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Cytokine regulation of MMP-9 in peripheral glia: Implications for pathological processes and pain in injured nerve

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Abstract

Matrix metalloproteinase-9 (MMP-9) is an extracellular protease that is induced in Schwann cells hours after peripheral nerve injury and controls axonal degeneration and macrophage recruitment to the lesion. Here, we report a robust (90-fold) increase in MMP-9 mRNA within 24 h after rat sciatic nerve crush (1 to 60 days time-course). Using direct injection into a normal sciatic nerve, we identify the proinflammatory cytokines TNF- α and IL-1 β as potent regulators of MMP-9 expression (Taqman qPCR, zymography). Myelinating Schwann cells produced MMP-9 in response to cytokine injection and crush nerve injury. MMP-9 gene deletion reduced unstimulated neuropathic nociceptive behavior after one week post-crush and preserved myelin thickness by protecting myelin basic protein (MBP) from degradation, tested by Western blot and immunofluorescence. These data suggest that MMP-9 expression in peripheral nerve is controlled by key proinflammatory cytokine pathways, and that its removal protects nerve fibers from demyelination and reduces neuropathic pain after injury.

Keywords

Schwann cell; Matrix metalloproteinase; TNF- α ; IL-1 β ; NGF; Glia; Myelination; MBP; Pain; Neuropathy

1. Introduction

Neuropathic pain is often a consequence of neuropathological and molecular changes resulting from peripheral nerve damage. Complex interactions of injured peripheral nerve fibers with activated glia (Schwann cells) and recruited immune cells is regulated by a number of immunomodulatory and trophic factors. Proinflammatory cytokines, such as tumor necrosis factor alpha (TNF- α) and interleukins (IL-1 β , IL-6), have been implicated in the pathogenesis of Wallerian degeneration and neuropathic pain, as they control axonal demyelination, degeneration, blood-nerve permeability, and immune cell recruitment (Stoll et al., 2002), and thus, represent model therapeutic targets in neuropathic pain (Myers et al., 2006). Recently, we have shown that some critical actions of TNF- α in injured nerve, such as macrophage recruitment, are mediated by matrix metalloproteinase-9 (MMP-9 or gelatinase B) (Shubayev et al., 2006).

MMP-9 belongs to a family of Zn²⁺-dependent extracellular proteases called matrix metalloproteinases (MMPs), that comprise collagenases, gelatinases, stromelysins, and membrane-type MMPs (Woessner, 1994). In the nervous system, MMPs produce

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neuroinflammation by controlling neurovascular permeability, immune cell recruitment, demyelination, cell necrosis, and apoptosis (Yong et al., 1998; Kieseier et al., 1999b, Rosenberg, 2002; Lee et al., 2004a, Yong, 2005). MMP-9 is upregulated in experimental peripheral neuropathy models (La Fleur et al., 1996; Kherif et al., 1998; Ferguson and Muir, 2000; Siebert et al., 2001; Hughes et al., 2002; Platt et al., 2003; Demestre et al., 2004) and in patients with symptomatic neuropathy (Leppert et al., 1999; Mawrin et al., 2003; Renaud et al., 2003; Gurer et al., 2004). We have recently shown that MMP-9 gene deletion or pharmacologic inhibition reduces injury-induced macrophage recruitment and protects nerves from axonal degeneration (Shubayev et al., 2006).

In the central nervous system, MMP-9 is fundamental to myelination (Yong, 2005), in part, by degradation of myelin basic protein (MBP) (Gijbels et al., 1993; Proost et al., 1993). While MBP constitutes only 10–20% of PNS myelin (Jacobs, 2005), it is critical to maintaining integrity and compactness of peripheral nerve in development (Martini and Schachner, 1997) and after injury (LeBlanc and Poduslo, 1990). The importance of MMP-9 in peripheral nerve demyelination has been documented (Redford et al., 1995, 1997; Kieseier et al., 1999a,b; Siebert et al., 2001), but the mechanism of its action has not been clarified.

The purpose of this study is to address whether activation of peripheral glia by proinflammatory cytokines induces MMP-9 expression *in vivo*, and to analyze the role of MMP-9 in controlling MBP levels and demyelination after peripheral nerve injury.

2. Methods

2.1. Animal surgery

Adult female Sprague–Dawley rats ($n = 133$; 250 g, Harlan Labs), MMP-9 knockout ($n = 25$, FVB.Cg-Mmp9^{tm1Tvu}/J) and wild-type mice ($n = 25$, FVB/NJ), TNFR1 ($n = 8$, B6.129-Tnfrsf1a^{tm1Mak}/J), TNFR1/2 knockout ($n = 8$, B6.129S-Tnfrsf1a^{tm1lmx} Tnfrsf1b^{tm1lmx}/J) and wild-type ($n = 8$, B6129SF2/J) mice were used. All mouse strains were obtained from Jackson Laboratory (Bar Harbor, ME). Anesthesia was induced with 4% Isoflurane (IsoSol; Vedco, St. Joseph, MO), the sciatic nerve was exposed unilaterally at the mid-thigh level, and crushed using fine, smooth-surface forceps twice for 5 s each to produce nerve crush. Nerve injections were made into uninjured rat sciatic nerves using a Hamilton syringe, a 30-gauge-needle and an injectate volume of 5 μ l. Animals were sacrificed using an intraperitoneal injection of a cocktail containing sodium pentobarbital (Nembutal, 50 mg/ml; Abbott Labs, North Chicago, IL) diazepam (5 mg/ml, Steris Labs, Phoenix, AZ) and saline (0.9%, Steris Labs) in a volume proportion of 1:1:2, respectively. All procedures were performed according to protocols approved by the VA Healthcare System Committee on Animal Research, and conform to the NIH Guidelines for Animal Use.

2.2. Antibodies and proteins

Recombinant rat TNF- α (R&D Systems), IL-1 β (Pierce) or NGF (Invitrogen) were delivered into sciatic nerve at 250 pg per rat, or bovine serum albumin (BSA, Sigma, 0.1%, vehicle) in 5 μ l volume as previously described (Wagner and Myers, 1996). The following antibodies were used for immunodetection: rabbit anti-MMP-9 (Torrey Pines Labs, 1:500), mouse anti-MBP (Abcam, 1:50), rabbit anti-S100 (Dako, 1:2000), and mouse anti- β -actin (Sigma, 1:10,000). Respective normal serum or IgG was used for negative control. All antibodies were diluted in 1% blocking serum in PBS.

2.3. Immunohistochemistry

Paraffin-embedded, 4% paraformaldehyde-fixed nerve sections (10- μ m-thick) were deparaffinized with xylenes, rehydrated in graded ethanol PBS and subjected to detection as previously described (Shubayev and Myers, 2002) and summarized below:

1. Dilaminobenzidine (DAB): endogenous peroxidase was blocked with 3% H₂O₂, antigen retrieval (Dako, Carpinteria, CA) (5 min at 95 °C and 20 min at room temperature), non-specific binding was blocked with 10% normal goat serum, followed by a rabbit anti-MMP-9 antibody incubation (see above) overnight at 4 °C, goat anti-rabbit IgG (Vector) and avidin-biotin complex (ABC Elite, Vector) application. Sections were developed with DAB (brown stain, Vector).
2. Immunofluorescence: 0.5% sodium borohydride in 1% dibasic sodium phosphate buffer was applied for 5 min to block endogenous aldehyde groups, followed by Dako antigen retrieval, non-specific binding block in 5% goat serum for 30 min, mouse anti-MBP antibody (see above) overnight at 4 °C, alexa goat anti-mouse 488 antibody for 1 h, and nuclear 4',6-diamidino-2-phenylindole (DAPI) stain (Molecular Probes, 1:20000, blue) for 5 min. Sections were mounted using Slowfade gold antifade reagent (Molecular Probes). PBS was used for all washes.

2.4. Real-time qPCR

Sciatic nerve fragments and L5/L4 DRG samples were pooled from 2 rats and stored in RNA-later (Ambion) at -20 °C. Total RNA was extracted with Trizol (Invitrogen) and treated with RNase-free DNase I (Qiagen). The RNA purity was verified by OD260/280 absorption ratio of about 2.0. cDNA was synthesized using SuperScript II first-strand RT-PCR kit (Invitrogen). Gene expression was measured by quantitative real-time polymerase chain reaction (qPCR, MX4000, Stratagene, La Jolla, CA) using 50 ng of rat cDNA and 2 $\times$ *Taqman* Universal PCR Master Mix (Applied Biosystems) with a one-step program (95 °C for 10 min, 95 °C for 30 s and 60 °C for 1 min for 50 cycles). Primers and *Taqman* probes for MMP-9 from Biosearch Technologies (Novato, CA) were optimized using injured sciatic nerve cDNA (amplification efficiency of 100.1–100.3%), as reported earlier (Shubayev et al., 2006). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene was used as a normalizer, and its expression was confirmed to be not regulated in injured and uninjured nerves. Duplicate samples without cDNA (no-template control) for each gene showed no contaminating DNA. Relative mRNA levels were normalized to GAPDH, five samples per group were quantified using the comparative *C_t* method (Livak and Schmittgen, 2001), and a fold change was determined by the MX4000 (Pfaffl, 2001).

2.5. Gelatin zymography

Nerves were lysed in non-reducing, protease-inhibitor-free Laemmli sample buffer, heated at 55 °C for 5 min and 50 μ g tissue per well was run on 10% SDS polyacrylamide gel containing 1 mg/ml of gelatin at 160 V for 90 min (Shubayev and Myers, 2000). The gels were washed in 2.5% Triton X-100, developed at 37 °C overnight in 50 mM Tris-HCl, 150 mM NaCl, 5 mM CaCl₂, 1 μ M ZnCl₂, and 0.2 mM sodium azide (pH 7.6) and stained with colloidal blue (Invitrogen), indicating gelatinolytic MMP activity as a clear band on a dark background of undegraded gelatin. Inverted images are presented. Recombinant human MMP-9 (Chemicon) was used for control. Zymograms were digitized using an EC3 Darkroom (UVP Imaging) and quantified by LabWorks 4.5. Data are expressed as relative optical density (OD) of gelatinolytic activity.

2.6. Western blotting

Nerves were lysed in Laemmli buffer containing 10 mM PMSF, 5 mM EDTA, protease inhibitor cocktail (Sigma) (pH 6.8) as previously described (Shubayev and Myers, 2000), reduced with 10% β -mercaptoethanol (Fisher), and 30–50 μ g of protein (BSA Protein Assay, Pierce) was run on 15% SDS–PAGE in a Laemmli system. Proteins were transferred to nitrocellulose at 50 V for 60 min in transfer buffer (12 mM Tris–base, 95 mM glycine, and 20% methanol, pH 8.3). Non-specific binding was blocked in 5% non-fat dry milk (Bio-Rad) followed by a primary antibody incubation overnight at 4 °C, HRP-tagged goat anti-mouse or anti-rabbit IgG, and detection with enhanced chemiluminescence (Amersham). Molecular weight was determined using HRP-tagged SDS–PAGE standards (Bio-Rad). Blots were digitized using an EC3 Darkroom (UVP Imaging) and quantified by LabWorks 4.5. Data are expressed as relative optical density (OD) ratios of experimental to control proteins.

2.7. Spontaneous pain behavior

Spontaneous pain behavior was measured according to the method described by Attal et al. (Attal et al., 1990; Paulson et al., 2002) in MMP-9 knockout ($n = 10$) and wild-type mice ($n = 10$) after sciatic nerve crush for 2 weeks. Each animal was placed in a plexiglass cylinder (19 cm $\times$ 31 cm) and allowed to habituate. One animal at a time was continuously observed for 2 min. This was repeated 2 more times within the next 2 h. Different positions of the injured hind paw were continuously rated, according to the following numerical scoring system: 0 = the paw is placed normally on the floor, 1 = the paw is placed lightly on the floor and the toes are in a ventroflexed position, 2 = only the internal edge of the paw is placed on the floor, 3 = only the heel is placed on the floor and the hind paw is in an inverted position, 4 = the whole paw is elevated, and 5 = the animal licks the paw. During each 2 min (120 s) test period, measurements were taken continuously by a tester blinded to the experimental groupings. In practical terms, this was done by pressing one of six (0–5) numerical keys on a computer keyboard. Only one key was pressed at a time, corresponding to the instantaneous behavior of the animal. This resulted in a continuous 120 s evaluation of the behavior that could be parsed off-line into seconds/behavior during the experimental period. An index for noxious behavior was calculated by multiplying the amount of time the mice spent in each behavior multiplied by a weighting factor for that behavior, and divided by the length of the observational period, using the formula: $[0t_0 + 1t_1 + 2t_2 + 3t_3 + 4t_4 + 5t_5]/120$ s, where t_0 – t_5 are the durations in seconds spent in behaviors 0–5, respectively. The three values corresponding to three blocks of 120 s were averaged to determine the spontaneous pain score for each mouse.

3. Results

3.1. MMP-9 expression in crushed rat sciatic nerve and corresponding DRG

The patterns of MMP-9 mRNA expression were analyzed during the course of Wallerian degeneration after rat sciatic nerve crush (Fig. 1). MMP-9 expression in nerve was robustly elevated (86.9 ± 7.78 -fold) at 1 day after crush, and gradually returned to baseline by 60 days post-crush. In the corresponding DRG, MMP-9 expression was moderately stable throughout the course of injury, showing a significant 2.65 ± 0.28 increase only at 2 weeks post-crush.

3.2. Cytokines regulate MMP-9 expression in peripheral nerve

Pro-inflammatory cytokines activate glia after nerve injury. MMP-9 in peripheral nerve is produced only after injury, predominantly by Schwann cells (Shubayev and Myers, 2000, 2002). Cytokines and trophic factors are known inducers of MMP-9 (Nagase, 1997). Twenty-four hours after we injected recombinant rat TNF- α , IL-1 β or NGF proteins into normal sciatic nerve, MMP-9 mRNA (Fig. 2A) and proteolytic activity (Fig. 2B) were analyzed. Day 1 crushed and uninjured nerves served as positive and negative controls, respectively. A

significant increase in MMP-9 mRNA was observed after NGF, TNF- α and IL-1 β injection relative to BSA (vehicle) injection and uninjured nerve. However, BSA injection did cause some MMP-9 induction relative to uninjured nerve. Immunohistochemical analysis of MMP-9 after TNF- α injection paralleled the mRNA and protein expression data, and identified myelinated Schwann cells as a chief source of MMP-9 in response to cytokine injections. Again, we observed a mild increase in MMP-9 after BSA injection, but a robust increase after TNF- α injection, comparable to that of Day 1 crush. Some axonal reactivity was noted in TNF- α -injected and crushed nerves, probably due to increased neuronal-glial interaction. The overall histopathological changes in cytokine-injected nerves were mild and comparable to that of crushed nerves.

To identify a specific pathway of TNF- α -mediated MMP-9 induction, we assessed MMP-9 activity in TNF- α receptor 1 (TNFR1) knockout and TNFR1 and TNFR2 double-knockout mouse nerves at Day 1 after crush (Fig. 3). Similar to TNF- α knockout (Shubayev et al., 2006), we observed only a mild decline of MMP-9 in TNFR1 and TNFR1/2 knockouts. There was no significant difference in MMP-9 activity between TNFR1 knockouts and TNFR1/2 double-knockout mice, suggesting that TNFR1 is the main TNF- α receptor to mediate MMP-9 expression. These data suggest that high MMP-9 levels in knockout cytokine nerve injury models is maintained due to compensatory activation of related mechanisms, such as IL-1 β . Together, these data support the hypothesis that Schwann cell activation by several important cytokine and trophic pathways results in MMP-9 induction.

3.3. MMP-9 influences neuropathic pain behavior

We sought to determine if MMP-9, as a cytokine-mediated factor, regulates neuropathic pain. Spontaneous pain behavior was scored in a blinded fashion in MMP-9 knockout and wild-type animals for 2 weeks after nerve crush (Fig. 4). MMP-9 knockout mice expressed less pain, as indicated by a statistically significant decline in the pain index relative to wild-type animals, at 2 days and at 8 and 10 days after crush. MMP-9 deletion, however, did not facilitate recovery from neuropathic pain, demonstrating the same score of 0.8 in both groups at 2 weeks after crush.

3.4. MMP-9 controls myelin protein content after nerve injury

While MMP-9 importance in regulating MBP turnover in CNS is well-accepted, its role in processing MBP in peripheral nerve has not been analyzed. MMP-9 knockout and wild-type mouse nerves were analyzed for MBP protein levels at 10 days after crush (Fig. 5). At this time-point, animals display reduced pain behavior (see Fig. 4), and MBP levels in wild-type injured sciatic nerve are normalized after initial demyelination (Gupta et al., 1988; LeBlanc and Poduslo, 1990); we confirmed the latter observation (not shown). MMP-9 gene deletion caused almost a 2-fold increase in unprocessed MBP (52 kDa) relative to wild-type (Fig. 5A and B). No change in S100 (common Schwann cell marker, 13 kDa) or β -actin (protein loading control, 42 kDa) was seen. Calibration of MBP to S100 levels indicates that MBP protection in MMP-9 knockout nerves is not related to the changes in Schwann cell viability. Immunofluorescence for MBP (green) and the nuclear stain, DAPI (blue) (Fig. 5C), paralleled observation of the Western blot, showing preserved MBP levels and myelin thickness after MMP-9 deletion.

These data indicate that in the PNS, MMP-9 regulates MBP turnover and myelin thickness, while MMP-9 gene deletion protects, concurrently, from neuropathic pain and myelin degradation.

4. Discussion

This study demonstrates that in peripheral nerve MMP-9 is induced within a day after injury in response to proinflammatory cytokines, and that MMP-9 gene deletion reduces neuropathic pain behavior in concordance with preserved myelin integrity.

MMP-9 increase within 1 day after nerve injury, preceding neuropathological evidence of degeneration, has been a consistent observation (La Fleur et al., 1996; Kherif et al., 1998; Ferguson and Muir, 2000; Shubayev and Myers, 2000, 2002, 2006; Platt et al., 2003). TNF- α induces MMP-9 in the CNS (Rosenberg et al., 1995), in injured sciatic nerve (Shubayev et al., 2006), and as shown here, in uninjured sciatic nerve. While this study emphasizes the importance of TNF- α , it also implicates IL-1 β and NGF in MMP-9 induction in peripheral nerve. IL-1 β upregulates MMP-9 in optic nerve (Zhang and Chintala, 2004) and brain (Vecil et al., 2000), and NGF is known to induce MMP-9 in cultured neurons (Muir, 1994; Shubayev and Myers, 2004). The ability of the vehicle injection to cause the increase in MMP-9 is consistent with observations of mild inflammatory response to sham surgeries (Kleinschnitz et al., 2005).

We observed that Schwann cells produce MMP-9 in response to TNF- α *in vivo*, in accordance with our earlier studies in cultured primary Schwann cells (Shubayev et al., 2006). However, other endoneurial cells can upregulate MMP-9 (Shubayev and Myers, 2002), and may do so in response to cytokines, as has been shown for fibroblasts (Singer et al., 1999) and endothelial cells (Genersch et al., 2000). It remains to be determined whether Schwann cells of different phenotypes equally respond to TNF- α challenge by increasing MMP-9 production. Central micro- and macroglia also produce MMP-9 in response to injury (Hughes et al., 2002; Rosenberg, 2002; Lee et al., 2004b).

MMP-9 is a critical mediator of demyelination in the central (Rosenberg, 2002) and peripheral (Kieseier et al., 1999b) nervous systems. It is known to control the breakdown of MBP (Chandler et al., 1995), a late component of myelin formation that is produced by Schwann cells in injured peripheral nerve (Gupta et al., 1988; LeBlanc and Poduslo, 1990). While MMP-9-dependent degradation of MBP has been shown in models of multiple sclerosis (Gijbels et al., 1993; Proost et al., 1993) and cerebral ischemia (Asahi et al., 2001; Cho et al., 2006), this is the first demonstration of this relationship in the PNS. Other MMPs, such as MMP-12 (Larsen et al., 2006) and MMP-3 (D'Souza and Moscarello, 2006), regulate MBP processing in the CNS and may play a role in peripheral nerve. MMP-9 is also involved in myelination via interaction with proteoglycans and growth factors (Yong, 2005). While MMP-9 promotes TNF- α -mediated macrophage recruitment into the injured nerve (Shubayev et al., 2006), neither TNF- α (Liefner et al., 2000) nor MMP-9 (Siebert et al., 2001) alter the myelin phagocytosing function of macrophages, suggesting that their roles in demyelination is not secondary to the ability to modulate macrophage recruitment.

Activation of Schwann cells has been implicated in the pathogenesis of neuropathic pain (McMahon et al., 2005; Myers et al., 2006). Here, we observed a delayed, mild but statistically significant reduction in pain behavior after MMP-9 gene deletion. The delay in mechanical allodynia is characteristic of other neuroprotective models, such as the spontaneous Wld^S mutant mouse model of delayed Wallerian degeneration (Sommer and Schafer, 1998), which fails to induce MMP-9 and TNF- α (Shubayev et al., 2006). The mild effect may point to the secondary role of MMP-9 in pain or compensatory mechanisms of MMP-9 knockout. To date, two other studies directly assessed the effect of MMP inhibition on neuropathic pain. MT5-MMP gene deletion virtually ablated mechanical allodynia associated with partial sciatic nerve ligation (Komori et al., 2004), and synthetic inhibitor TAPI significantly reduced thermal hyperalgesia and mechanical allodynia after chronic constriction injury in mice (Sommer et

al., 1997). TAPI inhibits TNF- α activation by chelating TNF- α converting enzyme (TACE) and, at higher doses, MMP-9 and other MMPs. MMP inhibition also improves electrophysiologic nerve conduction and motor performance (Leppert et al., 1999; Hsu et al., 2006).

Observation of unstimulated foot positioning is commonly done in the formalin test, and is used here to monitor long-lasting or tonic pain, the most common features of clinical painful neuropathy. This test correlates well with hyperalgesia to mechanical and thermal stimuli in major models of experimental neuropathy (Attal et al., 1990). Our study suggests that MMP-9 role in demyelination relates to the basic mechanisms of neuropathic pain. Demyelination of injured afferents is known to cause ectopic discharge and neuropathic nociception due to remodeling of the exposed axonal membrane, such as sodium channel insertion that is normally inhibited by myelin (Devor, 2006). Mechanical allodynia (signaled by myelinated afferents) associates with sciatic nerve crush (Lancelotta et al., 2003), but perhaps better assessed in models producing a more robust mechanical sensitization, e.g., spinal nerve ligation (Kim and Chung, 1992).

The present study utilizes mouse and rat species. While changes in MMP-9 expression after cytokine injections were correlated with the well-defined paradigm of nerve crush all in rat sciatic nerve, the use of mutant mouse nerve crush models allowed us to analyze specific mechanisms of MMP-9 expression (TNF- α receptor knockout) and function (MMP-9 knockout). Earlier work shows highly consistent changes in cytokine and MMP expression between mouse and rat nerve crush models, but certain signaling differences between the species might exist.

In conclusion, this study suggests that MMP-9 is a sensitive biomarker of peripheral nerve injury that is regulated by multiple cytokine pathways. MMP-9 deletion protects nerve fibers by preservation of MBP protein levels and myelin thickness and reduces spontaneous pain behaviors.

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Fig. 1. MMP-9 mRNA expression after sciatic nerve crush. Real-time Taqman qPCR for MMP-9 in nerve and ipsilateral DRG. Data are expressed as the mean fold increase $\pm$ SE in crushed relative to uninjured groups, $n = 10/\text{group}$, $*p < 0.05$, $**p < 0.01$, by one-way ANOVA and Tukey's post hoc. Note an 87-fold increase in MMP-9 mRNA in nerve at 1 day that is gradually reduced by 60 days after crush.

Fig. 2. Cytokine-induced MMP-9 expression in sciatic nerve. (A) Real-time Taqman qPCR for MMP-9. Data are expressed as the mean fold increase $\pm$ SE in injected relative to uninjured nerves, $n = 10$ /group. Statistics: (* $p < 0.05$, relative to uninjured nerve, # $p < 0.05$, relative to vehicle group by one-way ANOVA and Tukey's post hoc). Crushed (Day 1) nerves were used for positive control. (B) Gelatin zymography (inverted image) demonstrates increased MMP-9 activity in nerve after NGF, TNF- α and IL-1 β injection. Uninjured and crushed (Day 1) nerves are used as negative and positive controls, respectively ($n = 6$ /group). (C) MMP-9 immunoreactivity after TNF- α injection showing myelinated Schwann cell (arrow) reactivity is similar to the endogenous MMP-9 changes after crush. Micrographs are representative of four animals/group (100 $\times$ objective magnification).

Fig. 3. Partial reduction in MMP-9 in nerve after TNF- α receptor deletion. Gelatin zymography (inverted image) demonstrates partial reduction in MMP-9 activity in crushed (Day 1) TNFR1 knockout and TNFR1 and 2 double-knockout nerves. Densitometry (graph, $n = 6/\text{group}$, mean $\pm$ SE), * $p < 0.05$ knockout vs. wild-type by Student's t -test.

Fig. 4. MMP-9 gene deletion reduces painful behavior. Unstimulated pain score was recorded in MMP-9^{-/-} and control FVB mice for 2 weeks after sciatic nerve crush. Different positions of the injured hind paw were rated in each animal for 15 minutes (3 × 300 s) using 0–5 numerical scale (see methods); data expressed as mean ± SE. Note reduction in pain score in MMP-9^{-/-} animals, $n = 10/\text{group}$, $*p < 0.05$ knockout vs. wild-type by one-way ANOVA and Tukey's post hoc.

Fig. 5. MMP-9 regulates MBP turnover. Western blot (A) and immunofluorescence (B) for myelin basic protein (MBP) in crushed (day 10) MMP-9 knockout nerves showed preserved MBP (myelinating Schwann cell marker) and no change in S100 (Schwann cell marker) or β -actin (loading control). Densitometry was done in $n = 8$ /group. Dual-immunofluorescence for MBP (green) and nuclear stain DAPI (blue) shows preserved MBP and myelin thickness after MMP-9 deletion (B). Micrographs are representative of $n = 4$ /group (100 $\times$ objective magnification).