMP inhibitor with high selectivity for gelatinases. In this study, minocycline, AG3340 or vehicle were administered once daily for 6 days, beginning 24 hours after H-I, to determine whether gelatinase inhibition can limit H-I – induced injury. CD11b and glial fibrillary acidic protein (GFAP) immunopositive cells increased in ipsilateral cortex after treatment with vehicle alone, demonstrating microglia/macrophage recruitment and reactive astrogliosis, respectively. Cell death was indicated by Fluoro-Jade staining throughout the frontoparietal cortex and hippocampus. Treatment with minocycline or AG3340 inhibited microglia/macrophage recruitment, attenuated astrogliosis and reduced cell death when compared to vehicle alone. Thus, gelatin-degrading MMPs may be viable therapeutic targets to treat neonatal H-I injury.

Keywords: matrix metalloproteinase; inhibitor; AG3340; minocycline; hypoxia; ischemia; neonate; inflammation; microglia; macrophage
**Introduction**

Exposure to a hypoxic-ischemic (H-I) insult has distinctive consequences in the developing brain. Immature vasculature and low baseline blood flow render the immature brain susceptible to even modest changes in perfusion pressure (Baier 2006). The maladaptive neurobiological response can be severe, resulting in deep cerebral white matter injury and substantial neuronal loss (Volpe 2001). Similar to the human condition, neonatal rodent models recapitulating these injuries show cortical and subcortical infarctions, impaired motor function (Bona et al. 1997; Jansen and Low 1996; Ten et al. 2003) and cognitive deficits (Arteni et al. 2003; Ikeda et al. 2001; Young et al. 1986). Previous studies have linked oxidative stress (Back et al. 1998; Bernardo et al. 2003) and NMDA receptor activation (Kaur et al. 2006) to white matter injury, while glutamatergic blockade has been shown to reduce H-I–induced infarction (David et al. 2003; Dingley et al. 2006; Puka-Sundvall et al. 2000) and white matter damage (Follett et al. 2000). Though excitotoxicity and free radical production are key contributors to the neuropathology of these lesions, there is a growing interest in identifying additional therapies to limit the progressive neuroinflammation that accompanies ischemic injury.

Inflammation plays a key role in initiating pathological responses and potentiating neuronal damage after ischemia. The release of proteases from activated glia results in proteolytic degradation of basement membrane constituents (del Zoppo et al. 2007). This breakdown compromises the blood brain barrier, likely allowing entry of peripheral neutrophils and macrophages into the brain. These cell types, along with resident microglia, secrete proinflammatory cytokines and chemokines that further enhance microglia/macrophage recruitment to the injured site (Alvarez-Diaz et al. 2007). Matrix metalloproteinases (MMPs), the most well-studied extracellular matrix-degrading proteases, are capable of processing both TNF-\(\alpha\) (Gearing et al. 1994) and IL-1\(\beta\) (Schonbeck et al. 1998) to their biologically active forms. Several MMPs are elevated after cerebral ischemia and have been shown to degrade basement membrane proteins (Cunningham et al. 2005; Manicone and McGuire 2007). Gelatinases, in particular, are major contributors to ischemic pathology. In culture, MMP-2 – positive astrocytes produced MMP-9 when stimulated with either TNF-\(\alpha\) or IL-1\(\beta\) (Gottschall and Yu 1995). *In vivo,*
MMP-2 expression increased in astrocytic endfeet while MMP-9 expression was localized to neutrophils and endothelial cells (Rosenberg et al. 2001). Other data showed elevated gelatinolytic activity that colocalized with neuronal laminin degradation, effects that were attenuated after administration of a highly selective MMP inhibitor (Gu et al. 2005). In agreement with these data, mice lacking MMP-9 showed improved outcomes that were directly related to reduced microglial activation (Svedin et al. 2007), attenuated blood brain barrier degradation (Gidday et al. 2005) and limited white matter damage (Asahi et al. 2001).

In addition to cytokines and basement membrane proteins, MMPs are efficient at cleaving extracellular matrix (ECM) chondroitin sulfate proteoglycans (CSPGs). Lecticans, the family of aggregating CSPGs consisting of aggrecan, neurocan, versican, and brevican, are deposited as ECM in the brain, providing structural stability throughout the interstitial space and exerting effects on cellular adhesion and signaling (Yamaguchi 2000). Proteolytic processing of lecticans has been linked to H-I pathology in the rat neonate (Aya-ay et al. 2005). Furthermore, recent data indicates that white matter damage may be associated with reduced versican expression, while progressive neuronal damage correlates with lectican deposition in response to the injury (Leonardo et al. 2007). While lecticans are known substrates for ADAMTS (a disintegrin and metalloproteinase with thrombospondin repeats) proteases (Apte 2004; Dingley et al. 2006) and MMPs (Gottschall et al. 2005), little is known about the proteolytic regulation of lecticans after H-I.

Taken together, these data underscore the potential involvement of MMPs in ischemic pathology and point to a specific role in enhancing the inflammatory microenvironment, thereby exacerbating injury through proteolytic cleavage of various substrates. Rodent knockout studies have proven useful in identifying MMP substrates in the ischemic brain. Although data show improved neuropathological outcomes, the need for novel therapeutic interventions remains. In this study, two compounds were selected to determine the effects of MMP inhibition on neuroinflammation and cell death after H-I. Minocycline, a tetracycline derivative known for its anti-inflammatory properties, is neuroprotective in several rat injury models (Cai et al. 2006; Fan et al. 2006; Wasserman and Schlichter 2007) and has recently been shown to exert MMP inhibition both in vitro and in vivo (Machado et al. 2006). AG3340, a small molecule hydroxamate-
based inhibitor of MMPs, is efficacious in limiting tumor growth in rodent models (Alves et al. 2001; Price et al. 1999) and was shown to be neuroprotective in rodents exposed to chronic ischemia (Nakaji et al. 2006). While both compounds demonstrate good oral bioavailability, AG3340 is a potent MMP inhibitor with high nanomolar affinity for gelatinases, specifically, when compared to the broad anti-inflammatory actions of minocycline. Results here show that administration of either minocycline or AG3340 at a clinically relevant time point reduced neuroinflammation and cell death. These data provide evidence that MMPs modulate the neuroinflammatory response to H-I and highlight the potential of targeting MMPs when developing treatment-based therapies to combat neonatal H-I injury.

Materials and Methods

Induction of Hypoxia-Ischemia

All animal procedures were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals with a protocol approved by the Institutional Animal Care and Use Committee at the University of South Florida. Neonatal Sprague-Dawley rats were birthed from time-pregnant dams (Harlan Labs). Dams and litters were maintained on a 12 hr light/dark cycle (7 am – 7 pm) and given access to food and water *ad libitum*. Litters from 2 dams were culled to 10 pups per litter at postnatal day 1 (P1) and cross-fostered randomly between dams prior to each experiment. Two H-I experiments consisting of animals from 2 litters per experiment were used in this study. The H-I procedure was originally developed by Levine (Levine 1960) and reprised by Vannucci and colleagues (Rice et al. 1981; Vannucci et al. 1999) for use in the neonate. The H-I methodology was described in some detail previously (Leonardo et al. 2007). Briefly, the procedure entailed permanent unilateral ligation of the common carotid artery followed by transient exposure to hypoxia. P7 rats were anesthetized with 2.5% isoflurane, placed on a heating pad (37°C) and maintained at 350 ml/min of oxygen and 1.5% isoflurane with an interfaced scavenging system for the duration of the surgery. The right common carotid artery was exposed, isolated away from the vagus and ligated using a 6.0 nylon suture. The
musculature and skin were sutured, animals placed back with their corresponding dams for a 2 h
recovery period and subsequently exposed to 8% oxygen/N₂ balanced for 90 min. During
hypoxia, pups were placed into custom-made chambers that maintained a temperature of 37°C
while permitting the water-saturated oxygen mixture to be dispensed at a constant flow rate (Aya-
ay et al. 2005). Pups were then returned to their respective dams until initiation of the treatment
phase.

Drug Treatment

Animals exposed to H-I were randomly assigned to receive either vehicle, minocycline
(Sigma Aldrich, St. Louis, MO) or AG3340 (AG3340 was kindly provided by Dr. Peter Baciu,
Allergan, Irvine, CA). Vehicle consisted of 50% DMSO + 25% propylene glycol in distilled water.
Minocycline and AG3340 were dissolved in vehicle to obtain stock solutions of 13.5 mg/ml. Fresh
stock solutions of drugs were prepared every 2 days. Pups were weighed each day and weight-
based injection volumes were calculated to yield a final dose of 45 mg/kg for each animal.
Treatments were administered once daily (s.c.) for 6 days beginning 24 hours after H-I (P8).
Animals were evaluated daily for signs of pain or discomfort, and no adverse effects were
observed in response to either compound or vehicle alone. Following treatment, animals were
sacrificed for histochemical analyses.

Tissue Preparation

For histochemical evaluation, tissues were collected 7 days after H-I (P14) as previously
described (Leonardo et al. 2007). Animals were anesthetized with pentobarbital (60 mg/kg) and
intracardially perfused with phosphate-buffered saline (PBS, pH 7.4) followed by 4%
paraformaldehyde. Brains were removed and cryopreserved with increasing sucrose
concentrations (15%, 30%), sectioned coronally on a cryostat at 30 µm thickness and thaw-
mounted onto Superfrost slides (Fisher Scientific, Suwane, GA) to achieve maximum
preservation of morphology of the tissue surrounding the lesion. Serial sections were collected
throughout the brain beginning at approximately 1.2 mm rostral to bregma and ending at approximately 5.8 mm caudal to bregma.

**Histology and Immunohistochemistry**

Immunohistochemistry was performed as previously described (Leonardo et al. 2007) to assess reactive astrogliosis, MMP-cleaved proteoglycan fragment and microglia/macrophage recruitment to the injured site. Slides were rinsed with PBS, permeabilized and blocked for 60 min (3% Triton-X, 3% 1M Lysine, 10% NGS in PBS), incubated overnight with primary antibody at 4°C, incubated 60 min with secondary antibody at room temperature and coverslipped using Vectashield aqueous mounting media (Vector Labs, Burlingame, CA). Double-label immunohistochemistry was achieved by co-incubation with anti-mouse and anti-rabbit primary antibodies, and subsequent co-incubation with secondary antibodies conjugated to distinct fluorophores for each respective species of primary antibody.

Primary antibodies used in these studies were mouse anti-glial fibrillary acidic protein (GFAP) (Roche Applied Science, Indianapolis, IN; 1:1000), affinity-purified rabbit anti-PLPDSR (custom produced by Sigma Genosys, St. Louis, MO; 1:250, CCGGPLPDSR conjugated to keyhole limpet hemocyanin for immunization and the same conjugated to Pierce Sulfolink (Rockford, IL) for affinity purification) and mouse anti-OX-42 (Serotec, Raleigh, NC; 1:300). Primary antibodies were visualized using either Alexa Fluor 488 (green) or Alexa Fluor 594 (red) secondary antibodies (Molecular Probes, Eugene, OR). Working concentrations for secondary antibodies were 1:3000 for OX-42 and 1:1000 for all other primary antibodies.

The Fluoro-Jade stain identifies areas of cell death, predominantly degenerating neurons, and provides a positive quantitative marker as opposed to the absence of stain that is observed when using nissl stain. Fluoro-Jade was previously shown to be a more sensitive measure of cell death when compared to triphenyltetrazolium chloride (TTC). This method was adapted from Schmued and colleagues (Schmued et al. 1997) and subsequently detailed (Duckworth et al. 2005). Tissues were prepared and mounted on glass slides as described. Slides were sequentially placed in 100% ethanol for 3 min, 70% ethanol for 1 min and deionized water for 1
Sections were oxidized for 15 min using 0.06% KMnO₄ solution followed by 3 brief rinses in PBS. Slides were then immersed in a 0.001% solution of Fluoro-Jade (Histochem, Jefferson, AR) in 0.1% acetic acid for 30 min, rinsed with PBS, dried for 20 min at 45°C, cleared with xylene and coverslipped using DPX medium (Electron Microscopy Sciences, Ft. Washington, PA).

**Image Analyses and Quantification**

Images were acquired using a Zeiss Axioscope 2 (model #801572) microscope controlled by Openlab (Improvision Ltd, Lexington, MA) software, and photomicrographs were captured with a Zeiss Axicam Color (model #412-312) camera. All images subjected to direct comparisons were captured at the same exposure and digital gain settings to eliminate confounds of differential background intensity or false-positive immunoreactivity across sections. Immunoreactivity was quantified using NIH ImageJ software. Photomicrographs of sections from P14 rats were imported into ImageJ and two distinct methods were employed to best assess relative abundance of immunoreactivity. Histogram analyses were performed to assess astrogliosis. Total GFAP intensity values were calculated based upon the frequency of positive pixels that occurred within an intensity spectrum ranging from 0 (no immunofluorescence detected) to 256 (highest immunofluorescence intensity). For GFAP quantification, 2 cortical fields per section were selected for analyses. Cortical field selection was achieved by moving lateral from the most dorsal aspect of the corpus callosum (layers 1-4), and again moving medial-ventral to the adjacent field (layers 5-6). For cell death indicated by Fluoro-Jade stain, the entire cortex was traced and background subtraction was achieved by enhancing contrast until background particles were eliminated from the images. All sections were selected at fixed intervals throughout the bregma coordinates described previously.

**Statistical Analyses**

Data from all treatment groups were expressed as X ± SEM. For reactive astrogliosis and cell death, data were represented as arbitrary units (AUs) of total immunofluorescence intensity.
Group means were then subjected to a one-way ANOVA with “p value” set at 0.05. Pair-wise comparisons of group means were made using a Dunnett’s Multiple Comparison test.

**Results**

**Microglia/macrophages at the Cortical Lesion Site**

Exposure to H-I initiates deleterious biochemical cascades that facilitate recruitment of proinflammatory cells to the lesion site. Previous data from our laboratory identified reactive astrogliosis as a major component of the injury response (Leonardo et al. 2007). To expand on those data, immunohistochemistry was performed to determine whether microglia/macrophages play as large a role in this process. Because microglia express MMPs and are recruited to injured sites, sections from non-treated and vehicle-treated animals (N = 5 per group) were labeled with anti-OX-42, an antibody that binds CD11b and is a macrophage/microglial marker. These same sections were double-labeled with anti-PLPDSR, an antibody that recognizes an MMP-derived neopeptide fragment of the proteoglycan versican (Fig. 1). Robust labeling of CD11b was localized to regions containing PLPDSR-positive cells (Fig. 2). Intense OX-42 immunoreactivity was restricted to the ipsilateral cerebral cortex (Fig. 2B) and was markedly elevated when compared to the contralateral control region (Fig. 2A). Some labeling was detected in the contralateral hemisphere but was faint and diffuse by comparison. Anti-PLPDSR immunoreactivity was also elevated in ipsilateral cortex (Fig. 2D), appearing as clusters of cells that were difficult to identify specifically, although they appeared to be neuronal. Though fewer PLPDSR-positive cells were detected when compared to OX-42, the versican fragment was not detected in the contralateral control hemisphere (Fig. 2C) and each of the ipsilateral clusters was present within regions that showed intense OX-42 immunofluorescence (Fig. 2E,F). These effects were not observed in animals treated with minocycline or AG3340.
Cell Death Is Associated with MMP-Cleaved Versican and Microglia/Macrophage Recruitment

Hypoxia-ischemia often produces cortical and subcortical cavitary infarctions over time that are not present up to 4 days after insult. Tissues from vehicle-treated animals (N = 5) were collected 7 days after H-I and stained with Fluoro-Jade to determine the degree of cell death present at this time (Fig. 3). Fluoro-Jade staining was prominent in the ipsilateral hemisphere. Signal appeared as columns in lower cortical layers, was diffusely distributed throughout the striatum and labeled hippocampal CA1-CA3 pyramidal neurons intensely. All other hippocampal fields were comparatively signal-free (Fig. 3A). Importantly, Fluoro-Jade signal was present both in and adjacent to regions of OX-42 and PLPDSR immunoreactivity. The cortical neurodegeneration profile was consistent with elevated OX-42 and anti-PLPDSR immunoreactivity shown previously (Fig. 2). In hippocampus, OX-42 and anti-PLPDSR did not colocalize with Fluoro-Jade. Immunoreactivity for the MMP-cleaved fragment of versican was absent throughout Ammon’s horn, but appeared in the dentate gyrus (Fig. 3B). While increased OX-42 labeling was detected in the dentate gyrus (Fig. 3C), very little cellular colocalization was found with the PLPDSR antigen (Fig. 3D).

Delayed Treatment Dampens Reactive Astrogliosis

Previous data demonstrated a robust astroglial response early after H-I in the neonate, indicating that reactive astrocytes are key players in neuroinflammation and respond upstream of severe infarction (Leonardo et al. 2007). To test whether inhibition of MMPs can reduce astrocyte reactivity after H-I, immunohistochemistry was performed 7 days after H-I to assess GFAP upregulation (Fig. 4). In general, low levels of GFAP were detected in contralateral cerebral cortex (Fig. 4A,D,G). GFAP immunoreactivity showed robust increases in ipsilateral cortex and appeared to be elevated in layers 5-6 (Fig. 4C,F,I) relative to layers 1-4 (Fig. 4B,E,H). Animals treated with vehicle alone showed robust increases in GFAP in layers 1-4 (Fig. 4B) and layers 5-6 (Fig. 4C). This is consistent with the inflammatory response previously reported at an earlier endpoint. Astrogliosis was diminished after administration with either minocycline or AG3340.
While the observed effect of minocycline was most evident in cortical layers 1-4 (Fig. 4E), administration of AG3340 nearly abolished astrogliosis in layers 1-4 (Fig. 4H) and appeared more efficacious in layers 5-6 (Fig. 4I) when compared to minocycline (Fig. 4F). Quantification was performed by analyzing photomicrographs (N = 8 per group, 5 sections per animal) for total GFAP immunoreactivity. Results showed significant reductions in GFAP immunoreactivity in cortical layers 1-4 (Fig. 4J) and layers 5-6 (Fig. 4K) of animals treated with either minocycline or AG3340 compared to treatment with vehicle alone (p<0.05), as measured by One-way ANOVA followed by Dunnett’s post-hoc analysis. Despite a clear trend, there was no significant difference in efficacy between treatments.

**Delayed Treatment Reduces Cell Death**

Cell death was evident after H-I in animals treated with vehicle alone. To determine whether inhibition of MMPs provides neuroprotection, tissues were stained with Fluoro-Jade (Fig. 5). Staining was absent from the contralateral hemisphere and very little non-specific background staining was detected (Fig. 5A,C,E). Tissues from animals treated with vehicle alone showed pronounced Fluoro-Jade labeling in ipsilateral cortex, and to a lesser degree, in corpus striatum (Fig. 5B). While striatal staining was inconsistent among vehicle-treated animals, intense columnar-shaped immunofluorescence was evident throughout the cerebral cortex in sections that ranged from the initial emergence of lateral ventricles to ventral hippocampus. Treatment with minocycline abolished this effect, showing little or no cortical Fluoro-Jade labeling (Fig. 5D). Administration of AG3340 also reduced Fluoro-Jade staining in cerebral cortex (Fig. 5F) when compared to sections from animals treated with vehicle alone, though this compound appeared to be less efficacious than minocycline. Quantification of cortical Fluoro-Jade staining was performed by analyzing photomicrographs (N = 5 per group, 5 sections per animal) for total area occupied by stain. Results showed a significant reduction in Fluoro-Jade staining after treatment with both compounds (Fig. 5G) when compared to treatment with vehicle alone (p<0.05), as measured by One-way ANOVA followed by Dunnett’s post-hoc analysis. Efficacy did not differ significantly between minocycline and AG3340. Tissues examined from each of these treatment
groups showed inconsistent staining profiles in corpus striatum and hippocampus and therefore were not subjected to quantitative analyses.

**Delayed Treatment Ameliorates the Microglia/Macrophage Response**

To determine whether reduced cell death was associated with microglia/macrophage recruitment to the lesion site, immunohistochemistry was performed with anti-OX-42 to detect the CD-11b antigen present on cell membranes of microglia and macrophages (N = 5 per group). The results demonstrated a clear microglia/macrophage response to H-I (Fig. 6). Tissues from animals treated with vehicle alone showed increased OX-42 immunoreactivity in the ipsilateral cerebral cortex (Fig. 6B). CD11b labeling in contralateral control regions was faint and diffuse by comparison (Fig. 6A), indicating that OX-42 – positive cells were being recruited to the injured area. Treatment with minocycline ameliorated this effect, as ipsilateral OX-42 immunoreactivity (Fig. 6D) was indistinguishable from that observed in the contralateral control hemisphere (Fig. 6C). OX-42 – positive cells were also greatly reduced in ipsilateral cortex of animals treated with AG3340 (Fig. 6F) relative to contralateral control (Fig. 6E).

**Discussion**

In models that perturb blood brain barrier integrity, the injury response often includes activation of reactive astrocytes, infiltration of peripheral macrophages and activation of resident microglia. Subsequent release of pro-inflammatory cytokines and elevations in protease activity likely contribute to cell death and deep cerebral white matter injury after H-I. The present study evaluated the degree of reactive astrogliosis, microglia/macrophage recruitment and cell death in a neonatal rat model. Treatment for 6 days with either minocycline or AG3340 demonstrated that both compounds are neuroprotective. Inhibition of MMPs dampened key inflammatory components of H-I injury, and that inhibition of the inflammatory response was associated with reduced cell death.

Glial scar formation, an early neurobiological response to cell death, may enhance the inflammatory response over time and exacerbate injury via cytokine and chemokine release from
reactive astrocytes, resident immune cells and peripheral leukocytes. Robust elevations in GFAP immunoreactivity were prominent throughout the cerebral cortex and were restricted to the ipsilateral hemisphere of animals treated with vehicle alone. Fluoro-Jade histochemistry revealed marked cell death that was anatomically consistent with heightened GFAP and OX-42 labeling. OX-42 – positive cells were abundantly distributed throughout the cortical lesion site. These cells could have been either peripheral macrophages that entered the CNS through a compromised blood brain barrier or resident microglia that were recruited to and proliferated near the injured site. There is currently no antibody available that distinguishes between microglia and macrophages. Indeed, gelatinases were shown to degrade dystroglycan after autoimmune encephalomyelitis, thus allowing the entry of peripheral leukocytes into the brain (Agrawal et al. 2006). The majority of OX-42 – positive cells appeared to be ramified microglia, exhibiting spiny, dysmorphic morphology, yet many also displayed a rounder, amoeboid morphology indicative of an activated phenotype. While it is possible that the amoeboid cells were macrophages, there is some evidence indicating that microglia are the primary immune cells that respond to ischemic injury in the neonatal brain (Denker et al. 2007).

Both microglia and peripheral macrophages secrete pro-inflammatory molecules and MMPs. In addition to degrading blood brain barrier constituents and myelin proteins, it has become increasingly evident that MMPs participate in complex injury responses through interactions with various cytokines and chemokines (Van Lint and Libert 2007). One potential mechanism is through ‘sheddase’ activity, where MMPs cleave ectodomains of membrane-bound cytokines such as TNF-α and IL1-β (Gearing et al. 1994; Gottschall and Yu 1995), thus releasing activated forms into the ECM. Other data shows that gelatinases cleave SDF-1 and various MCP chemokines (Overall et al. 2002), and SDF-1 accelerates proteolytic processing of syndecans by MMP-9 (Brule et al. 2006). Taken together, these data offer a potential mechanism by which gelatinase activity enhances neuroinflammation after H-I by facilitating immune cell chemotaxis. Interestingly, treatment with minocycline or AG3340 blocked the recruitment of microglia/macrophages, and therefore it is possible that the observed neuroprotective effects
associated with MMP inhibition were due to reducing chemokine gradients or direct binding of pro-inflammatory cytokines to cell surfaces.

MMP-9 knockout mice showed reduced blood brain barrier degradation and white matter injury after experimental stroke (Asahi et al. 2001). Recent evidence also suggests that MMP-9 expression colocalizes with activated resident microglia (Svedin et al. 2007) and infiltrating leukocytes (Gidday et al. 2005; Romanic et al. 1998) in rodents subjected to cerebral ischemia. In agreement with these data, our results indicate that microglia/macrophage recruitment occurred at the lesion site where MMP activity was evident, i.e. adjacent to MMP-cleaved versican, after treatment with vehicle alone. The absence of immune cell recruitment observed after treatment provides further evidence that MMP activity potentiates the inflammatory response. Because gelatinases efficiently degrade basement membrane proteins, it is possible that this effect occurred, at least in part, through preservation of the blood brain barrier. However, the majority of OX-42 – positive cells resembled microglia and this would suggest that resident immune cells, alone, may be sufficient to exacerbate inflammation in the brain, potentially by enhancing the migratory efficiency of microglia via ECM proteolysis. This is an interesting potential mechanism which, to date, has not been investigated. Previous studies using in vivo stroke models have implicated MMPs in blood brain barrier disruption and cerebral infarction. Pretreatment with the broad spectrum MMP inhibitor BB-94 reduced rtPA-induced blood brain barrier opening and subsequent mortality (Pfefferkorn and Rosenberg 2003), while pretreatment with an MMP-9 neutralizing antibody reduced stroke-induced infarction (Romanic et al. 1998). A separate report demonstrated reductions in laminin degradation and neuronal apoptosis in the early phase of stroke after administration of the gelatinase-selective compound SB-3CT (Gu et al. 2005). Our data extend these findings by demonstrating a clear link between microglia/macrophage recruitment, astrocyte reactivity and gelatinase activity after H-I. Additionally, we showed that MMP inhibition at a delayed time point confers neuroprotection that persists 7 days after insult.

Minocycline is known for its broad anti-inflammatory actions and has recently been shown to inhibit MMP activity in an in vivo stroke model (Machado et al. 2006). In this study, we sought to determine the degree to which MMPs, specifically, influence H-I pathology by selecting
two distinct compounds that exhibit differential anti-inflammatory actions and affinities for MMPs. Data show that minocycline reduced microglia/macrophage recruitment and cortical cell death. Two additional neonatal H-I studies have demonstrated short-term (Cai et al. 2006) and long-term (Fan et al. 2006) protection after treatment with minocycline. These experiments showed reductions in H-I - induced necrosis, in agreement with the reductions in Fluoro-Jade staining observed here. Importantly, these experiments differed from the present study in that animals were pretreated prior to insult and again immediately after occlusion. In the present study, treatment did not begin until 24 hours after the insult. Both minocycline and AG3340 reduced astrogliosis, and a trend toward increased efficacy was observed after treatment with AG3340. These data suggest that MMPs, and particularly gelatinases, may be key contributors to cortical astrocyte reactivity.

Lecticans are well-know MMP substrates and are richly deposited in and around lesioned tissue in several rodent brain injury models (Morgenstern et al. 2002). Versican was demonstrated to be expressed throughout the cortical infarction after H-I, while it was reduced in deep cerebral white matter (Leonardo et al. 2007). MMPs cleave lecticans efficiently, and increased secretion and activation of MMPs should result in proteolytic degradation of the ECM substrates. An antibody was generated against the neoepitope (PLPDSR) of versican that is exposed upon proteolytic cleavage by MMPs. Indeed, anti-PLPDSR-positive cells labeled intensely and were located adjacent to microglia/macrophages within the lesion core of animals treated with vehicle alone. In contrast, little immunoreactivity was observed for the MMP-cleaved fragment of versican after treatment with minocycline or AG3340, indicating that each of these compounds inhibited MMP activity either directly or indirectly. These data support the idea that microglia/macrophages may be primarily responsible for secreting MMPs after H-I. The MMP-cleaved fragment of versican was detectable in white matter after H-I, yet the overall effects of treatment on PLPDSR immunoreactivity were subtle.

Taken together, these data provide strong evidence that MMPs potentiate H-I pathology through inflammatory mediators. Future studies aimed at defining the early time course for
efficacy and the specific pro-inflammatory molecules involved should help to elucidate the specific sequence of neuroinflammatory events that contribute to H-I injury.

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Figure 1
**Figure 1 Legend**

*Anti-PLPDSR.* Custom antisera was synthesized (Sigma Genosys) against MMP-cleaved versican. The antibody recognizes the peptide PLPDSR that is conserved within the hyaluronan binding region (HABR) of all versican isoforms. The custom peptide was designed using the versican V3 precursor protein (GenBank accession number [AAC26116](https://www.ncbi.nlm.nih.gov/nuccore/AAC26116)) and is located immediately upstream of the putative N-terminal MMP cleavage site (PLPDSR314 342FDAYCF). The specificities of the original antisera (Non-AP) and affinity purified antisera (AP) were verified by Western Blot using brain homogenate from adult rat and mouse. Results showed a specific band in rat homogenate that migrated at the predicted position of ~43 kDa on SDS PAGE, and a second band at ~86 kDa indicating a doublet of the MMP-cleaved fragment.
Figure 2

OX-42

PLPDSR

Merge
Microglia/macrophages are present with MMP-cleaved versican at cortical lesion site.

Exposure to H-I at P7 resulted in elevated microglia/macrophages, as measured by OX-42 immunoreactivity, in ipsilateral somatosensory cortex (B) compared to contralateral control (A). PLPDSR immunoreactive positive cells show MMP-cleaved fragment of versican at cortical lesion site (D) but absent in contralateral hemisphere (C). Merged image (E) demonstrates microglia/macrophages and MMP-cleaved versican fragment in the lesion core (F = 20x magnification). N = 5. Scale bars = 100 µm (A-E), 50 µm (F).
Figure 3

Fluoro-Jade

CA1

CA2

CA3

OX-42

PLPDSR

Merge
Figure 3 Legend

Cell death is associated with MMP-cleaved versican and microglia/macrophage recruitment. Exposure to H-I at P7 resulted in robust Fluoro-Jade staining throughout cerebral cortex and in CA1-CA3 pyramidal cell layers of hippocampus (A), indicating severe cell death. PLPDSR (B) and OX-42 (C) immunoreactivity show MMP-cleaved versican and microglia/macrophage labeling in the dentate gyrus of hippocampus. Merged image shows distinct temporal staining with little colocalization in dentate gyrus of hippocampus (D). N = 5. Scale bars = 50 µm (A), 100 µm (B-D).
Figure 4
**Figure 4 Legend**

*Delayed treatment dampens reactive astrogliosis.* P7 rat pups were exposed to H-I and treated with either vehicle, minocycline or AG3340 once daily for 6 days, beginning 24 hours after insult. (A-C) Astroglisis, as measured by GFAP immunoreactivity, was markedly elevated in cortical layers 1-4 (B) and layers 5-6 (C) compared to contralateral control (A) after treatment with vehicle alone. Administration of either minocycline (D-F) or AG3340 (G-I) 24 hours after H-I reduced GFAP immunoreactivity in layers 1-4 (E,H) and layers 5–6 (F,I) compared to control hemisphere (D,G). GFAP immunoreactivity was significantly reduced in cortical layers 1-4 (J) and layers 5-6 (K) of animals treated with either minocycline or AG3340 compared to treatment with vehicle alone. N = 8, * = p<0.05. Scale bar = 50 µm.
Figure 5

(A) cont   (B) ips  

(C) cont   (D) ips  

(E) cont   (F) ips  

(G) Fluoro - Jade Positive Area: Cerebral Cortex  

![Graph showing Fluoro - Jade Positive Area for different treatments. The graph indicates significant differences (*p < 0.05) between treatments.](image)
Figure 5 Legend

*Delayed treatment reduces cell death.* P7 rat pups were exposed to H-I and treated with either vehicle, minocycline or AG3340 once daily for 6 days, beginning 24 hours after insult. Animals treated with vehicle alone showed robust Fluoro-Jade labeling in ipsilateral cortex and corpus striatum (B). Fluoro-Jade staining was nearly abolished after treatment with minocycline (D) and greatly reduced after treatment with AG3340 (F) relative to vehicle alone (B). No labeling was detected in contralateral control regions (A,C,E). Quantification showed a significant reduction after treatment with either minocycline or AG3340 (G) compared to treatment with vehicle alone. N = 5, * = p<0.05. Scale bar = 100 µm.
**Figure 6 Legend**

*Delayed treatment ameliorates microglia/macrophage response.* P7 rat pups were exposed to H-I and treated with either vehicle, minocycline or AG3340 once daily for 6 days, beginning 24 hours after insult. Intense OX-42 immunoreactivity was evident throughout the ipsilateral cerebral cortex of animals treated with vehicle alone (B). Microglia/macrophage labeling was nearly abolished after treatment with minocycline (D) and was markedly reduced after treatment with AG3340 (F). OX-42 immunoreactivity in contralateral control regions was faint and diffuse by comparison (A,C,E). N = 5. Scale bar = 100 µm.
References


Chapter 3

Inhibition of Gelatinase MMPs Reduces Neurodegeneration in an Ex-Vivo Model of Oxygen Glucose Deprivation

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Abstract

Hypoxic-ischemic (H-I) insult during late gestational or early neonatal periods often manifests as cognitive and/or motor disturbances that appear early in development. Growing evidence indicates that targeting the neuroinflammatory response may be an avenue for therapeutic intervention. Resident microglia mediate the inflammatory response in brain by releasing proinflammatory molecules and proteases. Gelatinase matrix metalloproteinases (MMPs) activate proinflammatory cytokines and degrade basement membrane proteins after ischemia, thus permitting entry of peripheral monocytes/macrophages and enhancing neuroinflammation. To date, the relative contributions of resident and peripheral immune cells to brain injury in models that exhibit blood brain barrier degradation are not clear. Previous work showed that the selective gelatinase inhibitor AG3340 reduced neuroinflammation and neurodegeneration in vivo when administered at a delayed time point, yet the mechanisms and cell types involved remain unknown. The current study employed an ex-vivo model of oxygen glucose deprivation (OGD) to determine the cellular localization of MMP-9, and whether gelatinase inhibition could afford neuroprotection in the absence of peripheral immune cells. Organotypic hippocampal slices were exposed to 48 hrs of either normoxia or OGD in media containing vehicle, AG3340, or minocycline. Immunohistochemistry revealed that OX-42/MMP-9 – positive cells were prevalent after OGD, while few were observed after normoxia. Surprisingly, neuronal expression of MMP-9 was ubiquitous in both normoxic and hypoxic slices. In situ zymography revealed increased gelatinase activity after OGD in non-treated and vehicle-treated slices compared to normoxic slices. Treatment with AG3340 or minocycline reduced gelatinolytic activity and Fluoro-Jade staining after OGD, indicating that dampening gelatinase activity reduced subsequent neurodegeneration. These results suggest that resident microglia may be sufficient to enhance the neuroinflammatory response and neurodegeneration profile after H-I, potentially through upregulation or secretion of MMP-9. Additionally, these data lend support to the selective targeting of gelatinase MMPs as a potential therapeutic approach to combat H-I injury.
Keywords: matrix metalloproteinase; inhibitor; AG3340; minocycline; hypoxia; ischemia; neonate; inflammation; migroglia; macrophage
Introduction

Immature cerebral vasculature and low baseline blood flow render the immature brain inherently susceptible to changes in perfusion pressure (Baier 2006; Hamrick and Ferriero 2003). Because the developing vasculature adapts poorly, hypoxic-ischemic (H-I) insults that occur during the perinatal period initiate deleterious neurobiological responses. The resulting pathology includes cerebral white matter injury (Back et al. 2002; McQuillen and Ferriero 2004) and neurodegeneration (Vannucci et al. 1999), both of which are likely to result in loss of function. These injuries manifest early in development as motor disturbances and/or cognitive impairments (Back and Rivkees 2004; Folkerth 2005). Improvements in neonatal care have increased the number of infants who survive these insults, underscoring the need for novel approaches to combat H-I pathology. Similar to the human condition, neonatal rodents exposed to H-I exhibit cognitive deficits (Arteni et al. 2003; Ikeda et al. 2001; Young et al. 1986) and impaired motor function (Bona et al. 1997; Jansen and Low 1996; Ten et al. 2003). Previous work has demonstrated that oxidative stress (Back et al. 1998; Bernardo et al. 2003) and NMDA receptor activation (Kaur et al. 2006) contribute to white matter injury, while glutamateergic blockade immediately following H-I reduced white matter injury (Follett et al. 2000), H-I – induced infarction (Dingley et al. 2006) and DNA fragmentation (Puka-Sundvall et al. 2000). The destructive effects of excitotoxicity and free radical production are clear. Despite this fact, selective targeting of these mechanisms has failed to produce functional improvements in clinical trials. As the need for unique approaches is evident, accumulating evidence suggests that targeting neuroinflammatory mechanisms may be a promising avenue for therapeutic intervention.

The role of resident microglia in modulating the inflammatory response is well-documented in the developing brain (Chew et al. 2006). Following ischemic insult, activated microglia upregulate the expression of various cytokines and chemokines (Fukui et al. 2006; Kim 1996; Van Lint and Libert 2007). Blood brain barrier degradation permits peripheral monocyte/macrophage entry into the brain and further enhances microglia/macrophage recruitment to the injured site (Alvarez-Diaz et al. 2007). Data from numerous studies, both in vitro and in vivo, suggest that matrix metalloproteinases (MMPs) may be instrumental in the
production and maintenance of a proinflammatory microenvironment (del Zoppo et al. 2007). Astrocytes and microglia become activated within hours after ischemic insult. *In vitro*, MMP-9 expression was induced in astrocytes after stimulation with either TNF-α or IL-1β (Gottschall and Yu 1995) and in microglia after stimulation with LPS (Rosenberg et al. 2001). Conversely, MMPs have been shown to process both TNF-α (Gearing et al. 1994) and IL-1β (Schonbeck et al. 1998) to their biologically active forms, while other data demonstrated a role for MMPs in chemokine signaling (Overall et al. 2002). These data suggest a complex regulatory system by which the concerted actions of gelatin-degrading MMPs and proinflammatory molecules modulate the neuroinflammatory response.

Many groups have reported degradation of basement membrane proteins by MMPs after ischemia (Cunningham et al. 2005; Manicone and McGuire 2008). MMP-9 knockout mice showed improved outcomes that were associated with attenuated blood brain barrier degradation (Asahi et al. 2001; Gidday et al. 2005) and reduced microglial activation (Svedin et al. 2007). Other *in vivo* experiments revealed MMP-9 expression that was localized to neutrophils and endothelial cells, and showed elevated MMP-2 expression in astrocytic endfeet (Rosenberg et al. 2001). The latter expression profile places MMP-2 in the ideal position to proteolytically process basement membrane constituents. Indeed, cortical gelatinase activity increased after middle cerebral artery occlusion, and neuronal laminin degradation was associated with upregulated MMP-9 expression (Gu et al. 2005), evidence consistent with MMP proteolysis of basal lamina constituents.

MMPs also contribute to extracellular matrix (ECM) reorganization through the proteolytic processing of chondroitin sulfate proteoglycans known as lecticans (Gottschall et al. 2005). Lecticans provide structural stability throughout the interstitial space and exert effects on cellular adhesion and signaling (Yamaguchi 2000). Proteolytic processing of lecticans has been linked to H-I pathology in the rat neonate (Aya-ay et al. 2005), with alterations in expression correlating with progressive white and gray matter injury (Leonardo et al. 2008). Very little is known regarding the specific effects of ECM proteolysis in neonatal H-I injury, though potential mechanisms may include the induction of cellular anoikis or the facilitation of microglia/macrophage migration.
Despite this gap in knowledge, it is likely that the migration of microglia/macrophages to the lesion site is achieved, at least in part, through proteolytic activity of gelatin-degrading MMPs. Recent data from our laboratory demonstrated that rat neonates exposed to H-I showed reduced astrogliosis, microglia/macrophage recruitment and neurodegeneration after delayed treatment with either AG3340, a small molecule hydroxamate-based inhibitor with high selectivity for gelatinases, or minocycline, a tetracycline derivative with anti-inflammatory and MMP-inhibitory properties (unpublished data). However, it was not clear whether the immune cells identified were resident cells of the brain or peripheral cells that entered the CNS through a compromised blood brain barrier. Additionally, the immediate effects of gelatinase activity after H-I remain unknown. Therefore, the present study addressed these questions by utilizing an ex-vivo model that lacks the peripheral immune cell response to insult. These experiments were conducted to assess gelatinase activity, to determine the cellular localization of MMP-9 and to test the hypothesis that inhibition of gelatin-degrading MMPs can suppress OGD (oxygen glucose deprivation) – induced neurodegeneration.

Materials and Methods:
Organotypic Slice Culture

All animal procedures were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals with a protocol approved by the Institutional Animal Care and Use Committee at the University of South Florida. Neonatal Sprague-Dawley rats were birthed from time-pregnant dams (Harlan Labs, Indianapolis, IN). Dams were maintained on a 12 hr light/dark cycle (7 am – 7 pm) and given access to food and water ad libitum. Organotypic slice cultures were prepared using a method previously described (Stoppini et al. 1991), with slight adaptations. Rat pups (postnatal day 8 – 10) were removed from their dams, anesthetized with CO2, and decapitated. Brains were removed and intact hippocampi were dissected in cold isotonic buffer (136.89 mM NaCl, 5.37 mM KCl, 169 nM Na2HPO4, 22.04 nM KH2PO4, 27.52 nM glucose, 59.01 mM sucrose). Whole hippocampi were sliced at 400 µm thickness using a McIlwain Tissue Chopper (Mickle Laboratory Engineering Co. Ltd. Gomshall, Surrey, England). Slices were
quickly transferred back into a petri dish containing cold isotonic buffer. Mechanical separation was achieved by expelling 1 mL of isotonic buffer into the dish and repeating until the majority of slices appeared to be autonomous. Only slices that appeared thin and translucent were selected for culture. Using blunt forceps, individual slices were then transferred into droplets of isotonic buffer (1 slice per droplet) and chilled for 90 min at 4°C to seal membranes that were presumed to be damaged from the initial chopping. Cultures were maintained on Millicell CM (Millipore Corp., Billerica, MA) inserts that provide mechanical support for slice viability while allowing passive diffusion of media and tissue factors. Inserts were placed in 12 well plates containing 1.1 mL of organotypic slice culture media (OTCC; 500 mL neurobasal media, 10 mL B-27 supplement, 6.25 mL L-glutamine (Invitrogen, Carlsbad, CA)) per well and allowed 30 min to equilibrate. After equilibration, 15 µL of OTCC was added onto each insert. Slices were then carefully transferred into this media and allowed to settle onto the inserts. All slices were cultured for 14 days in a standard tissue culture incubator at 37°C and received partial media changes every 3-4 days (500 µL OTCC) prior to experimentation.

**Oxygen Glucose Deprivation**

Organotypic slices were subjected to 48 hrs of normoxia or oxygen glucose deprivation (OGD) to determine first, whether gelatinase activity is associated with neurodegeneration, and second, whether selective gelatinase inhibition can reduce cell death after OGD in the absence of peripheral immune cell effects. Slices were assigned to 1 of 2 exposures (normoxia or OGD) and 1 of 3 treatments (vehicle, AG3340, or minocycline). Immediately prior to exposure, inserts were transferred into new 12 well plates containing vehicle, AG3340 (AG3340 was kindly provided by Dr. Peter Baciu, Allergan, Irvine, CA), or minocycline (Sigma Aldrich, St. Louis, MO). Vehicle consisted of 0.1% DMSO in Delbecco’s Modified Eagles Medium (DMEM; Mediatech, Herndon, VA) for normoxia or DMEM without glucose (Invitrogen, Carlsbad, CA) for OGD. Stock solutions of minocycline and AG3340 were prepared, aliquotted and stored at -80°C prior to experiments. Fresh working solutions were prepared for each experiment. Stocks were diluted in the appropriate vehicles immediately prior to each experiment to yield final working volumes of 1.1
mL per well. Working concentrations were as follows: AG3340 = 3 µM; minocycline = 30 µM. Drug concentrations were selected based on doses that were previously shown to reduce neuroinflammation and neurodegeneration in vivo (unpublished data). Cultures were maintained in a standard tissue culture incubator during the normoxia exposure and a hypoxic chamber (CBS Scientific Co. Inc., Del Mar, CA) held at 37˚C during the OGD exposure.

**Histology and Immunohistochemistry**

Immunohistochemistry was performed as previously described (Leonardo et al. 2008) to determine the cellular localization of MMP-9 in organotypic hippocampal slices after exposure to normoxia or OGD. Immediately following the 48 hr exposure, inserts were transferred into 12 well plates containing 1.1 mL of 4% paraformaldehyde and slices were fixed for 24 hrs. Inserts were then submerged in phosphate-buffered saline (PBS, pH 7.4). Slices were carefully dissociated from the inserts using a small paint brush, mounted onto Superfrost Plus slides (Fisher Scientific, Suwane, GA) and dried overnight at 20˚C. For immunohistochemistry, slices were rinsed with PBS, permeabilized and blocked for 60 min (3% Triton-X, 3% 1M Lysine, 10% NGS in PBS), incubated overnight with primary antibody at 4˚C. On day 2, slices were washed with PBS, incubated 60 min with secondary antibody at 20˚C, washed again and coverslipped using Vectashield aqueous mounting media (Vector Labs, Burlingame, CA). Double-label immunohistochemistry was achieved by co-incubation with anti-mouse and anti-rabbit primary antibodies, and subsequent co-incubation with secondary antibodies conjugated to distinct fluorophores for each respective species of primary antibody.

Primary antibodies used in these studies were mouse anti-glial fibrillary acidic protein (GFAP) (Roche Applied Science, Indianapolis, IN; 1:1000), mouse anti-OX-42 (Serotec, Raleigh, NC; 1:300) and rabbit anti-MMP-9 (Chemicon, Temecula, CA, 1:200). Primary antibodies were visualized using either Alexa Fluor 488 (green) or Alexa Fluor 594 (red) secondary antibodies (Molecular Probes, Eugene, OR). Working concentrations for secondary antibodies were as follows: 1:3000 for OX-42; 1:1000 for GFAP and MMP-9.
Fluoro-Jade Staining

Fluoro-Jade staining was performed to determine whether treatment with AG3340 or minocycline improves organotypic hippocampal slice viability after exposure to OGD. The Fluoro-Jade stain identifies dead and degenerating neurons, thus providing a positive quantitative marker as opposed to the absence of labeling that is observed when using Nissl stain. Fluoro-Jade was previously shown to be a more sensitive measure of cell death when compared to triphenyltetrazolium chloride (TTC) (Duckworth et al. 2005). This method was adapted from Schmued and colleagues (Schmued et al. 1997) and subsequently detailed (Duckworth et al. 2005). Slices were prepared and mounted on glass slides as described for histology. Slides were sequentially placed in 100% ethanol for 3 min, 70% ethanol for 1 min and deionized water for 1 min. Sections were oxidized for 15 min using 0.06% KMnO₄ solution followed by 3 brief rinses in PBS. Slides were then immersed in a 0.001% solution of Fluoro-Jade (Histo-Chem, Jefferson, AR) in 0.1% acetic acid for 30 min, rinsed with PBS, dried for 20 min at 45°C, cleared with xylene and coverslipped using DPX medium (Electron Microscopy Sciences, Ft. Washington, PA).

In Situ Zymography

In situ zymography was performed to assess gelatinase activity in organotypic hippocampal slices after exposure to normoxia or OGD. Immediately following the exposure period, slices were dissociated from the inserts and mounted onto glass slides as described in histology. Slices were dried briefly prior to incubation. Slides were coated with 50 µl of DQ gelatin (Invitrogen, Carlsbad, CA), coverslipped and incubated 90 min at 37°C to allow for enzymatic cleavage of the gelatin. DQ gelatin is quenched with FITC molecules that emit fluorescence when the gelatin is enzymatically cleaved by MMP-2 or MMP-9. Following incubation, fluorescence was visualized at a wavelength of 480 nm.

Image Analyses and Quantification

For quantification, images were acquired using a Zeiss Axioscope 2 microscope controlled by Openlab (Improvision Ltd, Lexington, MA) software, and photomicrographs were
captured with a Zeiss Axicam Color camera. All images subjected to direct comparisons were captured at the same exposure and digital gain settings to eliminate confounds of differential background intensity or false-positive fluorescent signal across sections. Fluorescence was quantified using NIH ImageJ software. Photomicrographs of organotypic hippocampal slices were imported into ImageJ and background subtraction was achieved by enhancing contrast until background particles were eliminated from the images. To control for size differences between slices, fluorescence values were calculated by dividing the total fluorescence by the total area of the slice, thus yielding percent of total area occupied by fluorescent signal.

Additional high-power micrographs were captured for MMP-9 immunohistochemistry and in situ zymography to verify cell-specific expression of MMP and to better determine the cellular localization of gelatinase activity. These images were obtained using a Leica SP-2 confocal microscope and Leica LCS software.

**Statistical Analyses**

Data from all treatment groups were expressed as X ± SEM of the percent area occupied by fluorescent signal. Group means were then subjected to two-way ANOVAs with “p value” set at 0.05. Pair-wise comparisons of group means were made using Bonferroni post-hoc tests.

**Results**

**Cellular Localization of MMP-9**

Matrix metalloproteinases are known to be involved in the neuropathological response to ischemia. While MMP-2 activity contributes to peripheral monocyte/macrophage infiltration through blood brain barrier disruption, MMP-9 activity may contribute to neuronal damage by enhancing inflammation associated with resident microglia. Immunohistochemistry was employed to characterize the cellular localization of MMP-9 in an ex-vivo model of hypoxia-ischemia that lacks peripheral immune cell infiltration. Organotypic hippocampal slices were cultured for 14 days and exposed to normoxia or OGD. MMP-9 expression was ubiquitous in slices exposed to normoxia (Fig. 1). GFAP – positive astrocytes were prevalent throughout the slices and
predominantly displayed the typical morphology associated with the quiescent state (Fig. 1B,E). Immunoreactivity for MMP-9 was detected in some astrocytic cell bodies and processes (Fig. 1A-C). The majority of MMP-9 expression, however, did not appear to colocalize with astrocytes (Fig. 1D-F). To determine whether basal MMP-9 expression occurs in resident immune cells, slices were double-stained with anti-MMP-9 and anti-OX-42, an antibody that recognizes the cd11b antigen that is commonly expressed on the cell surfaces of microglia and macrophages. OX-42–positive cells displayed the classic amoeboid morphology associated with activated microglia (Fig. 1H), consistent with previous data indicating that these cells exhibit a heightened activation state under culture conditions. Interestingly, MMP-9 immunoreactivity did not appear to colocalize with microglia in most cases (Fig. 1G-I). Instead, MMP-9 expression appeared to be predominantly neuronal in normoxic slices.

The expression of MMP-9 was altered in slices that were exposed to OGD (Fig. 2). Astrogliosis and microglial activation are hallmarks of the neuroinflammatory response to H-I. Indeed, hypertrophic astrocytes were observed in slices subjected to OGD. GFAP immunoreactivity was intense in astrocytic cell bodies and processes throughout the slices (Fig. 2A). Similar to the expression profile observed in normoxic slices, MMP-9 immunoreactivity predominantly localized to cell bodies that were not GFAP–positive (Fig. 2B,C). Exposure to OGD resulted in increased OX-42 immunopositive cells (Fig. 2D). Clusters of these cells were generally observed adjacent to the hippocampal cell layers. MMP-9 expression was again observed in cells with neuronal morphology. Importantly, MMP-9 expression (Fig. 2E) colocalized with OX-42–positive microglia (Fig. 2F), indicating that exposure to OGD resulted in upregulated MMP-9 expression in these resident immune cells.

Treatment with AG3340 or Minocycline Attenuates Gelatinase Activity

To determine whether increased MMP-9 expression after OGD was associated with increased gelatinolytic activity, in situ zymography was performed on slices exposed to normoxia or OGD (Fig. 3). Gelatin-cleaved fluorescence was observed in slices exposed to normoxia (Fig. 3A,C,E), indicative of basal MMP-2 and/or MMP-9 activity in organotypic slice culture. Gelatinase
activity was primarily localized in the nuclei and throughout the cytoplasm, consistent with previous data (Amantea et al. 2008). Many of the fluorescent cell bodies appeared to have a twisted morphology indicative of a more activated phenotype when compared to the ramified phenotype characteristic of resting microglia. After exposure to OGD, slices showed increased fluorescent labeling (Fig. 3B,D,F). As in normoxic slices, gelatinase activity was found to be nuclear and cytoplasmic. However, compact, amoeboid cells resembling activated microglia were ubiquitous after OGD (Fig. 3D) in contrast to the twisted cells observed after normoxia. Additionally, a much greater fluorescent intensity was observed after OGD and was also localized to cellular processes when viewed at higher magnification (Fig. 3F). Gelatin-cleaved fluorescence in normoxic slices was faint by comparison and was limited to nuclei and cytoplasm (Fig. 3E).

Previous data from our laboratory showed that administration of either AG3340 or minocycline at a delayed time point after H-I conferred neuroprotection (unpublished data). To expand on those data, experiments were performed to assess the activity of gelatin-degrading MMPs in response to OGD, and to test the MMP-inhibitory efficacy of these compounds. In situ zymography was performed on organotypic slices that were exposed to 48 hrs of normoxia or OGD in media containing vehicle, AG3340, or minocycline. Quantification of gelatin-cleaved fluorescence was performed by analyzing photomicrographs (N = 2 cultures; 3 slices per group) for percent of total hippocampal area occupied by fluorescent signal (Fig. 4). Gelatin-cleaved fluorescence was increased in slices exposed to OGD (27.6% +/- 2.9%) relative to normoxia (15.5% +/- 4.1%; p<0.01) after treatment with vehicle alone. Furthermore, activity was significantly attenuated in slices exposed to OGD and treated with AG3340 (16.1% +/- 2.6%; p<0.01) or minocycline (20.1% +/- 3.4%; p<0.05) relative to those treated with vehicle alone. There were no significant differences between normoxia and OGD for either treatment, though trends for increased gelatin-cleaved fluorescence were evident.

Treatment with AG3340 or Minocycline Reduces Neurodegeneration

To determine whether gelatinase activity was directly associated with neurodegeneration after OGD, organotypic slices were exposed to 48 hrs of normoxia or OGD in media containing
vehicle, AG3340, or minocycline and were stained with Fluoro-Jade (Fig. 5). Basal levels of Fluoro-Jade stain were detected in normoxic slices after all treatments. Staining was most prominent in the hippocampal neuronal cell layers but was also present in surrounding tissue. In general, Fluoro-Jade staining was more intense and occupied larger areas in slices exposed to OGD and treated with vehicle alone (Fig. 5D) relative to their normoxic controls (Fig. 5A). Fluoro-Jade staining was elevated in slices exposed to OGD and treated with AG3340 (Fig. 5E) when compared to normoxic controls (Fig. 5B). In contrast, slices treated with minocycline and exposed to OGD showed faint staining that occupied smaller areas of total tissue (Fig. 5F) resembling normoxic controls (Fig. 5C).

Quantification of Fluoro-Jade staining was performed by analyzing photomicrographs (N = 3 cultures; 6 slices per group) for percent of total hippocampal area occupied by fluorescent signal (Fig. 6). Fluoro-Jade staining was significantly elevated in slices exposed to OGD (21.5% +/- 2.4%) relative to normoxia (8.0% +/- 1.0%; p<0.01) after treatment with vehicle alone. Quantification also revealed a significant increase in staining after OGD (10.7% +/- 1.2%) relative to normoxia (5.8% +/- 0.3%; p<0.05) in slices treated with AG3340, yet the degree of staining was significantly lower when compared to those treated with vehicle alone (p<0.01). Fluoro-Jade was also reduced after OGD in slices treated with minocycline (7.7% +/- 1.1%) relative to those treated with vehicle alone (5.1% +/- 1.1%; p<0.01), and the degree of staining was not statistically different from normoxic controls.

**Discussion**

H-I insults produce neural sequelae characterized by substantial white matter injury and neurodegeneration. Inflammation mediates the delayed injury response in ischemic brain. Both peripheral and resident immune cells may aid in proinflammatory signaling, yet the relative contributions of infiltrating leukocytes and resident microglia have yet to be elucidated. Gelatin-degrading MMPs proteolytically process endothelial cell junction proteins (Reijerkerk et al. 2006; Rosenberg and Yang 2007) and inflammatory cytokines (Ito et al. 1996; Schonbeck et al. 1998). Previous reports have demonstrated that gelatinases modulate neuroinflammation (Gidday et al. 2011).
blood brain barrier degradation (Asahi et al. 2001; Back et al. 1998) and cell death (Asahi et al. 2000; Lee et al. 2004) after ischemia. However, the association of MMPs with neuroinflammation and injury in the absence of peripheral immune cell effects has not been investigated to date. Therefore, the present study was conducted to determine whether selective gelatinase inhibition can confer neuroprotection in an ex vivo model of H-I that lacks peripheral immune cell involvement. Results showed that MMP-9 expression was upregulated in organotypic hippocampal slices after 48 hrs exposure to OGD. Furthermore, gelatinase activity and neurodegeneration increased in slices exposed to OGD, effects that were attenuated when slices were incubated with media containing either AG3340 or minocycline.

To our knowledge, this is the first study utilizing organotypic slice culture methodology to investigate the effects of ischemic insult in brain, and the first to link cell viability with MMP activity in the absence of peripheral immune cell effects. Immunohistochemistry revealed that MMP-9 expression was ubiquitous in organotypic slices and was associated with plasma membranes. Normoxic slices showed moderate expression in astrocytes and little expression in OX-42 – positive microglia. The size and morphology of MMP-9 – positive cell bodies, coupled with the general lack of colocalization with GFAP or OX-42, suggests that MMP-9 expression was predominantly neuronal. In contrast, MMP-9 colocalized with OX-42 – positive cell bodies and GFAP – positive astrocytic processes in slices exposed to OGD. MMP-9/cd11b - expressing cells were consistent with activated microglia such that the cell bodies were smaller and displayed an amoeboid morphology. These data are in agreement with previous studies that localized gelatinase activity to neurons and glia (Amantea et al. 2008; Lee et al. 2004; Lu et al. 2008). Additionally, the increased MMP-9 expression profile after OGD suggests that the insult induced microglial expression of MMP-9.

In situ zymography experiments demonstrated that there is basal gelatinase activity in organotypic hippocampal slices. Gelatin-cleaved fluorescence was predominantly limited to cells with neuronal morphology, consistent with the neuronal expression profile of MMP-9 in normoxic slices. Gelatinase activation in normoxic slices is likely a response to cellular injury from the slice
preparation itself. The presence of Fluoro-Jade staining in normoxic slices supports this explanation.

Upon exposure to OGD, gelatin-cleaved fluorescence was markedly increased. Gelatinase activity was not limited to neurons after OGD. Instead, activity appeared in cells with amoeboid microglial morphology and also localized to cellular processes. The cellular phenotypes associated with these processes were difficult to determine but were most likely astrocytes, as MMP-9 immunoreactivity appeared to localize to some astrocytic processes after OGD. Several groups have previously reported gelatinase expression in activated astrocytes in vitro (Apodaca et al. 1990; Muir et al. 2002; Rosenberg et al. 2001).

Both AG3340 and minocycline were efficacious in attenuating OGD – induced gelatinase activity and Fluoro-Jade staining. Gelatinase activity, therefore, correlated with increased neurodegeneration, and both compounds were able to reduce associated neuronal cell death. Consistent with these data, AG3340 was previously shown to be neuroprotective in the adult brain after ischemia (Nakaji et al. 2006). AG3340 is a small molecule hydroxamate-based MMP inhibitor with high selectivity for gelatinases. Minocycline has also been shown to possess MMP-inhibitory properties (Machado et al. 2006) but acts primarily as an anti-inflammatory agent. This compound has also improved outcomes in several rodent injury models (Cai et al. 2006; Fan et al. 2006; Wasserman and Schlichter 2007). Taken together with the cellular expression profile of MMP-9, these data suggest a direct link between gelatinase activity and neuroinflammation in response to H-I. While the selective inhibition of active MMP-9 and/or MMP-2 was sufficient to confer neuroprotection, the fact that minocycline was equally efficacious supports the notion that the resident immune cell response may facilitate MMP expression or activation.

Importantly, both compounds showed efficacy at concentrations that reduced recruitment of cd11b - positive cells and decreased neurodegeneration in an in vivo model of neonatal H-I (unpublished data). In the previous study, drug was administered 24 hrs after H-I and all data was collected 7 days post-insult. There are two differences worthy of mention. The former study consisted of a 6 day treatment period that primarily assessed the delayed immune response. Additionally, it could not be determined whether OX-42 – positive cells were of CNS origin, as
there is currently no available antibody that differentiates between resident microglia and peripheral macrophages. The present study exploited a model that lacks the peripheral immune cell response to determine whether gelatinase activity is of resident immune cell origin, and whether heightened MMP activity is associated with neuronal cell death during the early neuroinflammatory response to H-I. Other advantages to this model are that it is more conducive to mechanistic experimentation and more closely resembles the microenvironment in brain when compared to primary cell culture or mixed-glial culture. Taken together, these data indicate that gelatinase activity is linked to the resident microglial response within the first 48 hrs after OGD, and that inhibition of gelatinase activity reduces neurodegeneration in an ex vivo model of H-I injury. Thus, selective targeting of gelatin-degrading MMPs may be an avenue for therapeutic intervention to combat H-I neuropathies.

Acknowledgements

This work was supported by the Department of Molecular Pharmacology and Physiology at the University of South Florida College of Medicine.
Figure 1

![Image of a diagram showing different samples under normoxia conditions with labels for MMP-9 + GFAP and MMP-9 + OX-42.

Legend:
- MMP-9 + GFAP
- MMP-9 + OX-42

Samples:
- A
- B
- C
- D
- E
- F
- G
- H
- I

Annotations:
- Arrows pointing to specific areas on the images.
**Figure 1 Legend**

**Basal MMP-9 expression.** Confocal micrographs show cellular expression of MMP-9 (A,D,G), GFAP (B,E) and OX-42 (H) after 48 hrs exposure to normoxia. Basal MMP-9 expression was high and primarily localized to cell bodies (D,G). Merged images show MMP-9 expression in astrocyte processes and cell bodies (C), though the majority of astrocytes either did not express MMP-9 or expression was low (F). OX-42 – positive microglia display the activated phenotype in normoxic slices (H). Merged image shows OX-42 did not colocalize with MMP-9 (I). Magnification = 63x. Arrows indicate colocalization.
Figure 2
Figure 2 Legend

Neuroinflammatory response to oxygen glucose deprivation. Confocal micrograph shows hypertrophic astrocytes (A) after 48 hrs exposure to OGD, while MMP-9 expression is limited to distinct cell bodies (B). Merged image shows MMP-9 did not colocalize with astrocytes (C). OGD results in robust expression of cd11b (D) and MMP-9 (E). Merged image shows MMP-9 colocalizes with microglia (F). Magnification = 63x. Arrows indicate colocalization.
Figure 3 Legend

Gelatinase activity is elevated after oxygen glucose deprivation. Confocal micrographs show in situ zymography in organotypic hippocampal slices after 48 hrs exposure to normoxia or OGD. Basal gelatinase activity was detected in normoxic cells (A,C,E) and was increased after exposure to OGD (B,D,F). Gelatinase activity was localized to many distinct cellular morphologies after OGD (F) when compared to normoxia (E). Magnification = 20x (A,B); 40x (C,D); 63x (E,F).
Figure 4

Gelatinase Activity

- Vehicle
- AG3340
- Minocycline

Gelatin-Cleaved
Fluorescence (% Area)

Normoxia
OGD
Figure 4 Legend

Quantification of in situ zymography. Gelatin-cleaved fluorescence was quantified in organotypic hippocampal slices after exposure to normoxia or OGD in media containing vehicle, AG3340, or minocycline. Slices treated with vehicle alone showed a significant increase in fluorescent signal after exposure to OGD relative to normoxic controls (p<0.01). Gelatinase activity was significantly reduced in slices exposed to OGD and treated with either AG3340 (p<0.01) or minocycline (p<0.05) relative to those treated with vehicle alone. N = 6. * indicates significant from normoxia; # indicates significant from OGD + vehicle.
Figure 5

NM

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Neurodegeneration occurs after oxygen glucose deprivation. Organotypic hippocampal slices exposed to normoxia or OGD in media containing vehicle, AG3340, or minocycline were stained with Fluoro-Jade. Basal Fluoro-Jade staining was detected in slices exposed to normoxia after all treatments (A-C). Staining was markedly elevated in slices exposed to OGD and treated with vehicle alone (D) compared to normoxic controls (A), and this effect was attenuated in slices exposed to OGD and treated with either AG3340 (E) or minocycline (F). Scale bar = 100 µm.
Figure 6

Neurodegeneration

Fluoro-Jade Staining (% Area)

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**Figure 6 Legend**

*Quantification of Fluoro-Jade staining.* Fluorescent stain was quantified in organotypic hippocampal slices after exposure to normoxia or OGD in media containing vehicle, AG3340, or minocycline. Fluoro-Jade staining was significantly increased in slices exposed to OGD and treated with vehicle alone (p<0.01) or AG3340 (p<0.05) relative to their respective normoxic groups. There was no significant difference between slices treated with minocycline and exposed to either normoxia or OGD. Furthermore, the increase in Fluoro-Jade observed in slices treated with vehicle alone and exposed to OGD was significantly attenuated in those treated with either AG3340 (p<0.01) or minocycline (p<0.01). N = 6. * indicates significant from normoxia; # indicates significant from OGD + vehicle.
References


Conclusions

Improvements in medical care over past decades have increased the numbers of premature and low birth weight infants that survive H-I insults. Because there is a rising incidence in diseases associated with these events, it is critical to develop effective therapies to treat the various resulting neuropathies. The most common experimental model used to recapitulate clinical perinatal H-I insults produces neurodegeneration and deep cerebral white matter injury in the neonatal rat brain (Towfighi et al. 1995; Vannucci et al. 1999), leading to impaired cognitive and motor functioning. Excitotoxicity, free radical production, glial scar formation and neuroinflammation all contribute to the resulting pathology (Ashwal and Pearce 2001), yet the precise manner in which these processes are regulated has yet to be elucidated. ECM proteoglycans and matrix-degrading proteases exert effects on cell viability and may be associated with either protective or destructive processes after H-I. ECM constitutes the majority of brain parenchyma. Lectican deposition, in particular, is elevated in several rodent injury models (Beggah et al. 2005; Jones et al. 2003a; Jones et al. 2003b; Mayer et al. 2005; Vorisek et al. 2002). Turnover of ECM proteins is modulated by matrix-degrading metalloendopeptidases. Both ADAMTSs (Cross et al. 2006; Tian et al. 2007) and MMPs (del Zoppo et al. 2007; Gu et al. 2005; Rosenberg et al. 2001) have been associated with pathological states in brain. Despite these facts, the relative contributions of lecticans, ADAMTSs and MMPs to inflammation and cell death in the neonatal brain remain elusive.

The present study was designed to investigate several aspects of H-I pathology. The first series of experiments were conducted to characterize cellular damage and neuroinflammation in the P7 rat after exposure to H-I, and to determine if cell death and inflammation were associated with alterations in proteoglycan expression. Data from these experiments led to two additional objectives and subsequent series’ of experiments. The first of these was to determine whether treatment with AG3340, a selective inhibitor of gelatin-degrading MMPs, could confer
neuroprotection when administered at a delayed time point after insult, and to compare the efficacy with that of the well-known anti-inflammatory compound minocycline. These experiments showed that in addition to demonstrating efficacy, MMP inhibition significantly attenuated the classic neuroinflammatory response that is associated with progressive injury in this model, revealing a temporal relationship between the neuroinflammatory response and cell death within the first week after insult. The final series of experiments were designed to determine whether selective gelatinase inhibition could neuroprotect in an ex vivo H-I model that lacks peripheral immune cell involvement, thus providing insight into the relative contributions of resident microglia and gelatinase activity to the inflammatory sequelae. Data here showed that administration of AG3340 significantly reduced gelatinase activity and neuronal cell death after 48 hrs exposure to OGD. These data provide evidence that gelatinase activity originates from resident cells of the brain, thus lending support to the notion that gelatinases contribute to injury in the absence of peripheral leukocytes and through mechanisms distinct from blood brain barrier degradation.

**Brevican and Versican Deposition after H-I**

In response to H-I insult, energy failure resulting from reduced cellular uptake of oxygen and glucose leads to the production of free oxygen/nitrogen radicals and eventually cell death (Shalak and Perlman 2004). The delayed response to insult, though less understood, involves neuroinflammatory mechanisms initiated by both resident and peripheral immune cells, including the production and secretion of proinflammatory cytokines and chemokines, as well as glial scar formation. The glial scar acts as a barrier to separate injured tissue from surrounding viable tissue. Though considered to be an inherent protective mechanism, the glial scar restricts neuroplasticity and therefore reduces the capacity for regeneration of lesioned tissue (Silver and Miller 2004). Therefore, the first objective of the current study was to characterize the expression of brevican and versican at selected endpoints after insult (P11 (4 days), P21 (14 days), and P28 (21 days)), and to determine the association of these proteoglycans with neuroinflammation and cell death.
Immunohistochemical staining of reactive glia and fractin, the caspase-derived proteolytic fragment of \( \beta \)-actin, allowed for the detection of pathology at the cellular level in the absence of grossly observable infarctions. Animals sacrificed at later endpoints (P21 and P28) showed severe cortical and subcortical cavitory infarctions that were rarely present at P11, indicating that the resulting injury was progressive. Elevated immunoreactivity for GFAP and fractin was evident in cortex and hippocampus at P11 and was also observed at later endpoints. The fact that both GFAP and caspase-cleaved \( \beta \)-actin were observed at P11 and preceded cavitory infarction supports the notion that while reactive astrogliosis is associated with injury, apoptosis accounts for some degree of progressive cortical and hippocampal cell death. Loss of deep cerebral white matter was also observed at the early endpoint. Immunoreactivity for the putative pre-OL marker O4 was reduced in white matter of the external capsule, corpus striatum and hippocampal fimbria. A closer inspection revealed a population of cells in the external capsule that co-expressed the O4 and NG2 antigens. Pre-OLs are selectively vulnerable to H-I when compared to their mature, myelinating counterparts (Back et al. 2001). Data from a previous study indicated that OLs which co-express these antigens are likely to be either pre-OLs or immature OLs (Reynolds and Hardy 1997). Therefore, while disruptions of axoglial junctions may have accounted for the observed reductions, the presence of O4 and NG2 – positive cells at this stage in development suggests that at least a proportion of the total O4 immunoreactivity was specific to pre-OLs. The death of pre-OLs at this stage in development is detrimental, as myelination of neural tracts will be disrupted if these cells fail to differentiate into mature, myelinating OLs.

In general, lecticans inhibit neurite outgrowth and therefore might be expected to act as non-permissive substrates to tissue repair in this model (Asher et al. 2002). Brevican and versican are developmentally regulated in the rat neonate (Yamaguchi 2000). Upon investigation, the two proteoglycans showed distinct responses to H-I and alterations in expression were associated with distinct regions in brain. Brevican immunoreactivity was reduced at P11 in cortex and hippocampus, confirming data previously published by our laboratory (Aya-ay et al. 2005). Only modest changes in versican expression were detected in gray matter at P11 and were
limited to hippocampus. In contrast, versican expression was reduced in the external capsule at P11, where no changes in brevican immunoreactivity were detected.

A key feature of glial scar formation and maintenance is the upregulation of lectican expression, thus conferring inhibitory properties that restrict neural plasticity in several models of brain injury (Galtrey and Fawcett 2007). Intense immunoreactivity for both brevican and versican were evident in gray matter by P21. Previous reports have documented elevated lectican expression in the injured adult rat cortex. For example, the expression of both brevican (Jaworski et al. 1999) and versican (Asher et al. 2002) were increased in cortical stab wound tissue. This upregulated expression in response to injury, and in association with glial scarring, is consistent with the expression patterns shown here at P21 and P28. Despite these consistencies, it is noteworthy that the latter study reported increased versican expression 7 days after injury, while no changes in cortical versican were detected in the current study at P11. Because the current investigation did not include the P14 endpoint, it is difficult to interpret whether these data are, in fact, conflicting. However, reduced plasticity that is characteristic of adult rodents may result in an accelerated response to injury when compared to younger animals which display a greater potential for plasticity and repair. For this reason, care should be taken when comparing data from neonatal and adult injury models.

Though lectican deposition was clearly increased in ipsilateral cortex at P21 and P28, the presence of severe cavitary hippocampal infarcts at these endpoints prevented any direct comparisons between hemispheres. However, brevican and versican expression were detected in ipsilateral hippocampi, and deposition occurred along the perimeter of the remaining hippocampal structures. Thus, the general expression profile in gray matter at later endpoints, together with elevations in astrogliosis and caspase-cleaved β-actin, provides evidence that increased expression and deposition of these lecticans may aid in glial scar formation resulting from extensive gray matter injury.

Importantly, brevican and versican expression were altered in distinct regions of brain. Given the progressive injury that occurs in this model, cellular anoikis (Grossmann 2002) induced by proteolytic cleavage of lecticans may render neighboring cells susceptible to injury due to loss
of ECM signals or trophic support. O4 immunoreactivity was reduced in white matter at P11. Reduced white matter versican at both P11 and P21 supports the notion that versican may provide important signals that are necessary for immature OLs to differentiate into the mature, myelinating phenotype. Interestingly, fractin immunoreactivity was low in the selected white matter regions. These results would suggest that the loss of versican may contribute to cell death through mechanisms that are distinct from traditional caspase-mediated apoptotic signaling. A recent study in rat using a similar neonatal H-I model showed that the differentiation of pre-OLs was arrested 4 days after insult through mechanisms that were not associated with apoptosis. In these experiments, O4 – positive degenerating OLs were significantly increased up to 7 days after insult (Segovia et al. 2008). Although this group also reported significant increases in activated caspase-3 at this endpoint, there are important differences. In contrast to the current study, this group exposed rats to H-I at P3. By P10, caspase-3 immunoreactivity was reduced relative to P7 while OL degeneration remained elevated relative to controls. Therefore, when comparing this study to the current study, it is difficult to interpret whether the involvement of caspases is temporally related to the timing of the insult or the developmental stage of the animal. Regardless, the former study supports a mechanism that is distinct from caspase-mediated apoptosis and may explain the lack of fractin in white matter observed here. However, it is also possible that the anti-fractin antibody lacked the sensitivity to detect a lesser degree of β-actin cleavage than that which was observed in cortex and hippocampus. This explanation would also be supported by the heightened injury profile of these regions relative to that which was observed in white matter.

Another interesting finding was that no changes in brevican expression were detected in white matter. This may have been due to either lack of sensitivity or low expression levels at this developmental stage. However, another explanation may account for this unexpected finding. OL progenitors migrate from the SVZ to white and gray matter regions throughout the brain (Levison and Goldman 1993). In contrast to versican, which is secreted not only by OLs but also by astrocytes, OLs are the major source of brevican from P7 to P14 (Ogawa et al. 2001). Based on proximity, OLs arrive at deep cerebral white matter targets earlier than most gray matter regions.
Therefore, it is tempting to speculate that reduced brevican expression in gray matter at P11 was due, at least in part, to decreased OL migration resulting from the early loss of pre-OLs.

**ADAMTSs, Lectican Turnover and Pre-OL Susceptibility to H-I**

Data from the characterization experiments demonstrated that the deposition brevican and versican was altered after H-I. These results provided strong evidence that lecticans are involved in the formation and maintenance of the glial scar in this model, yet the unique spatiotemporal expression profiles observed at P11 prompted many questions as to the precise roles of these molecules in providing signaling and support to injured cells of the brain. As such, several additional studies were performed to gain insight into the mechanisms by which ECM lecticans are regulated following an ischemic event. Because lecticans are considered to be the major substrates for ADAMTS proteases (Flannery 2006; Porter et al. 2005), we hypothesized that activation or inhibition of ADAMTSs in response to the insult may have accounted for the altered expression profiles observed. Hence, the susceptibility of pre-OLs to H-I injury would be attributed to increased expression or activation of ADAMTSs, resulting in elevated ADAMTS-mediated proteolysis of lecticans in developing white matter. In contrast, inhibition of these proteases over time via compensatory negative feedback mechanisms would explain the accumulation of proteoglycan in glial scar.

The subsequent series of experiments were designed using multiple approaches. Immunohistochemistry was performed to determine whether ADAMTS cleavage of brevican was elevated in those regions that showed reduced expression at P11. For these experiments, an antibody was raised against the neoepitope of brevican (EAVESE) that is exposed in rat upon cleavage by ADAMTSs (Yuan et al. 2002). Regions of brain previously selected for immunohistochemical studies were dissected from a separate group of animals exposed to H-I, and biochemical assays were performed from brain homogenates to quantify cleaved fragments of brevican and versican. Concurrent with these experiments, primary pre-OLs were cultured to further investigate the role of CSPGs in pre-OL differentiation and susceptibility to OGD. In these experiments, proteoglycan stripe assays were performed in which OLs were seeded onto poly-L-
lysine coverslips that were previously streaked with known concentrations of purified proteoglycan from rat brain.

The data from these experiments, however, were not consistent with the expected outcomes. While it was hypothesized that EAVESE immunoreactivity would be elevated in cortex and hippocampus, supporting the notion that the reduction in brevican observed in these regions occurred as a result of proteolytic degradation by ADAMTSs, no changes in EAVESE expression were evident in cortex or hippocampus. Western Blots revealed that some animals showed increased cleavage fragments in ipsilateral regions relative to contralateral controls. Despite these data, the overall effects were variable and inconsistent. The final prediction was that pre-OL cultures would grow better on proteoglycan stripes relative to poly-L-lysine, consistent with the idea that proteoglycans are necessary substrates for pre-OLs as they differentiate into mature, myelinating cells. Although pre-OLs were able to grow on proteoglycan, quantification revealed that these cells actually grew better on the poly-L-lysine substrate. Taken together, these results demonstrated that ADAMTS activity is not likely to be a critical mediator of cell viability in this model, particularly at the early endpoint. Rather, a more plausible explanation for the expression data is that the reduced deposition of brevican and versican at P11 is simply an effect of cell death, whereby a proportion of OLs and/or astrocytes were no longer present to secrete these proteins into the ECM.

Despite these unexpected results, other data from our laboratory, as well as previous reports from various rodent injury models, indicated that MMP activity may exert detrimental effects on the developing brain by contributing to the neuroinflammatory response to injury. Although MMPs have been investigated in ischemic models, little work has been done to elucidate the precise mechanisms by which MMPs contribute to inflammation and cell death after H-I in the rat neonate.

The Effects of Anti-Inflammatory and MMP-Inhibitory Compounds in vivo

Hypoxic-ischemic insults produce neural sequelae characterized by white matter injury and neurodegeneration. Previous experiments from our laboratory showed that GFAP was
elevated in the ipsilateral cortex of animals sacrificed 24 hrs after H-I. In addition, dense pockets of microglia/macrophages were identified with anti-ED-1, an antibody that recognizes the cell surface antigen CD68 (supplemental Fig. 1), and were found to be localized to regions containing astrogliotic cells. These experiments not only demonstrated that the neuroinflammatory response had begun within the first 24 hrs after insult, but also that MMPs were upregulated within in the ischemic hemisphere in cells with microglial morphology (supplemental Fig. 2). Gelatin-degrading MMPs proteolytically process endothelial cell junction proteins (Reijerkerk et al. 2006; Rosenberg and Yang 2007) and inflammatory cytokines (Ito et al. 1996; Schonbeck et al. 1998), causing a second, delayed opening of the blood brain barrier which promotes extravasation and entry of peripheral monocytes and macrophages (Wetzel 2005). Taken together with the prolonged astrogliosis (elevations in GFAP at P21 and P28) and progressive infarctions that were observed in rats exposed to H-I at P7, it is likely that the neuroinflammatory response persists for weeks after the initial insult and promotes cell death. In the clinical population, the current lack of effective therapies to treat neonatal H-I highlights the importance of discovering new targets for pharmacological intervention. In experimental models, few compounds have shown efficacy when administered after the insult. As such, therapeutics that target key elements of the delayed immune response may prove beneficial in mitigating neural injury.

The second series of experiments evaluated the efficacy of two compounds in reducing reactive astrogliosis, microglia/macrophage recruitment and neurodegeneration in the rat neonate after H-I. Treatment for 6 days with either minocycline or AG3340, beginning 24 hrs after insult, demonstrated that both compounds were neuroprotective. Inhibiting inflammation, as with minocycline, or gelatin-degrading MMPs, as with AG3340, dampened key inflammatory components after H-I. Furthermore, inhibition of the inflammatory response was associated with reduced Fluoro-Jade staining, suggesting a link between immune cell recruitment and neurodegeneration. Immunohistochemistry revealed marked elevations in astrogliosis (GFAP) and microglia/macrophage recruitment (OX-42) throughout the ipsilateral cerebral cortex of animals treated with vehicle alone relative to contralateral controls. Fluoro-Jade staining indicated that cortical cell death was anatomically consistent with heightened GFAP and cd11b labeling,
suggesting that neurodegeneration was associated with the recruitment of immune cells to the lesion site. In addition, microglia/macrophage recruitment occurred in cortical regions where PLPDSR immunoreactivity was detected, demonstrating the presence of active MMPs in close proximity to these immune cells. These data are consistent with recent evidence indicating that MMP-9 expression colocalizes with activated resident microglia (Svedin et al. 2007). Little immunoreactivity was observed for the MMP-cleaved fragment of versican after treatment with minocycline or AG3340, indicating that each of these compounds inhibited MMP activity either directly or indirectly. Currently, there is no commercially available antibody that distinguishes between resident microglia and peripheral macrophages. While most cells that expressed cd11b appeared to be ramified microglia, many also displayed the amoeboid morphology indicative of an activated phenotype. Due to blood brain barrier disruption that most certainly occurs in this model, cells that expressed the cd11b cell surface antigen could have been either of these two cell types.

Both minocycline and AG3340 reduced astrogliosis and neurodegeneration, and a trend toward increased efficacy was observed after treatment with AG3340. These data suggest that MMPs, and particularly gelatinases, may be key contributors to cortical astrocyte reactivity. However, it cannot be determined whether astrogliosis is a response to cell death or contributes to cell death. Minocycline exerts anti-inflammatory actions and was previously shown to inhibit MMP activity in an in vivo stroke model (Machado et al. 2006). Specifically, this study reported reductions in H-I-induced necrosis. These data are consistent with the reductions in Fluoro-Jade staining shown here after treatment with minocycline, as well as with the MMP-inhibitor AG3340. Two additional neonatal H-I studies have demonstrated short-term (Cai et al. 2006) and long-term (Fan et al. 2006) protection after treatment with minocycline. In the present study, minocycline was selected for two specific purposes. As the neuroprotective effects of minocycline are well documented, this treatment group served as a positive control. More importantly, however, inclusion of the minocycline group allowed for a comparison of efficacy between a compound that exerts broad anti-inflammatory effects and a compound that specifically inhibits MMPs in the absence of any other known functions. Administration of minocycline reduced
microglia/macrophage recruitment and cortical neurodegeneration, in agreement with the previous studies. However, the present study differed significantly from those mentioned previously in terms of treatment. In the previous reports, animals were pretreated prior to insult and again immediately after occlusion. In the present study, treatment did not begin until 24 hours after the insult, a more clinically relevant time point when considering the nature of perinatal H-I injury and the difficulty in making an early diagnosis.

Previous experiments using in vivo stroke models have demonstrated not only that MMP activity compromises the blood brain barrier, but also that blocking MMP activity improves the neuropathological outcome. MMP-9 knockout mice showed reduced blood brain barrier degradation and white matter injury after experimental stroke (Asahi et al. 2001). Laminin degradation and neuronal apoptosis were reduced in the early phase of stroke after administration of the gelatinase-selective compound SB-3CT (Gu et al. 2005). Pretreatment with the broad spectrum MMP inhibitor BB-94 reduced rtPA-induced blood brain barrier opening and subsequent mortality (Pfefferkorn and Rosenberg 2003), while pretreatment with an MMP-9 neutralizing antibody reduced stroke-induced infarction (Romanic et al. 1998). In general, the neuroprotective effects of interfering with MMP activity shown previously are consistent with the improved outcomes demonstrated here after the administration of either minocycline or AG3340. Interestingly, the association between dampened immune cell recruitment and reduced neuronal cell death in the present study occurred after treatment with either of two distinct compounds, and both compounds showed similar efficacy. Therefore, data here extend previous findings by demonstrating that immune cell responsiveness and MMP activity may subserve either the same signaling pathways or distinct, but convergent signaling pathways that ultimately result in neuronal cell death. Additionally, these data show a clear association between microglia/macrophage recruitment, astrocyte reactivity and gelatinase activity after H-I, indicating that MMP activity likely promotes neuroinflammation after the initial injury.

In acknowledgement of previous data related to blood brain barrier degradation, it is possible that the effects of AG3340 were due, at least in part, to protection of the basement membrane. However, the majority of cd11b – positive cells resembled resting, inactivated
microglia. This would suggest that resident immune cells, alone, may be sufficient to exacerbate inflammation in the brain. Several other mechanisms may account for the protective effects of gelatinase inhibition. One potential mechanism by which this could occur is through reduced ECM proteolysis, thus diminishing the migratory capacity of microglia. This is a speculative possibility which, to date, has not been investigated. Despite this fact, the presence of intensely-labeled PLPDSR – positive cells in close proximity to microglia/macrophages within the cortical lesion site indicated that MMP cleavage of versican had occurred. Thus, the possibility that MMP-mediated ECM degradation could promote immune cell recruitment is, at the very least, plausible. This scenario would involve the secretion of MMPs by immune cells in response to immune cell activation, followed by MMP-mediated ECM proteolysis to allow for enhanced immune cell migration to injured sites within the brain.

Both microglia and peripheral macrophages secrete pro-inflammatory molecules and MMPs. In addition to degrading blood brain barrier constituents and myelin proteins, it has become increasingly evident that MMPs participate in complex injury responses through interactions with various cytokines and chemokines (Van Lint and Libert 2007). One potential mechanism is through ‘sheddase’ activity, where MMPs cleave ectodomains of membrane-associated effector molecules. For example, TNF-α (Gearing et al. 1994) is a substrate for gelatin-degrading MMPs. Conversely, both TNF-α and IL1-β have been shown to activate gelatinases (Gottschall and Yu 1995). Subsequent studies demonstrated that gelatinases cleaved SDF-1 and various MCP chemokines (Overall et al. 2002), and SDF-1 accelerated the proteolytic processing of syndecans by MMP-9 (Brule et al. 2006). Therefore, minocycline and/or AG3340 may exert effects on chemokine gradients through a variety of mechanisms. This scenario seems more likely for minocycline, a known anti-inflammatory agent, while much less is known regarding the effects of MMP inhibition on cytokine and chemokine signaling in vivo. Nonetheless, the relative efficacies of minocycline and AG3340 in reducing the recruitment of immune cells to the lesion site lend further support to these mechanisms of MMP-inhibition.

Taken together, data from these experiments provide strong evidence that attenuating the neuroinflammatory response provides neuroprotection at a delayed time point after injury.
Evidence also suggests that the efficacy of gelatinase inhibition is linked to neuroinflammatory responses, including reactive astrogliosis and the recruitment of resident microglia, peripheral macrophages, or both cell types.

**MMP Activity in Brain Contributes to Injury in the Absence of Peripheral Leukocytes**

Data from the previous experiments, while encouraging, prompted additional questions as to the precise role of the peripheral immune system after H-I. Gelatinases degrade endothelial cell junction proteins (Reijerkerk et al. 2006; Rosenberg and Yang 2007) and activate proinflammatory cytokines (Ito et al. 1996; Schonbeck et al. 1998). Previous reports demonstrated a role for these proteases in neuroinflammation (Gidday et al. 2005; Svedin et al. 2007), blood brain barrier degradation (Asahi et al. 2001; Back et al. 1998) and cell death (Asahi et al. 2000; Lee et al. 2004) after ischemia. Resident microglia are among the first cells to become activated in response to ischemic injury, acting as phagocytes to remove cellular debris from the brain parenchyma (Lai and Todd 2006) and releasing various cytokines (Fukui et al. 2006; Kim 1996). Activated astrocytes, characterized by increased expression of GFAP, also migrate to the injured site. Though the specific functions of reactive astrocytes are not clear, both astrocytes and microglia are capable of exacerbating the inflammatory response through the secretion of proinflammatory cytokines and chemokines (Chew et al. 2006). As such, these cells likely contribute to neonatal H-I injury. Therefore, while AG3340 and minocycline showed efficacy in reducing immune cell recruitment and neurodegeneration, the precise mechanisms by which these compounds achieve these effects are difficult to elucidate using *in vivo* models.

Recent evidence suggests that MMP-9 expression colocalizes with both activated resident microglia (Svedin et al. 2007) and infiltrating leukocytes (Gidday et al. 2005; Romanic et al. 1998) in rats subjected to cerebral ischemia. Each of these cell types elicits proinflammatory responses. Thus, in vitro models are necessary to determine the relative effects of the endogenous and exogenous immune cells on neuronal cell death after H-I.

The objectives of the final series of experiments were twofold: to determine whether minocycline or AG3340 confer neuroprotection in an *ex vivo* model of H-I that lacks peripheral...
immune cell involvement, and to determine the association of gelatinase activity with the cellular sources of MMP-9 in the ischemic brain. Results showed a shift in cellular MMP-9 expression that was concomitant with upregulated gelatinase activity in organotypic hippocampal slices after 48 hrs exposure to OGD. Furthermore, these measures were associated with increased neurodegeneration, as measured by Fluoro-Jade staining. Gelatinase activity was significantly attenuated in slices that were exposed to OGD and incubated with media containing either AG3340 or minocycline, and these compounds significantly reduced neurodegeneration.

*In situ* zymography in normoxic slices revealed that basal gelatinase activity is present in this culture system. Gelatin-cleaved fluorescence was predominantly limited to cells with neuronal morphology, consistent with the neuronal expression profile of MMP-9 in normoxic slices. Gelatinase activation in normoxic slices indicates a tissue response to injury from the slice preparation since basal gelatinase activity is low in the intact brain. Fluoro-Jade staining was also present in slices exposed to normoxia, supporting this contention. Gelatin-cleaved fluorescence was significantly increased in slices exposed to OGD. Activity appeared in cells that appeared to be of microglial and neuronal lineage, though cell types were not confirmed using immunohistochemical methods. Specifically, these included amoeboid cells typical of activated microglia and triangular cell bodies that may have been neurons or astrocytes. Cellular processes that appeared to be astrocytic also showed activity. In normoxic slices, localization appeared to be almost exclusively neuronal, in agreement with previous studies that localized gelatinase activity predominantly to neurons (Amantea et al. 2008; Lee et al. 2004; Lu et al. 2008).

While gelatinases include both MMP-2 and MMP-9, the latter is most associated with neural injury (Wetzel 2005) and has previously been shown to contribute to injury of hippocampal neurons in response to ischemia (Lee et al. 2004). In general, MMP-9 immunoreactivity was ubiquitous and localized to plasma membranes in organotypic slices. Interestingly, little colocalization was observed with the microglia cell surface antigen cd11b in normoxic slices. However, some GFAP – labeled astrocytes did express MMP-9. The vast majority of MMP-9 – positive cell bodies, however, appeared to be neuronal. This was determined based on the size and morphology of cell bodies, coupled with the general lack of colocalization with GFAP or...
cd11b. After OGD, a shift in expression was observed such that MMP-9 immunoreactivity colocalized with many cd11b – positive cells, though the majority of MMP-9 expression remained neuronal. MMP-9/cd11b - expressing cells were consistent with activated microglia such that the cell bodies were smaller and displayed an amoeboid morphology. MMP-9 immunoreactivity also localized to some astrocytic processes, consistent with previously studies that have reported gelatinase expression in activated astrocytes in vitro (Apodaca et al. 1990; Muir et al. 2002; Rosenberg et al. 2001). Additionally, the increased MMP-9 expression profile after OGD suggests that the insult expression of MMP-9. Some evidence suggests that microglia are the primary immune cells that respond to ischemic injury after MCAO in the neonatal rat brain (Denker et al. 2007), supporting the notion that microglia may initiate deleterious signaling cascades that are dependent upon MMP proteolysis.

To our knowledge, this is the first study utilizing organotypic slice culture methodology to investigate the effects of ischemic insult in brain, and the first to link cell viability with MMP activity in the absence of peripheral immune cell effects. Taken together, these data indicate that MMP-9 expression is linked to the resident microglial response within the first 48 hrs after OGD, and selective targeting of gelatin-degrading MMPs, most importantly MMP-9, may be an avenue for therapeutic intervention to combat H-I neuropathies.

**Implications and Future Directions**

Lectican deposition is upregulated in various injury models (Beggah et al. 2005; Mayer et al. 2005; Vorisek et al. 2002). These proteoglycans are key components of the glial scar (Silver and Miller 2004). The glial scar, a dense aggregation of proteoglycans, astrocytes and microglia, restricts plasticity through the inhibitory properties of lecticans and contributes to neuroinflammation through immune cell activity. The present study first investigated the specific pathology that is associated with neonatal H-I insult, and sought to determine whether lectican deposition was associated with the various elements of injury and inflammation.

In the P7 rat, exposure to H-I resulted in a neural sequelae characterized by the activation of reactive astrocytes, activation of cd11b – positive microglia/macrophages and
upregulated expression of MMPs. Neuroinflammation occurred within 24 hrs after the insult, the earliest endpoint examined in these experiments. The injury resulting from H-I was also progressive, as cavitary infarctions were seldom observed 4 days after H-I (P11) but were prevalent at later endpoints. This is an important feature of this model that is seldom mentioned in the literature. Consistent with this lesion profile, lectican deposition was elevated at later endpoints, indicating that these molecules aid in the formation and maintenance of glial scarring after neonatal H-I, consistent with their role in other injury models.

Another feature of neonatal H-I injury is damage to developing white matter. Deep cerebral white matter injury has been attributed to the inherent susceptibility of pre-OLs. Because lectican expression is developmentally regulated primarily by OLs, we hypothesized that cleavage of lecricans by ADAMTSs results in the loss of structural stability or cell signaling, thus rendering pre-OLs vulnerable to ischemic injury. Although the reduced expression of versican coincided with reductions in O4, the putative pre-OL marker, we were unable to detect changes in the cleaved fragment of versican. Similarly, no changes in brevican or the ADAMTS-derived cleavage fragment of brevican were observed in white matter. These data were also supported by the in vitro experiments which showed that pre-OLs did not preferentially grow on proteoglycan substrate. Upon considering these results, there are two possible explanations for the reductions in lectican expression at P11. The first is that the reduced expression was a consequence of cell death. This seems likely upon considering the role of lecricans after injury and the increased expression in glial scar at later endpoints. Another explanation, however, is that lecticans are cleaved by some other endogenous enzyme. As lecticans are the major substrates for ADAMTSs in brain and constitute the majority of ECM, the other likely proteases responsible for lectican cleavage are the MMPs. Indeed, MMP-9 expression was upregulated in the cerebral cortex and several white matter regions 24 hrs after insult. However, we did not detect alterations in cleavage fragments at the predicted molecular weights of those that would be produced from MMP proteolysis. Therefore, this scenario also seems unlikely.

Despite this fact, MMP activity has been implicated in several pathologies and is known to activate proinflammatory cytokines and contribute to blood brain barrier degradation via the
cleavage of basement membrane components. Additionally, previous studies with rodents have shown that inhibition of MMPs can improve the neuropathological and behavioral outcomes after ischemia. Importantly, most treatments have been administered prior to or immediately after insult. Although the initial injury occurs in large part from glutamate toxicity and oxidative stress, neuroinflammatory processes contribute to delayed cell death. Due to the fact that neural injury is progressive and early diagnosis is difficult, therapies that target the delayed immune response may prove clinically relevant in treating H-I – induced neuropathies. The involvement of neuroinflammatory processes and MMP activity in ischemic pathology, coupled with the growing need for alternative therapies, led to the second series of experiments that tested whether administration of an anti-inflammatory or MMP-inhibitory compound at a delayed time point could neuroprotect.

AG3340, a small molecule hydroxamate-based MMP inhibitor, exhibits high selectivity for gelatinases and demonstrates good oral bioavailability in rat (Santos et al. 1997). A previous study showed that this compound attenuated white matter injury in the adult rat brain when administered just prior to chronic cerebral hypoperfusion (Nakaji et al. 2006). Minocycline has also been shown to possess MMP-inhibitory properties (Machado et al. 2006) but acts primarily as an anti-inflammatory agent. This compound has also demonstrated neuroprotective effects after H-I in the rat neonate, including reductions in white matter injury and neurodegeneration (Cai et al. 2006), and improved behavioral outcomes (Fan et al. 2006). In these experiments, efficacy was achieved when the drug was administered 12 hrs prior to the insult and again immediately following the insult. Consistent with the protective effects of these compounds shown previously, we showed that both minocycline and AG3340 conferred neuroprotection when administered 24 hrs after H-I. Both compounds were effective in reducing neurodegeneration, astrogliosis and immune cell recruitment. Of interest, cd11b – positive microglia/macrophages were greatly reduced compared to vehicle alone, indicating that the recruitment of immune cells to the lesion site had been disrupted.

In the organotypic slice, both AG3340 and minocycline were efficacious in attenuating OGD – induced neurodegeneration. Furthermore, increased gelatinase activity correlated with
increased neurodegeneration, and both compounds were effective at reducing gelatinase activity as well. Data from these experiments also revealed an altered expression profile for MMP-9. Slices exposed to OGD upregulated MMP-9 expression in microglia, as identified with the microglial marker cd11b, and this colocalization was not observed in slices exposed to normoxia. Taken together, these data demonstrate a direct link between gelatinase activity and the inflammatory response to H-I. While the effects of AG3340 indicate that the selective inhibition of active MMP-9 and/or MMP-2 is sufficient to significantly reduce neurodegeneration in the absence of the peripheral immune system, the fact that minocycline was equally efficacious supports the notion that the resident immune cell response may be intimately connected with MMP expression or activation.

It is important to note that both minocycline and AG3340 showed efficacy in the ex vivo model at concentrations equivalent to the doses administered in the in vivo experiments. In vivo, administration of these compounds ameliorated the recruitment of cd11b - positive microglia or macrophages, reduced astrogliosis and decreased neurodegeneration 7 days after H-I, beginning 24 hrs after the insult. This is a critical distinction from previous reports that began treatment prior to or immediately following the insult, thus making the present data attractive in terms of potential clinical relevance. Collectively, the data from the in vivo and ex vivo models provides several insights into the ways in which neuroinflammation and MMP activity contribute to neurodegeneration after H-I. The in vivo experiments consisted of a 6 day treatment period that primarily targeted the delayed immune response. Additionally, it could not be determined whether cd11b – positive cells were of CNS origin, as there is currently no commercially available antibody that differentiates between resident microglia and peripheral macrophages.

The present study exploited a model that lacks peripheral immune cell responses and demonstrated that heightened MMP activity is associated with neuronal cell death during the early neuroinflammatory response to H-I. This model is also advantageous in that it is more conducive to mechanistic experimentation and more closely resembles the microenvironment in brain when compared to primary cell culture or mixed-glial culture. The fact that these experiments were performed on intact slices from neonatal rat brain, using drug concentrations
equivalent to *in vivo* doses from the previous experiments, further strengthens comparisons between the outcomes. An important difference with the slice culture experiments, however, is that these compounds were added to the media immediately prior to OGD exposure, which occurred for 48 hrs. Therefore, this model mainly assessed the early response to ischemic injury, whereas the *in vivo* study targeted the delayed immune response.

Previous studies have demonstrated that gelatinase upregulation is associated with blood brain barrier degradation (Cunningham et al. 2005; Manicone and McGuire 2008; Rosenberg and Yang 2007). Both peripheral and resident immune cells may contribute to cell death through inflammatory signaling, yet the relative contributions of infiltrating leukocytes and resident microglia have not been investigated in detail. Results from the present study show that resident microglia upregulate MMP-9 after OGD in intact brain slices, and that increased gelatinase activity and neurodegeneration are attenuated after treatment with AG3340. These data clearly demonstrate that gelatinase activity is likely connected with the early neuroinflammatory response in brain after H-I.

The present study showed that MMP inhibition is protective in the absence of peripheral leukocytes. This suggests that these proteases may enhance the inflammatory response through mechanisms other than blood brain barrier degradation. One candidate mechanism is “sheddase” activity, in which MMPs proteolytically cleave and activate cell surface cytokines, chemokines and apoptotic death receptors. Thus, gelatinase activity may facilitate cytokine release, alter chemokine gradients and/or initiate apoptosis through the extrinsic pathway. Several cytokines and chemokines have been identified as substrates for gelatin-degrading MMPs. Previous studies have shown that gelatinases process and activate TNF-α (Gearing et al. 1994) and IL1-β (Schonbeck et al. 1998), both of which are elevated after ischemia and are thought to contribute to cell death. The perinatal brain also expresses high levels of apoptotic enzymes, including caspase-3 (Hu et al. 2000), APAF-1 (Ota et al. 2002), Bax (Vekrellis et al. 1997) and Bcl2 (Merry et al. 1994). In fact, some data suggests that apoptosis is the primary mechanism of delayed cell death in the rat neonate after ischemia (Northington et al. 2001). TNF-α triggers apoptosis extrinsically through the activation of TNFR1. Thus, cleavage of TNF-α by gelatinases may be an
important event leading to cell death. Similarly, cleavage of FasL from cell surfaces could
increase apoptotic activity depending upon the specific microenvironment (Wetzel 2005). There is
data indicating that MMP-3 (Powell et al. 1999) and MMP-7 (Ethell et al. 2002) process FasL into
an activated soluble form, yet the overall effects on apoptotic activity depend upon the cell type.
To date, there is a paucity of data as to whether gelatinase cleavage of death receptors or death
receptor ligands occurs *in vivo* after H-I.

MMPs may also exacerbate the inflammatory response through proteolytic cleavage of
SDF-1α and various MCP chemokines, including MCP-1 (Overall et al. 2002). Another group
showed that SDF-1α stimulated gelatinase expression and MMP-9 activity, specifically, in a HeLa
cell line. These effects were associated with increased ectodomain shedding of syndecan 1 and
syndecan 4 that was not dependent on the SDF-1α receptor CXCR4. Additionally, RNAi silencing
of MMP-9 resulted in reduced cleavage of syndecan 1 and syndecan 4, demonstrating a major
role for MMP-9 in SDF-1α – mediated syndecan shedding. Expression of syndecans is high in
cerebral vasculature, and these proteoglycans modulate transendothelial migration of monocytes
across the brain endothelium (Floris et al. 2003). In the context of the present study, this
mechanism would be consistent with the *in vivo* experiments where minocycline and AG3340
ameliorated microglia/macrophage migration to the injured cortical lesion site. Future studies
aimed at defining the early time course for efficacy *in vivo*, as well as the specific pro-
inflammatory molecules and immune cell types involved in this response, are critical not only to
elucidate the specific sequence of neuroinflammatory events that contribute to H-I injury, but also
to develop effective therapeutics that can be administered at clinically relevant time points.
Figure 1

Cerebrosis in Neonatal Hypoxia-Ishchemia
ECM lecticans and gelatin-degrading MMPs contribute to neonatal H-I injury through multiple mechanisms. Necrotic cell death after H-I leads to blood brain barrier degradation, reactive astrogliosis and activation of resident microglia. Lecticans are deposited into the ECM in response to injury and contribute to glial scar formation, thus forming a barrier between injured and viable tissue that restricts cell migration into the lesion site. Immune cells of the brain increase expression and secretion of proinflammatory cytokines and chemokines. Gelatinase activity initiates a second, delayed opening of the blood brain barrier through proteolytic processing of basement membrane constituents. Peripheral macrophages infiltrate into the brain and further promote the immune response. Gelatinases may also activate cytokines and chemokines through sheddase activity. Ultimately, this feed-forward neuroinflammatory response potentiates apoptotic signaling and creates unfavorable conditions for neuroplasticity and repair.
Inhibition of gelatin-degrading MMPs reduces the inflammatory response and improves the neuropathological outcome after H-I in the rat neonate. Gelatinase inhibition is neuroprotective after H-I both in vivo and ex vivo. Treatment with AG3340 or minocycline significantly reduced neurodegeneration when administered 24 hrs after H-I. Additionally, these compounds ameliorated immune cell recruitment to the cortical lesion site. Organotypic slices exposed to 48 hrs of OGD in media containing these compounds also showed reduced neurodegeneration, effects that were associated with reduced gelatinase activity. Neuroprotection may occurs through several mechanisms. Gelatinase inhibition likely preserves the integrity of the blood brain barrier, restricting entry of peripheral macrophages into the brain, and may dampen microglial activation or migration to injured sites. Additionally, inhibiting gelatin-degrading MMPs could alter cytokine and chemokine signaling, thus attenuating neuroinflammation and apoptosis.
Supplemental Figure 1

Hippocampal Dentate Gyrus

Cont   Ips

A   B
C   D
Supplemental Figure 1 Legend

Activated microglia/macrophages recruit to hippocampal lesion site 24 hrs after H-I. Rats were exposed to H-I at P7 and sacrificed 24 hrs after insult. Micrographs show intense ED-1 immunoreactivity in the ipsilateral hippocampal dentate gyrus (B,D) compared to the contralateral control regions (A,C). CD68 expressing cells exhibit an amoeboid morphology consistent with activated microglia and macrophages.
Supplemental Figure 2

Corpus Striatum

Corpus Callosum

External Capsule

Cerebral Cortex
Supplemental Figure 2 Legend

**MMP-9 expression increases 24 hrs after H-I.** Rats were exposed to H-I at P7 and sacrificed 24 hrs after insult. Micrographs show increased MMP-9 immunoreactivity in the ipsilateral cerebral cortex (B), external capsule (D), corpus callosum (F) and corpus striatum (H) relative to respective contralateral control regions (A,C,E,G).
References:


About the Author

Christopher C. Leonardo received a Bachelor’s Degree in Psychology from Syracuse University in 2001. Having completed much coursework related to cognitive psychology and the physiology of behavior, his interest in studying brain function became the motivating force behind his future endeavors. Although he describes his undergraduate work as both interesting and rewarding, his desire to pursue experimental neuroscience steered him away from the social sciences and into the laboratory.

After completing a year of experimental research in a Neurology laboratory at the University of Rochester Medical Center, Christopher applied and was admitted to the University of South Florida College of Medicine to pursue a Ph.D. in Medical Sciences. He obtained a Master’s Degree in Medical Sciences upon successfully completing his Ph.D. Qualifying Examination, which consisted of designing and defending a series of proposed experiments that eventually led to the completion of his Doctoral dissertation. Christopher worked under the tutelage of several mentors, pursuing several different projects, throughout the course of his graduate experience. He describes his graduate work as a most rewarding experience, and the adversities he was forced to overcome as good learning experiences that have prepared him well for the obstacles he will encounter as an independent investigator in research academia. Christopher credits his success not only to extraordinary dedication and resolve, but also to the strong support network of family and friends whom encouraged him throughout the duration of his graduate studies.