Neuroscience 274 (2014) 331–340

UP-REGULATION OF MATRIX METALLOPEPTIDASE 12 IN MOTOR NEURONS UNDERGOING SYNAPTIC STRIPPING

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Abstract—Axotomy of the rodent facial nerve represents a well-established model of synaptic plasticity. Post-traumatic ''synaptic stripping'' was originally discovered in this system. We report upregulation of matrix metalloproteinase MMP12 in regenerating motor neurons of the mouse and rat facial nucleus. Matrix metalloproteinases (matrix metallopeptidases, MMPs) are zinc-binding proteases capable of degrading components of the extracellular matrix and of regulating extracellular signaling networks including within synapses. MMP12 protein expression in facial motor neurons was enhanced following axotomy and peaked at day 3 after the operation. The peak of neuronal MMP12 expression preceded the peak of experimentally induced synaptic plasticity. At the same time, MMP12 redistributed intracellularly and became predominantly localized beneath the neuronal somatic cytoplasmic membrane. Both findings point to a role of MMP12 in the neuronal initiation of the synaptic stripping process. MMP12 is the first candidate molecule for such a trigger function and has potential as a therapeutic target. Moreover, since statins have been shown to increase the expression of MMP12, interference with synaptic stability may represent one mechanism by which these widely used drugs exert their side effects on higher CNS functions. **Crown Copyright © 2014 Published by Elsevier Ltd. on behalf of IBRO. All rights reserved.**

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Abbreviations: ADAMs, a disintegrin and metalloproteases; ECM, extracellular matrix; GFAP, glial fibrillary acidic protein; MMPs, matrix metalloproteinases; PBS, phosphate-buffered saline; TBS-T, Tris-buffered saline with tween-20.

Key words: extracellular matrix, facial nerve axotomy, matrikines, metzincins, statins, synaptic plasticity.

INTRODUCTION

Matrix metalloproteinases (MMPs) are a family of multidomain zinc-dependent endopeptidases that are known to be involved in extracellular matrix (ECM) remodeling (Yong et al., 2001; Agrawal et al., 2008). Like other metzincin proteases they share a conserved zinc-binding motif in their catalytic active site (Loffek et al., 2011). Metzincins are increasingly recognized as fine modulators of nervous system physiology and pathology (Rivera et al., 2010). Other metzincin members are the well-known ADAMs (a disintegrin and metalloproteases), the ADAMs with a thrombospondin motif (ADAMTS), astacins and the bacterial serralysins (Klein and Bischoff, 2010). Also referred to as matrixins, the MMP subfamily of metzincins consists of 23 distinct enzymes in humans and 24 in mouse (Klein and Bischoff, 2010). Within the CNS, MMPs can be synthesized by endothelial cells, microglia, astrocytes and neurons (Rosenberg, 2002). It was originally thought that the main function of MMPs is to degrade ECM components but recent studies have demonstrated their significance as regulators of extracellular tissue signaling (Loffek et al., 2011). Specifically, MMPs influence signaling networks through the generation of matrikines (Maquart et al., 2005; Taddese et al., 2009). It is of particular interest that MMPs are capable of regulating signaling systems in synapses of the nervous system, including cadherins, BDNF/TrkB, ephrins/Ephs, and laminin/integrins that modify *N*-methyl-D-aspartate receptor activity (Ethell and Ethell, 2007).

MMP12 is a 54-kDa molecule, which in addition to elastin is able to cleave α1-antitrypsin, type I gelatin, type IV collagen, fibronectin, vitronectin, laminin, proteoclycan, myelin basic protein, and progranulin (Chandler et al., 1996; Yong et al., 2001; Cenik et al., 2012; Suh et al., 2012). MMP12 is regulated by a number of disease states in several organs. MMP12 expression may be particularly associated with severe Langerhans cell histiocytosis (Seo et al., 2012). Other examples include chronic obstructive pulmonary disease (Molet et al., 2005), vascular disease (atherosclerotic lesions, aneurysms) (Yamada et al., 2008), cancer (Yang et al., 2007), cutaneous granuloma (Vaalamo et al., 1999), traumatic spinal cord injury (Buss et al., 2007), multiple sclerosis (Vos et al., 2003), neonatal hypoxic ischemic injury (Svedin et al., 2009) and intracerebral hemorrhage

http://dx.doi.org/10.1016/j.neuroscience.2014.05.052
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(Power et al., 2003). MMP12 is best known as macrophage elastase and expressed by microglia in the CNS especially in their phagocytic state (Power et al., 2003; Vos et al., 2003; Nuttall et al., 2007). Known biological effects of MMP12 are permeability increases of the blood–brain/spinal cord barrier, recruitment of peripheral macrophages into CNS lesions and local stimulation of microglial activation at the site of injury (Wells et al., 2003). MMP12 also has a role in myelination (Lehmann et al., 2009) and in brain development (Milward et al., 2007). MMP12 exhibits some structural features by which it differs from other MMPs (Lang et al., 2001). A detailed comparison to other MMPs can be found in Nar et al., 2001 (Nar et al., 2001). MMP12 has been mentioned in the context of synaptic circuit remodeling (Huntley, 2012) but very little is known about MMP12 functions in nerve cells.

The facial nerve axotomy model permits the systematic and detailed study of the response of CNS neurons and their microenvironment to external stimuli. It does not involve a direct mechanical disturbance of the CNS or of its bony confinements, all manipulations are carried out remotely and there is no opening of the blood–brain barrier. The behavior of neurons and glial cells in this system has been studied extensively (Moran and Graeber, 2004), and the loss of afferent axonal terminals from the surface of axotomized nerve cells (''synaptic stripping'') was originally discovered here (Blinzinger and Kreutzberg, 1968). We have now studied the expression of MMP12 in this system.

EXPERIMENTAL PROCEDURES

Tissue samples

This study is based on 22 adult B6 mice and three Lewis rats. The rats were used for comparative purposes (Fig. 6). Approval of the surgical protocol was obtained from the animal ethics committee of the University of Sydney. Animals were anaesthetized using isoflurane (Southmedic Inc., Eagle Farm, Queensland, Australia, Cat No. 1-705-726-9383) and the right facial nerve was transected at its exit from the stylomastoid foramen (Moran and Graeber, 2004). The contralateral side was left intact and served as a paired internal control. Survival times studied were 2, 3, 4, 6, 7, 10, 14 and 21 days after peripheral nerve transection. Two independent series of animals were used (B6.SJL and C57BL/6, respectively). The reproduction cohort (C57BL/6) comprised post-operative days 2, 3, and 7 with four animals per time point. These time points have been characterized extensively in earlier studies. For cryostat sectioning, animals were euthanized with carbon dioxide and whole brains were removed and the brain stems frozen at −70 °C. For paraffin embedding, animals were injected with an overdose of pentobarbitone sodium (Virbac, Milperra, Australia, Cat No. LETHA450) and perfused transcardially with 3.7% phosphate-buffered, methanol-stabilized high-quality formaldehyde (Fronine, Australia, Code JJ014).

Fluorescence microscopy

Sixteen-micrometer-thick coronal cryosections were collected onto glass slides and allowed to thaw and dry for 5 min. They were then fixed in buffered (0.01 M phosphate-buffered saline (PBS), pH 7.4) formaldehyde solution followed by 50%, 100% and 50% cold acetone (4 °C) for 2 min each. After washes in 0.01 M PBS (2 X 5 min), sections were incubated with rabbit polyclonal anti-MMP12 antibody (1:100, Abcam, San Francisco, CA, USA, Cat No. AB66157) for two hours in a humid chamber at room temperature. Slides were subsequently rinsed in 0.01 M PBS for 5 min twice and incubated with Alexa 488-conjugated goat anti-rabbit secondary antibody (1:200, Molecular probes, Eugene, OR, USA, Cat No. A-11034) for 1 h at room temperature. At the end of the incubation, sections were rinsed twice in 0.01 M PBS for 5 min and cover-slipped with Fluoromount (Sigma–Aldrich, St. Louis, MO, USA, Cat No. F4680). The primary antibody was omitted as a labeling control. A second anti-MMP12 antibody from a different supplier (Origene, Rockville, MD, USA, Cat No. TA300810) was used following the same protocol to confirm specificity of MMP12 expression.

Double-labeling for markers of glial activation. For MMP12/GFAP and MMP12/MAC-1 double labeling, where anti-GFAP (glial fibrillary acidic protein) serves to detect reactive astrocytes (Graeber and Kreutzberg, 1986) and MAC-1 (CD11b) is used to detect activated microglia (Graeber et al., 1988) in the facial nucleus, the same staining procedure was followed as described above. Antibody dilutions were 1:100 for anti-MMP12 (Abcam), 1:20 for anti-GFAP (mouse monoclonal, Sigma–Aldrich, St. Louis, Cat No. G 3893) and 1:100 for anti-MAC-1 (rat monoclonal; AbD Serotec, Raleigh, NC, USA, Cat No. MCA74GA). Slides were subsequently washed in 0.01 M PBS for 5 min twice and incubated with the relevant secondary antibody for 1 h at room temperature (all at 1:200 dilution). The secondary antibodies were Alexa 488-conjugated goat anti-rabbit for MMP12 (Cat No. A-11034, Molecular Probes, OR, USA), Alexa 568-conjugated goat anti-mouse for GFAP (Cat No. A-11031, Molecular Probes, OR, USA) and Alexa 568-conjugated goat anti-rat for MAC-1 (Cat No. A-11077, Molecular Probes, OR, USA). Finally, sections were rinsed in 0.01 M PBS for 5 min twice and mounted using Fluoromount (Sigma–Aldrich).

Mounted sections were imaged on a Zeiss LSM 710 confocal microscope using a 5× EC Plan-Neofluar NA 0.16 and 20× Water Plan-Apochromat NA 1.0 objective. Alexa Fluor 488 was excited with the 488-nm laser, using a 488/561/633 dichroic mirror and emission captured from 495 to 580 nm. Alexa Flour 568 was excited with the 561-nm laser using a 488/561/633 dichroic mirror and emission captured from 570 to 710 nm.

Quantitative analysis of MMP12 immunolabelling. Fiji-ImageJ imaging software (http://pacific.mpi-cbg.de/) was used to carry out a quantitative assessment of MMP12 expression in axotomized motor neurons compared to

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