2016

The Role of Matrix Metalloproteinase 9 and Osteopontin in Synaptogenesis and Reinnervation of the Olfactory Bulb Following Brain Injury

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The Role of Matrix Metalloproteinase 9 and Osteopontin in Synaptogenesis and Reinnervation of the Olfactory Bulb Following Brain Injury

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

by

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Acknowledgement

I would first like to thank my advisor, Dr. Linda L. Phillips, for being a phenomenal mentor. I appreciate her confidence in my potential, as well as her tremendous patience as I learned and developed both personally and professionally. Her support, wealth of knowledge, and advice have been outstanding, and throughout the years, have shown me the traits of a great mentor who is genuinely vested in her students’ success. I would also like to thank my Ph.D. advisory committee members for being excellent sources of expertise to refine and improve my project. In particular, I thank Dr. Thomas M. Reeves for offering his assistance with statistics and graphic development and Dr. Patricia A. Trimmer for providing an opportunity for me to improve and learn new techniques in her laboratory. Dr. Dong Sun, Dr. Richard M. Costanzo, and Dr. Joseph Porter were instrumental in moving my project forward, and I value each of their respective contributions. In addition to the Department of Anatomy and Neurobiology as a whole, I am extremely grateful for the opportunities afforded me and the support for my career endeavors from the Neuroscience Program Director, Dr. John Bigbee, and the Chairman of our department, Dr. John T. Povlishock.

I also owe the members of my laboratory, both past and present, my gratitude for training me, assisting in experiments, and being overall great sources of camaraderie during the past 4 years. Raiford T. Black, Nancy N. Lee, Terry Smith, and Nicholas Russell have made my experience in the Ph.D. program memorable and I sincerely cherish their kindness, advice, and humor, which made the rough days much easier. My friends and family, near and far, including my sorority sisters, have been major sources of encouragement, and I appreciate each and every one of them. I am truly grateful for my Stepdad for always lending an ear, a helping hand, and advice when I needed it most, and my Dad for always believing in me. Finally, I would like to recognize my fiancé and son for being such critical parts of my amazing support system, keeping me happy, levelheaded, and motivated to conclude this incredible journey.

I dedicate these collective efforts to my Mom who has always been there for me, despite the circumstance, from the very beginning, and knows exactly what to say to inspire me to press on.
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List of Abbreviations

6-OHDA ................................................................. 6-hydroxydopamine
AD .............................................................................. Alzheimer’s disease
ADAM ........................................................................... a disintegrin and metalloproteinase
ALS .............................................................................. amyotrophic lateral sclerosis
AMPA .......................................................... α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
ANOVA ........................................................................ analysis of variance
APP ........................................................................... amyloid precursor protein
BBB ........................................................................... blood-brain barrier
BDNF ........................................................................... bone derived neurotrophic factor
cAMP ........................................................................... cyclic adenosine monophosphate
CCI ........................................................................... controlled cortical impact
CNS ........................................................................... central nervous system
CSF ........................................................................... cerebrospinal fluid
CSPG .......................................................................... chondroitin sulfate proteoglycan
CT ........................................................................... computerized tomography
CTE .......................................................................... chronic traumatic encephalopathy
DAI ........................................................................... diffuse axonal injury
DDTC ................................................................. sodium dimethyldithiocarbamate
EAA ........................................................................... excitatory amino acids
EAE.................................................................experimental autoimmune encephalomyelitis
ECM..............................................................extracellular matrix
EPL ...............................................................external plexiform layer
FGF ...............................................................fibroblast growth factor
FL .................................................................full length
FPI ...............................................................fluid percussion injury
GABA ............................................................gamma-aminobutyric acid
GAP-43 ...........................................................growth associated protein-43
GCL ...............................................................granule cell layer
GCS ..............................................................Glasgow Coma Scale
GFAP ............................................................glial fibrillary acidic protein
GL .................................................................glomerular layer
GPCR ..........................................................G protein-coupled receptors
HA ...............................................................hyaluronic acid
HRP ............................................................horseradish peroxidase
HSPG ...........................................................heparin sulfate proteoglycans
IBA1 ..............................................................ionized calcium-binding adapter molecule 1
IFN ..............................................................interferon
IGF ..............................................................insulin-like growth factor
IHC ...............................................................immunohistochemistry
IL ...............................................................interleukin
KO ..............................................................knockout
LCN2 ...........................................................Lipocalin-2
loss of consciousness
lateral olfactory tract
lipopolysaccharide
long-term potentiation
microtubule-associated protein
middle cerebral artery occlusion
mitral cell layer
monocyte chemoattractant protein-1
matrix metalloproteinase
1-methyl-4-phenylpyridinium
1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
magnetic resonance imaging
multiple sclerosis
membrane-type matrix metalloproteinase
negative evoked potentials
nuclear factor kappa-light-chain-enhancer of activated B cells
neutrophil gelatinase-associated lipocalin
N-methyl-D-aspartate
neurotransmitter
olfactory bulb
olfactory epithelium
olfactory ensheathing cell
olfactory marker protein
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>ON</td>
<td>olfactory nerve</td>
</tr>
<tr>
<td>ONL</td>
<td>olfactory nerve layer</td>
</tr>
<tr>
<td>OPN</td>
<td>osteopontin</td>
</tr>
<tr>
<td>ORN</td>
<td>olfactory receptor neuron</td>
</tr>
<tr>
<td>OTT</td>
<td>olfactory tract transection</td>
</tr>
<tr>
<td>PD</td>
<td>Parkinson’s disease</td>
</tr>
<tr>
<td>PTA</td>
<td>post-traumatic amnesia</td>
</tr>
<tr>
<td>PTSD</td>
<td>post-traumatic stress disorder</td>
</tr>
<tr>
<td>PTZ</td>
<td>Pentylenetetrazol</td>
</tr>
<tr>
<td>RMS</td>
<td>rostral migratory stream</td>
</tr>
<tr>
<td>ROD</td>
<td>relative optical density</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>SCI</td>
<td>spinal cord injury</td>
</tr>
<tr>
<td>SGZ</td>
<td>subgranular zone</td>
</tr>
<tr>
<td>SIBLING</td>
<td>small integrin-binding ligand, N-linked glycoprotein</td>
</tr>
<tr>
<td>SVZ</td>
<td>subventricular zone</td>
</tr>
<tr>
<td>TBI</td>
<td>traumatic brain injury</td>
</tr>
<tr>
<td>TEM</td>
<td>transmission electron microscopy</td>
</tr>
<tr>
<td>TGF</td>
<td>transforming growth factor</td>
</tr>
<tr>
<td>TIMP</td>
<td>tissue inhibitor of metalloproteinase</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>TUNEL</td>
<td>terminal deoxynucleotidyl transferase dUTP nick end labeling</td>
</tr>
<tr>
<td>UPSIT</td>
<td>University of Pennsylvania Smell Identification Test</td>
</tr>
</tbody>
</table>
WB .................................................................Western blot
WT .................................................................wild type
Abstract

THE ROLE OF MATRIX METALLOPROTEINASE 9 AND OSTEOPONTIN IN SYNAPTOGENESIS AND REINNERVATION OF THE OLFATORY BULB FOLLOWING BRAIN INJURY

By Melissa Ashley Powell, B.S.

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

Virginia Commonwealth University, 2016

Major Director: Linda L. Phillips, Ph.D.
Professor, Department of Anatomy and Neurobiology

Traumatic brain injury (TBI) is a serious health concern, causing cognitive, motor, and sensory deficits, including olfactory dysfunction. This dissertation explores the effects of TBI on synaptic plasticity within the olfactory system, seeking to define mechanisms guiding postinjury sensory reinnervation. Physical forces induced by TBI can axotomize olfactory receptor neurons (ORNs), which innervate olfactory bulb (OB). These axons regenerate OB projections after injury, a process involving growth through a complex extracellular matrix (ECM). As such, we investigated a potential molecular mechanism capable of modifying local OB ECM to support postinjury synaptogenesis. Since matrix metalloproteinases (MMPs) and their ECM substrates are recognized for TBI therapeutic potential, we explored the role of MMP9 and its substrate osteopontin (OPN) in promoting ORN reinnervation of the OB after mild fluid percussion injury (FPI). First, we confirmed that FPI deafferented the mouse OB. In Chapter 2, we showed concurrent activation of neuroglia, elevated spectrin proteolysis and reduction in ORN-specific
olfactory marker protein (OMP). As OMP normalized during regeneration, growth associated protein-43kD (GAP-43) peaked, marking OB entry of ORN growth cones. Ultrastructural analysis revealed ongoing ORN axon shrinkage and degeneration, glial phagocytosis of cellular debris, and a reorganization of synaptic structure. To explore ECM role in mediating postinjury OB reinnervation, we defined the time course of MMP9 activity and several downstream targets. Chapter 3 reports biphasic MMP9 activity increase during acute/subacute degeneration, accompanied by robust generation of 48kD OPN cell signaling peptide. OPN receptor CD44 also increased during the acute/subacute interval, suggesting potential interaction of the two proteins. Finally, we utilized MMP9 knockout (MMP9KO) mice to confirm MMP9 role in OB synaptogenesis. In Chapter 4, MMP9KO reversed FPI-induced lysis of 49kD OPN and altered postinjury expression of ORN axon degeneration marker OMP. Additional ultrastructural analysis verified delayed recovery of OB synaptic features within the injured MMP9KO. Overall, we demonstrated that mild FPI elicits ORN axotomy to induce OB reactive synaptogenesis, and that MMP9 supports reinnervation by processing OPN for activation of local glia, cells which reorganize the ECM for synapse regeneration.
CHAPTER 1

INTRODUCTION
TRAUMATIC BRAIN INJURY

Impact, Epidemiology, and Classification

Traumatic brain injury (TBI), one of the leading causes of death and disability in children and adults (Faul et al., 2010), remains a serious public health concern throughout the world. An average of 200 of every 100,000 people acquire a TBI (Bryan-Hancock and Harrison, 2010), amounting to over 1.7 million people each year in the United States alone (Faul et al., 2010). Falls are the most common cause of TBI, followed by motor vehicle accidents, and other blunt force traumas such as those involving sports or military combat (Langlois et al., 2006). Although people of any age, gender, or ethnic background may acquire a head injury, some populations, such as young children 0-4 years of age, adolescents, and older seniors over 75, are much more susceptible due to the high probability of toddlers and the elderly falling, and young adults exhibiting reckless behavior. The complexities of head trauma can produce both acute and chronic effects, with some injuries causing minimal or no loss of consciousness, while others require long-term rehabilitation (Hyder et al., 2007; Asikainen et al., 1998; Faul et al., 2016; Weightman et al., 2010). Roughly 52,000 patients with TBI in the United States die each year, while over 275,000 are hospitalized, and 1.3 million require emergency room visits (Rutland-Brown et al., 2006). In addition to the costs of hospitalizations, extensive psychological and physical therapy coupled with loss of productivity results in annual direct costs over $60 billion dollars (Langlois et al., 2006; Finkelstein et al., 2006; Rutland-Brown et al., 2006). Estimates for indirect social and economic costs such as reduced quality of life are much higher, surpassing $220 billion (Coronado et al., 2012). The emotional, physical, and financial burdens of TBI demonstrate the need for effective intervention.
TBI is defined as any mechanical force resulting in temporary or permanent, focal or diffuse, brain damage after trauma (Silver et al., 2005), including blunt force trauma, penetrating injuries, and coup-contre-coup (rotational) forces. Historically, TBI has been classified as mild, moderate, or severe, based on a clinical guide, the Glasgow Coma Scale (GCS). Clinicians assess patient brain function using the three part scale, divided into functions of eye opening, verbalization, and motor responses to stimuli. Summation of these scores dictates TBI classification: mild between 13-15, moderate 9-12, and severe 8 and below (Teasdale and Jennett, 1974; Teasdale and Jennett, 1976). In addition to the GCS, post-traumatic amnesia (PTA) and loss of consciousness (LOC) can also be used as measures of injury severity. If PTA persists for less than a day, the injury is considered mild, while 1 to 7 days is a moderate level injury, and more than 7 days of amnesia represents a severe TBI. LOC can also represent injury severity, as patients with mild TBIs exhibit 0-30 minutes of LOC, those with moderate level injuries experience 30 minutes to 24 hours of LOC, and the most severe cases lose consciousness for more than a day. Despite these classifications, which are utilized to improve approach to treating TBI patients, head trauma remains a devastating injury.

In addition to the 1.7 million reported cases, a much larger population is also afflicted by mild TBIs, such as a concussions, yet often these are not reported, leaving an unknown number of patients with progressive TBI consequences (Goldstein, 1990; Coburn, 1992), which can involve memory loss, changes in cognition, movement disorders, and sensory deficits. Although severe TBIs often result in death and moderate head trauma produces overt, devastating effects, mild TBI has recently gained increased public recognition for the chronic, delayed problematic functional deficits, especially in specific populations. Recent studies have focused on the impact
of TBIs on military combat troops and sports players (Pellman et al., 2006; Phillips and Woessner, 2015), implicating TBI in prolonged post-traumatic stress disorder (PTSD) (Benedictus et al., 2010; Greenwald et al., 2012), the development of chronic traumatic encephalopathy (CTE) (Goldstein et al., 2012; Saulle and Greenwald, 2012; McKee et al., 2009), and increased risk for neurological diseases later in life, such as epilepsy (Annegers et al., 1998; Ferguson et al., 2010; Frey, 2003), Parkinson’s (Bower et al., 2003; Goldman et al., 2006) and Alzheimer’s (Mortimer et al., 1985; Fleminger et al., 2003; Sivanandam and Thakur, 2012) diseases. Because soldiers can be exposed to multiple blast waves in one or more tours of duty and dedicated sports players often obtain more than one TBI attributed to prolonged game time, the additive effect of repetitive TBIs is also being explored as a contributing factor to the pathologies associated with injury (Goldstein et al., 2012; Saulle and Greenwald, 2012; McKee et al., 2009). Despite knowledge of the incidence of TBI and classifications that relay degree of injury, which do assist in helping clinicians with initial approaches for treatment, outcomes for TBI remain poor due to complexity and heterogeneity of injuries and the low sensitivity and specificity of the GCS. Fortunately, clinicians and research scientists are exploring the underlying mechanisms of TBI in order to more effectively address trauma-induced pathologies in a targeted manner.

Pathophysiology

Whether a TBI produces focal or diffuse damage, or a combination of the two, several physiological mechanisms are initiated and lead to the functional deficits that persist in patients. Initial primary injury is the result of a mechanical force applied to the brain in the form of a striking blow, penetrating object, or rapid acceleration and deceleration of the brain that can
involve skull fracture, contusion, and hemorrhage (Maas et al., 2007), directly affecting axons, causing brain damage (Werner and Engelhard, 2007). In mild TBIs, a prevailing mechanism of injury is rapid acceleration and deceleration, or coup contre-coup forces. This term represents the directional movement of the brain when the head strikes an immobile object. Momentum of the brain causes it to impact the skull (coup), the resulting force of which propels the brain to the side of the cranium opposite the initial blow (contre-coup; Allen, 1896; Goggi, 1941). Despite degree of TBI, forces inflicted upon injury can stretch, crush, and sever axons throughout the brain, which immediately compounds signal transduction. When these damaged neurons, which function as part of larger synaptic circuits, become dysfunctional, the consequences can be devastating. Additionally, the mechanical loading of primary injury impacts the entire neurovascular unit, comprised of neurons, blood vessels, and supportive glia, thus leading to disruption of the blood-brain barrier (BBB), overt vascular injuries, or hemorrhage (Davis, 2000), and increased intracranial pressure (Miller et al., 1977; Treggiari et al., 2007), all of which contribute to poorer outcomes.

Secondary injury or insult involves downstream effects of primary injury such as reactive oxygen species (ROS), axonal injury, neurotransmitter (NT) release, inflammation, with cytokine and chemokine production, and ionic dysregulation (Werner and Engelhard, 2007). These can collectively and progressively cause the significant chronic problems associated with TBI. Although mechanical force plays a role in facilitating TBI-induced brain damage, the subsequent abnormalities are often attributed to secondary injury molecular mechanisms. For example, one of the most common pathologies associated with TBI is diffuse axonal injury (DAI), which can result from direct force causing axon damage or in response to cellular ionic imbalances.
Secondary processes can cause more extensive physiological damage than primary insult by several mechanisms, which include but are not limited to persistent inflammatory responses maintained by neuroexcitation, reactive glia, axonal injury, and aberrant synaptic plasticity, all of which prevent functional recovery.

**Neuroexcitation**

Part of the complex secondary injury is pathological neuroexcitation, NT surge as a result of depolarization and nonspecific NT release (Hayes et al., 1992). Na⁺, K⁺, and Ca²⁺ are maintained in neurons at concentrations suitable for action potential generation and conduction, but the initial forces of TBI can alter that balance either by damaging the axolemma, the plasma membrane of axons, or by causing damage to oligodendrocytes, which hinders saltatory conduction (Mierzwa et al., 2015). Activation of Ca²⁺ channels can also lead to calcium-mediated effects such as cytoskeletal breakdown (Gennarelli and Graham, 1998) and the activation of some cell death initiating mechanisms with its role in mitochondrial dysfunction and ROS production (Starkov et al., 2004). In addition to ionic dysregulation, NT release can also be excitotoxic. For example, in severe TBIs, neurotransmitters are released into the brain parenchyma due to perforation of the axolemma. Considering NT release is typically tightly regulated, rapid, robust increases in excitatory or inhibitory NTs can cause serious imbalances in synaptic circuits, leading to dysfunction (Hayes et al., 1988; Lyeth et al., 1988; Faden et al., 1989; Miller et al., 1990). For example, release of excitatory NT glutamate can be excitotoxic (Rothman and Olney, 1986; Rothman and Olney, 1987; Bullock et al., 1998), as subsequent over activation of NMDA, AMPA, and kainite receptors can lead to increased intracellular calcium and cell death (Lee et al., 1999; Arundine and Tymianski, 2004). In milder injuries, mechanical
deformation can also depolarize cells, as extracellular $K^+$ is increased due to altered $K^+$ channels and membrane breakdown (Hayes et al., 1992). Release of excitatory amino acids (EAAs), glutamate, aspartate, and acetylcholine, all excitatory neurotransmitters, can lead to excessive, inappropriate excitatory input (Faden et al., 1989; Hayes et al., 1992; Lyeth et al., 1992) and altered neuronal function.

**Positive and Negative Inflammatory Response to TBI**

A physical and metabolic barrier between the periphery and the central nervous system (CNS), the BBB is an integrated network of endothelial cells and glial cells that maintains the necessary separation between brain and blood-borne components (Chodobski et al., 2011). Disruption of this homeostatic divide in the brain upon primary injury, when tight junctions of the BBB are dismantled, allows blood resident compounds and cells to cross the BBB (Morganti-Kossmann, 2001), prompting a swift and robust inflammatory response. Permeation of the BBB allows inflammatory cells like leukocytes, including neutrophils, lymphocytes, monocytes, and macrophages, to infiltrate the brain parenchyma (Chodobski et al., 2011) and migrate to sites of injury to protect against potential invading pathogens and produce pro-inflammatory cytokines and chemotactic cytokines, chemokines (Bellander at al., 2001; Fluitert et al., 2014; Lozano et al, 2015). Initial cytokine and chemokine production are critical for increasing BBB permeability and promoting a sustained inflammatory response (Chodobski et al., 2011). Common pro-inflammatory cytokines secreted and elevated in head injury paradigms are tumor necrosis factor α (TNFα), important for injury and repair mechanisms (Hofman et al., 1989; Shohami et al., 1994), interleukin-1 β (IL-1β), regulating the acute immune response (Rothwell and Luhesion, 2000), IL-6 (Breder et al., 1988; Frugier et al., 2010), IL-12 (Stahel et al., 1998), and interferon γ
Pro-inflammatory cytokines produced in acute phases of TBI have several benefits. Penkowa et al. (2000) demonstrated that reductions in IL-6 negatively impacted recovery, and eliminating TNFα did not improve long-term neurocognitive function (Scherbel et al., 1999; Sullivan et al., 1999). Some matrix molecules, such as osteopontin (OPN), can also act as a cytokine in certain CNS conditions (Shin, 2012), further enhancing pro-inflammatory cell cascades. Cell signaling mechanisms initiated with controlled, regulated cytokine release in turn activate transcription factors that eventually lead to more robust inflammatory responses, cell proliferation, differentiation, migration, apoptosis, and even phagocytosis of debris after TBI, all of which, in acute phases after injury, promote a more favorable environment for recovery processes to occur.

In addition to peripheral immune cells, which can invade the CNS in large numbers if BBB integrity is compromised, resident immune cells of the CNS, microglia and astrocytes, also produce cytokines, chemokines, and other components to facilitate proper injury repair mechanisms. Microglia are involved in both normal physiological processes such as embryonic development (Bilimoria and Stevens, 2015; Fiske and Brunjes, 2000) and normal synaptic stripping (Blinzinger and Kreutzberg, 1968; Kettenman et al., 2013), as well as pathological responses, as in a number of neurological diseases and conditions, including TBI. Acutely neuroprotective (Corps et al., 2015), microglia exhibit distinct phenotypic and marker profiles directly related to their role in response to TBI. These innate immune cells of the CNS can be classified, in the most general terms, as resting, surveillant, or ramified microglia, which possess small cell bodies, and abundant long, fine processes that frequently extend and retract to “survey” the environment, or activated, reactive microglia, which appear more amoeboid in
morphology, with large, round cell bodies, and shorter processes. Further, activated invading and resident macrophages, microglia, can exhibit two distinct marker profiles depending on their specific function in the inflammatory process, giving them a dual role in TBI pathology and recovery. For example, classically activated microglia (M1) are immediately responsive to injury stimuli like bacterial cell membrane component lipopolysaccharide (LPS) and TNFα, producing high levels of pro-inflammatory cytokines and oxidative metabolites, which are important for host defense (Ponomarev et al., 2007). Alternatively activated microglia (M2), which respond to anti-inflammatory cytokines such as IL-4 and IL-13 (Colton et al., 2006) and express markers associated with reducing the inflammatory response like Arginase-1, are thought to be involved in processes that promote recovery such as extracellular matrix (ECM) remodeling and tissue repair (Colton, 2009). Activation of both types of cells is key for appropriate inflammatory responses, however, it can become pathological and could play a role in long-term detrimental effects of inflammation (Holmin and Mathiesen, 1999; Bartfai et al., 2007; Ramlackhansingh et al., 2011). Excessive microglial activation in the CNS, which is damaging to healthy neurons (Block and Hong, 2005; Ponomarev et al., 2007), is linked to decreased neurogenesis (Lazarini et al., 2012), initiation of pro-apoptotic pathways (Block and Hong, 2007), development of auto-immune diseases such as multiple sclerosis (MS; Correale, 2014), and aberrant synaptic stripping (Ziebell et al., 2015), highlighting the duality of microglial functions in facilitating recovery processes.

Astrocytes, another glial cell important for synaptic function, also become reactive in TBI. This activation is characterized by elevated intermediate filaments vimentin and glial fibrillary acidic protein (GFAP), increased cell proliferation, and cell hypertrophy (Herrmann, 2008). These cells
typically produce and secrete fewer cytokines than microglia, but more growth factors and extracellular matrix components that contribute to postinjury recovery by supporting neuronal survival and protecting against cell death (Zhao et al., 2004). For example, brain derived neurotrophic factor (BDNF), critical to cell survival and axon growth, and chondroitin sulfate proteoglycans (CSPGs), which help form glial scars upon injury, protecting the site from further damage (Grumet et al., 1993; Harris et al., 2009; Kumar and Loane, 2012), are secreted by astrocytes. Another important role for astrocytes is the regulation of glutamate, reducing the contribution of excess glutamate to neurotoxicity after TBI (Schousboe and Waagepetersen, 2005; Kumar and Loane, 2012). Of note, when astrocyte function is altered or impaired after injury, neuron dysfunction is exacerbated (Myer et al., 2006). On the other hand, much like over activation of microglia, excessive astrocyte activation can lead to change in CSPG production, which can prevent and inhibit axon growth and regeneration (Cafferty et al., 2007).

Alternatively, in genetically modified mice with no vimentin or GFAP genes, axon growth and repair were improved after CNS injury (Menet et al., 2003; Wilhemsson et al., 2004), suggesting dichotomous roles for astrocytes after TBI as well.

Although the inflammatory response is a defense mechanism intended to improve the cellular environment such that further injury is prevented, prolonged secretion of pro-inflammatory cytokines from reactive glial cells caused by TBI does become neurotoxic, as excessive production of cytokines like IL-6 can increase neuron cell death (Almolda et al., 2014), and cytokine INFγ reduces number and length of dendrites, thus inhibiting synapse formation (Kim et al., 2002). For this reason, a number of studies have investigated anti-inflammatory drugs to explore their potential to reduce the negative effects associated with TBI and promote recovery.
While most primary injuries will occur despite preventive measures, some of the molecular processes of secondary insult, which exacerbate injury, may be preventable. For example, regulating the production of CSPGs and the formation of a glial scar such that new axons may be guided to appropriate targets without physical barriers may help restore some physiological function. Harris and colleagues (2010), for example, increased the number of GAP-43+ axon sprouts and slightly improved motor function after TBI using chondroitinase ABC, which breaks down inhibitory CSPGs. In experimental models of head injury, targeting robust pro-inflammatory cytokine TNFα with reduction resulted in enhanced BBB integrity, reduced edema, and improved neurological outcomes (Shohami et al., 1997). Additionally, IL-1β neutralization not only reduced production of pro-inflammatory cytokines but also decreased hippocampal damage after TBI, thus improving neurological behavior (Kumar and Loane, 2012). Anti-inflammatory drugs such as Minocycline, which suppresses production of pro-inflammatory cytokines, has been reasonably successful inhibiting microglial toxicity and improving neurological function in TBI studies (Bye et al., 2007; Chan et al., 2014; Kumar and Loane, 2012; Ng et al., 2012). Fortunately, there are also endogenous anti-inflammatory cytokines IL-4, IL-10 (Colton and Wilcock, 2010), and transforming growth factor β (TGFβ), that dampen the pro-inflammatory immune response and promote a more appropriate environment for recovery. For example, several studies have shown that IL-10 is neuroprotective in cases of head injury (Knoblach and Faden, 1998; Csuka et al., 1999; Kremlev and Palmer, 2005). Despite advances in understanding the inflammatory responses, preclinical success in the laboratory with immunosuppressants, steroids, statins (Weitz-Schmidtz, 2002), and treatments like hyperbaric chambers have largely failed in the clinic (Hellewell et al., 2015; Sanchez-Aguilar et al., 2013;
Loane and Faden, 2010), leaving the door open for investigators to continue exploration of other mechanisms to prevent neuropathology induced by TBI.

Similar to inflammatory cytokines/chemokines, ROS generated by infiltrating blood cells or reactive glia can have devastating effects in brain injury patients. ROS, molecules containing reactive oxygen atoms, are a normal physiological byproduct of cell metabolism, which include peroxides, superoxides, and singlet oxygen. Typically, these compounds are maintained in low concentrations because they are degraded by enzymes like peroxidase or neutralized with antioxidants. After TBI, antioxidant functions are often impaired (Wang et al., 2014), leading to an accumulation of ROS (Dasuri et al., 2013), which results in organelle and cellular dysfunction, and often, cell death (Lewen et al., 2000; Loh et al., 2006). Not only are impaired antioxidants problematic, but leukocytes such as neutrophils, which migrate into the brain after BBB breakdown, produce additional ROS as well as matrix metalloproteinases (MMPs), promoting BBB degradation and cytokine production to enhance inflammatory response (Scholz et al., 2007; Wang et al., 2014), leading to greater tissue destruction. Post-injury administration of antioxidants can, however, reduce damage and inflammation in models of brain injury if administered quickly (Wang et al., 2011; Choi et al., 2012), as in the cases of edaravone (Wang et al., 2011), apocynin (Choi et al., 2012), and glutathione (Roth et al., 2014).

Cell Death/Axonal Injury

In some cases of TBI, mechanical forces that induce the injury are sufficient to impair cytoskeletal networks and perforate the cell membrane, leading to influx of neurotoxic extracellular components, potentially causing cell death (Clark et al., 2000; Pike et al., 1998;
Programmed cell death, or apoptosis, occurs when a cell is no longer required for organ function, while necrosis is a cell death mechanism initiated in response to infection or injury. Apoptosis is characterized by DNA fragmentation, chromatin condensation, and cell shrinkage (Zhang et al., 2005), typically induced by two primary mechanisms. Extrinsic factors contributing to apoptosis involve cleavage and activation of several calcium activated neutral proteases (caspases), which are responsible for proteolysis of cytoskeletal proteins like αII-spectrin (Wang, 2000). Intrinsic mechanisms consist of the release of specific factors like cytochrome C from mitochondria, activating cell death signaling cascades (Zhang et al., 2005).

Cell death after TBI dates back to the 1940’s (Evans and Scheinker, 1944; Evans and Scheinker, 1945) with documented neuronal loss in cortex, hippocampus, cerebellum, and thalamus (Adams et al., 1985; Kotapka et al., 1993; Ross et al., 1993). Neurons, however, are not the only populations susceptible to cell death, as vulnerability to apoptosis in CNS cells after injury is present in most cell types but decreases from neurons to oligodendrocytes to astrocytes, with microglia least susceptible to apoptosis after injury (Conti et al., 1998). Interestingly, there have been differences in cell death mechanisms detected after TBI, largely dependent on injury severity. For example, mild TBI often produces apoptotic cells, which one might expect as a lower level of injury typically will not cause significant overt cell damage, while severe TBI promotes necrosis (Du et al., 1996; Charriaut-Marlaune et al., 1996), an expected outcome given the level of direct cell damage acquired in more robust injuries. Despite injury level, it has been shown that necrosis occurs first as a result of membrane disruption, metabolic disturbances, and excitotoxicity (Lenzlinger et al., 2001), while apoptosis is delayed (Liou et al., 2003). Interestingly, rat TBI studies utilizing caspase 3 inhibitors and transgenic mice overexpressing Bcl2, two manipulations developed to manipulate apoptosis, suggested 1/3 of postinjury cell
death was caspase-dependent apoptosis, 1/3 was characterized as caspase-independent apoptosis, and 1/3 could be attributed to necrosis (Zhang et al., 2005). When assessing extent of cell death, morphological analyses are often employed. Electron microscopy technology, which utilizes a high voltage electron beam to resolve intra and extracellular structures, can reveal characteristic apoptotic morphology of swollen axons with large mitochondria, vacuolated cytoplasm, and condensed chromatin (Dietrich et al., 1994; Raghupathi et al., 2000; Sutton et al., 1993). Immunohistochemical analyses, on the other hand, can facilitate the use of terminal deoxynucleotidyl transferase-mediated dUTP biotin nick end labeling (TUNEL) staining, which identifies DNA strand breaks in tissue (Gavrieli et al., 1992), an indication of cells undergoing programmed cell death. Injury-induced cell death is an important feature of TBI that promotes understanding of trauma-related neuropathology.

Another hallmark pathological feature of TBI is diffuse axonal injury (DAI). This term encompasses not only widespread damage to axons throughout the brain, but also perturbations distributed along the axon (Gennarelli et al., 1982, Adams et al., 1982). Previously, investigators and clinicians believed most axonal damage detected in TBI was caused by the initial mechanical forces which also induce cell death. It has since been established that this primary mechanism occurs primarily in severe injuries (Maxwell et al., 1993), and is likely not a major contributor of axon damage in moderate and mild TBIs. Instead, axon damage that occurs over hours or days has become accepted as the most accurate mechanism of TBI-induced axonal injury in milder head traumas (Povlishock and Christman, 1995). This delayed axotomy is a result of primary injury-induced perturbations of the axon related to local neurofilament change (Povlishock et al., 1983; Erb and Povlishock, 1988; Yaghmai and Povlishock, 1992). Altered neurofilament
organization extending from the axon initial segment, through nodes of Ranvier, down the length of the axon have been documented in several quantitative stereological studies with histological and transmission electron microscopy analysis, both in human and in experimental animals (Maxwell, 2015). When these critical cytoskeletal components become misaligned, anterograde transport is affected, causing accumulation of organelles in axonal swellings, which can later become retraction bulbs. These changes have been reported even in the absence of axolemma permeability (Gallant, 1992, Pettus et al., 1994), further highlighting its persistence even with mild TBIs, in which physical forces may not be sufficient to cause axolemmal damage. One defining characteristic of the axonal swellings that result is the presence of β-amyloid precursor protein (βAPP) (Sherriff et al., 1994), which in addition to organelles, accumulates within the axon (Shigematsu and McGeer, 1992) due to impaired transport, thus serving as a marker of injury-induced cytoskeletal change (Povlishock and Christman, 1995). Interestingly, injured axons can exist directly adjacent to uninjured axons (Adams et al., 1989; Mendelow and Teasdale, 1983; Saatman et al., 2009), further complicating our understanding of the pathobiology of TBI.

It is important to remember that DAI has several downstream effects. In mild TBI, as organelles and βAPP accumulate, tension of the growing axonal swelling can cause the axolemma to eventually pinch, leaving the retraction bulb attached to a proximal axon segment, while the distal axon segment stands alone and is then subject to Wallerian degradation and phagocytosis by reactive glia. In addition to intermediate-diameter neurofilaments, larger microtubules and smaller, actin-composed microfilaments of axons are also affected by TBI (Maxwell, 2015). The mechanical forces of a head injury can alter Na⁺ and Na⁺/Ca²⁺ exchanger channels (Fehlings and
Agrawal, 1995; Wolf et al., 2001; Agrawal and Fehlings, 1996), which ultimately disrupts ionic homeostasis and allows uncontrolled Ca\(^{2+}\) influx. Alpha II-spectrin is a protein that associates with the actin cytoskeletal components of microfilaments by binding to adhesion and adaptor molecules of the subaxolemma (Letourneau, 2009). Proteolysis of αII-spectrin is mediated by calcium-dependent proteases (caspases) or calpains, which become activated from the influx of Ca\(^{2+}\) upon axonal injury (Tuck and Cavalli, 2010), and studies show that after TBI, expression of αII-spectrin break down products is increased. The close association of αII-spectrin with the intact axon prompted investigators to explore αII-spectrin break down products in cerebrospinal fluid (CSF) of TBI patients (Ringger et al., 2004) as well as CSF and brains of experimental TBI animals (Pike et al., 2004) as a measure of axonal degradation and injury severity. In some models of TBI, αII-spectrin break down products were detected in injured tissues within cortical dendrites and subcortical white matter during acute phases of injury, up to 7 days (Saatman, et al., 1996; Reeves et al., 2010). To date, αII-spectrin has remained a reliable measure of axonal injury. Subsequent to DAI, as post-synaptic targets are deafferented, or lose signal input, the cell soma and proximal axon segment can undergo Wallerian degeneration, and eventually succumb to cell death (Maxwell, 2015). Considering the dense neuronal network of the brain, each cell is intimately integrated with others, and as a result, this axotomy and loss of contact between pre- and post-synaptic cells can strongly impair synaptic circuits, altering output. In addition to functional deficits as a result of axonal injury and cell death, cell fragments and debris produced by breakdown of axon terminals and cell bodies can intensify inflammatory processes, further complicating repair processes.
NEUROPLASTICITY AFTER TRAUMATIC BRAIN INJURY

Widespread axonal damage induced by primary and secondary injury processes of TBI, including DAI, leads to retraction of processes undergoing Wallerian degeneration and axotomy. Subsequent deafferentation results in synaptic disruption and functional deficits (Erb and Povlishock, 1991; Povlishock and Katz, 2005). While some brain regions such as the cortex, hippocampus, and thalamus (Adams et al., 1985; Kotapka et al., 1992; Ross et al., 1993), are more vulnerable than others after traumatic insult, the diffuse nature of most mild TBIs leaves the entire brain susceptible to damage. Early descriptions of the CNS rejected the possibility of successful regeneration or repair. Now, it is well understood that the CNS has a strong propensity for recovery, with evidence of axonal sprouting, niches of stem cells, and the formation of novel synapses upon the loss of previous connections. Synaptogenesis, the formation of new synapses between neurons, is a normal process that occurs during embryonic development, local synaptic plasticity accompanying long-term potentiation or depression, and in response to trauma. Interestingly, DAI, one of the main underlying pathologies of TBI, also drives structural synaptic plasticity (Grady et al., 1989; Levin, 1995; Povlishock, 1992), a process guided by a number of matrix proteins, glial cells, and secreted growth factors. Reactive synaptogenesis describes the specific process that occurs in response to loss of previously established synapses, which results from deafferentation caused by disease or trauma. Reorganization of these synapses occurs over a defined time course with described degenerative, regenerative, and stability phases (Figure 1).
Figure 1.1 Phases of Reactive Synaptogenesis
A-B. Deafferentation of normal synapses leads to an acute degenerative period, where damaged synaptic terminals \((d)\) are removed by reactive glia \((gl)\). C. Degeneration is followed by a regenerative phase in which new axons emerge \((open arrows)\). D. Nascent synapses then mature during the stabilization phase. (Steward et al., 1988)
Normal repair mechanisms are not always sufficient to regain function after TBI, evident in the persistence of functional deficits described earlier. Consequently, researchers continue to explore how such endogenous synaptic repair mechanisms might be improved, identifying viable targets for therapeutic intervention. Defined models of deafferentation and analysis of subsequent reactive synaptogenesis help clarify some of these recovery processes. Both the well-characterized synaptic organization and the vulnerability of the hippocampus to TBI have made it a fundamental brain region for studying TBI-induced synaptogenesis. The unilateral entorhinal cortex lesion has been a preferred model of deafferentation-induced synaptogenesis for several decades (Steward, 1989). In the model, the dentate gyrus of the hippocampus is deafferented of input from the entorhinal cortex, which stimulates axon sprouting and synaptic reorganization. Using this model, and other models of deafferentation, investigators have mapped the time course of a defined process of reactive synaptogenesis. During early phases of the recovery process (1-5 days after injury; Figure 1.1B), damaged axons degenerate and neuroglia facilitate the removal of cellular debris from the region. In the intermediate repair phase (6-15 days after injury; Figure 1.1C), nascent terminals are formed as axons begin sprouting and re-innervating deafferented regions (Lynch et al., 1976; Steward, 1976; Deller et al., 2007; Frotscher et al., 1997). When 15+ days have passed after injury, the process enters a period of stabilization (Figure 1.1D), where synapses mature and are both structurally and functionally strengthened (Steward and Vinsant, 1983). Although this time course is well established, and the sequence of events is relatively consistent across brain regions, the specific length of each phase can differ. For example, in the olfactory bulb (OB), the process is slower, occurring over a more extended period of 2 months (Graziadei et al., 1978; Graziadei et al.,
1979), rather than 2 weeks, revealing region-specificity and introducing the potential for exploring efficacy of therapies in brain regions with slightly different recovery periods.

Reactive synaptogenesis evokes several structural, molecular, and cellular changes in deafferented circuits. In experimental models assessing synaptic plasticity, a number of markers are often utilized to track the process of reinnervation. Microtubule-associated proteins (MAPs), for example, can detect morphological changes in synapses, as they are involved in axonal outgrowth (Gordon-Weeks and Fischer, 2000; Yamanouchi, 2005; Ma et al., 2000; Ramon-Cueto and Avila, 1999) and increase after axonal injury (Book et al., 1996). Pre-synaptic vesicle docking proteins, such as synaptophysin and synapsins, also change after insult, and can be measured to assess synaptic plasticity. Several studies have demonstrated reductions in Synapsin-I after ischemia (Kitagawa, 1992), deafferentation (Chan et al., 2014), and TBI in hippocampus and cortex (Ansari et al., 2008a, b), indicating loss of pre-synaptic terminals acutely after brain injury. During later recovery processes, Griesbach et al. (2004) showed increases in Synapsin-I, indicative of a reemergence of terminals. In addition to tracking the loss or appearance of physical components of the synapse, several other factors that contribute to regeneration and synaptogenesis can be assessed. Expression of growth factors like growth-associated protein-43 (GAP-43), which facilitates accumulation of F-actin in the neurite cytoskeleton (Aigner and Caroni, 1995; Dent and Meiri, 1998) and promotes axonal outgrowth, increases during plasticity. During critical periods of nervous system development, for example, GAP-43 expression is high (Shen et al., 2002; Latchney et al., 2014). Additionally, plastic brain regions with constant neurogenesis and integration of new synapses, such as the hippocampus and OB, also express high levels of GAP-43 (De la Monte et al., 1989), and increased expression
has been detected in the OB during regenerative periods (Verhaagen et al., 1993). Interestingly, change in GAP-43 expression occurs during reactive synaptogenesis as well (Benowitz et al., 1990). In multiple models of brain injury, GAP-43 expression is increased: during axon regeneration (Sachdeva et al., 2016, Williams et al., 2015; Wang et al., 2014), after SCI (Park et al., 2013), during regenerative axonal sprouting after stroke (Goto et al., 1994, Carmichael et al., 2005; Hulsebosch et al., 1998; Stroemer et al, 1993; Gorup et al., 2015), and in injured axons after TBI (Christman et al., 1997). Clearly, exploring altered post-injury expression of synaptic components can guide understanding of the processes underlying reactive synaptogenesis.

Inflammation and plasticity
TBI-induced production of pro-inflammatory mediators peaks during acute phases of injury, within the first 24-48 hours, while anti-inflammatory molecules are produced and secreted later to help facilitate post-injury recovery. Reactive neuroglia, such as microglia and astrocytes, respond to stimuli by clearing cellular debris and degenerating synaptic structures (Colton and Gilbert, 1987; Chao et al., 1992; Ramlackhansingh et al., 2011; Bechade et al., 2013; Bessis et al., 2007), producing and secreting growth factors, cytokines, and chemokines (Hanisch, 2002), and migrating through the brain parenchyma to affected brain regions (Carbonell et al., 2005; Imai et al., 1999). The functions of neuroglia in TBI, however, are not limited to injury response. Microglia also participate in maintaining the normal synaptic functions of the brain such as during cortical development, when both microglia and the rate of synaptogenesis are equally high (Steward and Falk, 1986), and Diniz et al. (2012) have shown that microglia produce factors like TGFβ to enhance synapse formation. When microglia function as first responders to sites of injury, after insult such as TBI, they serve beneficial roles, surveying
(Lafrenaye et al., 2015), engulfing and eliminating cell debris from the microenvironment and producing pro- and anti-inflammatory cytokines and growth factors, as discussed earlier. Further, in TBI specifically, the phagocytic properties of microglia make them critical in the removal of axonal debris and toxic substances to acquire homeostasis and promote tissue remodeling (Hanisch and Kettenmann, 2007). Pro-inflammatory cytokines secreted from microglia, such as TNFα, can also mediate synaptic scaling, or regulation of synaptic activity (Stellwagen and Malenka, 2006). While important components like bone derived neurotrophic factor (BDNF), which is involved in synaptic plasticity, are expressed by microglia after TBI (Nagamoto-Combs et al., 2007) and focal ischemia (Madinier et al., 2009), production of matrix molecules such as thrombospondin, which plays a role in synaptogenesis, has also been detected in microglia (Chamak et al., 1995; Moller et al., 1996; Christopherson et al., 2005). Moreover, studies from Chan et al. (2014) support a positive role for microglia in the process of synaptogenesis, documenting microglial reactivity and production of cytokine osteopontin, promoting synaptic organization in hippocampal regions of deafferentation-induced degeneration. One study showed that altering microglial proteins subsequently altered synaptic function as well (Bessis et al., 2007), and attenuating microglial response reduced synaptophysin, GAP-43, and BDNF levels (Madinier et al., 2009), documenting a strong association between proper microglial function and normal synaptic activity. Alternatively, microglia can also elicit negative responses during recovery processes (Byrnes et al., 2006; Ekdahl et al., 2009), particularly since they exhibit different roles in neuronal survival, depending on the microenvironment. The production of glutamate by microglia, for example, is neurotoxic, triggering neuronal apoptosis (Piani et al., 1991). In cases of brain injury, however, a certain level of these toxic stimuli may be beneficial, as death and removal of injured neurons allows
space for new terminals to sprout and form synapses. As an example, microglia have been
associated with both neuron death and synaptogenesis after axotomy (Moran and Graeber, 2004),
emphasizing the importance of proper microglial function in all phases of reactive
synaptogenesis induced by TBI.

Prolonged inflammation and neuroexcitation can exacerbate the effects of axotomy, an effect
that has been documented in maladaptive models of TBI (Phillips et al., 1994). Persistent
activation of microglia is one component of TBI-induced inflammation that can also be
detrimental to neuronal survival and synaptic repair. Several clinical and experimental studies
have reported long-term microglial activation after TBI (Johnson et al., 2013; Smith et al., 2013;
Gentleman et al., 2004; Faden, 2011; Engel et al., 2000), even up to 17 years post-injury
(Ramlackhansigh et al., 2011). Continued microglial activation in mice leads to several
detrimental consequences such as lesion expansion and hippocampal neurodegeneration (Loane
et al., 2014). Further, long term microglial activation in models of repetitive TBI produced white
matter degradation and cognitive deficits (Mouzon et al., 2014) as well as behavioral and
electrophysiological changes (Aungst et al., 2014). To combat these effects, some have
employed the use of anti-inflammatory treatments, which have attenuated not only the
inflammatory response, but also associated lesion volume and neuronal loss as well (Byrnes et
al., 2009; Loane et al., 2013). Minocycline, one such anti-inflammatory drug, has been shown to
reduce microglial activation and enhance neurogenesis, thus improving learning and memory
processes (Liu et al., 2007). Others, including Cyclosporin-A (Allessandri et al., 1999;
Allessandri et al., 2002; Colley et al., 2010; Scheff and Sullivan, 1999), Simvastatin (Wang et
al., 2007; Wang et al., 2014; Wu et al., 2010), and FK-506 (Fujita et al., 2011; Marmarou and
Povlishock, 2006; Oda et al., 2011) have also proven neuroprotective in TBI experimental brain injury paradigms improving cognition and motor skills, reducing histological damage and neuronal degeneration, and providing axonal protection, respectively. Recent evidence, however, points to select agents, such as Cyclosporin-A, producing variable to ineffective results when applied in several different TBI models (Dixon et al., 2016), yet controlling inflammation after injury still holds promise for improving functional deficits associated with head trauma.

Much like microglia, which become activated after TBI, astrocytes also adopt a reactive phenotype, as previously described, characterized by increased intermediate filament expression and secretion of components such as BDNF and chondroitin sulfate proteoglycans (CSPGs). BDNF is a prominent promoter of synaptogenesis, while CSPGs can either promote or hinder synaptic repair. Astrocytes secrete CSPGs to form an important component, the glial scar (Bignami and Dahl, 1976), to section off the site(s) of injury and prevent spread of the lesion to adjacent intact tissues (Matthews et al., 1979; Sofroniew, 2005; White and Jakeman, 2008). Unfortunately, these CSPGs can also be inhibitory for axonal growth (Matthews et al., 1976; Liuzzi and Lasek, 1987; Silver and Miller, 2004; Goldshmit and Bourne, 2010), thus limiting the capacity for synaptogenesis. On the contrary, BDNF production is largely beneficial. Expression of BDNF has been linked to synapse formation (Park and Poo, 2013), dendritic growth (McAllister et al., 1995), and axon branching (Cohen-Cory and Fraser, 1995), axon guidance, and neural development (Huang et al., 2001; Lu et al., 2013). The importance of neurotrophic factors like BDNF during synaptogenesis is represented in experimental models in which BDNF is absent, reducing synaptic proteins (Pozzo-Miller et al., 1999) and when BDNF is overexpressed, increasing the number of synapses (Aguado et al., 2003). Even in injury models
of ischemia and stroke, BDNF proves neuroprotective (Ferrer et al., 2001; Muller et al., 2008), demonstrating that the effects of some growth factors are indispensable in the process of reactive synaptogenesis. These analyses show that reactive neuroglia and the inflammatory processes they promote facilitate the formation of nascent synapses, both in normal conditions and during periods of synaptic repair, such as following TBI.

**EXTRACELLULAR MATRIX**

Although the tripartite synapse, which includes perisynaptic glia such as astrocytes, along with pre- and post-synaptic terminals, has long been accepted as the functional synapse, more recently, the tetrapartite synapse (Frischknecht and Gundelfinger, 2012; Dityatev et al., 2006; John et al., 2006; Dityatev et al., 2010) has been introduced, and includes the extracellular matrix (ECM). This hyaluronic acid-rich space (Frischknecht and Gundelfinger, 2012) between cells is inhabited by structural proteins, secreted factors, and matrix-shaping enzymes. Through cell-matrix interactions, this composition helps maintain neuronal and synaptic stability. Both glial cells and neurons produce components of the ECM including CSPGs, laminins, phosphacan, reelin, thrombospondins, as well as matrix-shaping enzymes such as proteases and hyaluronidases (Bandtlow and Zimmerman, 2000; Dityatev and Schachner, 2003; Christopherson et al., 2005; Dityatev and Fellin, 2008; Frischknecht and Gundelfinger, 2012), and structural components, which include collagens, proteoglycans, and glycoproteins (Dityatev and Schachner, 2003). Select ECM molecules can organize to form cartilage-like ECM, known as perineuronal nets (Fawcett, 2009), which surround cell bodies (Bruckner et al., 1993; Celio et al., 1998; Yamaguchi, 2000) for synapse stabilization. In normal physiological conditions, the ECM maintains the homeostasis of the extracellular space, highlighted by the compositional
maturity of the ECM from a diffuse matrix during embryonic development to a more condensed perineuronal net in adult brain for promotion of synaptic plasticity (Frischknecht and Gundelfinger, 2012). Evidence of this function is demonstrated by the presence of ECM interspersed between neurons and astrocytes (Frischknecht and Gundelfinger, 2012), and tightly wrapping synapses, with the ECM between synapses differing from the ECM surrounding them. For example, brevican is perisynaptic (Seidenbecher et al., 1997), while agrin is likely localized to the synaptic cleft, similar to its position in the neuromuscular junction (Matsumoto-Miyai et al., 2009). Some components such as hyaluronic acid (HA), serve not only structural purposes, but also have a direct impact on synaptic plasticity (Kochlamazashvili et al., 2010). Further, synaptic plasticity is impaired when the ECM is altered. Such is the case when loss of CSPGs brevican or neurocan leads to impairments in long-term potentiation (LTP; Zhou et al., 2001; Brakebusch et al., 2002). Similarly, adhesive protein N-cadherin has also been associated with LTP, as its muted elevation 15d post-injury in a maladaptive recovery model of deafferentation corresponds with a reduction in LTP (Warren et al., 2012), suggesting N-cadherin may be tightly associated with functional synaptic plasticity. Moreover, agrin protein is elevated during adaptive recovery, and reduced in a maladaptive model of synaptic repair (Falo et al., 2008), highlighting its importance in normal recovery processes. Thrombospondins have also been identified as critical for synapse development, for example, as interaction with cell adhesion molecule neuroligin 1 accelerates synaptogenesis (Xu et al., 2010). ECM component neuronal activity-regulated pentraxin (Narp) is involved in synapse formation and synaptic scaling as well and is increased at excitatory synapses (Chang et al., 2010; O’Brien et al., 1999). Matrix enzymes such as MMPs also associate with the process of synaptogenesis by, like other parts of the ECM, altering the conditions of the immediate environment, making it favorable to permit
synaptic plasticity. MMP-mediated processing of ECM components can both alter the stability of matrix structural components as well as produce fragments of important cell-signaling molecules, both of which can promote synaptogenesis and functional recovery. In pathological conditions such as TBI, these ECM proteins and enzymes are critical for proper synaptic reorganization and functional recovery.

Proteoglycans and Cell Adhesion Molecules/Extracellular Matrix Role in TBI

ECM proteoglycans, adhesion proteins such as calcium-based cell adhesion molecules (cadherins), and matrix receptors, play important roles in promoting synaptogenesis after TBI. Proteoglycans of the CNS such as brevican, neurocan, aggrecan, phosphacan, agrins, and tenascins, which are involved in synaptogenesis, are produced by neurons and glial cells (Bandtlow and Zimmerman, 2000; Heikkinen et al., 2014). CSPGs such as brevican, aggrecan, versican, and neurocan, are matrix proteins consisting of a protein core and a chondroitin sulfate side chain, and serve as structural components in tissues that help form glial scars and increase after injury to promote cellular processes such as cell adhesion, migration, and interaction with other ECM molecules. After injury, glia such as astrocytes, which secrete CSPGs such as brevican, versican, tenascin C, and neurocan, can inhibit axon growth (Fitch and Silver, 1997; Bradbury et al., 2002; Jones et al., 2002; Geissler et al., 2013; Siddiqui et al., 2009). Phosphacan, an isoform of receptor tyrosine phosphatase β/zeta receptor (Dobbertin et al., 2003), is one CSPG that has been explored in models of deafferented brain with mRNA expression peaking when synaptic reorganization is underway and protein localizing to dendrites (Harris, et al., 2011). This expression and relation to the time course of reactive synaptogenesis suggests a pro-reconstructive role for phosphacan in the synaptic recovery process. Conversely, inhibiting
CSPGs can also improve axon sprouting after TBI (Harris et al., 2010) and bone marrow stromal cell implantation downregulates neurocan, promoting axon growth and synaptophysin expression after TBI (Mahmood et al., 2014), supporting a dual role for CSPGs after TBI. Heparin sulfate proteoglycans (HSPGs) consist of 2 or 3 heparin sulfate chains that typically bind to different ligands to promote biological processes. Interestingly, HSPGs can stimulate synaptogenesis through interaction with polysialylated neuronal cell adhesion molecule (PSA-NCAM; Dityatev et al., 2004). The critical astrocyte response to injury may also be mediated by HSPGs through interaction with fibroblast growth factors, which occurs during neurogenesis and axon guidance (Yamaguchi, 2001). Interestingly, agrin, an HSPG, (Tsen et al., 1995; Cole and Halfter, 1996) is expressed in astrocytes, peaking 7d after injury, when axon sprouting in the hippocampus begins, and localizes with synaptophysin in nascent pre-synaptic terminals (Falo et al., 2008), demonstrating an association with synaptogenesis. Additionally, HSPG syndecan-2 is involved in spine maturation (Ethell and Yamaguchi, 1999). Overall, the ratio of permissive HSPGs to inhibitory CSPGs appears to be important in regulating axon growth (Tselnicker et al., 2014).

In addition to the proteoglycans, cadherins also have strong roles in maintaining cell contacts, with roles in axon guidance and synapse stabilization (Ranscht, 2000; Gerrow and El-Husseini, 2006). Several investigators have shown that N-cadherin plays a role in synaptogenesis. For example, it is involved in cell adhesion between neurons, and both N-cadherin and β catenin are important regulators of spine morphogenesis and synaptogenesis (Togashi et al., 2002; Okuda et al., 2007; Sagliette et al., 2007). In deafferented brain, N-cadherin increases around 15d post-lesion, during the regenerative phase of reactive synaptogenesis (Warren, et al., 2012), an indication of reestablished contact between neurons. More specifically, Wang et al. (2014)
showed that the ratio of precursor N-cadherin to mature N-cadherin is critical in regulating adhesion during synaptogenesis (Reines et al., 2012), as cleavage of the pro-domain is important for normal N-cadherin cell-cell adhesive functions (Latefi et al., 2009).

Several receptors exposed to the pericellular space also function as important mediators of matrix processes. Integrins are heterodimeric transmembrane receptor complexes of α and β subunit isomers, of which there are currently 18 α and 8 β subunits, and 24 known combinations (Wu and Reddy, 2012), each of which has distinct specificity. For example, the β₁ subunit can participate in at least 10 different combinations with α subunits, and β₃ ligands include fibronectin, fibrinogen, and vitronectin, depending on the paired α subunit (Guell and Bix, 2014).

Integrin receptors are responsible for initiating cell-signaling mechanisms through interaction with different ligands, many of which occur through an Arginine-Glycine-Aspartate (RGD) binding sequence, to promote cell adhesion, cell migration, and production of cytokines and chemokines to further enhance inflammatory response. Interestingly, several matrix enzymes can unmask the unique binding site on integrin ligands through proteolytic cleavage (Wang et al., 2008; Michaluk et al., 2009). Integrins serve pivotal roles in epithelial cells throughout the body, including cerebrovascular endothelial cells (Guell and Bix, 2014; Cousin et al., 1997; Jones, 1996; Grooms et al., 1993; Pinkstaff et al., 1999). Clegg et al. (2003) also demonstrated that activated integrins on microglia promote adhesion, endocytosis, and phagocytosis, while those on neurons support neural adhesion and regeneration. Notably, the process of embryogenesis requires integrins (Bronner-Fraser, 1986, 1994), more specifically, axon guidance, synaptogenesis, and astrocyte maturation and migration during brain development (Milner et al.,
In the adult brain, integrins, typically expressed on neurons and glia, have also been implicated in synaptogenesis.

Decreased dendritic spine density, defined by integrin antibody staining, suggests a role for integrins in synaptogenesis (Nikenenko et al., 2003), while integrin expression on astrocytes has been linked to axonal sprouting and synaptogenesis after neuronal cell loss (Fasen et al., 2003). Injury models also promote a functional role for integrins, as $\alpha_v\beta_3$ increases rapidly in the ischemic core after middle cerebral artery occlusion (MCAO; Abumiya et al., 1999). In focal ischemic stroke, $\alpha_v\beta_3$ interaction with OPN is involved in glial activation, organization, and repair (Ellison et al., 1999). Although the integrin receptors are composed of 2 subunits, some studies have explored the role of just one subunit in facilitating cellular processes. For example, $\beta_1$ integrin has been implicated in neurovascular remodeling (Lathia et al., 2010), and increases within nerve lesion models in the vicinity of the lesion, on growth cones and regenerating axons (Lefcort et al., 1992; Pinkstaff et al., 1998). Interestingly, integrins can also exhibit functions that contradict known properties, as the use of an $\alpha_4$ integrin antibody reduced infarct size and lowered neurocognitive deficit scores in a model of brain injury (Becker et al., 2001).

CD44, another receptor involved in several cell-cell and cell-matrix interactions, whose principal ligand is hyaluronic acid (HA), a core component of the ECM, can also promote processes such as adhesion, homing, aggregation of lymphocytes, and metastasis (Huet et al., 1989, Denning et al., 1990; Rothman et al., 1991; Jalkanen et al., 1986; Goodison et al., 1999), all of which can promote synaptogenesis. In addition to indirectly binding molecules such as HA, collagen, fibronectin, and laminin through chondroitin (Jalkanen and Jalkanen, 1992), CD44 also binds
directly to OPN, a matrix molecule with several signaling properties (Rangaswami et al., 2006). Localized on neurons or glia, CD44 may play a role in repair, as it is expressed after several different models of nerve transection (Jones et al., 2000), and a stab wound analysis also implicated CD44 in wound healing (Stylli et al., 2000). On the contrary, Wang et al. (2002) demonstrated that loss of CD44 resulted in smaller infarct size after MCAO, along with significant improvement in motor skills and lower pro-inflammatory IL-1β expression, suggesting CD44 expression may also be detrimental to recovery, under certain conditions. Interestingly, CD44 and integrins can form complexes for pro-MMP9 to dock (Redondo-Muñoz et al., 2008) promoting migration of macrophages, an essential part of ECM reorganization.

**Matrix Metalloproteinases**

Integrin binding can affect MMP activity, influencing membrane signaling and adhesion properties. The ADAM family of MMPs, classified as a disintegrin and a metalloproteinases, bind integrins to affect these cell responses and are found throughout the body, including the brain. Important functions for these ECM molecules have been discovered related to synaptogenesis, as ADAM21 is active in olfactory receptors neurons (ORNs), where neurogenesis and synaptogenesis are ongoing (Yang et al., 2005). Moreover, ADAM10 is expressed during dendritogenesis in the hippocampus (Pokorny and Yamamoto, 1981), while ADAM9 and ADAM10 mRNA are expressed during injury-induced sprouting after kainic acid injections (Ortiz et al., 2005). In a model of dentate gyrus deafferentation, the degenerative phase of reactive synaptogenesis is characterized by an increase in ADAM10 and membrane-type 5 matrix metalloproteinase (MT5-MMP; Warren et al., 2012), for which cell adhesion molecule N-cadherin is a substrate. Lastly, Seipel et al. (2010) showed that MT1-MMP,
ADAM10, CD44, MMP9, and αvβ3 act in concert to promote migration, an essential function for reactive glia promoting ECM reorganization. The ADAM and MT-MMP enzymes are members of a much larger, broader group of enzymes known as MMPs, which have been repeatedly implicated in TBI and synaptic recovery.

MMPs (Figure 1.2), which modulate the ECM, are zinc-dependent endopeptidases with highly conserved sequences and domains. Each has an amino-terminal signal peptide, carboxy-terminal hemopexin domain, propeptide region, which must be cleaved for activation, and a distinct catalytic domain, specific in each enzyme. Currently, there have been 24 secreted and MT-MMPs identified, grouped by substrate specificity (Nagase et al., 2006). Collagenases, stromelysins, matrilysins, gelatinases, and membrane-type MMPs are the main groups, which share similarities in catalytic functions, while some others are not strictly categorized. In general, stromelysins digest a number of ECM components and can activate other MMPs, while matrilysins lack a hemopexin domain, and membrane-type MMPs have an extra transmembrane domain to facilitate pericellular proteolytic processes. Collagenases break down collagens, structural components of meninges, and promote brain development through axon guidance and synaptogenesis (Fox, 2008; Hubert et al., 2009). Gelatinases (MMPs 2, 9) can also break down collagens, but further lyse gelatins in the ECM, cerebrospinal fluid, and blood serum (Abdul-Muneer et al., 2015). Each of the MMPs has several important physiological functions throughout the body, some of which also have important roles in the brain including during embryogenesis, bone remodeling, wound healing, and neurogenesis in normal circumstances, and BBB disruption, hemorrhage, and neuroinflammation in pathological conditions (Yong, 2005; Abdul-Muneer et al., 2015).
Figure 1.2 MMP Family Member Structural Domains
MMPs are zinc-dependent enzymes with highly conserved sequences designating a pro-peptide zone, hinge region, hemopexin domain, and an enzymatic site. Most of these endopeptidases can be grouped into families (listed in illustration) that share distinct catalytic functions, while others remained uncategorized. (Jaoude and Koh, 2016)
In the brain, MMPs are normally produced and secreted at low levels by neurons and reactive glia, while the enzymatic activities of MMPs are regulated by growth factors, cytokines, and a host of endogenous inhibitors. These include α2 macroglobulin and tissue inhibitors of metalloproteinases (TIMPs), of which there are 4, possessing tissue specificity, that inhibit the majority of MMPs (Sternlicht and Werb, 2001; Zhang et al., 2010; Abdul-Muneer et al., 2015). TIMPs 1 and 2 are the primary MMP inhibitors in the brain (Rivera et al., 2010) and maintain a physiologically relevant ratio with MMPs, such that overexpression of MMPs disrupts the inhibitor ratio, leading to potentially detrimental effects (Brew et al., 2000). MMP dysregulation, including aberrant elevation, has been described in several CNS models including stroke, hemorrhage, Alzheimer’s disease, and TBI (Abdul-Muneer et al., 2015; Van Hove et al., 2012; Verslegers et al., 2013). When the delicate balance between regulatory molecule and proteolytic enzyme becomes dysregulated, enzymatic functions can become pathological.

In injury paradigms, MMPs can be synthesized and released by astrocytes (Falo et al., 2006; Ogier et al., 2006), endothelial cells, oligodendrocytes (Oh et al., 1999; Uhm et al., 1998), and microglia (Peng et al., 2012) depending on injury type and CNS location (Zhang et al., 2010). After TBI, when a number of cellular processes are disrupted, MMP function is also altered and can lead to the progression of neuropathology. For example, high levels of MMPs can promote increased neuronal and tissue damage (Wang and Lo, 2003; Romanic et al., 1998; Lo et al., 2002; Yong et al., 2001; Rosenberg, 2002), and MMP8 concentration is associated with mortality in severe TBI patients (Roberts et al., 2013). Additionally, overactive MMP2, MMP3, and MMP9 proteolysis can cause BBB disruption, hemorrhage, neuroinflammation, intracranial pressure, edema, neurodegeneration, and cell death (Abdul-Muneer, et al., 2015; Lotocki et al.,
2009; Montaner et al., 2001; Pun et al., 2009), all of which promote poorer outcomes after injury. Moreover, MMPs can accelerate inflammatory processes and activate caspases, which leads to apoptosis (Abdul-Muneer et al., 2015). MMP1 interaction with integrins can also lead to apoptosis (Meerovitch et al., 2003), demonstrating that MMPs can attach to MT-MMPs, cell adhesion molecules, proteoglycans, and integrins (Yong, 2005; Dumin et al., 2001) to promote different cellular processes critical to recovery.

By redistributing proteins in the ECM, MMPs can promote synaptic plasticity (Phillips et al., 2014; Abdul-Muneer et al., 2015). Processing of some ECM components alter matrix structure, while breakdown of others leads to stabilization of synapses, as select MMP substrates can guide afferent axons during synaptogenesis (Treolar et al., 2009). For example, several proteoglycans are degraded by MMPs (Pizzi and Crowe, 2007; Hsu et al., 2008; Zuo et al., 1998), such as the case in MMP3 processing of agrin (Werle and VanSaun, 2003; Falo et al., 2008). MMP3 also targets other ECM proteins critical for synaptogenesis (Muir et al., 2002; Bejarano et al., 1988; Imai et al., 1995; Okada et al., 1987; VanSaun and Werle, 2000), and likely contributes to the process as it is upregulated for weeks after deafferentation and localizes to deafferented zones (Kim et al., 2005; Falo et al., 2006). After TBI, MMPs 2, 3, and 9, which can regulate extension of granule cell processes through cerebellar ECM (Vailant et al., 2003; Luo, 2005), are often activated, causing ECM degradation, BBB permeability, inflammation, and degeneration, which can be harmful or beneficial for synaptic plasticity (Phillips et al., 2014; Abdul-Muneer et al., 2015; Montaner et al., 2001). Several laboratories have shown that MMPs and their substrates promote reactive synaptogenesis in injured hippocampus (Phillips and Reeves, 2001; Kim et al., 2005; Falo et al., 2006; Warren et al., 2012; Chan et al., 2014), demonstrating the therapeutic
potential of targeting MMPs to improve recovery. Particular MMP inhibitors such as GM6001 (Sifringer et al., 2007; Wang and Tsirka, 2005; Warren et al., 2012), FN-439 (Falo et al., 2006; Kim et al., 2005; Reeves et al., 2003), and SB-3CT (Hadass et al., 2013) have proven useful for reducing MMP activity and subsequently affecting recovery and brain lesion size in experimental brain injury paradigms, but consistent and effective control of MMP activity after TBI has proven difficult because of the complexity of MMP functions and nonspecific effects of some experimental compounds (Zhang et al., 2010; Abdul-Muneer et al., 2015).

MMPs 2 and 9
Gelatinases A and B, or MMP2 and MMP9, typically localized in neurons, astrocytes, macrophages, and blood vessels (Yamaguchi et al., 2007; Noble et al., 2002; Hsu et al., 2006), are specific for both gelatin substrates as well as other ECM components. Gelatinase structure is similar to other MMPs (Van den Steen et al., 2002), with a signal peptide, pro-peptide for proteolytic activation, catalytic domain containing a zinc ion, three tandem repeats of fibronectin type-II inserts within the catalytic domain, proline-rich and heavily glycosylated linker region, and a hemopexin-like domain (Stute et al., 2003; Dziembowska and Wlodarczyk, 2012). Like other MMPs, MMP9 is produced as a zymogen and secreted into the ECM for cleavage into its active form by other MMPs or the plasminogen-plasmin system (Bruno and Cuello, 2006). Enzymatic activity of MMP9 is regulated by TIMP1 binding at the hemopexin domain (Olson et al., 1997; Yong, 2005), like other MMPs, as well as by neutrophil gelatinase-associated lipocalin (NGAL)/Lipocalin-2 (LCN2), which binds and protects MMP9 from degradation, enhancing enzymatic activity (Yan et al., 2001).
Several roles for gelatinases in CNS disorders have been uncovered. For example, MMP2 activity in TBI models degrades tight junctions of the BBB, promotes edema, and induces MMP9 activity (Abdul-Muneer et al., 2013; Jadhav et al., 2008), facilitating further pathological processes. Additionally, MMP9 activity is elevated in the neurofibrillary tangles and plaque accumulations associated with Alzheimer’s disease (Asahina et al., 2001) as well as in hippocampus during seizure/epileptic episodes, as it promotes abnormal recurrent mossy fiber sprouting and synaptogenesis (Konopka et al., 2013). Interestingly, ROS, which are produced in numerous pathological CNS conditions, increases both MMP2 and MMP9, and decreases TIMPS in vitro, altering the delicate ratio between enzyme and regulator (Rajagopalan et al., 1996; Haorah et al., 2007). Gelatinase activity is also correlated with glial scar formation (Duchossoy et al., 2001), which is consistent with MMP break down of CSPGs.

In response to spinal cord injury (SCI; Hsu et al., 2006; Hsu et al., 2008), olfactory bulb deafferentation (Costanzo et al., 2006; Costanzo and Perrino, 2008), TBI (Phillips and Reeves, 2001), and cortical contusion injury (CCI; Wang et al., 2000), gelatinase activity increases substantially, suggesting a role for their function in neuronal injury and recovery mechanisms. In the clinic, MMP2 and MMP9 are elevated in blood serum of subarachnoid hemorrhage patients (Horstmann et al., 2006), while in experimental models of injury, MMP9 activity peaks early (de Castro et al., 2000; Noble et al., 2002), followed by MMP2 in recovery phases (Goussev et al., 2003; Zuo et al., 1998; Hsu et al., 2006; Costanzo and Perrino, 2008). These results suggest that each gelatinase plays an important role in response to brain injury, with MMP9 potentially a critical mediator of the acute pathophysiological processes preceding synaptic repair.
Regarding individual function of MMP9 in TBI, investigators have associated apoptosis (Chintala et al., 2002; Sifringer et al., 2007; Jia et al., 2010), edema (Homsi et al., 2009; Hirose et al., 2013; Jadhav et al., 2008), inflammation (Homsi et al., 2009; Ralay Ranaivo et al., 2011; Suehiro et al., 2004), BBB degradation (Yu et al., 2008; Shigemori et al., 2006; Muradashvili et al., 2015; Abdul-Muneer et al., 2013; Higashida et al., 2011), and synaptogenesis with MMP9 activity (Chan et al., 2014; Phillips et al., 2014). In synapses, MMP9 mRNA and protein are localized to dendritic spines (Konopacki et al., 2007; Gawlak et al., 2009; Wilczynski et al., 2008), while enzymatic activity of MMP9 has also been found in mature synapses (Gawlak et al., 2009). Additionally, increased MMP9 can lead to spine enlargement (Wang et al., 2008), and depletion of MMP9 can impair functional synaptic plasticity (Frischknecht and Gundelfinger, 2012). Although normal MMP9 function may benefit synaptic plasticity in injury paradigms, MMP activity may become aberrantly high, causing synaptic reorganization to be maladaptive (Falo et al., 2006; Warren et al., 2012; Phillips et al., 2014). For example, assessing MMP9 role in synaptogenesis after injury, one study showed that post-injury MMP9 inhibition reduced lesion volume, increased dendritic spines, reduced glial reactivity, and provided enhanced cognitive and motor recovery (Hadass et al., 2013). Further, in a model of hippocampal deafferentation, MMP inhibition improved cognition and behavior (Falo et al., 2006; Warren et al., 2012), suggesting the specific inhibition of aberrant elevation of MMP9 activity after injury may prove useful. Further, time-dependent inhibition of MMP activity can shift the outcome of synaptogenesis after deafferentation (Reeves et al., 2003; Falo et al., 2006; Warren et al., 2012), as acute inhibition of MMP activity proves detrimental to recovery in adaptive models (Phillips et al., 2014), yet reducing MMP activity later, when pathology drives excessive enzymatic response, promotes better outcomes.
OSTEOPONTIN

Structure and Cell Signaling Properties

One important secreted ECM molecule involved in facilitating cellular responses, including those involved in inflammation, is osteopontin (OPN; **Figure 1.3**), also known as bone sialoprotein 1, secreted phosphoprotein 1 (spp1), or early component of type-1 immunity (Eta-1). Originally discovered as a bone matrix protein involved in bone resorption, and embryonically associated with bone formation (Nakase et al., 1994; Nomura et al., 1989), OPN is also expressed in bone, kidney, and epithelial linings (Giachelli and Steitz, 2000), as well as brain (Shin et al., 1999) of the adult. A multifunctional protein, OPN serves as both a secreted cytokine and immobilized matricellular protein (Singh et al., 2010; Frangogiannis, 2012; Wolak, 2014; O’Regan et al., 2000), and is a member of the SIBLING protein (small integrin binding ligand N-linked glycoprotein) family (Smith and Denhardt, 1987; Liaw et al., 1998; Asou et al., 2001). The size of full length OPN varies based on extent of post-translational modifications, and has been detected in SDS-PAGE migrating between 30 and 100 kDa (Patarca et al., 1995; Ashkar et al., 1993; Rodan, 1995; Sodek et al., 2000). Osteopontin is a highly acidic (Oldberg et al., 1988; Denhardt and Guo et al., 1993), pleiotropic protein with a moderately conserved amino acid sequence across species. Arginine-Glycine-Aspartate (RGD; 100% conserved), Serine-Valine-Valine-Tyrosine-Glycine-Leucine-Arginine (SVVYGLR; structurally conserved, SLAYGLR in mice and rats), and Glutamine-Leucine-Valine-Threonine-Aspartate-Phenylalanine-Proline-Threonine-Asparatate-Leucine-Proline-Alanine-Threonine-Glutamine (ELVTDFPTDLPATE; least conserved) sequences within the OPN protein confer binding capability to integrin receptors (Smith and Giachelli, 1998; Scatena et al., 2007). Cleavage of
Figure 1.3 Osteopontin Structure and Proteolysis

A. Osteopontin (OPN) structural features key to cell adhesion receptors. Proteolytic processing exposes integrin receptor binding sequences indicated by color (ELVTDFP: green; RGD: red; SVVYGLR: blue). Corresponding integrin receptors are listed adjacent to these cell adhesive regions and sites of peptide phosphorylation (P) along the protein sequence are indicated.

B. MMP and thrombin cleavage sites are identified within the human OPN sequence, notably the 166 site of MMP proteolysis which generates the N-terminal peptide with exposed RGD integrin binding residues.

C. MMP processing of OPN is shown. A 48kD amino-terminal OPN fragment containing the RGD integrin binding sequence is generated along with a carboxy-terminal 32kD OPN fragment, which is subject to further proteolytic processing. (Scatena et al., 2007; adapted from Takafuji et al., 2007)
OPN by matrix proteins including MMPs and thrombin (see again Figure 1.3) exposes the critical sequences concealed by the tertiary structure of the molecule, so that they may be uncovered to bind functional receptors and facilitate cellular responses such as chemotaxis, cell adhesion, metastasis, and immune regulation (Shin, 2012).

Thrombin proteolysis of OPN is well characterized, while the extent of MMP proteolysis of OPN is still being explored. The thrombin site of cleavage, immediately following the SVVYGLR sequence, generates amino terminal fragments that bind \( \alpha_\beta_3 \), \( \alpha_4\beta_1 \), and \( \alpha_9\beta_1 \) integrin receptors (Barry et al., 2000; Green et al., 2001; Bayless and Davis, 2001; Bayless et al., 1998), and also exposes the RGD sequence, which has \( \alpha_\nu \) specificity, and ELVTDFPTDLPATE, which binds \( \alpha_4\beta_1 \) (Bayless and Davis, 2001). Although some studies propose a single cleavage site for MMPs within the thrombin cleavage sequence, between the Glycine and Leucine residues (Gao et al., 2004), additional cleavage sites for MMP2, MMP3, MMP7, MMP9, and MMP12 have also been uncovered (Lindsey et al., 2015; Hou et al., 2004; Agnihotri et al., 2001). Even though different matrix enzymes can cleave OPN at unique sites, they all produce OPN fragments with similar binding properties, even considering that cleavage by certain MMPs may disrupt integrin-binding sequences. This occurs since the proteolytic conformational changes still expose critical integrin binding loci, one of which is the RGD sequence, which exhibits high affinity binding after OPN cleavage (Smith et al., 1996; Senger et al., 1994).

RGD, also found in fibronectin and vitronectin, confers cell-binding properties and is involved in the osteoblast OPN-mediated osteoclast migration process (Oldberg et al., 1986). OPN-receptor interactions have been addressed extensively in order to better understand the mechanisms
underlying OPN-mediated cellular functions. Liaw et al. (1995) found that the $\alpha_v\beta_3$ (vitronectin) receptor was needed for OPN-mediated migration, while $\alpha_v\beta_5$ or $\alpha_v\beta_1$ were sufficient to promote adhesion properties. Interestingly, $\alpha_v\beta_3$ is involved in other cell functions including apoptosis, and is expressed highest in osteoclasts, of which OPN promotes migration during bone matrix sculpting (Horton, 1996). OPN binding to several other integrin receptors, including $\alpha_8\beta_1$, $\alpha_4\beta_7$, $\alpha_6\beta_1$, and $\alpha_9\beta_1$, has also been documented (Scatena et al., 2007). While OPN binding to multiple integrin receptors is prominent, a somewhat unique interaction with hyaluronic acid receptor CD44 has also been discovered, where dose-dependent binding of OPN to CD44 is not mediated by chondroitin sulfate (Weber et al., 1996). Both integrin and hyaluronic acid receptors appear important relative to OPN role in synaptogenesis, as $\beta_1$ and CD44 antibodies blocked *in vitro* OPN-mediated neurite outgrowth (Plantman, 2012). Interestingly, the carboxy terminal fragment, which lacks integrin binding sites, does not mediate adhesion, as does the amino terminal fragment, and may actually suppress OPN-mediated adhesion (Smith et al., 1996; Gao et al., 2004; Maeda et al., 2001). There is also evidence that this smaller carboxy terminal fragment binds CD44 (Weber et al., 1997; Weber et al., 2002; Takahashi et al., 1998; Weber et al., 1996). The ability of OPN to bind several different matrix exposed receptors suggests its key role in mediating autocrine and paracrine response during tissue repair, making it a viable target for altering aberrant cell interaction throughout this process.

Several years of investigation have implicated OPN in cardiovascular disease (Singh et al., 2007), lung dysfunction (O’Regan, 2003), cancer (Shevde and Samant, 2014), and inflammation (Shin, 2012). Because OPN is involved in promoting several protective and pathogenic cellular responses, including cell proliferation and apoptosis, it has been explored as a therapeutic target.
for several models of disease and injury. For example, as cancerous growth involves uncontrolled cellular proliferation, OPN has been investigated in several oncology studies. In preclinical cancer treatment analysis, OPN has been successfully targeted with the commercial drug Simvastatin (Matsuura et al., 2011), which reduced OPN to both limit cell proliferation and increase apoptosis of cancer cells, demonstrating OPN could be successfully targeted for improved recovery.

Role in Inflammation and CNS Disease

Investigators have shown that OPN is consistently upregulated during CNS inflammation (Ellison et al., 1999; Hashimoto et al., 2007) as well as in neurodegenerative diseases promoted by inflammation (Maetzler et al., 2007; Comi et al., 2010). In the CNS, OPN has been detected in microglia (Albertsson et al., 2014; Tambuyzer et al., 2012), macrophages (Gliem et al., 2015), and astrocytes (Neuman, et al., 2014). When expressed in phagocytic microglia (Choi et al., 2004), it increases proliferation, while reducing ROS production (Tambuyzer et al., 2012). OPN is also involved with the promotion of macrophage infiltration (Crawford et al., 1998; Wang et al., 1996; Weber and Cantor, 1996; Yu et al., 1998). Such macrophages secrete IL-12, which may be T cell dependent (O’Regan et al., 2000a,b), reducing IL-10 levels and leading to type 1 immunity, often present with inflammatory disease (Ashkar et al., 2000; Weber et al., 1992). In studies using OPN KO mice, macrophages are less cytotoxic to tumor cells (Bourassa et al., 2004), suggesting a reduction in important inflammatory processes. Additionally, an incision model revealed OPN KO mice produced more residual debris, and less matrix reorganization (Liaw et al., 1998), an indication that OPN signaling is critical for proper macrophage directed wound healing. On a larger scale, OPN can serve as metric to assay immune-mediated disease,
indicating the extent of pathogenic effects in irritable bowel disease, hepatic disease, multiple sclerosis, emphysema, lupus, asthma, and allergies (Rittling and Singh, 2015). For example, OPN concentrations are elevated in blood plasma of patients with chronic inflammatory illnesses, such as Crohn’s disease, cancer, autoimmune diseases, and obesity (Scatena et al., 2007).

Osteopontin has also been associated with the underlying mechanisms of several neurodegenerative diseases that may include, but are not limited to, inflammation. In the chronic inflammatory condition multiple sclerosis (MS), a disease characterized by episodes of neurologic impairment followed by remissions, OPN expression marks the evolution of tissue pathobiology. Among others, Börnsten et al. (2011), found that OPN concentration was increased in CSF of MS patients during attacks, while Chabas et al. (2001) detected OPN within active plaques in MS patient brains, specifically in endothelial cells, microglia, astrocytes, and macrophages. In the experimental animal model of MS, experimental autoimmune encephalomyelitis (EAE), OPN expression was localized to T cells, macrophages, activated microglia, and astrocytes in EAE lesions (Kim et al., 2004), a finding consistent with the clinical presentation. Additionally, OPN was upregulated in rat brains with EAE, and not present in those protected from EAE. Near EAE lesions, OPN was largely expressed in microglia, but also present in neurons, although neuronal expression was exclusive to active disease states, absent in remission, suggesting the degree of OPN present may correlate with disease severity (Chabas et al, 2001). Loss of OPN in EAE models reduced the severity of disease (Chabas et al., 2001; Jansson et al., 2002), while Natalizumab treatment in relapsing remitting patients reduced OPN and as a result, improved cognitive function and reduced fatigue (Iaffaldano et al., 2014). Such
integration of OPN and CNS inflammatory disease highlight the importance of the molecule as a regulator of tissue inflammation, although OPN may influence other cell specific mechanisms in different CNS pathologies.

One such pathology is Parkinson’s disease (PD), which involves the progressive neurodegeneration of dopaminergic neurons in the substantia nigra (Bernheimer et al, 1973) and the distinct presence of intraneuronal Lewy bodies. Several cellular mechanisms can lead to PD including oxidative and nitratative stress, mitochondrial dysfunction, and excitotoxicity, all of which promote cell death (Jellinger, 2001). When challenged with LPS or dopaminergic toxin 6-hydroxydopamine (6-OHDA) to induce PD-like symptoms, expression of OPN and integrin and CD44 receptors are upregulated in the substantia nigra, suggesting a neuroprotective response to promote cell survival mechanisms (Ailane et al., 2013; Iczkiewicz et al., 2005, 2006). Further, the Iczkiewicz group found that both OPN and an RGD peptide were neuroprotective for neurons exposed to nigral cell neurotoxin, MPP+, in vitro (Iczkiewicz et al., 2010). Interestingly, OPN associates with microtubule stability proteins MAP1A and MAP1B in the substantia nigra and striatum (Long et al., 2012), an interaction that may underlie some of the neuroprotective effects of OPN in PD. In other PD studies, however, OPN expression was downregulated and exhibited anti-apoptotic activity (Kim et al., 2006). This is consistent with a report that OPN is localized within substantia nigra ECM and neurons, and not nigral glia, suggesting that OPN may play a key role in neuron survival (Iczkiewicz et al., 2006). Contrary to the beneficial effects of OPN, others have suggested the presence of OPN in PD patient serum and CSF is an indication of pathology, as OPN levels were highest in those with more severe motor dysfunctions (Maetzler et al., 2007). This group also found that OPN KO produced more surviving dopaminergic
neurons after treatment with MPTP, a neurotoxin used to model PD, further supporting the view of OPN as a pathogenic marker (Maetzler et al., 2007).

In other neurodegenerative diseases, such as HIV-associated dementia and amyotrophic lateral sclerosis (ALS), OPN expression in Iba-1+ microglia is high, while neurons and astrocytes also express the protein at lower levels (Silva et al., 2015), further pointing to OPN association with multiple CNS diseases. Moreover, Alzheimer’s disease (AD), a well-known and devastating neurodegenerative pathology can also be associated with altered OPN expression. AD is marked by progressive cognitive decline, as well as the presence of amyloid plaques and intraneuronal fibrillary tangles of Tau protein. Like PD, OPN is significantly increased in the CSF and serum of AD patients, however, this increase is associated with lower levels of cognitive decline (Comi et al., 2010; Sun et al., 2013). Nevertheless, OPN is not only higher in newly diagnosed AD patients (Sun et al., 2013), but it is also increased in AD pyramidal neurons (Wung et al., 2007), and OPN expression is significantly positively correlated with increased levels of tangle forming phospho-Tau (Heywood et al., 2015) as well as high amyloid β protein expression (Wung et al., 2007). In an experimental AD mouse model APP/PS1KI characterized by axon degeneration and neuronal loss, OPN was again increased along with other inflammatory mediators (Wirths et al., 2010). These findings strongly suggest involvement of that OPN could play either pathological or protective roles in neurodegeneration, depending on disease, disease state, or other confounding factors.
Role in CNS Injury and TBI

Studies utilizing different types of CNS injury models have highlighted the prominent, yet controversial role of OPN after CNS insult. For example, OPN may both enhance repair after adult brain injury (Doyle et al., 2008; van Velthoven et al., 2011; Chen et al., 2011), but potentiate injury in neonatal models (Albertsson et al., 2014), suggesting possible age-dependent effects, possibly attributed to differential integrin receptor expression. Additionally, much like the neurodegenerative diseases discussed above, OPN levels rise after brain injury (Plantman, 2012). Also, similar to observations in neurodegenerative disease brains, OPN can be expressed in neurons, such as in a cryolesion model, in which cortical neurons and reactive microglia both produce OPN (Shin et al., 2005). Exploring this association further, investigators found that macrophages and microglia produced OPN early after focal stroke, while $\alpha_v\beta_3$ vitronectin receptor expression was upregulated later (5-15 days post-injury) in astrocytes (Ellison et al., 1998). Similarly, Kang et al. (2008) found integrin receptors localized primarily in astrocytes, but also report that CD44 receptor expression peaked early after forebrain ischemia (2-3 days) and was localized to reactive microglia. Interestingly, both receptors co-localized with OPN expression, suggesting equally important roles for OPN within reactive glia during post-injury recovery processes in the brain. Interestingly, after ischemic stroke, OPN is localized to infiltrating macrophages, which can cause local astrocyte polarization and activation (Gliem et al., 2015). When OPN KOs were also subjected to ischemic stroke, animals possessed fewer reactive, polarized astrocytes. These observations highlight an important role for OPN in paracrine cell signaling to affect CNS glial response. In another stroke study, OPN was administered pre- or post-middle cerebral artery occlusion (MCAO), where it reduced lesion size and cell death, and improved motor recovery (Jin et al., 2014), likely through its RGD binding
sequence and downstream Akt or MAPK cell signaling pathways (Meller et al., 2005). Further, nasal administration of OPN through the FAK-PI3K-Akt pathway also reduced edema, cleaved capsase-3, and neuronal death after subarachnoid hemorrhage (Topkoru et al., 2013), again demonstrating overall neuroprotective effect of OPN in models of brain injury. Notably, genetic manipulation of OPN expression (OPN KO) support similar effects on tissue repair in a variety of injury models, with delayed wound healing (Miyazaki et al., 2008), worsened adaptive synaptic recovery after hippocampal deafferentation (Chan et al., 2014), and reduced glial reactivity and subsequently, more extensive histological damage after SCI (Hashimoto et al., 2007), all suggesting OPN expression and function is important in promoting CNS recovery processes after injury.

While OPN involvement in CNS injury has been explored, its specific role in TBI is still under investigation. OPN and one of its receptors, CD44, have been encountered in the perilesional area following TBI (Günther et al., 2015), localized to activated microglia and macrophages 1-4 days post-injury (von Gertten et al., 2005). Interestingly, it has been suggested that the OPN-CD44 interaction leads to poor outcome, while the integrin-OPN interaction is beneficial (von Gertten et al., 2005). In a model of hippocampal deafferentation, a common pathology occurring after TBI, OPN expression was highest during degenerative periods, and its transcript localized to microglia (Chan et al., 2014). The same study showed increased MMP9 lytic activity, aberrant synaptic protein expression, and altered glial migration within deafferented zones in the absence of OPN. These results suggest a critical role for OPN in mediating post-injury clearance of degenerating pre-synaptic axons and modification of synaptic cytoarchitecture during synapse reformation (Chan et al., 2014).
OPN Interaction with MMP9

OPN is proteolytically processed by thrombin, several MMPs, plasmin, and cathepsin-D (Christensen et al., 2010; Denhardt et al., 2001) into amino and carboxy-terminal fragments, which usually retain functional capabilities. The MMP9-OPN enzyme-substrate relationship, however, is a particularly important one. Notably, their interaction has been documented in a plethora of studies, often contributing to pathogenesis of disease. For example, in MS patients experiencing crises, not only is OPN expression increased (Börnsten et al., 2011), but OPN and one of its target enzymes, MMP9, are both elevated in CSF of MS patients (Braitch et al., 2008), an observation that has been correlated with MS disease severity (Romme Christensen et al., 2013). Increased expression of both MMP9 and OPN has been detected in investigations crossing numerous disease processes such as prostate cancer progression (Castellano et al., 2008) and periodontal tissue regeneration (Lima et al., 2008). Co-expression of these molecules is typically linked to critical cellular processes including cell adhesion (Wang et al., 2014), suggesting the MMP9/OPN link is important and the implications of this interaction are worth exploring.

In several studies, the MMP9/OPN relationship is characterized by OPN promoting MMP9 activity and downstream cellular processes. Generally, OPN level regulates MMP9 expression (Yang et al., 2011), facilitating cell matrix remodeling in muscular dystrophy and asthma disease models (Dahiya et al., 2011; Simoes et al., 2009). In smooth muscle cells, Liu et al. (2014) determined that OPN increased MMP9 protein, leading to enhanced smooth muscle functions such as proliferation and migration, while in an in vitro model of embryogenesis, OPN increased cellular invasion by influencing MMP9 secretion (Wu et al., 2015). Notably, Lai et al. (2006)
also demonstrated that the SVVYGLR sequence of OPN, exposed on the amino-terminal fragment of OPN after processing, can induce pro-MMP9 expression, offering a potential mechanism for MMP9 induction. Caers and colleagues (2006) suggested OPN interaction with CD44v isoforms mediated the upregulation of MMP9 and subsequent cell proliferation. A more exploratory investigation showed that carboxy terminal OPN binds CD44 to promote chemotaxis, while β3 integrin subunit activation, likely from amino terminal OPN, increases macrophage activation, pro-inflammatory cytokines, and MMP9 activity (Weber et al., 2002). In 2007, Desai and colleagues conducted a detailed study revealing that surface expression of CD44 was required for MMP9 secretion. They determined that the OPN/receptor interaction, especially with αvβ3, activates Rho GTPases to increase surface CD44 expression, which then promotes MMP9 activation, required for migration (Desai et al., 2007). Most impressive, a majority of studies focused on OPN interaction with one receptor or another, yet this study integrated understanding of how these are related in the same process.

Evidence of OPN-mediated MMP9 activity warrants discussion of upstream and downstream mediators of this interaction. Upstream, TNFα appears to play in important role. A significant positive correlation found among TNFα, OPN, MMP9, and a novel MMP9 regulator, LCN2 (Catalán et al., 2009) indicates interactive processes, while other studies more definitively elucidate the role of TNFα. For example, in the case of psoriasis, both OPN and MMP9 levels were enhanced by TNFα and reduced by anti-TNFα treatment (Buommino et al., 2012). Similarly, in diabetes, TNFα and OPN also enhance MMP9 activity, and loss of OPN abrogates MMP9 induction (Lai et al., 2006). As TNFα appears to be an upstream regulator several have also explored the specific pathways involved in OPN-mediated MMP9 increase.
Amino terminal OPN binding $\alpha_\beta_3$ to facilitate activation of downstream CamKII and ERK 1/2 to activate MMP9 (Pagano and Haurani, 2006) was demonstrated in one study, while OPN/$\alpha_\beta_3$ interaction promoted MMP9 activation through JNK1 phosphorylation in another (Rangaswami and Kundu, 2007). Rangaswami et al. (2004) also found that $\alpha_\beta_3$ interaction with OPN promoted NIK-dependent MEK-1 and ERK 1/2 phosphorylations, which activate NFkB, thus inducing MMP9 activity, and cell migration, which was blocked by MMP9 antibody. Interestingly, several studies implicate NFkB in MMP9 induction. In the pathogenesis of aortic aneurysms, for example, OPN upregulates MMPs through NFkB (Mi et al., 2011), while following similar logic, Liu et al. (2010) found that OPN inhibition reduced MMP9 and MMP2 expression through IKK-2 and NFkB. Several cell-signaling cascades appear to be involved in the intermediary processes of MMP9 induction.

Further downstream, MMP9 also promotes OPN actions through cleavage. Regardless of the specific mechanism of MMP9 activation, it is understood that OPN acts in a positive feedback mechanism, as enhanced MMP9 activity promotes cleavage of OPN to facilitate more OPN-mediated functions. Takafuji et al. (2007) explored the enzyme-substrate relationship between MMP9 and OPN, finding that MMP9 activity produces several OPN fragments, one of which increased tumor cell invasion, likely mediated by CD44. Similarly, Lindsey et al. (2015) discovered 9 cleavage sites for MMP9 within the OPN sequence, thus confirming MMP9 proteolysis of OPN. In vitro, MMP9-cleaved OPN increased macrophage migration (Tan et al., 2013), supporting evidence that MMP9 processing produces functional OPN fragments.
Despite the breadth of information following the reported positively correlated link between MMP9 and OPN, some investigational studies have shown conflicting results, particularly in those utilizing recombinant OPN to promote recovery. As expected, genetic ablation of OPN reduced MMP9 activity in a deafferentation model of brain injury (Chan et al., 2014). Other investigators, however, showed that MMP9 secretion was not affected by OPN KO (Koh et al., 2007), a finding which could suggest the role of OPN upstream MMP9 is to promote activity over secretion. In a lung injury model, recombinant OPN (rOPN) surprisingly increased TIMP1, yet did not alter MMP9 expression, attributed to promoting an appropriate regulator/enzyme ratio for recovery. Additionally, NFκB expression and lesion size were effectively reduced (Zhang et al., 2014), an indication of ongoing repair mechanisms. Similarly, rOPN also appeared to operate through this complex mechanism in brain injury models. Administration of rOPN after intracerebral hemorrhage attenuated increase in MMP9 and ROS and improved cognitive function (Wu et al., 2011), and pre-treatment with recombinant OPN administration early after subarachnoid hemorrhage deactivated NFkB and reduced MMP9 activation. Interestingly, these investigators further suggested that this may only occur in the presence of pro-inflammatory cytokines (Suzuki et al., 2010), and integrin receptors were likely involved (Suzuki et al., 2011). Collectively, these studies support a link between OPN and MMP9 both with OPN-guided MMP9 activity as well as MMP9-mediated cleavage of OPN, both interactions being critical to key cell functions in pathological and/or recovery conditions.
MODEL SYSTEM IN PRESENT STUDY: THE OLFACTORY BULB

Cellular Organization

Olfactory deficits are often overlooked in TBI patients, where there is potential for severe damage to the olfactory nerve, causing incapacitating loss of the sense of smell, or anosmia. Olfaction, the most primitive sensory system (Brodal et al., 2004), plays an essential role in the social behaviors of vertebrate animals, such as survival-dependent behaviors like food seeking, predator evasion, and maternal preference. Although the perception of smell is less keen in humans than in other mammals (Kandel et al., 2013), it remains extremely sensitive. Scents detected by this complex system are an essential part of life and can alert an individual to potential dangers or offer the pleasure to enjoy fragrant aromas. In the olfactory system, numerous cell types work in cohort to recognize and interpret an array of molecules that animals eventually perceive as discrete odors. Disease, aging, infection, and injury are some mechanisms by which olfaction can be impaired in humans (Mesholam et al., 1998; Sumner, 1964; Wilson et al., 2007), Parkinson’s disease (Doty, 2012) and head injury (Sumner, 1964) being two well documented examples. Given the low probability of long-term olfactory recovery after injury, it is important to understand the molecular details of olfactory system damage and address potential avenues for regaining function.

In general, the olfactory system involves the detection of specific molecules in the nasal cavity, transmission of signals through olfactory nerves, enhancement of output in the olfactory bulb (OB), projection through the lateral olfactory tracts, and eventually, interpretation of smell in the telencephalon. Of these constituents, the OB is a key component, considering a significant amount of processing in the olfactory system occurs in the OB. Similar to complex brain regions
and structures such as the neocortex and retina, cytoarchitectural lamination of the OB is crucial for its proper function. Although olfaction begins in the nasal mucosa, the first part of the olfactory system, input is refined in the layers of the OB (Figure 1.4A). In order, from the ventral portion of the OB, these layers include the olfactory nerve layer located just above the cribriform plate of the ethmoid bone, followed by the glomerular layer, external plexiform layer, mitral cell body layer, internal plexiform layer, and granule cell layer (Kandel et al., 2013). Each level possesses unique characteristics contributing to the fine discrimination necessary for interpreting a wide range of odors.

Pseudostratified columnar epithelia line the interior of the nasal cavity (Morrison and Costanzo, 1992), making up the nasal mucosa. Olfactory epithelia include ORNs, supporting or sustenacular cells, stem cells, microvillar cells, and Bowman’s glands (Costanzo et al., 2012; Kandel et al., 2013). Lamination of the OB begins in the olfactory nerve layer (ONL), the superficial OB region just above the cribriform plate, through which the ONs project to the glomerular layer (GL). In the GL, which ORN axons do not pass, oval-shaped regions densely populated with unmyelinated axons, dendrites, and glial processes, or neuropil, contain the axo-dendritic and dendro-dendritic synapses of various cell types. Chao et al. (1997) noted that these glomeruli are compartmentalized with sensory synaptic and central synaptic layers (Figure 1.4B). These observations were validated in 2001 when Kosaka et al. (2001) confirmed separate glomerular regions showing complex interdigitation, with differential cellular components and molecular characteristics. The sensory synaptic portion of each glomerulus is defined by ORN axons, which create axo-dendritic synapses with excitatory mitral and tufted cells and inhibitory
Figure 1.4 Organization of the Olfactory Bulb

A. Several cell types comprise the layers of the OB. Olfactory receptor neurons (ORNs), which originate in the olfactory epithelium (OE) and cross the cribriform plate, synapse onto mitral, tufted, and centrifugal cells in the olfactory bulb (OB) glomerular layer (GL). Inhibitory periglomerular cells surround these synapses with axo-dendritic connections, providing a source of signal modulation. The external plexiform layer (EPL) contains post-synaptic tufted cells, as well as dendrites of mitral cell bodies that lie within the mitral cell layer (MCL). Neurons of the deeper granule cell layer (GCL) offer an additional level of modulation before olfactory signals are transmitted through the lateral olfactory tract (LOT) to the olfactory cortex. B. Organization of the GL, densely populated with sensory axons, dendrites of relay (R) and periglomerular (PG) cells, and glia is shown. This area can be divided based on the specific synapses within each glomerulus: sensory ORN afferents (ORN) in the outer portion (sensory synaptic; SS), and centrifugal projections (cFA) localized to the interior regions of the glomerulus, toward the OB core (central synaptic; CS). The glomerular layer can be further divided by the absence (A⁻) or presence (A⁺) of astrocytes and blood vessels (BV), which are restricted to the CS compartment. (Chao et al., 1997; Simpson and Sweazey, 2013)
periglomerular cells. In contrast, the central synaptic layer does not contain the axons of ORNs, but is comprised of a variety of dendro-dendritic synapses between mitral, tufted, periglomerular, granule, and short axon cells, as well as axo-dendritic synapses with extrinsic fibers (White, 1965).

Each glomerulus is surrounded by two types of periglomerular (PG) interneurons that facilitate inter-glomerular communication. Type 1 PG cells are found in both the sensory and central synaptic layers and express \( \gamma \)-aminobutyric acid (GABA) and tyrosine hydroxylase (TH), the rate-limiting enzyme for the production of the catecholamine neurotransmitters (Kosaka et al., 2001). Conversely, type 2 cells only extend into the central synaptic region and express calbindin d28k and calretinin (Kosaka et al., 2001). Electron microscopic and immunohistochemical analyses showed that astrocytes are also distributed heterogeneously throughout the glomerular layer, although most astroglial cell bodies are integrated with periglomerular cells, delineating the GL from the ONL below and the external plexiform layer (EPL) above (Valverde and Lopez-Mascaraque, 1991). Astrocytes, which mediate a number of cellular processes to maintain the microenvironment of the OB, such as neurotransmitter uptake and ion exchange, extend processes into one or several glomeruli to contact periglomerular cell bodies, dendrites, and dendro-dendritic synapses (Chao et al., 1997). Interestingly, in the OB, astrocytes do not form triad synapses with sensory cell axo-dendritic connections as they do in other CNS structures. Although they are sparse in the sensory synaptic region, astrocytes comprise about 7% of all cell bodies in the GL (Chao et al., 1997). Information processing among astrocytes associated with a single glomerulus occurs through extracellular potassium produced by neuronal activity (Roux et al., 2011). One of the first suggestions for the function
of astrocytes in the bulb deemed them regulators of ORN axonal growth, as they promoted growth in the ONL and GL, but also inhibited axonal progression in deeper layers of the OB (Poston et al., 1991). In addition to these cell types, studies have shown that microglia and endothelial cells of capillaries are also present within glomeruli, but are likely restricted to the central synaptic compartment. Evidence suggests that microglia are present in all bulb layers, but populate non-glomerular layers more densely than the GL (Okere and Kaba, 2000), possibly playing a role in immune responses as well as non-glomerular synaptic function (Miyamoto et al., 2013; Murabe and Sano, 1982).

The next layer of the OB, the external plexiform layer (EPL) contains the length of the apical and secondary dendrites of mitral cells extending into glomeruli and protruding horizontally in the EPL, respectively (White, 1965). Many cell bodies of the axons that course the EPL are located in the mitral cell body layer (MCL), the next layer of the OB. Although the main cells in this layer are mitral cells, tufted and periglomerular cells are also present, and enhance the signals relayed from the olfactory epithelium to the olfactory cortex. Ezeh et al. (1993) noted two mitral cell types exist, some extending their dendrites from deep within the EPL, requiring high intensity input, while others are positioned more superficially, closer to the GL, effectively responding to lower intensity stimuli, demonstrating cell positioning in the OB is closely associated with function. Given that tufted cells are typically more superficial than mitral cells, they often respond more strongly to ON stimulation (Ezeh et al., 1993).

Horizontal axons of a variety of cell types are found in the internal plexiform layer (IPL). This layer is rich in fibers of tufted, centrifugal, and short axon cells (Shepherd, 1972). Processes of
granule cells also pass through this layer, but originate in the granule cell layer (GCL), the deepest layer of the OB. Granule cells, typically clustered the GCL, make reciprocal dendro-dendritic synapses on mitral cells. Similar to amacrine cells of the retina, granule cells have no identifiable primary axon, as all processes resemble dendrites (Shepherd, 1972). In 2002, granule cells, which have been classified as the most numerous cell type in the bulb (Shepherd, 1972), were confirmed as a prominent source of inhibition in the OB through dendro-dendritic synapses onto mitral cells, characterized by the release of GABA onto mitral cells, and the release of glutamate from mitral cells onto granule cells (Urban, 2002). Fibers of other cell types also appear in the GCL including the axons of mitral, tufted, and short axon cells, which converge to form the lateral olfactory tract (LOT). Additionally, perforating the GCL are extrinsic fibers from the telencephalon to the OB such as centrifugal fibers and projections from brain regions including the anterior olfactory nucleus and anterior commissure (Shepherd, 1972). The layers of the OB end with the GCL, from which the LOT projects to higher order brain regions for cognitive processing. 

The specialized bipolar sensory cells of the olfactory system, ORNs, are responsible for detecting odorants and transmitting appropriate information to the OB. Uniquely, these cells are the only first order sensory neurons that extend from the peripheral nervous system directly to the brain. At the apical end of ORNs, a single dendritic process bearing abundant cilia expressing odorant receptors projects into the nasal mucosa, while the basal end is characterized by a thin, unmyelinated axon that courses the epithelium and terminates in the OB (Morrison and Costanzo, 1992). In 1991, Richard Axel and Linda Buck revealed the odor detection mechanism of the olfactory system was similar to the visual system, mediated by G-protein coupled receptor
GPCR) signal transduction (Buck and Axel, 1991). These GPCRs, seven transmembrane domain receptors, are responsible for detection of odor molecules, or odorants, in the nasal mucosa. Activation of G_{olf}, the specific G protein localized in ORNs, triggers second messenger adenyl cyclase III and the subsequent production of cAMP (Menco et al., 1992) to propagate an action potential through the unmyelinated axon toward the OB, where the signal is relayed to other cells in glomeruli. Additionally, the ORN to glomerular ratio in the rodent is 2:1, and 16:1 in humans, as ORNs expressing the same receptor types converge on the same glomeruli (de Castro, 2009). Each ORN expresses a single odorant receptor gene, which can detect one or a small number of odorant molecules with similar molecular conformation (Buck and Axel, 1991). Although only one odor receptor type is expressed on each ORN, complex combinations of odorants result in the perception of distinct smells, termed a combinatorial receptor code (Malnic et al., 1999). Once odor transduction has begun, signals from the olfactory mucosa are relayed to the OB to undergo further processing.

Axons of ORNs extending from the olfactory epithelium form bundles of fibers, or fascicles, of which about fifty form the olfactory nerve (ON), also identified as cranial nerve I. Fascicles are defined by perineurial fibroblasts, within which lie groups of ORN axons wrapped by a special glial type, olfactory ensheathing cells (OECs), bound by a basal lamina, and separated by collagen fibrils (Field et al., 2003). Uniquely, S100$^+$ and P75$^+$ OECs exhibit similarities to both astrocytes and Schwann cells, but are restricted to the olfactory nerve layer (Ramon-Cueto and Nieto-Sampedro, 1992). OECs protect the vulnerable unmyelinated axons of ORNs and facilitate their passage from the peripheral nervous system into the CNS, extending along the length of the axons until they reach the OB, where astrocytes become the primary glial cell
Furthermore, OECs exhibit phagocytic properties, produce NFκB, TNFα, IL-1β, IL-6 as well as other inflammatory cytokines, and express macrophage markers such as toll-like receptors and OX-42 in response to lipopolysaccharide (LPS), a microglial activator (Su et al., 2013). Also present within each ON fascicle are Iba-1 and annexin A3 immunoreactive cells, likely microglia, the innate immune cells of the CNS, serving as the first line of defense against CNS invasion of pathogens (Smithson and Kawaja, 2010). Projecting from the olfactory epithelium, the ON pierces the cribriform plate of the ethmoid bone in the ventral portion of the cranium, and projects superiorly toward the ipsilateral OB, where ONs undergo terminal ramifications and synapse onto mitral and tufted cells in glomeruli. Each glomerulus contains numerous axonal arborizations from ONs, as ORNs only express one receptor type and subsequently only project to one glomerulus, yet several ORNs can express the same receptor, allowing the axonal endings of multiple ORNs to innervate each glomerulus (Buck and Axel, 1991). In the OB, ORNs synapse onto dendritic processes of mitral, tufted, and periglomerular cells. Axons of the mitral and tufted neurons eventually converge to form the lateral olfactory tracts (LOT), which project to the olfactory cortex.

**Synapses of the Glomerular Layer**

For many years, the basic cellular composition and synaptic organization among ORN, mitral, tufted, and periglomerular cells in the GL have been known (Pinching and Powell 1971a, b; Pinching and Powell 1972a,b; Price and Powell, 1970; Shepherd, 1972; White, 1965). In the GL, it has been established that olfactory axons terminate in axo-dendritic connections with characteristic post-synaptic densities, representative of excitatory synapses. Moreover, pre-synaptic marker synaptophysin colocalizes with olfactory marker protein (OMP), a marker for
ORNs. Furthermore, glutamate serves as the neurotransmitter relaying signals from ORNs to mitral, tufted, and periglomerular cells (Berkowicz et al., 1994; Trombley and Shepherd, 1993). This synaptic turnover is mediated by ionotropic NMDA, AMPA, and kainate receptors as well as some metabotropic mGluR receptors (Berkowicz et al., 1994; Berkowicz and Trombley, 2000). Most synapses in the GL are between ORNs and mitral or tufted cells. In decreasing abundance, the remaining synapses are those involving centrifugal fibers, ORNs onto periglomerular cells, periglomerular cells onto mitral cells, and lastly mitral and tufted cells onto periglomerular cells. For comparison, the number of periglomerular to mitral and tufted cell synapses is twice that of mitral and tufted to periglomerular cell synapses, emphasizing the large degree of inhibition in the GL (Kasowski et al., 1999).

Other important connections in the GL include those involving periglomerular and granule cells. Inhibition from these interneurons contributes to feature extraction, noise reduction, and contrast enhancement of olfactory input (Linster and Cleland, 2002). Periglomerular cells are excited by ORNs or mitral cells, and in turn, inhibit mitral cells in the same glomerulus or in adjacent glomeruli through lateral axonal projections (Linster and Cleland, 2002). Granule cells have reciprocal synapses with mitral cells in the EPL, on which mitral cell secondary dendrites excite and the granule cell dendrites inhibit. Interestingly, periglomerular and mitral cells are activated by nicotinic acetylcholine receptor agonists, and muscarinic acetylcholine receptor agonists inhibit granule cells, demonstrating that cholinergic fibers can both increase periglomerular activity and decrease granule cell inhibition (Linster and Cleland, 2002). The horizontal limb of the diagonal band of Broca provides a large source of cholinergic innervation and plays a role in olfactory short-term learning, olfactory habituation, and discrimination of overlapping olfactory
stimuli (De Rosa et al., 2001; Hunter and Murray, 1989; Ravel et al., 1994). Lesioning the acetylcholine tract increases perceptual overlap, strengthening the concept that cholinergic inputs fine-tune olfactory discrimination (Linster and Cleland, 2002).

Considering the OB is the largest dopaminergic system of the forebrain, with about 5% of all neurons in the olfactory bulb being dopaminergic interneurons (Cave and Baker, 2009), it is conceivable that dopamine plays a large role in olfaction. Dopaminergic periglomerular cells receive axo-dendritic inputs and subsequently inhibit other cells of the OB, while non-dopaminergic periglomerular cells, expressing calbindin and calretinin, only participate in dendro-dendritic synapses (Kosaka et al., 1995, Kosaka et al., 1998). About 10-16% of all periglomerular cells restricted to the glomerular layer are dopaminergic and coexpress GABA (Kosaka et al., 1995; Lledo et al., 2006; Parrish-Aungst et al., 2007). Hyperpolarizing neurons by mediating the influx of negatively charged chloride ions or the efflux of positively charged potassium ions, GABA is an important inhibitory neurotransmitter (Purves et al., 2008). Together, GABA and dopamine contribute to olfactory discrimination by modifying sensory information traveling from the bulb, proven by an enhanced responsiveness and inability to discriminate odors due to olfactory deprivation-mediated decreases in OB dopamine levels (Lazarini and Lledo, 2011). One purpose of dopamine in the GL is to modulate the release of glutamate from ORNs (Berkowicz and Trombley, 2000). Evidence of the importance of dopamine in the olfactory system is highlighted in Parkinson’s disease, in which the degeneration of dopaminergic neurons often results in a characteristic loss of olfaction (Doty, 2012). Interestingly, dopaminergic modulation of synaptic transmission occurs through pre-synaptic D2 receptors present on inhibitory neurons (Cave and Baker, 2009; Ennis et al., 2001).
Activation of these D2 receptors decreases cAMP and inhibits adenylyl cyclase, thus reducing the number of pre-synaptic vesicles released in the GL. Additionally, concentrations of tyrosine hydroxylase, dopamine production, GABA release, and glutamate decarboxylase mRNA expression are all reduced by D2 activation. These reductions result in decreased inhibition of mitral and tufted cell output, leading to enhanced synaptic transmission (Berkowicz and Trombley, 2000).

As briefly mentioned, synapsins are the major phosphoproteins localized to nerve terminals (Johnson et al., 1972), which can serve as functional synaptic markers. Both Synapsin-I and Synapsin-II form two isoforms that have been detected (Südhof and Jahn, 1991), all having identical amino terminal regions and variable carboxy-terminals. These synaptic proteins bind to elements of cytoskeleton including microfilaments, microtubules, and αII-spectrin (Baines and Bennet, 1985; Baines and Bennet, 1986; Bähler and Greengard, 1987; Petrucci and Morrow, 1987) to control synaptic vesicle release. Functionally, Synapsin-I in the OB is required in GABAergic interneurons for short-term habituation (Sadanandappa et al., 2013) and early odor memory formation (Knapek et al., 2010). A role for Synapsin-II has also been uncovered for pre-synaptic GABA release synchronization (Medrihan et al., 2015) through interaction with pre-synaptic calcium channels (Medrihan et al., 2013), which facilitates control of neurotransmitter release at inhibitory synapses. Interestingly, Synapsin-I expression has largely been found in the OB core, with mRNA mostly found in the MCL and GCL in adult rat (Melloni et al., 1993), while Synapsin-II has primarily been detected in the GL (Stone et al., 1994), where ORNs synapse onto their post-synaptic targets. Understanding the anatomical organization, synaptic
components, and mediators of plasticity of the OB provides a basis for addressing concerns regarding dysfunction of the olfactory system observed after injury.

**Induction of Post-Traumatic Anosmia**

Beginning as early as the 19th century, there have been documented cases of post-traumatic loss of smell, or anosmia, yet all processes underlying olfactory dysfunction and the mechanisms to restore olfaction remain elusive (Costanzo et al., 2012). Several case studies have been conducted over the past decades, demonstrating the persistence of TBI related anosmia. An estimated 15-20% of TBI patients experience anosmia when a cerebral contusion is involved (Hagan, 1967), along with 31% of patients with severe head injuries experiencing post-traumatic amnesia for more than 24 hours (Sumner, 1964). The incidence is drastically reduced to 3-8% for those who sustain injuries with no loss of consciousness or post-traumatic amnesia lasting less than one hour (Sumner, 1964). Costanzo and Becker (1986) reported that males between the ages of 15 and 24 make up a substantial percentage of reported cases of post-traumatic anosmia with most head injuries sustained by motor vehicle accidents. In 1991, Costanzo and Zasler (1991) reported incidence of post-traumatic anosmia around 25-30% of all severe head injuries, 15-19% of moderate injuries, and 0-16% of mild head injuries, which is in accord with older reports of olfactory disturbances associated with head trauma (Sumner et al., 1964). These data indicate a correlation between degree of TBI and the probability of acquiring anosmia. Most often, TBIs result from strikes to the frontal cortex, but TBIs caused by falls and other events leading to blows to the occipital lobe present a greater incidence of anosmia (Costanzo et al., 2012). Patients with occipital impact injuries appear five times more likely to suffer olfactory impairments than those with frontal head injuries (Sumner, 1964) due to the coup and contre-
coup forces involved. It has been established that complete loss of smell is often irreversible, but phenomena such as hyposmia, in which olfaction is diminished, or dysosmia, in which it is distorted, may resolve over time, indicating that post-injury synaptic plasticity is involved. Overall, changes in the ability to perceive odors can significantly reduce quality of life for TBI patients.

Head trauma can induce anosmia by three primary mechanisms. Trauma to the face, resulting in broken facial bones causes superficial damage to the olfactory epithelium and disruption of the sinonasal tract (Costanzo et al., 2012). Similar injuries can also follow medical treatments and processes such as the use of a nasal cannula to assist breathing, which can directly damage nasal epithelia, and some medications, which can inadvertently impede the function of smell. The most common mechanism of post-traumatic anosmia, however, may result as a consequence of the anatomical arrangement of the olfactory nerves, which makes them vulnerable to stretching and severing (Costanzo et al., 2012). Shearing forces caused by the involuntary rapid acceleration and deceleration of the brain can damage ORN axons at the point at which they course through the cribriform plate, leading to partial deafferentation of the OB, altered synaptic circuitry, and subsequent olfactory dysfunction. Additionally, notable damage to other olfactory structures in patients presenting with post-traumatic anosmia has been observed, with the most common sites of injury being the olfactory bulbs and olfactory tracts, followed by the interior frontal lobes (Yousem et al., 1996). Hagan (1967) showed that damage to the lateral olfactory tracts and cortical regions can cause disturbances in olfaction, although the incidence is low. As the OB sits beneath the cortex in the human brain, just superior to the cribriform plate, direct focal damage directly to the OB is not very common. Moreover, MRI studies have revealed that
complete anosmia due to cortical injury is unlikely, considering the lack of olfactory impairment linked to specific higher order brain regions of the olfactory cortex. Although there were no direct comparisons between TBI patients with and without anosmia in these MRI studies, the volume of bulbs and tracts in anosmic patients was reduced in comparison to normosmic or hyposmic patients (Yousem et al., 1996), possibly as a result of synaptic loss.

Development of anosmia following broken facial bones, use of nasal cannula, or medications may be remedied with surgery, nasal steroid sprays, or ceasing use of medication, respectively (Costanzo et al., 2012). Post-traumatic anosmia as a result of head injury, however, has a limited propensity for recovery. Literature suggests spontaneous recovery due to regeneration of olfactory receptor neurons occurs at a rate of around 30%, with Ikeda et al. reporting 24%, Costanzo and Becker suggesting 33%, and Doty et al. proposing 36% (Costanzo and Becker, 1986; Doty et al., 1997; Ikeda et al., 1995). Despite the breadth of information implicating post-traumatic anosmia as a pathological result of head injuries, experimental treatments have yet to prove effective. Clinical studies exploring potential therapeutics for post-traumatic anosmia have had limited success, but current experimental investigations in rodents show great promise. In addition to the heterogeneity of head injuries, obtaining human subjects or samples for experimentation has proven difficult, complicating translation from bench to bedside.

**Diagnosis of Anosmia**

Diagnosis of anosmia often offers a poor prognosis, although a few cases have reported functional recovery after long periods of time. Most clinical studies show there is a limited chance of recovery which typically occurs between twelve weeks and two years after head
trauma (Reiter et al., 2004; Sumner, 1964), while some have documented patients regaining a
sense of smell five, seven, and even nine years after diagnosis (Mueller and Hummel, 2009;
Sumner et al., 1964; Zusho, 1982).

Odorant identification provides an important avenue for diagnosis of anosmia following head
injury, as odor quality perception is altered following olfactory nerve transection (Yee and
Costanzo, 1998). Unfortunately, subjective olfactory tests can be compromised by cognitive
deficits or other effects of injury. In lieu of these tests, common neuroimaging technologies may
be utilized for assessing olfactory damage. High resolution CT scans are useful diagnostic tools
for evaluating damage caused by bones, while MRIs prove useful for analyzing soft tissue
damage (Costanzo et al., 2012). Recently, Rombaux et al. (2012) revealed OB volume at the
time of diagnosis of olfactory dysfunction may be prognostic in evaluating the likelihood of
recovery from post-traumatic or post-infectious olfactory loss. Gender, age, size of OB,
mechanism of olfactory loss, and specific olfactory dysfunction proved to play a role in the rate
of recovery, as women, young patients, and those with OBs larger than 40mm$^3$, showed that
postinfectious olfactory loss, and hyposmia, rather than anosmia, demonstrated the best chances
of recovery (Rombaux et al., 2012). There are a number of diagnostic methods for identifying
patients with post-traumatic anosmia, but effective treatments for olfactory impairment have yet
to be determined.

One of the most common diagnostic tests employed for post-traumatic anosmia is the University
of Pennsylvania Smell Identification Test (UPSIT), a simple test involving the identification of
forty olfactory cues, for which four different multiple choice options are provided for each
sample odorant. The number of correct choices determines patients’ scores, which are classified into one of six categories to confirm overall degree of olfactory deficit (Doty et al., 1984). In addition to differences found regarding age, race, and smoking habits, there are also slight differences related to gender, as a score of 34-40 is normal for men, and 35-40 is normal for women (Doty et al, 1997). Other less extensive smell tests have been developed from the UPSIT, including the 5-item pocket smell test and the 12-item brief smell identification test (Costanzo et al., 2012). Tests such as the UPSIT and the University of Connecticut Chemosensory Clinical Research Center Test are useful, assessing both detection and identification of odorants, while others only offer a strategy for detection. Some tests, such as the T&T Olfactometry and Alinamin tests, address recognition thresholds, while others like the Alberta Smell Test focus on laterality of olfaction (Costanzo et al., 2012). As some assessments of olfactory performance may be expensive, extensive, and time-consuming, methods such as Sniffin’ sticks have been developed to decrease time and costs for quick diagnosis (Kobal et al., 1996).

Post-Traumatic Anosmia and Quality of Life

Despite an understanding of the debilitating consequences of post-traumatic anosmia, few have detailed the specific ways in which quality of life can be reduced. Drummond et al. (2013) conducted a study evaluating the impact of anosmia in everyday tasks. Patients reported enduring a wide range of emotions from embarrassment to loss of identity. Depression has also been documented as a common consequence of the inability to smell (Atanasova et al., 2008; Callahan and Hinkenbein, 1999; Negoias et al., 2010). Those with anosmia who hold olfaction-dependent occupations such as chefs, florists, chemists, and firefighters are likely to experience a
greater degree of frustration, considering their lives are strongly based on the proper function of the olfactory system (Costanzo et al., 2012). Additionally, for those with careers that do not require a keen sense of smell, quality of life is still severely diminished considering daily activates involving safety, hygiene, and child care become exponentially more difficult.

**OB Plasticity and Approaches for Inducing Repair**

Published reports have shown that, even after injury, ORNs maintain and exhibit a clear regenerative capacity. For example, at 35d post olfactory nerve transection, a deafferentation model, small ovoid collections in the glomerular layer and smaller glomerular-like structures throughout the OB indicate ORN regeneration and, albeit distorted, reinnervation of the OB (Morrison and Costanzo, 1995). Studies injecting the olfactory epithelium with horseradish peroxidase to label pre- and post-synaptic terminals also suggest these regenerative ORNs are functional. Focusing more on specific stages of regeneration, Hirata et al. (2008) conducted studies utilizing sodium dimethyldithiocarbamate, or DDTC, which severely damages the olfactory epithelium. Electron microscopic analyses showed both recovery of olfactory vesicles with immature profiles at 3 weeks after DDTC injection, indicating ORN reinnervation of the OB, and mature, specialized ORN cilia appearing at 6 weeks, representing restoration of synapses (Hirata et al., 2008). Despite regeneration and extension into the OB, it has been proven that ORN axons can project to inappropriate OB layers, incorrectly and partially innervating multiple glomeruli instead of one, likely due to competition among axons to occupy synapses (Costanzo, 2005). Notably, ORNs must course a long path between the olfactory epithelium and the glomeruli of the OB, making synaptogenesis difficult and lengthening the time for recovery. Although ORNs are capable of regeneration into the OB, abnormal synaptic
connections may underlie the persistence of olfactory impairment after injury, suggesting mechanisms of this regenerative plasticity should be explored to help facilitate proper synapse formation to restore function.

As mitral cells are one of the main output cells of the OB projecting to the olfactory cortex, some recovery studies have focused on their functional repair instead of that served by input fibers. Olfactory tract transection (OTT) studies revealed 3-4 weeks after OTT, mitral cell numbers decreased significantly and returned to normal at 5 weeks. In a similar pattern, neurotrophic factors FGF-2, IGF-1, and BDNF were significantly increased at 2, 5, and 6 weeks after transection (Mansoor et al., 2012), suggesting these molecules may have a functional role in restoring cell counts. Mitral cell function after OTT has also been explored electrophysiologically. At 120 days after transection, structural and functional recovery were confirmed by synchronized firing between mitral cells and the piriform cortex (Anil et al., 2012), suggesting restored mitral cell function could be an additional factor in the spontaneous recovery seen in some anosmic patients.

Although olfactory deficits persist after TBI, the olfactory system has a strong propensity for regeneration from several sources, including stem cell replacement. Basal cells of the olfactory epithelium are a major source of stem cells for ORNs throughout life, yet the subventricular zone (SVZ) along the lateral ventricles contains the largest pool of neural stem cells produced into adulthood and also provides adult stem cells that eventually integrate into the OB. Neuroblasts originating in the SVZ travel through the rostral migratory stream (RMS) to the OB where majority become granule and periglomerular cell interneurons (Carleton et al., 2003; Kishi,
Many studies have focused on this neurogenesis as a source of functional recovery, but implications have yet to be fully defined. In models of TBI and stroke, neurogenesis has been largely examined in the context of SVZ migration into the injured OB (Kernie and Parent, 2010; Radomski et al., 2013). Increasing new interneurons in the OB enhances olfactory memory, and a decrease alters olfactory discrimination (Carleton et al., 2003), showing these cells play an important role in OB plasticity and could contribute to functional recovery of post-traumatic anosmia. In injury models such as cortical contusion, SVZ proliferation increased, yet olfactory discrimination deficits persisted at 30d (Radomski et al., 2013). Interestingly, Thored et al. (2009) found that after focal ischemia, neuroblasts from the SVZ are redirected from the RMS to the site of injury, therefore reducing the number of new neurons migrating to the OB, possibly contributing to the persistence of anosmia in TBI cases with clear vascular pathology. Conversely, others have documented transient expression of nestin+ radial glial cells after destruction of the olfactory epithelium (Hirata et al., 2008), which could indicate migration from the subventricular zone to degenerating terminals at the site of injury (Duggal et al., 1997) to promote recovery mechanisms.

In exploring the mechanism for regulation of OB cell replacement, some investigators have examined the effects of inflammation during neurogenesis. According to Lazarini et al. (2012), neuroprotective antibiotic Minocycline decreases inflammation and increases the survival of adult-born neurons by preventing the phagocytic properties of microglia, including impairment of neurogenesis. Suggesting the same concept of inflammation-mediated SVZ neuroblast inhibition, Tepavčević et al. (2011) demonstrated decreased SVZ neuronal supply to the OB in targeted experimental autoimmune encephalomyelitis (EAE) models of chronic inflammation,
which induced long term olfactory memory impairment, while short-term memory and odor
discrimination were unaffected. These findings not only demonstrate an inflammation-mediated
reduction of SVZ generated stem cells after injury, but also imply granule and periglomerular
interneurons produced by the migrating SVZ cells could be important factors involved in long-
term OB plasticity. While inflammation may play a role in SVZ neurogenesis, conflicting
evidence exists on the effects of reactive glia such as microglia in this process (Sierra et al.,
2010; Thored et al., 2009). Anti-inflammatory drugs have proven to increase survival of stroke-
generated neurobalasts (Hoehn et al., 2005), yet chronically activated microglia permitted
positive neuronal differentiation and survival in mouse cultures (Cacci et al., 2008).
Additionally, Yan et al. (2007) noted activated microglia and reactive astrocytes express
monocyte chemoattractant protein-1 (MCP-1), which promotes migration of neuroblasts.
Interestingly, MCP-1 is elevated in areas of the brain vulnerable to TBI (Woodcock and
Morganti-Kossmann, 2013). Although not immediately related to microglial function, the Yan
research group (2006) also reported IGF-1 as an important modulator of neural precursor cell
proliferation in the SVZ, and neural progenitors express IGF-1 receptors, an important find
considering secreted IGF-1 can act as a chemoattractant critical for olfactory nerve innervation
of the OB (Scolnick et al., 2008). Taken together, these studies showing that neurogenesis
affects OB regeneration provide insight as to how neurons and glia interact during restoration of
OB circuitry and function after TBI.

As an alternative approach, some investigators have suggested steroid treatment or the use of
Vitamin A to alleviate olfactory dysfunction after injury, but limited success has been achieved
(Ikeda et al., 1995). In one study, listing trauma as an exclusion factor, anosmic patients given
combined steroid treatment with systemic prednisolone and topical glucocorticoid fluticasone propionate demonstrated improved olfactory function (Blomqvist et al., 2003). Conversely, in a clinical investigation conducted by Jiang et al. (2010), which explored the efficacy of prednisolone for restoring the sense of smell in patients with head trauma, some cases showed signs of recovery, but the data were considered inconclusive due to small sample size and the inability to obtain all appropriate control groups. Additionally, the possibility of spontaneous recovery, a well-documented phenomenon, was not addressed in these results, and could be explained by the fact that some participants may have regained olfactory function after circuit regeneration. Similarly, a double-blind, placebo-controlled, randomized clinical trial assessing the potential of Vitamin A as a treatment for post-traumatic anosmia revealed no effect when administered orally (Reden et al., 2012), however, retinoic acid, a byproduct of Vitamin A and promoted extension of regenerated ORNs in the OB (Luxenhofer et al., 2008). Vitamin A treatment also improved recovery in an experimental rodent model of trauma, validated by a decreased latency for finding buried food (Yee and Rawson, 2000).

In another clinical trial, zinc sulfate, a compound which can enhance regeneration, appeared a more promising treatment for patients with post-traumatic anosmia rather than post-viral anosmia (Aiba et al., 1998). As post-traumatic anosmia is most often acquired from severance of select ORN axons and post-viral anosmia involves complete cell loss in the olfactory epithelium, enhanced regeneration supported by spared basal cells in patients with post-traumatic anosmia is more probable considering the olfactory epithelium is not completely destroyed. Although a variety of potential treatments may prove useful for some anosmic patients, many do not seem to significantly affect anosmia induced by TBI. One explanation is that those which are successful,
like steroids, act superficially on the nasal epithelium, reducing inflammation to permit regeneration. This is probably due to the fact that cell-mediated inflammation induced by TBI indirectly affects the nasal cavities, attenuating regeneration and undermining olfactory impairment after trauma.

**Treatment of Olfactory Dysfunction in Animal Models**

Regarding olfactory dysfunction after TBI, animal models present a challenge, as behaviors, especially those mediated by smell, are not identical to humans. However, experiments in the laboratory certainly offer invaluable insight for understanding TBI pathology. Considering the superior sense of smell in other mammals and rodents, similar animal models can in fact be excellent for testing olfaction. For example, application of steroids has not been particularly effective in the clinic, however, administration of steroids at early time points after injury have facilitated functional recovery in the rat (Kobayashi and Costanzo, 2009). Other drug treatments have also been explored in rodent models in order to facilitate olfactory bulb recovery. Minocycline, a known neuroprotective anti-inflammatory antibiotic, prevents decrease in bulb volume after injury (Siopi et al., 2012), suggesting the overall reduction in inflammatory processes also prevents some neurodegenerative processes. Similarly, Etazolate, an α secretase activator, also exhibited neuroprotective effects in the rodent OB after TBI by decreasing edema and IL-1β levels, increasing sAPPα and cAMP, and improving functional recovery (Siopi et al., 2013). Although administration of drugs seems the most likely treatment for TBI patients, some studies explore the effects of environmental enrichment in recovery. Johnson et al. (2013) found that environmental enrichment before TBI protects against functional deficits, increasing spatial memory and sensory discrimination after TBI. One study conducted by Yee and Costanzo
(1998) suggested that odor training could enhance olfactory discrimination, revealing that pharmacological intervention is not necessarily the only treatment mechanism capable of restoring the sense of smell. Each of these approaches to improve synaptic reorganization in animal models of TBI are feasible for clinical translation and have potential to serve as therapies for olfactory dysfunction after brain injury.

**OB Synaptogenesis after Injury**

Synaptic replacement in the OB is more complex than in most other areas of the CNS. It not only involves local axonal sprouting at sites of new synapse structure, but requires generation of new pre-synaptic neurons and growth of their axons over much greater distances to reach new post-synaptic sites. Basal stem cells of the nasal epithelium regenerate ORNs every month in the human (Moulton, 1974) – a process which leads to the differentiation of new pre-synaptic sensory cells and reinnervation of OB synapses. Despite a fairly intact olfactory epithelium after injury, IHC and ultrastructural analyses of biopsied tissue from patients with post-traumatic anosmia displayed olfactory epithelia distinctly different from those of control subjects (Jafek et al., 1989; Kern et al., 2000). Cells in the epithelium were less compact, enlarged, and nuclei were rearranged. ORNs of these patients bore no cilia for sensory reception, one clear morphological confirmation that olfaction was inhibited. Additionally, axon tangles appeared in the epithelium revealing passage of ORNs to the OB was inhibited (Jafek et al., 1989), possibly due to trauma-induced fibrotic scarring at the base of the cribriform plate, which often occurs if both the ORN axons and the OB are injured, and could underlie altered mapping of odorant patterns in the OB after injury (Costanzo, 2005; Kobayashi and Costanzo, 2009). Considering sensory ciliogenesis and establishment of contact between ORN axons and their post-synaptic
OB targets are concurrent processes (Cuscheri and Bannister, 1975), the limited reinnervation of the OB could explain the lack of cilia on the visualized ORNs.

From animal models of OB damage we know that axons of nascent ORNs are capable of growing into nonspecific target regions and forming synapses (around 20 days after bulbectomy in the mouse), despite having no appropriate mitral or tufted cell post-synaptic target (Graziadei et al., 1978). Axonal growth supporting synaptic reinnervation in the OB differs from local collateral axon sprouting that occurs to replace damaged terminals in other brain regions, as the olfactory sensory cells must differentiate, mature, extend axons that migrate a considerable distance to their targets and form synapses. This process takes between 60 and 120 days post-deafferentation in the rodent olfactory system (Graziadei et al., 1978; Graziadei et al., 1979), as terminal degeneration occurs 1-15 days after axotomy, regeneration between 15-60 days, and synapse stabilization extends well past 60+ days post-axotomy. Under normal conditions, axons of these neurons grow and re-innervate the post-synaptic mitral and tufted cell targets in the glomerular layer (Nagayama et al., 2014) either as part of typical physiological processes or in response to traumatic injury. Chemical ablation of ORNs with Triton X-100 (Nathan et al., 2001) and methyl bromide (MeBr; Bakos et al., 2010) has demonstrated that these cells can also regenerate after complete epithelial loss.

Olfactory nerve transection, which more accurately models the OB deafferentation after TBI, has also been explored, and functional assays have proven that regeneration can occur as ORNs form appropriate connections with post-synaptic targets. Early studies in the pigeon documented regrowth of sensory neurons after ON transection with recovered behavioral and
electrophysiological responses (Oley et al., 1975), and near normal synaptic organization manifesting 30-50d after axotomy (Graziadei and Okano, 1979; Morrison and Costanzo, 1995). Behavioral analyses supported these findings, with first instances of recovery at 19d, but normal behavior not exhibited until 40d post-transection (Yee and Costanzo, 1995). Similarly, utilizing horseradish peroxidase (HRP), which is transferred from a pre- to post-synaptic cell in functional synapses, and electrophysiology, comparing electrical input in pre-synaptic cells to output from post-synaptic cells, other investigators have shown that ORNs form functional synapses after injury. For example, following nerve transection, no HRP staining was detected 4d post-lesion (Morrison and Costanzo, 1995), while HRP was distributed in a uniform pattern much later, revealing 70% reinnervation by 32d and 100% reconnection by 64 and 96d (Jennings et al., 1995). Electrophysiology conducted to detect responses to odor stimuli presented to hamsters with transected ORNs similarly showed that longer recovery periods produced greater responses to stimuli (Costanzo et al., 1985). Later, Koster and Costanzo (1996) also demonstrated that negative evoked potentials (NEPs) were detected after transection, but failed to reach control amplitude, even by 120d post-injury, documenting potential for reconnection but persistent functional deficits.

Complimenting HRP and electrophysiological analyses of ORN recovery, OMP, a cytoplasmic protein specific for mature ORNs and integral to normal sensory neuron function, has also been measured as a marker of synaptogenesis. Reliably, independent of mechanism of denervation, OMP is reduced in several models of OB injury. Both Triton X-100 and MeBr reduced OMP 7 and 15d post-epithelial ablation, respectively, recovering expression by 40d (Bakos et al., 2010; Nathan et al., 2001), while complete bulbectomy reduced OMP at 11d, returning to control levels.
by 35-49d after loss of input (Tsukatani et al., 2003; Inamitsu et al., 1990). Nerve transection models demonstrate the sensitivity of OMP measurements, showing that OMP was lost 3-10d post-injury (Costanzo et al., 2006; Kobayashi et al., 2013), a quicker reduction and return than other models, reflecting a deafferentation-dependent and propensity for recovery. Interestingly, although OMP has been used as a marker of synaptogenesis because of its restricted presence in ORNs, it has also been associated with another marker of synaptogenesis, GAP-43. Tsukatani et al. (2003) demonstrated that when OMP was reduced most, GAP-43 levels in the olfactory epithelium peaked, while Griff and colleagues (2000) suggested a regulatory mechanism between the two, proposing that OMP downregulates GAP-43 such that when OMP is lost, GAP-43 is upregulated, promoting formation of several immature neurons.

Unfortunately, olfactory deficits commonly persist after TBI, despite ORN neurogenesis and reactive synaptogenesis. For example, reduced olfactory function persisted up to 120 days after olfactory nerve transection and OB deafferentation in the mouse, documented by negative evoked potentials, although synapse structure had been restored (Koster and Costanzo, 1996). In this case, it is likely that the ORNs do extend processes, attempting to form viable synapses, but part of the connections are inappropriate, lacking functional utility (Costanzo, 2005). While this process may only be aberrant in a subset of synapses, it still serves as an excellent model to study persistent maladaptive synaptogenesis and define mechanisms of regeneration that may be manipulated to improve olfactory outcome. Interestingly, this outcome is consistent with post-injury clinical observations. Despite aberrant connectivity after injury, the basic phases of reactive synaptogenesis still occur in the OB: terminal degeneration, axon sprouting and synapse reinnervation, and synapse stabilization (Monti Graziadei and Graziadei, 1979). Thus, a study of
the processes governing OB reinnervation may also offer additional answers as to which cellular mechanisms control synaptic plasticity in other brain regions.

Due to secondary injury induced by TBI, a number of molecules are upregulated after insult. As in other regions of the brain, ECM components, including matrix metalloproteinases have been implicated in post-injury synaptogenesis in the OB. In particular, gelatinases A and B (MMP2, 9) are robustly upregulated in deafferented OB, suggesting they may be important in post-injury synaptic reorganization. In nerve transection models, for example, MMP9 peaks at 1d, however, does remain elevated over controls throughout the entire OB degenerative period, up to 15d after injury (Costanzo et al., 2006; Kobayashi et al., 2013), while MMP2 expression peaks somewhat later, at 7d (Costanzo and Perrino, 2008), and returns to control levels more quickly. Olfactory epithelial loss, on the other hand, causes MMP9 to peak at 5d, slower than ON transection, and MMP2 to peak at 5d, sooner but less robust than transection (Bakos et al., 2010). The differential activation of the gelatinases with rapid MMP9 peak and later MMP2 peak, and MMP9 KO animals confirming that these are independent processes, suggests a degenerative function for MMP9 and greater role for MMP2 in regeneration and elongation (Costanzo and Perrino, 2008). Similarly, others have suggested MMP9 is more closely associated with deafferentation, while MMP2 plays a greater role in recovery (Zuo et al., 1998; Hsu et al., 2006; Phillips and Reeves, 2001). These rapid MMP peaks are followed by decreased levels of OMP, suggesting gelatinases may be important in mediating clearance of ORNs. Upregulation of these molecules, which are also linked to synaptic plasticity of the hippocampus (Dziembowska and Wlodarczyk, 2012; Phillips et al., 2014), suggests they play a similar role in promoting plasticity in the OB. Glial cells most likely contribute to this increase in MMPs after injury to the OB, as
olfactory ensheathing cells expressing MMP2 have been considered necessary for axonal regeneration in the OB (Pastrana et al., 2006), and microglia and astrocytes have historically been principal producers of MMPs, especially in injury paradigms. In addition to early investigations showing that phagocytic cells infiltrate 2-3d after nerve transection (Monti Graziadei and Graziadei, 1979), Costanzo et al. (2006) found post-knife lesion elevation in GFAP concurrent with OMP loss, yet surprisingly, the MMP9 peak preceded the GFAP increase and OMP reduction (Costanzo et al., 2006). These important molecules represent a number of factors involved in injury progression, and could potentially serve as targets for therapeutic intervention, as synaptogenesis of the OB may be mediated by these proteases.

**MOUSE OB AS A MODEL OF TBI-INDUCED REACTIVE SYNAPTOGENESIS**

**OB Deafferentation Models**

Different cells throughout the brain are axotomized after TBI. ORNs are subject to injury from head trauma by several mechanisms, one of which includes OB displacement along the cribriform plate, causing sensory axons traversing through the bony structure to become stretched and/or severed (Costanzo et al., 2012) at the point at which they cross. As a result, olfactory function can be severely impaired, as the injured axons often undergo Wallerian degeneration and subsequently retract synaptic input from the OB. The stem cells of the olfactory epithelium and the ability of differentiated nascent sensory neurons to innervate the OB provided the framework for the development of injury models that directly deafferent the OB to assess molecular processes promoting reinnervation after traumatic axotomy and denervation. One methodology which mimics ORN damage caused by severe TBI, the ON knife lesion, or transection, directly axotomizes the cells, as a blade is used to cut the ON just below the
cribriform plate, thus deafferenting the OB. Interestingly, the composition of the blade can dictate severity (Kobayashi and Costanzo, 2009), offering the benefit of graded injuries to distinguish traits of synaptogenesis that may be unique to level of injury. This model can damage both ORN axons as well as OB structures, modeling clinical aspects of traumatic impact where several components of the olfactory system are injured. Conversely, chemical ablation of the olfactory epithelium, which causes widespread ORN death without damaging the OBs (Bakos et al., 2010; Nathan et al., 2001), offers a more direct analysis of the process of OB reinnervation without confounding factors related to OB injury, such as glial scar formation.

Recently, Steuer and colleagues (2014) have proposed a rodent model that applies direct impact force to physically shift the OB perpendicular to ORN input, generating major ORN axon damage at the cribriform plate and significant loss of ORNs in the OE. While this model may mimic aspects of olfactory insult in more severe human TBI, the model directly damages the OB and ORNs, making the study of deafferentation-induced reactive synaptogenesis difficult. By contrast, the central fluid percussion injury model (FPI), which displaces brain matter using a fluid pressure pulse, causes a more diffuse, widespread axonal damage without contusional injury, permitting the assessment of synaptic repair in a variety of brain regions. Adapted from Dixon et al. (1987), FPI has been successfully modified for mice (Carbonell et al., 1998) and historically applied to study cortical, hippocampal, thalamic, and optic nerve damage. Our studies will utilize mild FPI to generate ORN axotomy and olfactory deafferentation without focal OB damage. We will test whether such FPI induces novel MMP9/OPN mediated cell reactivity to regulate TBI-induced OB reactive synaptogenesis.
MMP9 Knockout (KO) Mice, Brain Injury, and Neuroplasticity

Given the implications of MMP9 in CNS development and pathology, several laboratories have utilized MMP9 KO animals to study the role of MMP9 in CNS pathobiology and the neuroplasticity it induces. Genetically modified mice in which MMP9 expression is absent, yet who are phenotypically identical to the WT FVB/NJ background strain, were first generated in 1998 for studies of MMP role in angiogenesis and apoptosis (Vu et al., 1998). More recent studies have used these KO mice to show MMP role in the evolution of variety of CNS insults. For example, Mizoguchi et al. (2011) showed that seizure phenotype could be attenuated in MMP9 KO mice, attributing the effect to a reduced MMP9 processing of pro-brain-derived neurotrophic factor (BDNF), thereby diminishing abnormal recurrent mossy fiber sprouting in the hippocampus. Further, sensitivity to pentylenetetrazol (PTZ)-induced epileptogenesis was likewise decreased in MMP9 KO animals, likely through the same aberrant terminal sprouting mechanism (Wilczynski et al., 2008). MMP9 KO animals have also been used to confirm the enzyme’s role in pathogenesis resulting from certain types of brain injury, as well as the extent of functional deficits produced in these models. For instance, MMP9 KO mice exhibited slightly better motor skills and reduced lesion size acutely in the controlled cortical impact (CCI) model of TBI (Wang et al., 2000), and after transient global or focal cerebral ischemia, MMP9 KO attenuated cerebral damage (Copin et al., 2005; Lee et al., 2004). In these cases, it is likely that MMP9 KO prevented enzyme-associated disruption of ECM and vascular disorganization, resulting in more favorable outcomes. Alternatively, MMP9 may act in a positive role to promote neuroplasticity for improved synaptic function. Axotomy induced by TBI often results in deafferentation and synaptic loss. In this context, several studies have shown that loss of MMP9 significantly disrupts critical ECM reorganization necessary for successful synaptic
plasticity. For example, loss of MMP9 impairs long-term potentiation (Nagy et al., 2006), a cellular model for synaptic plasticity associated with learning and memory. Sensory deprivation in the rodent barrel cortex also causes collateral sprouting of spared fibers, a process which is attenuated in MMP-9 deficient mice (Kaliszewska et al., 2012). As specific mechanisms are still being explored for MMP9 role in reactive synaptogenesis, genetic models such as MMP9 KOs offer excellent experimental options for identifying those underlying processes. We propose that these genetically modified animals will be valuable for exploring the MMP9/OPN role in OB synaptic recovery.

Summary and Hypothesis for Present Study

The prevalence of head trauma, its persistent deficits and limited therapeutic options have encouraged decades of study, typically focusing on cortical injury and subsequent memory, motor, behavioral, and sensory loss. Anosmia is a common post-traumatic sensory dysfunction, attributed to OB deafferentation caused by both primary and secondary injury mechanisms. While such impaired olfaction has long been identified as an effect of TBI (Costanzo and Zasler, 1991; Sumner, 1964; Hagan, 1967; Costanzo et al., 2012), the processes underlying persistent anosmia and specific mechanisms of OB recovery from trauma-induced axotomy and deafferentation are poorly understood. Studies in both cortical brain regions and OB point to ECM components, like MMPs, as major mediators of repair following injury. Given that many aspects of MMP9-mediated plasticity are still unknown, and one of its novel substrates, OPN, has potential to improve neuronal integrity and function after brain injury, we believe that MMP9/OPN interaction is worth investigating in a TBI model of OB deafferentation. The regenerative capacity of the olfactory system, established MMP9 response to OB injury, and
OPN fragment-mediated glial cell reactivity to trauma all suggest that a MMP9/OPN pathway may be instrumental for OB synaptic plasticity. We hypothesize that TBI induces time-dependent changes in MMP9 activity, producing functional receptor-binding of OPN signaling fragments in the deafferented OB. Further, we posit that the binding of these fragments to matrix receptors leads to glial cell response which promotes reorganization of the ECM to facilitate reactive synaptogenesis.

We will employ the fluid percussion TBI model (FPI) in mice to administer widespread, diffuse damage to dissect the MMP9/OPN relationship in ORN reinnervation of the OB after a mild level of injury-induced denervation. In order to first document induction of injury and subsequent synaptogenesis, we will use Western blot protein analysis to assess axonal degeneration, as well as the loss and reemergence of several pre-synaptic markers. Additionally, we will analyze qualitative changes in synaptic and glial morphology using transmission electron microscopy (TEM) and immunohistochemistry (IHC). Gelatin zymography experiments will allow us to document the time course of MMP9 activity, which can be correlated with OPN fragment generation and receptor expression detected by Western blot. OB distribution of critical proteins in the MMP9/OPN pathway will be mapped with IHC. Our studies will examine 1, 3, 7 and 21d survival intervals, representing specific degenerative/regenerative phases of OB synaptic repair.

To confirm that TBI-induced synaptic changes and altered ECM protein expression are indeed dependent upon MMP9, we will probe the same molecular and structural outcome measures in MMP9 KO mice subjected to FPI and allowed to survive for the same postinjury intervals.
Changes in the progression of synaptogenesis will be compared with WT cases to confirm MMP9 role in OB deafferentation-mediated plasticity. We hypothesize that loss of MMP9 will significantly alter both the time course of WT synaptic recovery, as well as the OPN and glial cell changes induced by injury, allowing us to more precisely identify its role in OB reactive synaptogenesis.
CHAPTER 2

DIFFUSE FPI LEADS TO OB REACTIVE SYNAPTOGENESIS
ABSTRACT

Head trauma can cause olfactory disturbances in up to 30% of patients. The OB is a plastic brain region with strong regenerative capacity, however, olfactory deficits can persist long after TBI. Previous approaches to understanding injury-induced reactive synaptogenesis have largely focused on focal axotomy of the sensory ORNs, which is a primary mechanism for post-traumatic loss of smell. Here, we utilized diffuse FPI to induce OB deafferentation and reactive synaptogenesis. We hypothesized that FPI would damage ORN axons, leading to retraction of input to OB synapses and initiation of appropriate cellular responses to facilitate repair during a 21d recovery period, when axotomized ORN axons begin to reinnervate the OB. Analysis of OB protein extracts revealed calpain-cleaved 150kD αII-spectrin breakdown product elevated at 3d post-injury. This corresponded with a 3d reduction in protein expression and tissue distribution of a marker of mature ORNs, OMP. Normalization of OMP followed at 7 and 21d postinjury, during early onset of regeneration. While exploration of Synapsin-I and Synapsin-II did not show time-dependent injury changes in protein level, robust upregulation of GAP-43 at the 21d mark indicated initiation of synaptic sprouting. Immunohistochemistry (IHC) was employed to demonstrate that mild FPI was sufficient to produce an inflammatory response. We found that morphology of GFAP+ astrocytes and IBA1+ microglia changed most prominently 3d after FPI, indicative of glial activation. Lastly, we utilized transmission electron microscopy (TEM) to visualize synaptic morphology and axon cytoarchitecture following TBI and found extensive ORN axon disorganization, pre-synaptic terminal degeneration, and evidence of glial phagocytosis at 3 and 7d postinjury. By 21d, post-synaptic densities begin to become more pronounced, and pre-synaptic ORNs appeared to be shifting into a more normal arrangement. Together these data confirm a process of sensory neuron damage, clearance of cellular debris,
and synaptic repair. Overall, we have demonstrated that diffuse FPI can induce reactive synaptogenesis in the mouse OB, which provides a solid model for exploring mediators of plasticity in Chapters 3 and 4.

INTRODUCTION

TBI is a serious public health concern and financial burden in the United States. Immediate consequences of severe TBI can be devastating, but the long-term effects of mild TBIs can also prove problematic. It is well documented that TBI-induced axotomy will deafferent synaptic circuits and alter neuronal function (Phillips et al., 2014; Povlishock and Katz, 2005). In addition to cognitive, motor, and behavioral dysfunctions, sensory systems are also vulnerable to functional deficits resulting from altered synapses. The prevalence of olfactory dysfunction after head trauma (Costanzo and Zasler, 1991; Sumner, 1964; Hagan, 1967; Costanzo et al., 2012) demonstrates the widespread effects a TBI produces. Brain plasticity and the propensity to recover from multiple types of head trauma have been studied extensively in the hippocampus, cortex, cerebellum, and thalamus. Further, the plasticity of the OB, a site of constant neurogenesis, and where regenerating ORNs project new terminals under normal physiological conditions, has also been investigated in injury paradigms. ORNs, regularly reinnervate mitral and tufted cell synapses in the OB GL, serving as an excellent model to explore the molecular mechanism directing synaptic repair induced by TBI deafferentation of the OB. TBI-induced olfactory dysfunction has been studied in models of total olfactory epithelium ablation and focal OB deafferentation with olfactory nerve (ON) knife lesions. In several preclinical investigations of OB deafferentation, ORNs have been shown to reinnervate the OB and reestablish function once new GL synapses are formed (Graziadei et al., 1978; Koster and Costanzo, 1996; Costanzo,
1985; Morrison and Costanzo, 1995; Yee and Costanzo, 1995; Oley et al., 1975; Jennings et al., 1995). Although these studies successfully model OB deafferentation for analysis of subsequent synaptogenic processes, they do not address the partial axotomy likely produced by diffuse TBI. We utilized mild central FPI, a less focal, non-contusion TBI model, which does not produce complete ON axotomy, but rather offers insight to evolution of recovery in the clinical cases where only a subset of ORN processes are axotomized after a mild head injury.

We chose to explore the molecular pathways involved in TBI-induced reactive synaptogenesis, focusing on the role of extracellular matrix (ECM) proteins. In order to investigate OB synaptic reorganization after TBI, it was important to first establish that our FPI model, which injures cortex, hippocampus, corpus callosum, cerebellum, thalamus, and other brain regions (Sato et al., 2001), delivers a sufficient injury to the OB to drive reactive synaptogenesis. Utilizing FVB/NJ mice, we administered a mild level FPI, and collected OB samples at 1, 3, 7, or 21d postinjury or perfused OB tissue at 3, 7, or 21d after TBI. Based on literature analyses outlining the time course of OB reinnervation (Graziadei et al., 1978; Graziadei et al., 1979), these time points were chosen to reflect periods of acute/subacute degeneration (1-7d) and early regeneration (21d). Western blot technique was the primary assay used to document molecular changes in the OB after injury. We first demonstrated the extent of injury in the OB by measuring αII-spectrin fragment generation. Increase of mixed calpain and caspase derived 145-150kD fragments and a 120kD αII-spectrin peptide generated by caspase-3 have been used as benchmarks to confirm cytoskeletal disruption associated with axonal breakdown. Lysis of αII-spectrin has been a useful marker to confirm TBI axotomy over multiple injury models and brain regions (Hall et al., 2005; Newcomb et al., 1997; Park et al., 2007; Reeves et al., 2010; Saatman et al., 2003; Serbest
et al., 2007), suggesting it would be a valuable tool in our analyses. Early stages of synaptogenesis also require removal of dying pre-synaptic terminals, achieved through the induction of reactive gliosis. Although olfactory ensheathing cells (OECs) support the development and extension of ORN axons, and can engulf cellular debris, as well as produce important ECM molecules such as MMPs (Gueye et al., 2011; Pastrana et al., 2006; Su et al., 2013; Yui et al., 2014), OB microglia and astrocytes are more intimately associated with ORN synaptic sites and likely mediate ongoing synaptic plasticity in the structure. After injury, endogenous microglia and astrocytes shift from resting to activated forms, clearing degenerating axon terminals from the area and reorganizing the post-synaptic environment for synaptogenesis. To document the extent of FPI-induced gliosis in the OB, we employed IHC to detect morphological changes in microglia and astrocytes after TBI, with the goal of better understanding their role in both inflammatory cell-mediated processes and induction of synaptic recovery. As discussed in Chapter 1, primary and secondary injury processes can lead to axonal perturbations that may not be readily detectable. Ultrastructural examination of OB offers detailed analysis of cytoskeletal structure, axonal integrity and degeneration. To assess synaptic changes resulting from injury-evoked axon cytoskeletal destabilization, we employed TEM, to assay intra and intercellular changes in cytoarchitecture and connectivity of synapses, which will clarify the extent of OB injury with FPI.

Our qualitative approach for detecting synaptic change on an ultrastructural level warranted quantitative analyses of synaptic plasticity as well. Four molecular markers of OB neuroplasticity were used to confirm FPI-induced synaptic reorganization. Initially, we employed Western blot (WB) technique to assess postinjury generation of functional synapses by
measuring levels of Synapsin-I and Synapsin-II, synaptic vesicle docking proteins of the pre-
synaptic terminal, which can change after TBI. Reduction in Synapsin proteins typically
indicates a loss of synapses, as the pre-synaptic terminal is dysfunctional without these proteins.
The time course of the disappearance and reemergence of these proteins could also provide
insight to the time-dependent phases of synaptogenesis in the OB subjected to FPI. Both
synapsins are expressed in the OB, but Synapsin-II is more localized to the GL (Stone et al.,
1994). We propose to use this differential expression to our advantage, to hone in on GL-
specific synaptic change. Additionally, neuronal markers can be used to evaluate changes in
synaptic integrity. For our OB-specific investigation, we measured changes in OMP expression,
a cytoplasmic protein of mature ORNs that has been used extensively to label pre-synaptic cells
of the GL and track ORN axon regeneration (Bakos et al., 2010; Costanzo et al., 2006; Monti-
Graziadei et al., 1977; Inamitsu et al., 1990; Kobayashi et al., 2013; Nathan et al., 2001;
Tsukatani et al., 2003). Loss of OMP is detected in OB protein extracts as well as IHC of OB
tissue sections in other models of OB deafferentation, an indication that pre-synaptic input is
lost. Because we are interested in the extent of OB deafferentation after FPI, we measured OMP
protein levels in OB protein samples as well as distribution of OMP expression in the GL during
the defined postinjury time course outlined above. Time-dependent changes in OMP expression
promoted our understanding of GL-specific synaptic changes occurring in response to diffuse
brain injury. In order to more fully understand the process of synapse regeneration in the OB
after FPI, we also identified the extent of successful OB synaptic targeting using GAP-43. This
protein has been routinely used as a marker of axon terminal sprouting, and its presence an
indicator of the formation of new synapses (Benowitz et al., 1990; De la Monte et al., 1989;
Verhaagen et al., 1993; Sachdeva et al., 2016; Williams et al., 2015; Wang et al., 2014; Goto et
al., 1994; Carmichael et al., 2005; Hulsebosch et al., 1998; Stroemer et al, 1993; Gorup et al., 2015). Increase in GAP-43 after OB deafferentation suggests successful synaptic targeting of pre-synaptic neurons to post-synaptic cells. The prevalence of TBI and post-traumatic olfactory dysfunction coupled with the paucity of information available on OB injury in diffuse models of TBI support our proposed investigation. We hypothesize that FPI will induce injury in FVB/NJ mouse OB, deafferent the ORN target GL synapses, and will be confirmed with current, established methods of injury detection.

METHODS

Experimental Animals

All procedures met national guidelines for care and use of laboratory animals, and all experimental protocols were approved by the VCU Institutional Animal Care and Use Committee. FVB/NJ WT adult male mice (The Jackson Laboratory, Bar Harbor, ME) were housed (4 littersmates/cage) under a temperature (22°C) and humidity controlled environment, with food and water ad libitum, and subjected to a 12h dark-light cycle. WT mice (20-30g; 8-11 weeks old) were randomly selected and subjected to midline FPI. WT sham-injured cases served as control. Subsets of FPI and sham-injured groups [WT Sham (n=48), WT FVB TBI (n=53)] were allowed to survive for either 1, 3, 7 or 21d post-injury prior to molecular or histological analysis.

Surgery Preparation and Injury

Mice were anesthetized with isoflurane (4% in 100% O₂ carrier gas) and maintained on 2.5% isoflurane in carrier gas delivered by nose cone. Once stabilized in a stereotaxic frame, heads
were shaved, body temperature maintained at 40°C by Gaymar T/Pump water pump (Gaymar Industries Inc., Orchard Park, NY) and heart rate (bpm), arterial oxygen saturation (percent O₂), breath rate (brpm) and pulse/breath distension (µm) monitored by pulse oximetry (MouseOx; Starr Life Sciences, Oakmont, PA). Mice then received a midline incision and a 2.7 mm craniectomy prepared over the midline, centered between bregma and lambda. Without damaging the underlying dura, a Leur-Loc syringe hub was cemented to the skull surrounding the craniectomy and dental acrylic poured around the hub to stabilize the site. Topical anesthetic/antibiotic was applied to the incision site and the mice housed in recovery cages. After one hour of surgical recovery, mice were anesthetized for 4 mins (4% isoflurane, 100% O₂), and subjected to FPI as previously described (Dixon et al., 1987; Reeves et al., 2012). The device consisted of a 60 x 4.5 cm Plexiglas water filled cylinder, fitted at one end with a piston mounted on O-rings, with the opposite end housing a pressure transducer (EPN-0300A; Entran Devices, Inc., Fairfield, NJ). At the time of injury, the Leur-Loc fitting, filled with saline, was attached to the transducer housing. Injury was produced by a metal pendulum striking the piston, transiently injecting a small volume of saline into the cranial cavity and briefly deforming the brain tissue (20 millisecond pulse duration). The resulting pressure pulse was recorded extra cranially and registered 1.3±0.1 atm pressure. After injury, all mice were promptly ventilated with room air until spontaneous breathing resumed. The duration of suppression of the righting reflex (5.0±2.0 mins) was used as an index of traumatic unconsciousness. Once righting reflex was determined, mice were re-anesthetized for hub removal, scalp suture and topical anesthetic/antibiotic application. Sham-injured controls received the same surgical preparation, anesthesia and connection to the injury device, except that the intracranial pressure pulse was not
applied. All animals were returned to their home cages and assessed for weight loss, locomotion, and eye/nose exudate once per day until weights stabilized.

**Protein Extraction**

WT mice were anaesthetized with 4% isoflurane in carrier gas of 100% O₂ for 4 min, then sacrificed by decapitation at 1, 3, 7 or 21d after FPI or sham injury, with bilateral OBs dissected (n=4-7/group) for assessment of protein expression. Tissue samples were homogenized on ice in 100 µl of RIPA Lysis Buffer (EMD Millipore, Billerica, MA), and centrifuged at 14,000 x g for 20 min at 4°C. Supernatant was aliquoted and stored at -80°C. Prior to WB analysis, protein concentration was determined using Pierce BCA Protein Assay Reagent (Thermo-Fisher, Waltham, MA) and the FLUOstar Optima plate reader (BMG Labtech, Inc., Cary, NC).

**Western Blotting**

WB analysis was carried out utilizing Bio-Rad products (Hercules, CA). Twenty µg of protein was prepared in WB XT Sample Buffer and reducing agent (Bio-Rad Laboratories), then denatured at 95°C for 5 mins. Samples were electrophoresed on 4-12% or 12% Bis-Tris Criterion XT gels (200v x 45 min in MOPS running buffer), then protein transferred onto polyvinylidene fluoride (PVDF) membranes (1h at 100V). Post-blotted gels were stained with 0.1% Coomassie Brilliant Blue (Sigma-Aldrich, St. Louis, MO) in 40%MeOH+10% glacial acetic acid, then de-stained at RT to confirm protein load and even transfer. Membranes were rinsed with deionized water and Tris-buffered saline (TBS) before blocking with 5% milk TBS-Tween (mTBS-T). Blots were then incubated in 5% mTBS-T overnight (4°C) with individual primary antibodies to αII-spectrin (1:2,000, Enzo, Farmington, NY), Synapsin-I (1:500, Santa
Cruz, Dallas, TX), Synapsin-II (1:4,000, EMD Millipore, Billerica, MA), olfactory marker protein (OMP; 1:20,000, Wako, Richmond, VA), and growth associated protein-43 (GAP-43; 1:1,000, Santa Cruz, Dallas, TX). After primary incubation, membranes were washed with mTBS-T, then incubated with appropriate HRP-linked secondary antibodies [IgG bovine anti-goat (1:15,000, Santa Cruz, Dallas, TX), IgG goat anti-mouse (1:15,000, Rockland Immunochemicals Inc., Limerick, PA)] in mTBS-T for 1h at RT. Finally, blots were washed with mTBS-T and antibody binding visualized using Super Signal Dura West chemiluminescence substrate (Thermo-Fisher, Waltham, MA). WB images were captured with Syngene G:Box and positive band signal subjected to densitometric analysis (relative optical density, ROD) with Gene Tools software (Syngene, Frederick, MD). Protein data were expressed as percent change relative to paired WT Sham control cases run on the same transferred gel. Cyclophilin A (EMD Millipore, Billerica, MA) or beta actin (Sigma-Aldrich, St. Louis, MO) was used as load controls for signal detection.

**Immunohistochemistry**

At 3, 7 and 21d post-injury WT injured and WT sham mice (n=4/group) were prepared for fluorescent IHC analysis according to published protocol (Warren et al., 2012). Animals were anaesthetized with sodium pentobarbital (400mg/kg, i.p.), transcardially perfused with 0.9% saline followed by 4% paraformaldehyde in phosphate buffer (0.1M NaHPO₄, pH=7.4), after which brains with attached OBs were extracted and placed in fixative for 24 h before transfer to 0.03% NaN₃ in 1.0 M phosphate buffered saline (PBS). For IHC, fixed brains were cryoprotected in 30% sucrose for 3d, sucrose solutions exchanged after each day. Frontal lobes were blocked and attached bulbs mounted in Tissue Tek media (Thermo-Fisher, Waltham, MA) and stored at -
80°C. Coronal cryostat OB sections (13µM) were collected using a Cryostar™NX70; Cryotome™FSE; HM525 NX cryostat (Thermo-Fisher, Waltham, MA) and prepared for immunofluorescence visualization.

Free floating sections were first permeabilized in 5% peroxidase for 30 mins. After a wash with PBS, tissues were pre-incubated in blocking buffer (fish gelatin in PBS + 0.05% triton X-100) to prevent non-specific binding, and then incubated overnight in primary antibody (OMP, 1:20,000, Wako, Richmond, VA; glial fibrillary acidic protein [GFAP], 1:20,000, Dako; ionized calcium binding adaptor protein [IBA1] for microglia, 1:300, Wako, Richmond, VA) at 4°C. Sections were next washed with PBS, placed in blocking buffer for 30 mins, after which they were incubated with secondary fluorescent antibody (Alexa-Fluor 488 donkey anti-goat, 1:1000 and Alexa-Fluor 594 donkey anti-rabbit, 1:1000, Thermo-Fisher, Waltham, MA) in Blotto for 1h at RT. Slices were then PBS washed, equilibrated in phosphate buffer, and mounted on Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA) with Vectashield + DAPI (Vector Laboratories, Burlingame, CA). IHC signal was visualized on the Zeiss LSM 700 (Carl Zeiss, Thornwood, NY) confocal microscope (VCU Microscopy Core).

Transmission Electron Microcopy
Following FPI, WT injured and WT Sham-injured mice were randomly assigned to either 3, 7 or 21d survival groups (n=4/group). At time of sacrifice, mice were anesthetized with sodium pentobarbital (400mg/kg, i.p.), transcardially perfused with mixed aldehyde fixative (2% paraformaldehyde and 2.5% glutaraldehyde) in 0.1M phosphate buffer, pH-7.2. Brains were removed and post-fixed overnight at 4°C. OBs were next blocked in the sagittal plane and placed in 1% osmium tetroxide (0.1 M cacodylate buffer), and processed for embedment with
Epon resin (Embed; Electron Microscopy Sciences, Hatfield, PA). After curing, OB areas containing glomeruli were mounted and both semi-thin (0.5µm) and ultrathin (silver, 600 Å) sections were cut with a Leica EM UC6i ultramicrotome (Leica Microsystems, Wetzlar, Germany). Semi-thin sections were used to guide subsequent ultrastructural sampling. Ultrathin sections were collected on membrane Formvar-coated slotted grids and observed on a JEOL JEM-1230 electron microscope (JEOL USA, Inc., Peabody, MA), equipped with a Gatan UltraScan 4000SP CCD camera (Gatan, Inc., Pleasanton, CA).

Statistics

The effects of injury, and the time postinjury, on protein levels were evaluated using GLM SPSS v.23 (International Business Machines, Corp., Armonk, NY). These analyses used 2-way completely randomized ANOVAs. Specific pairwise comparisons were evaluated using the Duncan Post Hoc Test. Western blot and zymography results are reported as mean +/- SEM. An alpha level of 0.05 was used in all analyses.

RESULTS

Alpha II-spectrin proteolysis is increased after FPI

Investigation of reactive synaptogenesis in the OB requires that a valid model for induction of deafferentation injury be established. In order to determine the extent of ORN axonal damage within WT FVB/NJ mice following FPI, we first examined injury-induced production of αII-spectrin breakdown products, as the profile of these αII-spectrin proteolytic fragments within the OB after TBI was unknown. We and others have previously used level of αII-spectrin fragments to characterize extent of axonal injury, revealing increased expression after TBI (Reeves et al.,
Figure 2.1 OB αII-spectrin expression after FPI

A. Western blot (WB) analysis of αII-spectrin breakdown products in OB extracts. Acute, 1d expression of the 150kD calpain-derived αII-spectrin fragment was reduced by approximately 50%, but the effect was not statistically significant. However, by 3d postinjury, 150kD αII-spectrin was elevated over 3-fold, supporting a peak in FPI induced axon degradation at that time point. No other change in calpain-derived αII-spectrin proteolytic fragments (either 150 or 145kD) was detected. Post hoc analysis confirmed the time-dependent elevation of 150kD αII-spectrin between 1 and 3d postinjury. Notably, caspase-cleaved 120kD αII-spectrin remained unchanged across all time points. Results are expressed as percent of sham control relative optical density (*100% dashed line*). B. Representative blot images of 150kD, 145kD, and 120kD αII-spectrin lanes, with cyclophilin A and β-actin load controls, are illustrated below.

*p<0.05 day x vs. y, **p<0.01 Sham vs. TBI. N=4-8/group.
2010; Pike et al., 1998; Buki et al., 1999; Ringger et al., 2004; Thompson et al., 2006). With
WB methods, we examined changes in OB caspase and calpain-mediated αII-spectrin proteolysis
during postinjury intervals of 1, 3, 7, and 21d, a period mapping axon degeneration (1-7d) and
initial synaptic regenerative (21d) phases. Results suggest that mild diffuse FPI does produce
OB axotomy, however, significant change in αII-spectrin proteolytic fragment generation was
limited, and restricted to a large increase the 150kD fragment. For the calpain-derived 150,
145kD peptides, there was a non-significant trend toward reduction at 1d relative to sham
controls (52.68±11.90; 69.83±12.87), but no detectable shift in caspase-derived 120kD αII-
spectrin signal (90.00±11.71). By contrast, at 3d we found significant, nearly 4-fold elevation in
postinjury 150kD αII-spectrin relative to sham injured controls (380.77±129.58, p<0.01) (Figure
2.1). Levels of the 3d 145kD fragment (107.02±10.20) and 120kD fragment (104.14±8.18) were
not different from controls. These acute 3d postinjury changes in αII-spectrin proteolysis are
consistent with delayed onset of OB axon degeneration (Costanzo et al., 2006; Graziadei and
Monti Graziadei, 1980) and our observation that ORN axon marker protein OMP shows
significant reduction at the same 3d time point (see Figure 2.6 below). For the later
degenerative (7d) and early regenerative (21d) phases, we found no change in αII-spectrin
fragments at 150kD (130.50±26.46; 107.21±38.98) and 145kD (109.77±25.42; 102.98±16.50),
or at 120kD (119.27±18.85; 101.26±16.44). Post hoc comparison of the different survival time
points did show a significant change in 150kD αII-spectrin expression between 1 and 3d
(p<0.05), but failed to detect time-dependent differences for any other time comparisons. We
conclude that there is at least a 24h delay in expression of the αII-spectrin axon degeneration
marker within the OB after FPI induced axotomy and that the mechanism underlying production
of the 150kD breakdown product is predominant. This later 3d peak of αII-spectrin processing
could be attributed to the length of ORN axon projections from OE to the OB and the subsequently delayed/longer time course of synaptic reorganization. Further, no differences in 120kD αII-spectrin were detected, either relative to sham-injured cases or over time postinjury, suggesting little involvement of caspase-3 in OB cell death and axon terminal breakdown. This pattern of αII-spectrin proteolysis shows that time-dependent generation of OB axonal damage does occur in our TBI model. The time frame of this degeneration is consistent with that reported after complete ON axotomy and olfactory epithelium (OE) sensory neuron ablation (Costanzo et al., 2006; Costanzo and Perrino, 2008; Bakos et al., 2010; Nathan et al., 2001; Graziaidei and Monti Graziaidei, 1980), which follows a pattern of profound degeneration over the first week post-lesion.

**OB glia change morphology in response to FPI**

Understanding the time course of αII-spectrin expression leads to questions regarding the cellular mechanism underlying these degradative processes. Breakdown and accumulation of αII-spectrin, indicative of damaged OB axons, requires a process of removal these axons from the extracellular matrix (ECM) of affected brain regions before effective terminal sprouting can begin. In most cases of CNS trauma resulting in axonal injury, degeneration, and local synaptic deafferentation, reactive glia such as astrocytes and microglia are the principal cells responsible for ECM clearance to promote plasticity. Focusing on 3, 7, and 21d after injury, we next utilized IHC to examine cell morphological changes in OB neuroglia after FPI, as they can often contribute to axon breakdown and debris clearance. Interestingly, astrocytes, labeled with glial fibrillary acidic protein (GFAP) antibody, were most reactive at 3d postinjury (**Figure 2.2A**), exhibiting prominent hypertrophy with extended processes interdigitating deafferented
Figure 2.2 Immunohistochemistry (IHC) of OB glial phenotypes after diffuse FPI

A. Confocal imaging of sham cases shows GFAP labeled astrocytes principally within the GL, wrapping and infiltrating synaptic-rich glomeruli. At 3d postinjury, reactive astrocytes are observed within the deafferented GL, where their cell processes were thick and hypertrophic (arrows). Over time postinjury, astrocyte reactivity becomes more diffuse, with a less intense signal (7-21d).  

B. Sham IBA1 labeled microglial cells displayed a resting ramified morphology in GL and EPL, with long, thin processes and small cell bodies. At 3d after FPI, both regions appeared to contain greater numbers of activated microglia with rounded cell bodies and thick, bushy processes (arrows). At 7d, this microglial response was attenuated, with the reactive cells having less amoeboid morphology. By 21d postinjury, microglia were more ramified and resembled those in sham animals. Scale bar = 50µm. N=3-4/group.
glomeruli. Their processes were long and thick at 3d postinjury, their most reactive phenotype, gradually reducing over time, as they returned to a less reactive stellate-like appearance by 7d postinjury. Utilizing ionized calcium-binding adaptor molecule 1 (IBA1) antibody to mark microglia, we also found that these cells were highly reactive within the 3d injured OB when compared with paired sham controls (Figure 2.2B). Microglia shifted from a resting, ramified morphology detected in shams to an activated form, with enlarged, amoeboid cell bodies and lobular cytoplasmic processes. At 7d postinjury, microglial reactivity was reduced relative to the 3d response, but remained above that of sham-injured animals, possessing slightly longer processes and smaller cell bodies, as they began to return to a less reactive phenotype. By 21d, when early synaptic regeneration is reported in other models of OB deafferentation (Graziadei and Monti-Graziadei, 1980; Cummings et al., 2000), glial phenotypes were not different from sham cases. Astrocytes, both in sham and injured animals, were primarily localized to the GL, however, microglial response was not restricted, presenting in all layers of the OB. These results demonstrate that the predicted activation of endogenous OB glia (Bailey and Shipley, 2003; Lazarini et al., 2012) occurs in response to FPI axotomy, and suggest that synaptic morphology changes during the same postinjury intervals.

Expression of ORN marker OMP changes after FPI

To narrow our focus on the investigation of ORN-specific synaptic change, we next probed the same FVB/NJ OB extracts for change in expression of OMP, a cytoplasmic protein selective for mature ORNs and their axons (Monti-Graziadei et al., 1977; Cummings et al., 2000; Griff et al., 2000; Holtmaat et al., 1995; Kasowski et al., 1999; Stone et al., 1994). Taking OMP reduction as a metric for ORN axon loss (Figure 2.3), we found evidence for onset of axon damage at 3d
after FPI, consistent with our predicted induction of pathology due to diffuse axotomy, and the rather delayed time course of ORN degeneration seen in other models of OB deafferentation. At 1d postinjury, OMP protein was equivalent to control levels (100.84±11.17), but by 3d we found a significant reduction in the 19kD OMP signal to approximately 62% of control (61.84±10.09, p<0.05). Interestingly, at 7d after injury, OMP protein had returned to near sham control values (90.76±11.34) and reached a mean slightly higher than controls by 21d onset of the regenerative phase (108.69±7.54). This 3d reduction in OMP showed time-dependent differences from both 1d (p<0.05) and 21d (p<0.01) values, indicating that the 3d interval was a critical point in the evolution of OB postinjury axon degeneration. The fact that 7d OMP levels approach sham controls, but a 3d versus 7d comparison did not show significant temporal differences suggests that the 3-7d interval is critical for shifting from ORN degeneration to regeneration in our model. Further, this intriguing result opens the possibility that onset of OB regeneration may be a function of extent of injury and that more local sprouting could contribute to reinnervation in our model.

Similarly, IHC showed a detectable loss and later return of OMP in the GL beginning at 3d postinjury (Figure 2.4), while OMP staining remained strong across time points in the olfactory nerve layer. These findings suggest a time period of 3d is required for onset of axon degeneration, and that OMP positive terminals, marking synapse regeneration, could emerge as early as 7d after FPI. Our results also show that the time course of OMP reduction and restoration after FPI is similar to that observed during postnatal injury and ORN reinnervation of OB glomeruli (Gonzalez and Silver, 1994), as well as that described for OB synaptogenesis after
Figure 2.3 FPI induces OB time-dependent reduction of OMP

A. WB probe of ORN axon marker protein OMP showed significant postinjury reduction in the OB. While OMP expression did not change at 1d postinjury, by 3d it was significantly reduced to 60% of sham level. This supports subacute loss of mature ORN axons in the OB after FPI and is consistent with 3d peak in GL glial reactivity. However, by 7d OB OMP expression had increased to a level not different from controls. At 21d, predicted onset of OB reinnervation, OMP protein was slightly higher than sham cases. Post hoc comparison of time points showed that the 3d OMP reduction was significantly lower than both 1d and 21d protein levels, but not from 7d values. This result further supports 3d postinjury as the period of peak axon degeneration. Results are expressed as percent of sham control relative optical density (100% dashed line). B. Representative blot images of OMP, with cyclophilin A and β-actin load controls, are illustrated below. #p<0.05 day x vs. y, ##p<0.01 day x vs. y, *p<0.05 Sham vs. TBI. N=5-6/group.
Figure 2.4 IHC of OMP distribution in OB after FPI
A. Confocal imaging of OMP in sham controls shows heavy ONL labeling and signal within pre-synaptic ORN axons innervating OB glomeruli (GL; arrow). B. At 3d postinjury, GL staining of OMP is reduced, appearing punctate and diffuse, with fewer OMP+ ORN projections (arrows). This IHC pattern is consistent with 3d OMP reduction in WB experiments, further supporting injury-induced loss of GL synaptic input. Scale bar = 50µm. N=3/group.
ORN lesion and OE chemical damage (Costanzo et al., 2006; Cummings et al., 2000; Bakos et al., 2010).

**FPI does not significantly alter Synapsin protein expression**

Knowing that FPI induces an injury response in the OB, and GL synapses are affected, we next explored molecular markers of intact synaptic junctions to define the time course of ORN reactive synaptogenesis. First, we mapped postinjury expression of two synaptic vesicle regulatory proteins in FVB/NJ OB tissue extracts. Both Synapsin-I and II have functional roles related to synaptic vesicle release, particularly in inhibitory GABAergic neurons (Sadanandappa et al., 2013; Knapek et al., 2010; Medrihan et al., 2015). In several studies, Synapsin-I is altered in response to injury, and serves as a marker of synaptic plasticity in cortex and hippocampus (Kitagawa, 1992; Chan et al., 2014; Ansari et al., 2008a,b; Griesbach et al., 2004), suggesting FPI may alter Synapsin-I in OB as well. Interestingly, the OB contains both proteins, with Synapsin-I expressed throughout the OB core (Melloni et al., 1993) and Synapsin-II predominant in the glomerular region (Stone et al., 1994). This provides an opportunity to more specifically assess pre-synaptic changes in the vulnerable GL. Our WB analyses (Figure 2.5) detected both isoforms of Synapsin-I (80, 70kD), but revealed that neither showed significant difference from sham controls over time postinjury. At 1d after FPI, both the 80 and 70kD isoforms remained near control values (106.66±7.16; 103.26±18.37), as well as the 70kD Synapsin-I signal for 3d (107.94±17.93). However, we did observe a trend toward increase in the 80kD isoform at 3d postinjury (122.58±8.88, p=0.076). At the later survival intervals, 7 and 21d, we also failed to find significant differences in expression of either isoform relative to sham controls. For 80kD Synapsin-I, values were very close to controls (81.78±9.38; 94.78±5.21), however, the overall
Figure 2.5 Synapsin-I 80 and 70kD isoform expression in OB following FPI

A. WB analysis of OB Synapsin-I expression after FPI revealed no injury effect on the 80kD isoform compared to sham controls. However, time-dependent differences were found with post hoc analysis, showing that 1d 80kD Synapsin-I was higher than 7d expression, and 3d level was higher than protein levels at both 7 and 21d postinjury time points. These results suggest a trend toward lower 80kD Synapsin-I production during later postinjury time points. B. OB expression of 70kD Synapsin-I was not affected by FPI. It did not differ from controls at any time following injury, nor was time-dependent change in its expression detected by post hoc analysis. Results are expressed as percent of sham control relative optical density (100% dashed line).

C. Representative blot images of 80kD and 70kD Synapsin-I, with β-actin as a load control, are illustrated below. #p<0.05 day x vs. y, ##p<0.01 day x vs. y. N=4-7/group.
Figure 2.6 Synapsin-II 72 and 58kD isoform expression in OB following FPI

A. WB analysis of OB Synapsin-II expression after FPI revealed no injury effect on the either the 72 or 58kD isoform compared to sham controls. Similarly, results failed to show any significant differences in isoform expression over time postinjury. B. A trend toward increase in the 58kD Synapsin-II was observed at 1 and 3d after FPI (150% of shams). Although reasonable group numbers were used for these experiments, this trend suggests that larger sample size could yield a significant increase of injured samples over sham controls at the acute and early subacute time points. Results are expressed as percent of sham control relative optical density (100% dashed line). C. Representative blot images of 72kD and 58kD Synapsin-II, with cyclophilin A as a load control, are illustrated below. N=4-6/group.
pattern for the smaller 70kD isoform suggested a trend toward reduced expression during the late degeneration/early regeneration transition period (76.28±11.72; 67.19±9.16). When time-dependent changes in Synapsin-I expression were probed, we did find several significant differences in the 80kD isoform profile for the acute 1 and 3d intervals. Both of the early 80kD expression levels were higher than the 7d signal (p<0.05; p<0.01) and the 3d 80kD isoform was higher than the 21d level (p<0.05). As the smaller isoform seemed much less affected by FPI, it is possible that the 80kD Synapsin-I protein possesses a more important function in OB synapse repair.

Similarly, the two forms of Synapsin-II, expressed within mouse cortical and subcortical regions (Nesher et al., 2015), and detected in our OB extracts at 72kD and 58kD, were also not significantly affected by FPI when compared to sham injured animals (Figure 2.6). While postinjury levels were consistent with controls at 1, 3, 7, and 21d for 72kD Synapsin-II (108.21±7.33; 104.92±6.85; 90.86±5.55; 87.62±5.84), we did observe an average increase of about 58% in the 58kD form of Synapsin-II at 1 and 3d postinjury (160.64±46.97; 156.65±34.13), however, group variance was high and the data analysis failed to support these increases as significant (p=0.078; p=0.130). Similar to the 72kD isoform, the 7 and 21d signal for 58kD Synapsin-II was not different from sham injured cases (96.71±8.26; 84.57±10.21). Further, post hoc analysis did not show any significant time-dependent changes in OB Synapsin-II protein expression. Together, these results suggest that while OB expression of synaptic vesicle mobilization proteins are not substantially altered by FPI, subtle time-dependent shifts in Synapsin-I and II isoform level appear to occur after FPI, potentially marking transitions between early (1-3d) and later (7d) regenerative phases of OB synaptogenesis.
**GAP-43 increases during regenerative periods**

Finally, as a third probe for mapping synaptogenesis, GAP-43 expression was assayed over time postinjury to correlate our previous findings with a known protein marker of pre-synaptic sprouting and synapse regeneration. This growth factor is critical to axon growth and extension, as well as synaptic replacement in the OB (Cizková et al., 1995; Griff et al., 2000). It is also posited to be regulated by OMP expression (Griff et al., 2000), where, as axons are lost, OMP levels fall, removing inhibition of GAP-43 production. In our FPI model, we found that induction of GAP-43 expression was highly correlated with the onset of synaptic replacement at 21d postinjury. As would be predicted for the OB, we saw no significant change in the GAP-43 signal over the 1-7d postinjury period, covering the first week of the degenerative phase (Figure 2.7). In 1 and 7d samples, GAP-43 level was equivalent to sham controls (95.69±24.18; 105.20±10.99). At 3d after FPI, however, we detected a small 36% rise in mean levels of GAP-43 protein relative to controls (136.28±9.06), but this change failed to reach significance. Interestingly, such a shift toward GAP-43 increase would be predicted to accompany the observed 3d reduction in OMP if, indeed, the two have an inverse regulatory relationship with respect to expression (see again Figure 2.6). The fact that we did not find significant rise in GAP-43 associated with major loss of OMP protein could be attributed to the diffuse, partial extent of OB axotomy with FPI. Most notable was the significant, nearly 3-fold rise in GAP-43 over controls at 21d after FPI (297.19±58.77, p<0.001), a time interval corresponding to the onset of ORN axon regeneration after axotomy (Cizková et al., 1995; Graziadei et al., 1978; Graziadei et al., 1979). Moreover, this significant 21d elevation of GAP-43 was also statistically different from 1d (p<0.001), 3d (p<0.01), and 7d (p<0.001) expression, further supporting the importance of its role during the early regenerative phase of OB synaptic function.
Figure 2.7 OB GAP-43 is elevated at onset of synapse regeneration after FPI

A. WB analysis of changes in OB GAP-43 protein expression after FPI showed that the growth factor was not elevated over sham controls during the degenerative phase, 1, and 7d postinjury. At 21d, the time point characterized by ORN axon reinnervation and synapse reformation, a significant 3-fold elevation in GAP-43 was found. Post hoc analysis confirmed that this 21d change was significantly different from GAP-43 signal at all three of the other time points. Results are expressed as percent of sham control relative optical density (100% dashed line).

B. Representative blot images of GAP-43, with cyclophilin A and β-actin as a load controls, are illustrated below. ##p<0.01 day x vs. y, ###p<0.001 day x vs. y, ***p<0.001 Sham vs. TBI. N=4-6/group.
repair. Overall, postinjury changes in OMP and GAP-43 as two markers of OB neuroplasticity provide compelling support for the induction of OB injury and synaptic repair after TBI. Together, with the structural evidence of damage, these data show that FPI can serve as a good model for investigating OB reactive synaptogenesis following diffuse TBI.

Ultrastructure reveals axon and synaptic disruption postinjury

Our initial findings of increased αII-spectrin proteolysis and glomerular glial reactivity within the injured OB warranted a more precise exploration of glomerular synaptic morphology. In order to establish FPI effects on this morphology, we employed TEM to examine OB glomerular structure between 3 and 21 d after injury (Figure 2.8). Low magnification imaging of 1µm thick plastic embedded sections (panel A) revealed the gross organization of OB glomeruli seen in sham controls, where large groups of electron dense, dark ORN axons interdigitate throughout each glomerulus. At 7 and 21d after FPI, the low magnification view clearly shows pre-synaptic axon groups reduced in size relative to control cases. Subsequent ultrastructural analysis of sham control and 7d cases (panel B) revealed that bundles of ORN axons often encircled a central grouping of mitral and tufted cell dendrites. At 7d after injury, these ORN axon bundles were reduced in size and appear collapsed in shape. Higher magnification views of these cases (panel C) showed that, by 3d after FPI, there exists evidence of disrupted synaptic cytology, with ORN axon terminals beginning to exhibit early signs of degeneration relative to sham morphology. At 7d postinjury, ORN axons were visibly shrunken and collapsed, with multiple sites containing membranous debris reflecting tissue degeneration. Notably, glomerular glial processes were reactive and contained degenerated cell material at 7d after FPI. While 21d postinjury glomeruli still contained some areas of tissue degeneration, the normal structure of
Figure 2.8 Ultrastructure of OB glomeruli show ORN axon damage after FPI

A. Micrographs of semi-thin (0.5µm) sections from OB illustrate sham ORN axons (electron dense) within a glomerulus (black arrow) and bundled entering GL (white arrow). At 7d after FPI, these axons appear compressed and their grouping elongated in shape. By 21d, the sham ORN axon bundle structure begins to reemerge. B. Micrographs of ultrathin sections (600Å) show ultrastructure of ORN axon groups (ax) within a sham glomerulus, where the pre-synaptic processes interdigitate to make synapses (arrows) on multiple post-synaptic dendrites (d). By 7d postinjury, the ORN axon bundles appear irregular, collapsed (white arrows) and contain evidence of cytoplasmic degeneration (black arrows). C. Glomerular ultrastructure illustrates postinjury time-dependent change in axo-dendritic cytoarchitecture. Axons (black arrow) in sham show pre-synaptic vesicles grouped along membranes at synaptic junctions and thick post-synaptic densities (white arrow). At 3d, synaptic organization deteriorates (white arrows), with early signs of axon degeneration (black arrow). By 7d, ORN axons appear less compressed (black arrow), but retain poorly organized synaptic junctions (white arrows). Inset: Active phagocytosis of degenerative debris (black arrow) occurs within 7d reactive astrocytes. At 21d, glomerular synaptic structure mimics well-developed synaptic structure of controls (black arrows), even though ORN axons appear less electron dense and processing of degenerating membranes remains visible (white arrow). Bar in A=10µm; B sham=4µm; B 7d=2µm; C=1µm. N=2-3/group.
ORN axons and surrounding dendrites appeared to reemerge. These results are consistent with the observed 3-21 d IHC increases in OB glial reactivity after FPI and suggest that a protracted degenerative/clearance phase occurs following diffuse ORN deafferentation in this model. The delayed time course of axon loss (>3d after insult) matches that reported after knife cut ORN axotomy (Costanzo et al., 2006) and chemical ORN lesion (Cummings et al., 2000; Bakos et al., 2010).

DISCUSSION

Clinical presentation of olfactory dysfunction after TBI is well documented, yet the capacity for diffuse models of TBI to induce synaptic change in the OB is unknown. Although prior studies have used both hippocampal and OB deafferentation models to investigate time course of reactive synaptogenesis (Lynch et al., 1976; Steward, 1976; Deller et al., 2007; Frotscher et al., 1997; Monti Graziadei and Graziadei, 1979; Oley et al., 1975; Graziadei and Okano, 1979; Morrison and Costanzo, 1995), few studies have examined TBI effects on the OB. These TBI studies focused on direct impact injury to the OB structure, mapping injury response molecules in the OB and OE, as well as the time course of functional recovery, including effects of anti-inflammatory therapy (Siopi et al., 2012; Steuer et al., 2014). To our knowledge, none have focused on diffuse OB deafferentation caused by FPI, nor examined the role of MMPs and OPN cell signaling with OB deafferentation. Here we aimed to show that a mild injury delivered by central FPI is sufficient to cause damage to the FVB/NJ mouse OB and alter synapses. We hypothesized that FPI would cause a time-dependent injury-induced reactive synaptogenesis in the OB, based on changes in αII-spectrin breakdown, induction of glial reactivity, synapto-
dendritic ultrastructure, synaptic vesicle protein expression, OMP ORN marker protein level and elevation of GAP-43 with axonal regeneration.

Overall, our results showed that mild FPI does induce OB deafferentation, with a time course of synaptic reorganization similar to that reported for more severe ORN transection or OE disruption (Bakos et al., 2010; Costanzo et al., 2006; Cummings et al., 2000; Graziadei et al., 1978; Graziadei et al., 1979; Graziadei and Okano, 1979; Kobayashi and Costanzo, 2013; Graziadei and Monti Graziadei, 1980; Morrison and Costanzo, 1995). We examined acute 1d and subacute (3-7d) degenerative postinjury phases, as well as the early phase of synaptic reformation (21d). Currently, it is accepted that this process occurs in the OB over a longer time period than in other cortical brain regions. For example, the degenerative phase in hippocampus is 1-5d post-injury, the regenerative phase 6-15d and the synapse stabilization phase after the 15d mark (Lynch et al., 1976; Steward, 1976; Steward and Vinsant, 1978; Deller et al., 2007; Frotscher et al., 1997). By contrast, in the OB the same process is characterized by a degenerative phase of 1-15d, regeneration between 15-60d, and synapse stabilization beyond 60d (Graziadei et al., 1978; Graziadei et al., 1979; Cummings et al., 2000). In the present study, changes of the selected metrics of OB reactive synaptogenesis were observed after FPI, occurring along the predicted OB time intervals. Although reduced relative to more severe insult paradigms, changes in αII-spectrin breakdown fragments were detected. We also documented reactive glia (astrocytes and microglia) within the deafferented glomeruli and external plexiform layers of the OB. Molecular markers showing both acute reduction in pre-synaptic vesicle and OMP proteins, as well as a significant rise in GAP-43 at onset of OB synapse regeneration, all point to a predicted time course of deafferentation/reinnervation in animals subjected to FPI.
Thus, our evidence supports induction of OB synaptogenesis after a diffuse TBI, permitting subsequent exploration of how ECM molecules may regulate synaptic reorganization following trauma-induced OB deafferentation.

**Lysis of αII-spectrin marks subacute degeneration with FPI**

We first assessed changes in expression of breakdown products of αII-spectrin protein. Historically, αII-spectrin has been used as a marker of axonal degradation due to its membrane stabilizing function. Alpha II-spectrin binds to the cytoskeleton adjacent to the intact plasma membrane and is processed by Ca\(^{2+}\) activated enzymes calpain and several caspases. TBI leads to increased axon permeability, signal transduction, excessive neurotransmitter release and excitotoxicity. Increased [Ca\(^{2+}\)], activates these proteases, thus leading to the lysis of αII-spectrin and the accumulation of its breakdown products. These products have been used reliably to estimate extent of cytoskeletal breakdown as a result of TBI-induced axonal injury, where caspase and calpain proteolytic fragments are increased (Pike et al., 1998; Buki et al., 1999). For this reason, αII-spectrin expression can serve as a biomarker for tissue injury, such as when detected in the cerebrospinal fluid of TBI patients (Ringger et al., 2004), as well as for estimates of axotomy, as in the case of altered time course of αII-spectrin breakdown product expression in moderate and severe traumatic injuries (Thompson et al., 2006; Reeves et al., 2012). Interestingly, the level of FPI used in our experiments induced only a mild production of OB αII-spectrin lytic peptides. Significant elevation was observed only for the 150kD calpain fragment at 3d after injury. This result contrasts with prior reports from our laboratory (Reeves et al., 2010) and others (Harris and Morrow, 1988; Pike et al., 1998; Buki et al., 1999; Wang et al., 2000; Ringger et al., 2004; Thompson et al., 2006; Aikman et al., 2006), where animals
subjected to TBI show major (up to 10-fold higher) elevation of the 150 and 145kD fragments, as well as minor increases in the caspase 120kD peptide, within other regions of the injured brain (e.g., cerebral cortex, hippocampus, corpus callosum). Several factors could explain this difference in OB αII-spectrin proteolysis. Foremost, the rostro-caudal dissipation of injury forces from a midline, centralized injury site may result in a smaller subset of axotomized cells for the OB than in other parts of the FPI brain. It is also possible that more robust αII-spectrin breakdown could occur prior to the first 24 hr after FPI, the earliest postinjury interval examined here. In fact, an earlier study from our laboratory (Reeves et al., 2010) reported significant increase in 150, 145 and 120kD fragments in the cortex at 3 h postinjury. A third factor possibly responsible for this reduced production of OB αII-spectrin fragments would be that the source of FPI cytoskeletal proteolysis comes from a source other than ORN axons. As IHC analysis of αII-spectrin was not conducted in our studies, it is possible that the αII-spectrin breakdown products could result from cytoskeletal disruption within axons and dendrites of several types of OB neurons injured with FPI. Mitral and tufted cells, the principal post-synaptic targets of ORNs, are good candidates for injury sensitivity, as reported for a model of transient ischemia (Hwang et al., 2008). Further analysis of specific cell damage within the OB will be required to determine the exact source(s) of our detected αII-spectrin effects. To the best of our knowledge, this study provides the first documentation of time-dependent αII-spectrin proteolysis in the OB after diffuse TBI, directly linking deafferentation with a marker of axotomy.

FPI promotes glial reactivity in the OB

It is well documented that TBI generates a rapid and localized glial reactivity, concurrent with acute induction of inflammatory response (see Chapter 1). In addition to production of
cytokines, chemokines and reactive oxygen species, the inflammatory response alters glial function following brain insult. The cell signaling cascades initiated by these molecules are typically associated with reactive neuroglia, cells whose altered morphology reflects a role in both degenerative and regenerative phases of recovery. Importantly, these reactive glia are often associated with deafferented zones. For example, reactive microglia are juxtaposed to regenerating GAP-43+ axon terminals after auditory sensory deafferentation (Janz and Illing, 2014), and migrate to deafferented regions during reactive synaptogenesis in the hippocampus (Chan et al., 2014). While, such glial response after TBI has been established for other brain regions, the time course of OB glial reactivity to diffuse axotomy remained unclear. Nevertheless, it is established that the OB, like other parts of the CNS, contain responsive microglia and astrocytes, which often drive the different phases of synaptic plasticity induced by ORN damage (Bailey and Shipley, 1993; Chang et al., 2003; Lazarini et al., 2012). Our IHC results confirm FPI activation of both astroglial and microglial cells in the OB. We examined the same postinjury intervals as for αII-spectrin lysis (1, 3, 7 and 21d), observing a clear hypertrophy of glomerular astrocytes, and a shift of glomerular/outer plexiform layer microglia from resting to an activated state. This reactivity was greatest at 3d postinjury, matching the period when αII-spectrin lysis was detected and reduction of ORN axon marker OMP was maximal (discussed below). By 7d after FPI, this reactivity was attenuated, with cell morphology for each glial type returning to normal profile at 21d postinjury.

The time course of FPI glial response is in agreement with that reported by Lazarini et al. (2012) for OB microglia, where intranasal 2, 6-Dichlorobenzonitrile lesions revealed a peak response at 3d, with reduction of reactivity by 7d postinjury. Close examination of the immunostaining
patterns for OB astrocytes and microglia revealed additional detail as to their role in response to FPI. OB astrocytes were mostly confined to the glomerular layer in our model, and for this reason have been the focus of cellular response in other models of OB deafferentation (Bakos et al., 2010; Kobayashi et al., 2013). Few astrocytes appear outside this region. Our IHC results consistently show a postinjury GFAP+ hypertrophy, with larger cytoplasmic projections wrapping around individual glomeruli and finer processes extending into the surface zones of the glomeruli. Such a pattern is consistent with detailed ultrastructural studies, which show that astrocyte processes generate boundary zones between the axo-dendritic synapses of the central glomerular region and the juxtaglomerular neurons surrounding the core (Chao et al., 1997). This hypertrophic response to FPI suggests that astrocytes are more intimately involved in the early stage response to ORN deafferentation (1-3d postinjury) and are sensitive to the changes in synaptic organization required for OB reactive synaptogenesis. These findings are consistent with other reports of OB astrocyte response to injury, also showing confinement to the glomeruli and hypertrophy upon deafferentation. However, the temporal extent of astrocyte response after mild FPI appears to be attenuated relative to these other models. For example, methyl bromide exposure, which destroys ORNs, caused a 15d increase in GFAP (Bakos et al., 2010), while full ORN transection caused a steady increase in GFAP signal over 60d postinjury (Kobayashi and Costanzo, 2009; Kobayashi et al., 2013; Cummings et al., 2000).

Interestingly, most pro-inflammatory cytokines peak early after FPI, being chiefly produced by microglia and other invading immune cells. As predicted, we found that OB microglia also exhibited their most reactive phenotype at 3d post-injury. Such phenotypic change was similar
to that detected after injury of olfactory epithelium with zinc sulfate (Chang et al., 2003) and nerve transection induced macrophage reactivity (Bakos and Costanzo, 2011).

In contrast to astrocytes, the activated microglia in our study were not restricted to the deafferented glomerular layer, but appeared more uniformly distributed in the OB, particularly among the dendrites of the mitral and tufted neurons of the external plexiform layer. This would suggest that, under conditions of deafferentation, they may be involved in cell signaling for the entire OB structure. Acute microglial generation of pro-inflammatory molecules likely facilitates autocrine and paracrine cell signaling to locally activate other cells, including glomerular astrocytes (Shin et al., 2005; Doty, 2012). Although prolonged microglial reactivity has been reported for other brain regions after TBI (Johnson et al., 2013; Smith et al., 2013; Gentleman et al., 2004; Faden et al., 2011; Engel et al., 2000; Loane et al., 2014; Ramlackhansigh et al., 2011), we found that microglial morphology was identical to that of sham controls by 21d postinjury. This suggests a relatively rapid reduction of inflammatory response, possibly attributed to the reduced level of OB injury in our experiments and distance from injury site. Reactive glia are critical in altering the environment after injury. Therefore, documenting that OB microglia and astrocytes are responsive to FPI is imperative and defines the extent to which these reactive glia are challenged to promote reactive synaptogenesis. Our IHC analyses provide further insight into the time course of an important secondary injury/recovery mechanism.

**Olfactory marker protein reduction after FPI**

In order to determine the extent of glomerular pre-synaptic damage, we next examined the profile of OMP, a 19kD peptide selectively expressed in ORNs and their axons (Monti-Graziadei
et al., 1977). Cytoplasmic OMP is specific for mature ORNs, and is conserved across species (Keller and Margolis, 1976). It has been linked to functional odor responses (Kass et al., 2013), where OMP KO attenuates response to odor stimuli (Buiakova et al., 1996), reduces odorant sensitivity (Lee et al., 2011), lowers odor thresholds (Youngentob and Margolis, 1999), and decreases odor quality perception (Youngentob et al., 2001). While OMP role is still being fully defined, there is strong evidence to suggest it is involved in maturation of ORN function because it is not present in immature ORNs. Tracking gain and loss of OMP in models of OB injury and deafferentation has suggested the protein can be useful as a marker of axon damage and repair. For example, when glomerular synapses are deafferented by transection lesion, OMP levels drop significantly compared to controls (Costanzo et al., 2006), a clear indication that axonal input has been lost. In such cases, OMP expression generally falls anywhere between 3 and 15d after OB deafferentation as a function of the specific type of lesion (Bakos et al., 2010; Inamitsu et al., 1990; Kobayashi et al., 2013; Nathan et al., 2001; Tsukatani et al., 2003). Here we found significant reduction of OMP protein expression at 3d after FPI. In our mild diffuse injury model axon damage appears to be more limited, as predicted. When examined by WB, OMP levels did rise toward sham control values at 7d postinjury, but were not significantly different from 3d levels. By the 21d onset of synaptic regeneration, OMP expression was equal to normal levels.

In parallel IHC experiments, this 3d OMP reduction was confirmed, clearly associated with reduced protein localization and evidence of OMP disorganization within affected OB glomeruli. As expected, this 3d drop in OMP signal revealed time-dependent differences relative to early 1d postinjury response and the later 21d interval of axon regeneration. We conclude that this OMP
time course confirms acute ORN axon damage after FPI, likely within a subpopulation of pre-synaptic olfactory sensory axons.

Overall, our OMP findings are consistent with other OB deafferentation models discussed above, showing a protracted subacute degenerative period relative to other injured brain regions. However, OMP expression after FPI does indicate that a shorter degenerative phase may exist for milder, diffuse OB deafferentation. At 1d postinjury, we did not expect a large OMP reduction, since a subset of injured axons would not generate a large change in the marker immediately, but only after secondary injury processes are initiated, as discussed in Chapter 1. Further, even though OB degeneration in other models can extend through 15d, our data show initial OMP recovery by 7d. Nevertheless, reduced OMP expression after FPI was temporally correlated with 3d increase in the 150kD αII-spectrin fragment, which, taken together support ORN sensory axon degeneration. In addition, the fact that FPI failed to affect the generation of the calpain-derived 145 and caspase-derived 120kD αII-spectrin peptides, which also fits with a more diffusely injured axon population.

Another striking finding was the 3d postinjury correlation of OMP reduction and peak glial reactivity in the affected glomeruli, implicating participation of reactive OB neuroglia in the clearance of degenerated ORN axonal terminals and promotion of synaptic reorganization. Thus, 3d after FPI represents an important time point for significant ORN damage and the initiation of glial role in early clearance of axonal debris within the OB.
Pre-synaptic markers show minimal change after FPI

The loss of ORN axons indicated by our αII-spectrin and OMP data supports FPI induction of the acute degenerative phase of reactive synaptogenesis. This loss is frequently accompanied by disruption of synaptic vesicles within pre-synaptic terminals, thereby reducing the level of proteins which regulate synaptic vesicle movement and membrane fusion. Synapsins I and II are two such proteins, molecules which bind to and direct neurotransmitter vesicles. While a downward shift in Synapsin level has been used as an index of CNS synaptic deafferentation (Chan et al., 2014; Griesbach et al., 2009; Wu et al., 2011), the effect of TBI on its expression is varied and controversial. In some studies, it increases after injury (Griesbach et al., 2004a,b), while in others Synapsin expression is no different from controls (Griesbach et al., 2007; Xuan et al., 2015). Nevertheless, most reports support its acute reduction after brain insults (Wu et al., 2006; Aiguo et al., 2010; Wu et al., 2011; Shang et al., 2014; Ansari et al., 2008a,b; Chan et al., 2014). Over time, an increase in pre-synaptic protein markers like Synapsin would be expected during regenerative or stabilization periods, when reinnervation occurs.

Despite evidence for αII-spectrin lysis, OMP reduction, and synaptic disorganization in the OB after FPI, we failed to detect injury vs. sham control differences for either Synapsin-I or Synapsin-II over 1-21d of survival. There was, however, a trend toward reduction of both Synapsin-I isoforms, and the lower kD form of Synapsin-II. The only significant differences identified were time-dependent shifts in protein level of 80kD Synapsin-I, with the late degenerative/early regenerative phases showing reduced expression relative to one another. While tissue Synapsin level may be a reliable metric for other brain regions and more severe models of CNS axotomy, it does not appear to serve as robust marker of FPI-induced
deafferentation within the OB. This result is somewhat surprising since Synapsin-II is highly expressed within the glomerular layer, co-localizing with OMP (Stone et al., 1994), and Synapsin-I is found throughout the OB laminae, being less specifically associated with ORN axon marker OMP (Kasowski et al., 1999). The most likely explanation for our inability to detect changes in postinjury Synapsin expression would be that diffuse FPI effect was diluted due to sampling of protein extracts derived from the entire OB. In fact, the study by Stone and colleagues (1994), which used an intranasal Zn sulfate lesion, inducing a more severe OB insult, reported significant reduction of local glomerular Synapsin-II and OMP. We posit a smaller subset of axons are injured in our model, making the proportion of those with Synapsin loss low relative to the total synaptic content of the entire OB. In order to confirm our present Synapsin results, additional studies which limit sample dissection to the glomerular layers are needed.

**GAP-43 increase marks onset of OB reinnervation after FPI**

As a confirmation of successful axon sprouting and synaptic reformation after FPI, we conducted additional experiments probing for expression of GAP-43. This protein is known to be elevated over periods of axon growth and extension during development and in CNS regeneration (Shen et al., 2002; Latchney et al., 2014). Following peripheral nerve crush or transection, GAP-43 expression is upregulated in growth cones and sprouting axons undergoing regeneration (Knyihár-Csillik et al., 1992). Further, overexpression of GAP-43 has been linked to spontaneous formation of new synapses and enhanced sprouting after injury (Benowitz and Routtenberg, 1997). Additionally, Thompson et al. (2006) have demonstrated concurrent expression of GAP-43 and pre-synaptic synaptophysin, promoting GAP-43 as a marker of synaptogenesis. Relative to sham control samples, we found increased expression of GAP-43
restricted to 21d, the period when axon sprouting and synapse regeneration is reported to begin
within the deafferented OB. Moreover, these data are consistent with the stability of αII-spectrin
cytoskeleton at 21d after FPI, also supportive of regenerative processes directed by GAP-43. As
expected, this large elevation in GAP-43 showed significant time-dependent differences between
each of the other time points. Little change was seen in GAP-43 level over the three earlier time
points, however, the protein trends toward increase over controls at 3d postinjury, the same time
period when we see significant reduction of OMP signal. This slight shift is worth noting since
others propose that OMP inversely regulates GAP-43 production (Griff et al., 2000). That study
used OMPKO mice to show that loss of OMP increased ORN axon diameter and the number of
OB sensory axo-dendritic synapses, attributed to dysregulated rise in GAP-43 production. It is
possible that FPI may also alter OMP regulation of GAP-43 during specific phases of OB
reactive synaptogenesis. While our data does not currently show a significant rise in GAP-43 at
3d, more select sampling of glomerular regions could confirm such a relationship between OMP
and GAP-43.

Time-dependent differences in OB GAP-43 expression are clearly consistent with FPI pathology
driving synaptic regeneration in the structure. The significant increase of this growth-regulating
protein at 21d matches onset of synaptic restoration, as nascent terminals are guided to their post-
synaptic targets. This period is has been defined as the regenerative phase of OB
synaptogenesis, shown to occur between 15-60d post-injury (Graziadei et al., 1978; Graziadei et
al., 1979). Others have reported that newly formed glomeruli express increased GAP-43,
presumably during axonal re-growth (Cizková et al., 1995), which also supports our
interpretation of the role of elevated GAP-43 in deafferented OB at 21d after FPI. Given that
surrounding neuroglia could be responsible for GAP-43 upregulation, it is also interesting that the increase in this growth protein did not coincide with peak microglial and astrocyte reactivity identified by IBA-1 and GFAP. One possible explanation for this difference would be that a second wave of glial activation, not dependent on structural hypertrophy and occurring during a postinjury interval not sampled here, contributes to the 21d GAP-43 rise during later synapse regeneration. Alternatively, injury could drive increased expression of GAP-43 within olfactory ensheathing cells (OECs), typically restricted to ORN layers. One report exploring OEC transplantation into the cortical injury site of a weight drop model found increased GAP-43 signal in the adjacent tissue, correlating with higher synaptophysin and neurological protection (Wang et al., 2014). Thus, several lines of evidence show GAP-43 is involved in OB reactive synaptogenesis, but the specific cellular source(s) remain to be determined.

Axon and synapse integrity within OB glomeruli is compromised after FPI

Given that we observed molecular and IHC evidence for OB synaptic disruption following FPI, our last set of experiments focused on applying TEM methods to determine if these effects of diffuse axonal injury could be detected as change in glomerular synapto-dendritic structure. Overall, qualitative glomerular ultrastructure supported our other outcome measures, showing that FPI resulted in progressive structural degeneration of ORN sensory synapses over the same 1-7d postinjury interval. TEM revealed a similar series of events: first, axon terminal degeneration, then synaptic disruption and glial phagocytosis, followed by early stages of synapse reorganization at 21d. Consistent with other TEM studies of the OB (Chao et al., 1997; Valverde and Lopez Mascaraque, 1991; Kasowski et al., 1999) controls exhibited electron dense ORN axon terminals grouped around dendrites of mitral and tufted cells, forming well defined
synaptic structure. Pre-synaptic vesicles were seen opposed to synaptic membranes, which had thick post-synaptic densities. This profile was shifted toward a less organized and degenerating axo-dendritic structure as early as 3d after injury, consistent with the loss of OB OMP and increased αII-spectrin breakdown at that time. By 7d, acute pathology had evolved to form shrunken ORN axons with irregular shapes and sites with hypertrophied glial processes containing phagocytosed tissue debris. We interpret this increase in membranous material within OB astrocytes to be in line with a reduced expression of GFAP cytoskeletal protein, as the cells shift to a more phagocytic function at the 7-21d postinjury interval. While the majority of glomerular glial response appeared to be astrocyte in nature, reactive microglia were observed in the more peripheral zones, potentially serving as phagocytes within damaged axon bundles as seen after bullectomy (Smithson and Kawaja, 2010). Further, since WB data showed 7d OMP moving toward control levels, although not significantly different from 3d cases, we conclude that this intermediate time point is a highly transitional period between degeneration and regeneration for OB reactive synaptogenesis.

Summary
Collectively, these data show that the central FPI delivered to FVB/NJ mice is sufficient to cause ORN axotomy, induce deafferentation of OB glomeruli and generate overt synaptic disorganization. Reactive gliosis accompanies this injury sequence, contributing to removal of degenerative debris, and likely producing molecules which direct synaptic repair. These events occur despite the diffuse and somewhat mild nature of FPI, as well as the distance of the OB from injury epicenter. This injury profile is also consistent with the reported olfactory deficits in clinical TBI. Importantly, it also validates our experimental model for the further studies
described in Chapters 3 and 4, addressing the interaction between ECM proteins that regulate the recovery process.
CHAPTER 3

MMP9/OPN SIGNALING MARKS OB SYNAPTIC REORGANIZATION AFTER FPI
ABSTRACT

TBI has potential to axotomize ORNs, leading to deafferentation of OB synapses and olfactory deficits. In the plastic OB, ORNs are capable of OB reinnervation and synapse restoration after damage. ECM modulators like MMPs are upregulated in the OB after ORN axotomy and implicated in recovery processes. In Chapter 2, we showed that mild FPI was a viable model to induce OB injury and cause deafferentation. We posit that MMP9, an acute responder to TBI and ORN axotomy, mediates OB reinnervation by processing its substrate OPN. Proteolytic fragments of OPN express amino acid sequences with integrin and CD44 receptor binding capabilities, through which cell signaling pathways can promote glial migration, proliferation, and phagocytosis. Here, we profile the postinjury time course of MMP9 activity, OPN fragmentation, and CD44 receptor expression in order to determine whether MMP9/OPN interaction plays a role in OB synapse repair. Gelatin zymography analyses revealed significant elevations in MMP9 at 1 and 7d and MMP2 activity at 7d postinjury, highlighting both 1 and 7d as a critical periods in ECM modulation. Despite the fact that full length 66kD OPN did not change after FPI, however, we did observe increases in OB 48kD OPN at 1 and 7d, as well as a reduction in 32kD OPN at 7d. This result points to a time-dependent correlation between OPN processing and injury-induced gelatinase activity. IHC revealed that MMP9 was localized to reactive astrocytes and increased in these cells 7d after FPI, as well as within post-synaptic neuronal compartments. However, OPN did not co-localize with GFAP+ or IBA1+ cells, but labeled neurons in the EPL and MCL only. Co-labeling experiments with periglomerular cell marker tyrosine hydroxylase, supporting selective OPN expression in OB tufted and mitral cells. Further, IHC failed to show co-localization of neuronal OPN and microtubule associated protein-2, suggesting that intracellular OPN isoforms bound to microtubules were not predominant.
Interestingly, CD44 receptor protein was upregulated at both 3 and 7d, which was consistent with maximum glial activation observed in Chapter 2. We conclude that MMP9-generated OPN fragments are critical signals for the temporal progression of OB reactive synaptogenesis following FPI. Moreover, the generation of these OPN signals is likely sourced within both deafferented neurons and the ECM, where secreted MMP9 and OPN interact. Mapping of postinjury CD44 receptor expression suggests that an indirect OPN/CD44 interaction may foster cell signaling pathways to enhance glial mediation of successful OB synaptic plasticity after TBI.

INTRODUCTION

Adaptive and maladaptive models of synaptic recovery have shown that ECM components like proteoglycans and MMPs can direct the restoration of synaptic terminals after injury (Phillips and Reeves, 2001; Kim et al., 2005; Chan et al., 2014; Dityatev et al., 2004; Falo et al., 2008; Harris, et al., 2011; Harris et al., 2010; Mahmood et al., 2014; Warren, et al., 2012; Yamaguchi, 2001). Reactive synaptogenesis after TBI is a well-documented phenomenon, with stages representing injury-induced loss of synapses, glial-mediated removal of cellular debris and degenerating axons, sprouting of new terminals, and stabilization of nascent synaptic connections. While the process has been confirmed in focal models of deafferentation in hippocampus (Steward et al., 1988) and OB (Oley et al., 1975), whether synaptic repair could be induced in the OB following mild diffuse injury was previously unknown. In Chapter 2, we carefully documented the acute/subacute time course of OB deafferentation and reinnervation with mild FPI. Next, we aim to explore whether the posited MMP9/OPN interaction may contribute to successful TBI-induced OB synaptic plasticity. The link between ECM and plasticity is well documented (Frischknecht and Gundelfinger, 2012; Phillips et al., 2014;
In particular, several studies have focused on the dual roles of MMPs in head trauma, being either neuroprotective or causing detrimental consequences (Phillips et al., 2014). Consistently, time proves to be an important factor in MMP function, as early activation of MMPs usually promotes degenerative processes to clear injury debris, which are typically beneficial, but later, prolonged MMP activation can inhibit repair mechanisms. Understanding time-dependent activity of MMPs has been useful, yet therapeutics aimed at reducing MMP activity for TBI recovery have had little success in the clinic (Zhang et al., 2010; Abdul-Muneer et al., 2015). The reason for this outcome is largely considered to have been due to the pleiotropy and complexity of each enzyme, and limited specificity of MMP inhibitors. Nonetheless, therapeutic agents that focus on MMP activity remain viable options, given that appropriate molecular mechanisms are targeted.

As discussed in Chapter 1, gelatinases A and B (MMPs 2, 9) have been explored extensively in models of brain injury and functional recovery (Bakos et al., 2010; Costanzo et al., 2006; Costanzo and Perrino, 2008; Hsu et al., 2006; Hsu et al., 2008; Phillips and Reeves, 2001; Verslegers et al., 2013; Wang et al., 2000). In particular, several investigators have focused on the role(s) of MMPs 2, 9 in synaptogenesis of the deafferented OB. During the period of early reactive synaptogenesis (1-15d), following focal models of ORN axotomy, activity of both gelatinases increases (Bakos et al., 2010; Costanzo et al., 2006; Costanzo and Perrino, 2008). MMP9 generally peaks strongly and acutely within 5d (Bakos et al., 2010; Costanzo et al., 2006), emphasizing its importance in degenerative processes. Conversely, MMP2 expression is also upregulated following OB deafferentation, although its delayed peak suggests a more prominent role in facilitating later recovery. These differences highlight the importance of
understanding time course in molecular processes underlying synaptic repair. Injury-induced MMP9 elevation has consistently been detected in preclinical experiments and clinical trials (de Castro et al., 2000; Horstmann et al., 2006; Noble et al., 2002) as well as several models of axotomy (Costanzo et al., 2006; Chan et al., 2014), yet exact gelatinase-mediated mechanisms guiding synaptic recovery of the OB are still unknown. We posit that defining the time course of postinjury MMP9 activity in our OB model and testing a candidate substrate which could signal cell-based GL synaptic repair, will reveal important targets for development of therapeutics to support recovery of olfactory function.

One novel MMP substrate associated with such cellular signaling is the cytokine OPN. Interestingly, OPN is a dynamic phosphoprotein expressed in several tissues, with neuroprotective functions in models of brain injury (Chan et al., 2014; Hashimoto et al., 2007; Jin et al., 2014; Miyazaki et al., 2008; Topkoru et al., 2013). It is a substrate for several MMPs, including MMP9 (Lindsey et al., 2015; Takafuji et al., 2007; Tan et al., 2013). OPN is typically secreted into the ECM, and cleaved by matrix-dwelling enzymes. Upon cleavage, integrin receptor-binding RGD and SVVYGLR sequences are exposed, giving the OPN fragments important cell signaling capabilities. Signal transduction through integrin receptors (particularly the $\alpha_v\beta_3$ vitronectin receptor) is one of the primary mechanisms by which OPN facilitates its adhesion, migration, and proliferative properties. Both large and small OPN fragments, which are produced by MMP9 proteolysis, can be critical to the recovery process after FPI, so it is important to assess postinjury expression and localization of each. Further, interaction of OPN with hyaluronic acid receptor CD44 has also been well documented after CNS insult and is important for some OPN properties, such as migration and paracrine signaling between reactive
glial cells. While CD44 expression has not been examined during synaptic repair in many TBI models, the receptor has been reported to increase in tandem with OPN after cortical cryolesion (Shin et al., 2005), transient forebrain ischemia (Kang et al., 2008), and other central nerve injury models (Günther et al., 2015). Increased glial reactivity in the OB after FPI-induced axotomy, documented in Chapter 2, encourages exploration of change in CD44 expression, as altered glial function can often be mediated by cell surface receptors like CD44.

To determine MMP9-OPN-receptor role in OB reactive synaptogenesis after FPI, we first measured postinjury change of MMP9 enzyme activity. We then employed WB analyses to correlate the time course of MMP9 activity with expression of full-length and fragmented OPN, determining if an enzyme-substrate relationship existed over time postinjury. Additionally, we utilized IHC to explore OB tissue localization of OPN and MMP9 within in order to identify the cellular sources of these proteins during synaptogenesis. Lastly, CD44 receptor expression was also assessed by WB over the same 21d OB synaptic recovery period to determine if OPN fragmentation tracks with growth factor expression. Our results support a biphasic, time-dependent postinjury interaction between MMP9 and OPN, generating critical cell signaling peptides that show a complex relationship with glial reactivity and expression of CD44 cell surface receptors. Our analyses in Chapter 3 also revealed relationships between this MMP9/OPN cell activation mechanism and other markers of ORN axon damage (e.g., αII-spectrin and OMP) identifying, as yet unexplored, pathways potentially regulating synaptogenesis after FPI.
METHODS

Experimental Animals

All procedures met national guidelines for care and use of laboratory animals, and all experimental protocols were approved by the VCU Institutional Animal Care and Use Committee. FVB/NJ WT adult male mice (The Jackson Laboratory, Bar Harbor, ME) were housed (4 littermates/cage) under a temperature (22°C) and humidity controlled environment, with food and water ad libitum, and subjected to a 12h dark-light cycle. WT mice (20-30g; 8-11 weeks old) were randomly selected and subjected to midline FPI. WT sham-injured cases served as control. Subsets of FPI and sham-injured groups [WT Sham (n=48), WT FVB TBI (n=53)] were allowed to survive for either 1, 3, 7 or 21d post-injury prior to molecular or histological analysis.

Surgery Preparation and Injury

Mice were anesthetized with isoflurane (4% in 100% O₂ carrier gas) and maintained on 2.5% isoflurane in carrier gas delivered through a nose cone. Once stabilized in a stereotaxic frame, heads were shaved, body temperature maintained at 40°C by Gaymar T/Pump water pump (Gaymar Industries Inc, Orchard Park, NY) and heart rate (bpm), arterial oxygen saturation (percent O₂), breath rate (brpm) and pulse/breath distension (µm) monitored by pulse oximetry (MouseOx; Starr Life Sciences, Oakmont, PA). Mice then received a midline incision and a 2.7 mm craniectomy prepared over the midline, centered between bregma and lambda. Without damaging the underlying dura, a Leur-Loc syringe hub was cemented to the skull surrounding the craniectomy and dental acrylic poured around the hub to stabilize the site. Topical anesthetic/antibiotic was applied to the incision site and the mice housed in recovery cages.
After one hour of surgical recovery, mice were anesthetized for 4 mins (4% isoflurane, 100% O₂), and subjected to FPI as previously described (Dixon et al., 1987; Reeves et al., 2012). The device consisted of a 60 x 4.5 cm Plexiglas water filled cylinder, fitted at one end with a piston mounted on O-rings, with the opposite end housing a pressure transducer (EPN-0300A; Entran Devices, Inc., Fairfield, NJ). At the time of injury, the Leur-Loc fitting, filled with saline, was attached to the transducer housing. Injury was produced by a metal pendulum striking the piston, transiently injecting a small volume of saline into the cranial cavity and briefly deforming the brain tissue (20 millisecond pulse duration). The resulting pressure pulse was recorded extra cranially and registered 1.3±0.1 atm pressure. After injury, all mice were promptly ventilated with room air until spontaneous breathing resumed. The duration of suppression of the righting reflex (5.0±2.0 mins) was used as an index of traumatic unconsciousness. Once righting reflex was determined, mice were re-anesthetized for hub removal, scalp suture and topical anesthetic/antibiotic application. Sham-injured controls received the same surgical preparation, anesthesia and connection to the injury device, except that the intracranial pressure pulse was not applied. All animals were returned to their home cages and assessed for weight loss, locomotion, and eye/nose exudate once per day until weights stabilized.

**Protein Extraction**

WT mice were anaesthetized with 4% isoflurane in carrier gas of 100% O₂ for 4 min, then sacrificed by decapitation at 1, 3, 7 or 21d after FPI or sham injury, with bilateral OBs dissected (n=4-7/group) for assessment of protein expression. Tissue samples were homogenized on ice in 100 µl of RIPA Lysis Buffer (EMD Millipore, Billerica, MA), and centrifuged at 14,000 x g for 20 min at 4°C. Supernatant was aliquoted and stored at -80°C. Prior to or zymography analysis,
protein concentration was determined using Pierce BCA Protein Assay Reagent (Thermo-Fisher, Waltham, MA) and the FLUOstar Optima plate reader (BMG Labtech, Inc., Cary, NC).

**Western Blotting**

WB analysis was carried out utilizing Bio-Rad products (Hercules, CA). Twenty µg of protein was prepared in WB XT Sample Buffer and reducing agent (Bio-Rad Laboratories), then denatured at 95°C for 5 mins. Samples were electrophoresed on 4-12% or 12% Bis-Tris Criterion XT gels (200v x 45 min in MOPS running buffer), then protein transferred onto polyvinylidene fluoride (PVDF) membranes (1h at 100V). Post-blotted gels were stained with 0.1% Coomassie Brilliant Blue (Sigma-Aldrich, St. Louis, MO) in 40%MeOH+10% glacial acetic acid, then de-stained at RT to confirm protein load and even transfer. Membranes were rinsed with deionized water and Tris-buffered saline (TBS) before blocking with 5% milk TBS-Tween (mTBS-T). Blots were then incubated in 5% mTBS-T overnight (4°C) with individual primary antibodies to osteopontin (OPN; 1:500, R&D Systems, Minneapolis, MN; 1:500, Rockland Immunochemicals Inc., Limerick, PA) and CD44 (1:500, BD Biosciences, San Jose, CA). After primary incubation, membranes were washed with mTBS-T, then incubated with appropriate HRP-linked secondary antibodies [IgG bovine anti-goat, IgG bovine anti-rabbit (1:15,000, Santa Cruz, Dallas, TX), IgG goat anti-rat (1:15,000, Rockland Immunochemicals Inc., Limerick, PA)] in mTBS-T for 1h at RT. Finally, blots were washed with mTBS-T and antibody binding visualized using Super Signal Dura West chemiluminescence substrate (Thermo-Fisher, Waltham, MA). WB images were captured with Syngene G:Box and positive band signal subjected to densitometric analysis (relative optical density, ROD) with Gene Tools software (Syngene, Frederick, MD). Protein data were expressed as percent change relative to
paired WT Sham control cases run on the same transferred gel. Cyclophillin A (EMD Millipore, Billerica, MA) or beta actin (Sigma-Aldrich, St. Louis, MO) was used as load controls for signal detection.

**Immunohistochemistry**

At 3, 7 and 21d post-injury WT injured and WT sham mice (n=4/group) were prepared for fluorescent IHC analysis according to published protocol (Warren et al., 2012). Animals were anaesthetized with sodium pentobarbital (400mg/kg, i.p.), transcardially perfused with 0.9% saline followed by 4% paraformaldehyde in phosphate buffer (0.1M NaHPO₄, pH=7.4), after which brains with attached OBs were extracted and placed in fixative for 24 h before transfer to 0.03% NaN₃ in 1.0 M phosphate buffered saline (PBS). For IHC, fixed brains were cryoprotected in 30% sucrose for 3d, sucrose solutions exchanged after each day. Frontal lobes were blocked and attached bulbs mounted in Tissue Tek media (Thermo-Fisher, Waltham, MA) and stored at -80°C. Coronal cryostat OB sections (13µM) were collected using a Cryostar™NX70; Cryotome™FSE; HM525 NX cryostat (Thermo-Fisher, Waltham, MA) and prepared for immunofluorescence visualization.

Free floating sections were first permeabilized in 5% peroxidase for 30 mins. After a wash with PBS, tissues were pre-incubated in blocking buffer (fish gelatin in PBS + 0.05% triton X-100) to prevent non-specific binding, and then incubated overnight in primary antibody (OPN, 1:40, R&D Systems, Minneapolis, MN; MMP9, 1:100, R&D Systems, Minneapolis, MN; microtubule-associated protein 2 [MAP2], 1:500, EMD Millipore, Billerica, MA; tyrosine hydroxylase [TH], 1:300, EMD Millipore, Billerica, MA; glial fibrillary acidic protein [GFAP],
1:20,000, Dako; ionized calcium binding adaptor protein [IBA1] for microglia, 1:300, Wako, Richmond, VA) at 4°C. Sections were next washed with PBS, placed in blocking buffer for 30 mins, after which they were incubated with secondary fluorescent antibody (Alexa-Fluor 488 donkey anti-goat, 1:1000 and Alexa-Fluor 594 donkey anti-rabbit, 1:1000, Thermo-Fisher, Waltham, MA) in Blotto for 1h at RT. Slices were then PBS washed, equilibrated in phosphate buffer, and mounted on Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA) with Vectashield + DAPI (Vector Laboratories, Burlingame, CA). IHC signal was visualized on the Zeiss LSM 700 (Carl Zeiss, Thornwood, NY) confocal microscope (VCU Microscopy Core).

**Gelatin zymography**

Zymographic enzyme assay was performed using previously described methods (Chan et al., 2014). Briefly, 20 µg of protein from OB extracts described above were prepared with 2x Tris-glycine SDS sample buffer, then separated by gelatin electrophoresis (at 4°C, 45V for 1.5 h) on Novex® 10% zymogram gels (Thermo-Fisher, Waltham, MA). Gels were renatured in Novex® Zymogram Renaturing Buffer (Thermo-Fisher, Waltham, MA) for 30 min at RT before development in Novex® Zymogram Developing Buffer over 6 d at 37°C. Gelatin lysis was visualized with Coomassie brilliant blue (Sigma-Aldrich, St. Louis, MO) and purified enzyme run as positive control. Zymogram signal was captured on Syngene G:BOX, and densitometry analyzed as ROD with Gene Tools software (Syngene, Frederick, MD). Enzyme activity was expressed as percent change relative to paired controls run on the same gel.
Statistics

The effects of injury, and the time postinjury, on protein levels and gelatinase activity were evaluated using GLM SPSS v.23 (International Business Machines, Corp., Armonk, NY). These analyses used 2-way completely randomized ANOVAs. Specific pairwise comparisons were evaluated using the Duncan Post Hoc Test. Western blot and zymography results are reported as mean +/- SEM. An alpha level of 0.05 was used in all analyses.

RESULTS

MMP2, 9 activity changes in response to FPI

Prior studies in numerous brain injury models have confirmed that secreted MMPs and inflammatory cytokines are elevated postinjury, contributing to both acute pathophysiology and chronic repair mechanisms (Rosenberg et al., 2012; Phillips et al., 2014; Woodcock and Morganti-Kossmann, 2013). Time-dependent increases in OB gelatinases MMPs 2, 9 were reported during early postinjury intervals of pre-synaptic degeneration induced by either ORN knife cut axotomy or chemical lesion (Bakos et al., 2010; Costanzo et al., 2006; Costanzo and Perrino, 2008), however, OB MMP response to FPI has not been determined. Here, we first assessed whole OB protein extracts with gelatin zymography to determine whether our injury would increase each gelatinase over the 1-21 postinjury interval. Overall, we found elevated OB activity for both gelatinases MMP9 and MMP2 at 7d after FPI, with MMP9 also increasing in the acute postinjury period. At 1d postinjury, FVB/NJ WT mice showed a 2-fold rise in MMP9 activity versus sham controls (236.85±50.71, p<0.01). By contrast, 1d MMP2 lysis was the same as control values (99.48±10.63) and activity for both gelatinases remained equivalent to that of sham injured at 3d after FPI (117.40±17.71;107.69±10.89). Interestingly, at 7d after injury, the
Figure 3.1 FPI induces time-dependent increase of OB gelatinase activity

A. Zymographic assessment of postinjury OB gelatinase activity revealed prominent elevation of MMP9 relative to sham controls. As seen in other CNS insult models, acute increase of MMP9 activity occurred at 1d after injury. This effect was normalized by 3d, where enzyme lysis returned to control level. Surprisingly, we found a second phase of MMP9 activation at 7d postinjury, nearly 3-fold higher than sham proteolysis. By 21d, activity had again returned to control values. This biphasic pattern of OB MMP9 activity is novel in the context of diffuse TBI. Other brain regions and insults show predominant peak MMP9 activity only at the early postinjury intervals. Post hoc analysis confirmed the 7d peak MMP9 proteolysis to be different from its bracketed 3 and 21d intervals, where the enzyme was longer activated. B. Increase in MMP2 activity was also detected, but with two large differences: increase was confined to the 7d postinjury time point and this was only a modest elevation. MMP2 activity was not different from controls at 1, 3, or 21d postinjury. Results are expressed as percent of sham control relative optical density (100% dashed line). C. Representative zymogram images of Sham (left), Injured (right) MMP9, MMP2 are illustrated. #p<0.05 day x vs. y. *p<0.05 Sham vs. TBI; **p<0.01 Sham vs. TBI. N=4-10/group.
OB exhibited a significant elevation in both MMP9 (279.54±52.58, p<0.001) and MMP2 (130.75±10.02, p<0.05) (Figure 3.1). At this time point MMP9 activation was predominant (achieving a maximal rise of nearly 3-fold over sham cases), whereas MMP2 increase in activity only reached 31% over controls. By 21d, the period marking onset of ORN axon reinnervation, activity of each gelatinase was not different from baseline sham injured levels (111.79±10.42; 106.43±8.66). Notably, post hoc analysis showed that the MMP9 7d rise was also significantly increased relative to 3 and 21d postinjury time points (p<0.05), reflecting critical periods of enzyme activation at 1 and 7d postinjury. We conclude that, while both MMP2 and MMP9 were elevated at 7d, the differences in their time-dependent expression patterns suggest they may have unique roles in the recovery process. These findings indicate a critical gelatinase role during the early degenerative phases of OB reactive synaptogenesis following FPI deafferentation, and more specifically, a predominant role for MMP9 at days 1 and 7 postinjury.

**MMP9 localization supports glial response in deafferented OB**

Our molecular analyses suggested that during acute axon degeneration in the OB, secreted gelatinase MMP9 serves a key role, which likely includes processing intercellular signals for glial/neuronal reorganization. Prior studies have mapped MMP9 expression to both neuronal and glial cell types (Phillips et al., 2014). As reactive astrocytes closely associate with glomerular synapses, we performed IHC for cell specific localization of MMP9 in OB astrocytes after FPI. Our results showed that GFAP+ astrocytes surrounding glomeruli did contain MMP9, and that MMP9 was highly elevated within the GL and EPL at 7d postinjury (Figure 3.2). This not only supports the astrocyte as a primary glial source of elevated MMP9 in deafferented OB, but also reveals significant MMP9 upregulation within neuronal processes of deafferented GL
Figure 3.2 IHC of MMP9 protein distribution within OB following FPI
A. Confocal imaging of sham cases shows low level of MMP9 (green) labeling within the GL and EPL, appearing as punctate signal. Astrocytes labeled with GFAP (red) were localized to the GL and showed non-reactive phenotype (white arrows). Boxed sham area shown in high magnification at right, with no detectable MMP9 expression in GL astrocytes (white arrows).

B. MMP9 protein staining intensity was increased within the same OB layers 7d after FPI (yellow star=GL; red star=EPL) and visible within reactive astrocytes (white arrows). Boxed 7d area shown in high magnification at right, with no MMP9 expression clearly visible within reactive GL astrocytes (white arrows). These results are consistent with 7d rise in MMP9 activity assessed by gelatin zymography. Bar in A=50 µm; B=20 µm. N=3-4/group.
and adjacent OB layers. Together these two findings suggest a complex enzyme response to FPI, where astrocytes likely secrete MMP9 to process matrix proteins, including OPN, driving critical glial response, and affected neurons either produce and secrete MMP9 or utilize the enzyme intracellularly to process substrates like OPN.

**FPI does not change full length OPN expression**

Recent studies in deafferented hippocampus identified OPN as a mediator of synaptic plasticity, specifically linking its RGD integrin signaling fragment to glial reactivity, degenerative terminal removal and adaptive synaptic recovery (Chan et al., 2014). The observation that FPI causes OB glial reactivity (see Chapter 2), possibly induced by integrin signaling pathways, and a clear elevation of MMP9, which cleaves OPN to generate RGD exposed fragments (Lindsey et al., 2015; Scatena et al., 2007; Takafuji et al., 2007), lead us to examine full length OPN and OPN fragment generation in the deafferented OB. FVB/NJ WT OB protein extracts were again used to conduct WB probe for full length OPN (66kD) (Figure 3.3) and proposed OPN fragments (see Figure 3.4 below). Despite changes in MMP9 activity at 7d, full length OPN did not change for any time point after injury when compared with sham injured controls. Surprisingly, levels of 66kD OPN trended higher than sham controls at 1 and 3d postinjury (128.08±21.57; 124.84±10.12), however, these differences failed to reach significance. By contrast, full length OPN showed the opposite response at both 7 and 21d postinjury, trending lower than control values (77.33±12.39; 85.64±10.83), but again these changes failed to reach statistical significance. Nevertheless, post hoc comparison did reveal differences in 66kD OPN expression over time. The 7d level of this protein was found to be significantly lower than both 1 and 3d expression (p<0.05). These data suggest the possibility that small changes in full length OPN do
Figure 3.3 Full length OPN expression in OB is unchanged after FPI
A. WB analysis of full length OPN (66kD) in deafferented OB failed to show injury effect relative to sham controls. Nevertheless, a trend toward increase at 1 and 3d postinjury and decrease at 7d, was observed, but each time point failed to reach significance versus shams. Post hoc time point comparisons did reveal that 1 and 3d 66kD OPN expression was higher than 7d level. This shift toward 7d reduction is consistent with the significant MMP9 activity and OPN fragment generation observed at that postinjury time point. Results are expressed as percent of sham control relative optical density (100% dashed line). B. Representative blot images of 66kD OPN, with β-actin load controls, are illustrated below. *p<0.05 day x vs. y. N=4-7/group.
occur after FPI, and that a trend toward reduction in this form of the protein is consistent with the 7d interval of maximal MMP9 activation. It is possible that the overall non-significant change in 66kD OPN relative to sham controls occurs commensurate with altered OPN transcription to compensate for any injury-induced proteolysis, keeping the level of protein stable over time.

**OPN fragment expression is altered following FPI**

In order to examine the level of OB OPN signaling fragment generation after FPI, we utilized a commercial OPN antibody that specifically binds to its proteolytic fragments. According to prior studies (Lindsey et al., 2015; Scatena et al., 2007; Takafuji et al., 2007), MMP lysis of OPN should contribute to cleavage that exposes the RGD/SVVYGLR region of the protein, generating two principal signaling fragments: an amino terminal portion with the RGD sequence accessible, and a carboxy terminal peptide. When probing for OPN fragments, we observed the generation of these two principal OPN bands at 48 and 32kD (**Figure 3.4A**). The 48kD integrin signaling RGD fragment was significantly elevated over control levels at 1d (76%) and 7d (62%) after injury (176.06±22.81, p<0.01; 161.53±12.76, p<0.05), which was correlated with elevated MMP9 activity. By contrast, 3 and 21d levels of this fragment were not different from that of sham injured cases (123.22±18.77; 79.09±4.49). Analysis across the postinjury time intervals revealed that the low level of 48kD OPN at 21d was significantly different from both 1 and 7d elevations in the RGD signaling fragment (p<0.01). These findings support a variable, time-dependent expression pattern of the 48kD OPN signaling fragment, consistent with MMP9 activity. Further, the early increase in this fragment over 1-7d and subsequent stabilization at 21d, when synaptic regeneration is underway and MMP9 lysis normalized, would also support principal OPN signaling role during the period of acute degenerative change.
Figure 3.4 FPI induces time-dependent changes in OB OPN signaling fragments
A. WB analysis of OB 48kD OPN expression showed significant elevation of the RGD signaling fragment at 1 and 7d after FPI when compared with sham controls. At both 3 and 21d postinjury, 48kD OPN levels were not different from controls, however, 21d cases showed a trend toward reduction. Post hoc analysis revealed that the low 48kD OPN signal at 21d was significantly different from both 1 and 7d measures. This data is consistent with peak MMP9 activity at 1 and 7d after FPI and suggests that acute and subacute OPN RGD signaling is important for progression of OB synaptic repair. B. Expression the 32kD OPN peptide was not different from sham controls at 1 and 3d postinjury. By 7d, this fragment was half of sham level, and no longer different from control by 21d. Post hoc analysis pointed to the 7d 32kD reduction as significantly lower than both 1 and 3d signal, again consistent with the peak MMP9 activity at that time interval. Results are expressed as percent of sham control relative optical density (100% dashed line). C. Representative blot images of 48 and 32kD OPN, with cyclophilin A and β-actin load controls, are illustrated below. ##p<0.01 day x vs. y, ###p<0.001 day x vs. y, *p<0.05 Sham vs. TBI, **p<0.01 Sham vs. TBI, #p<0.05 day x vs. y. N=3-6/group.
A

OPN 48 kD

% of Control

1d  3d  7d  21d

B

OPN 32 kD

% of Control

1d  3d  7d  21d

C

48  32  LC

1d  3d  7d  21d

S  I  S  I  S  I  S  I
In contrast to 48kD OPN, the 32kD carboxy terminal fragment, lacking the RGD integrin-binding sequence, exhibited a different postinjury pattern (Figure 3.4B). Here, the only significant change from controls was a 53% reduction at 7d (52.52±7.26, p<0.05). While some variability in expression occurred for the 32kD OPN at 1, 3 and 21d after FPI, none of these changes were significant (117.90±14.02; 137.61±13.85; 78.11±18.10). Post hoc analysis also identified time dependent changes in this OPN fragment. Principally, the early 1 and 3d levels were different from the reduced 32kD OPN at 7d (p<0.01, p<0.001), and the 3d fragment expression different from that at 21d (p<0.01). Interestingly, the smaller carboxy terminal OPN peptide showed opposite injury-induced change at 7d versus the 48kD OPN signaling fragment. This may be due to the fact that increased activity of both gelatinases occurs at 7d, where MMP9 and MMP2 contribute to an elevation in the 48kD RGD-containing fragment, as well as a progressive proteolysis of the smaller non-RGD carboxy-terminal sequence. This correlation of postinjury OPN fragment expression with differential MMP activity is also supported by our observation that acute 1d response to FPI increases the 48kD peptide, but does not change the 32kD form. Such correlates highlight progressive changes in OPN expression during OB reactive synaptogenesis and a predominant role for 48kD in this model. In order to test whether 48kD OPN fragment generation was associated with OB cell signaling after FPI, we next mapped postinjury cellular localization of OPN.

**OPN localization supports neuronal response in deafferented OB**

Gelatin zymography and WB studies revealed time-dependent responses of MMP9 activity and OPN expression in deafferented OB. Following our detection of MMP9 in OB astrocytes, we initially performed IHC to assess OPN localization in OB tissue. Interestingly, we found strong,
selective OPN localization within two neuronal populations, external plexiform tufted and deeper mitral neurons (Figure 3.5A), two of the same populations reported to selectively contain OPN mRNA (Shin et al., 1999). These are the principal post-synaptic neurons affected by OB deafferentation, and their expression of OPN increased over time postinjury, peaking at the 7d interval and normalizing by 21d. Notably, this antibody binding intensity change matches the result of 48kD OPN postinjury fragment formation, implicating neurons as the primary source of OPN. To further substantiate our finding and confirm absence of glial OPN, we performed double label experiments with OPN and astrocyte and microglial markers GFAP and IBA1, respectively. Unlike other brain regions deafferented by FPI, we found no appreciable OPN protein signal within either reactive astrocytes or reactive microglia of the OB (Figure 3.5B).

While OPN signal appeared predominant within the cell bodies of these neurons, it remained unclear if this neuronal OPN served a secretory-peptide signaling function. Given that a variant form of OPN was reported to bind to intra-neuronal microtubule associated proteins (Long et al., 2012), we further probed these tissues to determine if MAP2/OPN co-localization was present in the processes of tufted and mitral neurons (Figure 3.6A). As predicted, we observed clear MAP2 labeling within neuronal processes (principally dendritic), however, little OPN co-localization with MAP2 dendritic marker was found, suggesting that its production in the deafferented OB neurons was more likely directed toward secreted, cell signaling function over bound intracellular role. In a second cell-based probe, we sought to clarify the identity of the OPN positive neurons found closely bordering deafferented glomeruli. To do this, OB tissues were exposed to paired tyrosine hydroxylase (TH) and OPN antibodies, with TH used to
Figure 3.5 IHC of OPN protein distribution within OB following FPI

A. Confocal imaging of sham cases shows OPN (green) localized to cells along the border between the GL and EPL, likely tufted neurons, as well as mitral neurons in the MCL (arrows). After FPI, OPN signal revealed time-dependent increase in these neurons, a modest rise at 3d and a robust increase within the neuronal cell bodies and primary dendrites at 7d postinjury. By 21d, OPN staining intensity was still higher than sham, but appeared reduced relative to the 7d interval. B. Double label experiments with OPN (green) + GFAP (red) at 3d, and OPN (green) + IBA1 (red) at 7d failed to show co-localization of signal, suggesting that OPN was not detectable in reactive glial cells of the deafferented OB. Scale bar = 50µm. N=3-4/group.
Figure 3.6 IHC of OPN expression in OB post-synaptic neurons following FPI
A. Confocal imaging showing the absence of interaction between OPN and microtubules (MAP2 labeled; red) at 7d postinjury. MAP2 signal is present in dendrites (yellow arrows) throughout the OB, higher within dendrite-rich EPL. OPN (green) did not colocalize with MAP2, but was restricted to cell soma of tufted and mitral neurons (white arrows). B. OPN was not found within tyrosine hydroxylase positive (TH; red) inhibitory periglomerular cells. While TH identified a large population of these cells surrounding glomeruli (yellow arrows), OPN (green) was not co-localized in them. This result suggests that OPN positive cells at the GL/EPL border are likely ORN post-synaptic tufted neurons. Scale bar = 50µm. N=3-4/group.
positively identify periglomerular inhibitory neurons forming boundaries around individual glomeruli. Results failed to show co-localization of the two proteins, confirming that the OPN positive cells near OB glomeruli were not TH containing periglomerular cells, but likely local tufted neurons (Figure 3.6B).

Taken together, our IHC results suggest that OB glia do not produce significant levels of OPN, but rather, may be targets of OPN signals generated by tufted and mitral neurons affected by injury. Further, since reactive astrocytes also contain MMP9, we posit that a complex cellular interaction occurs in the OB after FPI, which includes ECM processing of neuronal secreted OPN by astrocyte produced MMP9, activating local cellular response at sites of ORN terminal degeneration, and directing tissue response for reinnervation. Indeed, OB deafferented neurons may play a more precise role in directing this OPN tissue response than in other cortical regions.

FPI upregulates CD44 receptor expression

As a final approach to understanding the complex OPN-mediated OB cell interactions after FPI, we decided to probe for time-dependent postinjury expression of the cell surface receptor, CD44, which can bind OPN signaling fragments. From our observation that FPI induces acute OPN increase and pronounced glial reactivity within the deafferented OB, we posited that OPN and the CD44 receptor expression would show temporal correlation. When FVB/NJ WT OB protein extracts were probed for CD44, we found significant postinjury elevation in the receptor level at both 3d (364.96±16.96, p<0.001) and 7d (213.71±61.16, p<0.05) when compared to sham control cases (Figure 3.7). Interestingly, this early 3d rise in OB CD44 was nearly 3-fold over controls and persisted into the 7d period. We also observed an increase in CD44 protein at 1d
Figure 3.7 Diffuse FPI induces time-dependent increase of CD44 OPN receptor
A. WB analysis of CD44 protein expression showed significant elevation of the OPN receptor at 3 and 7d after FPI when compared with sham controls. By 3d, CD44 was over 3-fold higher than sham, and remained 2-fold higher at 7d. At 1d postinjury, CD44 trended toward increase relative to sham controls, but failed to reach significance, and at 21d, expression equaled control level. Post hoc analysis revealed significant differences in CD44 receptor protein level across time, with the 3d rise different from all other time points, and the reduction between days 3 and 7 also different. B. Results are expressed as percent of sham control relative optical density (100% dashed line). Representative blot images of CD44, with cyclophilin A and β-actin load controls, are illustrated below. ##p<0.01 day x vs. y, ###p<0.001 day x vs. y, *p<0.05 Sham vs. TBI, ***p<0.001 Sham vs. TBI. N=3-6/group.
(190.41±35.52), but this change failed to reach significance when compared with sham injured cases. By 21d postinjury, CD44 levels were again not different from controls (96.77±19.57).

After post hoc analysis, time-dependent differences were found between 3d CD44 and receptor levels at all three additional time points (p<0.01 for 3d vs 1and 7d; p<0.001 for 3d vs 21d). Acute rise in OPN-mediated receptor signaling is consistent with a reported 3d peak in CD44/OPN elevation after ischemia (Kang et al., 2008), and approximates the temporal rise documented after cryolesion (Shin et al., 2005). Notably, 48kD OPN fragment increase was observed at 1d following OB deafferentation, prior to the 3d induction of CD44, and both proteins are concurrently elevated by the 7d time point. Given that we also detected increase in MMP9 activity 1d postinjury and maximal enzyme activity at 7d, we posit that a molecular interaction between MMP9/OPN/CD44 is induced early after FPI to facilitate autocrine/paracrine cell signaling during the 1-7d degenerative phase of OB injury response.

**DISCUSSION**

A hallmark of TBI is diffuse axotomy, a pathology resulting in disruption of axon projections and deafferentation of synaptic circuits. Brain ECM plays an important role in maintaining the appropriate local environment to support structural integrity and function of these circuits (Marcoli et al., 2015). After TBI, collagens, proteoglycans and other glycoproteins, as well as MMPs, modify matrix structure around affected synapases. Further, synaptic repair induced by TBI deafferentation requires time-dependent adjustment of the surrounding ECM to facilitate removal of damaged axons, reshape post-synaptic sites and induce sprouting of re-innervating axon terminals. For example, in deafferented hippocampus, agrin and N-cadherin, matrix proteins which normally act to stabilize synaptic junctions, are reduced in their expression during
early periods of synapse deconstruction/reformation, and are subsequently increased during phases of nascent synapse stabilization (Falo et al., 2008; Warren et al., 2012). Interestingly, postinjury MMP activation is also correlated with these shifts in ECM protein level and, as such, is linked to progress of synaptic development and repair mechanisms (Ethell and Ethell, 2007; Kaliszewska et al., 2012; Verslegers et al., 2013; Phillips et al., 2014; Kelly et al., 2015).

Recently, our laboratory has discovered that OPN, an inflammatory signaling cytokine, is upregulated in the hippocampal glia after brain injury, and its activation is temporally correlated with increased proteolysis by local MMPs (Chan et al., 2014). While several studies suggest OPN may serve as a target for improving postinjury synaptic recovery in cortical brain regions (Shin et al., 2005; Plantman, 2012), it is not known if OPN plays a role in OB reactive synaptogenesis following TBI. Here, we investigate time-dependent MMP9 proteolysis and OPN expression within FVB/NJ mouse OB subjected to mild FPI, an insult which produces ORN axotomy and reactive synaptogenesis (detailed in Chapter 2). Based upon gelatinase (MMP2, 9) response to ORN transection in animal models (Costanzo et al., 2006; Costanzo and Perrino, 2008; Bakos and Costanzo, 2011), and the presence of clinical olfactory deficits after brain trauma (Costanzo and Becker, 1986; Costanzo and Zasler, 1991), we hypothesized that olfactory pathology generated by FPI would induce time-dependent increase in MMP2 and MMP9 activity, leading to OPN signal fragment production, which then directs OB cellular response during reactive synaptogenesis. We also posited that this OPN response would be mediated through reactive glial cells in deafferented glomeruli, which we expect to show time-dependent change in OPN receptor proteins.
Overall, our different outcome measures do support the first hypothesis, showing that MMP9 is the predominant OB gelatinase activated after FPI, and that the OPN RGD receptor binding fragment serves as a principal cell signal during the acute and subacute postinjury intervals. We also found MMP9 present in reactive astrocytes, similar to enzyme distribution in deafferented hippocampus. However, OPN protein localization was different from injured hippocampus, in that it was not present in reactive glia, but restricted to the deafferented mitral and tufted cells of the OB. Moreover, this neuronal OPN did not show the predicted association with cytoskeletal proteins, which characterizes cytoplasmic OPN isoforms, and failed to co-localize within periglomerular inhibitory neurons. We conclude that MMP9/OPN/CD44 signaling likely contributes to OB postinjury synapse degeneration and reinnervation after FPI through ECM processing of neuronal-derived, secreted OPN protein.

**Gelatinase activity shows time-dependent change after FPI**

We first assessed MMP2 and MMP9 proteolytic activity in OB protein extracts over the initial postinjury degenerative phase (1-7d) and at onset of the regenerative phase (21d) using gelatin zymography. These two MMPs are normally produced by both neurons and glia (Phillips et al., 2014; Abdul-Muneer et al., 2015), and assist in the redistribution of ECM substrate proteins, promoting plasticity in brain regions susceptible to axotomy by inducing axon sprouting and growth during synaptogenesis. While gelatinases can exhibit both pathological and beneficial properties within the injured CNS (Huntley, 2012), their time-dependent activity is linked to the success of specific phases of synaptic reorganization. In models of hippocampal deafferentation-induced synaptogenesis, MMP9 activity maximally rises during early degenerative periods, while MMP2 activity evolves later, during axonal sprouting and synapse reformation (Phillips
and Reeves, 2001; Phillips et al., 2014). This phase-related pattern has also been reported to occur for OB gelatinase protein expression following full ORN transection or nasal epithelium ablation (Costanzo et al., 2006; Costanzo and Perrino, 2008; Bakos et al., 2010).

Our study shows that diffuse FPI ORN axotomy can generate time-dependent change in gelatinase activity, however, the temporal pattern of this response appears to vary from more severe OB deafferentation models. Although these other studies assessed MMP9 protein rather than activity, comparison with our FPI model shows that the pattern of enzyme rise and recovery to normal activity is altered, and the relative contribution of the two gelatinases is different. Like FPI, full ORN transection (Costanzo et al., 2006) causes a rapid 1d stimulation of MMP9 protein, followed by reduction at 3d, however, no second peak in MMP9 response occurs with full nerve lesion as is observed with FPI. Even more interesting are the differences between FPI and OE ablation (Bakos et al., 2010). In that case, no OB MMP9 reaction to ORN cell death is detected at 1d postinjury, with only one peak in MMP9 protein generation occurring at 5d, more in line with our second peak in MMP9 activity at 7d. By 21d, we found no difference in MMP9 activity relative to controls, whereas both ORN transection and OE ablation maintain modest elevation in MMP9 protein during the 15-35d period. Overall, these results suggest multiple, perhaps distinct, roles for MMP9 throughout the acute/subacute injury process as a function of injury model. ORN transection and OE ablation are severe; FPI would not cause acute, massive axon damage. It is also interesting that diffuse OB deafferentation produced by FPI reveals two MMP9 activation cycles during the period of degeneration, and requires a longer time to maximally activate MMP9, perhaps due to a retarded rate or extent of axon/synaptic damage. In contrast to MMP9, we found MMP2 activity increase only at 7d postinjury, and to a much lower
extent. Again, this is in contrast to the more severe ORN transection, where the magnitude of the 7d peak in MMP2 protein was equal to that of 1d MMP9 (Costanzo and Perrino, 2008). A distinct difference with diffuse FPI is that 7d MMP9 activity is not equivalent to that of MMP2, but is elevated nearly 3 fold higher.

In parallel IHC experiments we probed for cellular localization of MMP9 in the OB. FPI not only caused an increase in enzyme signal within OB glomerular and external plexiform layers, but also showed clear upregulation within reactive astrocytes surrounding glomeruli. Notably, these changes were most pronounced 7d after FPI, matching our peak of zymography activity. This pattern of MMP9 increase is consistent with reported glial production and distribution of MMPs in and around other cortical synaptic sites deafferented by TBI (Rosenberg, 1995; Wang et al., 2000; Zhang et al., 2010). As discussed below, MMP9 distribution within reactive astrocytes surrounding disrupted synapses suggests an enzyme/substrate interaction between MMP9 and OPN to facilitate OB cell signaling for synaptic repair.

Overall, contrasting MMP9 and MMP2 activity in our FPI model emphasizes the importance of MMP9 over MMP2 following diffuse deafferentation of the OB. Considering that OB reactive synaptogenesis occurs over a longer period of time than in other brain regions, elevated gelatinase activity at both 1 and 7d is consistent with an extended synaptic degeneration/regeneration cycle occurring after diffuse axotomy. We posit that the 7d MMP9 activity represents peak enzyme targeting of critical cellular and molecular substrates that serve to facilitate the onset of synaptic repair. Osteopontin is potentially one such target.
Osteopontin cell signaling fragments increase following FPI

The multiple functions of OPN proteolytic fragments were discussed in Chapter 1, focusing on integrin and CD44 receptor binding which directs specific cellular responses. Most studies exploring OPN role after brain injury map such fragment generation, however, reports in several CNS trauma models do show robust elevation of full length 66kD OPN within 1d postinjury (Ellison et al., 1998; Hashimoto et al., 2007; Kang et al., 2008; Shin et al., 2011; Chan et al., 2014). For example, microglia surrounding stroke infarct show increased OPN mRNA well within 3h after injury (Ellison et al., 1998) and reactive microglia/macrophages exhibit significant rise in OPN by 4d after cortical cryolesion (Shin et al., 2011). In our laboratory, Chan et al. (2014) reported a multifold upregulation of both OPN mRNA and 66kD OPN 1-2d after entorhinal deafferentation of the hippocampus, with reduction to control levels by 7d. The present OB results do support a similar trend toward increase in full length OPN 1-3d and reduction at 7d after FPI, however, the overall analysis showed no significant changes relative to sham controls. This suggests that diffuse FPI insult of the OB fails to induce the multifold increases in 66kD OPN seen with severe hippocampal deafferentation. Again, we interpret this lack of significant effect to be a function of reduced axotomy in our model. Post hoc analysis did, however reveal a time-dependent reduction at 7d compared to 1 and 3d full length OPN, which fits with maximal MMP9 activity at 7d postinjury and could explain the shift toward less intact 66kD OPN at that time point.

Lysis of 66kD OPN can be achieved by thrombin and other proteases, including MMPs 3, 7 and 9 (Agnihtori et al., 2001; Lindsey et al., 2015; Scatena et al., 2007; Takafuji et al., 2007). Primary MMP cleavage occurs at a site near the internal RGD/SVYGLR sequence, generating
a larger amino-terminal fragment (~48kD) containing the exposed RGD integrin binding domain and a smaller carboxy-terminal fragment (~32kD) with hyaluronan receptor binding characteristics (Scatena et al., 2007). This 48kD amino-terminal OPN fragment has been explored in a model of CNS deafferentation, where its elevation has been documented (Chan et al., 2014) and linked to acute immune activation (Wang and Denhardt, 2008). It binds primarily to vitronectin (αvβ3 integrin) and other integrin receptor types, as well as inflammatory cell surface receptors, like the hyaluronan CD44 receptor, directing cell adhesion and migration (Scatena et al., 2007; Weber et al., 1996). Thus, the 48kD OPN fragment has significant potential to direct cellular reactivity and mobility after FPI, influencing early phases of reactive synaptogenesis. Following FPI, we first found a peak of 48kD OPN within the OB at 1d postinjury, an acute interval also reported to show fragment elevation after other forms of CNS injury (Chan et al., 2014). Surprisingly, we saw a second, later significant increase in 48kD fragment generation, coinciding with the peak of OB MMP9 activity at 7d. It became apparent that the pattern of 48kD OPN generation was temporally correlated with level of MMP9 proteolysis detected. The early elevation in enzyme activity at 1d and maximal peak of activity at 7d were matched by significant OPN cleavage, while the intervening 3d postinjury period showed no change in enzyme or 48kD OPN fragment. This suggests that OPN signaling through RGD integrin binding plays a principal role in the 1d acute and 7d subacute phases of FPI induced axon degeneration, likely directing inflammatory cell migration for debris removal and reorganization of local synaptic sites for subsequent onset of regeneration. Interestingly, tandem MMP9/OPN increase is also reported in multiple sclerosis, where patient CCR2+/CCR5+ T cells can be stimulated to produce high levels of both proteins (Sato et al., 2012). In our model, such cell reactivity was no longer visible at 21d, when the regenerative phase was underway,
apparently no longer requiring generation of OPN fragments by MMP9. Our results confirm this shift, with significant time-dependent differences between data for 21d and earlier time points. By contrast, we found that FPI had the opposite effect on OB expression of the 32kD OPN. Essentially no change was seen during the 1-3d postinjury period, but the fragment showed significant reduction below controls at 7d, when MMP9 activity was increased 3-fold. These results indicate a complex processing of this OPN peptide during the acute and subacute postinjury intervals. Specifically, its overall level is likely maintained early after FPI, with generation/degradation balanced. By 7d, however, high MMP9 activity appears to drive additional lysis of this smaller fragment, a recycling process already attributed to the enzyme (Lindsey et al., 2015; Takafuji et al., 2007). Together, these stages of 32kD processing suggest its role is more important during the early, acute phases and less significant during the subacute 7d period. Like the 48kD OPN signal, 32kD OPN shows no change from sham controls by the 21d regenerative phase, also supporting the decreased requirement for integrin or CD44 cell signaling activation.

Our parallel IHC experiments probing for OB cellular localization of OPN revealed rather unexpected results. Several studies, including those from our laboratory, have shown OPN protein within both neurons (Shin et al., 2005) and glia (Choi et al., 2007; Ellison et al., 1998; von Gertten et al., 2005), however, following CNS injury, increased OPN transcript is often prominent within microglia and elevated OPN protein in both microglia and astrocytes (Chan et al., 2014). Interestingly, we found no OPN localization within astrocytes and microglia of the OB glomerular and outer plexiform layers after FPI. Over 3-21d postinjury, OPN upregulation appeared within two neuronal populations, mitral and tufted cells, suggesting a direct OPN
response in the specific cells deafferented by FPI. Notably, the strongest neuronal OPN signal occurred at 7d, matching the peak MMP9 activity and significant increase in 48kD OPN. To our knowledge, this primary neuronal OPN response is unique to the OB among cortical regions deafferented by TBI. The majority of OPN signal was seen within the cell bodies of these neurons, with some protein extending into primary dendrites, presumably associated with ORN synapses. This result raised the possibility that changes in OPN protein after FPI might be mediated through cell to cell signaling originating within the actual deafferented neurons (i.e., mitral and tufted post-synaptic dendrites). Since a cytoplasmic OPN has been identified and associated with microtubule binding proteins of the cytoskeleton (Long et al., 2012), we explored whether our IHC signal reflected OPN and not secreted, lysed OPN. Based on the absence of OPN and MAP2 signal co-localization, we conclude that OB neuronal OPN exists primarily in a form which is secreted and cleaved within the ECM. It was also possible that some of the glomerular-associated neurons positive for OPN might be periglomerular inhibitory neurons, whose synapses would be indirectly affected by ORN axo-dendritic pathology. However, we failed to find OPN expression in these cells when co-localization experiments were performed with their cell marker, tyrosine hydroxylase. From this result, we conclude that the OPN+ neurons responding to FPI at the base of the OB glomerular layer are deafferented post-synaptic tufted neurons. Taken together, these IHC observations suggest that postinjury OPN signaling begins within the primary deafferented neurons, a process likely involving increased production and release of OPN locally. This posited release appears to pair with elevated neuronal and glial MMP9, generating OPN signaling peptides for critical autocrine/paracrine response to direct reactive synaptogenesis.
Elevation of OPN receptor CD44 after FPI

Our results show that time-dependent change in expression of OPN peptide fragments occurs within the OB after FPI. The two principal fragments we observed (48, 32kD) each have a high affinity for cell surface receptors which direct inflammatory activation, migration and axon growth (Plantman, 2012; Scatena et al., 2007; Weber et al., 1996). While 48kD amino terminal peptide can bind to multiple integrin receptors, including $\alpha_8\beta_1$, $\alpha_4\beta_7$, $\alpha_4\beta_1$, and $\alpha_6\beta_1$, (Scatena et al., 2007), Liaw et al. (1995) found that the $\alpha_\nu\beta_3$ (vitronectin) receptor supported OPN-mediated migration, while $\alpha_\nu\beta_5$ or $\alpha_\nu\beta_1$ promoted cell adhesion. There is also evidence that the smaller carboxy terminal 32kD fragment binds CD44 hyaluronan receptor (Takahashi et al., 1998; Weber et al., 1996; Weber et al., 1997; Weber et al., 2002). These integrin and CD44 receptors are most associated with OPN cell signaling in the injured CNS. Moreover, both appear important relative to OPN role in synaptogenesis, since $\beta_1$ and CD44 antibodies block OPN-mediated neurite outgrowth (Plantman, 2012), and interference with hyaluronan/CD44 signaling through genetic manipulation of Rac1/PKN$\gamma$ alters astrocyte cytoskeleton and cell migration (Bourguignon et al., 2007). Initially, we sought to probe OB postinjury expression of each receptor, however, antibodies tested for $\alpha_\nu\beta_3$ presented technical concerns, so our focus was shifted to mapping CD44. This receptor is acutely elevated after CNS injury, suggesting it as a candidate for OPN cell signaling following OB deafferentation. In addition, CD44 is expressed over the perilesional area after TBI (Günther et al., 2015) and increases within activated microglia and macrophages 1-4 days post-injury (von Gertten et al., 2005).

The present profile of OB CD44 expression revealed injury-induced upregulation at subacute intervals of 3 and 7d, with the greatest increase at 3d. This is striking, since the peak of CD44 is
early after injury and does not match the 7d peak of 48kD OPN. In fact, we found no change in the latter fragment at 3d after FPI. Nevertheless, it is possible that the generation of 48kD OPN at 1d, just prior to the CD44 3d peak expression, might contribute to the rapid upregulation of the receptor. Indeed, one *in vitro* study reports increase of CD44 surface membrane expression in the presence of OPN, and that OPN facilitates formation of specific phosphorylated variants of CD44 which interact with MMP9 at cell membranes to regulate enzyme activity (Desai et al., 2009). By contrast, the directional changes in CD44 and 32kD between 1-7d (upward, peak, downward), while not all significant, do show a consistent pattern of change. This pattern suggests the possibility that OB CD44 response to FPI may be closely linked to 32kD levels. Further, it points to CD44 as important for execution of the acute/subacute degeneration phase, and less so for the 21d reinnervation interval, which is similar to a pattern of CD44 increase reported after central and peripheral nerve transection (Jones et al., 2000). Interestingly, 32kD CD44 signaling could operate in tandem with 48kD vitronectin pathways to modulate the timing or extent of neurite outgrowth, a critical element for successful OB synaptogenesis. It is posited that the 32kD OPN, lacking integrin binding sites, may actually oppose 48kD OPN-mediated adhesion (Smith et al., 1996; Gao et al., 2004; Maeda et al., 2001), providing a means to balance growth at different stages of the degeneration/regeneration cycle. Notably, we observed a similar temporal pattern for glial reactivity and CD44 response, mapping maximal cell responsivity to the same 3d phase of the synaptogenic cycle. As stated above, the latter interpretation fits the post ischemia time sequence proposed by Kang et al. (2008), where early 2-3d microglial activation mediates CD44 glial mobility and cell adhesion. Further IHC studies mapping CD44 cell localization will be required to clarify whether acute neuronal or glial signals are responsible for OB CD44 activation after FPI.
Summary

Taken together, these data provide evidence that time-dependent change in gelatinase activity, OPN proteolysis and CD44 hyaluronan receptor expression each occur within the deafferented OB of FVB/NJ mice following central FPI. These changes suggest a temporally cued interaction between MMP9/OPN/CD44, which likely facilitates the initial degenerative phase of OB reactive synaptogenesis. Significant 7d elevation of MMP9 activity was correlated with generation of 48kD integrin binding OPN fragments, potentially signaling a second, critical cellular response to FPI axotomy. CD44 hyaluronan receptor upregulation occurs earlier at 3d after injury, revealing a pattern that may be more associated with the non-integrin binding 32kD OPN fragment. However, it is possible that early 1d rise in 48kD OPN could also underlie elevated CD44 at 3d postinjury. From additional IHC of MMP9 and OPN, we also found that FPI induces a unique OB pattern of response, where OPN signal could be generated both within deafferented post-synaptic neurons and ECM. OPN could be processed by MMPs in deafferented neurons and signal fragments released, or full length OPN released into the ECM around affected glomeruli for processing by astrocyte secreted MMP9. Our results support the hypothesis that MMP9 and OPN cooperate to provide cellular signaling that directs the early degenerative phase of OB reactive synaptogenesis. In Chapter 4 we will describe results from experiments which utilize MMP9 KO mice to manipulate this cooperative mechanism, directly testing its contribution to OB synaptic repair after FPI.
CHAPTER 4

MMP9 KO ALTERS PROGRESS OF FPI-INDUCED OB SYNAPSE REGENERATION
ABSTRACT

MMP9 has been implicated as a critical mediator of ORN recovery during axotomy-induced reactive synaptogenesis in the OB. We have proposed that MMP9 processes one of its substrates, OPN, generating signaling fragments which promote cellular responses necessary for synaptic repair. In Chapter 3, we documented postinjury biphasic elevation of MMP9 activity, which corresponded with increased levels of 48kD OPN peptide. In order to fully assess MMP9 role in postinjury ORN reinnervation of the OB, we subjected MMP9 KO animals to FPI, assessing the same outcome measures examined in WT mice. While full length 66kD OPN was similarly unaltered by loss of MMP9, generation of OPN fragments was significantly affected. Foremost, our WB data revealed that loss of MMP9 eliminated 1 and 7d injury-induced elevation of the 48kD OPN fragment. Paired IHC of MMP9 KO mice confirmed this result, with a clear reduction of OPN signal in tufted and mitral neurons at 7d postinjury. Moreover, WT patterns of OMP expression 3-21d after FPI were reversed by MMP9 loss, specifically showing a delayed initial loss and a persistent reduction in the protein marker of ORN axons. Together these two lines of evidence support direct OB MMP9/OPN interaction after FPI, indicating that the pathway provides critical cell signaling necessary for rapid clearance of injured ORN axon terminals and onset of reinnervation. Interestingly, the 7d injury-induced reduction of 32kD OPN in WT animals was attenuated in MMP9 KO cases, further validating the importance of MMP9/OPN interaction after FPI. Notably, measurement of MMP2 activity in MMP9 KO animals failed to show strain differences, even though WT 7d elevation was normalized to control level. This suggests that delayed degeneration associated with low OMP in the MMP9 KO caused attenuation of the 7d rise in MMP2 activity, normally linked to synapse regeneration.
As for WT mice, no injury effects on Synapsin-I or Synapsin-II expression were found in the MMP9 KO. However, small strain effects could be detected at 3d postinjury for Synapsin-I and at 21d postinjury for Synapsin-II, suggesting that a time-dependent role for MMP9 might exist. Glial reactivity, CD44 levels, and GAP-43 expression were all unchanged in the absence of MMP9, indicating that redundant molecular pathways exist for their activation to facilitate OB synaptogenesis. Finally, TEM analysis of MMP9 KO mice at 21d postinjury revealed persistent ORN axon degeneration, disorganization of pre-synaptic vesicles and underdeveloped synaptic junctions. We conclude that MMP9 plays an important role in OB postinjury cell signaling, a complex process that involves interaction with OPN to support ECM reorganization critical for ORN reinnervation.

INTRODUCTION

Reactive synaptogenesis is a complex process involving breakdown and clearance of axonal debris from injured neurons, emergence of nascent terminals, and stabilization of newly formed synapses. Matrix enzymes like MMP9 have several functions associated with modulating the ECM for synaptic repair and stabilization of synaptic function (Phillips et al., 2014). In OB synaptic recovery, however, the exact role(s) and mechanisms underlying their function are still being uncovered. Elevation of MMP9, a known mediator of synaptogenesis, is associated with critical stages of synaptic plasticity following FPI-induced OB deafferentation (see Chapter 3). Our results with diffuse FPI confirm MMP findings reported for focal OB injury models during reactive synaptogenesis. As with FPI, these other models show that MMP9 response is upregulated following OB deafferentation, induced by either ON transection (Costanzo et al., 2006) or chemical OE ablation (Bakos et al., 2010). Collectively, these data provide support for
the strategy of manipulating MMP9 to affect outcome during synaptic repair following diffuse brain injury. Thus, it is important to clarify specific properties of MMP9 associated with synapse reorganization in order to guide development of therapeutic interventions.

Data presented in the previous chapters reveals several time-dependent correlations of molecular response in the OB subjected to mild FPI: 1) αII-spectrin proteolysis and OMP reduction marking injury; 2) increased MMP9 activity and OPN fragment generation marking cell signaling; 3) activated glia and CD44 cell surface receptor upregulation marking autocrine/paracrine interaction; 4) GAP-43 elevation and OMP normalization marking reinnervation. Such interesting findings point to complex interaction among these different molecules within the damaged OB. Increased MMP9 expression or activity across several OB deafferentation models suggests the enzyme targets and processes a variety of ECM molecules in the OB, including OPN. Because OPN can be cleaved by several ECM enzymes to facilitate inter-cellular signaling during synaptogenesis, direct manipulation of MMP9 will determine whether this enzyme is critical to the production of OPN fragments after FPI and if it affects CD44 receptor expression for successful ORN synaptic recovery. Typical approaches to manipulate MMPs employ pharmacological inhibitors (Falo et al., 2006; Hadass et al., 2013; Hsu et al., 2008; Kim et al., 2005; Reeves et al., 2003; Warren et al., 2012; Zhang et al., 2011) or genetic knockout of MMP genes (Costanzo and Perrino, 2008; Copin et al., 2005; Hsu et al., 2008; Kaliszewska et al., 2012; Lee et al., 2004; Mizoguchi et al., 2011; Wang et al., 2000; Wilczynski et al., 2008; Van Hove et al., 2012; Vu et al., 1998; Zhang et al., 2011). Since MMP family members are highly homologous, and sites of enzyme inhibition can be non-selective for MMP type, MMP9 KO mice allow for a more specific test of the enzyme’s role in OB plasticity. We maintain a colony of MMP9 KO mice, generated on FVB/NJ background, which can be used
to test the effect of MMP9 loss on the OB synaptic degeneration/regeneration cycle and OPN expression. In other brain injury models, MMP9 KO reduces the efficacy of both structural (Kaliszewska et al., 2012) and functional (Nagy et al., 2006) synaptic recovery. The processing of OPN into key signaling fragments is also reported to be attenuated when MMP activity is pharmacologically inhibited (Chan et al., 2014; Phillips et al., 2014).

This chapter reports results of studies using MMP9 KO mice subjected to FPI in order to determine if OB MMP9 loss alters postinjury changes in OPN proteolysis and CD44 upregulation, ORN degeneration marker OMP, as well as expression of Synapsin vesicle proteins, and regeneration promoter GAP-43. Protein expression was again analyzed with WB and compared to WT injured cases generated in Chapter 3. IHC was also conducted to confirm any postinjury differences in glial reactivity, as well as cellular OMP and OPN localization due to MMP9 KO. To further test if synaptic recovery was altered by loss of MMP9, we also used TEM to probe for differences between MMP9 KO and WT synaptic reorganization at 21d postinjury. Based upon our results in Chapters 2 and 3, we hypothesized that MMP9 KO will significantly reduce OPN fragment generation, alter the pattern of ORN degeneration, as reflected by change in OMP reduction, and attenuate the expression of proteins linked to glial reactivity (CD44) and ORN reinnervation (GAP-43) within the OB deafferented by FPI. Our results clearly showed that postinjury MMP9 activity generates OB OPN fragments as well as altered the onset and progression of ORN axon degeneration marked by OMP reduction. The loss of OPN signaling shifted the time course of the early degenerative phase of OB reactive synaptogenesis, resulting in delayed debris removal from the ECM and poor synaptic reorganization at the onset of reinnervation. Our analysis in Chapter 4 also revealed that MMP9
loss did not affect OB CD44 receptor or GAP-43 expression after FPI, suggesting that redundant molecular pathways exist for their activation to facilitate OB synaptogenesis.

METHODS

Experimental Animals

All procedures met national guidelines for care and use of laboratory animals, and all experimental protocols were approved by the VCU Institutional Animal Care and Use Committee. FVB/NJ WT and matrix metalloproteinase 9 knockout (MMP9 KO) adult male mice (The Jackson Laboratory, Bar Harbor, ME) were housed (4 littermates/cage) under a temperature (22ºC) and humidity controlled environment, with food and water ad libitum, and subjected to a 12h dark-light cycle. WT mice (20-30g; 8-11 weeks old) were randomly selected and subjected to midline FPI. WT sham-injured cases served as control. Subsets of FPI and sham-injured groups [WT Sham (n=48), WT FVB TBI (n=53), MMP9 KO TBI (n=36)] were allowed to survive for either 1, 3, 7 or 21d post-injury prior to molecular or histological analysis.

In this study we compared the effect of MMP9 KO on OB response to injury using FVB WT sham animals as the control group. This design was intentional and based upon several factors. First, we sought to correlate our findings with published laboratory databases that compared KO effects relative to WT controls, specifically those focusing on MMP9 and OPN role in other brain regions following TBI (Chan et al., 2014). The design was also based upon preliminary profiling of MMP9 KO cases (Appendix C.1) which showed that: 1) glomerular axo-dendritic cytoarchitecture in the MMP9 KO OB was identical to that of FVB WT control cases, 2) MMP2 gelatinase activity in the OB of MMP9 KO sham mice was not different from that observed in
FVB WT sham cases, and 3) OPN protein expression in a second white matter tract injured by FPI (corpus callosum; Reeves et al., 2012) did not differ between FVB WT and MMP9 KO sham animals.

**Surgery Preparation and Injury**

Mice were anesthetized with isoflurane (4% in 100% O$_2$ carrier gas) and maintained on 2.5% isoflurane in carrier gas delivered by nose cone. Once stabilized in a stereotaxic frame, heads were shaved, body temperature maintained at 40ºC by Gaymar T/Pump water pump (Gaymar Industries Inc, Orchard Park, NY) and heart rate (bpm), arterial oxygen saturation (percent O$_2$), breath rate (brpm) and pulse/breath distension (µm) monitored by pulse oximetry (MouseOx; Starr Life Sciences, Oakmont, PA). Mice then received a midline incision and a 2.7 mm craniectomy prepared over the midline, centered between bregma and lambda. Without damaging the underlying dura, a Leur-Loc syringe hub was cemented to the skull surrounding the craniectomy and dental acrylic poured around the hub to stabilize the site. Topical anesthetic/antibiotic was applied to the incision site and the mice housed in recovery cages. After one hour of surgical recovery, mice were anesthetized for 4 mins (4% isoflurane, 100% O$_2$), and subjected to FPI as previously described (Dixon et al., 1987; Reeves et al., 2012). The device consisted of a 60 x 4.5 cm Plexiglas water filled cylinder, fitted at one end with a piston mounted on O-rings, with the opposite end housing a pressure transducer (EPN-0300A; Entran Devices, Inc., Fairfield, NJ). At the time of injury, the Leur-Loc fitting, filled with saline, was attached to the transducer housing. Injury was produced by a metal pendulum striking the piston, transiently injecting a small volume of saline into the cranial cavity and briefly deforming the brain tissue (20 millisecond pulse duration). The resulting pressure pulse was recorded extra
cranially and registered 1.3±0.1 atm pressure. After injury, all mice were promptly ventilated with room air until spontaneous breathing resumed. The duration of suppression of the righting reflex (5.0±2.0 mins) was used as an index of traumatic unconsciousness. Once righting reflex was determined, mice were re-anesthetized for hub removal, scalp suture and topical anesthetic/antibiotic application. Sham-injured controls received the same surgical preparation, anesthesia and connection to the injury device, except that the intracranial pressure pulse was not applied. All animals were returned to their home cages and assessed for weight loss, locomotion, and eye/nose exudate once per day until weights stabilized.

**Protein Extraction**

WT mice were anaesthetized with 4% isoflurane in carrier gas of 100% O₂ for 4 min, then sacrificed by decapitation at 1, 3, 7 or 21d after FPI or sham injury, with bilateral OBs dissected (n=4-7/group) for assessment of protein expression. Tissue samples were homogenized on ice in 100 µl of RIPA Lysis Buffer (EMD Millipore, Billerica, MA), and centrifuged at 14,000 x g for 20 min at 4°C. Supernatant was aliquoted and stored at -80°C. Prior to Western blot (WB), protein concentration was determined using Pierce BCA Protein Assay Reagent (Thermo-Fisher, Waltham, MA) and the FLUOstar Optima plate reader (BMG Labtech, Inc., Cary, NC).

**Western Blotting**

WB analysis was carried out utilizing Bio-Rad products (Hercules, CA). Twenty µg of protein was prepared in WB XT Sample Buffer and reducing agent (Bio-Rad Laboratories), then denatured at 95°C for 5 mins. Samples were electrophoresed on 4-12% or 12% Bis-Tris Criterion XT gels (200v x 45 min in MOPS running buffer), then protein transferred onto
polyvinylidene fluoride (PVDF) membranes (1h at 100V). Post-blotted gels were stained with 0.1% Coomassie Brilliant Blue (Sigma-Aldrich, St. Louis, MO) in 40% MeOH+10% glacial acetic acid, then de-stained at RT to confirm protein load and even transfer. Membranes were rinsed with deionized water and Tris-buffered saline (TBS) before blocking with 5% milk TBS-Tween (mTBS-T). Blots were then incubated in 5% mTBS-T overnight (4°C) with individual primary antibodies to Synapsin-I (1:500, Santa Cruz, Dallas, TX), Synapsin-II (1:4,000, EMD Millipore, Billerica, MA), olfactory marker protein (OMP; 1:20,000, Wako, Richmond, VA), growth associated protein-43 (GAP-43; 1:1,000, Santa Cruz, Dallas, TX), osteopontin (OPN; 1:500, R&D Systems, Minneapolis, MN; 1:500, Rockland Immunochemicals Inc., Limerick, PA), and CD44 (1:500, BD Biosciences, San Jose, CA). After primary incubation, membranes were washed with mTBS-T, then incubated with appropriate HRP-linked secondary antibodies [IgG bovine anti-goat, IgG bovine anti-rabbit (1:15,000, Santa Cruz, Dallas, TX), IgG goat antimouse, IgG goat anti-rat (1:15,000, Rockland Immunochemicals Inc., Limerick, PA)] in mTBS-T for 1h at RT. Finally, blots were washed with mTBS-T and antibody binding visualized using Super Signal Dura West chemiluminescence substrate (Thermo-Fisher, Waltham, MA). WB images were captured with Syngene G:Box and positive band signal subjected to densitometric analysis (relative optical density, ROD) with Gene Tools software (Syngene, Frederick, MD). Protein data were expressed as percent change relative to paired WT Sham control cases run on the same transferred gel. Cyclophillin A (EMD Millipore, Billerica, MA) or beta actin (Sigma-Aldrich, St. Louis, MO) were used as load controls for signal detection.
Gelatin zymography

Zymographic enzyme assay was performed using previously described methods (Chan et al., 2014). Briefly, 20 µg of protein from OB extracts described above for WT and MMP9 KO cases were prepared with 2x Tris-glycine SDS sample buffer, then separated by gelatin electrophoresis (at 4°C, 45V for 1.5 h) on Novex® 10% zymogram gels (Thermo-Fisher, Waltham, MA). Gels were renatured in Novex® Zymogram Renaturing Buffer (Thermo-Fisher, Waltham, MA) for 30 min at RT before development in Novex® Zymogram Developing Buffer over 6 d at 37°C. Gelatin lysis was visualized with Coomassie brilliant blue (Sigma-Aldrich, St. Louis, MO) and purified enzyme run as positive control. Zymogram signal was captured on Syngene G:BOX, and densitometry analyzed as ROD with Gene Tools software (Syngene, Frederick, MD). Enzyme activity was expressed as percent change relative to paired controls run on the same gel.

Immunohistochemistry

At 3, 7 and 21d post-injury WT injured and WT sham mice (n=4/group) were prepared for fluorescent IHC analysis according to published protocol (Warren et al., 2012). Animals were anaesthetized with sodium pentobarbital (400mg/kg, i.p.), transcardially perfused with 0.9% saline followed by 4% paraformaldehyde in phosphate buffer (0.1M NaHPO₄, pH=7.4), after which brains with attached OBs were extracted and placed in fixative for 24 h before transfer to 0.03% NaN₃ in 1.0 M phosphate buffered saline (PBS). For IHC, fixed brains were cryoprotected in 30% sucrose for 3d, sucrose solutions exchanged after each day. Frontal lobes were blocked and attached bulbs mounted in Tissue Tek media (Thermo-Fisher, Waltham, MA) and stored at -80°C. Coronal cryostat OB sections (13µM) were collected using a Cryostar™NX70;
Cryotome™FSE; HM525 NX cryostat (Thermo-Fisher, Waltham, MA) and prepared for immunofluorescence visualization.

Free floating sections were first permeabilized in 5% peroxidase for 30 mins. After a wash with PBS, tissues were pre-incubated in blocking buffer (fish gelatin in PBS + 0.05% triton X-100) to prevent non-specific binding, and then incubated overnight in primary antibody (OMP, 1:20,000, Wako, Richmond, VA; OPN, 1:40, R&D Systems, Minneapolis, MN; glial fibrillary acidic protein [GFAP], 1:20,000, Dako; ionized calcium binding adaptor protein [IBA1] for microglia, 1:300, Wako, Richmond, VA) at 4°C. Sections were next washed with PBS, placed in blocking buffer for 30 mins, after which they were incubated with secondary fluorescent antibody (Alexa-Fluor 488 donkey anti-goat, 1:1000 and Alexa-Fluor 594 donkey anti-rabbit, 1:1000, Thermo-Fisher, Waltham, MA) in Blotto for 1h at RT. Slices were then PBS washed, equilibrated in phosphate buffer, and mounted on Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA) with Vectashield + DAPI (Vector Laboratories, Burlingame, CA). IHC signal was visualized on the Zeiss LSM 700 (Carl Zeiss, Thornwood, NY) confocal microscope (VCU Microscopy Core).

Transmission Electron Microscopy

Following FPI, at 21d, select injured WT and MMP9 KO mice (n=4) were anesthetized with sodium pentobarbital (400mg/kg, i.p.), transcardially perfused with mixed aldehyde fixative (2% paraformaldehyde and 2.5% glutaraldehyde) in 0.1M phosphate buffer, pH-7.2. Brains were removed and post-fixed overnight at 4°C. OBs were next blocked in the sagittal plane and placed in 1% osmium tetroxide (0.1 M cacodylate buffer), and processed for embedment with Epon resin (Embed; Electron Microscopy Sciences, Hatfield, PA). After curing, OB areas
containing glomeruli were mounted and both semithin (0.5µm) and ultrathin (silver, 600 Å) sections were cut with a Leica EM UC6i ultramicrotome (Leica Microsystems, Wetzlar, Germany). Semithin sections were used to guide subsequent ultrastructural sampling. Ultrathin sections were collected on membrane Formvar-coated slotted grids and observed on a JEOL JEM-1230 electron microscope (JEOL USA, Inc., Peabody, MA), equipped with a Gatan UltraScan 4000SP CCD camera (Gatan, Inc., Pleasanton, CA).

Statistics

The effects of injury and MMP9 KO on protein levels and gelatinase activity were evaluated using GLM SPSS v.23 (International Business Machines, Corp., Armonk, NY). These analyses used 2-way completely randomized ANOVAs. Specific pairwise comparisons were evaluated using the Duncan Post Hoc Test. Western blot and zymography results are reported as mean +/- SEM. An alpha level of 0.05 was used in all analyses.

RESULTS

MMP2 elevation is attenuated by MMP9 KO

Time-dependent increases in OB MMP9 activity in both prior studies and ours (see Chapter 3) suggest critical MMP9 proteolytic functions for mediating ORN repair. In order to define MMP9 role, we utilized MMP9 KO animals to document consequences of genetically ablated MMP9 for postinjury OB reinnervation. In our initial MMP9 KO experiments, we measured MMP2 gelatinase activity to document a potential interaction between the two gelatinases during reactive synaptogenesis. Prior studies have shown that after ORN axotomy, MMPs 2, 9 exhibit distinct functions, as MMP2 protein levels are not affected by loss of MMP9 (Costanzo and
Perrino, 2008). Our model also axotomizes the ON and deafferents the OB (see Chapter 2), thus we chose to explore this interaction as well and potentially confirm and reproduce those findings. Similar to the nerve transection model, following TBI, MMP2 activity was not elevated in a compensatory manner in the absence of MMP9 (Figure 4.1). At 1, 3, 7, and 21d postinjury, MMP2 lytic activity in the MMP9 KO was no different from sham controls (103.17±9.47; 119.83±12.15; 113.22±11.82; 90.38±7.46). Notably, however, loss of MMP9 did attenuate the modest 7d increase in MMP2 detected in WT animals. While not significant, this effect suggests peak 7d MMP9 activity in injured OB indirectly contributes to MMP2 elevation and that loss of MMP9 impacts timing of MMP2 response during the subacute recovery period. To further investigate the extent of postinjury consequences attributed to MMP9 loss, we probed for protein expression of MMP substrate OPN.

**MMP9 KO alters FPI-Induced Full Length OPN Expression**

We posit that MMP9 elicits critical ECM reorganization for degenerative and regenerative periods of ORN recovery through OPN. As for WT cases, we first tested whether loss of enzyme activity with MMP9 KO affects expression of 66kD OPN during FPI-induced OB synaptogenesis. (Figure 4.2). Overall, no change in FL 66kD OPN was detected with loss of MMP9, as expression was not different from sham at each postinjury time point (93.23±17.90; 118.59±27.21; 88.25±9.08; 89.99±7.78). Likewise, no strain differences were found. However, post hoc analysis did show that WT time-dependent differences in 66kD OPN were no longer detected. This suggests that loss of MMP9 might have an effect on how FL 66kD changes over time postinjury, but the effect appears to be small and difficult to interpret. As for WT FPI, we shifted our focus to assay of change in the generation of OPN fragments within MMP9 KOs.
Figure 4.1 MMP9 KO effect on MMP2 response to FPI

A. Zymographic assessment of postinjury OB gelatinase activity in MMP9 KO mice was performed to assay whether loss of MMP9 generated a compensatory change of injury-induced MMP2 response. In WT cases, MMP2 activity showed a significant OB increase at 7d postinjury relative to sham controls, consistent with predicted role of this gelatinase in OB synaptic regeneration (WT data replotted for comparison). MMP9 KO did not affect MMP2 activity at 1, 3 or 21d after FPI. At 7d, loss of MMP9 did result in a normalization of MMP2 proteolytic response, making it no longer significantly elevated over controls. Despite this change, post hoc analysis failed to show strain differences at 7d. These data suggest that the two gelatinases interact during OB response to deafferentation, and a reduced MMP2 function could contribute to attenuated synaptic recovery at 21d postinjury (see Figure 4.12 below). 

B. Results are expressed as percent of sham control relative optical density (100% dashed line). 

C. Representative zymogram images of Sham WT (left) versus MMP9 KO Injured (right) MMP2 are illustrated. *p<0.05 Sham vs. TBI. N=3-8/group.
Figure 4.2 MMP9 KO does not alter OB expression of full length OPN after FPI

A. WB analysis of full length OPN (66kD) in deafferented OB failed to show MMP9 KO effect on injury response relative to sham controls. With loss of MMP9, 66kD OPN expression was not different from control level at any of the four postinjury time points examined. Post hoc time point comparisons also failed to detect any strain differences between WT and MMP9 KO. This results indicates that the postinjury expression of full length OPN remains stable whether or not MMP9 is involved in the injury response. Results are expressed as percent of sham control relative optical density (100% dashed line). B. Representative blot images of 66kD OPN, with β-actin load controls, are illustrated below. #p<0.05 day x vs. y. N=4-7/group.
Loss of MMP9 reduces OPN signaling fragments after FPI

An additional set of experiments probed MMP9 KO effect on FPI induced OPN expression, testing if generation of OPN signaling peptides can be altered when injury is delivered in the absence of MMP9. Since WT mice exhibited time-dependent elevation of the 48kD RGD cell signaling peptide and reduction of the carboxy terminal 32kD sequence, we focused on these two OPN fragments. In WT animals, generation of the 48kD fragment was correlated with elevated MMP9 activity at 1 and 7d postinjury (see again Figure 3.4). Here, we showed that loss of MMP9 reduced the injury-induced increase in 48kD OPN fragment generation at these two time points (Figure 4.3A), essentially returning expression level to that seen in control cases (115.21±12.58; 89.64±8.69). Surprisingly, we also found that MMP9 KO significantly reduced 48kD OPN by nearly 70% at 3d post-FPI (33.99±5.02, p<0.001), where, in WT cases, injury failed to have an effect on expression of this OPN form. By the 21d postinjury interval, loss of MMP9 did not produce a difference in 48kD OPN level vs sham controls (95.30±5.24).

Subsequent post hoc comparison revealed striking strain differences. Foremost, loss of MMP9 significantly reduced the 48kD RGD signaling fragment of OPN at 1, 3 and 7d after FPI when compared with WT injured cases (p<0.01, 1,7d; p<0.001, 3d).

For the 32kD carboxy terminal OPN fragment, we found little evidence of MMP9 KO effect. At 1, 3 and 21d after FPI, 32kD OPN levels in MMP9 KOs were not different from WT injured animals (111.72±18.87; 134.93±15.37; 86.45±15.07). The one exception was the MMP9 KO normalization of peptide expression at 7d postinjury (84.49±12.64), where WT cases did show a significant protein reduction versus sham control animals (Figure 4.3B). Nonetheless, post hoc comparison failed to identify any strain differences between WT and MMP9 KO 32kD OPN.
Figure 4.3 MMP9 KO alters OPN fragmentation after FPI

A. WB analysis revealed profound effect of MMP9 KO on 48kD OPN fragment expression 1-7d after FPI. Loss of MMP9 significantly attenuated WT injury-induced elevation of the fragment (WT data replotted for comparison) at 1 and 7d, normalizing expression to sham control levels. By contrast, loss of MMP9 significantly reduced 48kD OPN at 3d, to approximately 40% of control values. At 21d, the effect of MMP9 KO was lost, and the N-terminal fragment was no different from shams. Post hoc testing validated the 1-7d WT/MMP9 KO differences. These results point to direct MMP9/OPN interaction after FPI and suggest that at subacute time intervals when the enzyme is not elevated, its absence has a strong effect on the expression of 48kD OPN.

B. Expression of 32kD OPN fragment was much less affected by MMP9 KO. It was not different from sham values at all four time points and no post hoc strain differences were detected. MMP9 KO normalization of 7d 32kD OPN suggests loss of MMP9 reversed the reduction of 32kD OPN seen in 7d WT animals. Results are expressed as percent of sham control relative optical density (100% dashed line).

C. Representative blot images of OPN, with cyclophilin A and β-actin load controls, are illustrated below. *p<0.05 Sham vs. TBI, ***p<0.001 Sham vs. TBI, §§p<0.01 WT vs KO, §§§p<0.001 WT vs KO. N=4-6/group.
These results mapping MMP9 KO effect on OPN fragment generation after FPI show that if the contribution of MMP9 lytic activity is removed, then critical 48kD OPN signaling fragment production is significantly attenuated during the subacute degenerative phases of recovery, which must precede axon sprouting and synapse reformation at 21d. Overall, these data support our hypothesis that MMP9 activation after FPI is instrumental in producing and regulating cleavage of two critical inter-cellular signaling proteins.

**MMP9 KO affects OPN protein localization**

While a tissue immunobinding assay cannot discriminate between the 48 and 32kD forms of OPN, our histological results did reveal MMP9 KO effects on cell labeling which matched WB protein measures. IHC tissue staining supports the finding of attenuated OPN fragment generation in MMP9 KO cases (Figure 4.4). Qualitative staining differences consistent with the loss of 48kD OPN expression were observed in both mitral and tufted neurons, whose 3d postinjury OPN label intensity was strikingly reduced in the absence of MMP9. Further, at 7d after FPI, OPN labeling of the same cell populations in MMP9 KO mice was also visibly lower than in WT cases, with a signal close to that of sham controls, and consistent with 7d WB OPN fragment normalization. These morphological correlates support the hypothesis that MMP9 KO attenuates postinjury OPN response in deafferented neurons of the OB, likely affecting synaptic reinnervation of these cells.
Figure 4.4 IHC of OB OPN distribution in MMP9 KO animals following FPI

A. Confocal imaging of OPN in WT sham animals showed selective cell body labeling of tufted and mitral neurons (white arrows), which increased in intensity at 3d postinjury (Reproduced from Figure 3.5). MMP9 KO visibly reduced this neuronal OPN signal (right panel).  

B. Similar neuronal OPN staining is seen in 7d sham cases, with FPI induction of heavy OPN signal in WT animals 7d postinjury. Again, loss of MMP9 notably attenuated the OPN response. This IHC result is consistent with significant loss of 48kD OPN within the injured MMP9 KO cases. Scale bar = 50µm. N=3-4/group.
Loss of MMP9 fails to alter CD44 receptor expression after FPI

As for WT, we probed expression of OPN receptor protein CD44 in the OB of injured MMP9 KO animals. Overall, we found that loss of MMP9 failed to change the time course of OB CD44 protein expression after FPI (Figure 4.5). At 1 and 21d after injury, the level of CD44 protein in FPI MMP9 KO mice was not different from sham controls (131.57±11.30; 90.29±10.32). As for injured WT mice, expression of the OPN receptor in MMP9 KO at 3 and 7d postinjury intervals was significantly elevated over sham injured cases (267.77±33.03, p<0.001; 247.68±35.95, p<0.001). Nevertheless, post hoc tests failed to detect strain differences at any postinjury time point. In general, these results suggest that MMP9 does not significantly contribute to the postinjury signaling responsible for CD44 receptor elevation. Further, postinjury rise in cell surface CD44 receptors appears not to be dependent upon the generation of specific MMP9/OPN proteolytic signaling, which is reduced in the MMP9 KO mice. From these results we would now posit that OB OPN signaling after FPI likely occurs through other receptor pathways (e.g., vitronectin, αvβ3 integrin receptors), or that there exists another, indirect CD44 ligand whose signaling pathway compensates for the loss of MMP9 and OPN peptides, maintaining CD44 receptor expression after FPI. Further studies will be needed to clarify these possibilities.

MMP9 KO does not alter OB astrocyte reactivity

Given that we observed OB MMP9 to be associated with GFAP positive astrocytes (see again Figure 3.2), and that these glia are highly integrated among the GL synapses of the OB, we conducted IHC to determine if loss of MMP9 affects deafferentation-induced astrocyte reactivity. Importantly, astrocytes can express surface receptors for OPN paracrine signaling, raising the possibility that any manipulation altering OPN expression might affect cellular
Figure 4.5 Loss of MMP9 does not affect CD44 expression after FPI

A. WB analysis of OB CD44 receptor expression in MMP9 KO showed no difference from WT injured cases (WT data replotted for comparison). Significant elevation of CD44 protein at both 3 and 7d postinjury of WT cases was also seen in the MMP9 KO animals. Similarly, injury did not significantly alter 1 or 21d CD44. These results suggest that loss of MMP9 has little influence on postinjury CD44 expression. Results are expressed as percent of sham control relative optical density (100% dashed line). B. Representative blot images of OPN, with cyclophilin A and β-actin load controls, are illustrated below. *p<0.05 Sham vs. TBI, ***p<0.001 Sham vs. TBI. N=3-5/group.
reactivity to FPI. Loss of MMP9 would be one such manipulation. Upon analysis of OB tissue from injured MMP9 KO mice, we found that loss of MMP9 activity did not appreciably affect GL astrocyte response (Figure 4.6). Although the glia appeared slightly less hypertrophic in MMP9 KO cases at time points of 3 and 7d postinjury, when WT cellular reactivity was robust, there was only a minimal effect of MMP9 loss on astrocyte morphology. While OB glial reactivity and MMP mediated ECM disruption are critical to clearance of degenerating axons, as well as induction of growth factors for synaptic regeneration, MMP9 function in this process may be redundant with other OB ECM enzymes, resulting in a compensation for the loss of MMP9 function and little change in astrocyte reactivity.

**Loss of MMP9 alters molecular markers of OB synaptic plasticity**

We next profiled the expression of several proteins linked to synaptic stability and maturity (OMP, Synapsin-I, Synapsin-II) and establishment of axonal outgrowth (GAP-43) to track synaptic changes in MMP9 KOs subjected to FPI. The temporal pattern of postinjury OMP expression was dramatically altered as a result of MMP9 KO. While OMP level in the MMP9 KO was no longer different from that of sham injured controls at 3d (70.97±9.83), OMP expression was persistently reduced to 58% and 65% of sham control values at days 7 (58.94±9.44, p<0.05) and 21 (65.02±7.32, p<0.05), respectively (Figure 4.7). Similar to WT cases, 1d OMP levels were equal to controls (97.29±9.41). Notably, comparison of strain effect on OMP expression showed that the 7 and 21d return to sham control levels in WT cases, a shift associated with onset of axon outgrowth and synapse reformation, failed to occur in the MMP9 KO, where OMP protein remained significantly reduced relative to WT sham cases (p<0.05 at 7d; p<0.01 at 21d). These results indicate that MMP9 activity contributes to the time-dependent
Figure 4.6 IHC of OB astrocytes in MMP9 KO mice subjected to FPI
A. Confocal imaging of GFAP staining in 3d MMP9 KO samples was similar to WT injured cases (Reproduced from Figure 2.2), with astrocytes displaying only minimal change in process hypertrophy. Thus, loss of the gelatinase did not have an appreciable effect on astrocyte reactivity assessed by GFAP staining. B. As for the 3d interval, the 7d MMP9 KO animals subjected to FPI also showed little change in astrocyte reactivity relative to WT injured cases. GFAP labeling in 7d MMP9 KO OB exhibited a labeling pattern consistent with WT injured, slightly attenuated glomerular astrocyte reactivity relative to 3d postinjury. Scale bar = 50µm. N=3-4/group.
Figure 4.7 MMP9 KO causes persistent attenuation of OMP expression after FPI

A. WB analysis revealed that MMP9 KO had significant effect on OMP expression. While MMP9 KO did not alter 1d OMP expression relative to sham controls, the 3, 7, and 21d intervals were affected. WT 3d decrease in OMP (WT data replotted for comparison) was attenuated with loss of MMP9, no longer statistically differ from sham controls. WT restoration of OMP at 7 and 21d failed to occur in the MMP9 KO cases, with OMP now significantly reduced relative to sham controls at each of the two time points. These results suggest that loss of MMP9 compromised progression toward synaptic recovery and retarded reinnervation. Results are expressed as percent of sham control relative optical density (100% dashed line).

B. Representative blot images of OMP with β-actin as a load control. *p<0.05 Sham vs. TBI, §p<0.05 WT vs KO, §§p<0.01 WT vs KO. N=4-6/group.
**Figure 4.8 IHC of OB OMP distribution in MMP9 KO animals following FPI**

OB distribution of OMP in postinjury MMP9 KO animals showed changes consistent with WB shifts in OMP protein expression. **A.** Sham control (Reproduced from Figure 2.4) shows high OMP signal in the ONL and in ORN axons penetrating the glomeruli (GL; *arrow*). At 3d postinjury, MMP9 KO OMP tissue distribution was similar to the sham control, heavily staining the ONL and labeling pre-synaptic ORN axons throughout the GL (*arrow*). **B.** By 7d, a marked loss of OMP could be detected within the deafferented GL (*arrow*), consistent with the persistent reduction of OB OMP seen in WB analysis. These results, further show that loss of MMP9 has a real effect on the progression of pre-synaptic reinnervation within the deafferented OB. Scale bar = 50µm. N=3-4/group.
onset of ORN axon reinnervation after FPI, possibly involving cell pathways mediated by 7d MMP9 generation of OPN cell signaling fragments.

To confirm the identity of the cell regions exhibiting change of OMP expression in MMP9 KO animals, we performed IHC for the protein within the deafferented OB. As reported in Chapter 2, WT sham injured cases showed heavy localization of OMP protein in the surface ON layer of the OB, and along axon processes within glomeruli (Figure 4.8). As previously described for 3 and 7d postinjury WT cases, OMP protein remained high in the ON layer, however, its presence along 7d axon aggregates in the GL was of lower intensity, consistent with the WB reduction in OMP protein expression (see again Figure 2.3). Interestingly, 3d postinjury MMP9 KO mice no longer exhibited reduction in OB glomerular labeling, with signal intensity similar to that of sham controls and mimicking the shift toward control OMP expression seen in MMP9 KO WB experiments. ORN axon processes showed strong OMP signal within the GL region. These IHC data show consistency of MMP9 KO effects across outcome measures.

While our results support the hypothesis that loss of MMP9 produces a detectable effect on expression of those proteins associated with ORN-specific regeneration, however, changes in more general pre-synaptic markers were modest. Although MMP9 KO did not have a large effect on Synapsin-I and II expression, we did observe two modest time-dependent changes in protein expression following FPI. The 80kD form of Synapsin-I (Figure 4.9A), expressed as a percent of sham injured controls, showed a reduction of mean OB level relative to injured WT animals at 3d postinjury (96.30±6.84 vs 122.58±8.88, p<0.05). We also found that MMP9 KO slightly increased 21d expression of 72kD sham normalized Synapsin-II (Figure 4.10A) in the
deafferented OB, compared to WT cases (110.53±6.38 vs 87.62±5.84, p<0.05). However, MMP9 KO 1, 7, and 21d 80kD OB Synapsin-I levels, relative to sham controls (95.19±6.36; 95.87±10.34; 101.13±5.15), were not different from WT values, nor were any of the analyzed time point measures in the MMP9 KO 70kD Synapsin-I (100.59±13.38; 103.39±20.87; 90.53±15.41; 91.22±10.57) (Figure 4.9B). Further, loss of MMP9 failed to alter sham normalized 72kD Synapsin-II expression relative to WT in 1, 3, 7 d postinjury cases (108.21±7.33; 104.92±6.85; 90.86±5.55). As for Synapsin-I, measures of the lower 58kD Synapsin-II signal also did not show differences from WT values (119.70±20.73; 89.44±8.75; 137.07±25.95; 102.71±4.97). (Figure 4.10B) Interestingly, this rather modest effect of MMP9 KO on two synapsins occurred at postinjury time points where WT MMP9 activity was not different from sham controls (see again Figure 3.1), suggesting that the gelatinase is less likely to be associated with regulation of these proteins during OB reactive synaptogenesis.

In genetically modified MMP9 KO mice, overall GAP-43 protein expression relative to sham control cases was similar to injured WT, with only one detectable strain difference. Foremost, FPI in MMP9 KOs produced an elevation in 21d protein level similar to that of WT, (362.09±31.06 vs 297.19±58.77), each of which was significantly higher than sham controls (p<0.001; Figure 4.11). As in WT mice, levels of GAP-43 in MMP9 KO cases were also no different from sham controls at 1 or 7d postinjury (85.44±25.56; 145.59±27.22). Interestingly, we did find a shift to approximately 47% of uninjured control GAP-43 levels for the 3d postinjury MMP9 KO cases (47.16±4.38), but this reduction failed to reach significance. Nevertheless, post hoc analysis did reveal strain differences between WT and MMP9 KO values at this time point (p<0.05). In general, these results indicate that MMP9 KO does not
Figure 4.9 MMP9 KO does not affect OB Synapsin-I expression after FPI
A. WB analysis of 80kD Synapsin-I in MMP9 KO subjected to FPI failed to show any time-dependent effects on protein expression relative to sham control animals. Post hoc analysis detected only one strain related difference in this isoform, a reduction to control level at 3d in the MMP9 KO cases (WT data replotted for comparison). B. 70kD Synapsin-I expression was unaffected by the loss of MMP9 at each time point postinjury and no strain differences were observed. Results are expressed as percent of sham control relative optical density (100% dashed line). C. Representative blot images of 80kD and 70kD Synapsin-I with β-actin as a load control. §p<0.05 WT vs KO. N=4-5/group.
Figure 4.10 MMP9 KO does not affect OB Synapsin-II expression after FPI

A. Similar to Synapsin-I, WB probe of the larger 72kD Synapsin-II isoform did not show any time-dependent effects of injury in the MMP9 KO compared with sham controls. Post hoc analysis detected only one strain related difference in this isoform, a small, but significant increase at 21d in the MMP9 KO cases (WT data replotted for comparison). B. As for Synapsin-I, the lower, 58kD Synapsin-II isoform did not show injury effect relative to paired shams, nor did it have detectable strain differences compared with WT cases. Results are expressed as percent of sham control relative optical density (100% dashed line). C. Representative blot images of 72kD and 58kD Synapsin-II with cyclophilin A as a load control. §p<0.05 WT vs KO. N=4-6/group.
A

Synapsin-II 72kD

% of Control

1d | 3d | 7d | 21d

B

Synapsin-II 58kD

% of Control

1d | 3d | 7d | 21d

C

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1d | 3d | 7d | 21d
substantively alter the postinjury pattern of OB GAP-43 expression. However, it is intriguing that MMP9 KO shows a downward trend in GAP-43 protein at 3d, the same postinjury time when KO OMP expression increases toward sham control levels. An opposite, but inverse relationship between GAP-43 and OMP expression was also found in the WT cases at the same 3d survival interval. From these data we then speculate that these GAP-43/OMP protein changes are related to the posited inverse regulatory interaction between GAP-43 and OMP (Holtmaat et al., 1995; Margolis et al., 1991; Verhaagen et al., 1989; Verhaagen et al., 1990), and that MMP9 has an impact on such interaction.

Ultrastructure of OB glomerular synapses show effects of MMP9 KO

At 21d postinjury, our WB analysis of MMP9 KO OB showed a persistent reduction in OMP protein, contrary to the injured WT cases, where OMP returned to control levels. We reasoned that persistent attenuation of the ORN axon marker protein might represent aberrant progress toward synaptic reorganization in the MMP9 KO animals. To probe for this possibility, we performed TEM analysis of MMP9 KO OB tissues 21d after FPI (Figure 4.12). The ultrastructure of WT glomeruli showed re-emergence of normal morphology 21d after injury, with healthy ORN axon/dendritic synapses exhibiting multiple junctional sites and thick post-synaptic densities (Figure 4.12A). By contrast, glomeruli of the 21d postinjury MMP9 KO cases had fewer of these healthy synaptic profiles (Figure 4.12B). The identifiable ORN terminal synapses were poorly developed, with less mature post-synaptic density structure. Moreover, the 21d MMP9 KO contained frequent areas of persistent tissue degeneration, which correlated with a OMP loss at 21d, a time when synaptic regeneration and OMP reemergence
Figure 4.11 MMP9 KO does not affect OB GAP-43 expression after injury

A. WB analysis of GAP-43 response to injury in MMP9 KO animals was not significantly different from sham controls at 1, 3, or 7d postinjury. FPI caused a similar elevation of 21d GAP-43 in MMP9 KO (4-fold vs 3-fold in WT injured), and 7d MMP9 KO GAP-43 trended higher than in paired WT cases. Post hoc analysis confirmed a reduction in GAP-43 within the 3d MMP9 KO animals, although the 50% reduction failed to show statistical difference from sham controls. While major effects of MMP9 loss were not observed, effects at 3d suggest a potential role for GAP-43 attenuation during the acute postinjury phase. Results are expressed as percent of sham control relative optical density (100% dashed line). B. Representative blot images of GAP-43 with β-actin as a load control. ***p<0.001 Sham vs. TBI, §p<0.05 WT vs KO. N=4-6/group.
Figure 4.12 OB ultrastructure in MMP9 KO shows persistent damage after FPI

A. Micrographs of ultrathin sections (600Å) at 21d after FPI show normal reorganization of synapses. Active pre-synaptic ORN axon terminals, containing aggregates of synaptic vesicles (v; white arrows), form defined synaptic junctions with post-synaptic dendrites, exhibiting thick post-synaptic densities (black arrows). B. In the MMP9 KO animals, 21d postinjury synaptic morphology is less well developed. Synaptic junctions are loosely formed (black arrows), with pre-synaptic vesicles more diffusely distributed along the axon membranes and sites of active degeneration still present (red arrow). Inset: ORN axons form immature synaptic structure along dendritic shafts (black arrows). These qualitative differences further support the attenuated progression of OB synaptogenesis in the absence of MMP9 and the importance of subacute postinjury MMP9 activity in promoting successful synaptic repair. Bar =1µm. N=2-3/group.
should occur. Taken together, these cytoarchitectural features were consistent with MMP9 KO causing a disruption of the degeneration/regeneration cycle in the deafferented OB.

**DISCUSSION**

MMP9 has several established roles in CNS synaptic plasticity. Supportive evidence comes from studies showing enzyme regulation of different aspects of synaptic organization, including critical plasticity proteins (Phillips et al., 2014). For example, during brain development, MMP9 activity influences the evolution of spine morphology (Ethell and Ethell, 2007) and spine shape (Kelly et al., 2015). It also directs axon sprouting through growth factor processing (Shubayev and Myers, 2004; Sternlicht and Werb, 2001) and can affect structure, as well as membrane mobility, of predominant excitatory receptors like NMDAR (van der Kooij et al., 2014; Kelly et al., 2015). By modification of post-synaptic membranes, MMP9 is positively linked to efficacy of synaptic functional activity (Szepesi et al., 2013) and, more recently, to a supportive role in postinjury reactive synaptogenesis after CNS deafferentation of sensory pathways (Kaliszewska et al., 2012; Kelly et al., 2015). Traditionally, MMP9 investigation under conditions of CNS insult (e.g., spinal cord injury, TBI and stroke) has focused on the negative effects of postinjury enzyme activation, which generate tissue necrosis, blood brain/spinal cord barrier disruption, and excessive immune activation (Zhang et al., 2011; Hsu et al., 2008; Wang et al., 2000; Suofu et al., 2012; Noble et al., 2002; Lo et al., 2004; Ziebell and Morganti-Kossmann, 2010; Woodcock and Morganti-Kossmann, 2013). Nevertheless, our laboratory and others have provided evidence that MMPs can play a positive role over the course of CNS synaptic repair (Costanzo and Perrino, 2008; Hsu et al., 2006; Kaliszewska et al., 2012; Kelly et al., 2015; reviewed in Phillips et al., 2014). Only a few of these studies, however, have focused on MMP9 activity
during OB synaptic repair (Costanzo et al., 2006; Bakos et al., 2010), leaving its role in the molecular regulation of sensory plasticity after TBI relatively unexplored.

In Chapters 2 and 3, results mapping OB response to FPI showed that postinjury MMP9 enzyme activity increases in a time-dependent manner, correlated with acute/subacute degenerative phases of reactive synaptogenesis. More importantly, enzyme elevation was temporally associated with the production of OPN fragments capable of binding to cell surface receptors, like $\alpha_v\beta_3$ and CD44, to direct critical cell to cell signaling. MMP9 upregulation was also shown in deafferented zones populated with processes of glomerular neurons, as well as within reactive glia surrounding deafferented OB glomeruli during these same early stages of reactive synaptogenesis. In order to test whether these correlations were mechanistically dependent, we utilized our laboratory colony of MMP9 KO mice to see if the loss of MMP9 altered cellular and molecular components of the OB response to FPI. Based on the findings in Chapters 2 and 3, we hypothesized that MMP9 KO animals subjected to FPI will fail to show time dependent postinjury increases in cell signaling OPN fragments, along with reduced induction of CD44 receptor protein. We also posited that the reduction or absence of these molecular changes will attenuate ORN axon regeneration (tracked by OMP signal) and alter the expression of proteins critical to axonal growth (GAP-43) and synaptic function (Synapsins I and II). Results from these MMP9 KO experiments will further confirm the role of the MMP9/OPN/CD44 signaling pathway as a regulator of OB glomerular synaptic plasticity after TBI.

Overall, our results support the hypothesis that MMP9/OPN interaction is critical to the effective progression of OB reactive synaptogenesis following FPI. First, strain comparison with WT
mice showed that loss of MMP9 completely reversed time dependent generation of the 48kD OPN signaling fragment, pointing to specific OB enzyme/substrate interaction after brain injury. OB tissue staining for OPN in the MMP9 KO cases showed large signal reduction in both tufted and mitral neurons. More importantly, MMP9 KO animals revealed a persistent reduction in the ORN axon marker protein OMP at both 7 and 21d. This is in direct contrast to WT injured cases, where diffuse FPI deafferentation resulted in a postinjury OMP normalization toward control levels by 7d. Our parallel IHC results also support a glomerular reduction in OMP in the MMP9 KO. Thus, the transition between axonal loss and regeneration (Schwob et al., 1999) was disrupted without MMP9. Interestingly, WT vs. MMP9 KO comparison failed to show any effect on expression of CD44, not supporting our hypothesis that the OPN receptor protein induction is mediated through upstream MMP9/OPN interaction. Similarly, we found only minimal strain differences with respect to GAP-43 expression, which did not occur at the 21d postinjury interval where both strains remained equally and maximally elevated. As in the WT condition, MMP9 KO animals also failed to show significant change in Synapsin-I and Synapsin-II protein expression relative to control cases. From these data we conclude that a MMP9/OPN mediated response to FPI occurs during the early degenerative phase and has a direct correlation with the timing of initial ORN axon regeneration after injury. While it remains to be determined if OPN affects CD44 expression through alternative pathways not tested here, we can infer that MMP9 either does not directly affect downstream CD44 and synaptic protein expression, or other MMPs compensate for the loss of MMP9, producing the signals required to induce their postinjury response.
Loss of MMP9 reverses FPI generation of OPN proteolytic fragments

As described above, MMP9 could play multiple roles during repair of injured OB synapses. Since we observed significant correlation between MMP9 activity and production of OPN cell signaling peptides, we first assessed the effect of MMP9 KO on OB OPN protein expression following FPI. The same postinjury intervals focusing on acute/subacute degenerative and early regenerative phases sampled in Chapters 2 and 3 were examined here. As for WT cases, FPI failed to alter the expression of full length 66kD OPN in MMP9 KO mice when compared to uninjured controls. This result reinforces the interpretation that the diffuse level of axotomy produced by FPI is not sufficient to elevate OPN protein expression, in contrast to the increase of OPN in other brain regions subjected to deafferentation (Chan et al., 2014). Moreover, it suggests that MMP9 proteolytic activity does not significantly affect OB transcription processes, which would alter expression of nascent OPN. A very different result was observed for the 48kD OPN, where loss of MMP9 eliminated 1 and 7d postinjury elevation of the fragment seen in WT cases, which was no longer different from sham controls. Interestingly, MMP9 KO mice also revealed a vulnerability for large reductions in 48kD OPN at 3d after FPI, a time point where OPN did not change in WT cases. Parallel IHC of MMP9 KO animals confirmed a profound loss of OPN tissue signal in both tufted and mitral neurons, consistent with the significant drop in fragment generation. While other proteases, notably thrombin, can also lyse OPN (Scatena et al., 2007), the fact that increased generation of OB 48kD OPN no longer occurred in the absence of MMP9 strongly supports our hypothesis that critical RGD integrin cell signaling is mediated by MMP9/OPN interaction after FPI, and suggests this reduced OPN processing may occur within deafferented neurons. MMP9 KO also had an effect on 7d 32kD OPN expression, where loss of enzyme reversed WT reduction of this carboxy terminal fragment
to a level no longer different from sham controls. Since WT MMP9 activity is greatest at 7d, affecting OB expression of both 48 and 32kD OPN signaling peptides, and loss of MMP9 normalizes this response, we conclude that MMP9 targeting of OPN is principally responsible for generating critical cell to cell signaling fragments just prior to onset of synaptic regeneration. Such MMP9 influence on the process of synaptic repair is novel, in that the majority of CNS insults reveal induction of MMP9 expression and activity acutely, usually peaking well before 7d postinjury (Costanzo and Perrino, 2008; Phillips et al., 2014; Rosenberg, 1995; Zhang et al., 2010). Most of these studies show significant decline in MMP9 protein by the 10d post-lesion period, when the evidence of axon regeneration occurs. To our knowledge, no other studies with CNS models have tested OPN fragment generation in MMP9 KO mice, however, at least one model of ureteral obstruction kidney disease shows that MMP2/9 inhibitor and MMP9 antibody do significantly reduce the generation of MMP9-cleaved OPN (Tan et al., 2013).

**Loss of MMP9 fails to change FPI induction of CD44 receptor protein**

A reduction in OPN signaling fragments with MMP9 KO would suggest that upregulated expression of cell surface receptors targeted by these fragments might be attenuated without MMP9. In fact, we failed to observe such an effect. FPI induction of CD44 at 3 and 7d postinjury, while trending lower, was not significantly altered in the MMP9 KO animals. This result reinforces the importance of CD44 signaling in response to OB deafferentation and suggests that it is independent of MMP9 during the subacute degenerative phase of synaptic recovery. In Chapter 3, we posited that CD44/MMP9 membrane interaction could mediate local enzyme activity around affected synapses. Since MMP9 KO failed to change OB CD44 peptide, we now suggest that the receptor is likely regulated by alternative molecular signals, not directly
affected by MMP9. First, it remains possible that developmental loss of MMP9 allowed compensation by other homologous MMPs to maintain the CD44 response. In fact, it has been shown that membrane bound MT-1 MMP targets membrane tethered CD44 in glioma cells, cleaving its intracellular domain to function as a transcription factor for upregulation of CD44 transcript (Okamoto et al., 2001). However, a more plausible explanation of our results may be found in non-CNS cell models. For example, over expression of full length OPN can lead to increased membrane deposition of CD44 and potentially MMP9/OPN interaction on the cell surface of migrating prostate cancer cells (Desai et al., 2007). Further, this same group has reported increased membrane-associated activity of CD44 bound MMP9 linked to this OPN interaction (Desai et al., 2009). Such findings point to an alternative interpretation of our MMP9 KO results for CD44, suggesting that a steady 66kD OPN expression in the OB supports induction and maintenance of CD44 signal after FPI, even in the absence of MMP9. This possibility points to CD44 signaling and its OPN regulation occurring mainly within injured neurons of the OB, given that IHC principally localizes OPN in tufted and mitral cells. In the present studies, we did not perform CD44 IHC, but prior experiments in our lab do show CD44 expression in both neurons and glia (Powell and Doperalski, unpublished results). Since glia can also signal postinjury activation through CD44 receptors (Jones et al., 2000; Shin et al., 2005; Kang et al., 2008), our histological observations that WT OB astrocyte and microglial reactivity is not altered by MMP9 KO would be consistent with a maintained glial receptor level as well, even though these cells did not appear to contain significant OPN. However, it must also be considered that the phenotypic cellular changes we see in the OB glia could involve CD44 maintenance by local injury-induced release of inflammatory cytokines like TNFα and IL1β (Campo et al., 2012; Leir et al., 2003; Webb et al., 1990). Alternatively, OPN 48 and 32kD
fragments may act through other receptors (e.g., vitronectin integrin receptor) in response to FPI, a pathway we predict would be affected by MMP9 KO. Taken together, our results indicate that postinjury OPN receptor response is complex within the OB, involving multiple molecular mechanisms of regulation, potentially utilizing stable full length and elevated cleaved fragment OPN forms. It will be quite important to map $\alpha_v\beta_3$ receptor expression and binding to OPN after FPI in order to fully explain the role of these complex MMP9/OPN interactions.

**Loss of MMP9 persistently reduces olfactory marker protein after FPI**

In Chapter 2, we utilized OMP to identify ORN axons targeting OB glomeruli, confirming that FPI induced significant loss of this pre-synaptic sensory input. This loss supported the production of diffuse axotomy and deafferentation in our OB model. OMP loss was attenuated relative to more severe olfactory nerve insults (Cummings et al., 2000; Costanzo et al., 2006; Bakos et al., 2010) and occurred only at 3d postinjury. To clarify whether or not MMP9/OPN signaling might impact ORN terminal loss, we probed for OMP protein expression in MMP9 KO animals subjected to FPI. In WT animals, OMP returned to control levels by 7d postinjury, supporting a diffuse, milder FPI deafferentation and accelerated ORN axon regeneration. That loss of OMP was not temporally correlated with elevated MMP9 activity or 48kD OPN increase. However, our results in Chapter 4 showed the loss of MMP9 caused a persistent reduction of OB OMP protein, extending into the 7 and 21d postinjury intervals, where even severe ORN lesion models show protein recovery (Bakos et al., 2010; Costanzo et al., 2006; Cummings et al., 2000; Holbrook et al., 2014). More importantly, the persistent 7d loss of OMP was directly correlated with reduced 48kD OPN fragment generation, suggesting OPN cell signaling at that postinjury interval is critical to OMP normalization and onset of reinnervation.
These associated effects of MMP9 KO on OMP might be explained in two ways. First, loss of MMP9-generated OPN fragments, which activate local glia, could significantly attenuate removal of degenerative debris associated with ORN deafferentation, affecting ECM reorganization and prolonging or blunting ORN reinnervation. This OPN related glial activation has been documented after ischemia (Kang et al., 2008), cortical stab wound (Shin et al., 2005) and hippocampal deafferentation (Chan et al., 2014). In addition, MMPs can modify the ECM structure by targeting molecules that stabilize synaptic junctions (Ethell and Ethell, 2007; Monea et al., 2006; Warren et al., 2012) and vascular endothelium (Muradashvili et al., 2011). Loss of MMP9 likely interferes with this process, delaying clearance of degenerative debris from local OB synaptic environments. Alternatively, axonal growth and synapse regeneration could be reduced due to loss of MMP9/OPN generated signaling, resulting in a delayed return of OMP expression, as reinnervation occurs at a slower pace. Relative to ORN axon reinnervation, OPN expression is associated with increased CNS cell migration, principally shown in glioma (Lu et al., 2012) and astrocytoma (Ding et al., 2002) models. Loss of OPN at a critical axonal migration interval could delay reappearance of OMP+ ORN axons. Further, PC12 in vitro studies also link MMP9 to organization of α-tubulin in growing neurites, implying a role in axonal elongation (Shubayev and Myers, 2004). We also found evidence that loss of MMP9 causes a trend toward OMP protein increase at 3d, no longer different from uninjured controls. Although this result did not show strain difference, it may be related to the significant loss of 48kD OPN we observed at 3d in the MMP9 KO animals. At the least, this observation points to MMP9 influencing the stable expression of 48kD OPN level at 3d, which, in turn, may affect the extent of OMP loss. Notably, IHC of the MMP9 KO OB shows OMP tissue distribution
consistent with our protein measures, further validating a MMP9 role in the ORN axon degeneration/regeneration cycle after FPI. Together, these MMP9 KO results support our hypothesis that MMP9 generated 48kD OPN signal influences time course of ORN axon sprouting and glomerular synaptic plasticity. While we could find no prior studies of TBI plasticity using MMP9 KO mice to examine MMP9/OPN interaction, models of cortical barrel field and monocular deprivation have assessed sensory axonal plasticity in MMP9 KO mice. Their results support our OB findings. Kaliszewska et al. (2012) reported that loss of MMP9 caused significant decrease in experience-dependent synaptic plasticity within cortical layers II-IV, assessed by [14C]-2-deoxyglucose and c-Fos mapping. In a second study of monocular deprivation, ocular dominance plasticity was mapped in MMP9 KO mice, with results showing a clear reduction of spine and synapse density in visual cortex (Kelly et al., 2015). Interestingly, very limited histological change in reactive microglial morphology was also observed in that study, consistent with our findings in the OB.

**Loss of MMP9 only minimally affects OB synaptic markers**

In the characterization of our FPI OB model (Chapter 2), we examined the postinjury change in two pre-synaptic proteins critical to vesicular organization and transmitter release, Synapsins I and II. This approach was based on the fact that Synapsin reduction has been reported in several models of CNS insult, including ischemia (Kitagawa, 1992), deafferentation (Chan et al., 2014), and TBI (hippocampus and cortex; Ansari et al., 2008a, b). Over the first three weeks postinjury, FPI did not alter WT expression of either Synapsin-I or II relative to paired sham controls. Each of these proteins is localized to OB, with Synapsin-II most concentrated in the glomerular layer (Stone et al., 1994). Overall, in the MMP9 KO subjected to FPI, we failed to find any significant
changes in Synapsin-I or II expression relative to uninjured controls. Within strains, both isoforms of each protein were unaffected by MMP9 loss. This indicated that OB postinjury Synapsin stability was not dependent on MMP9 and reinforced our earlier conclusion that OB sampling procedure may have prevented detection of effect in a diffuse population of injured ORN axons. Nevertheless, post hoc analysis did identify two cases where small, but significantly different strain differences could be detected. In Synapsin-I 80kD, the small WT rise above controls at 3d was normalized in the MMP9 KO, and in Synapsin-II 72kD, a 21d shift from just below control in WT, to just above control level without MMP9, also achieved statistical significance. While these strain differences are difficult to interpret, and we could not find reported links between MMP9 and Synapsin protein expression, our results do raise interesting questions about MMP9 role in both the acute degenerative and regenerative phases of OB synaptic repair. In the MMP9 KO, 3d postinjury 80kD Synapsin-I trended toward normalization, correlating with a similar shift toward normalization of OMP protein. This relationship, although counterintuitive for the acute degenerative period, does suggest that loss of MMP9 may indirectly influence some aspect of axon stabilization, slowing ORN axon loss and reorganization. For Synapsin-II 72kD, MMP9 loss resulted in a trend toward increased expression at 21d, which correlated with GAP-43 increase relative to controls at that same postinjury interval (discussed below).

One explanation for these paired shifts might relate to Synapsin-II concentration in glomeruli and its involvement in the synchronization of GABA vesicular trafficking/release, controlling OB synaptic signaling (Medrihan et al., 2015). From this, we can speculate that loss of MMP9 may release some capacity for increase of local growth factors (e.g., GAP-43) to drive axon
regeneration and allow re-emergence of critical pre-synaptic terminal proteins. Additional experiments focusing on more accurate assessment of these proteins within deafferented OB glomeruli will be needed to determine if such MMP9 roles are valid.

Loss of MMP9 only minimally affects OB GAP-43 expression

Sprouting of new axon after CNS injury has long been characterized by growth factor induction (Benowitz and Routtenberg, 1997). Such neuroplasticity in the olfactory system is consistently associated with the upregulation of GAP-43. Whether induced by OE lesion or target bulbectomy, regenerating ORN axons have been marked by elevated GAP-43 expression, which is reduced upon synaptic contact and synapse formation with their OB targets (Verhaagen et al., 1990; Cizkova et al., 1995). In Chapter 2, we showed that WT mice subjected to FPI deafferentation exhibit a nearly 3-fold upregulation of OB GAP-43 at 21d, marking the active period of axonal regeneration into glomeruli. No change in WT GAP-43 was detected at any other postinjury time point. This finding suggests that the growth factor marked regenerating ORN axons are invading the OB, consistent with reported immuno-localization of GAP-43 in pre-terminal axons of the mouse olfactory bulb (Ramakers et al., 1992). Here, loss of MMP9 produced only minor effects on this GAP-43 response to FPI. While the 21d 3-fold upregulation of GAP-43 was essentially not different between the strains, MMP9 KO mice did show a trend toward increase. As discussed above, this trend was accompanied by a slight increase in 72kD Synapsin-II, which, together, may indicate progression toward synapse repair. Interestingly, we did see a strain difference at the 3d postinjury time point, a strong trend toward reduced GAP-43 in the MMP9 KO. While this decrease was not significantly different from uninjured controls, the nearly 50% reduction in GAP-43 was different from WT cases. The more straightforward
interpretation of these results would be that, overall, MMP9 does not substantively affect GAP-43 induction during onset of axon regeneration. In *vitro* studies using PC12 cells support this view, showing that NGF-induced increase in GAP-43 was not altered by MMP9 treatment or inhibition (Shubayev and Meyers, 2004). Nevertheless, MMP9 KO strain differences at 3d raise the interesting possibility that MMP9 may indirectly contribute to growth factor regulation during the early, subacute degenerative phase. With the loss of MMP9, OMP returns to a level not different from controls (discussed above), in tandem with a precipitous drop of GAP-43 protein. If MMP9 presence contributes to the decrease of WT ORN OMP at 3d, and this contribution is lost, then OMP levels might be abnormally maintained in MMP9 KO, driving a reduction in FPI induced GAP-43. This type of reciprocal OMP/GAP-43 expression has been described during postnatal development of the olfactory system (Verhaagen et al., 1989), and posited as a mechanism by which OMP null mice can increase immature ORN axon sprouting (Griff et al., 2000). Additional studies designed to map the tissue distribution of OMP and GAP-43 are needed to confirm whether this reciprocal relationship occurs in the OB following FPI.

**Loss of MMP9 attenuates OB synaptic reorganization following FPI**

In our biochemical analysis of MMP9 KOs subjected to FPI, OPN fragments were reduced across time, and OMP levels were persistently low, even into the period of axon regeneration. Together, these findings suggest a blunted postinjury synaptic recovery in the MMP9 KO vs. WT animals. As in Chapter 2, we used TEM ultrastructural analysis of the OB glomeruli to determine if such differences might be revealed in abnormal synaptic structure. Here we focused on the 21d time point, reasoning that the reversal of OPN fragment signaling at the onset of regeneration and abnormally low level of OMP well into active synaptic reinnervation phase
would have a maximal impact on synaptic structure at this postinjury interval. Overall, qualitative glomerular ultrastructure was consistent with the observed changes in OPN and OMP expression within the MMP9 KO, where synaptic architecture was clearly less organized at 21d after injury. Fine structure of ORN synapses revealed poorly organized junctions in the MMP9 KO mice. ORN terminal cytoskeleton appeared disrupted and pre-synaptic vesicles more diffusely distributed. Post-synaptic densities were also thinner and less defined. This was in contrast to WT 21d cases which had well organized synaptic structure, more like control OB glomeruli. There, pre-synaptic vesicles were easy to identify, seen within ORN axon terminals opposed to synaptic junctions containing thick post-synaptic densities. The latter WT profile is similar to the normal ultrastructure reported for ORN/mitral and ORN/tufted excitatory synaptic junctions (Kasowski et al., 1999; Kosaka et al., 2001). This same pattern of asymmetric vesicle-containing ORN terminals was reported after unilateral olfactory nerve transection in the hamster, where transneuronal tracer WGA-HRP labeled post-synaptic glomerular layers, confirming synaptic reinnervation (Morrison and Costanzo, 1995). In the only MMP9 KO mouse study with ORN transection, loss of MMP9 did not alter the time-dependent response of the other principal gelatinase, MMP2, which is highly expressed during glomerular reinnervation (Costanzo and Perrino, 2008). While no TEM was performed in that study, the time course of reinnervation was not affected by loss of MMP9, suggesting that OB reinnervation after full ORN axotomy does not show the same vulnerability to MMP9 KO as does FPI. As further evidence of poor recovery in our data, MMP9 KO glomeruli at 21d postinjury also contained sites of ongoing cellular degeneration. Membrane debris from either degenerating axons or post-synaptic dendrites was clearly visible. We interpret these TEM findings as evidence that loss of MMP9 significantly alters the time-dependent progress of terminal degeneration and ORN.
synaptic reinnervation after FPI. Such attenuation of degenerative debris clearance and impairment of ORN axon reinnervation would be expected to correlate with persistently low OMP levels. Loss of OPN signaling is posited to reduce local clearance of damaged ORN axon terminals, as well as attenuate other cell signaling pathways required for onset of OB reinnervation.

Summary

Taken together, our results in MMP9 KO mice confirms our hypothesis that the gelatinase MMP9 is critical to postinjury timing of the degenerative and regenerative phases of OB reactive synaptogenesis following FPI. Further, this role appears to be mediated through acute and subacute proteolysis of OPN, in particular the generation of the 48kD RGD containing cell signaling fragment. Loss of this fragment likely impairs clearance of injury generated debris and the maturation of regenerating ORN axons. A delayed increase of OMP during the regeneration phase and TEM evidence of poor synaptic recovery supports this interpretation. Surprisingly, loss of MMP9 does not alter FPI induction of the OPN receptor CD44 or growth factor GAP-43, the latter of which would be predicted given the altered time course for regeneration onset in the MMP9 KO. This suggests that FPI OPN signaling may be mediated through alternative integrin receptor pathways, quite possibly αvβ3. It also points to upregulation of GAP-43 through a MMP9-independent pathway, which is consistent with published studies (Fredrich and Illing, 2011; Shubayev and Myers, 2004).
CHAPTER 5

GENERAL DISCUSSION
SUMMARY OF STUDY AND FINDINGS

This is the first study documenting synaptic changes in the mouse OB resulting from mild diffuse brain injury induced with the fluid percussion model. We successfully demonstrated that the injury delivered over the sagittal suture adjacent to the cortex can distort brain tissue as distal as the OB. We hypothesized that TBI would cause axonal damage to ORNs, deafferenting synapses of the GL of the OB and activating molecular mechanisms that elicit a cellular response to induce reactive synaptogenesis. This process, we posited, would involve cellular signaling initiated by integrin and CD44 receptor binding of OPN fragments predominantly cleaved by MMP9. Our investigation of αII-spectrin proteolysis and TEM analysis, coupled with exploration of ORN specific marker OMP, supports FPI induction of reactive synaptogenesis in the OB. Assessment of the postinjury time course of glial activation, MMP9 activity, OPN fragmentation, and CD44 expression revealed a complex, interactive role of these proteins in OB reinnervation. Subsequent MMP9 KO experiments confirmed strong influence of MMP9 on OPN fragment generation and ORN axon recovery, marked by OMP expression. Together, our studies support the critical role of MMP9/OPN interaction in promoting OB ECM reorganization for synaptic repair.

In Chapters 2 and 3, we demonstrated that FPI was sufficient to cause ORN axotomy, leading to disruption and deafferentation of OB synapses, as well as onset of a temporal sequence of ECM associated cellular and molecular changes supporting OB synaptic recovery. We characterized FPI influence on OB using several markers of axonal injury and regeneration. First αII-spectrin was tested. This cytoskeletal protein is cleaved upon axonal injury and is used to measure degree of axotomy. In the OB, the calpain-cleaved 150kD αII-spectrin fragment was elevated
almost 4-fold at 3d postinjury, showing that axon degeneration occurs within injured OB at this subacute injury interval. To further characterize axonal damage, we assayed pre-synaptic protein markers. In our mild injury paradigm, analysis of Synapsins, which are vesicle associated proteins expressed in neuronal pre-synaptic terminals, did not reveal any WT postinjury change. We next focused on profiling the postinjury change in OMP expression, a protein serving as a marker of the ORN axon degeneration/regeneration cycle. The reduction of this mature sensory neuron marker at 3d after FPI, with a gradual return to normal levels by 21d, was consistent with the published time course of OB synaptic reinnervation in other models of ORN deafferentation. Importantly, spectrin proteolysis coincided with OMP loss at 3d, supporting this subacute interval as a critical period of axon degradation in the diffusely injured OB, and showing that axon breakdown is correlated with loss of mature ORN marker. In parallel with these molecular findings, IHC was used to map morphological changes in OB glia after FPI. Robust OB astrocyte hypertrophy and increase in the number of amoeboïd microglia at the 3d postinjury, pointed to the reactive glia as potential sources of MMP9 for OPN signal processing, which we posit directs removal of axon break down products. Moreover, TEM analyses showed OB glial phagocytosis is underway at 7d postinjury, supporting the predicted role for reactive glia in tissue debris clearance of GL synaptic regions. These phenotypic changes of astrocytes and microglia also indicate that FPI initiates an OB immune response, and GFAP labeling of astrocyte processes reveals an intimate association between these glia and synapses of the injured GL. From this we conclude that both glia are active in response to OB deafferentation, but GL astrocytes are likely to play a larger role in postinjury degenerative/regenerative stages. To explore the regenerative phase of OB synaptogenesis, the growth associated protein GAP-43 was employed to confirm the onset of ORN reinnervation at 21d after FPI. GAP-43 showed over 3-
fold elevation at that time point, yet was unchanged from control at the three earlier postinjury intervals, further supporting induction of synaptic reinnervation after FPI. Upregulation of GAP-43 also fits with WT TEM analyses, which demonstrated that evidence of damaged pre-synaptic terminals is almost completely removed by 21d, when more mature synapses begin to reappear. These data supported the use of our mild FPI model to induce OB reactive synaptogenesis, allowing the exploration of how ECM molecules might mediate the process of ORN synaptic recovery after TBI.

In Chapters 3 and 4, our results confirmed a time-dependent association between ECM molecular change and OB response to ORN deafferentation and dysfunction. Gelatinases are critical mediators of postinjury degradation and ECM modification, facilitating synapse recovery. While the full extent of MMP9 role in OB reactive synaptogenesis is open to further study, data from our experiments show that enzyme interaction with OPN, a MMP substrate, results in elevation of cleaved OPN fragments. These peptides can be released into the ECM, bind cell surface receptors and affect key cell signaling pathways in the injured OB. However, our study does reveal that this postinjury MMP9/OPN interaction is complex in the OB, with time-dependent change in OPN fragments representing distinct phases of reactive synaptogenesis. Interestingly, both MMP9 activity and OPN fragment expression were elevated at 1d after FPI, the acute degenerative phase, consistent with MMP9 response in other CNS insults. MMP9 activity is reduced to sham level at 3d postinjury, again similar to the predicted acute postinjury response pattern of this gelatinase. A novel finding in our study was the subsequent peak elevation of OB MMP9 activity at 7d after FPI, which was also associated with significant rise in OPN fragment processing (producing both elevated 48 and reduced 32kD proteins). The timing of this peak in
MMP9 lysis suggests that the enzyme serves a second functional role in facilitating OPN cell signaling at the late degenerative phase, perhaps aiding in the promotion of ORN axon growth associated with the 21d postinjury interval. While elevated MMP9 protein was found in the GL and EPL neuropil, as well as within reactive astrocytes at 7d postinjury, OPN was restricted to neuronal subtypes from 3-21d after FPI. Localization of OPN within deafferented mitral and tufted cells and the time-dependent upregulation of this neuronal OPN suggests a post-synaptic production and secretion of OPN into the ECM, priming cells in the local environment to prepare the synaptic site for reorganization. It remains possible, however, that glial expression of OPN may occur earlier, at 1d postinjury, a time interval which we did not examine with IHC. As OPN effects cellular reactivity through interaction with cell surface receptors, we probed hyaluronan receptor CD44, reported to bind with OPN. Robust upregulation of OB CD44 occurred at 3d and was sustained through 7d. Since this receptor is highly expressed on reactive microglia and astrocytes in the CNS, and reactive glia promote the stages of reactive synaptogenesis, we posited that OPN/CD44 interaction would be temporally correlated in the injured OB. Interestingly, we failed to see paired elevation of OPN and CD44 protein, but found time shifted peaks in the two, suggesting that OPN fragment rise at 1d may serve to signal a 3d increase of CD44 transcription and facilitate later positioning of CD44 within cell membranes for signaling purposes. Taken together, mapping of MMP9 activity, OPN fragment expression, OMP marking of ORN axon loss, and GAP-43 induction for axon growth, all point to a complex, multicellular interaction within the ECM that directs successful OB synaptic plasticity after FPI. In order to fully understand MMP9 role in the complex process, we conducted a second analysis of these proteins in MMP9 KO animals subjected to FPI.
Our studies utilizing MMP9 KO animals suggest that MMP9 activity is critical for the production of OPN signaling fragments at multiple points during the process of OB reactive synaptogenesis. Most notably, loss of MMP9 delayed reappearance of ORN axon marker protein OMP over time postinjury and significantly reduced OPN fragmentation at acute and subacute time points (up to one week postinjury). Interestingly, MMP9 loss failed to alter the time-dependent expression of OPN receptor CD44 after FPI, nor did it significantly change overall response of Synapsin-I and II or GAP-43 growth factor. While post hoc tests on measures of the latter three molecules did reveal interesting strain differences at some of the time points, these differences were small and likely do not reflect the prominent MMP9 effects related to OPN or OMP. Foremost, MMP9 KO effect on critical proteins marking efficacy of synaptic recovery suggests that MMP9 plays an important role in degeneration/regeneration of OB synapses after FPI. The limited effect of MMP9 activity on Synapsin expression, injury-induced astrocyte phenotype, as well as GAP-43 expression, all point to a more complex, indirect interaction of these molecules with the enzyme. The significant difference in OPN fragmentation between WT and MMP9 KO animals, however, indicates a strong association with acute, critical cell mediated signaling and later, a mediation of ORN axon growth and synaptogenesis. At 1d, when early cell activation is required to initiate ECM reorganization and debris removal, loss of MMP9 significantly reduces the 48kD OPN RGD fragment. This would potentially curtail this early activation of local glomerular glia. As noted above, we did not assay \textit{in vivo} glial reactivity at 1d postinjury, however, we did conduct pilot \textit{in vitro} assay of OB MMP9 KO glia. Results showed a more ramified microglial profile, consistent with attenuated OPN signaling between OB glia (Appendix B.1). At 7d postinjury, closer to the degeneration/regeneration transition phase, MMP9 production of 48kD OPN correlated with
high levels of CD44, suggesting MMP9/OPN interaction is important in this period of the recovery process. In the WT injured mice, it is this interval when MMP9 activity, OPN proteolysis and CD44 expression are concurrently high, and where OMP level starts to approach controls, pointing to onset of the reparative process. By contrast, MMP9 loss prevented elevation of 48kD OPN and attenuated reduction of 32kD OPN, as well as delayed the stabilization of OMP expression, which continued into the 21d time point. Additionally, OPN distribution within mitral and tufted cells was reduced in MMP9 KO animals, and TEM showed evidence of protracted damage to GL synapses, further confirming a significant role for MMP9 in mediating successful synapse recovery.

Overall, these results show that FPI induces OB injury sufficient to deafferent GL synapses, and MMP9 is an integral part of subsequent reactive synaptogenesis. Our data point to early OPN fragmentation induced by an acute immune response, which likely contributes to 3d CD44 upregulation and OB glial activation to mediate clearance of ORN axons/OMP protein. Regenerative processes may begin as early as 7d, when WT MMP9 peaks, OPN fragmentation is high, and OMP moves toward control levels, indicative of synaptic reorganization. This process is clearly delayed in MMP9 KO mice, pointing to a significant role for MMP9/OPN interaction in modulating cell biological processes for ORN reinnervation. This novel data for OB response to FPI details a mechanism that is consistent with published reports on more severe OB deafferentation and MMP9/OPN response to TBI. These studies offer new insight into gelatinase function in the OB following ORN axotomy.
FPI INDUCES ORN AXOTOMY AND SYNAPTIC REPAIR

Neurogenesis within the OE is well described (Moulton, 1974). ORNs exhibit continuous turnover, cycling through degeneration and replacement by OE basal stem cells. They extend their axons from the epithelium, through the cribriform plate and directly into the OB. This process of continuous OB reinnervation shows that GL environment supports synaptogenesis, making the region ideal for exploring post-traumatic synaptic plasticity. Mechanical forces and secondary injury mechanisms induced by head trauma can lead to ORN axonal injury and loss of GL synaptic input, resulting in loss of olfactory function (Costanzo et al., 2012; Sumner, 1964; Costanzo and Zasler, 1991; Hagan, 1967). Despite the ORN regenerative capacity, anosmia can persist in TBI patients, which could result from glial scar formation (Costanzo, 2005; Kobayashi and Costanzo, 2009) and/or aberrant synaptogenesis, with axons projecting to incorrect glomeruli, partially reinnervating the GL (Costanzo, 2005; Morrison and Costanzo, 1995).

Recently developed animal models of OB insult with TBI do show that severe closed head injury can reduce OB size, causing significant olfactory deficits up to 12 weeks postinjury (Siopi et al., 2012). However, it remains unknown how such functional loss relates to synaptic repair or whether similar OB effects could be generated with diffuse TBI. In order to address this question, we first established that our model of mild mouse FPI causes ORN axon damage and upregulates markers of OB deafferentation. This was important to show before testing the hypothesis that ECM enzymes, substrates, and receptors mediate OB reactive synaptogenesis induced by TBI. To do this, we probed for proteins associated with axonal damage (αII-spectrin) and presence of ORN axons (OMP), as well as markers of axon growth (GAP-43) and pre-synaptic vesicle trafficking (Synapsin-I and Synapsin-II).
After trauma, axolemmal Na$^+$ and Na$^+$/Ca$^{2+}$ channels are disrupted causing uncontrolled calcium influx and activating calcium-dependent caspases and calpains (Tuck and Cavalli, 2010). These enzymes can cleave αII-spectrin, disrupting stability of membrane tethered ankyrin-G and actin components of intact axons (Letourneau, 2009; Reeves et al., 2010), leading to disintegration of the axon cytoskeleton. Lysis of αII-spectrin after diffuse and focal injury in cortex, corpus callosum, and hippocampus has been documented (Pike et al., 1998; Reeves et al., 2010; Saatman et al., 1996; Saatman et al., 2003). Robust increases in 150kD and 145kD αII-spectrin fragments, as early as 2 hours and up to 4d postinjury, correlated with axotomy, particularly during acute recovery periods. Our studies probing for protein expression of αII-spectrin in the postinjury OB revealed a more limited amount of αII-spectrin breakdown, with only the calpain-cleaved 150kD αII-spectrin fragment significantly increased nearly 4-fold over controls 3d following injury, supporting an attenuated level of ORN axotomy with diffuse FPI. The fact that the 150kD αII-spectrin peptide did not change for any other time point, and that calpain-generated 145kD was unaffected, indicates that ORN axon degeneration in our model is less robust relative to other brain regions and likely peaks at 3d postinjury. This result would be predicted based upon the distance of OB from injury site and distribution of shearing forces reaching cortical regions rostral to the FPI focus. Further, since the caspase-produced 120kD αII-spectrin also did not change, we conclude that diffuse FPI does not generate αII-spectrin commensurate with significant cell death in the OB. More specifically, the predominant increase in calpain-cleaved αII-spectrin in our model suggests that calpain may be the more sensitive enzyme in the OB subjected to diffuse TBI, cleaving αII-spectrin extensively when activated by an indirect, diffuse injury.
Given that the OB contains several neuronal subtypes whose axons are also susceptible to TBI-induced damage, it was important for our OB deafferentation model to confirm that elevated αII-spectrin lysis, indicative of axotomy, was associated with axonal damage sustained by the more vulnerable ORN afferent axons. To identify injury-induced ORN loss, we probed OMP, a cytosolic protein that is only expressed in the first order sensory neurons of the OB (Monti-Graziadei et al., 1977). A mature ORN marker, OMP has been detected along the interface of the OE and OB as early as E16 (Valverde et al., 1993). Moreover, Verhaagen and colleagues (1989) documented OMP tissue distribution during a 6 month postnatal developmental period, revealing that OB maturation is characterized by emergence of OMP signal as ORN axons move from the OE to the ONL, approaching the target OB glomeruli. Since OMP expression marks OB innervation by labeling mature ORNs, it has been used to track the process of OB reinnervation after ORN damage. In models of OB deafferentation, OMP expression drops and recovers over a time frame largely dependent on severity and type of denervation. For example, when the ON is axotomized with knife transection, OMP is reduced 3-10d, decreasing and returning to control levels within 15d (Costanzo et al., 2008), while complete nasal epithelial loss, causes a later and more prolonged return of OMP to control levels, between 7-40d post-lesion (Bakos et al., 2010; Nathan et al., 2001). Interestingly, loss of OMP after diffuse FPI was consistent with the ON transection model, showing significant reduction at 3d after injury. Given that insult to the ORN axons is most likely the primary mechanism by which the OB is denervated after head injury, it is reasonable to predict a similar OMP expression pattern between FPI and the knife lesion. However, in contrast to the knife transection, OMP level at 7d postinjury was no longer different from controls, suggesting a more rapid restoration of mature ORN axons than in models of complete axotomy. This pattern of OMP reduction at 3d and
return to normal level by 7d suggests that, while diffuse FPI can cause axon damage and
deafferent the OB, the injury may also produce a less severe insult to a subset of ORN axons
which could be reversible. TEM analysis of the GL synaptic cytoarchitecture supports the
presence of both types of axon damage after FPI. First, we found evidence for the well described
progressive degenerative/regenerative response characteristic of OB reactive synaptogenesis:
disorganization of pre-synaptic ORN terminals surrounding post-synaptic dendrites at 3d;
shrunken, retracted terminals at 7d, with sites of glial phagocytosis; reemergence of normal
synaptic structure by 21d. However, we also observed disorganization of ORN axon
cytoskeleton and disruption of synaptic vesicle arrangement after FPI, which appeared to resolve
over time postinjury. The latter profile may represent a partial, reversible deafferentation or
remodeling without junctional separation in a subset of synapses. The revelation of a more
complex deafferentation profile is novel for TBI models. Future studies of OB synaptic
ultrastructure will be necessary to clarify the extent to which FPI generates different types of
ORN axon damage. In addition to our OMP analyses of axon damage, we assessed GAP-43
expression to verify the presence of growing axons and the induction of synaptic reinnervation.

Elevated growth factor GAP-43 is critical for synaptic sprouting, promoting neurite outgrowth
by guiding the reorganization of growth cone cytoskeletal structure (Benowitz and Routtenberg,
1997). These properties underlie the tight association of GAP-43 level and regeneration
(Sachdeva et al., 2016; Wang et al., 2014; Williams et al., 2015), as GAP-43 labels immature
neurons and increases within the CNS after several models of injury (Carmichael et al., 2005;
Christman et al., 1997; Park et al., 2013; Gorup et al., 2015; Goto et al., 1994; Hulsebosch et al.,
1998; Stroemer et al, 1993). Consequently, GAP-43 is high during periods of ORN axon growth
from the OE to the OB, then downregulated during ORN maturation and navigation toward OB targets (Margolis et al., 1991; Rodriguez-Gil et al., 2015; Steuer et al., 2014). In our FPI model, significant elevation in OB GAP-43 was not detected until 21d postinjury. This result is reasonable since growth cones of newly formed GAP-43 expressing ORN axons begin to enter and navigate the OB GL around this time after injury, moving toward their post-synaptic targets. Mapping of OB synaptogenesis after more severe insults shows that up to 60d is required for the process of reinnervation to be complete (Graziadei et al., 1978; Graziadei et al., 1979), making the 21d postinjury interval an early phase of such synaptic reorganization, perhaps requiring high levels of growth factor stimulation. Its significant elevation at 21d following FPI is also reasonable considering GAP-43 may control axon response to intrinsic OB signals for synaptic targeting (Holtmaat et al., 1995). In that context, strong upregulation of GAP-43 at 21d also suggests that sprouting terminals can now navigate a modified ECM which provides a favorable environment for ORN reinnervation (discussed below relative to MMP9 and its substrates).

Interestingly, OMP regulation of GAP-43 expression has been proposed (Griff et al., 2000; Holtmaat et al., 1995; Margolis et al., 1991; Verhaagen et al., 1989; Verhaagen et al., 1990), where low OMP expression, as in cases of OB injury, releases suppression of GAP-43, leading to increased GAP-43 expression for growth within immature neurons and axons. A similar inverse relationship between OMP and GAP-43 expression was recently reported in a new rodent model of OB insult, where direct OB displacement stretches ORN axons against the cribriform plate similar to human TBI (Steuer et al., 2014). Our results, however, show that robust GAP-43 increase is limited to 21d when OMP expression has returned to control level. On the surface, this result is somewhat difficult to interpret. One simple explanation is that the proposed
OMP/GAP-43 relationship is not expressed after diffuse FPI. Alternatively, intrinsic neurogenesis of the olfactory system might contribute to GAP-43 rise at 21d in our samples of whole OB, independent of OMP. For example, GAP-43 expression relative to ongoing neurogenesis in deeper OB layers might predominate over GL GAP-43 change. During development, the timing of OB entry for subventricular zone (SVZ)-generated progenitor cells is approximately 15-30d (Petreanu and Alvarez-Buylla, 2002), which would be consistent with the observed 21d GAP-43 peak expression in our study. Further, TBI studies mapping progenitor generation do show increased postinjury neurogenesis in the SVZ (Sun, 2014), however, OB targeting for these newly induced cells after brain injury has not been confirmed. While progenitor contribution cannot be ruled out at this point, at least three lines of evidence suggest that this pathway is not a major contributor to GAP-43 profile in our study. First, we see no change in GAP-43 at other postinjury time points, during which ongoing neurogenesis would presumably also affect growth factor expression. In addition, GAP-43 protein expression peaks at the documented time point of ORN axon reentry. Further, more severe CNS injury models can increase proliferation in the SVZ, yet they are reported to reduce neurogenesis in the OB, suggesting migrating neuroblasts are diverted from the rostral migratory stream (RMS) to other injured cortical areas (Radomski et al., 2013; Sundholm-Peters et al., 2005). Additional studies are needed to clarify the relationship between OB OMP and GAP-43 after FPI. Mapping each protein’s expression during later postinjury intervals could determine if GAP-43 is actually reduced during synapse maturation and correlated with a surge in OMP level, indicating that the two proteins interact to promote the successful reestablishment of stable GL synapses.
In order to further verify trauma-induced degeneration of ORN axons, we probed for changes in OB pre-synaptic vesicle markers after FPI. Synapsins are involved in the docking and release of neurotransmitter vesicles, with Synapsin-I present throughout the OB core and Synapsin-II localized to the GL (Melloni et al., 1993; Stone et al., 1994). Interestingly, improved spatial memory is associated with increased 80kD Synapsin-I and 72kD Synapsin-II (John et al., 2009). Further, 72kD Synapsin-II also regulates GABA inhibition by affecting glutamatergic synaptic vesicle clustering and release (Medrihan et al., 2013; Orenbuch et al., 2012; Song and Augustine, 2015). Synapsin-II role in controlling the size of the synaptic vesicle reserve pool within glutamatergic terminals (Gitler et al., 2008) could underlie its reported association with the GL, where both ORNs and mitral cells transmit glutamate (Cave and Baker, 2009). After zinc sulfate-induced OB deafferentation, OMP and Synapsin-II were not only co-localized in the GL, but also reduced to a similar degree at 3-4d post-injury (Stone et al., 1994). This finding suggests that ORN axon terminals can be marked by both OMP and expression of proteins controlling their terminal vesicles. Since association between the two OB proteins appeared strong, we predicted that FPI would show parallel reductions in the two proteins over time postinjury. Surprisingly, we did not find this correlation in our studies, as OMP was significantly reduced in WT animals at 3d relative to sham controls, but the 72 and 58kD isoforms of Synapsin-II were not affected. One simple explanation for lack of Synapsin-II effect could be a dilution of signal in our whole OB samples. It is possible that GL Synapsin-II appeared unaltered by diffuse FPI as a result of its lower percentage expression within a larger, heterogeneous OB population of pre-synaptic structures. This includes ORN axons as well as GABAergic terminals of periglomerular and granule cells, which we posit are less vulnerable to axotomy after TBI. Another possible interpretation of our result comes from GL TEM data.
There, we found that synaptic vesicles could still be visualized within many of the disorganized pre-synaptic terminals after FPI. This observation suggests that while some pre-synaptic ORN axons degenerate, others are simply disorganized, retaining a population of terminal vesicles with Synapsin-II protein.

We also measured protein expression of the 80 and 70kD isoforms of Synapsin-I after TBI, again observing no difference from sham controls throughout the 21d recovery period. Since 80kD Synapsin-I promotes GABA inhibition through synaptic vesicle trafficking (Song and Augustine, 2015), the failure to detect change in its expression might indicate limited FPI-induced damage to inhibitory GABAergic interneurons. Even if some of these cells are affected by diffuse injury, normal Synapsin-I expression within the intact periglomerular or granule cell terminals could mask detection of a minimal Synapsin-I loss in the ORN axons. Nevertheless, we found mean expression of both Synapsin-I isoforms trending lower during the 21d period of early synapse regeneration, with post hoc tests supporting a clear reduction between 3d and both 7 and 21d time points. Generally, in the context of CNS trauma, Synapsin-I expression is reduced acutely following injury (Kitagawa, 1992; Chan et al., 2014; Ansari et al., 2008a, b), and increases later, during postinjury recovery (Griesbach et al., 2004). Again, our OB data did not completely fit this prediction, which we interpret to be due to the mild nature of the injury.

Overall, we conclude that even mild OB insult to the mouse brain, occurring distal to the focal point of diffuse TBI, can set into motion neuroplasticity of ORN projecting axons, and induce time-dependent production of molecules mediating synaptic reorganization. Our results
documenting such reactive synaptogenesis supported further exploration of molecular mechanisms underlying this OB reinnervation.

**MMP9 IS ACTIVATED DURING POSTINJURY OB SYNAPTIC PLASTICITY**

As modulators of ECM proteins, MMPs can promote both pathogenesis and recovery from different CNS insults, with their time course of expression a function of injury type and brain region (Abdul-Muneer et al., 2015; Montaner et al., 2001; Phillips et al., 2014). Interestingly, aberrant MMP elevation has been observed with stroke, cerebral hemorrhage, Alzheimer’s disease, and TBI (Abdul-Muneer et al., 2015; Van Hove et al., 2012; Verlegers et al., 2013). This elevation contributes to evolution of excessive neuropathology by increasing glial activation and further degrading the ECM. By contrast, time-dependent upregulation of several MMPs promotes reactive synaptogenesis after neuronal injury (Chan et al., 2014; Kim et al., 2005; Falo et al., 2006; Warren et al., 2012; Phillips and Reeves, 2001), where ordered processing of targeted substrates support terminal sprouting, synapse formation, and synapse stabilization.

Acute rise in MMP 2, 3, and 9 expression is consistently detected after injury, where the enzymes contribute to degenerative and regenerative processes involved with synaptic plasticity, providing a rationale for exploring OB MMP9 in our study. For instance, OB deafferentation by full ORN knife lesion induces acute (1d) elevation of MMP9 protein expression (Costanzo et al., 2006), a response that gradually declines over a 20-60d recovery period. A second study by the same group showed that direct MeBr gas ablation of ORNs in the OE also induced OB MMP9 expression, however, the peak protein was seen at 5d, not 1d postinjury, and rapidly decreased between 5-10d after lesion (Bakos et al., 2010).
In the present study, we did not assay MMP9 protein, but used zymography to measure postinjury enzyme activity. Nevertheless, comparison of FPI OB MMP9 response to these prior studies shows some interesting similarities and differences. For example, diffuse FPI to the OB did produce a 1d rise in MMP9 activity, consistent with the elevated MMP9 protein reported after full ORN transection. No such elevation of MMP9 protein was reported with OE insult. This supports a common response to local ORN axotomy near the OB. At 3d postinjury, when spectrin proteolysis peaked and OMP was lowest in our model, OB MMP9 activity was essentially equal to control cases. Initially, this result was surprising, however, both ORN transection and OE lesion were in line with such reduction. Interestingly, our data was most consistent with the 3d baseline MMP9 protein level observed with OE lesion, whereas the ORN transection model retained some MMP9 protein elevation at 3d after lesion. These model similarities and differences point to an important, yet complex, role for MMP9 in the deafferented OB. It suggests that ORN injury locus and type may differentially alter the time course of ECM and cell signaling response in the distal synaptic regions targeted by these neurons. After FPI, our data suggests that acute postinjury MMP9 activity serves to promote clearance of degenerating axon terminals by locally activated glia, primed for their peak reactive state at 3d. OB MMP9 activity would then return to control levels, concluding the acute phase role. By 7d postinjury, we observed a second, more robust OB MMP9 activity, pointing to the subsequent, critical role for increased MMP9 activity in ECM reorganization, just prior to axonal regeneration. Notably, FPI causes axon degeneration and pre-synaptic terminal loss, as well as acute inflammatory response (Abdul-Muneer et al., 2015; Erb and Povlishock, 1991; Werner and Engelhard, 2007). Each pathway could drive MMP9 elevation, producing time-dependent cleavage of inflammatory cell signaling proteins like OPN to program 3-7d glial reactivity.
detected in our studies. For example, elevated OPN at 1d may signal a spike in CD44 cell surface receptors on activated glia at 3d (Desai et al., 2007), contributing to increased MMP9 activity at the 7d mark. Successful ECM clearance of ORN axonal debris through increased microglial and astrocyte phagocytosis would result, preparing the local OB environment for synaptic reinnervation. Our observation of extensive glial phagocytosis in the GL with TEM analysis would further support this interpretation.

While both ORN transection and MeBr OE ablation show increase of MMP9 protein, only the ablation model (Bakos et al. 2010) identifies the later MMP9 rise as a peak response to insult, similar to our observations with FPI. This suggests that the 7d MMP9 peak activity after FPI could reflect more substantive damage to ORN cells and their axons, generating a pattern of response similar to deafferentation without major axotomy near the OB. It remains possible that diffuse FPI also causes direct damage to ORNs within the OE, which would be consistent with the timing of enzyme response after MeBr OE ablation. Whether from direct axotomy at the cribriform plate or ORN damage within the OE, the 7d peak MMP9 activation appears to represent important substrate processing during the transition from degenerative to regenerative phases of recovery. Within the 21d recovery window, we saw no evidence of prolonged or aberrant MMP9 activity which could lead to disorganized reorganization. This result differs from those of maladaptive recovery models (Falo et al., 2006; Warren et al., 2012; Phillips et al., 2014), where prolonged elevation of MMP activity leads to aberrant synaptic repair. In summary, our data points to biphasic periods of OB enzyme increase after diffuse FPI, suggesting a well-timed, beneficial role for MMP9 activation in the processing of substrates critical to ECM reorganization and successful synaptic plasticity.
Given that CNS trauma typically produces time-dependent elevation in both protein and enzymatic activity of MMPs 2 and 9 (Costanzo et al., 2006; Costanzo and Perrino, 2008; Horstmann et al., 2006; Hsu et al., 2006; Hsu et al., 2008; Phillips and Reeves, 2001; Wang et al., 2000), we also assessed the effect of OB FPI on MMP2 activity. Despite similar effects on ECM protein reorganization, it has been proposed that the two gelatinases mediate different critical periods of synaptogenesis, principally due to the postinjury time course of their expression/activation. For example, in the deafferented hippocampus and FPI corpus callosum, our laboratory has documented single acute peak MMP9 elevation at 1d postinjury, while MMP2 increases during the 7-14d period (Phillips and Reeves, 2001; Phillips et al., 2014; unpublished data). Similarly, in the ORN transection model, a 7d MMP2 peak protein expression in the OB was reported (Costanzo and Perrino, 2008). In Chapter 3, we showed that OB MMP2 activity in WT mice subjected to FPI peaked at the 7d postinjury interval, in tandem with the second peak of MMP9 activity. However, MMP2 elevation above sham was much lower than that of MMP9 (130% vs. 280% of sham), suggesting a differential, and perhaps specific, role for each enzyme during synaptic recovery. This observation of an increased gelatinase activity is consistent with MMP role in regeneration, however, early MMP9 activation is usually followed by its decline in tandem with later elevation of MMP2 (Goussev et al., 2003; Zuo et al., 1998; Hsu et al., 2006; Costanzo and Perrino, 2008), implicating MMP9 mediation of the acute degenerative phase and a MMP2 role in regeneration. This pattern was true for gelatinase protein expression following OB deafferentation by ORN transection (Costanzo et al., 2006; Costanzo and Perrino, 2008), where rise in MMP2 was not concurrent with MMP9 elevation. Interestingly, Bakos et al. (2010) did report concurrent MMP9 and MMP2 elevation after OE ablation, at 5d rather than 7d
postinjury, but with relative change in gelatinase level similar to what we observed for activity. The similarity between gelatinase change after FPI and OE ablation further supports the possibility that FPI may cause additional damage to ORNs, engaging a combined MMP9/MMP2 response greater than that predicted for ORN axon transection alone. From these data, we interpret our 7d phase of gelatinase activation to mean that FPI to the OB enlists both MMP9 and MMP2 in the pre-reinnervation stages of synaptic recovery. Peak 7d activity of both enzymes also suggests similar roles for the two gelatinases during the OB regenerative phase, with MMP9 providing greater support than in other injury paradigms.

In summary, we observed a predicted, time-dependent increase in OB gelatinase activity following FPI, consistent with a documented role for MMP9 in preparing the ECM for axon degeneration and for MMP2 fostering the onset of synaptic reinnervation. Our results revealed a novel 7d combined MMP9/MMP2 response to FPI, raising the possibility that distal injury to ORNs may be influencing OB gelatinase activation in addition to local axotomy.

**MMP9/OPN INTERACTION MARKS TIME COURSE OF OB PLASTICITY**

In WT animals, time-dependent postinjury activation of MMP9 showed correlation with some, but not all, of the selected molecular markers used to map OB reactive synaptogenesis. Consistent with our hypothesis that a MMP9/OPN signaling mechanism is induced after OB deafferentation, we found a direct temporal correlation between increased MMP9 activity and generation of OPN 48kD RGD fragment at 1 and 7d after FPI. Further, at 7d peak in MMP9 activity, the 32kD OPN fragment, particularly vulnerable to MMP9 digestion (Takafuji et al., 2007), was significantly reduced relative to sham controls. Moreover, during periods of control
level MMP9 activity (3, 21d postinjury), no change in either OPN fragment was detected. We interpret these data as evidence for a predominant interaction of OB MMP9 with OPN during acute degenerative and subacute pre-regenerative phases, and that the RGD containing OPN peptide is produced as a major mediator of critical cell surface signaling. Maximal MMP9 activation at 7d also likely drives significant breakdown of the non-RGD containing OPN fragment, further supporting the important role for MMP9 at this postinjury interval.

We also found a temporal relationship between MMP9 and markers of axon degeneration in WT cases, where acute 1d rise in MMP9 and 48kD OPN could generate cell signals to hasten ORN axon degeneration. This process was positively identified at 3d postinjury by 150kD αII-spectrin increase and OMP loss, as well as with TEM evidence of GL axon disruption. Interestingly, other models of OB deafferentation document elevated MMP9 protein preceding OMP loss (Bakos et al., 2010; Costanzo et al., 2006), which matches the relationship between increased 1d MMP9 activity and reduced OMP observed in our studies. Alternatively, the 3d OMP loss may be independent from 1d MMP9/OPN change, resulting from either the diffuse nature of OB axotomy causing secondary injury, or a delay in response due to ORN damage in the OE, requiring more than 24 hours for cell body injury signals to affect axons in the OB.

Interestingly, the same MMP9/OPN fragment relationship was found at 7d, where ORN axon marker OMP shifted back to control levels. Although OB OMP returns to control levels much faster with FPI than after ORN transection or OE ablation (Bakos et al., 2010; Costanzo et al., 2006), each of the latter models fail to show MMP9 peak protein expression at the time of OMP normalization. Taken together, we conclude that it is less likely a direct signaling interaction between MMP9 derived OPN peptides causes the OMP restoration at 7d, but it cannot be ruled
out that other, as yet unidentified, indirect signaling between MMP9/OPN and OMP could be involved.

Our WT mouse results also pointed to no temporal correlation between elevated MMP9 activity and either CD44 or GAP-43 protein increase. With respect to CD44, we interpret these results as evidence of a complex signaling pathway controlling its OB postinjury expression. For example, MMP9/CD44 interaction could be mediated through acute OPN signaling, generated by MMP9 activation at 1d after FPI. OPN is reported to influence both CD44 transcription and its insertion within cell surface membrane (Desai et al., 2007). Membrane bound CD44 has potential to bind and facilitate MMP9 activation at the cell surface (Desai et al., 2007; Desai et al., 2009), leaving open the possibility that its peak at 3d postinjury contributes to later high MMP9 activity at the 7d time point. There was also no evidence for time-dependent OB MMP9 interaction with GAP-43 after FPI. At the 21d time point of growth factor rise in response to OB deafferentation, no change in MMP9 was found. This suggests that GAP-43 elevation is not time-matched by MMP9-associated signaling and is linked with the regenerative phase of synaptogenesis after FPI. Nevertheless, it remains possible that MMP9/OPN/CD44 stimulus of GAP-43 expression could have occurred at an earlier time point, due to change in the pathway proteins. Although less likely, the 1-7d increase in these proteins might influence long term glial activation to produce maximal GAP-43 expression at 21d. Alternatively, significant elevation of the MMP9/OPN/CD44 pathway may exist between 7 and 21d postinjury, an interval not measured in this study.
Finally, we did assess whether MMP2 activity showed temporal correlation with the molecules of our proposed signaling pathway after FPI. Overall, MMP2 response showed little association with acute changes in αII-spectrin, OMP, OPN or in the subsequent rise in expression of its receptor CD44, nor did the enzyme activity match upregulation of GAP-43. Although we did see a significant increase in MMP2 activity at 7d after FPI, this was the only interaction with the MMP9/OPN/CD44 pathway found in our study. While OB MMP2 change in activity did occur when MMP9 response was maximal, the proportional amount of MMP2 lysis was relatively small. A later, regenerative associated role for MMP2 has been postulated in both knife transection and MeBr OE ablation models (Bakos et al., 2010; Costanzo and Perrino, 2008), which we conclude is also likely after FPI to the OB. Moreover, after cochlear ablation, the presence of GAP-43 positive growth cones was required for MMP2 ECM redistribution during reinnervation of the cochlear nucleus (Friedrich and Illing, 2011). It remains possible that at postinjury intervals beyond 21d, after GAP-43 containing ORN axon invasion, MMP2 protein and activity may be higher than observed here. Nevertheless, the rather modest 7d MMP2 activity in OB could be important to the onset of regeneration, but we conclude that its role is secondary to that of MMP9 during the time periods we examined.

**MMP9 KO ALTERS MARKERS OF OB SYNAPTIC PLASTICITY**

While MMP9 KO mice have been used to explore postinjury tissue damage in both TBI and SCI models (Noble et al., 2009; Wang et al., 2000), prior studies tested how removing MMP9 would alter evolution of acute pathobiology and affect extent of functional recovery. These studies also applied more severe forms of each injury paradigm, not examining how loss of MMP9 might change synaptic reorganization. As discussed above, WT OB response to diffuse FPI does
suggest that MMP9 plays a critical role in timing of synaptic recovery. We performed proof of principal studies utilizing MMP9 KO mice to test whether MMP9 indeed mediates OB synaptogenesis after TBI. Our findings provide strong evidence to support that conclusion. A direct assessment of MMP9 influence on ORN reinnervation was made by mapping how MMP9 KO affected OMP in the deafferented OB. Loss of MMP9 attenuated the 3d OMP reduction of WT mice, and also caused a persistent decrease in OMP expression at both 7 and 21d. The latter change was in direct contrast to the WT injured animals, where 7 and 21d OMP levels were equal to controls, indicative of movement toward OB sensory reinnervation. The attenuation of OMP loss at 3d, coupled with persistent OMP reduction at 7 and 21d, further supports MMP9 involvement in both promoting cellular clearance of axon debris, as well as ORN axon reinnervation of OB targets. These results were supported by 21d TEM analyses that revealed persistent disruption of pre-synaptic terminal and post-synaptic density structure in the MMP9 KO animals, suggesting delayed reactive synaptogenesis.

In order to confirm how a delay in synaptic recovery might alter molecular markers of synaptic organization, we again probed for change in Synapsins I and II in MMP9 KO animals subjected to FPI. We discovered that loss of MMP9 had minimal effect on the amount and timing of expression for each Synapsin, detectable only as small strain differences in select isoforms. For example, at 3d, 80kD Synapsin-I was significantly reduced in the MMP9 KO mice relative to WT injured animals, and 21d 72kD Synapsin-II in the MMP9 KO cases was elevated compared to WT injured mice. Neither of these shifts in Synapsin expression were different from paired sham controls. Overall, these results indicate that loss of MMP9 did not alter the stable postinjury measures of Synapsin expression in the WT mice. Nevertheless, a potential
regulatory role for MMP9 might be inferred from the two time-dependent strain differences we found. Additional studies with more localized GL analysis are needed to determine if these minor effects on Synapsin-Isoform expression are relevant to OB synaptic recovery.

As a third index of how MMP9 loss affects synaptic reinnervation, we mapped postinjury GAP-43 expression within the injured MMP9 KO. Generally, GAP-43 and MMP9 expression in neurons exhibit parallel response to CNS trauma (Juan et al., 2014; Keilhoff et al., 2016). In regenerating nerves, MMP9 and GAP-43 colocalize (Shubayev and Myers, 2004), although a direct link between the two proteins during reinnervation has not been established (Fredrich and Illing, 2011; Shubayev and Myers, 2004). Thus, MMP9 and GAP-43 could play key, but separate, roles in CNS regeneration. Surprisingly, following FPI of the OB, WT MMP9 activity and GAP-43 expression did not map together over postinjury time. MMP9 activity was elevated at 1 and 7d postinjury, when GAP-43 was not changed, and at 21d, when GAP-43 showed a 3-fold elevation, MMP9 activity was equal to controls. Despite these differences, MMP9 KO GAP-43 levels were reduced to nearly 50% of control at 3d postinjury, when degenerative processes appear to peak and was different from WT GAP-43 expression. At 21d, peak GAP-43 in MMP9 KO mice did not show strain difference relative to WT cases, but there was a trend toward higher growth factor expression in the absence of MMP9. It should be noted that we did, however, find that reinnervation of GL synapse cytoarchitecture was hindered without the enzyme, showing that MMP9 function plays a role in the full restoration and stabilization of OB synapses following injury.
These results suggest that MMP9 activity is not required for the induction of major OB GAP-43 expression during later stages of ORN axon growth. Several alternative pathways could independently drive OB GAP-43 upregulation, including other MMPs such as the regeneration-associated MMP2, multiple injury-induced cytokines released to stimulate ORN growth factor production, or GAP-43 production by reactive glia within deafferented GL. Thus, local OB synaptic sprouting could continue to occur in the absence of MMP9, pointing to an indirect relationship between MMP9 and GAP-43 during GL reinnervation.

As discussed above, several investigators have proposed a relationship between OMP and GAP-43, where the growth factor is expressed in the absence of OMP (immature neurons), while upregulation of OMP (when ORNs mature) suppresses GAP-43 (Griff et al., 2000; Holtmaat et al., 1995; Margolis et al., 1991; Verhaagen et al., 1989; Verhaagen et al., 1990). Evaluation of OMP and GAP-43 in MMP9 KO mice also revealed potential interaction between the two proteins within the OB after FPI. This type of inverse expression was not observed in our injured WT animals, however, MMP9 KO subjected to FPI had persistent OMP reduction, revealing patterns of OMP/GAP-43 expression consistent with their inter-dependent regulation. For example, at 3d postinjury, WT OMP was significantly reduced, with GAP-43 just trending toward elevation over controls. Loss of MMP9 reversed that expression pattern, with a normalization of OMP and a large reduction of GAP-43 signal, for which strain differences could be detected. Similarly, sharp OMP reduction at 7 and 21d in the MMP9 KO was paired with trends toward increased GAP-43 levels. While these patterns suggest that OMP could serve as an inverse regulator of OB GAP-43, the WT 21d GAP-43 upregulation, with normal OMP level, was not consistent with that interpretation. Clearly, MMP9 does influence the extent of
detectable OMP/GAP-43 interaction, however, the relationship of the two proteins at 21d postinjury argues that another pathway predominates for GAP-43 regulation in postinjury OB.

Finally, we did assay for postinjury change in MMP2 activity in response to loss of MMP9. As predicted by a prior study using MMP9 KO in the knife transection model of OB deafferentation (Costanzo and Perrino, 2008), we found no compensatory increase in MMP2 lysis and no specific strain differences in MMP2 enzyme activity across time postinjury. However, loss of MMP9 did reduce the 7d elevation of MMP2 activity to a level equivalent with sham controls, suggesting that activation of MMP2 may be linked to MMP9 or its downstream effects within the deafferented OB. For example, we found OMP persistently reduced at 7 and 21d in the MMP9 KO mice, indicating a more protracted degenerative phase. If MMP2 serves to drive the synapse regeneration, a blunted enzyme response would be predicted as the degenerative phase lagged.

Overall, our studies in the deafferented OB support the hypothesis that MMP9 activity is correlated with organized synaptic loss and axon degeneration following FPI. Data showing the effect of MMP9 KO on ORN plasticity further demonstrates that MMP9 plays a critical role in the temporal progression of OB postinjury synaptic repair.

**MMP9/OPN INTERACTION FACILITATES OB SYNAPTIC RECOVERY**

The studies presented here identify a complex enzyme(substrate) interaction between MMP9 and OPN that facilitates reactive synaptogenesis. Changes in level of MMP9 activity and OPN expression over time postinjury are confirmed with multiple outcome measures, supporting our
hypothesis that MMP9/OPN interaction mediates progression of early degradative processes and the initiation of terminal sprouting for synapse reformation. Activated microglia and astrocytes are a primary source of injury or disease-induced OPN expression (Albertsson et al., 2014; Ellison et al., 1999; Neuman, et al., 2014; Silva et al., 2015; Tambuyzer et al., 2012). Upon brain injury, these cells can upregulate OPN, which is then processed for binding to integrin and/or CD44 receptors to promote reactive glial function. After FPI, such functions include increasing pro-inflammatory cytokines and chemokines for a robust immune response, and promoting cellular migration, proliferation, and cell adhesion, which can mediate synaptic repair. Here, we also detected an increase of OPN in deafferented OB neurons 7d after injury, and posit that this increase signals ECM modification promoting glial reactivity as well as navigation of nascent synaptic sprouts toward their appropriate post-synaptic targets.

We have provided evidence that the interaction between MMP9 and OPN occurs during OB deafferentation-induced synaptic plasticity. OPN signaling can precede and drive MMP9 expression (Caers et al., 2006; Lai et al., 2006; Liu et al., 2014; Wu et al., 2015; Yang et al., 2011) to promote matrix remodeling. While OPN elevation would normally be followed by MMP9 activity, parallel expression of OPN and MMP9 has also been reported in several pathologies including MS and prostate cancer (Braitch et al., 2008; Castellano et al., 2008), as well as in beneficial conditions such as periodontal tissue regeneration and cell adhesion (Lima et al., 2008; Wang et al., 2014). While we failed to detect changes in full length 66kD OPN, paired, time-dependent elevation of 48kD OPN and MMP9 activity was observed in WT deafferented OB, consistent with correlated expression of the two proteins reported in the literature. At 1d postinjury, MMP9 activity was significantly elevated along with peak 48kD OPN RGD fragment production, suggesting the two proteins interact during acute postinjury
phases to promote cell surface signaling, critical to effective glial clearance of degenerating presynaptic axons. The early 3d degenerative period is marked by peak activation of OB astrocytes and microglia, increased 150kD αII-spectrin expression, OMP reduction and onset of GL synaptic breakdown. Taken together with the rapid MMP9/OPN response, these cellular changes suggest that OB reactive neuroglia are significant contributors to ECM reorganization required for synaptogenesis, principally through OPN-mediated migration and phagocytosis of ORN debris. In fact, the biphasic expression of OPN signaling fragments has been posited to mediate time-dependent shifts in glial reactivity with other CNS insults. For example, OPN transcript is also upregulated in a model of forebrain ischemia in a cell and time-dependent, biphasic pattern (Choi et al., 2007). There, at 3d postinjury, OPN mRNA is detected in CD44+ microglia, while protein interaction with integrin receptors is localized to astrocytes around 10d, suggesting time-dependent production of OPN to mediate distinct cellular responses. Early microglial synthesis of OPN might also occur in the injured OB, prior to the 1d postinjury interval, and could contribute to the OPN RGD fragment pattern we detect across time intervals.

Interestingly, MMP9 activity and 48kD OPN level were not different from controls at the 3d postinjury interval, when glia are most responsive. We interpret this shift in MMP9/OPN response to mean that acute 1d rise in MMP9/OPN first serves to prime local glia in preparation for active degenerative response at 3d, after which the two proteins return to normal activity/expression. However, MMP9/OPN also show a second paired increase at 7d, suggesting the two are subsequently upregulated to promote onset of repair processes at 21d. Thus, MMP9/OPN show a biphasic pattern of response to OB deafferentation, indicating a dual role in the synaptic repair process, regulating aspects of both degeneration and regeneration phases.
Indeed, it is possible that the 21d GAP-43 increase associated with ORN axon growth and reinnervation of the OB may be promoted by the cell and ECM changes signaled through MMP9/OPN elevation two weeks earlier.

In order to confirm the importance of MMP9-mediated processing of OPN in OB plasticity, we compared the effects of MMP9 loss in FPI induced synaptogenesis using MMP9 KO animals. Concurrent robust elevation of MMP9 activity and 48kD OPN protein expression in WT animals at 1 and 7d postinjury was lost in MMP9 KO animals subjected to FPI, and 3d expression of 48kD OPN was significantly reduced below control levels. Although thrombin and cathepsin-D can cleave OPN and produce functional 48 and 32kD OPN proteolytic fragments (Christensen et al., 2010; Denhardt et al., 2001), our results with MMP9 KO mice support the hypothesis that MMP9 enzyme activity is responsible for the acute and subacute postinjury generation of OPN cell signaling fragments in the deafferented OB. It also points to critical MMP9 role in driving OPN fragment signaling pathways to regulate cytokine production, phagocytosis, and cell migration through CD44 and integrin receptors.

A second major effect of MMP9 KO was the reversal of OMP protein expression 3-21d after OB deafferentation. MMP9 KO attenuated the 3d postinjury peak of OMP loss, while at the same time induced OMP reduction at 7 and 21d. Since OMP loss/return shift marks ORN axon degeneration/regeneration cycling, our OMP data at 3 and 7d point to MMP9/OPN signaling as a regulator of ORN plasticity after FPI. Surprisingly, OMP is significantly reduced by MMP9 KO at the 21d mark relative to WT animals, but OPN is unaltered. This result suggests that MMP9 may be independently involved in fostering a shift to ORN reinnervation at that time point. Such
a response could occur by MMP9 influence on the ECM for regrowth or through some role in the maturation of the pre-synaptic OMP+ terminals as they reenter the GL. It also appears likely that the importance of OPN fragment processing as signal for ORN OMP change is restricted to 1-7d postinjury. This interpretation fits with the literature, since OPN has been identified as an acute mediator of inflammation, eliciting its effect during early recovery periods. Overall, these MMP9 KO data show that loss of MMP9 disrupts progress toward synapse reformation. They further highlight a strong role for MMP9 in producing OPN fragments for postinjury degeneration, in addition to processing, as yet unidentified, substrates important for ORN axon maturation and stabilization of pre-synaptic terminals.

Since we posit that MMP9/OPN interaction promotes effective cell signaling for ECM clearance during synaptogenesis, a second approach for proof of principle would be to test whether the manipulation of OPN also changes OB response. In addition to assessing MMP9 KO-induced changes in OPN fragmentation, we also conducted pilot experiments with OPN KO mice to look for differences in OB MMP9 activity and OMP recovery after FPI (Appendix A). While loss of MMP9 significantly reduced OPN fragment generation, we found that MMP9 activity actually increased in OPN KO mice at 1 and 7d postinjury when compared with injured WT C57Bl/6 animals (OPN KO background strain). This result differs from change in MMP9 activity reported for OPN KO mice after hippocampal denervation. Chan et al. (2014) showed that when OPN is absent, MMP9 activity is significantly reduced to roughly half the level displayed at 2d post-lesion. Given that we observed a tight temporal interaction between MMP9 and its OPN substrate in the deafferented OB, it might be predicted that loss of this substrate would downregulate enzyme production and gelatinase activity. For example, OPN protein may itself
invoke cell transduction signaling to drive transcription factors that upregulate MMP9 after injury. Since OPN binds receptors that initiate that transduction, loss of OPN would then attenuate injury-induced MMP9 production and reduce MMP9 activity, as in deafferented hippocampus. In the injured OB we observed the opposite result, a significant increase in MMP9 activity (Appendix A.1). One explanation would be that loss of OPN may cause an overexpression of other pro-inflammatory cytokines, resulting in a hyper-elevated MMP9 production and activity. Alternatively, the MMP9 rise could simply be due to the absence of postinjury elevation in WT C57Bl/6 MMP9 activity (discussed further below). More OB studies in the injured OPNKO mouse model will be required to test such possibilities, including a detailed look at potential variation of FPI response due to mouse strain differences.

Based on our WT mouse data comparison between C57Bl/6 and FVB/NJ strains, we conclude that differences exist in OB MMP9 response to FPI as a function of mouse strain. Unlike the WT FVB/NJ mice, where we found clear time-dependent injury elevation of MMP9 activity, no change in enzyme activity was seen in OB of C57Bl/6 injured mice (see again, Appendix A). Similarly, we found WT strain differences in postinjury OMP expression when C57Bl/6 and FVB/NJ mice were compared. For example, the 3d postinjury OMP reduction in WT FVB/NJ cases was absent for the WT C57Bl/6 strain, and OMP expression was largely unaffected by injury in OPN KO. Nevertheless, 7d protein trended toward reduction in the OPN KO, consistent with 7d OMP reduction in our MMP9 KO data and potentially supporting MMP9/OPN interaction in ORN recovery. Interestingly, strain differences in the extent of pathology and inflammatory response following TBI have been reported (Witgen et al., 2006), and our studies support these findings. Overall, our experiments with KO mice point to complex interactions that are not easily defined by complete gene inactivation. A more targeted approach
involving inducible knockout to control expression over time or conditional knockout mice with either OPN or MMP9 gene ablation in specific cell types (microglia, astrocytes, or neurons) could be used to help clarify whether specific cell sources of MMP9 or OPN protein are responsible for the differences we see between WT strains and individual KO mice.

As OPN exhibits cytokine properties through binding of its fragments to cell membrane receptors, we explored changes in principal OPN receptor expression. Both integrin and CD44 receptors are capable of recognizing amino acid sequences on OPN fragments, with 48kD OPN expressing RGD and SVVYGLR sites primarily for binding integrin receptors (Scatena et al., 2007; Smith and Giachelli, 1998), and carboxy terminal 32kD OPN binding CD44 in an RGD-independent manner (Weber et al., 2002). This interaction is not strict, however, with evidence that CD44 also binds the 48kD amino terminal OPN fragment, inducing cell survival through activation of the PI3K and Akt pathway (Lin and Yang-Yen, 2001). Such alternative binding is consistent with our results, in that 48kD OPN expression at 1d postinjury may prompt the 3d CD44 upregulation, increasing potential interaction between even normal levels of OPN and CD44. We expect that if we had been able to probe for time-dependent correlations between postinjury expression of 48kD OPN and the \( \alpha_v\beta_3 \) integrin receptor, we would have observed a more robust interaction in the deafferented OB. Nevertheless, OPN co-localizes with CD44 in some studies (Zohar et al., 2000), and we did find temporal correlations between CD44 receptor and its 32kD OPN binding partner, as post hoc analysis showed 3d postinjury expression of both proteins significantly higher than at 7d. While OPN/CD44 interaction may be important at 3d postinjury, such interaction could also occur with control 32kD OPN levels, indicating that
CD44 ligands other than 48kD OPN could more readily bind to the hyaluronan receptor to promote OB reactive glial cell functions.

In line with our hypothesis, MMP9 KO caused significant changes in ORN recovery, as well as OPN fragmentation, which could be mediated through activated OB glia. In MMP9 KO animals, injury-induced OMP reduction was persistent throughout the early regenerative phases of OB recovery, while 48 and 32kD OPN were attenuated 1-7d. By contrast, CD44 expression was not different between WT and MMP9 KO cases at any time point. Notably, GFAP positive astrocytes showed no obvious change in IHC assessed reactivity when MMP9 was genetically ablated. Hsu et al. (2008) demonstrated that loss of MMP9 negatively impacted migration of astrocytes, but not proliferation, which could explain why no apparent change in extent of OB glial activation was observed in the GL of injured MMP9 KO animals. Although our IHC showed that reactive OB astrocytes express MMP9 at 7d, which fits with other studies documenting the same type of activation (Candelario-Jalil et al., 2009; Zhao et al., 2006), loss of MMP9 did not drastically change astrocyte morphology, suggesting OB glial activation may be less sensitive to loss of MMP9. This indicates that the MMP9/OPN interaction, while it likely influences synaptic plasticity through an OPN receptor signaling mechanism, may not directly alter astrocyte hypertrophic response during the first week of OB reactive synaptogenesis. One explanation for this result would be that MMP9 and OPN more readily interact with OB microglia (through CD44 receptor) during subacute (3, 7d) stages of synaptic repair. Other CNS insult studies do support a biphasic, differential postinjury interaction between OPN and CD44/integrin receptors (Choi et al., 2007; Kang et al., 2008; Shin et al., 2005). They provide evidence for acute (3-4d) CD44 upregulation and OPN binding within reactive microglia, while
OPN interaction with integrin receptors is delayed, occurring in reactive astrocytes at later postinjury intervals (14-28d). Although we observed hypertrophic GL astrocytes at 21d and ultrastructural evidence of continued astrocyte phagocytosis, the delayed time course of reactive synaptogenesis within the injured OB suggests that we may possibly detect robust astrocyte reactivity beyond our 21d time point. Such reactivity could correlate with elevated integrin receptor expression. We suggest this possibility given that astrocytes significantly contribute to growth factor production for axon guidance during synaptic reinnervation. In this case, extending our time course and probing for integrin receptors could prove useful to determine exact influence of processed OPN in OB astrocyte response to injury.

In the present study, we have focused on a molecular mechanism whereby MMP9 cleaves OPN into signaling fragments that bind CD44/integrin cell surface receptors to induce signal transduction and glial cell reactivity. There is evidence, however, that OPN can induce formation of a CD44/MMP9 membranous complex to promote MMP9 activity at the cell surface (Desai et al., 2007). In the injured OB, OPN increases at 1d postinjury and may foster CD44/MMP9 interaction at 3d, where CD44 receptor protein peaks in the context of normal MMP9 activity levels. If promotion of peak MMP9 activity is required for 7d OPN signaling, then higher 3d cell surface complexing between elevated CD44 receptors and MMP9 could enhance MMP9 activity, leading to the 7d peak we observed after FPI. This would support the increased 7d OPN processing we see as both 48kD elevation and 32kD reduction. In addition, Desai and colleagues (2007) suggest that OPN itself can direct an increase in CD44 production. This interpretation is again plausible in our OB model since highest OPN 48kD elevation occurs at 1d after FPI, just before CD44 protein peaks. Thus, enhanced OPN and CD44 receptor cell
signaling permits increased cell reactivity to promote a more effective degenerative phase and foster onset of orderly synaptic regeneration. Interestingly, MMP9 activity, OPN fragment generation and CD44 expression all return to control levels by 21d postinjury, suggesting that our posited role for these proteins in OB synaptogenesis is focused within the initial degenerative and early ORN regenerative phases.

One interesting feature of OPN injury response in the OB is the apparent effect of FPI on neuronal OPN expression. While reactive glia express OPN, several models of CNS insult document OPN localization within neurons (Borges et al., 2008; Shin et al., 2005). In our FPI model, cellular OPN was restricted to post-synaptic mitral and tufted neurons, where it is increased 7d after insult, a finding consistent with our whole OB protein analyses. Interestingly, only a subset of post-synaptic excitatory cells express OPN in our model, while interneurons like the TH+ periglomerular cells surrounding GL synapses do not contain OPN. Few investigators specify whether such postinjury OPN+ neurons are inhibitory or excitatory, but morphologically these cells are pyramidal in shape, suggesting they are excitatory neurons. It is likely that these are the neurons directly affected by loss of ORN input, in which OPN can drive autocrine or paracrine signaling to implement local cell response to injury. This pattern is consistent with strong OPN expression in dentate granule cells deafferented by entorhinal cortical lesion (Chan et al., 2014). Developmentally, OPN is retained in neuronal soma, not only appearing during differentiation and increasing with maturation in brainstem and cerebellum (Lee et al., 2001), but also reappearing in injured neurons of adult brains (Borges et al., 2008; Shin et al., 2012). In neurons, there is evidence for an intracellular form of the protein, OPN\textsubscript{i}, which is not secreted but binds with microtubule-associated proteins 1A (MAP1A) and 1B (MAP1B). These MAP
proteins act to stabilize microtubules of the cytoskeleton, which could introduce a role for OPN in synapse formation, neurite outgrowth, and axonal transport (Chan et al., 2014; Long et al., 2012). While we did not directly assess OPN/MAP1A/MAP1B binding, we did use IHC to probe for OPN association with another microtubule-associated protein (MAP2), which has a similar role in cytoskeletal stabilization as MAP1 (Huber and Matus, 1983), and is often used to mark post-synaptic dendritic integrity. Expression of OPN was essentially limited to the cell bodies of mitral and tufted cells, but also visible within a few primary dendrites. Importantly, IHC failed to show co-localization of OPN and MAP2 in either neuronal cell bodies or within the limited OPN positive dendrites. Given the strong cell soma localization of OPN and the lack of OPN signal overlap with MAP2, we posit that the postinjury response in OB neurons likely represents increase in production and secretion of the cell signaling form of OPN to promote OB synaptic recovery.

One potential source of OPN upregulation could be the mechanical stress induced by FPI. This stress may directly injure the post-synaptic neurons to promote their OPN expression. Further, it is possible that distal deafferentation, rather than direct injury to the cell soma underlies the elevation of mitral and tufted cell OPN, similar to OPN upregulation within neurons peripheral to sites of experimental cryolesions (Shin et al., 2005). Alternatively, it may also be that OPN is rapidly produced by glia during an acute immune response, and secreted from these reactive cells too early for detection in our 3-21d IHC studies. Interestingly, we performed preliminary in vitro studies of OB glia derived from P2 pups and did find OPN present in a subset of microglia, but not in astrocytes (Appendix B). This OPN signal was reduced in microglia derived from MMP9 KO mice, further supporting MMP9/OPN interaction in the OB. Since these experiments
suggest potential OPN production in OB glia, and we did not examine acute microglial IHC (<3d after FPI), it remains possible that microglia activated 1d or earlier after injury could also express OPN in the OB. Early (1d) in situ hybridization experiments, which would identify cells responsible for synthesizing OPN through mRNA localization, could help clarify these possibilities.

Another way FPI could lead to high neuronal OPN expression might be explained by the fact that the injury generates an environment in which neurons are subject to excitotoxicity, excessive calcium influx, and potential degeneration (see Chapter 1). Park et al. (2012) showed that OPN interacts with calcium to bind the cell surface of degenerating neurons after transient focal brain ischemia, leading to OPN-mediated phagocytosis, which could explain the presence of OPN in a subset of mitral and tufted cells after FPI. Similarly, OPN appeared in degenerating neurons after pilocarpine induction of status epilepticus, however, it failed to play a role in regulating cell death (Borges, et al., 2008). Other studies show that OPN can be localized to mitochondria of degenerating neurons, as well as within macrophages as phagocytosed particles (Kim et al., 2015), suggesting OPN may mediate phagocytosis in damaged neurons. Although interesting, the association of OPN with OB neuronal cell death seems less likely in our study since diffuse, mild FPI appears to have little effect on OB neuronal ultrastructure. Rather, our results point to a more prominent OPN role in post-synaptic cellular signaling of deafferented neurons, perhaps for axon guidance during later postinjury phases, as reported in stab wound injury models (Plantman et al., 2012). Finally, exactly how this neuronal OPN might be processed and directed toward sites of regeneration is not yet clear. Based on the fact that the OPN antibody used in our IHC recognized both full length and major OPN fragments, several possibilities might be
considered: 1) OPN is both produced and cleaved within the neuron, as MMP9 has been detected in dendrites (Konopacki et al., 2007; Gawlack et al., 2009; Wilczynski et al., 2008), then secreted as signaling peptides, 2) full length OPN is produced in deafferented neurons, then secreted into the ECM for signal peptide processing, or 3) OPN is transiently produced by acute glial inflammatory response, secreted for cleavage by matrix MMPs, then taken up by injury affected neurons. Future studies will be necessary to determine whether MMP9/OPN interaction occurs in one or more of these potential locations in order to generate critical cell signaling proteins for OB synaptogenesis.

MMP9/OPN DIRECT OB SYNAPTOGENESIS: A WORKING HYPOTHESIS

Our studies have highlighted a role for MMP9/OPN processing in postinjury reinnervation of the OB. Acute changes in synaptic integrity and protein expression have revealed a novel role for ECM modulators in FPI-induced OB reactive synaptogenesis. Here, we outline our working hypothesis for MMP9 and OPN-mediated events leading to ORN repair following TBI (illustrated in Figure 5.1).

We have defined a time course of critical cellular and molecular responses that contribute to OB plasticity. Within the first 24 hours of injury, initiation of inflammatory processes and membrane perturbation lead to axon damage. Given the diffuse nature of FPI, evidence of OB axonal injury is not immediately apparent, however, the acute immune response activated by head trauma upregulates MMP9 activity 2-fold 1d after injury. Moreover, MMP9 processes OPN, contributing to an elevation of 48kD OPN. The RGD-containing 48kD, and to an extent,
the carboxy-terminal 32kD OPN, are then available to bind integrin and CD44 receptors localized to microglia and astrocytes, promoting glial activation and synaptic repair. Loss of the enzyme caused a significant reduction in 48kD OPN, further confirming its role in OPN fragmentation at the 1d postinjury interval.

During the 3d postinjury period, we have documented clear evidence of axon degeneration. Our TEM analyses showed that synaptic vesicle reserve pools were disorganized and arrangement of GL synapses was altered after injury. Additionally, calcium-dependent calpain caused an elevation of spectrin breakdown products, while protein expression of ORN marker OMP dropped significantly. These results suggest important molecular processes facilitating the degradation of injured ORN terminals are underway. Although CD44 expression was significantly upregulated 3d after TBI, both MMP9 activity and expression of 48kD OPN returned to control levels, which could be a real temporal shift or, in part, reduced antibody access due to high CD44 or integrin-bound OPN. Interestingly, OPN was localized to post-synaptic mitral and tufted cells, likely inducing both paracrine and autocrine signaling to pre-synaptic neurons and neuroglia. At the 3d interval, microglia were less ramified, increasing in number with larger cell bodies and shorter processes, while astrocyte processes were hypertrophied and thickened as fibrillary protein increased, both indicative of significant cell reactivity. Their activated states promote further cell migration, proliferation, and clearance of tissue debris from the GL and EPL matrix through phagocytosis. Surprisingly, MMP9 KO did not attenuate CD44 elevation or glial reactivity. We posit that, in absence of OPN fragment elevation, cytokines and other ligands compensate, maintaining both CD44 and glial response. OMP loss was, by contrast, attenuated by MMP9 KO, suggesting degradative processes
mediated by 48kD OPN were inhibited or delayed without MMP9. Overall, there is strong evidence of an acute 3d period of axonal degradation mediated by MMP9/OPN interaction, directing downstream cellular responses to facilitate FPI-induced synaptogenesis.

One week after FPI appears to mark a transition phase between degenerative and regenerative phases. OMP expression approaches control level, pointing to the onset of ORN axon recovery in the GL. CD44 receptor protein is reduced, suggesting a less important role for the cell surface receptor at the 7d time point. Increases in 48kD OPN and a 50% reduction in 32kD OPN fragment expression can be attributed to significantly elevated MMP9 activity at this same time. High levels of OPN protein in mitral and tufted neurons points to a direct effect of FPI deafferentation on these cells, which is confirmed with their increased IHC signal for OPN at 7d. This could potentially indicate both direct response to deafferentation and increased expression due to cell signaling through CD44. Notably, MMP9 protein was detected in astrocytes at 7d postinjury. The cellular distribution of MMP9 and OPN suggests they could be produced within cells and secreted for interaction in the OB matrix. However, MMP9 also heavily labeled the GL and EPL neuropil, pointing to the possibility that the enzyme may be present in post-synaptic dendrites, where MMP9/OPN interaction might also occur. TEM analyses revealed that removal of degenerating tissue was underway, indicating that OB glia remained active and play a pivotal role in modifying the ECM for new synapse formation.

We also predict that time course of ORN reinnervation is significantly affected by MMP9, as OMP reduction failed to normalize 7d after injury in MMP9 KOs subject to FPI, specifically correlated with reduced 48kD OPN production. By contrast, CD44 and glial morphology were
no different between WT and MMP9 KO animals, suggesting other cytokines, enzymes, and ECM proteins compensate for absence of increase in OPN fragments. Finally, since MMP2 is normalized in 7d postinjury MMP9 KO mice, its potential role in synaptic regeneration is likely attenuated with loss of MMP9, pointing to clear interaction between the two gelatinases in OB response to FPI.

The 21d postinjury period is marked by onset of OB reinnervation, where the predominant effect of FPI is the significantly upregulated GAP-43, supporting reentry of immature ORN terminals. In tandem, OMP continues to rise, slightly exceeding control signal. IHC staining at 21d suggests that microglia and astrocytes no longer exhibit reactive profiles. TEM analysis at 21d postinjury shows a reduction in glial phagocytosis, indicating that critical glial-mediated degeneration is nearing completion. At the same time, synaptic structure is reorganizing, paired with control level of MMP9 activity and OPN fragment expression. Since loss of MMP9 persistently inhibits OMP recovery and has no effect on OPN fragment generation, we posit that an additional MMP9-dependent mechanism, independent of the MMP9/OPN interaction, can affect the timing of ORN reinnervation. Beyond 21d, when OB reinnervation persists, we predict that GAP-43 expression would return to normal levels as ORNs continue to mature and synapses are stabilized.

**FUTURE DIRECTIONS**

The studies of this dissertation provide support for the hypothesis that MMP9/OPN interaction mediates the progress of OB reactive synaptogenesis following FPI. We addressed gelatinase processing of OPN to promote OB plasticity over the first 3 weeks postinjury using molecular
Figure 5.1 Working hypothesis for ECM-mediated synaptic change in the OB after TBI
Illustrated are four panels representing the time-dependent postinjury changes in the OB microenvironment during deafferentation and reinnervation of an OB glomerlus. Alpha II-spectrin labeling shows the breakdown and reemergence of pre-synaptic OMP+ axons. Late appearance of GAP-43+ growth cones marks entry of nascent synaptic terminals. Concurrently, changes in astrocyte and microglial activation are shown, as well as representative levels of MMP2 and MMP9 activity, OPN protein levels, and CD44 receptor expression at each respective time point. Specific ECM interactions and influence on glial reactivity and synaptic morphology are detailed within the text.
and structural endpoints, and have considered additional experiments that would complement our results and enhance understanding of this complex plasticity process.

Here we focused on acute molecular interactions (1-21d after injury). As OB reinnervation typically requires up to 60d, extending the studies to cover the entire recovery interval would provide a better understanding of MMP9/OPN role in the GL regeneration process. In addition, our whole OB protein extracts do not permit assay within the GL alone, where synaptic reinnervation occurs. Enriching our extracts for the synaptic-rich GL would allow us to confirm whether our whole OB gelatin zymography and Western blot analyses are reproduced in that target layer. Such enriched samples would clarify how much the GL contributes to the large OB GAP-43 change at 21d postinjury. Given that we failed to find postinjury changes in OB Synapsins, a more precise GL dissection might clarify presence of injury effect. We could also add analysis of other synaptic marker proteins in OB and GL extracts. One good choice would be synaptophysin, which is localized to a majority of pre-synaptic terminals (Wiedenmann and Franke, 1985) and co-expressed with OMP in the OB (Kasowski et al., 1999). Finally, since the 48kD RGD fragment of OPN primarily binds the $\alpha_\nu\beta_3$ receptor, probing for either $\alpha_\nu$ or $\beta_3$ in the injured OB and GL would help clarify the signaling pathway through which OPN might regulate OB synaptogenesis. It is possible that such OPN/integrin receptor interaction will promote glial growth factor production for guidance of ORN axons to their synaptic sites. Each of these more refined assays could be used in studies comparing WT with MMP9 KO and OPN KO mice.

The use of genetically modified animals is a powerful tool, which we have already applied to understand MMP9/OPN interaction in the deafferented OB. Loss of MMP9 alters both OPN
fragmentation and postinjury recovery of OMP expression. We also piloted studies in OPN KO mice, revealing interesting postinjury differences from MMP9 KOs. Adding samples for later survival intervals and filling in critical acute postinjury time points in each KO are needed to complete the proof of principle analysis for MMP9/OPN interaction. Comparison of additional synaptic markers, glial reactivity, and synaptic cytoarchitecture in both MMP9 KO and OPN KO animals will help to determine the relative contribution of each protein to ORN reinnervation. Ideally, we could also generate double MMP9/OPN knockouts or double conditional knockout mice, to more accurately assess the postinjury consequences of their interaction.

Additional mapping of tissue protein and mRNA would provide new and important molecular data. Expanding our IHC time course to include the 1d time point would reveal more detail regarding acute glial changes. Both IHC at 1d and in situ hybridization at each postinjury time point would allow identification of OPN synthesizing cells and the level of postinjury transcript induction. To characterize the OPN+ cells in our current studies, we could employ the use of mitral and tufted cell markers such as T-box brain protein 1 (Tbr1) and Tbr2 (Bulfone et al., 1998; Imamura and Greer, 2013; Mizuguchi et al., 2012) and cholecystokinin, or CCK (Ma et al., 2013; Nagayama et al., 2014). IHC probe for OB CD44 and αvβ3 in the OB would identify cells responsible for their injury-induced elevation, as the receptors are found on both neuroglia and neurons. We can also probe for GAP-43 in OB tissue sections, which would label and identify the cells responsible for the 21d upregulation.

It remains unclear whether OB deafferentation induced by FPI produces reversible behavioral deficits or how MMP9 KO and OPN KO affect olfactory function. A few models of OB injury
have addressed these questions. In zebrafish, amino acid administration provided clues about olfactory function after OB deafferentation (Paskin and Byrd-Jacobs, 2012), while behavioral analyses were also utilized to determine the effect of growth factors on rat OB reinnervation (Herzog and Otto, 2002). Interestingly, olfactory discrimination tests were administered and deficits revealed in a focal, cortical injury model (CCI), but Radomski and colleagues (2013) focused their study on the effects of injury on OB neurogenesis and granule cell formation rather than ORN recovery. These observations suggest we could apply olfactory functional testing to assay mild-severe forms of FPI. Electrophysiological assessment of OB synaptic function is also used to measure extent of OB injury/recovery. That approach is feasible in our FPI model, however, interpretation of results could be difficult given new synapses may show normal postsynaptic potentials even with aberrant perception of smell. While testing olfaction in mild-moderate FPI will likely require a more sensitive assay, experiments addressing specific effects on olfactory function would make our study much more comprehensive.

Our study provides novel information about a molecular pathway that could identify therapeutic targets to improve recovery of OB synaptic circuits after head injury. Nasal steroid sprays are commonly prescribed for treatment of clinical anosmia (Costanzo et al., 2012), reducing inflammation associated with glial scarring that inhibits OB reinnervation after injury (Costanzo, 2005; Kobayashi and Costanzo, 2009). In the context of our link between OPN and successful OB reinnervation, intranasal OPN has been applied to improve cortical function and recovery after CNS trauma (Albertsson et al, 2014; Doyle et al., 2008; Jin et al., 2016; Joachim et al., 2014; Topkoru et al., 2013). This would support future experiments designed to test whether
intranasal OPN treatment might induce greater OB reinnervation after TBI and if that effect is altered in MMP9 KO or OPN KO mice.

**FINAL REMARKS**

In conclusion, the studies in this dissertation reveal an important molecular mechanism involved in OB reactive synaptogenesis. Using the FPI model, our data support the hypothesis that the OB is deafferented after injury, and ORN reinnervation of the GL can be mediated through proteolytic processing of cytokine OPN by MMP9. We successfully documented a postinjury time course of altered synaptic morphology and OMP expression, local glial activation, and increased production of critical matrix and synaptic components supporting reinnervation. In conclusion, the findings presented not only offer insight regarding OB recovery after head trauma, but also reveal a potential neuroplasticity mechanism that may be targeted to facilitate repair of other brain regions where CNS insult disrupts synaptic circuits.
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Appendix A.1 MMP9 activity increases in OPN KO mice following FPI
C57/B16 WT mice and OPNKO mice (C57/B16 background strain) were subjected to the identical FPI protocol as for FVB/NJ WT and MMP9KO mice and OB samples used for gelatin zymography assay. Postinjury intervals of 1, 7 and 21d were sampled and MMP9 activity measured as in Chapters 3 and 4. In contrast to WT FVB mice, we found no significant increases in MMP9 activity at 1 or 7d postinjury relative to sham controls, but did find a solid trend toward increased enzyme activity at 21d, which just missed significance (p=0.057). In mice lacking OPN, the pattern of MMP9 activity was reversed. We found significant elevation (2-3-fold over sham controls) at 1 and 7d after FPI. Post hoc tests confirmed that this MMP9 injury response differed between strains. These results make two important points: mild FPI affects OB in the WT C57/B16 and FVB/NJ strains quite differently, and loss of OPN appears to release MMP9 from some type of activity inhibition after C57/B16 brain injury. *p<0.05 Sham vs. TBI; **p<0.01 Sham vs. TBI; §p<0.05 WT vs KO. N=3-4/group.
MMP9 Activity

% of Control

1d  7d  21d

WT  OPNKO

$\$  **  $  *  $  p=0.057

360
Appendix A.2 OMP expression is not altered in OPN KO mice following FPI
C57/Bl6 WT mice and OPNKO mice (C57/Bl6 background strain) were subjected to the identical FPI protocol as for FVB/NJ WT and MMP9KO mice and OB samples used for WB probe for OMP expression as a metric of ORN axon damage. All four survival intervals sampled in FVB/NJ experiments were used (1, 3, 7 and 21d postinjury). Surprisingly, we found no effect of mild FPI on OMP expression in either WT or OPNKO cases for any time point examined. All levels of OMP signal were not different from sham control cases. This result is consistent with the absence of MMP9 activity in C57/Bl6 WT after FPI, which we posit is due to attenuated OB injury in these mice relative to FVB/NJ animals. The reason for this difference is not yet clear. Loss of OPN also had no effect on this lack of OMP response. These data support further exploration to ascertain what appears to be significant differences in OB response to TBI between the two mouse strains. N=4-5/group.
Appendix B.1 MMP9 KO alters in vitro expression of OPN within OB microglia

Mixed neuronal/glial cultures derived from P2 olfactory bulbs of FVB/NJ WT or MMP9KO mice were grown on MatTek dishes and subjected to confocal labeling for OPN (green) in combination with microglial marker IBA-1 (red; A,D), astrocyte marker GFAP (red; B,E) or neuronal marker TUJ1 (red; C,F). Loss of MMP9 failed to alter phenotype or lack of OPN signal in OB astrocytes or neurons. Interestingly, OB microglia grown in vitro did show OPN expression (unlike microglia from 3-21d in vivo). MMP9KO also did not change microglial phenotype, but significantly reduced OPN expression in the glia. These results raise the possibility that OB microglia may be a source of OPN, expressed at undetectable levels with IHC, but detectable under the stress of in vitro conditions. Bar=20µm; N=2 separate cultures.
Appendix C.1 MMP9 KO does not alter OB glomerular cytoarchitecture

A. TEM analysis of OB glomeruli from MMP9 KO naïve cases (n=2) showed axo-dendritic structure matching that of FVB WT sham controls (compare with Figure 2.9). B. R.O.D. volume measures from gelatin zymograms (n=3/strain) and WB probe (n=3-4/strain) of FVB WT and MMP9 KO sham cases revealed no difference in either OB MMP2 activity or white matter OPN 66kD protein (corpus callosum) between the two sham control groups. These data support the choice of FVB WT sham as the control for the investigation of OB FVB WT vs. MMP9 KO strain differences after FPI. Bar=4µm.
A
MMP9 KO Naive Glomerulus

B
FVB WT vs. MMP9 KO Sham

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<th>FVB WT Sham</th>
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<td>1277706</td>
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Data expressed as R.O.D. volume

n = 3, MMP2
n = 3-4, OPN 66 kD

366
Appendix C.2 Group n values for Chapters 2-4
Animal number for each group subjected to statistical analysis is shown for experiments in Chapters 2-4.
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<td>Experiment</td>
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</table>
Vita

Melissa Ashley Powell was born on September 11, 1989 in Williamsburg, Virginia. She graduated from Springside School in Chestnut Hill, Pennsylvania in 2007. She received her Bachelor of Science from Hampton University, Hampton, Virginia in 2011, and promptly entered the Biomedical Sciences Doctoral Portal at Virginia Commonwealth University. She anticipates a graduation date of December 2016.