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Matrix metalloproteinase-9 is associated with acute inflammation after olfactory injury

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Abstract

We previously reported an increase in matrix metalloproteinase-9 (MMP-9) levels in the olfactory bulb immediately following nerve transection, however its role remains unknown. In this report, we determined the source of MMP-9 by monitoring the infiltration of inflammatory leukocytes in the olfactory bulb following nerve transection. We used myeloperoxidase to identify neutrophils and CD68 to identify macrophages at days 1, 7, and 10. MMP-9 co-localized with neutrophils at all three time points but was not contained in macrophages. This is the first report to demonstrate that MMP-9 is associated with the early inflammatory response following olfactory injury and provides insight into mechanisms underlying olfactory injury and recovery processes.

Keywords

olfaction; matrix metalloproteinase; nerve injury; regeneration

Introduction

The olfactory system has the remarkable capacity to regenerate neurons and establish functional synaptic connections following injury, a unique property within the central nervous system (CNS). Basal cells in the olfactory epithelium are responsible for maintaining the population of olfactory sensory neurons [1-5]. Regeneration of olfactory sensory neurons by basal cells allows for the restoration of odor detection, though the discrimination of odorants is often impaired. This is due to disruptions in the rewiring of sensory neuronal connections in the olfactory bulb, the first site of synaptic connection between detection and identification of odors by the olfactory system [6]. The molecular mechanisms responsible for this disrupted targeting of sensory neuron projections to the olfactory bulb following injury remain unknown.

Recently, a family of enzymes, the matrix metalloproteinases (MMP), has been the focus of CNS injury and recovery studies. MMPs are classically described as remodelers of the extracellular matrix, though their role has expanded to include cytokine regulation and cell migration (reviewed in [7]). A subclass of MMPs, the gelatinases (MMP-2 and MMP-9) have important roles in CNS injury. Specifically, MMP-9 is elevated immediately in

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different injury models including stroke, spinal cord injury, and multiple sclerosis, and is associated with the inflammatory response [8-10]. We previously reported that MMP-9 levels increased in the olfactory bulb immediately after transection of olfactory sensory nerve fibers, and remained elevated for up to two weeks following injury [11]. However, the source of this MMP had not been previously investigated. In this present study, we examine the inflammatory response following nerve transection, using markers for neutrophils and macrophages, as a potential source for MMP-9, leading to further understanding the role it plays in the response to olfactory injury.

Methods

Surgical procedures

A total of 9 P2-IRES-tau-lacZ mice (3 mice at each time point examined) between the ages of 3-6 months were anesthetized with sodium pentobarbital (80 mg/kg, intraperitoneal). P2-IRES-tau-lacZ mice were used to allow for comparison from previous experiments' data [6]. After anesthesia, the left olfactory bulb was exposed and a thin Teflon cutting blade was inserted between the left bulb and cribriform plate (Figure 1A). The Teflon blade was then manipulated around to outer surface of the olfactory bulb to transect all the olfactory axons that connect to the left bulb. The right olfactory bulb was left intact and served as a control. The Teflon surface of the blade produced minimal damage to the bulb and cribriform plate. After nerve transection, the skin incision was sutured and mice were monitored postoperatively to assure full recovery before returning to their home cage. All procedures were approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University.

Immunohistochemistry

Immunohistochemical staining for MMP-9, myeloperoxidase (neutrophils), and CD68 (macrophages) was performed on olfactory bulb tissue sections at different recovery time points following nerve transection: days 1, 7, and 10. 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, and then immersed in 0.3% ethylenediaminetetraacetic acid for a week to decalcify the skulls. Following decalcification, skulls were placed in phosphate-buffered saline (Invitrogen, Carlsbad, CA) containing 30% sucrose for a week and then frozen in a minus 80°C freezer. Horizontal sections (n=60/mouse) through a mid-region of the olfactory bulb (Figure 1B) were cut at 10µm thickness on a Microm HM 550 series Cryostat (MICROM International GmbH, Walldorf, Germany) and placed on Superfrost® Plus VWR® Micro Slides (VWR, West Chester, Pennsylvania). Slides were stored in a minus 20°C freezer.

Microwave antigen retrieval was performed as previously described [12], with some modifications as described below. Each slide was allowed to reach room temperature before being washed in phosphate-buffered saline for 10 minutes. 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 phosphate-buffered saline, followed by incubation for 1 hour in a blocking solution that contained 10% normal donkey serum (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) and 0.2% Triton X-100 (LabChem Inc. Pittsburgh, PA) in phosphate-buffered saline. Half of the sections (n=30) were exposed to goat anti-MMP-9 (1:10; R & D Systems, Minneapolis, MN) and rabbit anti-myeloperoxidase (1:10; Abcam, Cambridge, MA), while the other half of sections were exposed to rabbit anti-MMP-9 and rat anti-CD-68 (1:100; AbDSeroTec, Raleigh, NC) in blocking solution. Two sections from each mouse were randomly assigned as control

sections and were not exposed to the primary antibodies (data not shown). The following day, slides were washed 3 times in phosphate-buffered saline and placed in species appropriate donkey secondary antibody for 1 hour. MMP-9 was visualized with CY3 (1:300; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) while myeloperoxidase and CD-68 were visualized with Alexa Fluor 488 (1:100; Invitrogen). The slides were cover slipped using Vector mounting media containing 4',6-diamino-2-phenylindole (DAPI; Vector Laboratories Inc., Burlingame, CA).

All sections of tissue were examined for immunohistochemical staining in the olfactory bulb at high magnification (62x) on a Leica TCS-SP2 AOBs confocal microscope (Leica Microsystems, Inc., Bannockburn, IL). Representative confocal images were arranged using Adobe Photoshop version 7.0 (Adobe Systems, Inc., San Jose, CA).

Results

Myeloperoxidase stained neutrophils in the injured bulb

Myeloperoxidase stained neutrophils were identified in the olfactory bulb by confocal imaging (Figure 2). All sections at the 3 recovery time points had myeloperoxidase positive cells located in the anterior region of the lesioned (left) olfactory bulb. In this region, approximately 5-12 myeloperoxidase positive cells were observed when examined using high magnification confocal imaging (62.8 $\mu\text{m} \times 65.6 \mu\text{m}$ area: see Figure 2). The control (right) bulb did not show myeloperoxidase staining at any of the time points measured (data not shown). This demonstrated that neutrophils had infiltrated the injured bulb within 24 hours following nerve transection and were present through day 10. MMP-9 positive cells were seen as early as day 1 and remained present through day 10. Merged images of myeloperoxidase and MMP-9 positive cells showed that these molecules co-localized to the same cells. DAPI nuclear staining confirmed that the myeloperoxidase/MMP-9 positive cells have polymorphic nuclei characteristic of neutrophils. Occasionally, myeloperoxidase positive cells failed to co-localize with MMP-9 (Figure 2: asterisks), suggesting that not all neutrophils in the bulb contained MMP-9.

Macrophage migration into the injured bulb

To determine if macrophages infiltrated the bulb and contained MMP-9, CD68 staining, a marker mainly associated with macrophages, was performed. Representative confocal images of the injured bulb are shown in Figure 3. Approximately 1-5 CD68 positive cells were visualized when viewed with high magnification confocal imaging in the lesioned bulb except at day 1 (62.8 $\mu\text{m} \times 65.6 \mu\text{m}$ area: see Figure 3). CD68 staining was not observed in the control (right) bulb (data not shown). As in the myeloperoxidase data, MMP-9 was seen in all three recovery days. In the merged images at days 7 and 10, MMP-9 failed to co-localize with CD68. This suggests that macrophages are not a source of MMP-9 following olfactory nerve injury.

Discussion

The regenerative capacity of olfactory sensory nerves is unique among neurons of the CNS; however, the significant processes for this ability remain unknown. This study represents a new approach directed at uncovering the molecular mechanisms important for neuronal recovery in the olfactory bulb following nerve transection. We previously reported increased levels of MMP-9 in the bulb immediately after injury and now suggest that neutrophils, an acute inflammatory leukocyte, are primarily responsible for the elevated MMP-9 (Figure 2). MMP-9's association with the acute inflammatory response following nerve transection has been shown in other CNS injury models [9,13]. In both spinal cord injury and stroke,

MMP-9 increased immediately and is thought to promote migration of neutrophils into damaged tissue. MMP-9 degrades many components of the blood-brain barrier and extracellular matrix (ECM), obstructions to neutrophil migration. MMP-9 is stored within the tertiary granules of neutrophils, which may not actively express MMP-9 at the site of injury [14-18]. This may explain our observation that some neutrophils did not contain MMP-9 (Figure 2: asterisks), perhaps having completely secreted their stored levels of MMP-9.

MMP-9's association with the acute inflammatory response in different CNS injury models suggest that MMP-9 may have a ubiquitous role following injury regardless of the mechanism of trauma. MMP-9 knockout experiments have revealed important roles for MMP-9 in CNS injury and recovery [3, 15, 17]. If MMP-9 has a ubiquitous role in CNS injury and recovery, MMP-9 knockout experiments could provide data to support the development of new strategies to improve olfactory neuronal recovery following nerve transection. Although the olfactory system has the ability to regenerate neurons and reestablish synaptic connections in the bulb, functional recovery of odor discrimination is often impaired. Yee and Costanzo (1995) demonstrated that hamsters were able to detect, yet unable to identify previously learned odors following recovery from nerve transection [19]. An additional learning period was necessary before animals could distinguish between previously learned odors. The authors concluded that alterations of sensory perception were responsible for changes in odor qualities. Further studies demonstrated that in mice, regenerated olfactory axonal projections to the bulb are distorted, which may explain the inability to discriminate odors following injury [7]. Future experiments modulating MMP-9 levels could support the development of new therapeutic options for improved recovery from olfactory injury.

Interestingly, CD68 positive macrophages were not found to be a source of MMP-9 in the olfactory bulb following nerve transection (Figure 3). This suggests that in our model of injury, MMP-9 is not associated with later inflammatory processes. There are conflicting reports as to whether macrophages contain MMP-9 following CNS injury [8,20,21]. This discrepancy may be due to the fact that there are multiple phases of macrophage maturation once they are activated (reviewed in [22]). CD68 is associated with the phagocytic phenotype of macrophages, suggesting that MMP-9 may not have an important role in phagocytosis. Though MMP-9 was not localized to macrophages following nerve transection, this does not exclude the possibility that MMP-9 may be stored and released by macrophages at some point before they enter the site of injury during the recovery process.

Conclusion

This study demonstrated that neutrophils, and not phagocytic macrophages, are the source of increased MMP-9 in the olfactory bulb during the acute period following nerve transection. The data suggest that MMP-9 plays an important role during the time period immediately following injury. Future studies aimed at modulation of MMP-9 during the acute period following olfactory nerve injury may provide novel strategies to restore olfactory function.

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Figure 1.

Diagram of the olfactory bulb and nasal cavity. **A.** A Teflon blade was inserted between the olfactory bulb and cribriform plate, which completely severed the olfactory sensory axons as they traversed from the nasal cavity to the bulb. **B.** The dotted line illustrates the location where horizontal sections were obtained to monitor the inflammatory response and MMP-9 by immunohistochemistry.

Figure 2.

Confocal images of neutrophils in the olfactory bulb after olfactory nerve transection injury. Three time points representing the acute response to injury (days 1, 7, and 10) show the presence of both myeloperoxidase (MPO)-positive cells (medium grey), an enzymatic marker for neutrophils, and matrix metalloproteinase-9 (MMP-9)-positive cells (light grey) in the lesioned olfactory bulb. Merged images demonstrate that MPO and MMP-9 are colocalized to the same cells. 4',6-Diamino-2-phenylindole staining (dark grey) revealed that the MPO-positive cells/MMP-9-positive cells have polymorphic nuclei characteristic of neutrophils. These data show that MMP-9 is present in neutrophils, although not all neutrophils (asterisks) contain MMP-9. Scale bar: 10 μ m.

Figure 3.

Confocal images of macrophages in the olfactory bulb after nerve transection. CD68 macrophages (medium grey) were observed at recovery days 7 and 10. Matrix metalloproteinase-9 (light grey) was seen at days 1, 7, and 10. The merged images reveal that CD68 and MMP-9 are not localized to the same cell, suggesting that MMP-9 is not found in CD68 macrophages. 4',6-Diamino-2-phenylindole staining confirmed that the MMP-9-positive cells had polymorphic nuclei characteristic of neutrophils. Scale bar: 10 μm .