Metalloproteinase MT5-MMP is an essential modulator of neuro-immune interactions in thermal pain stimulation

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Peripheral interactions between nociceptive fibers and mast cells contribute to inflammatory pain, but little is known about mechanisms mediating neuro-immune communication. Here we show that metalloproteinase MT5-MMP (MMP-24) is an essential mediator of peripheral thermal nociception and inflammatory hyperalgesia. We report that MT5-MMP is expressed by CGRP-containing peptidergic nociceptors in dorsal root ganglia and that Mmp24-deficient mice display enhanced sensitivity to noxious thermal stimuli under basal conditions. Consistently, mutant peptidergic sensory neurons hyperinnervate the skin, a phenotype that correlates with changes in the regulated cleavage of the cell-cell adhesion molecule N-cadherin. In contrast to basal nociception, Mmp24$^{-/-}$ mice do not develop thermal hyperalgesia during inflammation, a phenotype that appears associated with alterations in N-cadherin-mediated cell-cell interactions between mast cells and sensory fibers. Collectively, our findings demonstrate an essential role of MT5-MMP in the development of dermal neuro-immune synapses and suggest that this metalloproteinase may be a target for pain control.

inflammation | mast cell | N-cadherin

Sensory neurons that innervate the skin are the primary mediators in the perception of external noxious and non noxious stimuli. Under physiological conditions, sensory neurons known as nociceptors are activated by acute painful stimuli and, in situations of nerve damage or inflammation, sensitization of primary sensory neurons leads to persistent neurogenic or inflammatory pain, respectively (1, 2). It is becoming increasingly clear that nociceptive processing in these pain states requires the contribution of glial and immune cells (3). Therefore, the elucidation of signaling relationships between nociceptive primary afferents and peripheral immune cells appears necessary to understand pain physiology and to develop therapeutic strategies for pain control.

Proteolytic remodeling of the extracellular matrix (ECM) is an essential event in the development of both peripheral and central nervous systems (4). MMPs (matrix metalloproteinases) and ADAMs (a disintegrin and metalloproteinase) have the ability to cleave all major ECM protein components under physiological conditions (4, 5). A concerted action of different proteases may, therefore, be highly relevant to the construction and physiology of neural circuits (6, 7). Numerous studies have implicated ADAMs in the formation of neural connections in the developing nervous system (8–11). In contrast, the potential role of MMPs in axonal elongation and guidance through regulated cleavage of ligands and/or receptors involved in axon navigation has been more elusive (7, 11–13).

MT5-MMP (Mmp24) is mainly expressed in neuronal cells of both central and peripheral nervous systems, although its presence has also been detected in inflammatory cells (14–18). MT5-MMP can degrade several ECM components, such as inhibitory chondroitin sulfate proteoglycans, thereby promoting neurite outgrowth in vitro (16, 19). Moreover, it appears capable of mediating the cleavage of cell-adhesion molecule N-cadherin in heterologous cells (20). On this basis, together with the absence of Aβ-fiber sprouting after sciatic nerve injury in a mouse strain lacking MT5-MMP, it has been suggested that this metalloproteinase may contribute to the ECM degradation associated with neuronal plasticity (21). However, at present, very little information is available about the in vivo functional relevance of MT5-MMP. In this work, we have generated mutant mice deficient in MT5-MMP (Mmp24$^{-/-}$) to further characterize the in vivo role of this protease in both physiological and pathological processes.

Results

Generation of Mmp24-Deficient Mice. To analyze the in vivo role of MT5-MMP, we generated mice with a targeted disruption in the Mmp24 gene by replacing a 6-kb fragment containing exons 5–7 of Mmp24 with an antisense-oriented PGK-neo cassette (Fig. 1*A*). This deletion results in a loss of the catalytic domain and part of the hemopexin domain of the protein. Chimeric founder mice derived from two independent ES cell lines were mated to C57BL/6J mice to generate heterozygous mice that were identified by Southern blot analysis. After intercrossing heterozygous mice from the F1 generation, we obtained Mmp24$^{-/-}$, Mmp24$^{+/-}$, and Mmp24$^{+/+}$ mice (Fig. 1*B*) in the expected Mendelian ratios. Northern blot analysis of total RNA obtained from tissues of Mmp24$^{-/-}$ and Mmp24$^{+/+}$ mice revealed the presence of a shorter Mmp24 transcript corresponding to the expected targeted deletion in cerebellum from knock-out mice (Fig. 1*C*). To support an absence of functional MT5-MMP in Mmp24$^{-/-}$ mice, we expressed in COS-7 cells a recombinant protein (ΔMT5-MMP) lacking those domains encoded by the exons removed in Mmp24$^{-/-}$ mice, and analyzed its ability to activate proMMP-2, a characteristic enzymatic function of the

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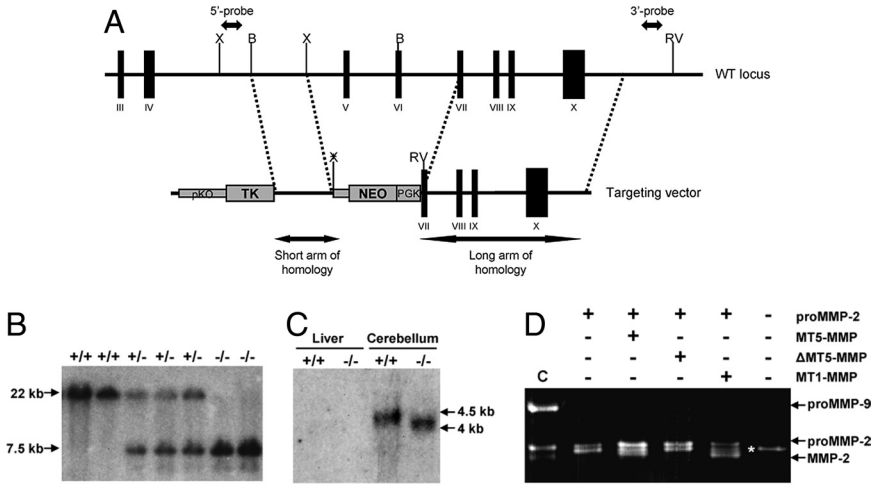

Fig. 1. Gene targeted disruption of the MT5-MMP gene. (*A*) Restriction maps of the MT5-MMP (Mmp24) gene region of interest (top) and the targeting construct (bottom). B, *Bam*HI; RV, *Eco*RV; X, *Xho*I; X with superimposed X, destroyed *Xho*I. (*B*) Southern blot analysis of Mmp24$^{+/+}$, Mmp24$^{+/-}$, and Mmp24$^{-/-}$ mice. (*C*) Detection of Mmp24 mRNA in cerebellum by Northern blot analysis. Liver tissue was loaded as a negative control. (*D*) Analysis of proMMP-2 activation by transient transfections of the full-length Mmp24 wild-type cDNA and the ΔMT5-MMP truncated form, obtained from cerebellum of knock-out mouse. The asterisk indicates an unspecific gelatinolytic activity present in the conditioned medium of untransfected COS-7 cells. C, conditioned medium, used as a control, from the human fibrosarcoma HT1080 cell line that endogenously produces both pro-MMP-2 and pro-MMP-9.

MT-MMPs (22). As shown in Fig. 1*D*, co-transfection of COS-7 cells with full-length proMMP-2 and MT5-MMP expression plasmids resulted in proMMP-2 activation, as assessed by the generation of the 62-kDa band corresponding to active MMP-2. Similar results were obtained when COS-7 cells were transfected with an expression vector for MT1-MMP, whose ability to activate proMMP-2 is well-established (22). In contrast, co-transfection of the same cells with proMMP-2 and ΔMT5-MMP expression vectors failed to demonstrate active MMP-2 (Fig. 1*D*). Taken together, these results indicate that in the unlikely event that Mmp24$^{-/-}$ mice produce a small amount of truncated MT5-MMP, this mutant protein should be devoid of enzymatic activity. Physiological characterization delineated that mutant mice were viable, developed normally, and showed no overt abnormalities. Further, males and females were fertile and their long-term survival rates were indistinguishable from those of their wild-type littermates.

Altered Nociceptive Thermal Response in Mmp24$^{-/-}$ Mice Is Characterized by Enhanced Sensitivity to Noxious Heat. According to the Mmp24 expression pattern in neural tissues together with the absence of mechanical allodynia from neuropathic origin in another strain of Mmp24$^{-/-}$ mice (21), we performed a detailed analysis of sensory nociceptive responsiveness in Mmp24-mutant mice. As can be seen in Fig. 2 *A* and *B*, nociceptive tests determined that Mmp24$^{-/-}$ mice exhibit an increased response to thermal noxious stimuli. Thus, in the unilateral hot plate test (set at 52.00 ± 0.50 °C), wild-type mice exhibited thermal withdrawal latencies of about 14 s while these values were significantly lower in Mmp24-deficient mice (Fig. 2*A*). Furthermore, the mean temperature at which 50% of wild-type mice withdrew their paws from the hot plate was 51.20 ± 0.35 °C whereas in Mmp24-deficient mice this value was significantly lower (46.60 ± 0.52 °C, $P < 0.01$) (Fig. 2*B*). Further, no differences were observed in the mechanical nociceptive tests between Mmp24$^{-/-}$ and Mmp24$^{+/+}$ mice (Fig. S1). These results indicate the involvement of MT5-MMP in the detection of acute thermal noxious stimuli.

Hypertrophy of Nociceptive Cutaneous Innervation in Mmp24$^{-/-}$ Mice. Neurons involved in pain perception are located in sensory ganglia and can be divided into NGF-dependent trkA+ peptidergic and GDNF-dependent c-ret+ non-peptidergic subtypes, selectively recognized with antibodies to CGRP/SP and the lectin IB4, respectively (1, 23). We demonstrated by immunofluorescence that neurons in adult DRGs express MT5-MMP (Fig. 3*A*). Furthermore, double staining with antibodies to CGRP or the IB4 lectin revealed that all MT5-MMP-positive cells are peptidergic sensory neurons, whereas non-peptidergic neurons do not exhibit detectable levels of this metalloproteinase. This suggests that effects of the Mmp24-deficiency would likely be restricted to NGF-dependent nociceptive neurons (Fig. 3 *B* and *C*). Immunohistochemical analyses of skin innervation in adult mice footpads with antibodies to the general neuronal marker PGP9.5 and the specific peptidergic C-fiber marker CGRP, identified a significant increase in the density of PGP9.5+ and peptidergic fine nerve endings in Mmp24 mutants compared to wild-type littermates (Fig. 3 *D–F*). Moreover, close inspection of the epidermis of Mmp24-deficient mice indicated that single PGP9.5+ fine nerve fibers were not only more numerous but more ramified, with secondary branches which often traveled parallel to the skin surface for relatively long distances, a feature never observed in the wild-type skin (Fig. S2). As the number of sensory neurons remained unaltered by

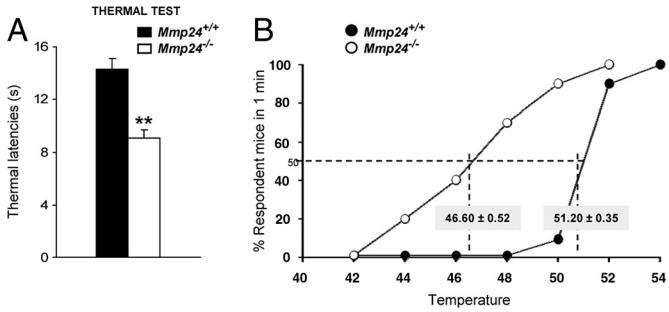

Fig. 2. Enhanced sensitivity to noxious thermal stimuli in Mmp24-deficient mice. (*A*) Thermal nociceptive responses of Mmp24$^{+/+}$ ($n = 10$; black bars) and Mmp24$^{-/-}$ ($n = 9$; white bars) mice were assessed by using the unilateral hot plate (UHP) test set at 52.0 ± 0.5 °C (**, $P < 0.01$). (*B*) Percentage of Mmp24$^{+/+}$ ($n = 6$; black circles) and Mmp24$^{-/-}$ ($n = 5$; open circles) mice which respond to a different set of temperatures in 1 min ($F_{6,94} = 795{,}09$; **, $P < 0.01$).

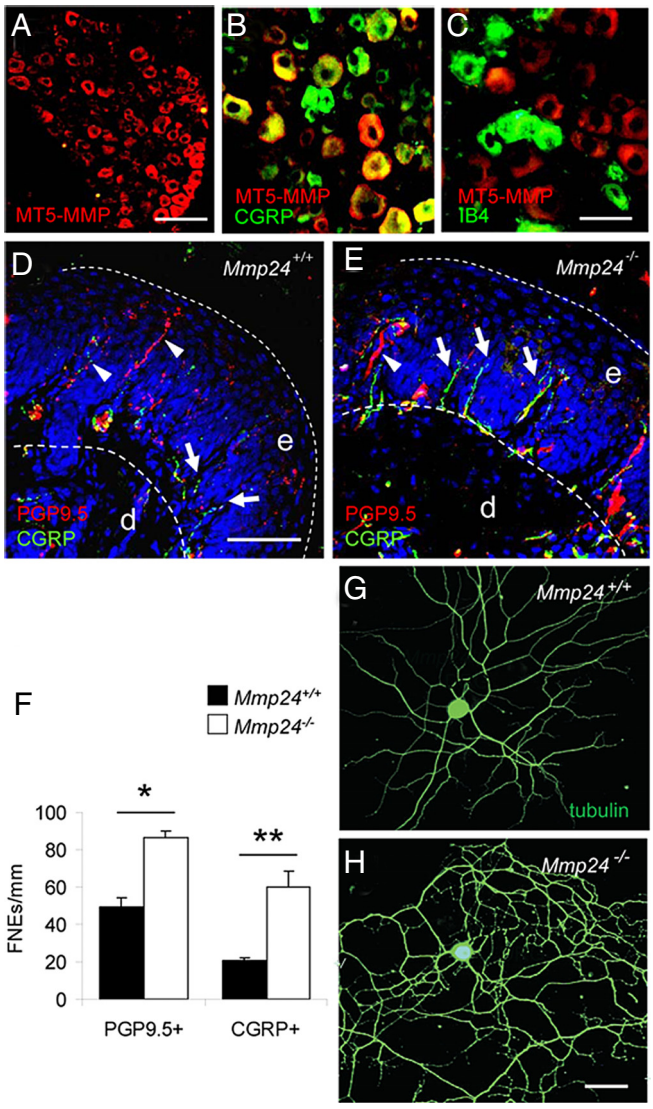

Fig. 3. MT5-MMP expression and hyperinnervation of the skin in footpads of wild-type and *Mmp24*-deficient mice. (*A–C*) Sections through lumbar DRGs immunostained for MT5-MMP (red) alone (*A*) or in combination with antibodies to CGRP (*B*) or with the IB4 lectin (*C*) (green). Notice that MT5-MMP+ neurons are CGRP+ but not IB4+. (*D* and *E*) Confocal micrographs showing the innervation of the epidermis and upper dermis at comparable locations in the footpads of $Mmp24^{+/+}$ and $Mmp24^{-/-}$ adult mice as shown by double immunofluorescence with anti-CGRP (green) and anti-PGP9.5 (red). The structure of the tissue is revealed by the fluorescent histochemical DNA marker TOPRO (blue). The broken lines indicate the limit between upper dermis (d) and epidermis (e) and the skin surface. Arrowheads indicate examples of fine nerve endings in the epidermis that are only labeled with anti-PGP9.5 antibodies. Arrows point at fine nerve endings that are also labeled with anti-CGRP antibodies. (*F*) Quantification of the innervation to the epidermis in footpads of $Mmp24^{+/+}$ and $Mmp24^{-/-}$ mice. Because mutant and wild-type skins do not differ in epidermal thickness, the quantitative analyses were carried out on three grids of fixed width per section on four sections per animal and the mean of each parameter was obtained. Mean numbers of fine nerve endings (FNE)/mm ± SEM of three mice of each genotype are shown. The parameters analyzed were the number of PGP9.5+ and CGRP+ FNE. (*G* and *H*) Isolated neonatal DRG neurons from $Mmp24^{+/+}$ and $Mmp24^{-/-}$ mice plated on collagen and cultured for 2 days in the presence of 10 ng/mL NGF, fixed and immunostained with antibodies to neuronal tubulin. (Scale bars in *A*, 50 μm; *B* and *C*, 25 μm; *D* and *E*, 40 μm; *G* and *H*, 25 μm.)

the mutation, these results collectively indicate that peptidergic sensory C-fibers in the skin of $Mmp24^{-/-}$ mice are more highly branched, suggesting that MT5-MMP is essential for determining the size of the receptive field of cutaneous nociceptive neurons. To further determine whether the cutaneous hyperinnervation phenotype of $Mmp24^{-/-}$ mice could be the result of enhanced neuritic outgrowth of their nociceptive neurons, we cultured neonatal DRG neurons in the presence of 10 ng/mL NGF. NGF-dependent neurons from $Mmp24^{-/-}$ neonatal mice exhibited increases in neurite ramifications and varicosities compared to neurons from $Mmp24^{+/+}$ mice (Fig. 3 *G* and *H*). We quantitated neurite growth in neurons seeded on collagen and observed significant increases in the number of branch points and in the total number of secondary neurites per neuron in $Mmp24^{-/-}$ cultures in comparison to the wild-type ones (Fig. S3*A*). Further, the same branching phenotype was demonstrated by neurons labeled with anti-CGRP (Fig. S3*B*), but not by those labeled with the IB4 lectin (Fig. S3*C*), suggesting that the CGRP+ fiber hypertrophy observed in mutant skin results from a more profuse branching activity by sensory neurons.

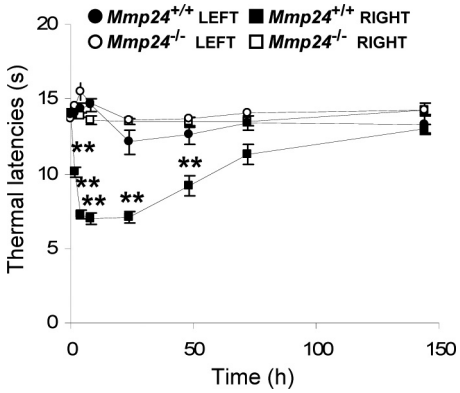

Fig. 4. Altered hyperalgesic behavior in Mmp24-deficient mice after intraplantar injection of carrageenan. Carrageenan-induced inflammatory thermal hypernociception in wild-type ($n = 5$) and Mmp24-deficient mice ($n = 5$, **, $P < 0.01$). Right, right paw (injected); Left, left paw (sham).

Absence of Thermal Inflammatory Hyperalgesia in $Mmp24^{-/-}$ Mice. It has long been appreciated that peptidergic CGRP+ neurons contribute to the enhanced pain responsiveness that follows target tissue injury, as is the case for inflammation (1). Considering the cutaneous hyperinnervation phenotype observed in $Mmp24^{-/-}$ mice, we further analyzed how this increase in the sensory fibers might affect the hyperalgesic responses under inflammatory conditions. Peripheral inflammation was induced in $Mmp24^{-/-}$ and $Mmp24^{+/+}$ mice by carrageenan intraplantar injection and thermal withdrawal latencies were measured. As the thermal latencies obtained in basal conditions were lower in $Mmp24^{-/-}$ mice compared to wild-type animals, the hot plate temperature was adjusted in each case to yield basal values (non-inflamed) of 14 s. The magnitude of the inflammation, as determined by the volume of the paw injected with carrageenan (right), was similar in both wild-type and $Mmp24^{-/-}$ mice (Fig. S4*A*). In agreement, histological examination of the inflamed paws showed no overt differences (Fig. S4*B*). We next analyzed the hyperalgesic response to thermal stimulation. As expected, wild-type mice exhibited lower withdrawal latencies in the inflamed (right) paws compared to the corresponding non-inflamed (left) paws when measured after carrageenan administration. However, inflamed and non-inflamed hindpaws of Mmp24-deficient mice showed similar withdrawal latencies at the different times studied (Fig. 4). The unanticipated diminishment of hyperalgesia development in $Mmp24^{-/-}$ mice following inflammatory injury, led us to evaluate the effect of factors released during the inflammatory process. As shown in Fig. S5 *A–E*, the withdrawal latencies obtained in the hind paws

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of wild-type mice receiving intraplantar injection of TNF-α (30 pg/25 μL), IL-1β (30 pg/25 μL), IL-6 (30 pg/25 μL), NGF (1 μg/25 μL), or PGE_2 (100 ng/25 μL) were significantly lower ($P < 0.05$) than those obtained in the non-injected contralateral paw. However, only the administration of IL-6 and PGE_2 produced hyperalgesia in $Mmp24^{-/-}$ mice, whereas TNF-α, IL-1β, and NGF had no effect, suggesting that these proteins act through an inflammatory pathway which is altered in knock-out mice.

Abnormal Interactions between $Mmp24^{-/-}$ Mast Cells and Sensory Neurons. The absence of an inflammatory response to TNF-α, IL-1β, or NGF in $Mmp24^{-/-}$ mice, prompted us to analyze the cellular mechanisms involved in peripheral sensitization during inflammation. Although NGF can directly sensitize nociceptors, the NGF-induced hyperalgesic effect can be reduced by preventing mast cells degranulation, suggesting that mast cells are required to enable NGF to have its full effect on nociceptive terminals (24, 25). Accordingly, we analyzed the dynamics and characteristics of mast cells present in skin samples from $Mmp24^{+/+}$ and $Mmp24^{-/-}$ adult mice. Electron microscopy analysis demonstrated a significant increase in the number of degranulated mast cells in mutant skin when compared to wild-type samples (61.5 ± 3.1% vs. 41.2 ± 2.0%, respectively, $n = 3$ animals per genotype, $P < 0.01$; Fig. S6 *A* and *B*). Degranulation of mast cells can be experimentally induced by topical application of capsaicin to mesentery preparations (26), as such, we used this approach to functionally evaluate mast cell degranulation in $Mmp24^{-/-}$ mice in vivo. First, under basal conditions we observed the same phenotype as that detected in the mutant skin. Although a similar number of mast cells were present in $Mmp24^{+/+}$ and $Mmp24^{-/-}$ mice, the percentage of mast cells which were degranulated was significantly higher in samples from mutant mice than in wild-type samples (62.1 ± 4.0% vs. 17.4 ± 0.7%, respectively, $n = 3$ animals per genotype, $P < 0.001$; Fig. S6 *C* and *D*). Furthermore, wild-type mast cells responded to exogenous administration of capsaicin as the percentage of degranulated mast cells increased around 50% (27.2 ± 2.6%, $n = 3$, $P < 0.05$) following the treatment, in agreement with previous data (26). Significantly, we did not observe any increases in the mutant samples (52.5 ± 1.7%, $n = 3$, $P = 0.07$), suggesting that abnormal mast cell degranulation in $Mmp24^{-/-}$ mice may prevent the action of exogenous inflammatory stimuli.

To study potential interactions between mast cells and neurons, as well as to analyze the effects of the Mmp24 mutation on the mast cells directly, we obtained primary cultures of bone marrow-derived mast cells (BMMCs) from wild-type and mutant mice. In cultures of isolated BMMCs, we observed that the percentage of degranulated cells was similar in wild-type (16.3 ± 2.5%, $n = 3$ cultures from independent animals per genotype) and mutant cultures (19.5 ± 3.5%, $n = 2$), an indication that lack of MT5-MMP does not directly affect mast cell basal degranulation. Therefore, to analyze if the degranulating phenotype observed in mutant mice was due to abnormal interactions between mast cells and sensory neurons, we performed co-culture experiments between mast cells and sensory neurons. In co-cultures of wild-type BMMCs and wild-type neurons, immune cells appeared to extend cellular processes which approached sensory fibers, although they maintained spatial distance from the mast cells. However, in co-cultures of mutant neurons and BMMCs, fibers were always closely apposed to mast cells (Fig. S6*E*). To evaluate whether these morphological changes were functionally relevant, we studied BMMC degranulation in the different co-culture conditions. In co-cultures of wild-type neurons and wild-type BMMCs the percentage of degranulated mast cells was very low (Table S1) and virtually identical to that found in wild-type or mutant BMMC-only cultures (see above). Treatment with capsaicin or NGF resulted in the degranulation of the majority of mast cells present in the wild-type/wild-type cultures. Remarkably, in co-cultures of mutant neurons with mutant BMMCs the percentage of degranulated BMMCs was over 70% under basal conditions (Table S1), and could not be increased with the subsequent capsaicin or NGF treatment. These results indicate that the lack of hyperalgesic responses in vivo after inflammation may be due to the inability of mast cells to release mediators required for fiber sensitization. Consequently, to determine whether the abnormal degranulating phenotype was due to altered physical contact between mutant neurons and mutant mast cells, we indirectly co-cultured mutant DRG sensory neurons and mutant BMMCs using transwell insets. Under these conditions, we did not observe abnormal degranulation of mast cells in mutant/mutant co-cultures (70 degranulated cells out of 182 total cells in wild-type neuron/wild-type BMMC co-cultures; 79 out of 177 cells in mutant neuron/mutant BMMC co-cultures; 70 out of 171 in mutant neuron/wild-type BMMC co-cultures; and 80 out of 198 in wild-type neuron/mutant BMMC co-cultures), an indication that the altered physical contact between mutant neural and immune elements underlies the mast cell ectopic release response. These results indicate that cellular interactions between mast cells and sensory fibers are altered in the absence of MT5-MMP and result in an abnormal physiological response.

Changes in N-Cadherin Underlie Effects of MT5-MMP in Mast Cell-Neuron Interactions. To gain an insight into the mechanism by which MT5-MMP regulates the interaction between immune and neural cells, and hence inflammatory pain, we studied the potential actions of this metalloproteinase in the regulation of cell adhesion. Interestingly, N-cadherin plays a role in the stabilization of neurite branches, is expressed by mast cells and its trafficking to sites of interaction with the neurites appears to be induced by neurites, suggesting its implication in the establishment of neuro-immune synapses (27, 28). Our experiments revealed that mutant neurons exhibited increased accumulation of N-cadherin labeling than wild-type neurons (Fig. 5 *A* and *C*). Although this result could be the consequence of mutant neurons having more branching points, western blot analysis of homogenates from sensory neuron cultures of both genotypes indicated that the absence of functional MT5-MMP significantly reduces the level of cleaved intracellular C-terminal fragments of N-cadherin. This suggests that mutant neurons have higher levels of full length N-cadherin at the membrane (Fig. S7). In agreement with the observed changes in N-cadherin processing, levels of β-catenin in the mutant neurons were significantly increased relative to wild-type neurons (Fig. 5 *B* and *D* and Fig. S7). MT5-MMP+ mast cells also expressed N-cadherin. Both molecules exhibited similar distributions in both neurons and BMMCs (Fig. 5 *E–G*). Interestingly, we could observe that at contact sites between neurites and BMMCs, the labeling for N-cadherin and MT5-MMP became more homogeneous and intense (Fig. 5*G*). As in neurons, the lack of MT5-MMP in BMMCs also resulted in an abnormal processing of N-cadherin and in increased levels of β-catenin, as assessed by western blot analysis (Fig. S7).

To test whether N-cadherin and MT5-MMP play a role in the functional interaction between neurons and mast cells, we treated co-cultures of neurons and mast cells of different genotypes with anti-N-cadherin function blocking antibodies or with control IgGs, and analyzed the BMMC degranulating phenotype by cresyl violet staining. In agreement with previous experiments showing that degranulation requires direct contact between neural and immune cells (27), our results indicated that normal degranulation requires N-cadherin-mediated cell-cell interaction between mast cells and neurites, as no degranulation could be elicited by capsaicin in anti-N-cadherin antibody-treated wild-type/wild-type co-cultures (Table S2). Interestingly, addition of N-cadherin antibodies, but not of the control IgGs, rescued the abnormal degranulating phenotype observed in mutant/mutant co-cultures (Table S2). Thus, N-cadherin modulates neuro-immune synapses and regulation of this cell-adhesion molecule by MT5-MMP appears essential for inflammatory pain responses.

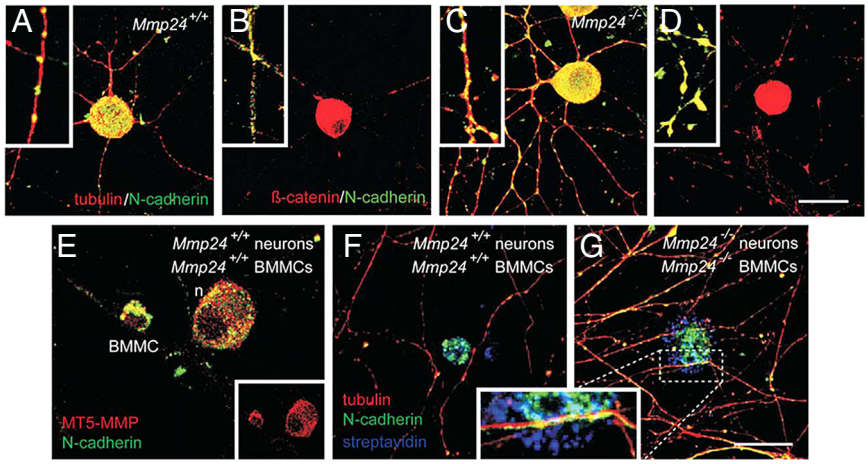

Fig. 5. Increased N-cadherin levels in the absence of MT5-MMP. (*A* and *C*) Isolated DRG neurons from Mmp24$^{+/+}$ (*A*) and Mmp24$^{-/-}$ (*C*) mice plated on collagen and cultured for 2 days in the presence of 10 ng/mL NGF, fixed and co-immunostained with antibodies to neuronal tubulin (red) and N-cadherin (green). Insets show a stretch of a labeled neurite at higher magnification. (*B* and *D*) Isolated neonatal DRG neurons from Mmp24$^{+/+}$ (*B*) and Mmp24$^{-/-}$ (*D*) mice plated on collagen and cultured for 2 days in the presence of 10 ng/mL NGF, fixed and co-immunostained with antibodies to N-cadherin (green) and to β-catenin (red). Insets show a stretch of a labeled neurite at a higher magnification showing co-localization at varicosities. (*E*) Co-culture of wild-type BMMCs and DRG neurons labeled with antibodies to MT5-MMP (red) and to N-cadherin (green). (*F* and *G*) BMMCs and DRG neurons in wild-type/wild-type (*F*) and mutant/mutant (*G*) co-cultures labeled with antibodies to neuronal tubulin (red) and to N-cadherin (green) as well as with Cy3-conjugated streptavidin (blue) which specifically labels mast cell granules. (Scale bars in *A–D*, 20 μm; *E–G*, 15 μm.)

Discussion

We show herein that MT5-MMP plays an important role in cell-cell interactions between nociceptive neurites and mast cells. Our data demonstrates that the absence of MT5-MMP leads to a phenotype of hyperinnervation and enhanced sensitivity to noxious thermal stimuli in Mmp24$^{-/-}$ mice. The absence of MT5-MMP in nociceptive peptidergic sensory neurons alters fiber outgrowth in a cell autonomous way. As each mutant sensory neuron supplies larger cutaneous territories, it is likely that a high number of these neurons will be recruited by the noxious thermal stimulation, increasing the final sensory inflow that reaches second-order neurons at the central nervous system.

We have found that virtually all MT5-MMP expressing neurons in DRGs are CGRP+ and that, conversely, MT5-MMP is not expressed by IB4+ neurons. It is generally accepted that the peptidergic population is involved in the enhanced pain responsiveness that follows inflammatory processes (1). In agreement with MT5-MMP expression in peptidergic neurons, our behavioral analysis of Mmp24$^{-/-}$ mice has shown that MT5-MMP is essential for the development of inflammatory hyperalgesia. However, the finding of diminished thermal pain responsiveness after carrageenan injection resulted rather surprising in light of our previous observation that CGRP+ fibers hyperinnervate the skin in mutant animals. A detailed analysis of the effect of different factors released during the inflammatory process (29, 30) revealed that only the administration of IL-6 and PGE_2 produced hyperalgesia in Mmp24$^{-/-}$ mice, whereas TNF-α, IL-1β, and NGF had no effect, suggesting that these proteins act through an inflammatory pathway which is altered in the mutant mice. As the action of these factors during the inflammatory process can be organized into an amplification cascade, whereby NGF would be the last protein effector (31), we focused on the evaluation of the hyperalgesia induced by NGF in the inflammatory setting. Although NGF can directly sensitize nociceptors, pharmacological approaches have indicated that mast cells are necessary mediators in the hyperalgesic phenotype through the release of several neuroactive molecules in response to NGF stimulation (24, 32–34). We have observed an abnormal increase in degranulated mast cells in Mmp24$^{-/-}$ samples under basal conditions. Furthermore, the increase precluded the degranulation-inducing effect of either capsaicin or NGF and correlated with a lack of hyperalgesia after NGF administration in vivo. Therefore, our results are consistent with previous reports describing that mast cell degranulation is a necessary intermediate in inflammatory hyperalgesia.

Our results also indicate that mast cell degranulation requires physical cell-cell interaction with sensory neurites, and that MT5-MMP regulates this interaction by a mechanism involving N-cadherin, the expression of which has been reported to be concentrated at the sites of contact mediating nerve-mast cell synaptic interaction (27). In addition, it has been previously shown that MT5-MMP activity generates a C-terminal fragment from N-cadherin cleavage in heterologous cells (20). Consistently, we have shown that N-cadherin is a substrate of MT5-MMP in vivo. Moreover, the finding that the blockade of N-cadherin rescues the abnormal interactions between Mmp24$^{-/-}$ mast cells and neurons supports the hypothesis that the Mmp24$^{-/-}$ phenotype is a consequence of altered N-cadherin activity in both cell types. Changes in cell adhesion are likely mediators of the hyperinnervating phenotype. Neuritogenesis is a highly dynamic process involving constant extensions and retractions. N-cadherin participates in this process by stabilizing neurite branches (28). It has been demonstrated that overexpression of N-cadherin and β-catenin in hippocampal neurons enhances their dendritic arborization (35). Consequently, we have observed that Mmp24 mutant DRG sensory neurons display high levels of β-catenin. Therefore, changes in N-cadherin processing and distribution, as well as the higher levels of β-catenin observed in Mmp24$^{-/-}$ DRG cultures and BMMCs, may underlie both the neuritic growth and the nerve-mast cell interaction phenotypes.

In conclusion, our results provide causal evidence that the absence of a MMP family member, specifically MT5-MMP, causes a cutaneous phenotype of hyperinnervation and a morphological and functional alteration in neuro-immune interactions. Further, we demonstrate that these changes have complex consequences on nociceptive thermal responses, under physiological and pathological conditions. Many reports have evidenced the importance of the MMP family of proteolytic enzymes in the pathology of the peripheral and central nervous system. The increased expression and detrimental contribution of these enzymes to a number of pathologies such as blood–brain barrier dysfunction, demyelination, neuroinflammation, spinal

cord injury, multiple sclerosis, and haemorrhagic stroke has suggested the use of metalloproteinase inhibitors for the treatment of neurological diseases (7, 36, 37). Beyond these previous proposals, and based on the results presented herein, we conclude that some metalloproteinases may also constitute relevant targets for pain control (3).

Materials and Methods

Refer to *SI Material and Methods* for detailed histological and immunocytochemical methods and for a description of the knock-out construct and mouse characterization.

Generation of Mmp24-Deficient Mice. For detailed methods refer to *SI Material and Methods*. Briefly, a PGK-neo cassette with the transcriptional orientation opposite to that of Mmp24 gene replaced a 6-kb fragment containing exons 5–7 of the gene. The heterozygous stem cells 14.1 (129/Ola derived) obtained by homologous recombination were aggregated to CD1 morulas. Chimeric males generated were mated with C57BL/6J females and the offspring was screened by Southern blot analysis of tail genomic DNA. The heterozygous littermates were mated to obtain homozygous mutant mice.

Behavioral Tests. Experimental procedures were approved by the Comité Ético de Experimentación Animal, Universidad de Oviedo, Spain. For detailed methods refer to *SI Material and Methods*. Briefly, thermal nociceptive responses were assessed by using the unilateral hot plate test, as described (38). Mechanical sensitivity was assessed using a modification of the Randall-Selitto test (39) and von Frey filaments as described (40). For intraplantar injections, drugs were dissolved in 25 μL and administered in the plantar side of the right hindpaw.

DRG Neuron Culture. Wild-type and mutant neonatal mice were decapitated and around 50 DRGs per animal were dissected out and collected in L15 media. Ganglia were incubated at 37 °C with 2 mg/mL collagenase followed by 0.05% trypsin. Approximately 2,000 neurons were plated in 1 mL defined medium (refer to *SI Material and Methods*) using 24-well plates with coverslips coated with poli-DL-ornithine (0.5 mg/mL; Sigma) plus collagen I (1.5 mg/mL; Invitrogen). Neurons were maintained in culture for 48 h at 37 °C in a humidified incubator under 5% CO_2. For immunolabeling experiments refer to *SI Material and Methods*.

Nerve-Mast Cell Co-Culture. For detailed methods refer to *SI Material and Methods*. Briefly, bone marrow-derived mast cells (BMMCs) were isolated from femurs and tibias of adult wild-type and mutant mice and cultured in the presence of 10 ng/mL recombinant mouse IL-3 (PreProtech) for 4–9 weeks at 37 °C in 5% CO_2 to allow for the differentiation of differentiation of BMMCs (41). For co-cultures, BMMCs (1×10^4 cell/mL) were added to 5-day-old dissociated DRG cultures and incubated in supplemented Hams F-12 medium including 10 ng/mL IL-3. Some BMMCs were cultured alone as controls.

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