

Mouse mast cells and mast cell proteases do not play a significant role in acute tissue injury pain induced by formalin

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Abstract

Subcutaneous formalin injections are used as a model for tissue injury-induced pain where formalin induces pain and inflammation indirectly by crosslinking proteins and directly through activation of the transient receptor potential A1 receptor on primary afferents. Activation of primary afferents leads to both central and peripheral release of neurotransmitters. Mast cells are found in close proximity to peripheral sensory nerve endings and express receptors for neurotransmitters released by the primary afferents, contributing to the neuro/immune interface. Mast cell proteases are found in large quantities within mast cell granules and are released continuously in small amounts and upon mast cell activation. They have a wide repertoire of proposed substrates, including Substance P and calcitonin gene-related peptide, but knowledge of their *in vivo* function is limited. We evaluated the role of mouse mast cell proteases (mMCPs) in tissue injury pain responses induced by formalin, using transgenic mice lacking either mMCP4, mMCP6, or carboxypeptidase A3 (CPA3), or mast cells in their entirety. Further, we investigated the role of mast cells in heat hypersensitivity following a nerve growth factor injection. No statistical difference was observed between the respective mast cell protease knockout lines and wild-type controls in the formalin test. Mast cell deficiency did not have an effect on formalin-induced nociceptive responses nor nerve growth factor-induced heat hypersensitivity. Our data thus show that mMCP4, mMCP6, and CPA3 as well as mast cells as a whole, do not play a significant role in the pain responses associated with acute tissue injury and inflammation in the formalin test. Our data also indicate that mast cells are not essential to heat hypersensitivity induced by nerve growth factor.

Keywords

Pain formalin, transgenic, mast cell, protease

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Introduction

When tissue injury occurs, a cascade of responses including inflammation and pain sensation is initiated to minimize harm and facilitate healing. Subcutaneous formalin injection serves as a translational model for tissue injury-induced pain where formalin causes injury and inflammation by interacting with the transient receptor potential (TRP) A1¹ and by covalently cross-linking proteins in a nonspecific fashion, thus disrupting cell membranes.² TRPA1 is expressed on a subpopulation of TRPV1-positive primary afferents,^{3,4} and activation leads to action potential formation and subsequent transmitter release in both the central and peripheral terminals of the afferents, thereby causing pain sensation

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and activation of immune cells, including mast cells. In the skin, this process is generally referred to as cutaneous neurogenic inflammation⁵ (also reviewed in the study by Gouin et al.⁶)

Nerve growth factor (NGF) is a neurotrophic factor essential for the development and survival of sympathetic and sensory neurons.⁷ NGF injections have been used in a translational model of inflammation-induced hypersensitivity toward both thermal and mechanical stimuli, a hypersensitivity that persists for hours and days, respectively, after injection.^{8,9} NGF activates the high-affinity tropomyosin kinase A receptor (TrkA) on nociceptive neurons, promoting upregulation and intracellular modifications of TRPV1 receptors via the mitogen-activated protein kinase and phosphatidylinositol 3-kinase signaling pathways,^{10,11} which subsequently leads to thermal and mechanical hypersensitivity.

Mast cells are immune cells that are considered to be of particular importance in tissue injury responses, as they are widely distributed throughout the body, express receptors capable of recognizing various different substances of exogenous and endogenous origin, and can respond quickly by producing and releasing appropriate mediators, many of which are inflammatory, such as cytokines.^{12,13} Besides cytokines, mast cells also contain proteases that are stored in their active form in large amounts within the mast cell secretory granules, which are released upon mast cell activation.^{14–16} Furthermore, granular content is continuously released at baseline conditions by the so-called piecemeal degranulation.¹⁷ Consequently, mast cell proteases are found in the circulation¹⁸ as well as in biologically active form^{19,20} on mast cell surfaces at baseline conditions and can have an impact *in vivo* in the absence of mast cell stimuli. This has for instance been demonstrated for chymase, which has been shown to increase intestinal permeability at homeostatic conditions²¹ and to decrease bone mass in the absence of any mast cell activation stimulus.²² Hence, mast cell proteases are biologically active in the tissue both in inflammatory conditions as described below and in the absence of stimuli that cause mast cell degranulation.

The mast cell proteases are generally divided into three groups based on their peptide bond cleavage specificities: chymases, tryptases, and carboxypeptidases.²³ The mouse mast cell proteases (mMCPs) mMCP4 (chymase), tryptase mMCP6, and carboxypeptidase A3 (CPA3) are considered the closest functional homologs to the human mast cell chymase, tryptase, and carboxypeptidase and are all expressed in connective tissue mast cells.²⁴ Chymases and tryptases have an extensive repertoire of proposed substrates, mostly based on *in vitro* experiments, while the cleavage profile of CPA3 is more limited.²³ Several of the mast cell protease substrates are pro-inflammatory mediators, which are

inactivated upon protease cleavage. Mast cell protease substrates also include pro-inflammatory precursor proteins, which the proteases activate by enzymatic cleavage. For instance, mast cell chymase has been shown to degrade the neuropeptides Substance P (SP) and vasoactive intestinal peptide (VIP),²⁵ pro-inflammatory mediators that are released from primary afferents upon their activation, and can induce mast cell degranulation.²⁶ Chymase also degrades bradykinin,²⁷ the interleukin (IL) precursors Pro-IL-18 and Pro-IL-1 β ^{28,29} as well as IL-6, IL-13, IL-33, HMGB1 (high mobility group protein B1), and heat shock protein 70.^{30,31} Mast cell tryptase cleaves VIP^{25,32} and is efficient in degrading the neuropeptide calcitonin gene-related peptide (CGRP).³² Additionally, tryptase activates the protease-activated receptor 2 (PAR2), which mediates inflammation as well as itch^{33,34} and has been reported to generate NGF by cleaving its precursor peptide pro-NGF.³⁵ CPA3 is the least studied of the three proteases but has been shown to cleave endothelin-1 (ET-1),³⁶ a neurotransmitter capable of transmitting pain and itch by activating the ET_A receptor on sensory neurons^{37,38} as well as degranulating mast cells.³⁹

Information about the exact *in vivo* role of the mast cell proteases is limited; however, they have been linked to both protective and harmful effects in inflammatory conditions.^{16,23,40} Studies in mice have shown that mMCP4 has a pro-inflammatory role in arthritis⁴¹ and contributes to skin inflammation together with the elastase mMCP5 after epidermal burn injury.^{42,43} In allergic airway inflammation, however, mMCP4 modulates IL-33 levels and has a protective effect.⁴⁴ PAR2 activation by mMCP6 contributes to intestinal inflammation in mice,³³ and human tryptase contributes to joint inflammation via the same receptor when injected in mice.⁴⁵ Based on the reported roles of the mast cell-specific proteases in neurotransmitter degradation and inflammatory conditions, the individual contributions of chymase mMCP4, tryptase mMCP6, and carboxypeptidase CPA3 to tissue injury-induced pain behavior were investigated in this study by performing the formalin test on *mMCP4*^{-/-}, *mMCP6*^{-/-}, and *CPA3*^{-/-} mice. The role of intact mast cells was studied as well by performing the formalin test on the mast cell-deficient mouse line *Mcpt5*^{Cre+};R-DTA. The same mouse line was used in investigating the role of mast cells in heat sensation and heat hypersensitivity by performing the Hargreaves test coupled with a subcutaneous NGF injection.

Materials and methods

Generation of transgenic animals and genotyping

Mouse lines deficient in the mast cell proteases mMCP4,⁴⁶ mMCP6,⁴⁷ and CPA3⁴⁸ as well as a mast cell-deficient

mouse line (Mcpt5^{Cre+};R-DTA (diphtheria toxin A))⁴⁹ were generated as previously described. In the Mcpt5^{Cre+};R-DTA mice, Cre recombinase expression is controlled by the Mcpt5 promoter, inducing diphtheria toxin gene expression through Cre-mediated deletion of a loxP-flanked stop cassette. As Mcpt5 (coding for the chymase mMCP5) is exclusively expressed by connective tissue mast cells, only those cells are affected and undergo cell death as a consequence of activated diphtheria toxin expression.⁴⁹ The *mMCP4*^{-/-}, *mMCP6*^{-/-}, and *CPA3*^{-/-} lines were all backcrossed for a minimum of 10 generations to the wild-type strain used (C57BL/6) and were maintained in a homozygous state. To avoid genetic drift, the respective knockout strains were routinely rebackcrossed to the wild-type C57BL/6 line. For detecting mast cell-deficient mice, the following primers were used: 5'-ACAGTGGTATTCCCGGGGAGTGT-3' (forward), 5'-GTCAGTGC GTTCAAAGGCCA-3' (reverse), and 5'-TGAGAAGGGCTATGAGTCCCA-3' (reverse, mutant allele). For detecting mMCP4-, mMCP6-, CPA3-null genotypes, the following primers were used: mMCP4: 5'-CAAGGTCCA ACTA ACTCCCTTTGTGCTCC-3' (forward), 5'-GGTGATCTCCAGATGGGCCATGTAAGGGCG-3' (reverse), and 5'-GGGCCA GCTCATTCTCCCACTCATGATCT-3' (reverse, mutant allele); mMCP6: 5'-TTTAGC TGGA CTCAGGCTGTGCTCCTCACT-3' (forward), 5'-CTCCTGAATTGG AGCTAACCCCTGGGATTCT-3' (reverse), and 5'-GACCATGTGATCGCGCTTCT-3' (reverse, mutant allele); and CPA3: 5'-GGACTG TTCATCCCCAGGAACC-3' (forward), 5'-CTGGCG TGCTTTTCATTCTGG-3' (reverse), and 5'-GTCC GGACACGCTGA ACTTG4-3' (reverse, mutant allele).

Behavior

All behavioral tests were performed on adult (>7 weeks old) male mice with the exception of the NGF-associated heat hypersensitivity test where one female couple was used together with five male couples. The tests were performed in the day (light) part of the cycle and by the same female investigator. The mast cell-deficient mice were observed by two female investigators in the formalin test. Controls for protease-deficient mice were age-matched wild-type mice (C57BL/6) housed in the same animal room; for the mast cell-deficient Mcpt5^{Cre+};R-DTA mice, littermate Mcpt5^{Cre-};R-DTA mice were used as controls. All behavior analyses were performed in a controlled environment of 20°C–24°C, 45%–65% humidity, and 12-h day/night cycle. All animal procedures were approved by the local ethical committee in Uppsala and followed the Directive 2010/63/European Union of the European Parliament and of the Council, The Swedish Animal Welfare Act (SFS (Svensk författningssamlingar) 1988:534), The Swedish Animal Welfare Ordinance (SFS

1988:539), and the regulations regarding the use of animals for scientific purposes: SJVFS (Statens jordbruksverks författningssamlingar) 2017:40 (L150). The observers were blinded to the genotype when measuring pain behavior in the mast cell-deficient mice. However, this was not possible when working with the protease-deficient animals due to the organization of the animals in the facility, so the risk of observational bias cannot be excluded.

Formalin test

Each mouse was gently restrained using a paper towel before 20 µl of 5% formalin (37% formaldehyde (Sigma-Aldrich, St. Louis, MO) diluted in 0.9% saline) was injected subcutaneously into the plantar surface of the right hind paw with a microsyringe (1710 TLL (PTFE luer lock) 100 µl; Hamilton Central Europe, Ghiroda, Romania) using a 30-G needle (BD Microlance, Beckton, Dickinson & Co. Ltd., Drogheda, Ireland). The mouse was then observed in a transparent cage surrounded by mirrors on three sides for the following 60 min to measure pain behavior defined as licking and biting of the injected paw, using a stopwatch. The results were expressed as the mean amount of pain behavior (in seconds) for each group during every 5-min interval ± standard error of mean (SEM) and during the first 10 min (Phase 1), the following 50 min (Phase 2), and over the whole hour.

Hargreaves test

Prior to the experiment, the mice were acclimatized to the Hargreaves setup (transparent acrylic glass chambers with a glass floor) on two separate occasions for 30 min in order to minimize stress caused by new surroundings. On the day of the experiment, the mice were acclimatized in the setup for 60 min or until no exploratory behavior was observed. The Hargreaves heat source (IITC Life Science, Woodland Hills, CA) was placed with the guide light pointing toward the plantar surface of the right hind paw, and the thermal beam was started. Paw withdrawal would stop the test, and the response time was noted. The cut-off time was set to 20 s. The test was repeated two or three times per animal, allowing at least 5 min between each measurement.

NGF-evoked hypersensitivity

Baseline measurements were performed using the Hargreaves test as previously described. The mice were then injected subcutaneously with 50 ng human recombinant β-NGF (MBL International, Woburn, MA) dissolved in 20 µl 0.9% saline into the plantar surface of the right hind paw with a microsyringe (1710 TLL 100 µl, Hamilton Central Europe, Ghiroda, Romania) using a 30-G needle (BD Microlance, Beckton, Dickinson & Co. Ltd., Drogheda, Ireland). Hargreaves measurements

were repeated at 30 min and at 1, 2, 4, and 24 h after NGF injection to follow the development of hypersensitivity. The test was repeated two or three times per animal at each time point, allowing at least 5 min between measurements. The results were expressed as the mean withdrawal latency time at each time point for each group \pm SEM. Development of hypersensitivity for each group was evaluated as the difference between the mean response time at baseline (before the NGF injection) versus the mean response time at each time point after the injection.

Toluidine blue staining

Naïve adult Mcpt5^{Cre+};R-DTA mice and Cre-negative controls (one female and one male per genotype) were sacrificed and plantar surfaces of hind paws were collected and fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 2 h. Subsequently, the skin was transferred to a stepwise gradient of sucrose solutions in PBS, ending with 30% sucrose overnight at 4°C. Skin samples were embedded in optimal cutting temperature (OCT) compound (Richard-Allan Scientific, Kalamazoo, MI) on dry ice and cut using a cryostat (CryoCut 1800, Leica Reichert-Jung, Wetzlar, Germany) into 12- μ m-thick sections. Staining of tissue sections started with 10-min incubation in acetone at -20°C, followed by rehydration in PBS (twice for 5 min) and stained in toluidine blue (Sigma-Aldrich, St. Louis, MO) solution (0.1% toluidine blue in 171 mM NaCl (pH 2)) for 2 min. Tissue sections were washed in distilled water, dehydrated, and mounted. Mast cells were observed with a Nikon Eclipse 90i bright field microscope (Nikon Instruments Europe, Amsterdam, Netherlands) using a 10 \times and 20 \times objective.

Statistics

For all sets of data, normality of variance was assessed by a Shapiro–Wilks test. When comparing two groups, parametric calculations were conducted with two-tailed Student's t test, and nonparametric calculations were performed using a two-tailed Mann–Whitney test. Nonparametric calculations of p values (>2 groups) were conducted with Kruskal–Wallis (one-way analysis of variance (ANOVA)) followed by Dunn's post hoc test. For the NGF/Hargreaves test, development of hypersensitivity for each group was compared using repeated measurement ANOVA with Dunnett's post hoc multiple comparison. All calculations were performed using Prism version 5.04 (GraphPad Software, Inc., San Diego, CA). Values of $p < 0.05$ were considered significant.

Results

mMCP4^{-/-}, mMCP6^{-/-}, and CPA3^{-/-} mice do not differ significantly from controls in pain behavior at any time point of the formalin test

To evaluate if single mast cell protease deficiency plays a role in the pain responses to tissue injury and acute inflammation, the formalin test was performed on mast cell protease knock-out mice *mMCP4^{-/-}*, *mMCP6^{-/-}*, and *CPA3^{-/-}*, together with age-matched wild-type controls (Figure 1). The accuracy of the three knockout lines has been evaluated in previous analyses where each null mutation was shown to result in absence of the respective protein.^{46–48} The *mMCP4^{-/-}* mice did not differ significantly from the controls at any 5-min interval, but a slight trend toward higher pain responses during the last 15 min of the formalin test was observed; at 45–50 min, they demonstrated 124.0 ± 17 s of pain behavior versus 69.5 ± 20 s for controls (Figure 1(a)). The *mMCP6^{-/-}* mice did not differ significantly from controls either at any time point, but contrary to the *mMCP4^{-/-}* mice, the *mMCP6^{-/-}* mice had a tendency to show lower pain responses than controls during the inflammatory phase; at 40–45 min, they exhibited on average 54.8 ± 16 s of pain behavior versus 101.2 ± 18 s for controls (Figure 1(b)). The pain behavior of *CPA3^{-/-}* mice did not differ from controls in either direction (Figure 1(c)). The total pain responses in Phase 1 and Phase 2 were also compared, with no difference observed between genotypes (Figure 1(d)).

Mast cell deficiency does not affect pain responses in the formalin test, Hargreaves test, or inflammation-induced heat hypersensitivity

After establishing that the individual mast cell proteases do not contribute significantly to the behavioral phenotype observed in the formalin test, we wanted to investigate if mast cell deficiency would have an effect. To study the role of mast cells in formalin-induced nociception, the connective tissue mast cell-deficient line (Mcpt5^{Cre+};R-DTA) was used. Previous experiments have shown that Mcpt5^{Cre+};R-DTA mice have an almost complete depletion of mast cells in the peritoneal cavity as well as in the skin of the abdomen, back, and ears.⁴⁹ In this study, it was confirmed that mast cells are also absent in plantar hind paw skin (Figure 2(a)). Moreover, an earlier study has shown that Mcpt5^{Cre+};R-DTA animals completely lack mMCP6, which is exclusively expressed by connective tissue type mast cells.⁵⁰ The pain responses of mast cell-deficient mice (Mcpt5^{Cre+};R-DTA) and controls were not significantly different at any time point (Figure 2(b)). However, slightly lower pain responses for mast cell-deficient

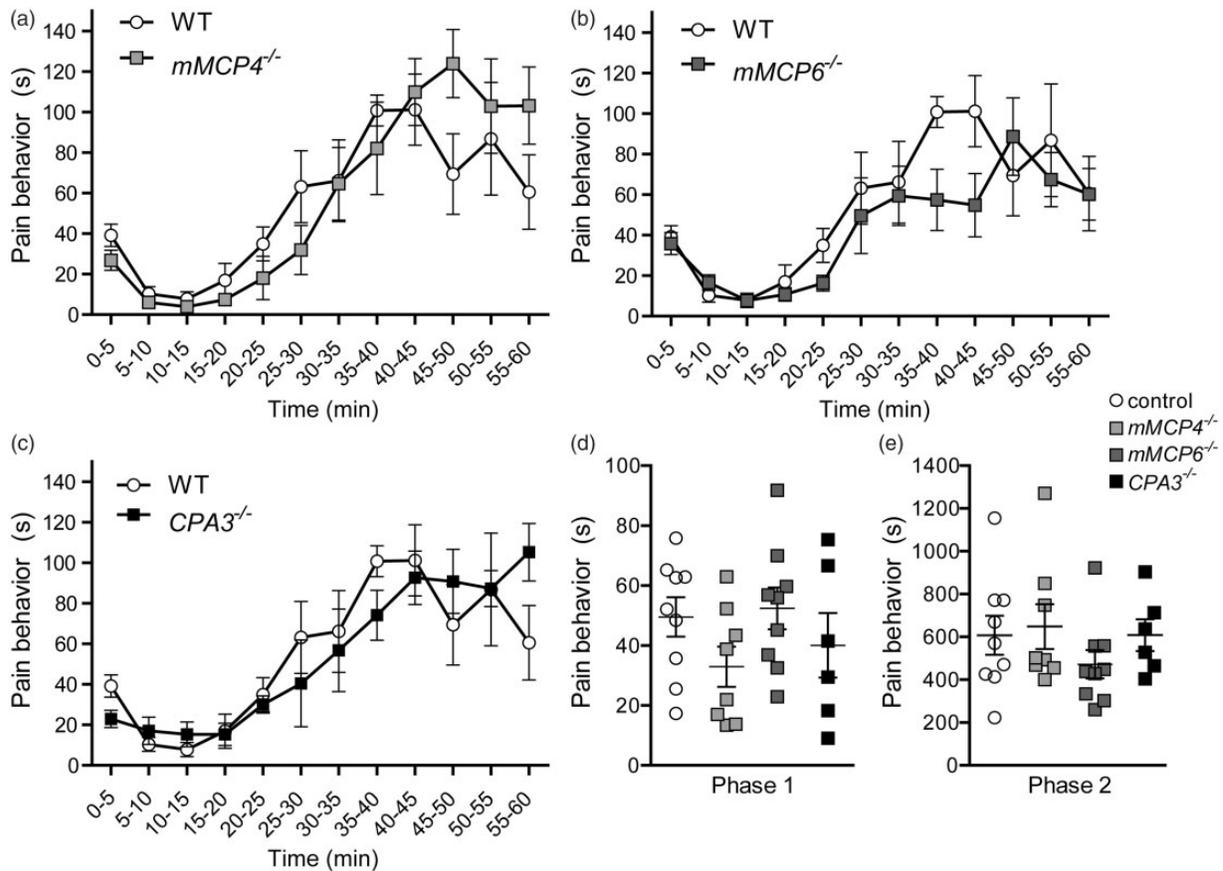


Figure 1. *mMCP4*^{-/-}, *mMCP6*^{-/-}, and *CPA3*^{-/-} mice do not differ from controls in the formalin test. Twenty microliters of 5% formalin were injected subcutaneously into the right hind paw and the mouse was then observed for pain behavior for 60 min. (a) *mMCP4*^{-/-} mice ($n = 8$) show similar pain behavior to controls ($n = 9$) over the time course of the formalin test, except for a slight trend for higher pain responses in the last 15 min of the test. (b) In contrast to the *mMCP4*^{-/-} mice, the *mMCP6*^{-/-} mice ($n = 9$) had a slight tendency to show lower pain responses than controls during the inflammatory phase, but the difference was not significant ($p > 0.05$). (c) The pain behavior of *CPA3*^{-/-} mice ($n = 6$) was not significantly different from controls. (d) and (e) No differences were observed between genotypes in the total pain behavior in Phase 1 (0–10 min) or in the inflammatory Phase 2 (10–60 min). The same set of control mice ($n = 9$) was used in all comparisons and presented in (a), (b), and (c) to facilitate visual presentation. Kruskal–Wallis and Dunn’s post hoc tests were used. Data are presented as mean \pm SEM. WT: wild type.

mice were observed at 30–35 min, where they demonstrated on average 30.1 ± 8.5 s of pain behavior versus 55.8 ± 11 s for controls, but the difference was not significant. When the total pain responses in Phase 1 and Phase 2 were compared between genotypes, no differences were seen (Figure 2(c) and (d)).

To investigate if mast cell deficiency has an effect on heat sensation and inflammation-induced heat hypersensitivity, the Hargreaves test coupled with an injection of the TrkA ligand β NGF was performed on *Mcpt5*^{Cre+};R-DTA mice and controls. No differences between genotypes were observed in the baseline response times before injection; 5.7 ± 1.0 s for mast cell-deficient mice and 6.4 ± 1.0 s for controls (Figure 2(e) and (f)). To follow the development of heat hypersensitivity, Hargreaves measurements were repeated at 30 min, 1 h, 2 h, 4 h, and 24 h after the injection and compared with the baseline

values. The controls did not develop significant hypersensitivity, though there was a trend toward shorter response times post-injection, with the shortest response time measured at 2 h, 4.9 ± 1.0 s (Figure 2(e)). The *Mcpt5*^{Cre+};R-DTA mice did develop hypersensitivity at 4 h, with a response time of 3.8 ± 0.3 s (Figure 2(e)). Although the *Mcpt5*^{Cre+};R-DTA mice had a trend toward shorter response times than controls (Figure 2(f)) and developed significant heat hypersensitivity at one time point, no statistically significant differences were observed between the genotypes.

Discussion

Here, we have investigated the role of mast cell-specific proteases and mast cells in the pain response associated with the first (acute) phase and second (inflammatory)

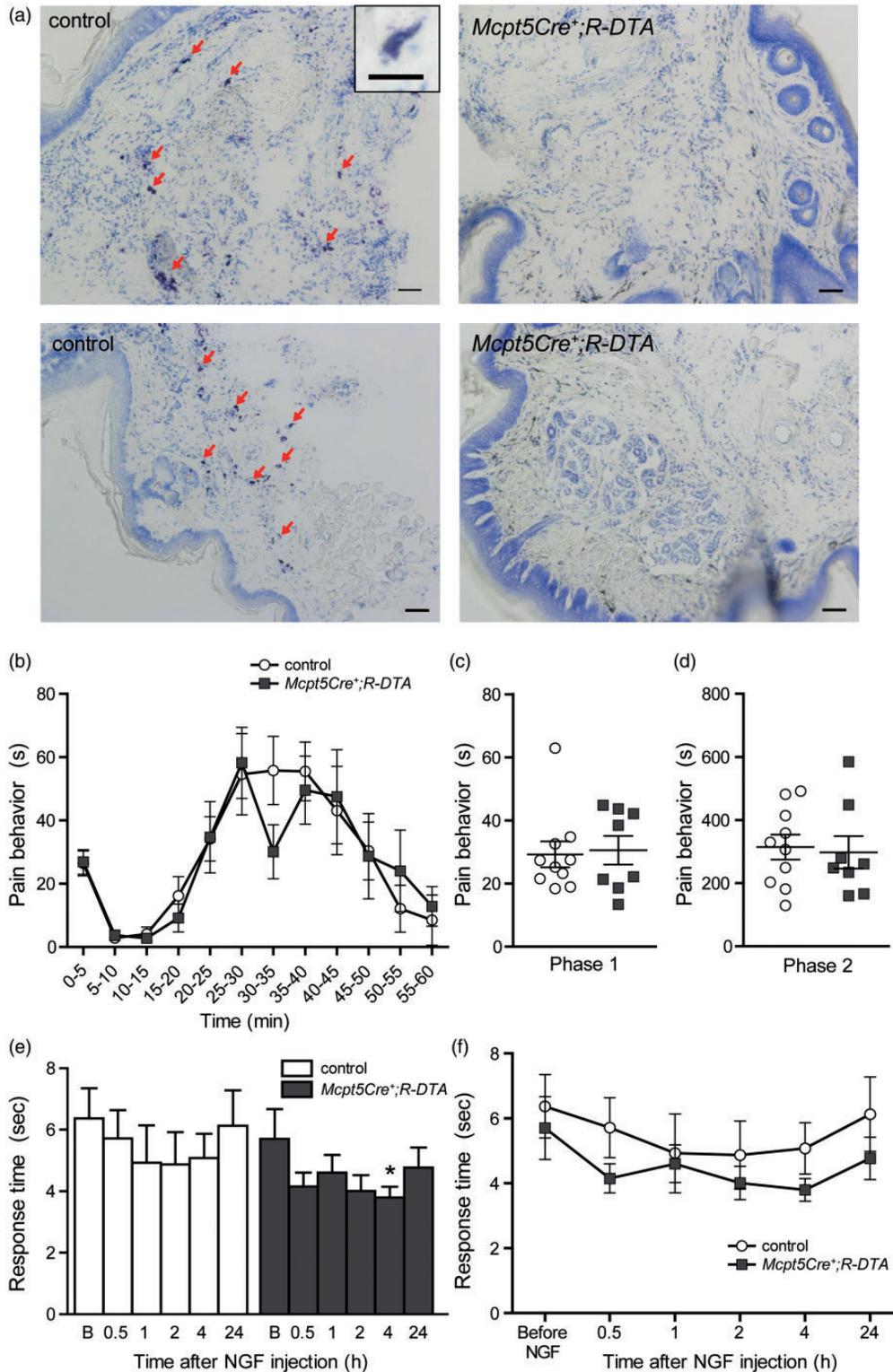


Figure 2. Mast cell-deficient mice do not differ from controls in the formalin test or in heat hypersensitivity induced by NGF. (a) Toluidine blue-stained cryo sections of hind paws. Plantar surfaces of hind paws of naïve *Mcpt5^{Cre+};R-DTA* (controls, $n = 2$) and *Mcpt5^{Cre+};R-DTA* ($n = 2$) were fixed with PFA, embedded in OCT compound, cut into 12- μ m-thick sections, and subsequently stained with toluidine blue. Red arrows point to metachromatically stained mast cells in control tissue, which are absent in tissue of *Mcpt5^{Cre+};R-DTA* mice. 10 \times objective, scale bar = 50 μ m. Inset represents metachromatically stained mast cell, 20 \times objective, scale bar = 20 μ m. (b) The pain behavior of mast cell-deficient mice (*Mcpt5^{Cre+};R-DTA*, $n = 8$) does not differ from controls ($n = 10$) over the time course of the formalin test.

(Continued)

phase of tissue injury induced by formalin, as well as the role of mast cells in heat sensation and inflammation-induced heat hypersensitivity. No significant differences in pain responses following the formalin test were observed in neither mast cell protease-deficient nor mast cell-deficient mice, compared with control mice. In addition, noxious heat sensation and inflammation-induced heat hypersensitivity as measured by the Hargreaves test coupled with NGF injection were not affected by mast cell deficiency.

Formalin as a translational model for tissue injury-induced pain

Formalin, an aqueous solution of formaldehyde, is a tissue fixative that covalently crosslinks proteins in a nonspecific fashion, thus disrupting cell membranes,² causing irreversible tissue injury. Furthermore, it has been suggested that it can cause mast cell degranulation directly.⁵¹ When formalin is injected into the mouse hind paw, the paw quickly becomes inflamed and a two-phased pain response can be observed. The pain response is usually measured by the amount of time mice spend licking or biting their paw^{52,53} as was done in this study, or by quantifying the flinching of the injected paw.¹ Moreover, in the initial phase, it has been shown that formalin immediately causes pain by directly activating TRPA1,¹ which is highly expressed in a subset of C-fiber nociceptors that also express TRPV1.^{3,4} The secondary pain phase usually appears between 20 and 30 min after formalin injection⁵² where cells injured by formalin crosslinking release damage-associated molecular patterns that can activate immune cells to initiate inflammation.^{13,54,55} Inflammatory mediators released from immune cells or directly from damaged tissue continue to activate TRPA1-expressing primary afferents, contributing to pain sensitization of the central nervous system.^{1,56} The formalin response is also dependent on SP transmission. For instance, *Tachykinin 1* (SP precursor gene)-deficient mice show markedly reduced responses to formalin in both the first and the second phase⁵⁷ and the SP antagonist sendide attenuates the formalin response.⁵⁸ SP is stored in and released from primary afferents⁵⁹ and contributes to the second/inflammatory phase of the formalin response

by relaying the nociceptive signal to the central nervous system and by interacting with immune cells such as mast cells,^{5,60} thus promoting the inflammation.

mMCP4 in formalin-induced pain

Mast cell chymase (canine version of mMCP4) has been shown to degrade the neuropeptides SP and VIP,²⁵ which are pro-inflammatory mediators released by primary afferents that can induce mast cell degranulation.²⁶ Mast cell chymase has also been shown to degrade bradykinin in vitro,²⁷ an oligopeptide which also activates TRPA1.^{61,62} Tissue injury activates the kallikrein-kinin cascade, where the precursor kininogen is converted to the active pain mediator bradykinin by the serine protease kallikrein.⁵³ Bradykinin has been shown to mediate pain in the formalin test by acting through the bradykinin 1 and 2 receptors expressed on peripheral nociceptors.⁶³ Furthermore, it has been reported that mMCP4 degrades IL-33,^{31,44} which has been shown to have a role in mediating formalin-induced pain.⁶⁴ Taken together, the slight trend toward an increase in nociceptive behavior observed in the later stages of the inflammatory phase in *mMCP4*^{-/-} mice, although not significant, may be explained by the reported roles of mMCP4 in degradation of pro-inflammatory mediators.

mMCP6 in formalin-induced pain

Tryptase also has a role in the kallikrein-kinin pathway, as it has been demonstrated that human tryptase can cleave prekallikrein, generating kallikrein and thus contributing to bradykinin formation.⁶⁵ It has been shown that pain responses and paw edema in mice in both phases of the formalin test can be greatly diminished by inhibiting kallikrein.⁵³ Also, human tryptase can directly generate bradykinin by cleaving kininogen.⁶⁵ The involvement of mMCP6 in the kallikrein-kinin pathway might explain the statistically nonsignificant trend of *mMCP6*^{-/-} mice having lower pain responses in the later phase of the formalin test. Despite the capabilities of mast cell tryptase to cleave inflammatory neuropeptides CGRP and VIP in vitro,³² it has not been shown that tryptase can have protective properties in inflammatory conditions in vivo; it mainly has pro-inflammatory effects in that context.⁶⁶

Figure 2. Continued

Slightly lower responses were observed at 30–35 min, but the difference was not significant (Mann–Whitney: $p = 0.10$). (c) and (d) When total pain behavior in Phase 1 (0–10 min) or in the inflammatory Phase 2 (10–60 min) was analyzed, no differences were observed between genotypes. (e–f) Hargreaves test coupled with NGF injection on *Mcpt5*^{Cre+};R-DTA mice ($n = 7$), and controls ($n = 6$). (e) The baseline Hargreaves values before NGF injection (“B” on the x-axis) did not differ between the two genotypes (Student’s *t* test: $p = 0.64$). After NGF injection, only *Mcpt5*^{Cre+};R-DTA mice developed heat hypersensitivity compared with their baseline values, at 4-h post-injection (one-way repeated measurement ANOVA: $p < 0.05$). (f) Despite the absence of significant hypersensitivity in the controls, there was no difference observed between the two genotypes at any time point before or after the injection (Student’s *t* test: $p > 0.05$). All data are presented as mean \pm SEM. NGF: nerve growth factor.

CPA3 has no apparent effect in formalin-induced pain responses

It has been suggested that IL-33 can initiate a hypernociceptive signaling cascade, by upregulating the production of TNF α which in turn triggers IL-1 β \rightarrow interferon γ (IFN γ) \rightarrow ET-1 \rightarrow prostaglandin E2 (PGE2) production.⁶⁷ CPA3 can cleave ET-1,³⁶ and ET-1 has been shown to induce sensitization to formalin-induced nociception in mice, as well as contributing to paw edema.⁶⁸ In this study, however, there was no indication that CPA3 deficiency had any effect on formalin-induced pain behavior, suggesting that the role of CPA3 in ET-1 cleavage is of little consequence in the pain responses observed in the formalin test. Studies of the in vivo function of CPA3 are currently limited⁶⁶ and mainly indicate that it has protective effects by degrading toxins found in bee and snake venom⁶⁹ but no apparent connection to inflammation. As previously mentioned, CPA3^{-/-} mice also lack the elastase mMCP5,⁴⁸ which has been shown to contribute to skin inflammation after burn injury in mice.^{42,43} This might suggest that lacking mMCP5 would have protective effects in inflammation resulting from formalin-induced tissue injury, However, no such effect was observed here.

Mast cells are not essential for tissue injury-induced pain by formalin nor NGF-induced heat hypersensitivity

Given the central role for mast cells in inflammatory conditions,^{70,71} the close proximity between mast cells and primary afferents and the ability of primary afferent transmitters to activate mast cells, it was surprising to find that mast cell deficiency did not affect the behavioral phenotype induced by formalin injection. However, mast cell-deficient mice were recently shown to develop normal levels of heat and mechanically evoked hypersensitivity associated with NGF or complete Freund's adjuvant-induced inflammation.⁷² This supports the theory that mast cells do not significantly contribute to pain-associated inflammatory processes originating in the skin. In this study, mast cell-deficient mice (Mcpt5^{Cre+}; R-DTA) and their Cre-negative littermates were also tested for their sensitivity to heat using the Hargreaves test followed by NGF injection. Hargreaves testing was then repeated to follow the development of heat hypersensitivity. No difference was detected between the two genotypes at any time point. The fact that the mast cell-deficient mice did eventually develop heat hypersensitivity following the NGF injection but their littermates did not indicates that mast cells are not essential for the development of inflammation-induced heat hypersensitivity. Furthermore, other studies have found that mast cells

do not express NGF receptors^{72,73} and would thus have a negligible role in NGF-evoked hypersensitivity.

Conclusion

Here, we have tested for the first time how acute inflammation and tissue injury pain responses in a formalin-based model are affected by the deficiency of mMCPs: mMCP4, mMCP6, and CPA3, as well as mast cell deficiency. Additionally, mast cell-deficient mice were tested in a model for noxious heat sensation and inflammation-induced heat hypersensitivity. Based on the results, neither the proteases mMCP4, mMCP6, CPA3 nor mast cells as a whole were found to be essential for the defined pain behavior.

Author Contributions

EIM performed the behavioral experiments. MG performed histological analysis, genotyping and handled the mouse colony. AR, KH and GP contributed the transgenic mouse lines. EIM, GP and MCL designed the study and wrote the manuscript. All authors have approved the manuscript.

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Declaration of Conflicting Interests

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