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The Expression of Matrix Metalloproteinase-9 and -2 in Olfactory Injury and Recovery

Stephen Bakos
Virginia Commonwealth University

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THE EXPRESSION OF MATRIX METALLOPROTEINASE-9 AND -2 IN OLFACTORY INJURY AND RECOVERY

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

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<td>Central Nervous System</td>
</tr>
<tr>
<td>CPA</td>
<td>Cyclophilin A</td>
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<tr>
<td>°C</td>
<td>Degree Celsius</td>
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<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
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<tr>
<td>EPL</td>
<td>External Plexiform Layer</td>
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<td>GCL</td>
<td>Granule Cell Layer</td>
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<td>GL</td>
<td>Glomerular Layer</td>
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<td>IPL</td>
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<td>MeBr</td>
<td>Methyl Bromide Gas</td>
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<td>ML</td>
<td>Mitral Layer</td>
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<td>MMP</td>
<td>Matrix Metalloproteinase</td>
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<tr>
<td>NL</td>
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<tr>
<td>NTx</td>
<td>Nerve Transection</td>
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<tr>
<td>NTx-TB</td>
<td>Nerve Transection with Teflon Barrier</td>
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<td>OB</td>
<td>Olfactory Bulb</td>
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<tr>
<td>OE</td>
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<tr>
<td>OMP</td>
<td>Olfactory Marker Protein</td>
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<tr>
<td>OSN</td>
<td>Olfactory Sensory Neuron</td>
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<tr>
<td>V</td>
<td>Volt</td>
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Abstract

THE EXPRESSION OF MATRIX METALLOPROTEINASE-9 AND -2 IN OLFACTORY INJURY AND RECOVERY

By Stephen Robert Bakos, B.A.

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

Virginia Commonwealth University, 2010

Director: Richard M. Costanzo
Professor, Physiology and Biophysics

The olfactory system has the remarkable capacity for neurogenesis following injury. However, the molecular mechanisms important for reinnervation of the olfactory bulb (OB) remain unknown. The matrix metalloproteinases (MMPs) are important components in many central nervous system (CNS) injury paradigms, yet remain unexplored in olfactory injury and recovery. To address the role of MMPs, the temporal expressions of MMP-9 and MMP-2 were examined in 3 olfactory injury models: nerve transection (NTx), methyl bromide gas (MeBr) exposure, and nerve transection with Teflon barrier (NTx-TB). Each injury model represents a different degree of olfactory injury and neuronal recovery. In NTx, sensory axons are lesioned, leading to neuronal degeneration and subsequent reinnervation of the OB. MeBr exposure damages the cell
bodies of sensory neurons in the peripheral olfactory epithelium (OE), leading to
degeneration and reinnervation of the OB without direct trauma to the OB. In NTx-TB,
sensory axons are lesioned and a barrier is inserted following injury that blocks
regenerated neurons from reinnervation of the OB.

Following NTx, MMP-9 increased immediately in the OB and was localized to
neutrophils, an inflammatory leukocyte. The elevated levels of MMP-9 corresponded to
neuronal degeneration. To confirm this relationship, MMP-9 expression was measured
following MeBr injury. MMP-9 increased during neuronal degeneration in the OB and
was localized to neutrophils in the area of sensory axon degradation. These
experiments demonstrated that MMP-9 is important for both neuronal degeneration and
the acute inflammatory response following olfactory injury.

In NTx injury, MMP-2 expression peaked at day 7 and corresponded to the
transition between degeneration and reinnervation of the OB. MMP-2 was localized to
the granule cell and external plexiform layers in control and day 7 bulbs. Following
NTx-TB, MMP-2 remained low and was not expressed by regenerated axons. The
absence of a MMP-2 peak in the NTx-TB injury suggests that this peak depends on
reinnervation of the OB.

This study demonstrates a temporal correlation between MMP-9 and
degeneration and MMP-2 and reinnervation following olfactory injury. These findings
provide new insight into the molecular mechanisms underlying olfactory nerve injury.
Modulation of MMPs could provide novel therapeutic interventions to improve neuronal
recovery following injury.
1.1 The olfactory system

Among sensory systems in the body, olfaction is unique, yet remains largely overlooked by both society and the scientific community. To interact and receive information about the external environment, humans depend less on the olfactory system than the visual and auditory systems (Malnic et al., 2004). However, as the French author Marcel Proust eloquently described in *Remembrance of All Things Past*, a simple odor can evoke vivid memories of past events. This experience, termed the Proust phenomenon, illustrates how olfactory stimuli are strong cues of autobiographical memories.

Although olfaction is often unnoticed in the daily experience of humans, anosmia (loss of smell) can be detrimental to the quality of life. Patients with olfactory dysfunction often suffer from depression and are unable to detect hazards such as smoke, gas leaks, and spoiled foods (Deems et al., 1991; Miwa et al., 2001; Santos et al., 2004; Seiden, 1997). Currently, few therapeutic options are available for anosmic patients, which illustrates the need for further studies in olfactory injury and recovery.

The 19th century neuroscientists, Golgi, Cajal, and Retzius, were the first to examine the anatomy of the olfactory system. Later in the 20th century, researchers discovered a unique property of olfactory sensory neurons (OSN). These neurons
regenerate throughout life, the only nerves in the central nervous system (CNS) with this capability. Subsequent studies demonstrated that OSNs regenerated following injury, making the olfactory system a unique CNS injury and recovery model.

1.1.1 The anatomy of olfaction

The olfactory epithelium (OE) is the peripheral component of olfaction and is located along the roof of the nasal cavity. It consists of three main cell types: OSNs, supporting cells, and basal cells (Cuschieri and Bannister, 1975; Morrison and Costanzo, 1992; Graziadei and Monti Graziadei, 1979). The OSNs are located along the basal or middle portion of the OE, depending on their age. Younger neurons tend to located along the basal aspect of the OE, while older neurons move towards the middle (Graziadei and Monti Graziadei, 1977). These bipolar neurons project an unbranched dendritic process to the apical epithelium that ends in a swelling termed the olfactory knob. Extending from this knob are 10 to 25 cilia, which express the receptor that interacts with odorant molecules in the nasal cavity (Morrison and Costanzo, 1990). The mechanism of odorant and receptor interaction remains unknown, although supporting cells secrete small proteins into the mucus that are thought to assist in odor detection (Pevsner et al., 1985).

There are two main types of supporting cells in the OE: Bowman’s glands and sustentacular cells. These cells provide a supportive environment for the unprotected dendrites in the nasal cavity. Bowman’s glands secrete mucus that prevents drying of the OE and contains ions that may enhance olfactory function (Morrison and Costanzo, 1992; Getchell et al., 1984). The sustentacular cells phagocytize debris and have a
variety of P450 enzymes that degrade toxins in the nasal cavity (Chen et al., 1992; Ding and Coon, 1988). Although Bowman’s glands and sustentacular cells maintain a supportive environment, the OSNs require constant turnover. Basal cells in the OE differentiate into new neurons and are responsible for maintaining the neuronal population during normal turnover (Schwartz et al., 1991; Calof and Chikaraishi, 1989).

Sensory axons, the smallest in the CNS (0.7 to 1.0 μm in diameter), exit the OE basally into the basal lamina (reviewed in Farbman, 1992). These axons converge to form fascicles that are blanketed by olfactory ensheathing cells. The fascicles pass through foramina of the cribriform plate and project to the olfactory bulb (OB). The OB consists of six morphologic layers (superficial to deep): nerve (NL), glomerular (GL), external plexiform (EPL), mitral (ML), internal plexiform (IPL), and granule cell (GCL). The axons of the OSNs form the NL and terminate on the dendrites of second order neurons (e.g., mitral and tufted cells) in a spherical neuropil called the glomerulus. The axons of the second order neurons serve as the main output of the OB to structures in the cerebral cortex (Doty, 2009). The anatomical organization of the olfactory system is unique in that it is the only sensory system without a primary thalamic relay. As axons leave the OB, via the olfactory tract, they project to cortical areas collectively termed the olfactory cortex. This region includes the anterior olfactory nucleus, piriform cortex, anterior cortical nucleus of the amygdala, periamygdaloid complex, and the rostral entorhinal cortex. The olfactory cortex serves to identify odors, while also associating the odors with specific emotions and memories. Although the olfactory cortex is important in the identification of odors, it is thought that odor encoding begins in the OE and OB.
1.1.2 Topographical organization of the olfactory system

Many sensory systems, such as vision and somatosensation, are highly organized to detect and interpret stimuli in the environment. The visual system sends information from the retina along neuronal pathways to the lateral geniculate body (LGB) of the thalamus. The LGB has six layers, with layers 2, 3 and 5 receiving input from the ipsilateral retina, while layers 1, 4 and 6 from the contralateral retina. Further topographical organization is found in the striate cortex of the occipital lobe. This structure contains layers that correspond to specific regions of the visual field. In the somatosensory cortex, regions of the body project to specific locations in the postcentral gyrus, often represented as the sensory homunculus.

Similar to vision and somatosensation, the olfactory system is topographically organized to detect and discriminate odors (Figure 1.1). The rodent genome contains over 1000 genes dedicated to encoding unique G-protein olfactory receptors (Buck and Axel, 1991; Zhang and Firestein, 2002). The olfactory receptors are randomly dispersed within 4 distinct zones of the OE (Strotmann et al., 1992; Vassar et al., 1993). These zones are arranged along the dorsoventral and medioventral axes of the epithelium and project to precise regions in the bulb. The olfactory receptors respond to a variety of odors, but at different intensities and send electrical stimuli to the OB. Mombaerts et al. (1996) demonstrated that a subclass of sensory receptor neurons, expressing one olfactory receptor in the OE, send axons to one or two specific glomeruli in the OB. The pattern of glomerular activation by an odorant may be important for the olfactory cortex to interpret and identify odor stimuli.
1.1.3 Turnover and replacement of olfactory neurons

Early neuroscientists believed that CNS recovery was limited because mature neurons lacked the ability to regenerate. Since peripheral nerves were observed to regenerate, early researchers concluded that mature CNS neurons lost this intrinsic ability after development. This idea went largely uncontested for over a century until mitotically active basal cells were observed to generate new neurons in the OE (Graziadei and Monti Graziadei, 1978).

The turnover of OSNs is thought to occur every 30-45 days, however longer periods have been reported (Graziadei and Monti Graziadei, 1978; Hinds et al., 1984; Mackay-Sim and Kittel, 1991). The continual replacement of neurons may be necessary because the dendritic processes of OSNs are exposed to mechanical trauma, toxic compounds, and infectious diseases in the nasal cavity. The OSNs are replaced by globose basal cells in the OE, maintaining the neuronal population throughout life (Graziadei and Monti Graziadei, 1979; Schwartz et al., 1991; Calof and Chikaraishi, 1989). The new OSNs send axons to the OB and reestablish functional synaptic connections with second order neurons (Graziadei and DeHan, 1973). Normal turnover of OSNs maintains the topographical organization of the olfactory system until old age (Costanzo and Kobayashi, 2010).

The olfactory system provides a unique model to study CNS neurogenesis due to its capacity to regenerate OSNs following injury. Graziadei and Hans (1973) demonstrated that following olfactory nerve transection (NTx), there is a rapid and complete degeneration of OSNs. Neuronal degeneration is first observed along the rostro-ventral axis of the OB and subsequently extends to the dorso-caudal region.
Figure 1.1: Topographical organization of the olfactory system. A subclass of sensory neurons send axons through the CP to one specific glomerulus (G) in the OB. These axons terminate on dendrites of second order neurons. The axons of second order neurons project to structures in the olfactory cortex. CP: cribriform plate; G: glomerulus; OB: olfactory bulb.
(Graziadei and Monti Graziadei, 1980). Glial cells in the OB enlarge following injury and phagocytize axonal debris, while sustentacular cells in the OE engulf apoptotic neuronal cell bodies (Graziadei and Monti Graziadei, 1980; Suzuki et al., 1995; Suzuki et al., 1996).

1.1.4 Olfactory injury and recovery

The morphology of the OB remains largely intact following NTx, however the dendrites of second order neurons are swollen (Graziadei and Monti Graziadei, 1980). In the OE, basal cells increase their mitotic activity and differentiate into new neurons (Graziadei, 1973). These regenerated neurons send axons to the NL and GL of the OB.

The time course of neuronal degeneration and reinnervation following NTx varies among species. In frogs, degeneration of sensory axons occurs over a two week period, while neuronal regeneration is first measured by day 20 (Graziadei and DeHan, 1973). Regeneration of sensory neurons in pigeons is evident by 32 days following NTx (Jennings et al., 1995). In rodents, degeneration is evident 15-24 hours following injury and peaks between days 3 and 4. Regenerated axons are apparent in the bulb by day 20, but shorter recovery periods have been reported (Graziadei and Monti Graziadei, 1980; Costanzo et al., 2006). Early studies of NTx inserted a rigid steel blade between the OB and cribriform plate to lesion sensory axons. Costanzo (1985) developed a new technique that used a flexible Teflon blade to transect sensory axons, leading to reduced injury to the cribriform plate and the surface of the OB. Neuronal regeneration in the OB of mice is observed as early as day 15 following NTx with the Teflon blade (Costanzo et al., 2006).
Regenerated axons reestablish functional synapses in the OB, as demonstrated by both electrophysiological and behavioral experiments. In salamanders, electro-olfactogram and extracellular unit activity recordings measured decreased sensory neuronal activity 10 days following NTx, indicating OSN degeneration (Simmons and Getchell, 1981a; Simmons and Getchell, 1981b). As early as 24 days after injury, neuronal activity was observed and by day 100 returned to control levels, signifying reinnervation of the OB. A similar response to electrical stimuli has been reported in other species. Negative evoked potential studies measured activity in hamster OB as early as day 20 following NTx and returned to control levels by day 30 (Koster and Costanzo, 1996). This demonstrated that sensory neurons reestablished functional synaptic connections with the dendrites of second order neurons.

Behavioral studies indicate that animals have the capacity to detect and distinguish odors following nerve injury. Pigeons and goldfish detect odors within two weeks following NTx (Oley et al., 1975; von Rekowski C. and Zippel, 1993). This recovery period is more rapid than in mammals and is likely due to the more simple anatomy of olfaction in lower species. The olfactory nerves in pigeons and goldfish form a single nerve projection to the bulb, whereas in mammals, the organization is more extensive. Yee and Costanzo (1995) reported that mice were able to detect odors within 40 days following NTx. However, the ability to discriminate individual odors was impaired and mice required an additional learning period to distinguish odors (Yee and Costanzo, 1995; Yee and Costanzo, 1998). This impaired discrimination may be related to distortion of the topographical organization of the olfactory system following NTx. Costanzo (2000) demonstrated that regenerated OSNs converge on multiple glomeruli.
in the OB after injury. Following olfactory injury, patients complain of pleasant odors (e.g. flowers) taking on unpleasant qualities, such as rotting trash. Since the topographical map is distorted, the glomerular activation by an odor is altered, and therefore, the odor is misidentified by the olfactory cortex.

The morphological changes associated with NTX have been well studied, but little is known about the molecular mechanisms responsible for the reinnervation of the OB. Although the topographical map is maintained during normal turnover of OSNs, it becomes distorted following injury. This suggests that injury-related changes in the OB are responsible for the altered projections of sensory axons. Examining the processes present in the OB following NTx can lead to a better understanding of how the olfactory system responds to injury and may uncover novel therapeutic interventions aimed at improving neuronal recovery.

1.2 Introduction to the matrix metalloproteinases

The matrix metalloproteinases (MMPs) are a family of enzymes important in the remodeling of the extracellular matrix (ECM). Gross and Lapiere (1962) first described MMP activity in tadpole tissue, later named MMP-1. The ability to modulate the ECM has implicated MMPs in different physiological processes including development, angiogenesis, and neuronal growth (Nagase and Woessner, Jr., 1999). To date, there are 25 enzymes in the vertebrate MMP family divided into 6 categories (Table 1.1) based on substrate specificity and structural properties (Sternlicht and Werb, 2001).
1.2.1 MMP structure and domains

The structure of MMPs varies between categories, however most have 5 conserved domains: prepro, pro, catalytic, hinge, and hemopexin-like. The preprodomain signals the translocation of the MMP to the endoplasmic reticulum and is cleaved upon arrival (Sternlicht and Werb, 2001). The prodomain maintains the enzyme as a zymogen, or inactive enzyme. The interaction of a cysteine moiety in the prodomain with the catalytic divalent cation renders the enzyme inactive. This interaction is called the cysteine switch, and its disruption is necessary for enzyme activity (Becker et al., 1995; Nagase et al., 1991; Springman et al., 1990). The catalytic domain contains the conserved sequence HEXXHXXGXXHZ, in which the 3 histidines (H) anchor the catalytic Zn$^{2+}$. The Zinc moiety, bound to a water molecule, is polarized by the glutamyl carboxylate (E), leading to nucleophilic attack on scissile peptide bonds (Stocker et al., 1995). Between the catalytic and hemopexin-like domains is the hinge region, which stabilizes the enzyme and assists in collagenolytic activity (Knauper et al., 1997). The C-terminal hemopexin-like domain assists in recognition and cleavage of substrates (Patterson et al., 2001). The endogenous inhibitors of MMPs, tissue inhibitor of metalloproteinase (TIMPs), bind to and inactivate MMPs in this domain (Sternlicht and Werb, 2001).

The MMPs are a diverse family of enzymes, mainly due to their different structures. Although most MMPs are secreted, there exists a membrane bound category. The membrane-type matrix metalloproteinases (MT-MMP) have either a single pass transmembrane domain (MMP-14, -15, -16, -24) or a C-terminal glycoposphatidylinositol signal (MMP-17 and -25) that anchors the enzyme to the membrane (Itoh et al., 1999; Kojima et al., 2000). MMP-9 and MMP-2 have a
Table 1.1: The vertebral matrix metalloproteinases

<table>
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<tr>
<th>Category</th>
<th>MMP</th>
<th>Common Name(s)</th>
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<td>MMP-13</td>
<td>Collagenase-3</td>
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<td></td>
<td>MMP-18</td>
<td>Collagenase-4 (Xenopus)</td>
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<td>Gelatinase B</td>
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<td>MMP-25</td>
<td>MT6-MMP</td>
</tr>
<tr>
<td><strong>Other</strong></td>
<td>MMP-12</td>
<td>Macrophage metalloelastase</td>
</tr>
<tr>
<td></td>
<td>MMP-20</td>
<td>Enamelysin</td>
</tr>
<tr>
<td></td>
<td>MMP-21</td>
<td>XMMP (Xenopus)</td>
</tr>
<tr>
<td></td>
<td>MMP-22</td>
<td>CMMP (chicken)</td>
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<td></td>
<td>MMP-23</td>
<td>Cysteine-array MMP</td>
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<td></td>
<td>MMP-27</td>
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<td></td>
<td>MMP-28</td>
<td>Epilysin</td>
</tr>
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</table>
fibronectin type II domain insert in the catalytic region that assists in binding and cleavage of substrates, while MMP-7, -23 and -26 lack a linker domain (Murphy and Nagase, 2008; Steffensen et al., 1995). These structural differences allow for proteolytic cleavage of a wide variety of substrates.

1.2.2 Activation of MMPs

Due to their potential destructive capability towards the ECM, MMPs are strictly regulated at multiple levels. Most MMPs require transcriptional activation, which can be induced by various molecules such as the inflammatory cytokines: TNF-α, IL-1β, and IL-8 (Galis et al., 1994; Pugin et al., 1999). These cytokines activate intracellular cascades that induce expression of various proteins, one of which is the transcriptional factor activator protein-1 (AP-1). This molecule binds to specific regions of DNA to promote or enhance transcription (Agrawal et al., 2008).

Another important level of regulation is disruption of the cysteine switch. There are two ways to remove the cysteine switch: removal of the prodomain or S-nitrosylation. Many enzymes have the ability to remove the prodomain to activate MMPs including furin (Pei and Weiss, 1995; Pei and Weiss, 1996), plasmin (Carmeliet et al., 1997), and other MMPs (Fridman et al., 1995; Ogata et al., 1992). A unique example of prodomain removal is found in MMP-2 activation. Initially, MT1-MMP binds to TIMP-2, forming a complex that binds a latent MMP-2 at the C-terminal domain (Strongin et al., 1995; Sato et al., 1994). The MT1-MMP cleaves a fragment of the latent MMP-2 prodomain. The final step requires a previously activated MMP-2 to be recruited to the
complex, which removes the remaining portion of the prodomain and activates the enzyme (Deryugina et al., 2001).

Studies have shown that removal of the prodomain is not necessary for MMP activation. The cysteine switch can be disrupted by S-nitrosylation in which a nitric oxide (NO) molecule reacts with the inhibitory cysteine to form a cysteine derivative. This derivative releases the catalytic Zn$^{2+}$, rendering it available for proteolytic cleavage of substrates (Van Wart and Birkedal-Hansen, 1990; Gu et al., 2002).

The activity of MMPs can be regulated by endogenous inhibitors called TIMPs. These proteins bind to the C-terminus of MMPs, rendering the MMP inactive. Although there exist only 4 TIMPs, each MMP is inhibited by at least one TIMP, forming a tight 1:1 complex (Nagase and Woessner, Jr., 1999).

### 1.2.3 MMPs in the normal CNS

In the developing and normal CNS, only a few MMPs are expressed and their importance is not fully understood (Anthony et al., 1998). During development, MMP-9 plays an important role in neurite growth and the vascularization of the cortex (Canete et al., 1995). Vaillant et al. (2003) reported that the granular layer of the cerebellum was enlarged in MMP-9$^{-/-}$ mice. They concluded that MMP-9 assisted in the fine tuning of the cerebellar neuronal networks by controlling granular cell apoptosis during development. Several MMPs are important in the targeting of axons during development by degrading axonal guidance molecules. This suggests that a balance between MMPs and guidance cues are necessary for normal development (Galko and Tessier-Lavigne, 2000). During learning and memory, MMP-3 and MMP-9 are localized to the hippocampus and are
thought to have a role in synaptic remodeling to process the new information (Meighan et al., 2006). MMP-9 and MMP-2 have been localized to microglia, astrocytes and neurons in the normal CNS, but the importance of this observation remains unclear (Cuzner et al., 1996; Anthony et al., 1997). Although more research is needed, MMPs seem likely to have critical roles in the developing and normal CNS due to their ability to remodel the ECM.

1.2.4 The role of MMPs in CNS injury and recovery

When mature CNS neurons are provided a peripheral nerve graft, they were observed to grow long distances (Benfey and Aguayo, 1982; Huebner and Strittmatter, 2009). This suggests that CNS neurons retain the capacity to regenerate after development. Therefore, injury-related changes to the ECM may be responsible for limited CNS recovery. There are two classes of neuronal inhibitors in the CNS: myelin-associated inhibitors (MAIs) and chondroitin sulfate proteoglycans (CSPGs). Following CNS injury, oligodendrocytes express MAIs that interact with Nogo-66 receptor on neurons, attenuating the growth of axons (Huebner and Strittmatter, 2009). CSPGs are a component of the astroglial scar and function to wall off the injured tissue, which prevents secondary injury. However, CSPGs are also a physical barrier to neuronal growth and may limit recovery in the CNS (Morgenstern et al., 2002). Studies showed that MMPs are capable of proteolytic degradation of MAIs and CSPGs (Pizzi and Crowe, 2007). This led researchers to focus on the role of MMPs in CNS injury and recovery.
Interestingly, the gelatinases (MMP-9 and MMP-2) are temporally expressed in many models of CNS injury including spinal cord injury (SCI), stroke, and multiple sclerosis (Anthony et al., 1997; Noble et al., 2002; Rosenberg et al., 1996). Generally, MMP-9 is increased immediately following injury, while MMP-2 expression is delayed for up to a week. Although these two MMPs have a similar structure and substrate specificity, the different temporal expression suggests MMP-9 and MMP-2 have different roles in CNS injury and recovery. The immediate expression of MMP-9 is associated with the early inflammatory response. Neutrophils, an inflammatory leukocyte, release MMP-9 in both stroke and SCI, which may have a role in neutrophil diapedesis and migration through the ECM (Justicia et al., 2003; de, Jr. et al., 2000; Murphy et al., 1991). Later in neuronal recovery, macrophages gradually replace neutrophils in the injured tissue. There are conflicting reports as to whether macrophages contain MMP-9 (Fleming et al., 2006; Larsen et al., 2003; Anthony et al., 1997). These different observations may be related to multiple factors including animal species, injury model, and multiple phenotypes of macrophages.

The importance of MMP-9 in neuronal injury and recovery has been demonstrated by targeted genetic disruption in mice. Following SCI, MMP-9−/− mice have less disruption of the blood-spinal cord barrier, attenuated neutrophilic infiltration, increased NG2 proteoglycan levels, and have increased functional recovery (Noble et al., 2002). The latter observation suggests that MMP-9 may have a detrimental role in CNS recovery. MMP-9 degrades both adhesion molecules important for neuron-ECM interaction and components of myelin (Gijbels et al., 1993; Zalewska et al., 2002). Proteolytic degradation of these neuronal components leads to nerve apoptosis.
Therefore, MMP-9 may contribute to a secondary trauma by damaging neurons that escaped the initial injury.

The importance of MMP-2 in CNS injury and recovery is not well understood. In vitro, MMP-2 is secreted by the growth cones of neurites, while in vivo MMP-2 is expressed in glial cells and neurons following nerve injury (Muir, 1994; Planas et al., 2001; Zuo et al., 1998). MMP-2−/− mice have impaired functional recovery and increased glial scar formation, suggesting that MMP-2 is important for neuronal recovery in the CNS (Hsu et al., 2006). MMP-2 is thought to degrade CSPGs, allowing regenerated neurons to pass through the glial scar and reestablish synaptic connections in the CNS.

1.3 Project goals, hypotheses, and specific aims

The goal of this research is to determine the importance of MMP-9 and MMP-2 in olfactory injury and recovery. To accomplish this, 3 olfactory injury models will be examined: 1) nerve transection (NTx), 2) methyl bromide gas (MeBr) exposure, and 3) nerve transection with Teflon barrier (NTx-TB). These injury models produce varying degrees of CNS trauma and neuronal recovery. Comparing the temporal expression of MMP-9 and MMP-2 in each model will determine the importance of these MMPs in olfactory injury and recovery processes. Previous studies have demonstrated that MMP-9 has a role in the initial inflammatory response following CNS injury, while MMP-2 is important for neuronal recovery (Noble et al., 2002; Rosenberg et al., 1996). Therefore, it is hypothesized that MMP-9 will be associated with the inflammatory response following olfactory injury, while MMP-2 will be important for neuronal recovery in the OB.
**Hypothesis 1:** MMP-9 and MMP-2 are increased at critical time periods in the OB during neuronal degeneration and reinnervation following NTx.

**Specific aim 1a:** Determine the time course of neuronal degeneration and reinnervation of the OB following NTx.

To establish the time course of neuronal degeneration and reinnervation following NTx, changes in olfactory marker protein (OMP) expression, a marker for mature sensory axons, will be measured by Western blot. A decrease in OMP will indicate neuronal degeneration, and a subsequent increase in OMP will signify reinnervation of the OB.

**Specific aim 1b:** Determine the temporal expression of MMP-9 and MMP-2 in the OB following NTx.

The temporal expression of MMP-9 and MMP-2 following NTX will be measured by Western blot. The changes in MMP levels will be compared with the neuronal degeneration and reinnervation time course established in specific aim 1a. This will test the hypothesis that MMP-9 and MMP-2 are associated with neuronal degeneration and reinnervation of the OB.

**Hypothesis 2:** MMP-9 is associated with the inflammatory response in the OB following NTx.
Specific aim 2a: Determine the time course of inflammatory leukocyte infiltration into the OB following NTx.

Inflammatory leukocyte infiltration into the OB will be monitored by immunohistochemistry at different recovery time points. Neutrophils will be identified with myeloperoxidase (MPO), an enzyme that is stored in the azurophilic granules of these cells (Klebanoff, 2005), while macrophages will be identified with CD68, a glycoprotein expressed by this leukocyte.

Specific aim 2b: Localize MMP-9 to the inflammatory leukocytes

To determine if the inflammatory leukocytes contain MMP-9, colocalization studies will be performed on the recovery days that correspond to leukocyte infiltration, as determined in specific aim 2a. Colocalization of MMP-9 with the leukocyte markers identified in specific aim 2a will test the hypothesis that neutrophils and macrophages are a source of MMP-9 following NTx.

Hypothesis 3: MMP-9 is associated with neuronal degeneration

Specific aim 3a: Determine the time course of neuronal degeneration and reinnervation of the OB following MeBr.

MeBr damages the cell bodies of sensory neurons in the OE, leading to neuronal degeneration and subsequent reinnervation of the OB, without direct trauma to the CNS (Schwob et al., 1995). To determine the time course of degeneration and reinnervation,
changes in OMP expression in the OB will be monitored by Western blot at different
time points following MeBr exposure.

**Specific aim 3b:** Determine the time course of MMP-9 in the OB following MeBr.

Changes in MMP-9 levels in the OB following MeBr will be measured by Western
blot. Comparing the temporal expression of MMP-9 with the neuronal degeneration
determined in specific aim 3a will test the hypothesis that MMP-9 is associated with
degeneration of sensory axons.

**Specific aim 3c:** Localize MMP-9 in the OB following MeBr.

Following MeBr, degradation of sensory axons should be evident in the nerve
and glomerular layers of the OB. Therefore, it is hypothesized that MMP-9 will be
localized in these layers. Immunohistochemical analysis of MMP-9 will be performed in
the OB at the time periods corresponding to neuronal degeneration, as established in
specific aim 3a.

**Hypothesis 4:** MMP-2 expression is associated with reinnervation of the OB

**Specific aim 4a:** Determine the expression of MMP-2 in the OB following NTx-TB.

MMP-2 has been reported to play an important role in neuronal recovery
following CNS injury (Rosenberg *et al*., 1996). To test the hypothesis that MMP-2 is
associated with reinnervation of the OB, a Teflon barrier will be inserted to block
regenerated axons following NTx. Changes in MMP-2 expression will be measured by Western blot at different recovery time points.

**Specific aim 4b:** Demonstrate blocking of regenerated axons with the Teflon barrier following NTx.

The use of a Teflon barrier to block regenerated axons from reaching the bulb is a novel model to study olfactory injury and recovery. The blockage of regenerated axons by the barrier will be confirmed by histological analysis of the OB. Changes in OMP, monitored by Western blot, will demonstrate the effect of the Teflon barrier on reinnervation of the OB.

Successful completion of these specific aims will provide important information regarding the roles of MMP-9 and MMP-2 in olfactory injury and recovery. In addition, the comparison of 3 olfactory injury models represents a novel method to determine the importance of specific molecular processes in CNS injury and recovery.
Chapter 2
Expression of Matrix Metalloproteinase-9 and -2 Following Olfactory Nerve Transection

2.1 Introduction

The olfactory system is a unique model for neurogenesis due to its remarkable capacity to regenerate and replace neurons throughout life. Basal cells located in the OE divide and differentiate into new neurons, replacing injured nerves over the course of 30-45 days (Graziadei and Metcalf, 1971; Graziadei and DeHan, 1973; Graziadei and Monti Graziadei, 1978; Hinds et al., 1984; Moulton, 1974). New neurons send axons to the OB and establish functional synapses with second order neurons in structures called glomeruli.

The projections of sensory axons to the bulb are highly organized, creating a topographical map (Buck and Axel, 1991; Mombaerts et al., 1996; Ressler et al., 1994; Vassar et al., 1993). Although the olfactory bulb contains over 1800 glomeruli (Royet et al., 1988), subclasses of axons, based on the olfactory receptor expressed in the OE, converge on only two specific glomeruli located on the medial and lateral aspects of the bulb. This topographical organization is thought to have an important role in odor discrimination and is maintained throughout normal turnover of neurons (Bozza and Kauer, 1998; Mombaerts et al., 1996; Royet et al., 1988; Wang et al., 1998).
Following injury, basal cells in the OE differentiate into new neurons, replacing the injured OSNs. The time course of neuronal degeneration and recovery following olfactory nerve injury has been established (Costanzo, 1985; Costanzo et al., 2006; Graziadei and DeHan, 1973). The degeneration of sensory axons occurs during the first two weeks, followed by the regenerative phase. New neurons send axons to the bulb and reestablish functional synaptic connections with second order neurons by day 40 (Yee and Costanzo, 1995). However, the axons converge on multiple glomeruli, distorting the topographical organization (Costanzo, 2000). The distortion of the topographical map is likely due to injury-related processes because sensory axonal projections to the OB are maintained during normal turnover. Although the morphological changes associated with olfactory nerve injury is well characterized, little is known about the molecular mechanisms important for neuronal regeneration and reinnervation of the OB.

This study focuses on the expression of MMP-9 and MMP-2 in the OB following NTx. These MMPs are important for degeneration and recovery processes in different models of CNS injury. MMP-9 expression increased immediately following injury and associated with the initial inflammatory response, glial scar formation, and blood-brain barrier disruption (Gijbels et al., 1993; Larsen et al., 2003; Noble et al., 2002; Planas et al., 2001; Rosenberg et al., 1996). In contrast, MMP-2 is often delayed for up to a week after injury and correlates with the transition from neuronal degeneration to recovery (Hsu et al., 2006; Rosenberg et al., 1996). MMP-2 is expressed in growth cone and cell bodies of neurons, suggesting a role in neuronal regeneration and reinnervation (Muir, 1994; Planas et al., 2001).
This chapter introduces the NTx as a model for neuronal injury and recovery, and examines the response of MMP-2 and MMP-9 in the OB following NTx. It is hypothesized that MMP-9 will increase immediately after NTx while MMP-2 expression will be delayed, corresponding to critical periods of neuronal degeneration and recovery. The goal of these experiments is to determine the temporal expression of MMP-9, MMP-2, neuronal degeneration, and reinnervation of the OB following NTx.

2.2 Materials and Methods

**Surgical procedure.** Adult P2-IRES-tau-lacZ mice were anesthetized with sodium pentobarbital (80 mg/kg, intraperitoneal). The P2 sensory neurons in these mice express a tau-lacZ fusion protein that allows for observing axonal projections from the OE to the OB. After anesthesia, the left OB was exposed and a thin, Teflon cutting blade was inserted between the bulb and the cribriform plate. The use of a Teflon blade resulted in transection of all the olfactory axons that connect to the left olfactory bulb and minimal damage to the olfactory bulb and cribriform plate (Figure 2.1). The right bulb was not transected and served as an internal control (CTRL) for histological analysis. After transection, the skin incision was sutured, and each animal was observed postoperatively before returning to its cage. All procedures were approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University.

**Tissue preparation for histology.** Mice were anesthetized with sodium pentobarbital and perfused transcardially with saline followed by 4% paraformaldehyde (ICN Biomedicals, Inc., Aurora, OH). The skulls were removed and placed in 4%
**Figure 2.1:** Diagram illustrating the NTx procedure and tissue collection for Western blot analysis. **A.** The Teflon blade is inserted between the CP and OB, leading to complete transection of sensory axons. **B.** After different recovery time points, the OB is cut (represented by the dotted line), and the anterior-ventral portion is collected. A: anterior; CP: cribriform plate; G: glomerulus; OB: olfactory bulb; V: ventral.
paraformaldehyde for 30 minutes, then rinsed with tap water for 5 minutes and immersed in Decal overnight (Decal Chemical Corp, Tallman, NY). The following day, skulls were placed in Invitrogen (Carlsbad, CA) phosphate-buffered saline (PBS) containing 30% sucrose for a week, then frozen in a minus 80°C freezer. Horizontal sections were cut on a Microm HM 550 series Cryostat (MICROM International GmbH, Walldorf, Germany) and placed on Superfrost® Plus VWR® Micro Slides (VWR, West Chester, Pennsylvania). The slides were stored in a minus 20°C freezer until processed.

**Hematoxylin and eosin histology.** Before processing, each slide was allowed to reach room temperature and then washed with PBS for 10 minutes. Sections were immersed for one minute intervals in a series of ethanol solutions (70%, 95%, 100%, 95%, 70%). Slides were placed in Harris Modified Hematoxylin (Fisher, Fair Lawn, NJ) for 5 minutes, rinsed in running tap water for 3 minutes and then placed in a 1% acid alcohol solution (1% hydrochloric acid in 70% alcohol) for 2 seconds. Following a 3 minute rinse in tap water, the sections were placed in an eosin solution (Fisher) for 5 minutes and rinsed for 5 minutes in running tap water. The slides were then mounted with Cryoseal™ 60 (Richard-Allan Scientific, Kalamazoo, MI) and visualized on an Eclipse E600 microscope (Nikon Inc, Melville, NY).

**DAB immunohistochemistry.** 3,3'-diaminobenzidine (DAB) staining for OMP was performed on CTRL and day 60 transected bulbs to observe changes in glomerular morphology following NTx. After washing with PBS for 10 minutes, sections were placed in 0.01 M citric acid solution with steam for 10 minutes, followed by a wash in PBS for 10 minutes. Sections were then immersed for 1-minute intervals in a series of alcohol
solutions (70%, 95%, 100%, 95%, and 70%) and placed in PBS wash for 5 minutes. Nonspecific binding was blocked with solution containing 10% normal rabbit serum, 4% bovine serum albumin, 5% nonfat dry milk, and 0.2% Triton X-100 in PBS for 1 hour. The slides were then incubated with goat anti-OMP primary antibody (1:10; Wako Chemicals, Richmond, VA) in the blocking solution overnight. The following day, the slides were washed 3 times in PBS for 5 minutes then incubated in peroxidase-conjugated rabbit anti-goat IgG secondary antibody (1:50; Rockland Immunochemicals, Inc., Gilbertsville, PA) in blocking solution, then exposed to DAB (Vector Laboratories, Inc., Burlingame, CA). Sections were counterstained with Harris Modified Hematoxylin (Fisher) and visualized on an Eclipse E600 microscope (Nikon, Inc., Melville, NY).

**Western blot tissue sampling and preparation.** Protein expression of MMP-9 and MMP-2 was measured by Western blot analysis. At 5 hours and 1, 3, 7, 10, 15, 35 and 60 days after the NTx, mice were anesthetized with sodium pentobarbital and killed by rapid decapitation. The left olfactory bulb tissue was carefully removed along with left bulb tissue from CTRL animals (receiving no surgical treatment). Although the right bulb of transected mice serves as an internal CTRL, occasionally some injury is observed in this bulb. Therefore, CTRL animals were used to ensure that no injury was present in CTRL samples. The left bulbs were immediately placed in protein extraction buffer (50 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 1% sodium dodecylsulfate [SDS], 1% DOC) and homogenized with a motor-driven plastic homogenizer. The tissue was incubated on a rotating platform for 20 minutes at 4°C and centrifuged at 16,000g for 30 minutes at 4°C. Solubilized proteins in the supernatant were quantified with a DC protein assay reagent kit (Bio-Rad Laboratories, Hercules, CA), using bovine serum albumin as a
Protein measurement. Quantification of protein expression was performed using Quantity One Analysis software (Bio-Rad Laboratories). Protein expression was measured as a function of both band density and band area. This density–area measurement for MMP-9, MMP-2, and OMP at each recovery time point was then standardized against the corresponding density–area measurement for CPA in the same gel. The protein measurement of MMP-9, MMP-2, or OMP was then expressed as

standard. Protein measurements were made at 720 nm on a μQuant plate reader (BioTek Instruments Inc., Winooski, VT). Equal protein samples and purified murine MMP-9 or MMP-2 (Chemicon, San Francisco, CA) were loaded onto Bis-Tris 4–12% density gradient gels and separated using NuPAGE MES [2-(N-morpholino) ethane sulfonic acid] reducing buffer system (Invitrogen) for 1 hour at 200 V and 4°C. Protein was transferred to nitrocellulose membranes for 2 hours at 25 V and 4°C. Nonspecific binding was blocked with 5% bovine nonfat dry milk in Tris-buffered saline and 0.05% Tween-20 for 1 hour. Goat anti-MMP-9 and goat anti-MMP-2 primary antibodies (1:200) were obtained from R&D Systems (Minneapolis, MN). Goat anti-OMP (1:20,000; Wako Chemicals) was used as a marker of axonal degeneration and regeneration in the bulb. Rabbit anti-cyclophilin A (CPA) (1:5000; Upstate, Lake Placid, NY) antibody was used to standardize protein loading. Nitrocellulose membranes were incubated overnight in primary antibody at 4°C. Membranes were then treated with species-specific peroxidase-conjugated IgG secondary antibodies (Rockland Immunochemicals, Inc.) for 1 hour, then incubated for 1 minute with Western Lightning Plus reagent (Perkin Elmer, Wellesley, MA) and exposed to Blue Sensitive Autoradiography film (Marsh BioProducts, Rochester, NY).
a ratio of CPA (amount of protein divided by amount of CPA) and normalized to CTRL levels (amount of protein/CPA ratio divided by amount of CTRL protein/CPA ratio).

**Gelatin zymography.** MMP-9 activity was measured by gelatin zymography. Purified murine MMP-9 and equal amounts of protein from each recovery period (5 hour, 1, 3, 7, 10, 15, 35, and 60 days) were loaded onto a 10% zymogram (gelatin) gel (Invitrogen) and separated using Novex Tris-glycine SDS buffering system (Invitrogen) for 3 hours at 135 V and 4°C. The gel was then incubated in Novex Zymogram Renaturing Buffer (Invitrogen) for 1 hour and transferred to developing buffer (0.5% coommassie blue, 10% acetic acid, 10% ethanol) for 24 hours at 37°C. The following day, the gels were placed in a destaining buffer (10% acetic acid, 10% ethanol) for 1 hour and visualized on a light box. Images were captured with a Nikon D60 camera.

**Statistical analysis.** Protein levels at each of the Western blot recovery time points were compared to CTRL values using 2-way ANOVA and a Holm-Sidak 2-sided post hoc test. A probability of less than 0.05 was considered statistically significant. All statistical analysis was performed using SPSS software (IBM, Chicago, IL).

### 2.3 Results

**Neuronal recovery following NTx.** The normal olfactory bulb has 6 distinct morphological layers: nerve, glomerular, external plexiform, mitral, internal plexiform, and granule cell (Figure 2.2A). Sensory axons from the epithelium passed through the cribriform plate to the nerve layer and terminated in the glomerular layer. The day 1 NTx bulb showed disruption of the nerve, glomerular, and anterior portion of the external plexiform layers, while the deeper layers and posterior bulb remained intact (Figure
Figure 2.2: Comparison of the OB from CTRL and NTx mice. A. The six distinct morphological layers are evident in CTRL bulbs. Sensory axons (arrows) from the OE pass through the CP to the outer NL, terminating on the GL. B. Following NTx, the NL, GL and anterior EPL are disrupted (asterisk), leaving the deeper layers (ML, IPL, and GCL) and posterior bulb intact. This indicates that the transection procedure selectively cuts the sensory axons. The corresponding CTRL bulb remains relatively intact, however injury is occasionally seen. In this bulb, injured tissue is limited to the anterior NL (arrowhead). CP: cribriform plate; CTRL: control; EPL: external plexiform layer; GCL: granule cell layer; GL: glomerular layer; IPL: internal plexiform layer; ML: mitral layer; NL: nerve layer; NTx: nerve transection; OB: olfactory bulb; OE: olfactory epithelium. Scale bars: 500 μm.
Figure 2.3: Injury-related changes in OMP expression in the OB following NTx. A. Representative Western blot measuring OMP expression at different recovery time points. Lane 1 represents OMP levels in untreated, control mice. CPA levels serve as gel loading control. B. Plot of the mean OMP expression, normalized to CTRL levels, from four separate Western blots. OMP remained near CTRL expression 5 hours following NTx. By day 1, OMP expression decreased and reached a minimum by day 10. This represents the degeneration of sensory axons in the OB. Between days 10 and 15, OMP increased to CTRL levels, which indicates reinnervation of the OB, and remained elevated through day 60. Data points represent the mean ± SEM (n=4 at each time point). ANOVA analysis with a Holm-Sidak post hoc for each time point compared to CTRL is given in Table 2.1. CPA: cyclophilin A; CTRL: control; NTx: nerve transection; OMP: olfactory marker protein.
Figure 2.4: Changes in P2 odorant receptor projections following NTx. Whole mount preparations showing the medial aspect of the OB and OE. P2 axons (dark blue) project from the OE to the OB, converging on two discrete loci called glomeruli (arrows). Several days following NTx, sensory axons degenerate and are not apparent in the bulb (day 7). Some faint staining is evident in the OE, although it is unclear if these neurons are degenerating or newly regenerated. After long recovery periods (days 35 and 60), regenerated sensory neurons are observed in the OE and OB. However, the axons converge on three or four glomeruli (arrows), indicating an altered projection of P2 axons to the OB. NTx: nerve transection; OB: olfactory bulb; OE: olfactory epithelium. Scale bars: 1 mm.
Figure 2.5: OMP expression in CTRL and NTx bulbs demonstrating changes in glomerular morphology. **A.** Low power image showing the CTRL and day 60 NTx bulbs. OMP positive axons (brown) project to the outer NL and terminate in the GL. **B.** High power image illustrating the normal morphology of glomeruli, having a homogenous, spherical appearance (asterisk). **C.** Following neuronal reinnervation of the OB, glomeruli are punctated and have a heterogeneous appearance (arrowheads). Asterisk and arrows in A correspond to glomeruli in B and C. CTRL: control; GL: glomerular layer; NL: nerve layer; NTx: nerve transection; OMP: olfactory marker protein. Scale bars: A: 500 μm; B&C: 100 μm.
This indicated that the NTx selectively transected olfactory sensory axons while minimizing damage to the bulb. The corresponding right bulb at day 1 is not transected and serves as an internal CTRL, however some injury was occasionally seen along the nerve layer. To establish the time course of neuronal degeneration and reinnervation of the OB, OMP, expressed by mature sensory axons, was monitored by Western blot analysis (Figure 2.3A). The relative amounts of OMP expression are plotted in Figure 2.3B. OMP levels decreased at day 1 and remained below CTRL levels for two weeks, indicating neuronal degeneration. Between days 10 and 15, OMP returned back to CTRL, signifying reinnervation of the OB by regenerated sensory axons. The changes in OMP expression demonstrated that the degeneration of sensory axons was observed during the first two weeks following NTx, while reinnervation of the OB is apparent by day 15.

Although reinnervation of the OB is observed following NTx, the projection of sensory axons are distorted (Figure 2.4). In CTRL mice, P2 receptor axons originate in the OE and converge on two discrete loci in the OB, called glomeruli. After NTx, the sensory axons are completely degenerated in the bulb by day 7. Some dark blue staining is evident in the OE, although it is unclear if these neurons are degenerating or newly regenerated. Following long recovery periods (days 35 and 60), regenerated P2 axons project from the OE to the OB and converged on multiple glomeruli. This demonstrated that the topographical map is distorted following recovery from NTx. OMP histological analysis of day 60 transected mice showed altered glomerular morphology in the NTx bulb compared to the corresponding internal CTRL bulb (Figure 2.5). The
CTRL glomeruli had a uniform, spherical pattern (Figure 2.5B), while glomeruli in the injured bulb were punctuated and vary in size (Figure 2.5C).

**Matrix metalloproteinase expression after NTx.** The changes in MMP-9 and MMP-2 were measured at different recovery time periods that reflect neuronal degeneration and reinnervation of the OB. Figure 2.6A shows a representative Western blot illustrating the change in MMP-9 levels following NTx. CTRL bulbs did not have detectable MMP-9. The relative amounts of MMP-9 at the different recovery time points are plotted in Figure 2.6B. MMP-9 increased by 5 hours after injury, and its levels peaked at day 1. The elevation of MMP-9 was observed for two weeks following NTx. By day 15, MMP-9 started to decrease and returned to CTRL levels by day 60. The time course of increased MMP-9 corresponded to decreased OMP expression (Figure 2.3). As OMP increased at day 15, MMP-9 levels decreased. This suggests a correlation between MMP-9 and degeneration of sensory axons. The antibody used in the Western blot analysis was unable to detect the active form of MMP-9. Therefore, gelatin zymography was performed to determine MMP-9 activity (Figure 2.7). The band corresponding to the active form of MMP-9 was observed during the first two weeks of injury (days 1-10), although a band at day 15 was seen in gel 3. This demonstrates that MMP-9 is active during the first two weeks following NTx.

Western blot analysis of MMP-2 levels in the OB following NTx is shown in Figure 2.8A. In CTRL bulbs, MMP-2 was detectable, confirming previous reports that MMP-2 is constitutively expressed in the CNS (Anthony et al., 1997; Cuzner et al., 1996). The relative amounts of MMP-2 at different recovery days are plotted in Figure 2.8B. Shortly after injury (5 hours), there was an injury-related increase in MMP-2 that
Figure 2.6: Changes in MMP-9 expression in the OB following NTx. A. Representative Western blot demonstrating changes in MMP-9 after injury. Lane 1 contains purified murine MMP-9. CTRL bulbs have no detectable MMP-9. CPA levels serve as gel loading control. B. Plot of the mean MMP-9 expression, normalized to CTRL levels, from four separate Western blots. MMP-9 expression was increased 5 hours following injury. The levels peaked at day 1 and remained elevated through day 10. At day 15, MMP-9 expression decreased and returned to CTRL levels by day 60. Data point represent mean ± SEM (n=4 at each time point). ANOVA analysis with a Holm-Sidak post hoc for each time point compared to CTRL is given in Table 2.1. CPA: cyclophilin A; CTRL: control; OB: olfactory bulb.
**Figure 2.7:** Gelatin zymography illustrating changes in the pro and active forms of MMP-9 following NTx. **A.** 3 zymography gels show the band corresponding to the active form of MMP-9 between days 1 and 10 following NTx. An active MMP-9 band is observed at day 15 in the lower gel (3). **B.** Table summarizing changes in the active form of MMP-9 following NTx. +: active MMP-9 observed; Ø: no active form of MMP-9 detected. NTx: nerve transection.
Figure 2.8: Changes in MMP-2 levels in the OB following NTx. A. Representative Western blot demonstrating MMP-2 expression at different recovery time points. Lane 1 contains purified murine MMP-2. CTRL mice (lane 2) have detectable levels of MMP-2. CPA levels serve as gel loading control. B. Plot of the mean expression of MMP-2, normalized to CTRL levels, from four separate Western blots. A 5-fold increase in MMP-2 was detected within 5 hours after injury and remained at this level for the first few days. At day 7, MMP-2 expression rapidly increased and peaked at 11.5-fold above CTRL levels. By day 10, MMP-2 decreased back to the day 3 levels and returned to CTRL at day 35. Data points represent the mean ± SEM (n=4 at each time point). ANOVA analysis with a Holm-Sidak post hoc for each time point compared to CTRL is given in Table 2.1. CPA: cyclophilin A; CTRL: control; NTx: nerve transection; OB: olfactory bulb.
**Table 2.1:** Table summarizing the significant Western blot recovery points compared to CTRL for OMP, MMP-9, and MMP-2

<table>
<thead>
<tr>
<th>Recovery</th>
<th>OMP $p$</th>
<th>MMP-9 $p$</th>
<th>MMP-2 $p$</th>
</tr>
</thead>
<tbody>
<tr>
<td>5H</td>
<td>0.598</td>
<td>0.207</td>
<td>0.038 *</td>
</tr>
<tr>
<td>D1</td>
<td>0.073</td>
<td>&lt;0.001 **</td>
<td>0.065</td>
</tr>
<tr>
<td>D3</td>
<td>0.087</td>
<td>&lt;0.001 **</td>
<td>&lt;0.001 **</td>
</tr>
<tr>
<td>D7</td>
<td>0.016 *</td>
<td>0.001 **</td>
<td>&lt;0.001 **</td>
</tr>
<tr>
<td>D10</td>
<td>0.014 *</td>
<td>&lt;0.001 **</td>
<td>0.215</td>
</tr>
<tr>
<td>D15</td>
<td>0.755</td>
<td>0.058</td>
<td>0.002 **</td>
</tr>
<tr>
<td>D35</td>
<td>0.964</td>
<td>0.515</td>
<td>0.948</td>
</tr>
<tr>
<td>D60</td>
<td>0.912</td>
<td>0.883</td>
<td>0.653</td>
</tr>
</tbody>
</table>

*: $p<0.05$; **: $p<0.01$
was sustained for the first few days following NTx. However, between days 3 and 7, MMP-2 increased rapidly and peaked at 11.5 fold higher than CTRL levels. By day 10, MMP-2 decreased back to the day 3 levels and returned to CTRL at day 35.

Table 2.1 shows 2-way ANOVA and Holm-Sidak post-hoc analysis demonstrating the Western blot recovery time points that are significant compared to CTRL levels for OMP, MMP-9, and MMP-2 expression.

2.4 Discussion

This report demonstrated the temporal expression of MMP-9 and MMP-2 following NTx. MMP-9 increased rapidly after injury and remained elevated for up to two weeks. This time period corresponded to the degeneration of sensory axons. A peak in MMP-2 expression was observed at day 7 and may correlate with the transition between neuronal degeneration and reinnervation of the OB.

**MMP-9 expression following NTx.** MMP-9 has been shown to play an important role in CNS injury-related processes such as gliosis, inflammation, and degradation of neurons. In NTx, reactive gliosis is observed in the bulb during the first few days after injury and remained elevated for over two months (Costanzo et al., 2006). Gliosis is initiated by reactive astrocytes, the resident glial cells of the CNS. Astrocytes have many important roles after injury including phagocytosis of neuronal debris and formation of the glial scar (Kimelberg, 2010; Stichel and Muller, 1998). Specifically, the glial scar has dichotomous roles in neuronal injury and recovery. It functions to isolate the injured areas and prevent secondary trauma to the surrounding tissue. However, components of the scar are inhibitory to neuronal growth including
chondroitin proteoglycans (Morgenstern et al., 2002). Although olfactory sensory neurons regenerate, the new axons converge onto multiple glomeruli in the bulb, distorting the topographical map (Figure 2.4). In contrast, normal turnover of sensory neurons maintains this map. This suggests that injury-related processes, such as the glial scar, may play an important role in distortion of the topographical map.

During the immediate period following NTx, MMP-9 increased rapidly and peaked by day 1 (Figure 2.6). The early expression of MMP-9 precedes the reported rise in reactive gliosis in the OB after NTx. MMP-9 degrades components of the glial scar, including CSPGs and may limit the extent of scarring (Hsu et al., 2008). Larsen et al. (2003) demonstrated that MMP-9−/− mice have increased glial scarring following CNS demyelination injury, demonstrating that MMP-9 may regulate glial scar formation. Although this implies that MMP-9 serves a beneficial role in neuronal recovery, others report that MMP-9−/− mice have improved functional outcomes following stroke and SCI (Lee et al., 2004; Noble et al., 2002). These studies indicate that although MMP-9 has multiple roles after injury, it may have an overall detrimental role in neuronal recovery.

The time course of MMP-9 elevation correlates with decreased OMP expression, suggesting that MMP-9 may play a role in neuronal degeneration. MMP-9 degrades components of myelin and adhesion molecules important for neuron-ECM interaction, leading to neuronal apoptosis (Gijbels et al., 1993; Zalewska et al., 2002). The expression of MMP-9 following injury may lead to indiscriminate proteolytic degradation of both injured and the surrounding healthy neurons. Newman et al. (2001) demonstrated that the injection of exogenous MMP-9 leads to neuronal injury, indicating
that MMP-9 is detrimental to neurons. This may explain the observation that MMP-9KO mice have improved neuronal recovery in CNS injuries.

One source of MMP-9 following CNS injuries is the inflammatory leukocytes, neutrophils and macrophages (Fleming et al., 2006; Justicia et al., 2003; Noble et al., 2002). The early rise in MMP-9 following NTx corresponded to the reported time course of neutrophils infiltration in other CNS injuries (Carlson et al., 1998; Dusart and Schwab, 1994). During later stages of recovery, macrophages gradually replace neutrophils in the injured tissue and are reported to contain MMP-9 (Larsen et al., 2003). The immediate increase in MMP-9 following NTx may represent inflammatory leukocytes recruitment in the OB, but the inflammation response following NTx has not been studied.

**MMP-2 expression following NTx.** MMP-2 was measured in CTRL bulbs, confirming previous reports that this protein in constitutively expressed in the CNS (Anthony et al., 1997; Cuzner et al., 1996). Following NTx, MMP-2 expression increased above CTRL and may indicate immediate injury-related changes in the OB (Figure 2.8). The peak expression of MMP-2 observed at day 7 may signal the transition between neuronal degeneration and reinnervation of the OB. Mature regenerated axons expressing OMP were measured in the bulb between days 10 and 15. However, the transition between immature and mature axons, and therefore, the expression of OMP may take up to a week (Miragall and Monti Graziadei, 1982). This indicates that immature axons may initially contact the bulb at day 7, leading to the peak expression of MMP-2. MMP-2 is important for neuronal recovery, as MMP-2⁻/⁻ mice have impaired functional recovery following SCI (Hsu et al., 2006). Future studies are needed to
determine the source of MMP-2 in the OB, which will determine, in part, the role of MMP-2 in olfactory injury and recovery.

**Conclusion.** The results from the NTx experiments demonstrate a temporal expression of MMP-9 and MMP-2 at critical periods during neuronal degeneration and reinnervation of the OB. MMP-9 was expressed immediately following injury and remained elevated for two weeks. This period corresponded to the degeneration of sensory axons. MMP-2 levels initially increased following NTx, with a large peak observed at day 7. This peak may correlate to the transition between neuronal degeneration and reinnervation of the OB. Future studies aimed at targeted disruption of MMP-9 and MMP-2 can determine the importance of these MMPs in olfactory injury and may uncover novel therapeutic options to improve neuronal recovery.
Chapter 3
MMP-9 is Associated with the Acute Inflammatory Response Following Olfactory Nerve Transection

3.1 Introduction

The matrix metalloproteinases (MMPs) are a family of proteinases important in the regulation of the extracellular matrix (ECM). These enzymes have the capacity to degrade many prominent components of the matrix and play critical roles in development, angiogenesis, and cellular migration (Canete et al., 1995; Itoh, 2006; Zhou et al., 2000). However, if left unregulated, MMPs have the potential to promote widespread tissue damage. Uncontrolled MMP activity initiates or exacerbates pathological processes such as rheumatoid arthritis, metastatic cancer, and multiple sclerosis (Gijbels et al., 1993; Melchiori et al., 1992; Brinckerhoff, 1991). In the CNS, a category of MMPs, the gelatinases (MMP-9 and MMP-2) are frequently reported to have increased temporal expression following different models of injury. MMP-2 is constitutively expressed in the CNS and is increased following injury, although its importance is not fully known (Rosenberg et al., 1996; Planas et al., 2001). MMP-9 is immediately elevated following SCI, stroke, and traumatic brain injuries and is associated with the initial inflammatory response (Hayashi et al., 2009; Justicia et al., 2003; Noble et al., 2002).
We have previously reported the expression of MMP-9 following olfactory NTx. The olfactory system is a unique model for neurogenesis due to its capacity for neuronal regeneration throughout life and following injury. Basal cells located in the epithelium replace injured neurons and reestablish synaptic connections in the CNS. The time course of neuronal degeneration and recovery following NTx has been previously reported (Costanzo et al., 2006). Degeneration of sensory axons occurs during the first two weeks, followed by neuronal recovery and establishment of functional synaptic connections in the OB by day 40 (Yee and Costanzo, 1995). MMP-9 expression increased rapidly after NTx and remained elevated for two weeks, corresponding to neuronal degeneration and glial scar formation (Costanzo et al., 2006). As regenerated axons contacted the bulb, MMP-9 levels decreased, suggesting this protein plays an important role during acute injury events following NTx.

Although the time course of MMP-9 expression following NTx has been defined, little is known about its role in olfactory neuronal degeneration and reinnervation of the OB. In this study, we examine the cellular source of MMP-9 expression after injury. Given previous reports that MMP-9 is involved in the acute inflammatory response following different CNS injuries, we hypothesize that MMP-9 will be localized to inflammatory leukocytes during the first two weeks following NTx. In addition, this report will provide the first look at the inflammatory response in the OB after nerve injury.

3.2 Material and Methods

Surgical procedures. Adult P2-IRES-tau-lacZ mice were anesthetized with sodium pentobarbital (80 mg/kg, intraperitoneal). After anesthesia, the left OB was
exposed and a thin, Teflon cutting blade was inserted between the left bulb and the cribriform plate. The use of a Teflon blade resulted in transection of all the olfactory axons that connect to the left bulb with minimal damage to the bulb and cribriform plate. The right bulb was not transected and served as an internal control (CTRL) for histological analysis. After transection, the skin incision was sutured and each animal was observed postoperatively before returning to its cage. All procedures were approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University.

**Immunohistochemistry.** Immunohistochemical staining for MMP-9 and myeloperoxidase (MPO) was performed at different recovery points following NTx: 1, 7, 10, and 60 days. CD68 labeling was performed on CTRL and days 1, 3, 7, 10, 15 and 60 NTx bulbs. Mice were anesthetized with sodium pentobarbital and perfused with saline, followed by 4% paraformaldehyde (ICN Biomedicals, Inc., Aurora, OH). The skulls were removed and placed in 4% paraformaldehyde for 30 minutes, rinsed with tap water for 5 minutes, followed by immersion in either Decal (Decal Chemical Corp, Tallman, NY) overnight or 0.3% ethylenediaminetetraacetic acid (EDTA) for a week. Decal interfered with the 4,6-diamidino-2-phenylindole (DAPI) signal, and therefore, DAPI staining was performed on skulls decalcified with EDTA. After decalcification, skulls were placed in Invitrogen (Carlsbad, CA) phosphate-buffered saline (PBS) containing 30% sucrose for a week, then frozen in a minus 80°C freezer. Horizontal sections were cut on a Microm HM 550 series Cryostat (MICROM International GmbH, Walldorf, Germany) and placed on Superfrost® Plus VWR® Micro Slides (VWR, West Chester, Pennsylvania). The slides were stored in a minus 20°C freezer until processed.
Before processing, each slide was allowed to reach room temperature, then washed with PBS for 10 minutes. Microwave antigen retrieval was performed as previously described with some modifications (Stone et al., 1999). Sections were heated to 65°C for 8 minutes and allowed to cool for 20 minutes at room temperature. Following antigen retrieval, slides were washed 3 times in PBS, then incubated for 1 hour in a blocking solution containing 10% normal donkey serum (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) and 0.2% Triton X-100 (LabChem Inc. Pittsburgh, PA) in PBS. The sections were then placed in blocking solution containing primary antibodies overnight: goat anti-MMP-9 (1:10; R & D Systems, Minneapolis, MN), and rabbit anti-MPO (MPO; 1:10; Abcam, Cambridge, MA), or goat anti-MMP-9 (R & D Systems) and rat anti-CD-68 (1:100; AbD Serotec, Raleigh, NC). The following day the slides were washed 3 times in PBS and placed in the species appropriate donkey secondary antibody. MMP-9 was incubated with CY3 conjugated donkey anti-goat secondary antibody (1:300; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) while MPO and CD-68 were incubated with AlexaFluor 488 conjugated donkey anti-species appropriate secondary antibodies (1:100; Invitrogen) for 1 hour. The slides were mounted using Vector mounting media containing DAPI (Vector Laboratories Inc., Burlingame, CA) and visualized on a Leica TCS-SP2 AOBS confocal microscope (Leica Microsystems, Inc., Bannockburn, IL).

**DAB immunohistochemistry.** 3,3'-diaminobenzidine (DAB) staining for MMP-9 was performed on day 1 NTx bulbs. Tissue processing was identical to the immunohistochemical protocol until the blocking step. Sections were blocked for 1 hour in a solution containing 10% normal rabbit serum, 4% bovine serum albumin, and 5%
nonfat dry milk in PBS. The sections were then incubated in goat anti-MMP-9 (1:10, R & D Systems) primary antibody in block solution overnight. The following day, the slides were washed 3 times in PBS and placed in peroxidase conjugated rabbit anti-goat secondary antibody (1:50, Rockland Immunochemicals Inc., Gilbertsville, PA) for 1 hour. The slides were washed 3 times in PBS, exposed to DAB (Vector Laboratories, Inc.) and visualized on an Eclipse E600 microscope (Nikon Inc., Melville, NY).

3.3 Results

MMP-9 immunohistochemical staining at 4 recovery time points (days 1, 7, 10, and 60) following NTx is shown in Figure 3.1. MMP-9 was apparent during the acute period following injury (days 1, 7 and 10) only in the NTx bulb. The corresponding CTRL bulbs at each time point did not contain MMP-9. This suggests that MMP-9 is associated with an injury-related process. By day 60, MMP-9 staining was not observed in either the CTRL or NTx bulb.

To determine the source of MMP-9, co-localization of MMP-9 and myeloperoxidase (MPO), a marker predominately for neutrophils, was performed (Figure 3.2). At 3 recovery days (1, 7, and 10), MPO and MMP-9 signals were apparent in the same cell. Nuclear staining with DAPI demonstrated that these cells have a polymorphic nuclear morphology, which is characteristic of neutrophils. DAB staining on day 1 bulbs further indicated that MMP-9 was localized to neutrophils, having the characteristic polymorphic nucleus (Figure 3.3). Taken together, this strongly suggests that neutrophils are the source of MMP-9 during the acute period following NTx.
To determine if macrophages, another inflammatory leukocyte, are present in the injured bulb following NTx, CD68 localization was monitored by immunohistochemistry. The infiltration of CD68 positive cells, associated with the phagocytic phenotype of macrophages (Micklem et al., 1989; Ramprasad et al., 1996), at different recovery time points is shown in Figure 3.4. CD68 positive cells were not evident in the CTRL or day 1 OB. By day 3, CD68 positive cells were increased in the NTx bulb while absent in the corresponding CTRL bulb. CD68 cells remained in the transected bulb at days 7 and 10, while absent in the CTRL bulbs. This indicates that macrophages are associated with an injury-related response. Colocalization studies at three recovery days (7, 10, and 15), when CD68 levels were maximal, show that CD68 positive macrophages do not contain MMP-9 (Figure 3.5). Nuclear staining with DAPI demonstrated the cells that contain MMP-9 are neutrophils, having the characteristic polymorphic nuclear morphology.
Figure 3.1: MMP-9 is localized only to the NTx bulb. At days 1, 7, and 10, MMP-9 (arrows) is evident in the NTx bulb, while no labeling is apparent in the corresponding CTRL bulb. This suggests that MMP-9 is associated with the NTx injury. By day 60, MMP-9 expression is not observed in either the CTRL or NTx bulb. CTRL: control; NTx: nerve transection. Scale bar: 100 μm.
Figure 3.2: Colocalization of MMP-9 with neutrophilic makers in the OB following NTx. Representative images from three recovery days (1, 7, 10) demonstrate that MPO (green), a marker for neutrophils, and MMP-9 (red) are localized to the same cell (yellow). Nuclear staining with DAPI demonstrates that the MPO/MMP-9 positive cells have a polymorphic nucleus, the characteristic nuclear morphology of neutrophils. Taken together, this indicates that neutrophils are a source of MMP-9 expression at these three recovery days. However, some neutrophils do not contain MMP-9 (asterisks). DAPI: 4′,6-diamidino-2-phenylindole; MPO: myeloperoxidase; NTx: nerve transection. Scale bar for all images: 10 μm.
Figure 3.3: DAB and hematoxylin staining demonstrates that neutrophils contain MMP-9 in the OB following NTx. A. Low power images of the day 1 NTx bulb show disruption of the normal morphology of the OB. MMP-9 positive cells (brown) are distributed throughout the injured tissue (arrows). B. High power images confirm that the cells expressing MMP-9 are neutrophils, having the characteristic polymorphic nuclei. Arrows in (A) correspond to cells shown in (B). DAB: 3,3'-diaminobenzidine; NTx: nerve transection; OB: olfactory bulb. Scale bars: A: 50 μm; B: 10 μm.
Figure 3.4: Injury related changes in CD68 positive cells following NTx. In CTRL and day 1 NTx bulbs, CD68 cells, mainly associated with macrophages, are not evident. At day 3, CD68 positive cells (arrows) are observed in the NTx bulb while absent in the corresponding CTRL bulb. The day 7 and 10 images demonstrate CD68 cells remained localized to the NTx bulb. This suggests that CD68 cells are associated with an injury-related response. By day 60, CD68 cells are not observed in the CTRL or NTx bulbs. CTRL: control; NTx: nerve transection. Scale bar: 100 μm.
**Figure 3.5:** CD68 positive cells do not contain MMP-9 following NTx. Three representative images at different recovery days (7, 10, 15) demonstrate that CD68 positive cells (green), associated mainly with macrophages, fail to co-localize with MMP-9 (red). This indicates that CD68 positive cells do not contain MMP-9 in the bulb following NTx. Nuclear staining with DAPI indicates that the MMP-9 positive cells have polymorphic nuclei, indicating these cells are neutrophils. DAPI: 4',6-diamidino-2-phenylindole; NTx: nerve transection. Scale bar for all images: 10 μm.
3.4 Discussion

This study demonstrated that MMP-9 is localized to neutrophils in the injured bulb during the acute period (days 1-15) following NTx. CD68 positive cells, a marker for phagocytic macrophages, infiltrated the bulb by day 3 and remained elevated through day 15. These cells do not contain MMP-9, suggesting that neutrophils are the main source of MMP-9 in the OB following NTx.

Inflammation in the CNS. Barker and Billingham proposed that the CNS was an immune privileged site and protected from inflammation following injury (Scholz et al., 2007). One reason for this designation is the blood-brain barrier (BBB) prevents trafficking of molecules between the vasculature and CNS. Transcellular transport is inhibited because endothelial cells of the BBB have decreased pinocytosis and the BBB lacks fenestrations (Mooradian, 1988). In addition, the endothelial cells are connected together by adherens and tight junctions, preventing paracellular transport of molecules (Pun et al., 2009). These characteristics of the BBB were thought to prevent inflammatory cytokines from recruiting leukocytes to the CNS, however current research has challenged this dogma.

Inflammatory leukocytes infiltrate damaged tissue during the acute periods (1-3 days) following different CNS injuries including stroke, SCI, and traumatic brain injury (Carlson et al., 1998; Jin et al., 2010; Holmin et al., 1995). In the minutes to hours following these injuries, the damaged tissue and glial cells secrete proinflammatory cytokines, such as TNF-α and IL-1 (Witko-Sarsat et al., 2000; Cheret et al., 2008; Barone et al., 1997; Rothwell et al., 1997). These cytokines induce expression of adhesion molecules in the adjacent endothelial cells of the BBB and circulating
inflammatory leukocytes (Qian et al., 2007). The adhesion molecules interact, leading to rolling of the leukocytes along the vasculature and eventual tight binding to the endothelium (Yilmaz and Granger, 2008). Once adjacent to the injured tissue, the leukocytes need to penetrate through the BBB basal membrane, which includes collagen, fibronectin, and laminin (Scholz et al., 2007). MMP-9 has the capability to degrade these molecules and may have a role in diapedesis of neutrophils. In this study, we demonstrated that neutrophils contain MMP-9 shortly after NTx (Figures 3.2 & 3.3), which is observed in other models of CNS injury (Larsen et al., 2003; Lee et al., 2004). Beyond the BBB, neutrophils migrate through the ECM by haptotaxis, a gradient of insoluble chemoattractants bound to structural components of the matrix (Witko-Sarsat et al., 2000). It has been suggested that neutrophils secrete MMP-9 to degrade components of the ECM, allowing for migration to the injured tissue (Murphy et al., 1991). However, Mandeville et al. (1997) demonstrated an alternate method in which neutrophils can reversibly distort the structural component of the ECM and squeeze through gaps in the matrix without proteolytic degradation. This process has been termed “amoeboid-type” migration (Wolf et al., 2003). However, this does not exclude the possibility that, depending on the environment, the structural component of the ECM can be either flexible or tight (Itoh, 2006). MMP-9 may have a role in the migration through the tight ECM. Nobel et al. (2002) demonstrated that MMP-9−/− mice have decreased infiltration of neutrophils following SCI. This suggests that MMP-9 is important for neutrophil migration from the vasculature to the injured tissue.

Once at the injured tissue, neutrophils secrete oxidative enzymes and proinflammatory cytokines. These molecules may promote a secondary trauma to the
surrounding tissue, although this has yet to be conclusively determined (Emerich et al., 2002). The oxidative enzymes, including myeloperoxidase and NADPH oxidase, increase the levels of oxygen free radicals, resulting in increased intracellular calcium and cell damage (Arnhold and Flemmig, 2010). In addition to these enzymes, neutrophils secrete MMP-9 at the site of injury, which may damage healthy neurons. MMP-9 cleaves components of myelin and adhesive molecules important for neuron-ECM interaction, leading to apoptosis of the neuron (Gijbels et al., 1993; Zalewska et al., 2002). MMP-9 may also exacerbate the inflammatory response of the injured tissue. IL-8, an inflammatory cytokine, is cleaved by MMP-9 at the N-terminus (Gijbels et al., 1993; Van Den Steen et al., 2000). This truncated molecule is a strong stimulus for neutrophils, leading to an increased inflammatory response. While MMP-9 may serve some beneficial roles in CNS injury and recovery, its overall activity may be inhibitory to neuronal recovery. MMP-9−/− mice have improved functional recovery following different CNS injury models, confirming that MMP-9 is detrimental to CNS recovery (Lee et al., 2004; Noble et al., 2002).

Macrophages represent the chronic inflammatory response and are often measured in injured tissue for weeks or months following injury (Dusart and Schwab, 1994; Fleming et al., 2006). CD68 positive cells, associated with the phagocytic phenotype of macrophages, infiltrate the transected bulb by day 3 following injury and are observed through day 10 (Figure 3.4). The corresponding CTRL bulb at each recovery period did not have detectable levels of CD68. This suggests that CD68 cells are involved in an injury-related process. Figure 3.5 demonstrated that CD68 positive macrophages do not contain MMP-9. There are conflicting reports regarding the
expression of MMP-9 by macrophages which may be related to morphologic changes of these cells (Anthony et al., 1997; Fleming et al., 2006; Larsen et al., 2003). Unlike the short-lived neutrophils, macrophages undergo multiple morphologic phenotypes during their lifetime in damaged tissue (Adams and Hamilton, 1984). Therefore, macrophages may contain MMP-9 only during specific phenotypic stages, such as transmigration from the vasculature. Further studies are needed to determine if other macrophage phenotypes contain MMP-9.

**Conclusion.** This is the first report to demonstrate that neutrophils are a source of MMP-9 in the OB following NTx. Neutrophils infiltrated the injured bulb during the acute period (days 1-15) after injury, while CD68 positive macrophages gradually entered the NTx bulb by day 3. Although CD68 macrophages do not contain MMP-9, this does not exclude the possibility that other macrophage phenotypes express MMP-9. Future studies with MMP-9−/− mice can determine the role of MMP-9 in neuronal degeneration, inflammation and its potential as a therapeutic target to improved recovery.
Chapter 4
Matrix Metalloproteinase-9 and -2 Expression in the Olfactory Bulb Following Methyl Bromide Gas Exposure

4.1 Introduction

Matrix metalloproteinases (MMPs) are a family of over 20 structurally related enzymes comprised of a propeptide and a Zn$^{2+}$-binding catalytic domain. These enzymes are implicated in the degradation of extracellular matrix (ECM) components including laminin, collagen IV, and elastin (Yong, 2005). In normal physiological processes, such as angiogenesis, wound healing, learning and memory, MMPs serve an important role in the remodeling of the ECM (Nagase and Woessner, Jr., 1999; Yong, 2005). Though their enzymatic action is necessary for tissue restructuring and repair, the MMPs are highly regulated due to their potential destructive capability towards the ECM. Most MMPs are secreted as zymogens and require cleavage of the prodomain and association of a Zn$^{2+}$ in the catalytic domain to become fully activated. Studies have shown that uncontrolled activation of these enzymes can result in certain pathologies including arthritis, multiple sclerosis and Alzheimer’s disease (Yong et al., 1998; Yoshihara et al., 2000).

In the central nervous system (CNS), several MMPs are modulated after neuronal injury. Two specific MMPs, MMP-9 and MMP-2, have defined temporal expression patterns following different models of neuronal injuries (Romanic et al.,
1998; Rosenberg et al., 1996; Wang et al., 2000; Wang et al., 2002). For example, after SCI, MMP-9 expression increased within hours whereas MMP-2 expression was delayed for up to a week (de, Jr. et al., 2000; Goussev et al., 2003). The early presence of MMP-9 correlates with inflammation, degradation of blood vessel walls, edema and neuronal death. The delay in MMP-2 expression is important for remodeling the ECM and breakdown of scar, ensuring the proper conditions necessary for neuronal recovery (Hsu et al., 2006; Goussev et al., 2003; Zuo et al., 1998).

We have previously reported the expression of MMP-9 and MMP-2 following direct trauma to olfactory neurons using a NTx injury model. In this model, the axons are lesioned as they penetrate through the cribriform plate before entering the olfactory bulb. The NTx injury is not limited to the axons alone but also injures other CNS structures including the anterior-ventral portion of the bulb and blood vessels. Following NTx, there is a temporal expression of these two specific MMPs, similar to other CNS neuronal injuries. MMP-9 is elevated within hours following NTx whereas MMP-2 levels did not increase until a week after injury (Costanzo et al., 2006; Costanzo and Perrino, 2008). The temporal expression of these two enzymes provides insight into the molecular processes associated with olfactory degeneration and regeneration. Though the expression of MMP-9 and MMP-2 in CNS injury has been established, these models involve some component of trauma. It is unknown if MMP-9 and MMP-2 are upregulated in the absence of direct trauma or toxic injury, i.e. by deafferentation alone.

Unlike most models of CNS injury, the olfactory system is unique in that the cell bodies of sensory neurons are located outside the CNS, in the peripheral olfactory epithelium and project their axons to the olfactory bulb, a CNS structure. This
organization allows for the study of direct (NTx) injury to the CNS vs. isolated deafferentation via the destruction of the neuronal population. The latter is achieved through methyl bromide gas (MeBr) exposure. MeBr is passively inhaled, filling the nasal cavity and leading to destruction of over 90 percent of cells in the olfactory epithelial, including olfactory neurons (Schwob et al., 1995). This leads to deafferentation of the olfactory bulb without direct trauma.

In this study we examined the expression levels of MMP-9 and MMP-2 in the olfactory bulb following MeBr exposure to determine if these MMPs are associated with neuronal deafferentation in the absence of direct CNS trauma. A comparison of findings from both these indirect (MeBr) and direct (NTx) CNS injury models provides a new approach to investigating the role of MMPs in neuronal injury and recovery processes.

4.2 Materials and Methods

**Methyl bromide gas exposure.** Adult C57/BL6 mice were exposed to MeBr gas as previously described (Chen et al., 2004; Schwob et al., 1995). Mice were placed in a wire enclosure measuring 15 X 15 X 15 cm centered within a plexiglass box measuring 30 X 30 X 30 cm and exposed to 180 ppm MeBr gas in purified air at a flow rate of 10L per hour for six hours. Mice exposed to room air served as controls (CTRL). All procedures were approved by the Institutional Animal Care and Use Committees of Virginia Commonwealth University and Tufts University School of Medicine.

**Tissue sampling and preparation.** At 1, 3, 5, 7, 10, 15, 40 and 60 days after MeBr gas exposure, mice were anesthetized with sodium pentobarbital and sacrificed by rapid decapitation. The OBs were removed from the skull, flash frozen in liquid
nitrogen and processed as previously described (Costanzo et al., 2006). The anterior-ventral portion of the bulbs and samples from frontal cortex were removed for protein measurements. All tissue samples were placed in protein extraction buffer (50 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 1% sodium dodecylsulfate, 1% DOC) and homogenized with a motor-driven plastic homogenizer. The tissue was incubated on a rotating platform at 4°C for 30 minutes, homogenized and centrifuged at 4°C for 30 minutes at 16,000 rpm. Samples were then placed in a minus 80°C freezer for storage. Solubilized proteins in the supernatant were quantified using DC protein assay reagent kit (Bio-Rad Laboratories, Hercules, CA) using bovine serum albumin as a standard. Protein measurements were made at 750 nm on a μQuant plate reader (BioTek Instruments Inc., Winooski, VT). Equal amounts of protein (20 μg for MMP-9 and 40 μg for MMP-2) from the tissue extracts and purified murine MMP-9 and MMP-2 (R and D Systems, Minneapolis, MN) were loaded onto 4-12% Bis-Tris density gradient gels and separated using NuPAGE MES [2-(N-morpholino) ethane sulfonic acid] reducing buffer system (Invitrogen, Carlsbad, CA) for 1 hour at 200 V and 4°C. Protein was transferred to nitrocellulose membrane for 2 h at 25V and 4°C. 5% bovine nonfat dry milk in Tris-buffered saline and 0.05% Tween-20 was used to block nonspecific binding for 1 hour. Goat anti-MMP-9 and Goat anti-MMP2 (1:200, R and D Systems), goat anti-OMP (1:20000, Wako Chemicals, Richmond, VA), and rabbit anti-glial fibrillary acidic protein (1:50000, DakoCytomation, Glostrup, Denmark) primary antibodies were used. Rabbit anti-CPA (1:7000, Upstate, Lake Placid, NY) primary antibody was obtained to control for protein loading. Nitrocellulose membranes were incubated in the primary antibody overnight at 4°C. Membranes were then exposed to the appropriate species
peroxidase-conjugated IgG secondary antibody (Rockland, Gilbertsville, PA) for 1 hour. The membranes were incubated for 1 minute in Western Lightning Plus reagent (Perkin Elmer, Wellesley, MA) and exposed to Blue Sensitive Autoradiography film (ThermoScientific, Rockford, IL).

Protein measurements. Quantification of protein expression on the nitrocellulose membrane was performed using Quantity One Analysis software (Bio-Rad Laboratories). The protein bands were measured both by band density and band area. The density-area measurements for MMP-2, MMP-9, OMP and GFAP at each recovery time point were then divided by the corresponding density-area measurement for CPA in the same gel to adjust for differences in protein loading. These protein ratios were then divided by CTRL samples to obtain normalized expression levels.

Immunohistochemistry. Immunohistochemical staining for MMP-9 and MMP-2 was performed on horizontal sections at 3 different time points after MeBr: days 1, 5 and 10. After washing with Invitrogen Phosphate Buffered Saline (PBS) for 10 minutes, sections were placed in 0.01M Citric acid with steam for 10 minutes, and washed in PBS for 10 minutes. Sections were then immersed for 1-minute intervals in a series of alcohol solutions (70%, 95%, 100%, 95%, 70%) and placed in PBS wash for 5 minutes. This was followed by incubation with 10% normal rabbit serum, 4% bovine serum albumin, 5% non-fat dry milk and 0.5% Triton X-100 in PBS for 1 hour and placed with goat anti-MMP-9 or goat anti-MMP-2 primary antibody (1:10) overnight. Sections were then incubated in peroxidase-conjugated rabbit anti-goat immunoglobulin (1:50) and exposed to 3, 3’-diaminobenzidine (DAB, Vector Laboratories, Inc., Burlingame, CA). Sections were counterstained with Harris Modified Hematoxylin (Fisher Scientific, Fair
Lawn, NJ) and visualized on an Eclipse E600 microscope (Nikon Inc., Melville, NY).

**MMP-9-labeled cell counts.** Digital images of immunostained histological sections were used to obtain cell counts of MMP-9 positive cells located within different layers of the olfactory bulb. For each olfactory bulb section, the total number of MMP-9 positive cells was counted within each of four defined regions: the combined nerve and glomerular layer, the external plexiform layer, the combined mitral and internal plexiform layer, and the granular cell layer. The criteria used to define a MMP-9 labeled cell was: positive DAB staining and visualization of the cell nucleus. Three separate bulb sections were used to obtain the mean number of cells for each region. We also measured the average area (mm$^2$) for each bulb region using ImageJ analysis software (National Institutes of Health, MD).

**Statistical analysis.** A comparison of protein levels at each of the different recovery time points relative to CTRL was performed using 2-Way ANOVAs and a Holm-Sidak 2-sided post-hoc test. A probability of less than 0.05 was considered statistically significant. All statistical analysis was performed using SPSS software (IBM, Chicago, IL).

**4.3 Results**

MMP-9 and MMP-2 protein expression was measured in the olfactory bulb shortly after MeBr gas exposure (day 1) and during neuronal degeneration (day 1-15) and regeneration (day 15-60) time periods. Western blot analysis of MMP-9 and MMP-2 expression following MeBr is shown in Figure 4.1. Density-area measurements of MMP protein bands were compared with that of CPA (Figure 4.1A) to determine the relative
amounts of MMP-9 and MMP-2 in the bulb at each time point. The mean values normalized to CTRL levels from 4 separate experiments are plotted in Figure 4.1B and C. In CTRL samples, MMP-9 expression was absent and MMP-2 levels were barely detectable. MMP-9 remained at CTRL levels during the first 3 days, increased rapidly between days 3 and 5, and reached maximum expression, 33-times higher than CTRL values, at day 5. MMP-9 remained elevated for 2 weeks after MeBr then returned to CTRL by day 40. MMP-2 expression was low in CTRL animals and remained near CTRL following MeBr injury, though an increase in expression (2.1-times higher than CTRL) was detected on day 5.

To determine if the MeBr injury leads to a widespread activation of MMP-9 in the CNS, we monitored MMP-9 expression in the frontal cortex (Figure 4.2). At each recovery time point, MMP-9 expression in frontal cortex remained at CTRL levels, suggesting that the MeBr injury response was limited to the olfactory system in contrast with other systemically administered toxins (Colin-Barenque et al., 2008). This finding is consistent with other studies of MeBr injury (Schwob et al., 1995).

In addition to MMP expression, we monitored changes in OMP and glial fibrillary acidic protein (GFAP) within the olfactory bulb (Figure 4.3). OMP, a protein expressed in mature olfactory neurons, was used to monitor deafferentation and subsequent reinnervation of the bulb. The mean OMP values from four separate experiments normalized to CTRL levels are plotted in Figure 4.3A. OMP reached its lowest levels by day 15, corresponding to deafferentation of the bulb. By day 40, OMP levels had increased approaching CTRL levels, indicating reinnervation of the bulb by regenerated olfactory neurons.
Figure 4.1: Changes in MMP-9 and MMP-2 protein expression in the OB following MeBr. A. Representative Western blots illustrating changes in MMP-9 and MMP-2 expression at different time points following injury. Lane 1 shows purified murine MMP-9 and MMP-2 standards (STD). Lane 2 shows an absence of MMP-9 and minimal detection of MMP-2 in CTRL tissue. CPA bands for each lane were used as controls for protein loading. B&C. Plots of the relative amounts of MMP-9 and MMP-2 expressed as a percentage of CPA and normalized to CTRL levels. MMP-9 increased rapidly on day 5, reaching 33 times the expression levels in CTRL samples. MMP-9 remained elevated for 10 days and then returned to CTRL levels by day 40. MMP-2 expression was detectable but at low levels following injury. At day 5, MMP-2 expression was increased two fold above background CTRL levels. The doublet bands in the MMP-2 Western blot represent the pro and active forms of MMP-2. Data points represent the mean normalized to CTRL ± SEM (n=4 for each time point). ANOVA analysis with a Holm-Sidak post hoc for each time point compared to CTRL is given in Table 4.2. CPA: cyclophilin A; CTRL: control; MeBr: methyl bromide gas; OB: olfactory bulb.
Figure 4.2: Comparison of MMP-9 expression in the OB and frontal cortex following MeBr. Bar graphs represent the relative amount of MMP-9 expressed as a ratio of CPA normalized to CTRL levels. The expression of MMP-9 in the olfactory bulb (black) remains at CTRL levels through day 3 following MeBr. Between days 3 and 5, MMP-9 levels increase rapidly and reach maximum expression. MMP-9 levels begin to decrease by day 7 and return to CTRL levels by day 40. In the frontal cortex (grey), MMP-9 expression did not increase above CTRL levels. Data points represent the mean normalized to CTRL ± SEM (n=4 for each time point). CTRL: control; MeBr: methyl bromide gas; OB: olfactory bulb.
Monitoring GFAP levels allowed for the assessment of reactive gliosis following injury. GFAP is an intermediate filament protein present within astrocytes that increases in response to CNS injury (Silver and Miller, 2004). Mean values of GFAP normalized to CTRL levels from four separate experiments are plotted in Figure 4.3B. After MeBr gas exposure, GFAP expression remained near CTRL levels through day 3. GFAP levels increased by day 10 and achieved maximal expression at day 15. By day 40, GFAP expression started to decrease, although at day 60 was still slightly elevated.

Histological sections of the olfactory bulb following MeBr injury are shown in Figure 4.4. The low power image (Figure 4.4A) provides orientation to the distinct morphological layers of the bulb. Figure 4.4B shows immunohistochemical staining for MMP-9 and MMP-2 during the degeneration of pre-existing olfactory axons (days 1, 5, and 10). No MMP-9 signal was detected on day 1, confirming the Western blot analysis. At day 5, MMP-9 immunoreactivity was observed in cells in all regions of the bulb, though the cells labeled with MMP-9 were highly concentrated in the olfactory nerve and glomerular layers where the olfactory axons traverse and terminate, respectively (Figure 4.4B & Table 4.1). By day 10, MMP-9 immunoreactivity decreased although still detectable within the bulb. MMP-2 labeled cells were not observed at any of the three time points. The combination of 3, 3’-diaminobenzidine (DAB) and hematoxylin staining (Figure 4.5A & B) was used to demonstrate that MMP-9 was localized to neutrophils, which were identifiable on the basis of their lobulated nuclei.

Table 4.2 shows 2-way ANOVA and Holm-Sidak post-hoc analysis demonstrating the Western blot recovery time points that are significant compared to CTRL levels for MMP-9, MMP-2, OMP, and GFAP expression.
**Figure 4.3:** Changes in OMP and glial fibrillary acidic protein in the OB following MeBr. Plots represent relative amount of protein for OMP and glial fibrillary acidic protein (GFAP) expressed as a ratio of CPA normalized to CTRL levels in four separate experiments. A. OMP is a marker for degeneration and regeneration of olfactory neurons. OMP levels declined slowly following MeBr injury, reaching a minimum at day 15, indicating deafferentation of the bulb. By day 40 OMP begins to increase towards CTRL levels, corresponding to reinnervation of the bulb. B. GFAP, representing astrocytic activation and gliosis, remained low until day 5, slowly increased and reached a maximum level at day 15. Data points represent the mean normalized to CTRL ± SEM (n=4 for each time point). ANOVA analysis with a Holm-Sidak post hoc for each time point compared to CTRL is given in Table 4.2. CPA: cyclophilin A; CTRL: control; GFAP: glial fibrillary acidic protein; MeBr: methyl bromide gas; OB: olfactory bulb; OMP: olfactory marker protein.
Figure 4.4: Coronal sections of the mouse OB following MeBr. A. Diagram (left) illustrates approximate location of histological images. Image (right) shows the different anatomical layers of the olfactory bulb. B. Images stained for MMP-9 and MMP-2 at recovery days 1, 5 and 10. MMP-9 expression was not observed at day 1. MMP-9 labeled cells were detected in all layers of the OB, concentrated within the NL and GL at day 5. Fewer MMP-9 labeled cells were observed at day 10. MMP-2 labeled cells were not observed at day 1, 5 or 10. EPL: external plexiform layer; GCL: granular cell layer; GL: glomerular layer; IPL: internal plexiform layer; MeBr: methyl bromide gas; MCL: mitral cell layer; OB: olfactory bulb; ONL: olfactory nerve layer.
Figure 4.5: Localization of MMP-9 to inflammatory cells in the OB at day 5. **A&B:** At low magnification (40x), MMP-9 labeled cells are observed in the NL and GL of the OB. Lower panels: At high magnification (60x), MMP-9 labeling was observed in neutrophils, having the characteristic polymorphic nuclei. Arrows in A and B (low magnification) identify cells shown below at high magnification. GL: glomerular layer; NL: nerve layer; OB: olfactory bulb.
Table 4.1: Average number of MMP-9 labeled cells and areas of OB layers

<table>
<thead>
<tr>
<th>Bulb Layer</th>
<th>Day 1</th>
<th>Day 5</th>
<th>Day 10</th>
<th>Area (mm^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NL &amp; GL</td>
<td>15.0 ± 3.0</td>
<td>236.0 ± 29.8</td>
<td>39.3 ± 11.8</td>
<td>0.171 ± 0.029</td>
</tr>
<tr>
<td>EPL</td>
<td>5.0 ± 1.7</td>
<td>72.0 ± 3.6</td>
<td>17.3 ± 10.5</td>
<td>0.163 ± 0.050</td>
</tr>
<tr>
<td>ML &amp; IPL</td>
<td>1.7 ± 0.6</td>
<td>10.3 ± 2.1</td>
<td>3.3 ± 2.5</td>
<td>0.057 ± 0.025</td>
</tr>
<tr>
<td>GCL</td>
<td>2.7 ± 2.1</td>
<td>49.0 ± 8.9</td>
<td>9.7 ± 6.5</td>
<td>0.187 ± 0.096</td>
</tr>
</tbody>
</table>

OB: olfactory bulb; NL: nerve layer; GL: glomerular layer; EPL: external plexiform layer; ML: mitral layer; IPL: internal plexiform layer; GCL: granule cell layer
Table 4.2: Table summarizing the significant Western blot recovery points compared to CTRL for MMP-9, MMP-2, OMP, and GFAP

<table>
<thead>
<tr>
<th>Recovery</th>
<th>MMP-9</th>
<th>MMP-2</th>
<th>OMP</th>
<th>GFAP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>p</td>
<td></td>
<td>p</td>
<td></td>
</tr>
<tr>
<td>D1</td>
<td>0.679</td>
<td>0.934</td>
<td>0.907</td>
<td>0.755</td>
</tr>
<tr>
<td>D3</td>
<td>0.995</td>
<td>&lt;0.001 **</td>
<td>0.205</td>
<td>0.866</td>
</tr>
<tr>
<td>D5</td>
<td>&lt;0.001 **</td>
<td>&lt;0.001 **</td>
<td>0.258</td>
<td>0.103</td>
</tr>
<tr>
<td>D7</td>
<td>0.014 *</td>
<td>0.040 *</td>
<td>0.313</td>
<td>0.056</td>
</tr>
<tr>
<td>D10</td>
<td>0.139</td>
<td>0.120</td>
<td>0.199</td>
<td>0.001 **</td>
</tr>
<tr>
<td>D15</td>
<td>0.032 *</td>
<td>0.102</td>
<td>0.002 **</td>
<td>&lt;0.001 **</td>
</tr>
<tr>
<td>D40</td>
<td>0.948</td>
<td>0.217</td>
<td>0.134</td>
<td>0.008 **</td>
</tr>
<tr>
<td>D60</td>
<td>0.905</td>
<td>0.003 **</td>
<td>0.136</td>
<td>0.102</td>
</tr>
</tbody>
</table>

*: p<0.05; **: p<0.01
4.4 Discussion

**Comparison of the two injury models.** Following trauma, the CNS uses different mechanisms to prevent further injury and to initiate a recovery process. However, some mechanisms may be important for only a specific component of injury such as neuronal deafferentation. Most CNS injury models include both deafferentation and direct trauma and therefore are unable to distinguish between the two. This limits our understanding of how the CNS responds to and resolves deafferentation vs. direct trauma. The olfactory system offers a unique opportunity to assess mechanisms following both deafferentation (MeBr) and direct traumatic injury (NTx) in the CNS. These two olfactory injury models result in deafferentation followed by a reinnervation of the olfactory bulb. MeBr is a peripheral deafferentation injury for which there is no direct lesion to the CNS since the olfactory epithelium is separated from the bulb by the bony cribriform plate. In contrast NTx injury includes direct trauma to CNS tissue including the olfactory bulb and nerve layer in addition to bulb deafferentation. Comparing these two injury models allows for the identification of processes associated with deafferentation and/or trauma. Expression of MMPs in both injury models suggests they play a common role in deafferentation of the olfactory bulb.

**MMPs in CNS injury.** Recovery from CNS injury requires the remodeling of the ECM and breakdown of scar tissue for successful recovery. MMPs have become the focus of recent studies of neuronal injury due to their ability to degrade many prominent components of the ECM and scar. Previous reports have demonstrated MMP-9 and MMP-2 have temporal expression patterns following different CNS trauma models including olfactory NTx, spinal cord injury, and stroke suggesting an important role in
neuronal injury and recovery (Costanzo et al., 2006; Costanzo and Perrino, 2008; de, Jr. et al., 2000; Romanic et al., 1998). MMP-9 expression increases early in response to these CNS injuries, corresponding to inflammation, vascular breakdown, edema, and neuronal death. The inflammatory leukocytes are co-localized with MMP-9. These cells may use MMP-9 to penetrate the blood vessel walls and migrate through the ECM to reach the site of injury (Busch et al., 2009; Fleming et al., 2006; Rosell et al., 2008). MMP-9 has also been shown to disrupt the ECM and neuronal connections, leading to neuronal degeneration and death (Gu et al., 2002; Siebert et al., 2001). MMP-2 expression increases within a week following many CNS injuries. This corresponds to the reparative phases of neuronal recovery when MMP-2 participates in the remodeling of the ECM, digests scar components, and participate in angiogenesis (Hsu et al., 2006; Montaner et al., 2001; Zuo et al., 1998).

The inhibition of these enzymes has demonstrated the importance of MMPs in the injury and recovery process. In the absence of MMP-9 there is improved recovery in stroke injury models whereas the inhibition of MMP-2 is detrimental to neuronal recovery (Asahi et al., 2001; Hsu et al., 2006; Lee et al., 2004; Noble et al., 2002). The modulation and temporal expression of MMP-9 and MMP-2 during olfactory injury and recovery provides potential targets for therapeutic intervention and improved outcome.

**MMPs in olfactory injury and recovery.** In this report, we demonstrate increased MMP-9 and MMP-2 expression in the olfactory bulb after MeBr exposure (Figure 4.1). Between days 3 and 5, MMP-9 rose rapidly and reached expression levels 33-times higher than in CTRL samples at day 5. This increase following MeBr is isolated to the olfactory system, as demonstrated by the absence of MMP-9 expression in the
frontal cortex following MeBr exposure (Figure 4.2), confirming that MeBr injury does not have a diffuse MMP response in the CNS. The increase in MMP-9 expression in MeBr injury is delayed when compared to the 5 hour increase with direct CNS injury such as olfactory NTx. This delay in MMP-9 expression may reflect the time necessary for the degeneration process to reach the axon terminals within the bulb. In contrast, the rapid response of MMP-9 following NTx could be due to components of direct injury specifically vascular disruption and trauma to CNS structures such as the anterior-ventral surface of the bulb. With vascular injury, components of clotting cascade are increased, including tissue-plasminogen activator (tPa) and urokinase plasminogen activator (uPa), both known regulators of MMP-9 expression (Menshikov et al., 2002; Wang et al., 2003). Since MeBr injury occurs within the olfactory epithelium, a site distant from the olfactory bulb, bleeding is unlikely to occur within the CNS, and therefore, vascular-induced MMP-9 expression is not expected.

The constellation of findings in the olfactory bulb after MeBr exposure are similar in many respects to direct CNS injury (i.e., NTx), and therefore, MMP-9 may be playing a common role. During the initial recovery period following neuronal injury, two processes that rely on MMP-9, glial scar formation and leukocyte infiltration, are increased at the site of injury (Hsu et al., 2008). We demonstrated that MMP-9 is localized to neutrophils concentrated in the region of the injured nerve axonal projections following MeBr (Figures 4.4, 4.5, & Table 1). This suggests that the signal for MMP-9 expression is the deafferentation injury. MMP-9 may allow these inflammatory leukocytes to penetrate through the vascular wall and ECM to reach and degrade the injured axons. Likewise following MeBr injury, astrocytic activation is
observed within the bulb as demonstrated by high levels of GFAP at day 10 and in previous immunohistochemical analysis (Schwob et al., 1999). The increased expression of GFAP paralleled the elevated levels of MMP-9. This association is also demonstrated following olfactory NTx, where GFAP increased 3 days following injury during elevated MMP-9 expression. This suggests similar processes are involved in both MeBr and NTx, leading to increases in MMP-9 expression.

MMP-2 expression was detectable but low in both CTRL and MeBr injury samples, though a 2-fold increase was observed on day 5. Low levels of MMP-2 detection may reflect its constitutive expression in the CNS (Rosenberg, 2002). The response of MMP-2 following MeBr is minimal compared to NTx, where up to a 20-fold increase in expression has been reported (Costanzo and Perrino, 2008). This dramatic increase in MMP-2 following NTx occurs 7 days after injury, corresponding to the transition between deafferentation and reinnervation of the bulb. In MeBr injury, this transformation was observed between days 15 and 40, as demonstrated by changes in OMP (Figure 4.3A). The transition between deafferentation and reinnervation of the bulb following MeBr is delayed compared to NTx and may explain the relatively low MMP-2 expression in our MeBr injury model. The small increase in MMP-2 at day 5 may represent changes in the olfactory bulb with second order neurons, specifically the mitral and tuft cells. As mitral and tuft cells synaptic connections are lost in the glomerular layer, these neurons may secrete MMP-2 in order to modify the ECM in an attempt to create new synapses. Further work will be necessary to determine if MMP-2 is being expressed by the regenerated first order neurons, or the second order neurons, in an attempt to create new synaptic connections.
Conclusion. A comparison of MeBr and NTx injury models has proven useful in uncovering important information underlying neuronal injury and recovery in the olfactory system. In both injury models, we demonstrated an early expression of MMP-9 corresponding to inflammatory processes. This suggests that, regardless of the mechanism of injury, inflammation is a key component of neuronal injury and recovery. Though the importance of MMP-2 is still unclear, we did observe differences in the timing of MMP-2 expression between the two injury models. This is the first report demonstrating that MMP-9 expression is associated with deafferentation in the absence of direct trauma. Further studies are needed to determine the underlying mechanisms and contributions of MMP-9 and MMP-2 to neuronal injury and recovery processes.
Chapter 5

The Peak Expression of MMP-2 Following Nerve Transection is Dependent on Reinnervation of the Olfactory Bulb

5.1 Introduction

In Chapter 2, MMP-2 expression peaked sharply at day 7 following NTx, suggesting an important process is occurring at this time point (Figure 2.8). Previous reports have localized MMP-2 expression to macrophages, glial cells, and neurons in the normal and injured CNS (Anthony et al., 1997; Planas et al., 2001). Following NTx, both macrophages (Chapter 3: Figure 3.4) and gliosis (Costanzo 2006) are increased in the NTx bulb by day 3, suggesting that these processes may not be responsible for the peak expression of MMP-2. Therefore, MMP-2 may be related to regenerated axons initially contacting the bulb at day 7. The time course of neuronal degeneration and recovery in the bulb has been previously defined by monitoring OMP expression, a marker for mature sensory neurons (Chapter 2: Figure 2.3). Following NTx, OMP levels decreased at day 1 and remained below CTRL levels through day 10, signifying the degeneration of sensory axons. By day 15, OMP expression increased back to CTRL, indicating regenerated axons penetrating the OB. However, reinnervation of the OB is likely to occur before this period, as the transition from immature and mature neurons may take up to a week (Miragall and Monti Graziadei, 1983). This suggests that
immature axons may initially contact the bulb at day 7 but delay expressing OMP until day 15.

This chapter has two objectives: 1) determine the source of MMP-2 in CTRL and day 7 NTx bulbs, and 2) establish a relationship between MMP-2 and regenerated axons at day 7. The hypothesis of the first objective is that MMP-2 will be expressed by resident neurons (granular cells and second order neurons) in both CTRL and day 7 NTx bulbs. At day 7, regenerated axons in the bulb will also express MMP-2, leading to the peak expression observed. The hypothesis of the second objective is that the peak expression of MMP-2 at day 7 will be attenuated if regenerated axons are blocked from reaching the bulb. Blockage of sensory axons will be accomplished by insertion of a Teflon barrier following NTx. These experiments will identify the cellular source of MMP-2 and begin to uncover the importance of this MMP in neuronal injury and recovery.

5.2 Materials and Methods

**NTx surgical procedure.** Adult P2-IRES-tau-lacZ mice were anesthetized with sodium pentobarbital (80 mg/kg, intraperitoneal). After anesthesia, the left OB was exposed and a thin Teflon cutting blade was inserted between the left bulb and the cribriform plate. The use of a Teflon blade resulted in transection of all the olfactory axons that connect to the left bulb, and minimal damage to the bulb and cribriform plate. After transection, the skin incision was sutured and each animal was observed postoperatively before returning to its cage. The right bulb was not transected and serves as an internal CTRL for histological analysis.
**Teflon barrier surgical procedure.** The NTx protocol was performed as described above. Following NTx, a thin piece of Teflon was inserted between the left bulb and cribriform plate. The skin incision was sutured, leaving the barrier in place until the animal was sacrificed. All procedures were approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University.

**Immunohistochemistry.** Immunohistochemical staining for MMP-2 was performed on CTRL and day 7 NTx bulbs. Hematoxylin and eosin and immunohistochemical staining for MMP-2 and GAP-43 were performed on nerve transection with Teflon barrier (NTx-TB) bulbs at day 7. Mice were anesthetized with sodium pentobarbital and perfused with saline followed by 4% paraformaldehyde (INC Biomedicals, Inc., Aurora, OH). The skull was removed and placed in 4% paraformaldehyde for 30 minutes, then rinsed with tap water for 5 minutes and immersed in Decal overnight (Decal Chemical Corp, Tallman, NY). The following day, skulls were placed in PBS containing 30% sucrose for a week then frozen in a minus 80°C freezer. Horizontal sections were cut on a Microm HM 550 series Cyrostat (MICROM International GmbH, Walldorf, Germany) and placed on Superfrost® Plus VWR® Micro Slides (VWR, West Chester, PA). The slides were stored in a minus 20°C freezer until processed.

The slides were allowed to reach room temperature before washing with Invitrogen (Carlsbad, CA) phosphate-buffered saline (PBS) for 10 minutes. Microwave antigen retrieval was performed as previously described (Stone et al 1999) with some modifications. Sections were heated to 65°C for 8 minutes and allowed to cool for 20 minutes at room temperature. After washing 3 times in PBS, the slides were blocked
and permeabilized for 1 hour in a solution containing 10% normal donkey serum (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) and 0.2% Triton X-100 (LabChem Inc. Pittsburgh, PA) in PBS. The sections were incubated with primary antibody in blocking solution overnight: goat anti-MMP-2 (1:10; R&D Systems, Minneapolis, MN) and rabbit anti-GAP-43 (1:500; Novus Biologicals. Littleton, CO). The following day the slides were washed 3 times in PBS and placed in the species appropriate donkey secondary antibody. MMP-2 was visualized with CY3 conjugated donkey anti-goat (1:100; Jackson ImmunoResearch, Inc., West Grove, PA) and GAP-43 visualized with AlexaFluor 488 conjugated donkey anti-rabbit (1:100; Invitrogen). The slides were analyzed on an Eclipse E600 microscope (Nikon Inc, Melville, NY).

**Western blot tissue sampling and preparation.** At 5 hour and 1, 3, 7, 10, 15, and days after surgery, mice were anesthetized with sodium pentobarbital and killed by rapid decapitation. The left OB tissue was carefully removed along with left bulb tissue from CTRL animals (receiving no surgical treatment). Although the right bulb of transected mice serves as internal CTRL, occasionally some injury is observed in this bulb. Therefore, CTRL animals were used to ensure that no injury was present in CTRL samples. Left bulbs were immediately placed in protein extraction buffer (50 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 1% sodium dodecylsulfate, 1% DOC) and homogenized with a motor-driven plastic homogenizer. The tissue was incubated on a rotating platform for 20 minutes at 4°C and centrifuged at 16 000g for 30 minutes at 4°C. Solubilized proteins in the supernatant were quantified with a DC protein assay reagent kit (Bio-Rad Laboratories, Hercules, CA), using bovine serum albumin as a standard. Protein measurements were made at 720 nm on a μQuant plate reader (BioTek
Instruments Inc., Winooski, VT). Equal protein samples and purified murine MMP-2 (Chemicon, San Francisco, CA) were loaded onto Bis-Tris 4–12% density gradient gels and separated using NuPAGE MES [2-(N-morpholino) ethane sulfonic acid] reducing buffer system (Invitrogen) for 1 hour at 200 V and 4°C. Protein was transferred to nitrocellulose membranes for 2 hours at 25 V and 4°C. Nonspecific binding was blocked with 5% bovine nonfat dry milk in Tris-buffered saline and 0.05% Tween-20 for 1 hour. Goat anti-MMP-2 primary antibody (1:200) was obtained from R&D Systems. Goat anti-OMP (1:20,000; Wako Chemicals, Richmond, VA) was used as a marker of axonal degeneration and regeneration in the bulb. Rabbit anti-CPA (1:5000; Upstate, Lake Placid, NY) antibody was used to standardize protein loading. Nitrocellulose membranes were incubated overnight in primary antibody at 4°C. Membranes were then treated with species-specific peroxidase-conjugated IgG secondary antibodies (Rockland, Gilbertsville, PA), incubated for 1 minute with Western Lightning Plus reagent (Perkin Elmer, Wellesley, MA) and exposed to Blue Sensitive Autoradiography film (Marsh BioProducts, Rochester, NY).

**Protein measurement.** Quantification of protein expression was performed using Quantity One Analysis software (Bio-Rad Laboratories). Protein expression was measured as a function of both band density and band area. This density–area measurement for MMP-2 and OMP at each recovery time point was then standardized against the corresponding density–area measurement for CPA in the same gel. The protein measurement of MMP-2 or OMP was then expressed as a ratio of CPA (amount of protein divided by amount of CPA) and then normalized to CTRL levels (amount of protein/CPA ratio divided by amount of CTRL protein/CPA ratio).
**Statistical analysis.** Protein levels at each of the Western blot recovery time points were compared to CTRL values using 2-way ANOVA and a Holm-Sidak 2-sided post hoc test. A probability of less than 0.05 was considered statistically significant. All statistical analysis was performed using SPSS software (IBM, Chicago, IL).

5.3 Results

**MMP-2 localization in CTRL and day 7 NTx bulbs.** To determine the cellular source of MMP-2 expression, immunohistochemical analysis was performed on CTRL and NTX (day 7) bulbs (Figure 5.1). In the CTRL bulb, MMP-2 was apparent in the deep granule cell layer. This layer is composed mainly of granule cells (small inhibitory neurons) and glial cells. At high power, MMP-2 is observed in long processes that extend from the deep layers of the bulb, through the external plexiform layer and terminate in the glomerular layer. Contained in the external plexiform layer are the dendrites of second order neurons (mitral and tufted cells) and glial cells. In the day 7 bulb, MMP-2 was localized to the granule cell and external plexiform layers, similar to the CTRL bulb. MMP-2 was not apparent along the anterior aspect of the bulb, where injured tissue is observed or the nerve and glomerular layers, the location of regenerated axons.

**Teflon barrier.** To determine if MMP-2 expression is associated with reinnervation of the OB, a Teflon barrier was inserted between the cribiform plate and
Figure 5.1: Comparison of MMP-2 labeling in CTRL and transected OB. In the low power image, MMP-2 is apparent throughout the GCL of the CTRL OB. MMP-2 is also observed in the EPL. High power of the EPL shows MMP-2 expression in long processes (arrow) extending through this layer and terminating in the glomerular layer (GL). The day 7 NTx OB at low power shows a similar pattern of MMP-2 labeling compared to CTRL. MMP-2 expression is apparent in the GCL, however is not seen along the anterior aspect of this layer. This portion of the bulb is damaged during the NTx procedure and contains injured tissue. High power image of the EPL shows MMP-2 in processes extending from the EPL to the GL, similar to the CTRL image. CTRL: control; EPL: external plexiform layer; GCL: granule cell layer; GL: glomerular layer; NTx: nerve transection; OB: olfactory bulb. Scale bars: low magnification: 100 μm; high magnification: 50 μm.
OB. This barrier serves to block regenerated axons as they pass through the cribiform plate. Figure 5.2A shows the site of Teflon barrier insertion between the bulb and cribiform plate. Occasionally, the barrier fell off the slide during tissue processing, leaving a blank space (Figure 5.2A right panel).

As axons reach the barrier, they are unable to contact the bulb and change their path (Figure 5.2B arrows). Some axons are seen circumventing the block along the medial and lateral edges of the barrier (Figure 5.2B asterisks).

To verify that the Teflon barrier was successful at blocking regenerated axons from reaching the bulb at the gross level, whole mount preparations were compared between NTx and NTx-TB bulbs at day 35 (Figure 5.3). The Teflon barrier was inserted between the anterior OB and cribiform plate. The barrier does not encompass the entire bulb, and therefore, it is not seen in the medial view of the whole mounts (Figure 5.3B). Regenerated P2 axons are evident in the anterior portion of the day 35 NTx bulb. In contrast, the NTx-TB mice have considerable depletion of P2 axons in the bulb. Axons that are blocked converge along the anterior aspect of the barrier (Figure 5.3B asterisks). Some axons are observed circumventing the barrier and penetrate the OB (Figure 5.3B arrows).

To confirm that the Teflon barrier blocked the majority of regenerated sensory axons, OMP expression was monitored by Western blot (Figure 5.4). OMP initially decreased between days 1 and 3 following NTx-TB and remained below CTRL levels through day 15 (Figure 5.4B). This indicates the degeneration of sensory axons. In contrast, OMP expression increased by day 15 following NTx (Figure 5.4C). This
Figure 5.2: Teflon barrier blocks regenerated axons from contacting the OB. A. Horizontal sections at day 7 show the site of Teflon barrier insertion between the cribriform plate and OB. The left panel illustrates the barrier in a horizontal section. During tissue processing, the barrier occasionally fell off the slide, leaving a blank space (right panel). B. High power images comparing the trajectory of sensory axons in CTRL and NTx-TB OB. Sensory axons normally pass through the cribriform plate projecting directly to the NL and terminate on the GL (left two panels). The right panels demonstrate that the insertion of a Teflon barrier prevents the axons from contacting the bulb. The axons that originate from the anterior epithelium appear to alter their path at the barrier (arrows). Some axons are observed bypassing the block and contacting the OB along the medial and lateral aspects of the barrier (asterisks). CTRL: control; GL: glomerular layer; NL: nerve layer; NTx-TB: nerve transection with Teflon barrier; OB: olfactory bulb. Scale bar: A. 500 μm; B. 100 μm.
Figure 5.3: Comparison of P2 axonal projections at day 35 following NTx or NTx-TB. **A.** Diagram comparing horizontal views of the NTx and NTx-TB. The Teflon barrier (red) is inserted between the cribriform plate and OB. The barrier encompasses only the anterior portion of the bulb and therefore it is not seen in the medial view in the whole mount images. **B.** Whole mount preparations showing the differences in P2 axonal projection in NTx (left) and NTx-TB (right). P2 axons are apparent in the bulb following NTx and converge onto multiple glomeruli by day 35. In NTx-TB, most of the P2 axons are blocked from entering the bulb. These blocked axons are observed along the cribriform plate, in front of the barrier (asterisks). Some axons are seen bypassing the barrier and penetrate the bulb (arrow). NTx: nerve transection; NTx-TB: nerve transection with Teflon barrier; OB: olfactory bulb. Scale bar: 500 μm.
demonstrates that the barrier was successful at blocking the initial reinnervation of the OB in NTx-TB mice. Between days 15 and 35, OMP expression in the NTx-TB mice increased towards CTRL, indicating that some regenerated axons bypassed the barrier and penetrated the bulb.

Having established that the Teflon barrier successfully blocked regenerated axons, MMP-2 expression was measured by Western blot analysis (Figure 5.5). In CTRL animals, MMP-2 levels were detectable. Following NTx-TB, a 5-fold increase in MMP-2 expression was observed, which was sustained through day 35 (Figure 5.5B). In contrast, a large peak in MMP-2 levels was evident at day 7 following NTx (Figure 5.5C). This indicates that the peak expression of MMP-2 at day 7 is associated with regenerated neurons contacting the bulb.

To determine if regenerated axons contain MMP-2, immunohistochemical analysis of MMP-2 and growth associated protein-43 (GAP-43) was performed on day 7 NTx-TB bulbs. GAP-43 is a molecule expressed in the growth cone of developing and regenerated axons. At day 7, histological analysis demonstrates sensory axons immediately anterior to the barrier (Figure 5.6 H&E low power and high power). GAP-43 is expressed by these axons, signifying that they are regenerated and migrating towards the OB. However, MMP-2 was not observed in the area of the axons, indicating that MMP-2 is not contained in regenerated axons following NTx-TB.

Table 2.1 shows the ANOVA and Holm-Sidak post-hoc analysis indicating the Western blot recovery time points that are significant compared to CTRL levels for OMP and MMP-2 expression.
Figure 5.4: Changes in OMP expression following NTx-TB. **A.** Representative Western blot illustrating OMP expression at different recovery time points. CPA levels serve as a gel loading CTRL. **B.** Plot of the mean OMP expression, normalized to CTRL levels, from four separate Western blots. Following NTx-TB, OMP levels remain near CTRL through day 1. By day 3, OMP expression decreased, indicating degeneration of sensory neurons. This decrease continues through day 15, when OMP levels are minimal. Between days 15 and 35, OMP levels increase, suggesting some of the regenerated axons bypass the barrier. **C.** In NTx, OMP expression was increased by day 15, signifying regenerated axons penetrating the bulb. This indicates that the barrier successful at blocking the initial regenerated axons from reaching the bulb. Data points represent the mean ± SEM (n=4 at each time point). ANOVA analysis with a Holm-Sidak post hoc for each time point compared to CTRL is given in Table 5.1. CPA: cyclophilin A; CTRL: control; NTx: nerve transection; NTx-TB: nerve transection with Teflon barrier; OMP: olfactory marker protein.
Figure 5.5: Expression of MMP-2 following NTx-TB. A. Representative Western blot showing changes in MMP-2 expression at different recovery time points in the bulb. Lane 1 contains purified pro and active murine MMP-2. CTRL lane demonstrated detectable levels of MMP-2. CPA levels serve as a gel loading CTRL. B. Plot of the mean expression of MMP-2, normalized to CTRL levels, of four separate Western blots. A 5-fold increase in MMP-2 expression is detected as early as 5 hours following NTx-TB. This level was sustained through the recovery time period measured. C. In NTx, there was a large peak in MMP-2 expression observed at day 7. This suggests that the peak in MMP-2 expression following NTx is dependent on regenerated sensory axons in the OB. Data points represent the mean ± SEM (n=4 at each recovery time point). ANOVA analysis with a Holm-Sidak post hoc for each time point compared to CTRL is given in Table 5.1. CPA: cyclophilin A; CTRL: control; NTx: nerve transection; NTx-TB: nerve transection with Teflon barrier.
Figure 5.6: Regenerated axons do not express MMP-2 following NTx-TB. Three examples (A, B, C) showing axons (asterisk) projecting from the OE to the Teflon barrier (TB) at day 7. The high power images demonstrate the sensory axons (asterisks) that are analyzed in the immunohistochemical images. The sensory axons are labeled with growth associated protein-43 (GAP-43: green), a marker for growing axons, signifying that these axons projecting to the TB are regenerated. MMP-2 expression (red) was not observed to colocalize with GAP-43, indicating that regenerated axons do not express MMP-2 as they project to the TB. The immunofluorescent analysis (GAP-43 and MMP-2) was performed on sections 10 μm adjacent to the histological sections (low and high power). GAP-43: growth associated protein-43; OE: olfactory epithelium; TB: Teflon barrier. Scale bars: low magnification: 1 mm; high magnification: 10 μm.
Table 5.1: Table summarizing the significant Western blot recovery points compared to CTRL for OMP and MMP-2

<table>
<thead>
<tr>
<th>Recovery</th>
<th>OMP</th>
<th>MMP-2</th>
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<tbody>
<tr>
<td></td>
<td>p</td>
<td>p</td>
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<tr>
<td>5H</td>
<td>0.516</td>
<td>0.007  **</td>
</tr>
<tr>
<td>D1</td>
<td>0.553</td>
<td>0.003  **</td>
</tr>
<tr>
<td>D3</td>
<td>0.329</td>
<td>0.009  **</td>
</tr>
<tr>
<td>D7</td>
<td>0.011 *</td>
<td>0.017 *</td>
</tr>
<tr>
<td>D10</td>
<td>0.054</td>
<td>0.004  **</td>
</tr>
<tr>
<td>D15</td>
<td>0.001 **</td>
<td>0.050 *</td>
</tr>
<tr>
<td>D35</td>
<td>0.088</td>
<td>0.042 *</td>
</tr>
</tbody>
</table>

*: p<0.05; **: p<0.01
5.4 Discussion

Based on emerging data that MMP-2 plays an important role in neuronal recovery, it was hypothesized that MMP-2 is expressed by regenerated axons following NTx. Immunohistochemical analysis demonstrated that MMP-2 is observed in the granule cell and external plexiform layers in CTRL mice. Although the Western blot of MMP-2 demonstrated a large peak at day 7 following NTx, surprisingly the histological analysis failed to show differences in MMP-2 labeling between CTRL and day 7 bulbs. To determine if there is a relationship between the large peak in MMP-2 expression and regenerated axons reaching the bulb at day 7, a Teflon barrier was inserted between the cribriform plate and OB following NTx. This barrier was successful at blocking the initial innervation of the bulb by regenerated axons. The peak in MMP-2 expression was not observed in NTx-TB bulbs. MMP-2 did not localize to the regenerated axons, suggesting that regenerated axons may trigger processes in the OB that increase the expression of MMP-2 at day 7.

MMP-2 expression in CTRL and day 7 NTx bulbs. The olfactory bulb is a highly dynamic structure that requires constant remodeling of the ECM. In normal mice, there is constant turnover of sensory neurons and synaptic connections with second order neurons (e.g. mitral and tufted cells). The deep layer of the OB is also dynamic with small inhibitory neurons, called granule cells, continuously replaced by neuroblasts derived from the subventricular zone of the cortex (reviewed in Curtis et al., 2009). These cells migrate along the rostral migratory stream to the granule cell layer of the OB. The turnover of these two neuronal populations likely involves the remodeling of the ECM. In CTRL animals, MMP-2 was localized to cells in the granule cell layer and
processes extending through the external plexiform layer to the glomerular layer (Figure 5.1). As the granule cells migrate along the rostral migratory stream and enter the granule cell layer of the bulb, they may express MMP-2 to degrade components of the ECM. This would allow these neurons to easily migrate to their target tissue. The constant renewal of sensory axons leads to turnover of synaptic connections with second order neurons in the glomerular layer. MMP-2 may have an important role in dendrite migration through the external plexiform layer to establish synaptic connections with new sensory axons.

An alternative source of MMP-2 expression in the granule cell and external plexiform layers may be the glial cells. In the normal CNS, glial cells, specifically astrocytes, express MMP-2 (Cuzner et al., 1996). Astrocytes are found throughout the CNS, including the OB and play a role in synaptic pruning and remodeling (Barres, 2008; Christopherson et al., 2005; Stevens et al., 2007). The high turnover of neurons in the CTRL bulb likely requires constant modulation of synapses, in which MMP-2 may have an important role.

Histological analysis failed to show differences in MMP-2 labeling between the CTRL and day 7 NTx bulbs. This is surprising because a large peak in MMP-2 expression was measured at day 7 by Western blot (Figure 5.5C). Western blot analysis is a measure of the global changes in protein levels, i.e. samples are taken from the antero-ventral OB. In contrast, immunohistochemical analysis examines a small portion (10 μm) of the OB. There may be subtle changes in MMP-2 expression in the granule cell and external plexiform layers that are undetectable by immunohistochemistry.
Teflon Barrier. To determine if the peak expression of MMP-2 at day 7 is dependent on reinnervation of the OB, regenerated axons were blocked with a Teflon barrier. Regenerated axons travel along a very narrow and defined course, having to pass through the small foramina of the cribriform plate. A barrier can be inserted anterior to the OB, which blocks the axons as they emerge from the cribriform plate. The Teflon barrier was successful at blocking regenerated neurons from reaching the OB. In histological images, blocked sensory axons were evident along the anterior edge of the barrier (Figure 5.2), while whole mount preparations showed the majority of P2 axons did not reach the OB after 35 days recovery (Figure 5.3). Western blot analysis demonstrated that OMP expression reached a minimum at day 15 following NTx-TB (Figure 5.4). In contrast, OMP levels increased by day 15 following NTx, indicating reinnervation of the OB. Taken together, these experiments indicate that the placement of the Teflon barrier between the cribriform plate and OB was successful at blocking regenerated axons. With the NTx-TB injury model established, MMP-2 expression was monitored by Western blot (Figure 5.5). An injury-related increase in MMP-2 was observed following NTx-TB and remained at this level through the time period measured. This indicates that the peak expression of MMP-2 following NTx is dependent on regenerated axons reaching the OB. Immunohistochemical analysis demonstrated that the regenerated axons do not contain MMP-2 following NTx-TB (Figure 5.6). This suggests that the regenerated axons may trigger processes in the OB that increase MMP-2 expression. MMP-2 may modify the ECM to create a supportive environment for regenerated axons to reestablish synaptic connections with second order neurons. Since MMP-2 has the capability to degrade many prominent
components of the ECM, is seems likely that it has an important role neuronal recovery. However, further studies aimed at targeted disruption of MMP-2 are needed to determine the importance of MMP-2 in olfactory injury and recovery.

**Conclusion.** The results presented in this study demonstrate that the peak expression of MMP-2 at day 7 following NTx is dependent on regenerated axons reaching the OB. However, MMP-2 was not expressed by the regenerated axons. In CTRL and day 7 NTx bulbs, MMP-2 was localized to the granule cell layer and processes extending through the external plexiform layer to the glomerular layer. This suggests that MMP-2 is found in resident cells of the bulb (e.g. granule cells, second order neurons, and glial cells). These resident cells may increase the expression of MMP-2 at day 7 to remodel the ECM, providing a supportive environment for regenerated axons to reestablish functional synapses. Future studies aimed at targeted disruption of MMP-2 are needed to determine the role of MMP-2 in neuronal injury and recovery and to assess its potential as a target for therapeutic intervention.
Chapter 6
General Discussions

6.1 Summary of important findings

This project uncovered differences in the temporal expression of MMP-9 and MMP-2 following 3 olfactory nerve injury models (Figure 6.1). These injury models: 1) nerve transection (NTx), 2) methyl bromide gas (MeBr) exposure, and 3) nerve transection with Teflon barrier (NTx-TB), produced different degrees of CNS trauma and neuronal recovery. The changes in MMP-9 and MMP-2 in each model revealed a temporal correlation between MMP expression and neuronal degeneration and reinnervation of the OB.

Chapter 2 presented data that MMP-9 increased immediately in the OB following NTx and remained elevated for two weeks (Figure 2.6). The temporal expression of MMP-9 coincided with decreased OMP expression (Figure 2.3). Chapter 3 presented evidence that neutrophils were a source of MMP-9 following NTx (Figure 3.2). These experiments suggest that MMP-9 is associated with both the acute inflammatory response and neuronal degeneration following NTx. To confirm that the increased MMP-9 level was associated with neuronal degeneration, the temporal expression of MMP-9 following MeBr exposure was measured (Chapter 4). MeBr is a toxic compound that ablates the olfactory epithelium, leading to neuronal degeneration and subsequent regeneration, without direct trauma to the CNS. Following MeBr exposure, MMP-9
expression increased in the OB at day 5 and remained elevated through day 15, while neuronal degeneration was maximal between days 3 and 15 (Figures 4.1B & 4.3A). MMP-9 was localized to neutrophils primarily in the nerve and glomerular layers of the OB (Figures 4.4 & 4.5; Table 4.1). The temporal expression and location of MMP-9 corresponded to neuronal degeneration in the OB, demonstrating that MMP-9 is associated with the degradation of sensory axons.

MMP-2 was constitutively expressed by resident cells in the granule cell and external plexiform layers of the bulb (Figure 5.1). An injury-related increase in MMP-2 was observed following NTx, with a significant peak apparent at day 7 (Figure 2.8). This recovery day corresponded to the transition between neuronal degeneration and reinnervation of the OB (Figure 2.3). To determine if the day 7 peak in MMP-2 expression was dependent on regenerated axons in the OB, a Teflon barrier was inserted between the OB and cribriform plate. The Teflon barrier successfully blocked the initial reinnervation of the bulb at day 7 (Figures 5.2, 5.3, & 5.4) and, consequently, MMP-2 levels did not peak (Figure 5.5B). This demonstrated that the peak levels of MMP-2 following NTx are dependent on the reinnervation of the OB at day 7. Surprisingly, MMP-2 was not localized to the regenerated axons (Figure 5.6). Therefore, regenerated axons contacting the OB likely trigger processes that increase expression of MMP-2 at day 7.

This is the first study to associate the temporal relationship of MMP-9 with neuronal degeneration and MMP-2 with reinnervation of the OB by comparing 3 olfactory injury models. Future studies aimed at targeted disruption of MMP-9 and
**Figure 6.1:** Diagram illustrating the temporal correlation between MMP-9 and degeneration, and MMP-2 and the onset of reinnervation. In all 3 injury models (A, B, & C), increased MMP-9 levels (blue) correlated with neuronal degeneration (red). In NTx injury (A), a large peak in MMP-2 (grey) expression was observed at day 7 and correlated with the transition between degeneration and reinnervation (green). In MeBr injury (B), the transition from degeneration to reinnervation occurred between days 15 and 35, recovery time periods not studied (shaded area). In the NTx-TB model (C), regenerated axons were blocked from reaching the OB and MMP-2 expression remained low. The absence of a MMP-2 peak in NTx-TB suggests that the peak is dependent on reinnervation of the OB. MMP-2 (grey) is constitutively expressed in control bulbs and present prior to each injury (arrow). MeBr: methyl bromide gas; NTx: nerve transection; NTx-TB: nerve transection with Teflon barrier; OB: olfactory bulb.
MMP-2 can determine the role of these two MMPs following olfactory injury and may uncover novel therapeutic targets to improve neuronal recovery.

6.2 Future Directions

This report describes the temporal correlation of MMP-9 with neuronal degeneration and MMP-2 with the initial transition to recovery. However, the functional role of these two MMPs remains unclear. Future studies aimed at targeted disruption of expression or activity can determine the significance of the MMP-9 and MMP-2 in olfactory injury and recovery. Studies with gene knockout mice have demonstrated important roles for MMP-9 and MMP-2 in other models of CNS injury. Following both stroke and spinal cord injuries, MMP-9−/− mice have improved functional recovery (Lee et al., 2004; Noble et al., 2002). In contrast, MMP-2−/− mice have diminished neuronal recovery after SCI (Hsu et al., 2006). Behavioral studies with wild-type, MMP-9−/−, and MMP-2−/− would be a simple way to determine whether these MMPs contribute to neuronal recovery following olfactory injury. If either MMP is important for neuronal regeneration, then genetic knockout would attenuate the detection and discrimination of odors following olfactory injury. In addition, changes to the topographical map can be monitored with the development of a P2-tau lacZ/MMP−/− mouse. This mouse would demonstrate changes in the topographical map compared to wild-type mice following injury, depending on the role of MMP-9 and MMP-2 in neuronal recovery.

Other MMPs in olfactory injury and recovery. Accumulating evidence points towards other MMPs having important roles in CNS injury and recovery. The findings from this present study can direct future research towards other MMPs that may have
important role in olfactory neuronal regeneration. Previous studies have shown that the prodomain of MMP-9 is cleaved by MMP-3 (stromalysin-1), a key step in the activation of MMP-9 (Ogata et al., 1992; Rosenberg et al., 2001). This represents a possible mechanism for MMP-9 activation following olfactory injury. In other models of CNS injury, including SCI and traumatic brain injury, MMP-3 increased early and was localized to glial cells and apoptotic neurons (Falo et al., 2006; Grossetete et al., 2009; Kim et al., 2005). MMP-3 can induce the generation of inflammatory cytokines and recruitment of inflammatory cytokines and may represent an early trigger for the inflammatory response (Kim et al., 2005). As the initial inflammatory leukocytes infiltrate the injured tissue, MMP-3 can activate MMP-9 secreted by neutrophils. MMP-3/- and MMP-9/- mice have similar neuroprotection following global ischemia, suggesting that MMP-3 may be key regulator of MMP-9 in vivo or has a similar role in neuronal injury and recovery (Walker and Rosenberg, 2009). Another MMP that may be present following olfactory injury is MT1-MMP, an important activator of MMP-2. The mechanism of converting latent MMP-2 to the active form by MT1-MMP has been previously described (Chapter 1, section 1.2.2). Following NTx, both the pro and active forms of MMP-2 are increased in the OB at day 7. Therefore, it can be logically assumed that MT1-MMP will be located in the bulb following injury. MT1-MMP is also known to be important in cellular migration and processing of cell adhesion molecules, however its importance in neuronal degeneration and recovery in the CNS has yet to be investigated (Itoh, 2006).

The infiltration of neutrophils into the injured bulb represents another source for MMPs following olfactory injury. The granules of neutrophil contain both MMP-9 and
MMP-8 (neutrophil collagenase) and secrete these MMPs in response to injury (Toft-Hansen et al., 2004; Murphy et al., 2002). To this point, the study of MMP-8 in the CNS has been limited to the inflammatory response following injury. Finally, research has started to focus on MMP-12 as an important protein for CNS injury and recovery. Buss et al. (2007) demonstrated that MMP-12 increased in response to SCI and was localized to macrophages. MMP-12−/− mice have improved neuronal recovery after injury. Although still in its beginning stages, MMP-12 research represents renewed interest into the importance of MMPs in the CNS.

6.3 Significance of this project

This project represents the first steps in understanding the molecular mechanisms important for olfactory injury and recovery. Currently, it is estimated that over two million people in the United States suffer from olfactory disorders (Hoffman et al., 1998; Costanzo and Zasler, 1992). Olfactory dysfunction can be dangerous, with patients unable to detect dangers such as smoke, gas leaks, or spoiled food (Seiden, 1997; Santos et al., 2004). Up to 40 percent of patients have experienced a hazard event due to an olfactory disorder (Santos et al., 2004). In addition, people with impaired olfactory function are more likely to report a diminished quality of life and suffer from depression (Miwa et al., 2001; Deems et al., 1991). Currently, there are limited treatment options for olfactory dysfunction, leading to frustration of clinicians and often misunderstanding of olfactory problems. Determining the molecular mechanisms important for olfactory injury can lead to future research modulating these processes, perhaps leading to improved neuronal recovery. Based on previous studies, modulation
of MMP-9 and MMP-2 leads to increased neuronal recovery. Therefore, future studies aimed at targeted disruption of these two MMPs may reveal novel therapeutic options for patients suffering from olfactory dysfunction.
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Vita

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Stephen has presented his research both nationally and internationally and has one first author publication in *Chemical Senses*. He plans to submit work in this dissertation for two peer-reviewed publications.