The serine protease inhibitor SerpinA3N attenuates neuropathic pain by inhibiting T cell–derived leukocyte elastase

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Neuropathic pain is a major, intractable clinical problem and its pathophysiology is not well understood. Although recent gene expression profiling studies have enabled the identification of novel targets for pain therapy1–4, classical study designs provide unclear results owing to the differential expression of hundreds of genes across sham and nerve-injured groups, which can be difficult to validate, particularly with respect to the specificity of pain modulation5. To circumvent this, we used two outbred lines of rats6, which are genetically similar except for being genetically segregated as a result of selective breeding for differences in neuropathic pain hypersensitivity7. SerpinA3N, a serine protease inhibitor, was upregulated in the dorsal root ganglia (DRG) after nerve injury, which was further validated for its mouse homolog. Mice lacking SerpinA3N developed more neuropathic mechanical allodynia than wild-type (WT) mice, and exogenous delivery of SerpinA3N attenuated mechanical allodynia in WT mice. T lymphocytes infiltrate the DRG after nerve injury and release leukocyte elastase (LE), which was inhibited by SerpinA3N derived from DRG neurons. Genetic loss of LE or exogenous application of a LE inhibitor (Sivelastat) in WT mice attenuated neuropathic allodynia (Fig. 1c) as compared to sham treatment (Fig. 2a). Quantitative real-time PCR (qPCR) analysis demonstrated an upregulation of the expression of mouse SerpinA3n mRNA in ipsilateral L3–L4 DRGs at day 1 after SNI as compared to sham treatment (Fig. 2a), but we did not observe this in contralateral lumbar DRGs (Fig. 2a) or ipsilateral thoracic DRGs after SNI (Supplementary Fig. 2a). We next rescreened all eight initial ‘gene hits’ on biologically distinct RNA samples via slot-northern blotting with the criterion of injury-induced differential regulation between the rats with high and low pain sensitivity (Fig. 1c and Supplementary Fig. 1). Only two transcripts, encoding rat serine protease inhibitor 3 (Serpin3a3n, formerly known as Spin2c) (Fig. 1c) and family with sequence similarity 111, member A (Fam111a) (Supplementary Fig. 1) were differentially expressed. Nerve injury induced Serpin3a3n expression in rats with low pain sensitivity to a greater extent than it did in rats with high neuropathic pain sensitivity (Fig. 1c,d). This was also validated via mRNA in situ hybridization in the injured L4 DRG, which revealed a primarily neuronal expression of Serpin3a3n (Fig. 1e).

Upregulation of Serpin3a3n expression also occurred in mice and in the spared nerve injury (SNI) model (Fig. 2). Quantitative real-time PCR (qPCR) analysis demonstrated an upregulation of the expression of mouse Serpin3a3n mRNA in ipsilateral L3–L4 DRGs at day 1 after SNI as compared to sham treatment (Fig. 2a), but we did not observe this in contralateral lumbar DRGs (Fig. 2a) or ipsilateral thoracic DRGs after SNI (Supplementary Fig. 2a). In comparison with nine other related Serpin3 genes in the mouse DRG, Serpin3a3n is the most abundantly expressed serpin isoform, followed by Serpin3g, whereas Serpin3h,
SerpinA3k and SerpinA3f show very low abundance (Supplementary Fig. 2b). After SNI, we found that SerpinA3g and SerpinA3k were upregulated at days 1 and 3 after nerve injury (Supplementary Fig. 2c). Expression of SerpinA3N protein was increased at days 1 and 3, and it declined to sham levels at day 7 after SNI, as compared to sham-treated mice (Fig. 2b and Supplementary Note 1).

Immunohistochemical analysis of mouse L3–L4 DRGs with an antibody raised against SerpinA3N in combination with co-staining for the identification of DRG neuronal subpopulations revealed SerpinA3-like staining (Supplementary Note 1) in neurofilament 200 (NF200)-positive large myelinated Aβ fiber neurons and in a large fraction of calcitonin gene–related protein (CGRP)-positive peptidergic nociceptive neurons, and it showed that SerpinA3-like staining was less abundant in isolectin B4 (IB4)-positive nonpeptidergic nociceptor neurons (Fig. 2c,d and Supplementary Fig. 3a). The percentage of CGRP-positive neurons with SerpinA3-like immunoreactivity increased in the DRG after SNI, as compared to sham controls (Fig. 2d). This was not due to a change in the distribution pattern of CGRP in DRG neurons after injury, as the total percentage of neurons with SerpinA3-like immunoreactivity increased at day 3 after SNI (Supplementary Fig. 3b). This was further supported by histograms showing cell size versus frequency, demonstrating that neurons with a diameter of 10–20 μm exhibit increased SerpinA3-like immunoreactivity 3 d after SNI as compared to sham treatment (Supplementary Fig. 3c). Using activat-

Figure 1  SerpinA3n is upregulated in rats with low neuropathic pain sensitivity. (a) Schematic diagram of the microarray analyses on L4–L5 DRGs from rat strains demonstrating low neuropathic pain or high neuropathic pain behavior after spinal nerve ligation. (b) Chi statistical test versus interaction size plot showing the regulated transcripts detected 3 d after SNL. Filled blue and green triangles represent SerpinA3n and Fam111a, respectively. Empty red triangles represent false-positive genes. (c) Northern blot analysis of SerpinA3n mRNA in low-neuropathic and high-neuropathic rats at 3 d after SNL (normalized to PPIA, encoding cyclophilin A), n = 3. (d) Quantitative estimation of SerpinA3n mRNA regulation analyzed via Affymetrix expression array and northern blots (n = 3). For northern data, two-way analysis of variance (ANOVA) (P = 2.9e–6); *P < 0.05 as compared to sham, †P < 0.05 between low-pain and high-pain rats after SNL, post hoc Tukey’s test. (e) In situ hybridization using digoxigenin-labeled antisense riboprobes for SerpinA3n expression on L4 DRG from low- and high-pain rats, 3 d after SNL and sham treatment; image at the bottom right represents sense control probe. Scale bars, 100 μm. Error bars are means ± s.e.m.
Figure 2 Serpina3n is upregulated in mouse lumbar DRGs after spared nerve injury, a model of neuropathic pain. (a) Real-time qPCR analysis of Serpina3n expression in L3–L5 DRGs after SNI, with PPIA serving as reference gene (n = 4 mice per time point; *P < 0.05 compared to sham DRG; one-way ANOVA, Tukey’s post hoc test). (b) Western blot analysis and quantification of SerpinA3n expression (55-kDa band) in L3–L4 DRGs after SNI normalized to α-tubulin expression (n = 3–5 mice per time point, one-way ANOVA, Tukey’s post hoc test). (c) SerpinA3-like immunoreactivity in peptidergic (CGRP) and non-peptidergic (IB4-binding) nociceptors and large-diameter neurons (NF200) in L3–L4 DRG sections from naive mice (arrows indicate areas of colocalization). (d) Quantitative analysis of SerpinA3-like immunoreactivity in L3–L4 DRGs at day 3 after SNI as compared to sham treated (n = at least 3 DRG sections/mouse, 3 mice per treatment group; *P < 0.05 compared to sham; two-tailed unpaired t-test) and examples of upregulation in the CGRP-expressing population. (e) SerpinA3 and ATF3 staining in L3–L4 DRGs at day 1 after SNI showing SerpinA3+/ATF3+ (white arrows), SerpinA3+/ATF3− (white arrowheads) and SerpinA3−/ATF3+ (yellow arrowheads) neurons. (f) Western blot analysis of SerpinA3n expression in lysates and medium from cultured DRG neurons. (g) Western blot analysis and quantification of SerpinA3n expression in lumbar spinal cord after SNI (n = 3–5 mice per time point, *P < 0.05 compared to sham, one-way ANOVA, Tukey’s post hoc test). Scale bars, 100 µm (c–e). Error bars are means ± s.e.m.

Serpins exert their function by binding and inhibiting specific serine proteases, and several serine proteases can be substrates for SerpinA3n (Supplementary Note 2). However, consistent with studies showing a role for LE in activating MMP-9 (refs. 16,17), we observed that in comparison with BSA (35 nM) (Fig. 4a), other serine proteases such as thrombin and matrix metalloproteinase 9 (MMP-9), which are associated with nociception14,15, were not affected by SerpinA3n (Supplementary Fig. 9a,b). Inhibition of LE using Sivelestat18, administered 8 d after SNI via a single, low-dose i.t. injection (200 pmol) rapidly and considerably reduced mechanical allodynia compared to BSA-containing vehicle (Supplementary Fig. 6i). These results suggest that LE is functionally involved in the induction as well as to a brush stimulus applied to the plantar surface were stronger in Serpina3n−/− mice post-SNI than in WT littermates (Supplementary Fig. 6i), suggesting that upregulation of SerpinA3n during the early post-injury phase acts as an endogenous brake that specifically affects the transition from acute to chronic neuropathic mechanical hypersensitivity. To address this hypothesis further, we overexpressed Serpina3n cDNA in L3–L4 DRGs of adult WT mice by intraganglionic injection of recombinant adeno-associated virions (rAAVs) carrying Serpina3n cDNA or GFP cDNA as a control (Fig. 3d). Overexpression of SerpinA3n in the DRG did not alter basal nociceptive sensitivity, but it decreased mechanical allodynia at day 3 and 7 after SNI, but not thereafter, as compared to GFP-expressing mice (Fig. 3e).

To determine whether exogenous SerpinA3n would reduce established neuropathic tactile allodynia, at 8 d after SNI, we administered rSerpinA3n (10 pmol) or PBS containing 10 pmol of bovine serum albumin (BSA) as vehicle intrathecally (i.t.) to WT mice with mechanical allodynia (Fig. 3f,g). rSerpinA3n substantially reduced mechanical allodynia compared to PBS within 1 h after the first injection, which was extended until 72 h upon using the dosing scheme shown in Figure 3f,g. The protective effects of rSerpinA3n were dose dependent (Fig. 3h–j). A single i.t. injection of SerpinA3N (10 pmol) at day 18 after SNI, when chronic hypersensitivity is established, did not decrease mechanical hypersensitivity as compared to BSA-containing vehicle (Supplementary Fig. 6j). The anti-allodynic effects of i.t. rSerpinA3N during early post-SNI stages were not accompanied by any damage to spinal neurons, microglia and astrocytes (Supplementary Fig. 7), nor did rSerpinA3N influence any parameters of nerve injury–related neuroinflammation in the spinal cord or the DRG at 2–7 d after SNI (Supplementary Fig. 8 and Supplementary Note 2).

SNI-induced mechanical allodynia was reduced in mice deficient in the gene encoding LE (Bleace−/− mice) as compared to WT littermates, up to 21 d after nerve injury (Fig. 4c,d). Inhibition of LE using Sivelestat18, administered 8 d after SNI via a single, low-dose i.t. injection (200 pmol) rapidly and considerably reduced mechanical allodynia compared to controls (Fig. 4e,f). Sivelestat blocked LE activity in vitro at doses comparable with its effects on mechanical allodynia in vivo (Supplementary Fig. 9c). These results suggest that LE is functionally involved in the induction as well as...
Figure 3 SerpinA3N attenuates mechanical allodynia after nerve injury. (a,b) SerpinA3n mRNA in situ hybridization (a) and western blot analysis (b) of SerpinA3N (55-kDa band) in L3–L4 DRGs from SerpinA3n-null mice (SerpinA3n−/−) 1 d after SNI. Scale bar, 100 μm. (c) Paw withdrawal thresholds to plantar von Frey application in SerpinA3n+/− and WT mice (n = 16 mice per group). (d,e) Western blot analysis of SerpinA3n expression in L3–L4 DRGs (d) and mechanical thresholds (e) in mice receiving intraganglionic injection of AAVs expressing SerpinA3n or GFP (n = 8 mice per group). (f,g) Paw withdrawal responses (f) and area under the curve (AUC; g) of mechanical responses (0.04–2 g; h) or AUC (0.04–2 g; j) at day 8 after SNI (n = 10–12 mice per dose or vehicle). In c and e, *P < 0.05 compared to basal values and †P < 0.05 compared to the corresponding control. In f–j, *P < 0.05 compared to day 8 after SNI; †P < 0.05 compared to vehicle. In all panels: two-way ANOVA of repeated measures, Tukey’s post hoc test. Error bars are means ± s.e.m.

the maintenance of long-term tactile hypersensitivity and that its pharmacological blockade is effective when peak neuropathic pain is established.

LE is produced in blood leukocytes, mainly neutrophils. Although Elane mRNA is expressed in mouse-derived L3–L4 DRGs or spinal cord tissue, Elane mRNA was not detectable in neuronal cultures derived from the DRG or the spinal cord. Elane mRNA was also not expressed in many non-neuronal cells that have been associated with neuropathic pain, including astrocytes, microglia, macrophages or Schwann cells, but it was found in T lymphocytes (Fig. 4g). We found that T cells are present in the mouse DRG in vivo under naïve conditions, and they increase in frequency after nerve injury (Supplementary Fig. 10a–f), which is consistent with previous reports. Although neutrophils identified via Gr-1 immunostaining are found in the vicininity of the DRG and they increase in frequency after nerve injury, they remain primarily localized to the borders rather than invading the parenchyma of the DRG in large numbers (Supplementary Fig. 10g). We found that LE activity was increased in L3–L4 DRGs 1 and 7 days after SNI as compared to sham-treated mice (Fig. 4h,i).

To address whether T cells function as a source of LE in neuropathic pain, T cells derived from WT or Elane−/− mice were adoptively transferred into recombination activating gene 2–null (Rag2−/−) mice. Mechanical allodynia was reduced after SNI in Rag2−/− mice compared to WT mice (Fig. 4j), consistent with previous reports on Rag1−/− mice which similarly lack T cells. Adoptive transfer of WT T-cells in Rag2−/− mice restored mechanical allodynia (Fig. 4j), whereas Rag2−/− recipients of LE-deficient T cells did not develop mechanical allodynia after SNI (Fig. 4j). These differences were not due to differential efficiency of adoptive transfer of T cells derived from Elane−/− or WT mice (Supplementary Fig. 11).

SerpinA3N is a part of a large superfamily that is preserved and differentially amplified across species, whose members exert myriad functions, ranging from coagulation to chromatin condensation to control of immune cell function, with specificity being imparted by distinct expression patterns and substrate recognition. Our data suggest that the SerpinA3 cluster expressed and induced in peripheral sensory neurons may be a key endogenous regulator of the acute-to-chronic switch in pain hypersensitivity. This anti-allodynic activity of SerpinA3N is not linked to modulating glial reactions post-injury, nor does SerpinA3N prevent immune cells from infiltrating the DRG; rather, SerpinA3N unfolds its actions a step further down by acting on proteases derived from immune cells in the DRG. Indeed, LE represents a novel effector for nociceptive modulation in the DRG, particularly via T lymphocytes, underscoring their contributions in mechanical allodynia post-injury.

Unlike the close functional links observed between SerpinA3N, LE and resident as well as infiltrating T cells over induction of mechanical allodynia (i.e., the first 7 d after SNI), we observed a
Figure 4 Leukocyte elastase (LE) is a substrate for SerpinA3N, and it promotes neuropathic allodynia. (a,b) LE activity (a) or MMP9 activity (b), as assessed by in vitro fluorometric assays. AFU, arbitrary fluorescence units; *P < 0.05 compared to protease alone, †P < 0.05 compared to BSA control. (c–f) Paw withdrawal thresholds (c,e) and AUC of responses to von Frey filaments (d,f) in LE-null (Elane−/−) and WT mice (c,d) or in WT mice after injection of the LE inhibitor Sivelestat or vehicle 8 d after SNI (e,f); n = 8–9 per group; *P < 0.05 compared to basal (c,d) or vehicle (e,f), 1P < 0.05 between genotypes (c,d) or compared to vehicle (e,f). (g) Elane mRNA expression in different cell types in vitro or tissues from WT mice. (h) Quantification and staining (i) of LE activity in L4 DRGs of Elane−/− and WT mice; n = 4–5 experiments, 4 mice per data point; *P < 0.05 compared to sham, 1P < 0.05 between genotypes, one-way ANOVA, post hoc Tukey’s test. Scale bars, 100 µm. (j) Mechanical allodynia in Rag2−/− and WT mice after adoptive transfer of WT- or LE-deficient T cells 6 d before SNI; n = 10 mice per group; *P < 0.05 compared to basal values, 1P < 0.05 compared to Rag2−/− mice without T cell transfer. Unless otherwise indicated, we used two-way ANOVA of repeated measures, Tukey’s post hoc test. Error bars are means ± s.e.m.

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AUTHOR CONTRIBUTIONS
I.V., D.E.S., A.L., K.K.B., M.S., D.H., S.P., P.R., R.S.G., C.N., S.G. and M.C. performed experiments and analyzed data; R.K., M.C., C.J.W., S.D.L., M.D., B.A., M.A.M. designed and supervised experiments; R.K., I.V. and M.G. primarily wrote the manuscript.

COMPETING FINANCIAL INTERESTS
The authors declare competing financial interests: details are available in the online version of the paper.

METHODS
Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.
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ONLINE METHODS

Genetically modified mice. We generated conditional Serpina3n-knockout mice using a BAC recombination strategy. The 129svBAC clone (bMQ-266h22) containing Serpina3n was obtained from BioScience. Primers were designed to retrieve a 10.3-kb region containing Serpina3n, insert an upstream loxp site within intron 1, and insert a downstream FRT-Neo-FRT-loxp cassette within intron 2. exon 2 was chosen for deletion because it contains the translation start site, encodes the signal sequence for secretion, and accounts for over 50% of the Serpina3n coding sequence. The final targeting vector, with a loxp-flanked region of 1.1 kb and homology arms of 4.7 kb and 4.5 kb, was linearized with AattI, electroporated into W4/129S6-derived embryonic stem (ES) cells, and selected with G418. DNA from G418-resistant ES cells was analyzed by long range PCR and Southern blot. Validated ES cells were injected into blastocyst and implanted into C57BL/6 pseudo-pregnant female mice. Male chimeric animals were bred for germline transmission with (1) germline FLPe-expressing females (Gr(Rosa)26Sor1(Flp)31) and (2) germline Cre-expressing females (Tg(Eliaa-cre) C5379Lmgd) to generate heterozygote null mice (Serpina3n+/-). F1 heterozygotes were interbred, yielding WT, heterozygous, and homozygous mice in the expected 1:2:1 ratio. In subsequent generations, the Eliaa-cre allele was bred out of the null line. For all behavioral and histology experiments, mixed background Serpina3n+/- and WT littermate controls were used. Serpina3n+/- mice are viable and fertile. Mice were genotyped by PCR using the following primers: F1 (mutant forward) 5′- TCTGGATTCAGGCCACGGTTT3′; F2 (WT forward) 5′- AGGCATTGATGGTGCTGGT3′; R1 (common reverse) 5′- CCTACAGGCGATGGATATTTCC 3′. The WT and mutant alleles were detected as fragments of 459 bp and 518 bp, respectively.

Mice lacking the leucocyte elastase (referred to here as Elane+/- mice) (B6.129X1-Elane+/-;S12)11 were bought commercially (Jackson Laboratories) and genotyped by PCR on mouse genomic DNA using the primers oIMR7064 (WT forward), oIMR7065 (common) and oIMR8162 (mutant reverse). Homozygote mice were identified by a 310-bp fragment corresponding to the mutant Elane gene. WT mice were identified by a 230-bp fragment corresponding to the WT allele. Heterozygote animals were identified by the presence of both fragments. Primer sequences are available on the corresponding website of the source (Jackson Laboratories).

High neuropathic pain and low neuropathic pain strain production. High-neuropathic and low-neuropathic rats were derived from outbred Wistar-based Sabra strain rats by genetic selection for high- versus low-autotomy behavior in the neuroma model of neuropathic pain. The animals used for the high neuropathic pain and low neuropathic pain substrain production, was originally described by Devor and Raber6. High-pain and low-pain sub strains were produced using a BAC recombination strategy. The 129svBAC clone (bMQ-266h22) containing Serpina3n was obtained from BioScience. Primers were designed to retrieve a 10.3-kb region containing Serpina3n, insert an upstream loxp site within intron 1, and insert a downstream FRT-Neo-FRT-loxp cassette within intron 2. exon 2 was chosen for deletion because it contains the translation start site, encodes the signal sequence for secretion, and accounts for over 50% of the Serpina3n coding sequence. The final targeting vector, with a loxp-flanked region of 1.1 kb and homology arms of 4.7 kb and 4.5 kb, was linearized with AattI, electroporated into W4/129S6-derived embryonic stem (ES) cells, and selected with G418. DNA from G418-resistant ES cells was analyzed by long range PCR and Southern blot. Validated ES cells were injected into blastocyst and implanted into C57BL/6 pseudo-pregnant female mice. Male chimeric animals were bred for germline transmission with (1) germline FLPe-expressing females (Gr(Rosa)26Sor1(Flp)31) and (2) germline Cre-expressing females (Tg(Eliaa-cre) C5379Lmgd) to generate heterozygote null mice (Serpina3n+/-). F1 heterozygotes were interbred, yielding WT, heterozygous, and homozygous mice in the expected 1:2:1 ratio. In subsequent generations, the Eliaa-cre allele was bred out of the null line. For all behavioral and histology experiments, mixed background Serpina3n+/- and WT littermate controls were used. Serpina3n+/- mice are viable and fertile. Mice were genotyped by PCR using the following primers: F1 (mutant forward) 5′- TCTGGATTCAGGCCACGGTTT3′; F2 (WT forward) 5′- AGGCATTGATGGTGCTGGT3′; R1 (common reverse) 5′- CCTACAGGCGATGGATATTTCC 3′. The WT and mutant alleles were detected as fragments of 459 bp and 518 bp, respectively.

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Microarray analysis and secondary screen. In adult high-pain and low-pain rats, induction of peripheral nerve injury, sample collection, DRG total RNA extraction, and microarray hybridization were performed as previously described10. RAE230A arrays (Affymetrix) were used for biologically independent triplicate samples. Background correction and quantile-quantile data normalization were followed by probe set intensity computation, all using the RMA algorithm within R as implemented in the Bioconductor package11–13. For each probe, a linear model with one-way effects for sub-strain (high or low pain) and injury (sham or SNL), and sub-strain by injury interaction was fit using iteratively reweighted least-squares. For each probe, the chi statistic34, comparing the residual sum of squares of the full model to the model without interaction effect, was calculated. Graphical analysis of outliers in a plot of chi statistic versus interaction effect magnitude allowed identification of probes whose regulation potentially differed between substrains. Each of the eight outlying genes within the top left and top right sections of Figure 1b were subject to a secondary screen on biologically independent triplicate DRG total RNA samples, distinct from those used in the array analysis. Of the eight genes, two showed reliable differential regulation between the two substrains in this secondary screen. These were Serpina3n (Fig. 1c) and Fam11a (Supplementary Fig. 1).

Spared nerve injury. In the spared nerve injury (SNI) model for neuropathic pain55, mice were anesthetized under 2% isoflurane, and the fur of the lateral part of the left thigh was removed. The skin on the lateral surface of the thigh was incised and a section was made directly through the biceps femoris muscle exposing the sciatic nerve and its three terminal branches: sural, common peroneal and tibial nerves. The common peroneal and tibial nerve were tightly ligated with 5.0 silk and sectioned distal to the ligation, removing 2–4 mm of the distal nerve stump. These two nerves were subsequently cut and the sural nerve was left intact. Muscle and skin were closed in two layers. The mice were housed under standard conditions in cages for 3 d before the isolation of tissues or perfusion of animals. In some experiments, Minocycline (Sigma), was injected i.p. into mice at a concentration of 30 mg/kg (dissolved in H2O) 16 and 0.5 h before SNI operations and then twice daily for the duration of the experiment.

Generation of recombinant adeno-associated virions (rAAVs) expressing Serpina3N. In order to overexpress SerpinA3N unilaterally in L3 and L4 DRG of mice, the mouse SerpinA3n cDNA sequence was amplified from RNA isolated from DRG of C57Bl/6 mice using AccuPrime Pfx SuperMix (Invitrogen). The DNA fragment corresponding to SerpinA3n was gel-purified and TOPO-cloned using the Zero Blunt TOPO PCR Cloning Kit (Invitrogen). The TOPO-cloned Serpina3n was transformed into competent bacteria and minipreps of single colonies from this transformation reaction were prepared. After sequencing the clones, one single colony containing the right Serpina3n open reading frame (ORF) sequence was excised from the TOPO vector using appropriate digestion enzymes and then cloned into the pAM-MCS-QERK-stop vector.

Chimeric AAV2/8 virions (a mixture of serotypes 2 and 8) carrying the Serpina3n ORF as described above or GFP as a control were produced according to the protocol described in Luo et al.36.

Intraganglionic (DRG) injections. We have previously described intraperi-neural injections into mouse DRG in vivo35. Mice were deeply anaesthetized with sleep mix (Dormicum, Domitar and Fentanyl). The back hair was removed using a rodent trimmer and the eyes were covered with eye ointment (Bepanthen) to prevent them from drying out. Mice were fixed firmly in a stereotactic frame and placed on a heating plate to maintain the body temperature constant throughout the surgery. The iliac crest and last rib were identified through palpation of the dorsal surface of mice and the area between them was sterilized by wiping the skin with 70% (vol/vol) ethanol. L3 and L4 lumbar vertebral columns were identified from the last rib and iliac crest and a small incision directly above the iliac crest and on top of L3 and L4 level was made using a sterile scalpel. The lateral processes of the vertebral bones were exposed by moving the corresponding muscles right at the inter-vertebral junctions. A small portion at the edge of the lateral process right on the top of the DRG was removed in order to expose a small portion of the DRG and a small glass pipette (tip diameter: 25 µm) filled with viral solution containing 0.01% Fast Green was inserted in the middle of the DRG. The quality of injection was determined from a gradual increase in the coloration of the DRG and any leakage was detected by visualization. After the injection, the exposed DRG was covered by saline-soaked gelatin foam and muscles were moved back to their original positions.

Intrathecal delivery of drugs. To enable i.t. delivery of pharmacological agents at the level of lumbar spinal segments, mice were deeply anaesthetized with an intraperitoneal injection of 0.65 µl/g body weight of sleep mix (0.23 µg/μl Sedator (Eurovet International), 3.08 µg/μl Dormicum (Roche), 0.01 µg/μl Fentanyl-Janssen (Janssen-Cilag)) and a polyethylene catheter (Biomedical Instruments) was stereotactically inserted through an opening in the cisterna magna into the lumbar subarachnoid space at the L3–L4 segment. The tip of the catheter was located near the lumbar enlargement of the spinal cord. The volume of dead space of the i.t. catheter was 10 µl. Mice were allowed to recover for 3 d after surgery and animals showing any motor
abnormalities were excluded from further experiments. The correct placement of the catheter was verified at the end of the experiment by i.t injection of 5 μl 1% Evans blue and performing a laminectomy. Drugs and recombinant proteins were administered intrathecally in the indicated dosage using a microinjection syringe (Hamilton) in a volume of 5 μl separated from an 8-μl volume of saline through a 1-μl air bubble. For every experiment, mice were randomly allocated to experimental treatments (vehicle or test substance, for example, recombinant proteins or drugs) by a statistician independent from the experimenter.

Reverse-transcription PCR. Total RNA was extracted from mouse L3–L4 DRGs, lumbar L3–L4 spinal cord segments and ipsilateral paw skin isolated from mice at 8 d after SNI using the phenol/chloroform extraction method; similar methods were employed for extracting RNA from cultured DRG neurons and cultured embryonic spinal cord neurons. Total RNA from acutely isolated mouse dorsal spinal cord neurons and T-cells and from primary cell cultures of Schwann cells, astrocytes, microglia and macrophages were purchased from 3H Biomedical, Uppsala, Sweden. The RNA was reverse-transcribed using RevertAid M-MuLV Reverse Transcriptase (Fermentas), random hexamer primers (Roche and Invitrogen, respectively) according to standard protocols. PCR reactions were then performed on cDNA from the aforementioned cell types and tissues using the following primers:\(5\prime-\text{GGC CCT TGG CAG ACT ATC CAG C-3}\prime\),\(5\prime-\text{ACC TGC ACG TTG TGG TTA ATA G-3}\prime\),\(5\prime-\text{AGT TAT GGG TTC TTT GGT GTC TC-3}\prime\),\(5\prime-\text{GTT ATT TTT GGT CAC TAC CTC CC-3}\prime\). Paw tissue from WT mice 1 d after SNA served as a positive control, 18s RNA served as a housekeeping gene and reverse-transcription reactions performed without (–) cDNA served as a negative control.

Real-time quantitative PCR. qPCR reactions were performed on cDNA samples using the TaqMan Gene Expression Assay (Invitrogen) including predesigned primers for \textit{Serpina3n}, GAPDH and Cyclophilin A (Invitrogen). The reactions were performed using a LightCycler 96 Real Time PCR System (Roche) and the data analyzed using the corresponding software.

Gene expression analysis of \textit{Serpina3} isoforms was performed on DRG cDNA using the iQ SYBR Green Supermix system (Bio-Rad Laboratories). Primers were directed against the non-conserved 3′ region of the transcripts, which encodes the protease interaction domain that defines target specificity. To ensure primer specificity, 10 of the 11 proposed functional \textit{Serpina3} transcripts were cloned from mouse tissue and each primer pair was tested for its ability to amplify only the correct place of the transcripts, which encodes the protease interaction domain that defines target specificity. To ensure primer specificity, 10 of the 11 proposed functional \textit{Serpina3} transcripts were cloned from mouse tissue and each primer pair was tested for its ability to amplify only the correct place. The PCR reactions were performed using a LightCycler 96 Real Time PCR System (Roche) and the data analyzed using the corresponding software.

Antibodies. For western blot analysis, we used a goat polyclonal anti-\textit{Serpina3N} antiserum (1:500; R&D Systems, cat. no. AF4709, no references available) and a mouse monoclonal anti-\(\alpha\)-tubulin antibody (1:2,000; Sigma, cat. no. T9026)\(40\). For immunohistochemistry, we used a goat polyclonal anti-\textit{Serpina3N} antiserum (1:200; R&D Systems, cat. no. AF4709), a rabbit polyclonal anti-CGRP antiserum (1:2,000; Immunostar, cat. no. 24112)\(41\), a rabbit polyclonal anti-NF200 antiserum (1:500; Sigma, cat. no. N4142)\(42\), a rat monoclonal anti-CD3 (clone 17A2) antibody (1:100; BD Biosciences, cat. no. 555273)\(43\), a rabbit polyclonal anti-NF-200 antibody (1:500; Sigma, cat. no. N4142)\(42\), an anti-\(\beta\)-Tubulin Isotype III (clone SDL31D10) monoclonal antibody (1:800; Sigma, cat. no. T0576)\(44\), an anti-NeuN (clone A6) monoclonal antibody (1:200; Millipore, cat. no. MAB377)\(45\) and a donkey anti-rabbit-\(\gamma\)-Cy3 antiserum (1:300; Jackson ImmunoResearch, cat. no. 711-165-152)\(46\) For FACS analysis, the following antibodies were used: FITC Rat Anti-Mouse CD4 (Clone RM4-5) (1:100; BD Biosciences, cat. no. 553046)\(47\), Alexa Fluor 647 anti-mouse cDNA 80i (1:100; BioLegend, cat. no. 108416)\(48\) and Pacific Blue anti-mouse/human cDNA11b (1:100; BioLegend, cat. no. 101224). Tissue preparation. For immunohistochemistry, animals were perfused transcardially with PBS followed by 4% PFA. Spinal cord segments at lumbar level L3–L4 and the L3 and L4 DRGs were then removed. Paw skin punches were taken out using a biopsy punch. Tissues were post-fixed for up to 16 h in 4% PFA at 4 °C. Spinal cords, DRGs and paw skin punches were stored in 0.5% PFA at 4 °C for up to 2 weeks and incubated in 30% sucrose solution overnight at 4 °C for cryostat sectioning (25 μm, 16 μm and 20 μm, corresponding to the thickness per section, respectively).

DAB immunohistochemistry. The immunohistochemical staining procedure started with the incubation in 1% hydrogen peroxide (in PBS:methanol 1:1) followed by 3 times of 15 min washing with PBS and 30 min incubation in 7% normal horse serum in 0.02% PBS (blocking solution). Sections were incubated overnight with the desired primary antibody in blocking solution at 4 °C. The next day, sections were washed three times for 15 min with 0.02% PBS, followed by incubation with the secondary biotinylated antibody for 30 min (Vectastain Elite ABC Kit, Vector laboratories). After three 15-min washing steps with 0.02% PBS and incubation for 30 min in an avidin-biotin complex solution (Vectastain Elite ABC Kit), sections were washed two times for 15 min in 0.02% PBS and twice for 15 min in PBS before being stained with 3,3′-diaminobenzidine tetra hydrochloride (DAB) solution (DAB substrate kit for peroxidase, Vector laboratories, Burlingame, CA, USA). Staining reactions were terminated upon visual inspection by adding and washing with water. Sections were finally air-dried and mounted with Mowiol (Sigma). Bright-field colored images were captured with an automated Leica DM4000 B microscope coupled to a MF8 CX9000 camera (MBF Bioscience) and displayed with PictureFrame software (Leica).

In situ hybridization. For rat DRG, in situ hybridization was performed on 10-μm cryosections of the L4 dorsal root ganglia using digoxigenin-labeled antisense riboprobes, as previously described\(30,38\).

In situ hybridization was performed on mouse DRG sections as follows. DRGs L4–L5 were harvested from WT and \textit{Serpina3n}−/− mice 1 d after SNA, cryosectioned (10 μm) mounted on Superfrost Plus slides (VWR) and frozen at −80 °C until use. A digoxigenin-labeled anti-sense cRNA probe against the floxed exon of \textit{Serpina3n} was generated by a T7 (Roche) in vitro transcription reaction using a \textit{Serpina3n} cDNA (5′-ACTGGAGACACAGAGGATGGCCCTF; TCACCAGCACCATGAATGCTTTTTTT). In situ hybridization was performed as previously described\(39\). Following overnight hybridization, slides were incubated with alkaline phosphatase–conjugated anti-digoxigenin antibody (Roche, 1:200) for 1 h at RT. After several washes in PBST, slides were incubated in NBT/BCIP chromogenic substrate (Roche) according to manufacturer’s specifications for 5 h. Slides were coverslipped and bright-field images were captured on a Nikon 80i upright microscope.

Northern blotting. Blotting was performed according to the protocol described by Costigan et al.\(27\).

\textit{In situ} hybridization. For rat DRG, in situ hybridization was performed on 10-μm cryosections of the L4 dorsal root ganglia using digoxigenin-labeled antisense riboprobes, as previously described.\(27,30,38\).
glycine in PBS and two washings for 15 min in PBS. Afterward the slides were blocked for 1 h in 10% NHS in 0.5% PBST and incubated overnight at 4 °C with the appropriate primary antibody in 1% NHS in 1% PBST. The next day, sections were washed 3 times for 15 min in PBS. Alexa Fluor 488- or Alexa Fluor 594–conjugated secondary antibodies (Life Technologies) were diluted 1:1,000 in 1% NHS in 0.1% PBST and incubated on the sections for 1 h in the dark. Sections were then washed 3 times for 10 min with PBS and incubated for 10 min in 10 mM Tris-HCl pH 8.0 in the dark before mounting with Mowiol. For CD3 staining, we followed a previously described protocol13. For immunostaining of ATF3 on in situ hybridization sections for Serpina3n, at completion of in situ hybridization the slides were washed in H2O and blocked in blocking buffer (1% BSA, 0.1% Triton X-100) for 1 h. Slides were then incubated with rabbit-anti-ATF3 antibody overnight at 4 °C. Slides were afterward washed in PBST and incubated in anti-rabbit-Cy3 antiserum for 1 h at RT. After images were captured, the in situ hybridization signal was pseudocolored green and overlaid on ATF3 staining.

Fluorescence images were obtained using a laser-scaning spectral confocal microscope (Leica TCS SP2 AOB, Bensheim, Germany) and Leica Confocal Software (v2.61) or a Nikon A1 confocal laser microscope system and the corresponding software. For GFP and TRITC fluorescence, a sequential-scan mode was used to rule out bleed-through between channels.

Quantitative analysis of stained cells. For images derived from co-immunostaining between SerpinA3N and NF200, Iba1, C4GR or ATF3, the number of double-positive cells was determined using Fiji (ImageJ) software. The cell size of SerpinA3-like immunoreactive cells corresponded to Feret’s diameter, which is defined as the longest distance between any two points along the selection boundary. All images from the same experiment were processed similarly. Afterward, every positive cell within a tissue section was labeled with a number and those cells exhibiting positive immunoreactivity for two antibodies were considered double-positive. Quantification of Iba-1 immunoreactivity in spinal cord sections was done using Fiji software (Fiji) and corresponded to the average cell number of 3 representative ROIs (regions of interest) of 100–2 microns from each section. For these images, a deconvolution step was included to reduce background noise. The number of CD3-positive cells within a tissue section was estimated by counting them directly from the microscope. The final value corresponded to the average number of CD3-positive cells within every section from the same experimental group.

Western blotting. DRGs and spinal cord segments at the lumbar level L3–L4 were mechanically homogenized and lysed in ice cold RIPA buffer with complete, Mini, EDTA-free Protease inhibitor tablets (Roche) for 1 h at 4 °C. Lysates were then centrifuged for 20 min at 13,000 r.p.m. at 4 °C to remove insoluble material. Protein concentration was then determined using the BCA method (BCA Protein Assay Kit, Thermo Scientific). The samples were separated on 10% SDS-polyacrylamide gels and transferred to a nitrocellulose membrane. The membranes were blocked in 5% milk in TBST, incubated overnight with the primary antibody in the same solution and washed 3 times for 15 min in TBST. The membranes were incubated with the secondary antibody for 1 h and washed again as before. For the α-tubulin loading control, the membranes were incubated 30 min with stripping buffer (200 mM glycine, 0.1% SDS, 1% Tween-20, pH 2.2) at RT, blocked, and incubated with the primary and secondary antibodies as described above. Detection of immunoreactive bands was performed using the enhanced chemiluminescence detection system (ECL, GE Healthcare). The intensity of immunoreactive bands was quantified using ImageJ software (http://rsbweb.nih.gov/ij/).

Culture of DRG neurons. 5–8 weeks old female and male C57BL/6 mice were anesthetized with CO2 and killed by decapitation. DRGs were quickly collected in cold PBS and transferred to F12 medium without serum containing 1% penicillin/streptomycin. After removing axons and meningeal tissue, DRGs from each animal (30–40) were digested for 30 min at 37 °C in 1 ml of an enzymatic solution (0.25 mg/ml trypsin (6000BAEE units/mg, Sigma), 1 mg/ml collagenase (300 units/mg, Sigma) and 0.2 mg/ml DNase I (2,580 Kunitz units/mg solid, Sigma) in F12 medium without serum) using a thermomixer (max. velocity 14,000 r.p.m.). After mechanical dissociation with a glass pipette pre-coated with Sigmacoat solution (Sigma), the digestion was stopped by adding 120μl of 10× Trypsin inhibitor (2.5 mg/ml, 10,000 BAEE units/mg protein, Sigma) and 100 μl FBS. Cells were mixed well and centrifuged at 1,000 r.p.m. for 3 min. The supernatant was removed and the cells were carefully resuspended in 2 ml of F12 medium containing 10% FBS. The cell suspension (2 ml, max. 2 mice) was carefully loaded in a tube containing a discontinuous Percoll gradient (1.5 ml 35% Percoll (Percoll; 90% Percoll (Amersham) + 10% 10X) in F12 medium plus 2.5 ml 25% Percoll in DMEM medium) to allow separation of neurons from non-neuronal cells. Following centrifugation at 1,000g for 15 min, the upper part of the supernatant was removed and the lower 1–1.5 ml portion was left intact. The pellet was resuspended and mixed with F12 medium up to 13.5 ml and an additional 1.5 ml 10% FBS was added and mixed with the solution. After 10 min centrifugation at 500g, the supernatant was removed and cells were resuspended in 6 ml of F12 medium plus 10% FBS. The cell suspension was mixed carefully and centrifuged 7-8 min at 500g. The cells were resuspended in 400 μl of F12 medium plus 10% FBS and plated on a Petri dish (35 mm diameter). After a short time, 1.6 ml medium was added to the cells and they were incubated overnight at 37 °C with 5% CO2. The next day the medium was changed with F12 medium plus 10% FBS containing a mixture of growth factors (BDNF (10 ng/ml), NGF (10 ng/ml), GDNF (5 ng/ml), NT3 (5 ng/ml), Sigma) and the mitotic inhibitor AraC (5 μM, Sigma). Cells were incubated at 37 °C for 4–7 d until treatment.

To study release of SerpinA3N from cultured DRG neurons, all DRGs from one 5-week-old male WT mouse were collected, split into three wells of 12-well plates and resuspended in 1 ml of F12 medium plus 10% FBS per well. 4 d later, 750 μl of medium was removed from each well and 500 μl of serum-depleted medium containing KCl or vehicle alone was added into each well and incubated at the desired concentrations and incubation times. Cell supernatants were analyzed by western blotting. For quantification, intensity values of bands corresponding to SerpinA3N in the supernatants were normalized to tubulin expression in the corresponding cell lysates to ensure equal quantities of cellular input.

Embryonic spinal cord cultures. E16 embryos were removed and placed in a 10-cm Petri dish containing 0.33 M glucose/PBS. After removing of the dura, spinal cords were collected in 0.33 M glucose/PBS and cut in small pieces. The dissociated tissue (two or three spinal cords) was transferred to 1 ml of enzyme solution (the same used for DRG cultures) and digested for 15 min at 37 °C. The digestion was stopped with Trypsin inhibitor (the same used for DRG cultures), mixed gently and centrifuged for 10 min at 1,000 r.p.m. The supernatant was removed and the cells resuspended in 0.5–1 ml complete medium (Neurobasal medium A, 2 mM Glutamax, 1× B27 supplement and 1× penicillin/streptomycin). Cells were counted using a Neubauer chamber, diluted to 60,000 cells per ml and plated on a 12-well plate (1 ml cells per well). The day after, half of the medium was changed with new medium containing 5-Fluorouracil and Uridine (15 μg/ml and 35 μg/ml respectively, Sigma). Cells were kept in culture at least 3 weeks, changing half of the medium every 3–4 d.

Adoptive transfer experiments. For purification of splenic T cells, MACS cell sorting was performed using Dynabeads (untouched mouse T cells, Invitrogen) and neutrophils were depleted using biotinylated rat anti-Gr-1 antibody (clone RB6-8C5) as described in details previously54. Mice were injected i.v. with 200 μl of purified T cells in sterile PBS at a concentration of 1 × 107/200 μl, 6 d before SN1. Surface staining was performed via flow cytometry according to standard procedures. Data acquisition was carried out with an eight-color flow cytometer (Canto II, BD Biosciences) and analyzed with FlowJo software (Treestar).

Fluorometric measurements of protease activity. The proteolytic activity of the desired proteases was measured through the fluorescence released after protease-induced cleavage of specific fluorogenic substrates. With the exception of the dose-response assay of Sivelestat effect over rLE activity (which was performed in an Infinite 200 PRO multimode reader from Tecan), all fluorescence measurements were performed using a Fluoroskan Ascent (Thermo Electron LED) and expressed as AU (arbitrary fluorescence units). For all experiments, the following parameters were used: kinetic-mode, 20 ms integration time, 1 s lag time, 30 s interval time. Ascent Software 2.4.2 (Thermo
region of interest. About 8 slices were analyzed per DRG and FRET intensity accumulated unspecifically along the outer rim of all sections, this region which the activity was quantified – these were uniformly applied across all ground subtraction, exclusion of saturated pixels, smoothing with a median processed with ImageJ 1.38r software (http://rsb.info.nih.gov/ij/) using back-

The activity of mouse recombinant LE (R&D Systems) was measured using the fluorogenic substrate MeOsucaAAPV-AMC (Bachem) as described by the manufacturer. Pre-mixes containing a fixed amount of rLE and increasing concentra-
tions of rSerpinA3N or Sivelestat (Sigma) were prepared in assay buffer (50 mM Tris, 1 M NaCl, 0.05% (wt/vol) Brij-35, pH 7.5). The solutions containing the proteins were mixed with equal volumes of substrate in assay buffer reaching a final substrate concentration of 100 µM. The samples were placed in the multi-well plate and the reading was measured immediately with the fluorescence reader at excitation and emission wavelengths of 380 nm and 460 nm (respectively) for 30 min.

The activity of purified active mouse alpha thrombin (Cell Systems Biotechnology) was measured by its ability to cleave the fluorogenic peptide substrate benzoyl-FVR-AMC (Merck). Pre-mixes containing a fixed amount of thrombin and increasing concentrations of mouse rSerpinA3N (R&D Systems) in assay buffer (30 mM Tris-HCl, 300 mM NaCl, pH 8.2) were prepared in different tubes and incubated at RT for 10 min. The solutions containing the proteins were mixed with equal volumes of substrate in assay buffer reaching a final substrate concentration of 50 µM. The samples were placed in the multi-well plate and the reading was measured immediately with the fluorescence reader at excitation and emission wavelengths of 380 nm and 460 nm (respectively) for 20 min.

The activity of MMP-9 was measured using the FRET substrate Mca-PLGL-Dap(Dnp)-AR-NH2 (MobiTec). To test SerpinA3N effect on MMP-9 activity, mouse recombinant pro-MMP-9 (10 ng/µl, MobiTec) was first activated in 1 mM APMA, followed by incubation at 37 °C for 2 h. Pre-mixes containing a fixed amount of active MMP9 and increasing concentrations of rSerpinA3N were prepared in pre-warmed assay buffer (50 mM Tris, 10 mM CaCl2, 150 mM NaCl, 0.05% Brij-35, pH 7.5) and incubated at 37 °C for 10 min. For the HLE-induced MMP-9 activity assay, samples containing a fixed amount of HLE and increasing concentrations of SerpinA3N were prepared in assay buffer. For MMP9 activation, every sample was mixed with pre-MMP-9 (10 ng/µl) in pre-warmed assay buffer and incubated for 2 h at 37 °C. For both assays, the solutions containing the proteins were mixed with equal volumes of substrate in assay buffer reaching a final substrate concentration of 10 µM. The final mixtures were added to the plate and the reading was performed at excitation and emission wavelengths of 320 nm and 400 nm (respectively) for 20 min.

**Measurement of LE activity in ex vivo DRG explants.** The activity of LE in DRG was measured using the LE ratiometric probe NEmo-2, a ratiometric FRET-based monitoring LE reporter that detects the activity of membrane-associated LE, but not soluble LE35. Briefly, L4 DRGs from operated mice were isolated and incubated immediately with 50 µM NEmo-2 dissolved in F12 medium for 60 min at 37 °C protected from light. DRGs were briefly washed with PBS, fixed for 48 h in 4% PFA and postfixed for 16 h in 0.5% PFA followed by overnight incubation in 30% sucrose for cryosectioning. DRGs were cut in 40-µm sections and images from every section were taken using a Leica SP8 confocal microscope (Leica Microsystems, Wetzlar, Germany) equipped with a 20x 1.4 dry objective with the following settings: The donor coumarin 343 of NEmo-2 was excited with a 405-nm diode laser and emission wavelengths of 380 nm and 460 nm (respectively) for 20 min. The donor/acceptor LE, but not soluble LE55. Briefly, L4 DRGs from operated mice were isolated and incubated immediately with 50 µM NEmo-2 dissolved in F12 medium for 60 min at 37 °C protected from light. DRGs were briefly washed with PBS, fixed for 48 h in 4% PFA and postfixed for 16 h in 0.5% PFA followed by overnight incubation in 30% sucrose for cryosectioning. DRGs were cut in 40-µm sections and images from every section were taken using a Leica SP8 confocal microscope (Leica Microsystems, Wetzlar, Germany) equipped with a 20x 1.4 dry objective with the following settings: The donor coumarin 343 of NEmo-2 was excited with a 405-nm diode laser and emission wavelengths of 380 nm and 460 nm (respectively) for 20 min...

**Nociceptive tests.** All animal procedures were approved by the local governing bodies (Regierungspräsidium Karlsruhe, Germany, local ethics committees at Harvard University, USA and University of Jerusalem, Israel). For all experiments involving behavior, operations and delivery of drugs, 8–10 weeks old female and male (equal numbers) mice of the C57BL/6 strain were used. All experimenters were blinded to the identity of the mice from which the DRGs were derived for measurement of LE activity.

**Application of mechanical force using von Frey filaments (static punctate mechanical stimuli).** Before testing, mice were habituated in a small plastic (7.5 × 7.5 × 15 cm) cage for 1 h. Mechanical sensitivity was determined with a graded series of nine von Frey filaments that produced a bending force of 0.02, 0.04, 0.07, 0.16, 0.4, 0.6, 1, 1.4 or 2 g. The stimuli were applied within the sural nerve territory (lateral part of the hind paw). Each filament was tested 10 times in increasing order starting with the filament producing the lowest force. Von Frey filaments were applied at least 3 s after the mice had returned to their initial resting state. For baseline mechanical sensitivity test all filaments were applied and the number of withdrawals was recorded. For tactile allodynia: the minimal force filament for which animals presented either a brisk paw withdrawal and/or an escape attempt in response to at least five of the ten stimulations determined the mechanical response threshold. Mechanical thresholds were defined as the minimum pressure required for eliciting 60% or 40% of withdrawal responses out of five stimulations and measured in grams (force application). To represent responsiveness to all filament strengths, stimulus intensity (force)–response frequency curves were constructed per time point tested and the integral (area under the curve) thereof was represented at the point time. Responses to plantar pinprick stimulation and plantar acetone were recorded as duration of the time spent by the animals licking, flinching or biting their paws.

**Contact heat pain (hot plate test).** Mice were placed on a metallic plate heated to a set temperature (30, 49.52 or 55 °C) within an acrylic container (Bioseb, France), and the latency for flinching, licking one of the hind paws, or jumping was measured. Mice were sequentially tested for 30, 52, 55 and 49 °C. One temperature was tested per day.

**Radiant heat pain (Hargreaves test).** Before testing, mice were placed on an elevated glass surface and habituated in their individual cages for 30 min. Then a radiant heat source (beam intensity set to cause baseline latencies in C57BL/6J mice ~1 s) targeted at an individual paw and the latency to paw withdrawal measured. The radiant heat apparatus used was from Ugo Basile (Italy).

**Brush test (dynamic mechanical stimuli).** Mice were habituated in a small plastic (7.5 × 7.5 × 15 cm) cage for 1 h and then three successive gentle touch stimuli were applied with a paint brush of round head diameter of 2 mm (Princeton Brush Co.) onto the sural territory of the paw. Each stimulation was applied from the middle of the foot to its distal part and lasted approximately 1 s. The total time spent flinching or licking the paw was measured with a stopwatch.

**Statistics.** For all measurements, data was calculated and presented as mean ± s.e.m. Sample size was chosen to ensure adequate power to detect effect sizes using the G-POWER program. We used a two-tailed unpaired t-test, one-way ANOVA followed by post-hoc Tukey's test, or two-way ANOVA for random measures or repeated measures followed by Tukey's post-hoc test to determine statistical significance. P ≤ 0.05 was considered significant. For all statistical analyses, the appropriate statistical tests were chosen, the data met the assumptions of the test and the variance between the statistically compared groups was similar. SigmaPlot (Systat Software Inc.) or ORIGIN (OriginLab) were used for statistical analyses.

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