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RESEARCH PAPER

Evidence for the role of neurogenic inflammation components in trypsin-elicited scratching behaviour in mice

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Background and purpose: We investigated the mechanisms underlying the pruritogenic response induced by trypsin in mice, to assess the relevance of neurogenic inflammation components in this response.
Experimental approach: Itching was induced by an intradermal injection of trypsin in the mouse neck. The animals were observed for 40 min and their scratching behaviour was quantified.
Key results: Trypsin-induced itching was blocked by the lima bean trypsin inhibitor, the selective proteinase-activated receptor-2 (PAR-2) antagonist FSLLRY and PAR-2 receptor desensitization. An important involvement of mast cells was observed, as chronic pretreatment with the mast cell degranulator compound 48/80 or the mast cell stabilizer disodium cromoglycate prevented scratching. Also, trypsin response was inhibited by the selective COX-2 inhibitor celecoxib and by the selective kinin B_2 (FR173657) and B_1 (SSR240612) receptor antagonists. Moreover, an essential role for the mediators of neurogenic inflammation was established, as the selective NK_1 (FK888), NK_3 (SR142801) and calcitonin gene-related peptide ($CGRP_{8-37}$ fragment) receptor antagonists inhibited trypsin-induced itching. Similarly, blockade of transient receptor potential vanilloid 1 (TRPV1) receptors by the selective TRPV1 receptor antagonist SB366791, or by genetic deletion of TRPV1 receptor reduced this behaviour in mice. C-fibre desensitization showed a very similar result.
Conclusions and implications: Trypsin intradermal injection proved to be a reproducible model for the study of itching and the involvement of PAR-2 receptors. Also, trypsin-induced itching seems to be widely dependent on neurogenic inflammation, with a role for TRPV1 receptors. In addition, several other mediators located in the sensory nerves and skin also seem to contribute to this process.
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Abbreviations: CGRP, calcitonin gene-related peptide; PAR-2, proteinase-activated receptor-2; TRPV1, transient receptor potential vanilloid 1

Introduction

In recent years, understanding of the basis of pruritus has grown following the discovery of proteinase-activated receptors (PARs). These receptors belong to a subfamily of G-protein-coupled receptors that are activated by serine proteinases that cleave their extracellular terminal sequence and expose a new NH_2 terminus. This new portion acts as a tethered ligand, activating the cleaved receptor molecule (Macfarlane *et al.*, 2001; Cottrell *et al.*, 2003; Steinhoff *et al.*, 2005; Ramachandran and Hollenberg, 2008). Recently, it has been suggested that PAR-2 are closely related to the onset and maintenance of both inflammatory and pain states (Steinhoff *et al.*, 2000; Seeliger *et al.*, 2003; Paszcuk *et al.*, 2008). PAR-2 is highly expressed in the skin (Steinhoff *et al.*, 1999) and can be activated by both tryptase (from mast cell degranulation) and trypsin (from pancreatic and/or extra pancreatic sources) (Corvera *et al.*, 1997; Cottrell *et al.*, 2004). Moreover, this receptor is located throughout the sensory system (Steinhoff *et al.*, 2000; Seeliger *et al.*, 2003; Vergnolle *et al.*, 2003) and has been indicated as an important effector of pruritogenic processes (Thomsen *et al.*, 2002; Steinhoff

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et al., 2003; Shimada *et al*., 2006; Ui *et al*., 2006; Sun and Chen, 2007). Although pruritus may be triggered by PAR-2 activation following tryptase action, there is little work on the role of other proteinases in this context.

Neurogenic inflammation is another physiological mechanism that could be involved in pruritus (Ikoma *et al*., 2006; Zegarska *et al*., 2006). The process of neurogenic inflammation involves the participation of some neuropeptides, including tachykinins (substance P, neurokinin A and neurokinin B) and calcitonin gene-related peptide (CGRP) (Holzer, 1998). In addition, recent evidence suggests that the transient receptor potential vanilloid 1 (TRPV1) is important in neurogenic inflammation. TRPV1 is a ligand-gated non-selective cation channel present in a subset of nerve fibres (C and Aδ), and when it becomes activated, it can lead to the release of vasoactive peptides such as substance P and CGRP. Interestingly, recent studies have reported that TRPV1 receptors can be sensitized by PAR-2 activation, leading to inflammatory and pain states (Cottrell *et al*., 2004; Amadesi *et al*., 2006).

Thus, the current view is that PAR-2 activation by tryptase provokes neuropeptide release, which, in turn, may contribute to itching processes (Ikoma *et al*., 2006; Paus *et al*., 2006; Shimada *et al*., 2006; Ui *et al*., 2006). Interestingly, it has recently been demonstrated that trypsin or the selective peptide PAR-2 agonist SLIGKV is capable of eliciting itching in humans (Thomsen *et al*., 2002; Steinhoff *et al*., 2003). Furthermore, trypsin can be released from neuronal tissues (Bunnett, 2006) and is capable of activating human and mouse keratinocytes both *in vitro* and *in vivo* assays (Wakita *et al*., 1997; Babiarz-Magee *et al*., 2004; Meyer-Hoffert *et al*., 2004).

Therefore, we decided to evaluate the ability of an intradermal trypsin injection to induce pruritus in mice and evaluate its potential as a model to study itch. Furthermore, we attempted to investigate, by means of selective antagonists and inhibitors, or by using TRPV1-deficient mice, some of the mechanisms involved in pruritus, with emphasis on components of neurogenic inflammation and TRPV1 receptor activation.

Methods

Animals

Male adult Swiss mice (8–10 weeks) housed in controlled room temperature (22 ± 2 °C) and humidity (around 60–80%) under a 12:12-h light–dark cycle (lights on 0600 hours) were used in this study. Food and water were provided *ad libitum* except during the experiments. Experimental procedures were carried out in accordance with the National Institutes of Health Animal Care Guidelines (NIH publications no. 80-23) and were approved by the Ethics Committee of the Federal University of Santa Catarina (protocol number PP00032). In some experiments, C57BL/6 wild-type and TRPV1 knockout ($TRPV1^{-/-}$) mice were used. Wild-type and $TRPV1^{-/-}$ mice were kindly donated by Merck Sharp and Dohme (Harlow, UK) and were generated by replacing the exon that encodes part of the fifth and the entire sixth transmembrane domain (including the interconnecting p-loop) of the receptor with a neomycin gene, as described by Caterina *et al*. (2000). These experiments were conducted in accordance with the Animals (Scientific Procedures) Act of 1986.

Induction of scratching behaviour

The experimental protocols were similar to the method described by Hayashi and Majima (1999) with minor modifications. Two days before the experiments, the hair at the back of the mouse neck was shaved. On the day of the experiments, the animals were placed individually in a clear acrylic cage with a floor area of 170 cm^2, for at least 1 h, to acclimatize them to the experimental environment. After this period, each mouse was briefly removed from the chamber and given an intradermal injection of saline (50 μl) containing trypsin (30–500 μg per site) or the selective peptide PAR-2 receptor agonist SLIGRL-NH_2 (100 μg per site) (Shimada *et al*., 2006). Immediately after trypsin or SLIGRL-NH_2 treatment, the animals were put back into the same cages. The animals were observed for 40 min and their scratching behaviour was quantified by counting the number of scratches with forepaws and hindpaws close to the injected site. Scratching behind the ears, but not on the face, was also counted. When a mouse scratched continuously for about 1 s without stopping and repeated it more than once, this episode of scratching was counted as one. The results were expressed as the number of scratches in 40 min. Saline-treated animals (50 μl per site) were used as control. To exclude any nonspecific action of trypsin, in a separate experiment the intradermal injections of boiled (for 5 min) and denatured trypsin were also evaluated. The mean of the trypsin effective dose (ED_{50}) was estimated and this value was used in subsequent experiments. Consequently, the dose of trypsin selected for subsequent experiments was 200 μg per site.

Neonatal-capsaicin treatment

To explore the role of capsaicin-sensitive C-fibres in the scratching behaviour induced by trypsin, neonatal mice (2 days old) received either capsaicin (50 mg kg^{-1}, s.c.) or vehicle alone (10% ethanol, 10% Tween-80 and 80% PBS), as described previously (Salt and Hill, 1983; Ferreira *et al*., 2004). The animals were used 6–7 weeks after treatment with capsaicin or vehicle (used as control). In an attempt to determine whether a complete degeneration of the C sensory fibres had occurred after neonatal treatment with capsaicin, the animals were first submitted to an eye-wiping test. For this purpose, a 20 μl capsaicin solution 0.01% (w/v) was instilled into the eye and the number of wiping movements that occurred for 1 min was counted. The animals that wiped their eyes no more than five times were considered to be desensitized by the neonatal capsaicin treatment.

Pharmacological treatment protocol

To analyse the contribution of the serine proteolytic activity of trypsin for the scratching behaviour observed in mice, we used the specific lima bean trypsin inhibitor (100–500 μg per

site). To confirm the activation of PAR-2 receptors and their contribution to the trypsin-elicited scratching behaviour, mice were treated with the selective PAR-2 receptor antagonist FSLLRY (100 μg per site) (Al-Ani *et al*., 2002). The involvement of PAR-2 was further confirmed using a desensitization protocol in which animals received a single daily intradermal injection of trypsin (200 μg per site) for 7 successive days before the experiments.

To assess the role of mast cell degranulation in trypsin response, separate groups of mice were pre-treated with the mast cell stabilizer disodium cromoglycate (8 mg kg^{-1}, i.p., once a day, 6 days) or the mast cell degranulator compound 48/80 (1, 3, 10 and 10 μg per site on the 1st, 2nd, 3rd and 4th days, respectively). The possible involvement of different mast cell degranulation products was assessed by treating animals with the selective H_1 receptor antagonist pyrilamine (10 mg kg^{-1}, s.c.), the mixed serotonergic/histaminergic antagonist cyproheptadine (20 mg kg^{-1}, s.c.) or the non-selective protease inhibitor gabexate mesylate (10 mg kg^{-1}, s.c.). The participation of the products of the arachidonic acid pathway was evaluated by treatment with the selective COX-1 and COX-2 inhibitors SC560 (5 mg kg^{-1}, i.p.) and celecoxib (10 mg kg^{-1}, p.o.), respectively. The contribution of the kinin pathway was determined by treatment of the animals with the selective non-peptide kinin B_2 or B_1 receptor antagonists FR173657 (30 mg kg^{-1}, i.p.) or SSR240612 (1 mg kg^{-1}, i.p.), respectively. The relevance of neuropeptides was assessed by treatment with the selective NK_1 (FK888, 1 mg kg^{-1}, i.v.), NK_2 (SR48968, 1 mg kg^{-1}, i.v.), NK_3 (SR142801, 3 mg kg^{-1}, i.v.) or the selective CGRP ($CGRP_{8-37}$, 300 μg kg^{-1}, i.v.) receptor antagonists. The importance of TRPV1 receptor to trypsin response was assessed by pretreatment with the selective TRPV1 receptor antagonist SB366791 (500 μg kg^{-1}, s.c.).

Most of the drugs, except SB366791 and SC560, were prepared daily in 0.9% (w/v) NaCl (saline) solution before use. SB366791 and SC560 were diluted in a 2% ethanol solution (in saline) and in a 1% Tween 80 solution (in saline), respectively. Control groups received the corresponding vehicle at the same time points depending on the experimental protocol. Subcutaneous and intraperitoneal treatments were both given 30 min before trypsin (200 μg per site) injection. Drugs were administered orally and intravenously 1 h and 15 min before trypsin, respectively. Local doses (per site) were co-injected with trypsin. The doses of all drugs were selected from literature data (Calixto *et al*., 2003; Chen *et al*., 2006; Costa *et al*., 2006; Ui *et al*., 2006; Paszcuk *et al*., 2008) or from previous studies (data not shown).

All receptors and channel nomenclature are in accordance with the *British Journal of Pharmacology*'s Guide to Receptors and Channels (Alexander *et al*., 2007).

Drugs and reagents

The following drugs were used: trypsin (from porcine pancreas), trypsin inhibitor from *Phaseolus limensis* (lima bean), disodium cromoglycate (cromolyn), compound 48/80, pyrilamine, cyproheptadine, gabexate mesylate, aprotinin, SC560, calcitonin gene-related peptide fragment 8–37 ($CGRP_{8-37}$), SB366791 and capsaicin all from Sigma Chemical Company (St Louis, MO, USA). Celecoxib was obtained from Merck (Rio de Janeiro, Brazil). FK888 and FR173657 were kindly donated by Fujisawa Pharmaceutical Co. (Osaka, Japan). SSR240612, SR48968 and SR142801 were kindly supplied by Sanofi-Aventis R&D (Montpellier, France). FSLLRY and SLIGRL-NH_2 were synthesized by Dr Luis Juliano (Universidade Federal de São Paulo, São Paulo, Brazil).

Data analysis

The results are presented as the mean ± s.e.mean of 6–10 animals, except for the estimated ED_{50} values (that is, the dose of trypsin required to produce 50% of the maximal scratching behaviour response) that are given as the geometric means accompanied by the 95% confidence limit. Statistical comparison of the data was performed by one-way ANOVA, followed by Dunnett's or Newman–Keuls tests when appropriate. *P*-values of less than 0.05 were considered significant. The ED_{50} value was determined by linear regression from individual experiments using linear regression GraphPad Software (GraphPad Software, San Diego, CA, USA).

Results

Trypsin-induced scratching behaviour in mice

A dose-related effect of trypsin on inducing scratching in mice is shown in Figure 1a. The effective dose ranged from 100 to 500 μg per site, being maximum at 300 μg per site. The estimated mean ED_{50} value (accompanied by 95% confidence limit) for this effect was 97 (67–140) μg per site. Consequently, the dose of 200 μg per site was chosen for the following experiments, as this was the closest dose to the ED_{50} value capable of inducing reproducible effects with less variance. The intradermal injection of heat (boiled for 5 min)-inactivated trypsin (200 μg per site) did not cause any significant alteration to the scratching behaviour in comparison with the saline-treated group (Figure 1b). Co-treatment with the specific Lima bean trypsin inhibitor (100–500 μg per site) consistently inhibited trypsin-induced scratching behaviour in a dose-dependent manner (maximal inhibition of 106 ± 8%) (Figure 1c).

To investigate whether the effects of trypsin on the generation of scratching were mediated by PAR-2 activation, we assessed the effect of a selective PAR-2 receptor antagonist, FSLLRY. Trypsin-induced pruritus was inhibited by treatment with FSLLRY (100 μg per site), resulting in 91 ± 7% inhibition (Figure 2a). We also assessed the PAR-2 activation role by using a PAR-2 desensitization protocol. Further studies of the scratching behaviour induced by both trypsin (200 μg per site) and the peptide PAR-2 receptor agonist SLIGRL-NH_2 (100 μg per site) were significantly reduced by previous PAR-2 desensitization, with 80 ± 12% and 83 ± 10% inhibition, respectively (Figures 2b and c).

Mechanisms of trypsin-induced scratching behaviour in mice

In this set of experiments, we analysed some of the possible mechanisms and mediators implicated in the scratching behaviour evoked by trypsin injection in mice. First, we

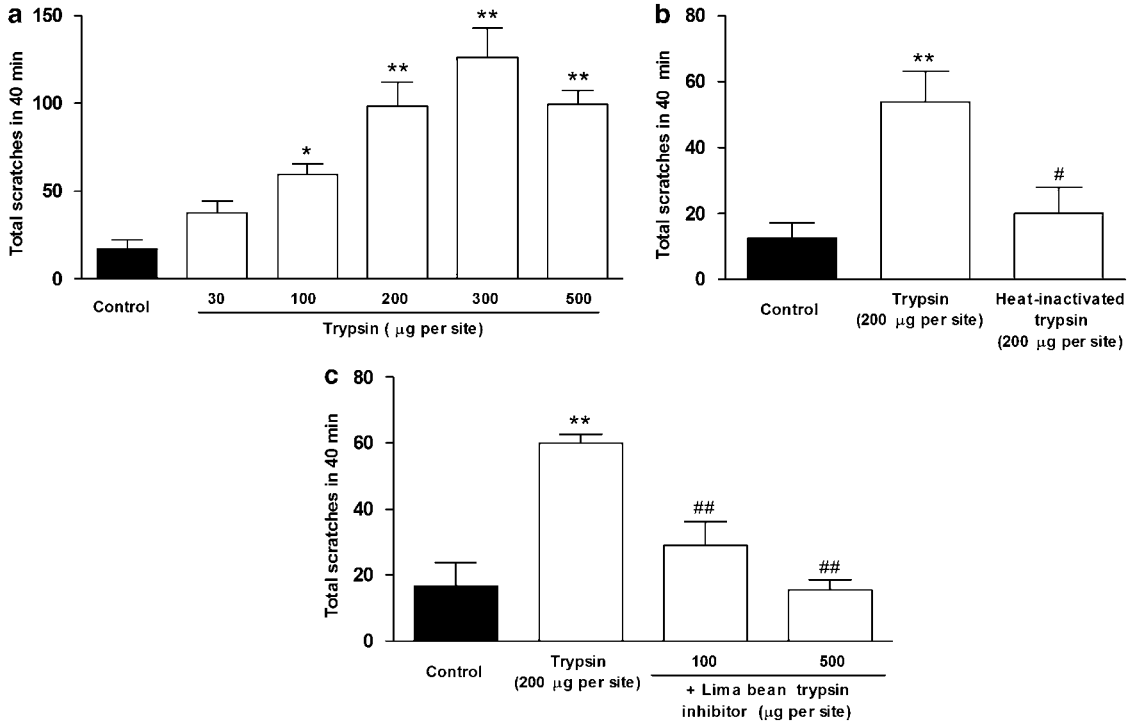

Figure 1 (**a**) Dose–response curve for scratching behaviour elicited by trypsin (30–500 μg per site, i.d.) in Swiss mice. (**b**) Effect of heat-inactivated trypsin (200 μg per site, i.d.) injection. (**c**) Effect of treatment with the specific trypsin inhibitor from lima bean (100–500 μg per site, co-injection) on the trypsin (200 μg per site)-elicited scratching behaviour in Swiss mice. Each column represents the mean of 6–10 animals and the vertical bars represent the s.e.mean. Significantly different when compared with the saline group (*$P<0.05$ or **$P<0.01$) and the trypsin-treated group ($^{\#}P<0.05$ or $^{\#\#}P<0.01$).

observed a significant inhibition of the trypsin-induced scratching behaviour after pretreatment with the mast cell stabilizer disodium cromoglycate (8 mg kg^{-1}, i.p., 6 days) or the mast cell depletor compound 48/80 (1–10 μg per site, 4 days) with inhibitions of 59 ± 18% and 73 ± 10%, respectively (Figure 3a). In contrast, pretreatment with the selective H_1 receptor antagonist pyrilamine (10 mg kg^{-1}, s.c., 30 min) was not able to interfere significantly with the scratching response elicited by trypsin (Figure 3b). Interestingly, the mixed serotonergic/histaminergic antagonist cyproheptadine (20 mg kg^{-1}, s.c., 30 min) significantly inhibited trypsin-induced pruritus (63 ± 8% inhibition) (Figure 3b). In addition, a marked reduction of both the trypsin-induced and PAR-2 selective agonist SLIGRL-NH_2-induced pruritus was found in the animals pre-treated with the non-selective protease inhibitor gabexate mesylate (10 mg kg^{-1}, s.c., 30 min) with inhibitions of 72 ± 9% and 53 ± 20%, respectively (Figures 3c and d).

Treatment with the selective COX-2 inhibitor celecoxib (10 mg kg^{-1}, p.o., 1 h) significantly diminished the itching response induced by trypsin (65 ± 12%) (Figure 4a). Conversely, a partial, but not statistically significant, inhibition (45 ± 10%) of trypsin-elicited scratching was also observed with the selective COX-1 inhibitor SC560 (5 mg kg^{-1}, i.p., 30 min) (Figure 4a). Furthermore, the selective non-peptide kinin B_2 receptor antagonist FR173657 (30 mg kg^{-1}, i.p., 30 min) had a significant inhibitory effect on the trypsin-elicited scratching behaviour in mice (74 ± 20% of inhibition). Similarly, the selective non-peptide kinin B_1 receptor antagonist SSR240612 (1 mg kg^{-1}, i.p., 30 min) was able to significantly affect the itching response induced by trypsin (76 ± 20% of inhibition) (Figure 4b).

The results depicted in Figure 4c show a marked inhibitory effect of the scratching behaviour induced by trypsin after pretreatment with the selective NK_1 FK888 (1 mg kg^{-1}, i.v., 15 min) and NK_3 SR142801 (3 mg kg^{-1}, i.v., 15 min) receptor antagonists, with inhibitions of 84 ± 14% and 79 ± 5%, respectively. Similarly, the selective CGRP receptor antagonist $CGRP_{8-37}$ (300 μg kg^{-1}, i.v., 15 min) caused a significant reduction (70 ± 19%) of the trypsin-induced scratching behaviour. Surprisingly, the selective NK_2 receptor antagonist SR48968 (1 mg kg^{-1}, i.v., 15 min) had no effect in this model (Figure 4c).

In a series of experiments to determine the contribution of C-fibre activation to the trypsin-induced scratching behaviour in mice, neonatal animals (2 days old) were subcutaneously treated with capsaicin (50 mg kg^{-1}) and submitted to the trypsin-induced scratching behaviour protocol when they became adults. Mice treated with capsaicin showed a markedly reduced number of scratches in comparison with vehicle-treated animals following trypsin (200 μg per site) injection (74 ± 11% reduced) (Figure 5a). A similar effect was observed in animals that were pre-treated with the selective TRPV1 receptor antagonist SB366791 (500 μg kg^{-1}, s.c., 30 min; 76 ± 10%) (Figure 5b). Furthermore, TRPV1-deficient mice ($TRPV1^{-/-}$) showed a markedly diminished frequency of scratching bouts compared with the response of wild-type mice ($TRPV1^{+/+}$) after trypsin treatment (95 ± 19% reduced) (Figure 5c).

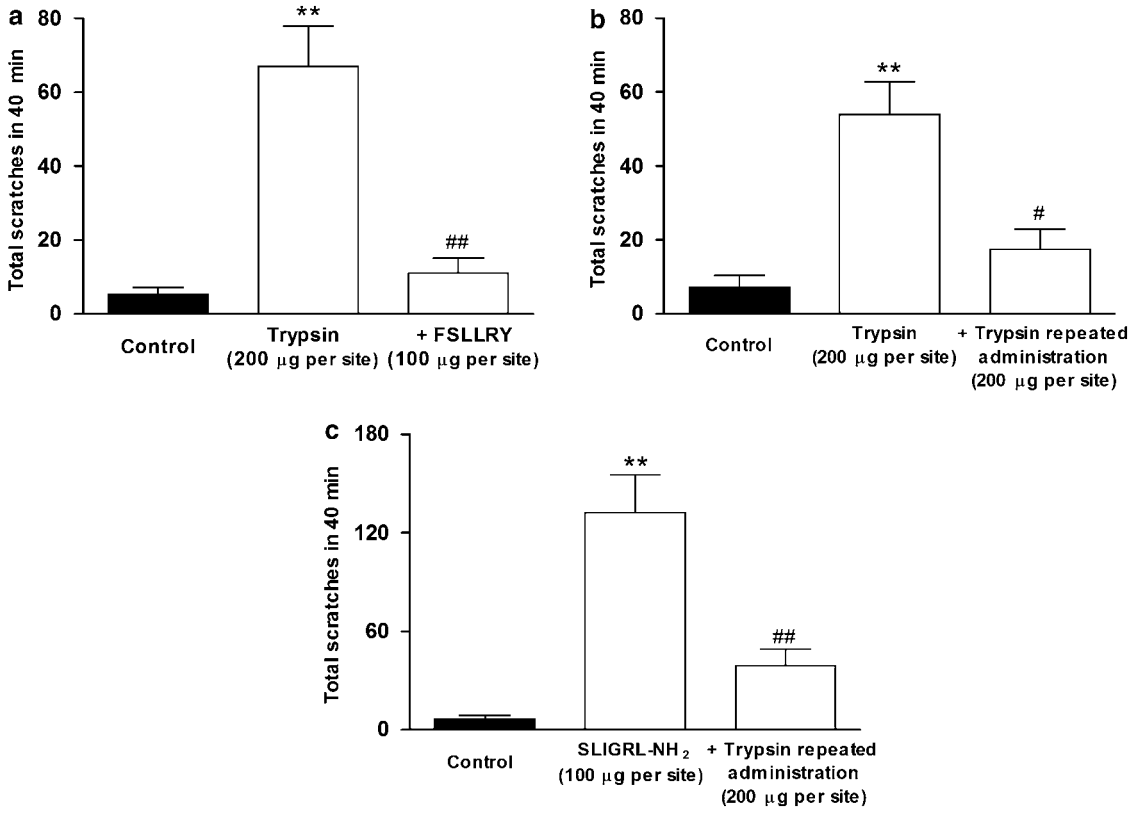

Figure 2 (**a**) Effect of treatment with the selective peptide proteinase-activated receptor-2 (PAR-2) antagonist FSLLRY (100 μg per site, i.d., co-injected) or (**b**) trypsin-repeated injections (200 μg per site, i.d., once a day, 7 days) on the trypsin (200 μg per site)-elicited scratching behaviour in Swiss mice. (**c**) Effect of treatment with trypsin-repeated injections (200 μg per site, i.d., once a day, 7 days) on the SLIGRL-NH_2 (100 μg per site)-elicited scratching behaviour in Swiss mice. Each column represents the mean of 6–10 animals and the vertical bars represent the s.e.mean. Significantly different when compared with the saline group (**$P<0.01$) and the trypsin-treated group ($^{\#}P<0.05$ or $^{\#\#}P<0.01$).

Discussion and conclusions

Trypsin is a serine proteinase that can cleave and activate PAR-2 receptors (Schmidlin and Bunnett, 2001). In this study we demonstrated the ability of trypsin to evoke itching when injected at the back of the mouse neck. This effect was observed in different mouse strains (Swiss and C57BL/6), allowing us to suggest that the trypsin intradermal injection can be used as a reproducible model to evaluate itching. We showed that the pruritogenic effect of trypsin is dependent on the preservation of serine proteolytic activity, as the injection of heat-inactivated trypsin or treatment with the Lima bean trypsin inhibitor markedly reduced the number of scratches by trypsin-treated mice.

Significantly, despite the known ability of trypsin to activate PAR-1 receptors, our results strongly suggest that trypsin-induced itching is triggered by PAR-2 activation, as the scratching behaviour induced by trypsin was reduced by treatment with the selective PAR-2 receptor antagonist FSLLRY and by trypsin-induced PAR-2 receptor desensitization. In fact, recent studies have shown that a single intradermal injection of the selective PAR-2 agonist SLIGRL-NH_2 or tryptase is capable of eliciting pruritus in mice (Shimada *et al.*, 2006; Ui *et al.*, 2006; Sun and Chen, 2007). Furthermore, Paszcuk *et al.* (2008) demonstrated that trypsin-induced paw inflammation and nociception are mediated, at least in part, by PAR-2 receptor activation. Although our results clearly suggest that the effects of trypsin on scratching behaviour are essentially dependent on serine proteolytic cleavage of PAR-2 and subsequent receptor activation, the actions of trypsin at other sites cannot be excluded.

We also investigated some of the potential mechanisms and mediators that could be implicated in the itching response induced by trypsin. Mast cells can express PAR-2, and these cells may play a pivotal role in the itching processes. Mast cells store a range of mediators in their intracellular granules, such as histamine, 5-HT, proteases (mainly tryptase and chymase) and prostanoids, that might contribute to the itching processes (Steinhoff *et al.*, 2005, 2006; Zegarska *et al.*, 2006). Our results suggest a key role for mast cells in trypsin-induced pruritus. The scratching behaviour of mice is decreased after pretreatment of the mice with the mast cell stabilizer disodium cromoglycate or the mast cell degranulator compound 48/80. These results are in good accord with those of a recent study showing that trypsin-evoked nociceptive effects are also dependent on mast cell degranulation (Paszcuk *et al.*, 2008).

We then evaluated the involvement of some mast cell degranulation products in trypsin-induced pruritus. Trypsin-elicited itching was not affected by pretreatment with the selective histamine H_1 receptor antagonist pyrilamine. This

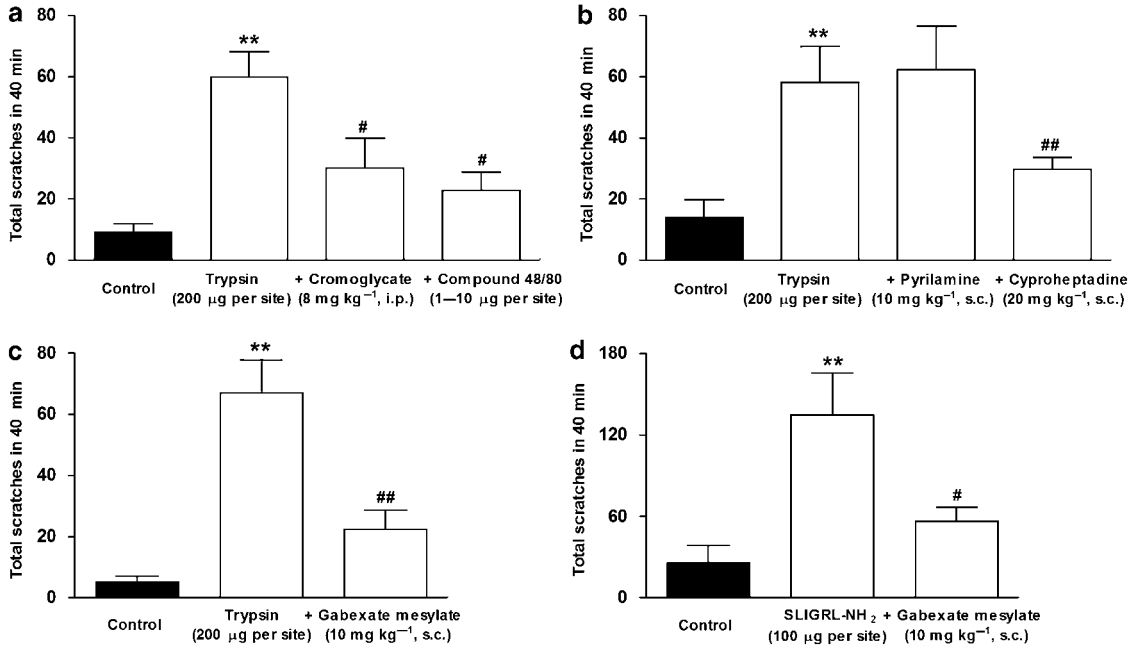

Figure 3 (**a**) Effect of treatment with the inhibitor of mast cell degranulation, disodium cromoglycate (8 mg kg^{-1}, i.p., for 6 days, once a day) or the mast cell degranulator, compound 48/80 (1–10 μg per site, i.d., for 4 days, once a day) on the trypsin (200 μg per site)-elicited scratching behaviour in Swiss mice. (**b**) Effect of treatment with the selective histamine H_1 receptor antagonist pyrilamine (10 mg kg^{-1}, s.c., 30 min before) or the mixed 5-HT/histamine antagonist cyproheptadine (20 mg kg^{-1}, s.c., 30 min before). (**c**) Effect of treatment with the non-selective protease inhibitor gabexate mesylate (10 mg kg^{-1}, s.c., 30 min before). (**d**) Effect of treatment with the non-selective protease inhibitor gabexate mesylate (10 mg kg^{-1}, s.c., 30 min before) on the SLIGRL-NH_2-induced scratching behaviour in Swiss mice. Each column represents the mean of 6–10 animals and the vertical bars represent the s.e.mean. Significantly different when compared with the saline group (**$P<0.01$) and the trypsin-treated group ($^{\#}P<0.05$ or $^{\#\#}P<0.01$).

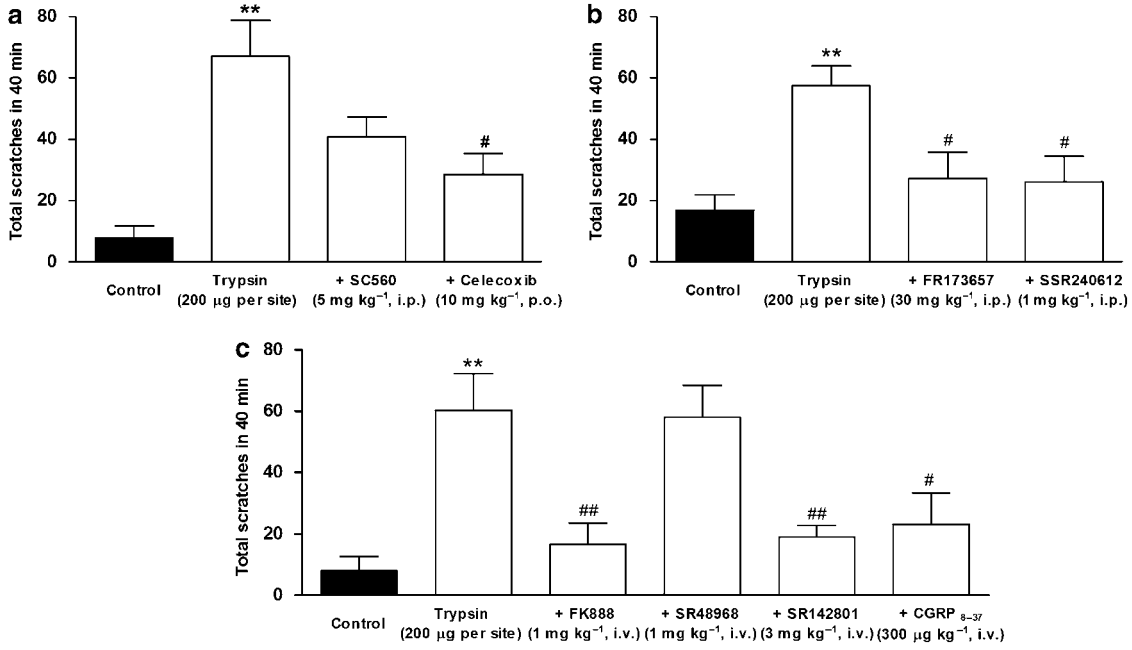

Figure 4 (**a**) Effect of treatment with the selective COX-1 or COX-2 inhibitors SC560 (5 mg kg^{-1}, i.p., 30 min before) and celecoxib (10 mg kg^{-1}, p.o., 1 h before), respectively. (**b**) Effect of treatment with the selective kinin B_2 or B_1 receptor antagonists FR173657 (30 mg kg^{-1}, i.p., 30 min before) and SSR240612 (1 mg kg^{-1}, i.p., 30 min before), respectively. (**c**) Effect of treatment with the selective NK_1 FK888 (1 mg kg^{-1}, i.v., 15 min before), the selective NK_2 SR48968 (3 mg kg^{-1}, i.v., 15 min before), the selective NK_3 SR142801 (3 mg kg^{-1}, i.v., 15 min before) or the selective calcitonin gene-related peptide (CGRP) $CGRP_{8-37}$ fragment (300 μg kg^{-1}, i.v., 15 min before) receptor antagonists on the trypsin (200 μg per site)-elicited scratching behaviour in Swiss mice. Each column represents the mean of 6–10 animals and the vertical bars represent the s.e.mean. Significantly different when compared with the saline group (**$P<0.01$) and the trypsin-treated group ($^{\#}P<0.05$ or $^{\#\#}P<0.01$).

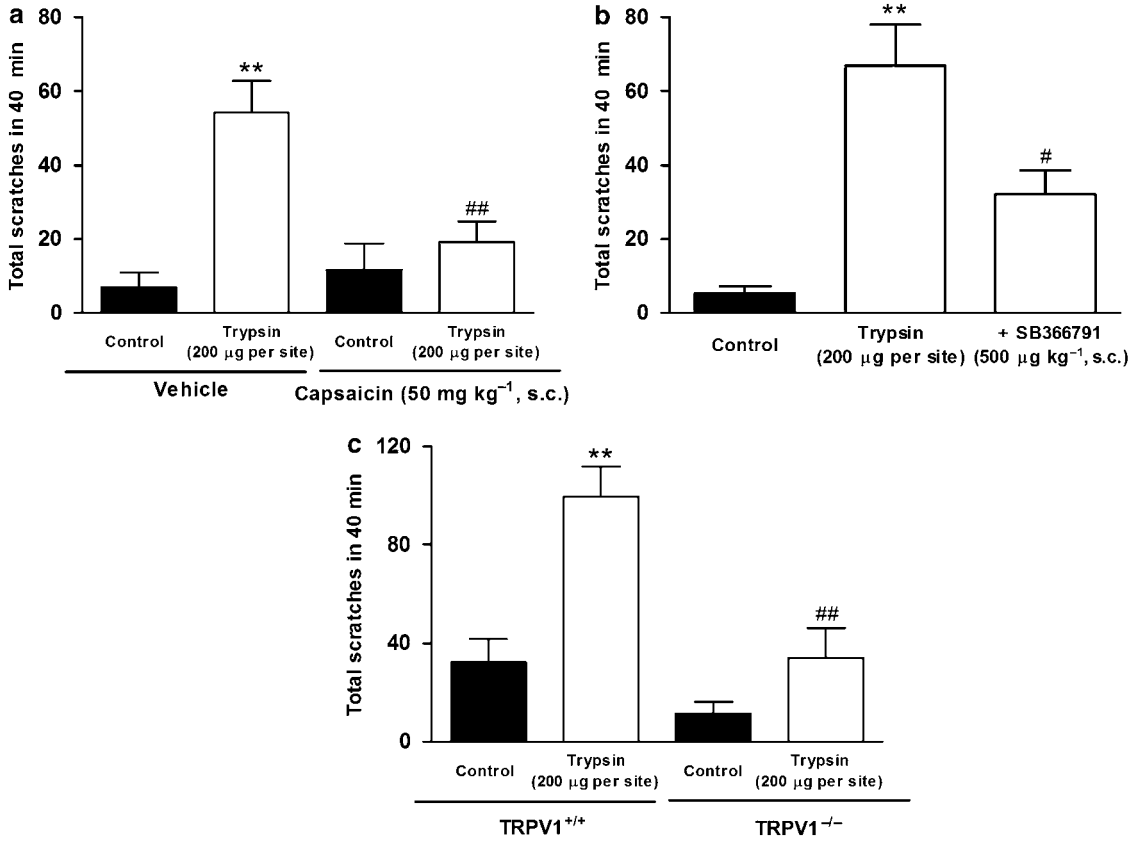

Figure 5 Trypsin-induced scratching behaviour in (**a**) capsaicin (50 mg kg^{-1}, s.c.) neonatal-treated Swiss mice and (**c**) in TRPV1$^{-/-}$ C57BL/6 mice. (**b**) Effect of treatment with the selective transient receptor potential vanilloid 1 (TRPV1) receptor antagonist SB366791 (500 μg kg^{-1}, s.c., 30 min before) on the trypsin (200 μg per site)-elicited scratching behaviour in Swiss mice. Each column represents the mean of 6–10 animals and the vertical bars represent the s.e.mean. Significantly different when compared with the saline group ($^{**}P<0.01$) and the trypsin-treated group ($^{\#}P<0.05$ or $^{\#\#}P<0.01$).

result is in agreement with those of a previous study demonstrating that the scratching behaviour caused by the PAR-2 agonist SLIGRL-NH_2 is not dependent on the histamine H_1 receptor (Shimada *et al.*, 2006). Although histamine plays an essential role in a range of skin diseases acting via the H_1 receptor, this pathway does not seem to contribute to trypsin effects. However, it is important to note that a new subtype of histamine receptors, the H_4 receptor, is now being suggested as an alternative target for the treatment or prevention of itching responses (Bell *et al.*, 2004; Dunford *et al.*, 2007; Thurmond *et al.*, 2008).

Our data also suggest that 5-HT release is important as the trypsin pruritus was reduced by cyproheptadine. However, human mast cells do not contain 5-HT so that the effects of cyproheptadine in this model may be rodent-specific.

Gabexate mesylate, the non-selective protease inhibitor, inhibits the trypsin effects in this model suggesting a role for the additional release of proteases from mast cells. However, this compound is also capable of inhibiting different proteases including thrombin, trypsin, tryptase, kallikreins, plasmin and chymotrypsin (Wisner *et al.*, 1987). But gabexate mesylate also markedly inhibited pruritus induced by the selective PAR-2 receptor agonist SLIGRL-NH_2, suggesting that trypsin activity may lead to a secondary release of proteases. Mast cells can express and release a variety of proteases including tryptase, chymase and cathepsins, among others (Steinhoff *et al.*, 2006).

Recently, it has been suggested that PAR-2 activation by tryptase from mast cells can sensitize C-fibres and thus contribute to the itching sensation (Ikoma *et al.*, 2006; Paus *et al.*, 2006; Ui *et al.*, 2006). However, trypsin can activate not only mast cells but also keratinocytes and neuronal cells. High levels of trypsin and its receptor PAR-2 can be constitutively detected in human skin (Babiarz-Magee *et al.*, 2004). However, more investigation is needed to determine the sequence of cell activation induced by trypsin in our model and/or the parallel activation of different pathways.

A role for prostanoids in human itching processes has been suggested (Steinhoff *et al.*, 2003). Our studies also support the notion that these eicosanoids are involved in the development of pruritus after trypsin injection. This involvement seems to be dependent on COX-2 rather than on COX-1, as the trypsin effect was significantly reduced only by the selective COX-2 inhibitor celecoxib. Indeed, studies have shown that central PAR activation stimulates intracellular messengers associated with the activation of COX and thereby prostaglandin production (Koetzner *et al.*, 2004). In addition, it has been reported that prostaglandin E_2 can induce neurotrophin release from keratinocytes, an event that is important for the transmission of the itching sensation throughout the nervous system (Kanda *et al.*, 2005; Steinhoff *et al.*, 2006).

Another interesting and new result observed in our study is the involvement of both kinin B_2 and B_1 receptors in

trypsin-induced scratching behaviours in mice. We have shown that the selective blockade of the kinin B_2 (by FR173657) or B_1 (by SSR240612) receptors elicited a significant decrease in the frequency of trypsin-induced scratching. In fact, mast cell activation can release kallikreins (Imamura *et al*., 2004), which might be responsible for the cutaneous kinin production. Moreover, bradykinin (a selective B_2 receptor agonist) has potent pruritogenic effects in patients with atopic dermatitis (Hosogi *et al*., 2006). Also, Hayashi and Majima (1999) have demonstrated that treatment with the selective kinin B_2 receptor antagonists FR173657 or HOE140 is effective in inhibiting sodium deoxycholic acid-induced scratching behaviour in mice. Interestingly, the same authors also reported that the selective antagonism of kinin B_1 receptor by des-Arg^9-[Leu^8]-bradykinin was not able to reduce the frequency of scratching (Hayashi and Majima, 1999). Conversely, our results showed a clear involvement of the kinin B_1 receptor in trypsin actions. Our results also apparently constitute the first literature data suggesting the involvement of PAR-2 activation in the kinin B_1 receptor upregulation in pruriginous processes.

PAR-2 receptors are widely expressed in sensory nerves, and a direct action of these receptors in eliciting inflammation and pain by means of neurogenic pathways has already been shown (Steinhoff *et al*., 2000; Vergnolle *et al*., 2001; Cottrell *et al*., 2003; Seeliger *et al*., 2003; Obreja *et al*., 2006; Paszcuk *et al*., 2008). In this context, we explored the possible involvement of neuropeptides in the trypsin-induced pruritus. Our results indicate that the effects of trypsin are mediated by the release of neurokinins (substance P and neurokinin B) and CGRP, probably from the peripheral endings of spinal afferent neurons. These findings are in line with previous data demonstrating that the PAR-2 activators, trypsin and thrombin, can directly stimulate neurokinin and CGRP release from the rat skin (Obreja *et al*., 2006). Moreover, Steinhoff *et al*. (2000) have demonstrated that trypsin and tryptase, acting on PAR-2 receptors, stimulate the release of neuropeptides from slices of the spinal cord. Also, substance P release has been implicated in skin irritation mainly for its property of inducing plasma extravasation, an event markedly potentiated by CGRP, which acts as a potent vasodilator (Newbold and Brain, 1993). Although neurokinin A can itself cause itching in humans (Thomsen *et al*., 2002), this mediator does not seem to be important for the trypsin-induced pruritus in mice, as the selective NK_2 receptor antagonist SR48968, in an appropriate dose, was not able to reduce the effects of trypsin.

Finally, we investigated whether the TRPV1 receptor, located on the C-fibre afferents, is involved in the amplification of the effects of trypsin. Our data show that TRPV1 plays a central role in the itching process mediated by trypsin. First, the postnatal capsaicin treatment promoting C-fibre destruction significantly attenuated the pruritus induced by trypsin. Similarly, pretreatment with the selective TRPV1 receptor antagonist (SB366791) consistently diminished the number of scratches in trypsin-treated mice. Lastly, our studies showed that the genetic deletion of TRPV1 receptor almost completely reduced the effect of trypsin. These data support the notion that PAR-2 can lead to the activation and/or sensitization of TRPV1 receptors (Cottrell *et al*., 2004; Amadesi *et al*., 2006). Similarly, several inflammatory mediators, such as bradykinin, ATP, prostaglandin E_2 and nerve growth factor, are also able to directly sensitize TRPV1 (Ferreira *et al*., 2004). In this context, we suggest that proteases from mast cells (such as tryptase), along with kinins and prostaglandins, can exert an important role in the itching induced by trypsin, probably by sensitizing TRPV1 receptor sensory nerve endings.

Overall, the present results suggest that the intradermal injection of trypsin in the mouse neck is a reproducible model for the study of mechanisms involved in PAR-2-mediated itching. We also suggest a sequence of events leading to the scratching behaviour induced by trypsin (Figure 6). Following trypsin injection, there is activation of PAR-2 receptors that could be located on neuronal, kerati-

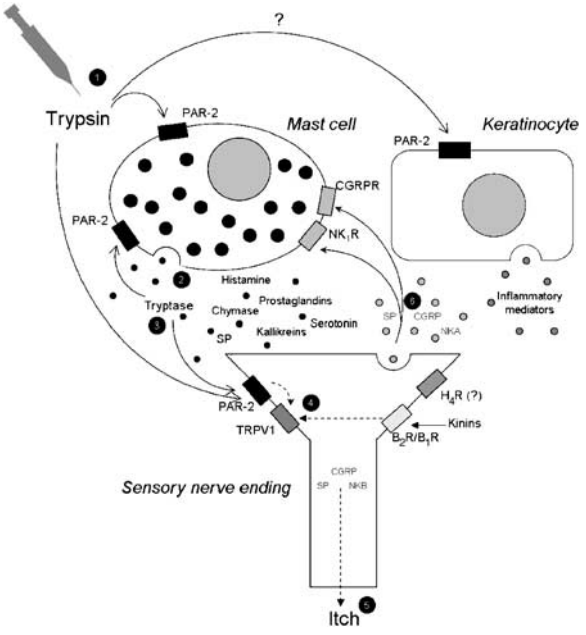

Figure 6 Schematic representation of the neurogenic mechanisms of trypsin-induced scratching behaviour in mice. (1) Intradermally injected trypsin cleaves PAR-2 at the plasma membrane of sensory nerve endings, mast cells and/or keratinocytes to expose a tethered ligand domain that binds and activates the cleaved receptor. (2) Activation of PAR-2 on mast cells stimulates the release of several mast cell mediators. (3) Tryptase released from degranulated mast cells activates proteinase-activated receptor-2 (PAR-2) on sensory nerve endings and/or on their own. (4) Mediators from mast cells and other inflammatory cells (such as tryptase, kinins and/or prostaglandins) may sensitize transient receptor potential vanilloid 1 (TRPV1) receptors on sensory nerves. (5) At the level of the spinal cord, the activation of sensory nerves leads to the release of CGRP and tachykinins (e.g., SP, neurokinin B), transmitting itch sensations to the central nervous system (CNS) and leading to the scratching behaviour. (6) Sensory nerve endings stimulation promote the release of CGRP and tachykinins (e.g., SP), which interacts with their specific receptors on mast cells (promoting mast cell degranulation) and/or on blood vessels (causing hyperaemia and plasma extravasation), establishing the neurogenic inflammation scenario. B_2R, bradykinin B_2 receptor; B_1R, bradykinin B_1 receptor; CGRPR, calcitonin gene-related peptide receptor; H_4R, histamine H_4 receptor; NKA, neurokinin A; NKB, neurokinin B; NK_1R, neurokinin receptor 1; serotonin, 5-HT.

nocytes and mast cells. PAR-2 activation leads to the release of different molecules, such as substance P, neurokinin A, CGRP, 5-HT, prostaglandins, kinins and proteases, that act together to lead to sensitization of sensory nerves and to transmit the itch sensation to the brain.

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Conflict of interest

The authors state no conflicts of interest.

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