Supplementary online materials for the manuscript:
The serine protease inhibitor SerpinA3N inhibits T cell-derived leukocyte elastase and modulates neuropathic pain

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Supplementary online notes:

Note 1: A newly described commercial antibody, which recognizes a recombinant version of mouse SerpinA3N (rSerpinA3N), displaying a band of ~56 kDa, detected 2 bands of ~55 kDa and ~58 kDa in DRG lysates (**Fig. 2b**) and an additional band of ~56 kDa in spinal cord lysates (**Supplementary Fig. 4c**). SerpinA3N corresponds to the ~55 kDa band, since the endogenous protein lacks the 6-residue C-terminal polyhistidine-tag present in rSerpinA3N, which accounts for the ~1 kDa difference in MW observed between them (**Fig. 2b; Supplementary Fig. 4c**). That this band corresponds to endogenous SerpinA3N was confirmed upon its specific loss in *Serpina3n*$^{-/-}$ mice (**Fig. 3b**). In experiments performed to test the hypothesis that SerpinA3N is released from DRG neurons, the SerpinA3N immunoreactive band from the culture medium exhibited a slightly reduced MW of ~53 kDa compared to the ~55 kDa band from the cell lysates (**Fig. 2f**, left panel), which would correspond to the cleaved secreted protein lacking the predicted signal peptide.

The expression levels of SerpinA3N protein were much higher in the DRG than the spinal cord, as revealed by comparing the quantity of protein loaded and the corresponding loading controls in **Supplementary Fig. 4c.** This is also evident upon comparing **Fig. 2b** with **Fig. 2g**, in which the same quantity of recombinant protein

was loaded as a positive control, but different exposure times were employed during developing the gels in order to be able to visualize the bands under the saturation level.

The 58 kDa band and other bands recognized by the antibody in all likelihood represent additional members of the *Serpina3* cluster (**Supplementary Fig. 2**). Because immunohistochemistry, unlike Western blotting, does not enable demarcating SerpinA3N immunoreactivity from other SerpinA3 isoforms in the cluster, which are potentially recognized by the antibody, we represent positive immunohistochemical staining as 'SerpinA3-like immunoreactivity'. Moreover, unlike the loss of the band corresponding to about 55 kDa in *Serpina3n*$^{-/-}$ mice in Western blot analysis, immunoreactivity is preserved because the design of the KO strategy is such that a small, truncated transcript is maintained in the KO mice which retains the epitope for the antibody.

Note 2: We analyzed the impact of SerpinA3N on neuroinflammation and glial reaction in the spinal cord and DRG by analyzing immune cell infiltration, upregulation of glial markers (astrocytes, microglia and satellite cells) and morphological changes in glial cells as described previously [1]. Experiments were performed in the SNI model and mice received daily single intrathecal injections of 10 pmol rSerpinA3N (a dose which attenuates allodynia) or BSA as a control injected throughout the period post-SNI until diverse immunohistochemical analyses of DRG and spinal cord tissue were performed.

To address the effect of i.t. rSerpinA3N administration on nerve injury-induced glial reaction, we first evaluated the expression of the monocyte- and microglial-marker, Iba-1, which enables visualizing microglial proliferation in the spinal cord following nerve injury, as described previously [2]. At day 7 post-SNI, a time point when Iba-1

expression increases markedly in ipsilateral spinal cord, we did not observe any significant difference in Iba-1 expression between rSerpiNA3N- and BSA-injected mice (**Supplementary Fig. 8a**). In contrast, minocycline, employed as a positive control, blocked the microglial reaction. We also evaluated the expression of GFAP, which labels astrocytes in the spinal cord and satellite glial cells in the DRG. At day 2 and at day 7 post-SNI revealed an increase in GFAP immunoreactivity in the spinal dorsal horn in SNI-treated mice as compared to sham-treated mice, which, however, occurred to a comparable magnitude across rSerpinA3N- treated and BSA-treated mice (examples of images are shown in **Supplementary Fig. 8b**). To study the glial reaction in the DRG, we performed costainings of GFAP and the neuronal marker ß-tubulin III at day 7 post-SNI, where we observed increased GFAP immunoreactivity in satellite cells surrounding ß-tubulin III-positive DRG neurons (**Supplementary Fig. 8c**), as reported previously [3]. However, we did not observe a clear difference in this reactivity of satellite cells between rSerpinA3N-treated and BSA-treated mice (**Supplementary Fig. 8c**).

Neuroinflammation in the DRG was studied by analyzing nerve injury-induced infiltration of macrophages, T-cells and neutrophils in the DRG, using antibodies against Iba-1 (**Supplementary Fig. 8d**), CD3 (**Supplementary Fig. 8e**) and Gr-1 (**Supplementary Fig. 8f**) respectively. Although clear changes were observed post-SNI, comparison between mice intrathecally injected with rSerpinA3N or BSA did not reveal any impact of rSerpinA3N on neuroinflammation in the DRG at day 7 post-SNI (**Supplementary Figs. 8d,e,f**).

These data thus indicate that the anti-allodynia effects of SerpinA3N are unlikely to be linked to glial reactions post-injury. Moreover, these data show that SerpinA3N does not actually prevent immune cells from infiltrating the DRG, but rather unfold its actions a step further down in that it acts on LE derived from T-cells (and potentially

neutrophils) in the DRG, as indicated by the other data presented in this study.

Supplementary references:

1. Ji, R.R., Berta, T. & Nedergaard, M. Glia and pain: is chronic pain a gliopathy? *Pain* 154 Suppl 1, S10-28 (2013).

2. Suter, M.R., Wen, Y.R., Decosterd, I. & Ji, R.R. Do glial cells control pain? *Neuron glia biology* **3**, 255-268 (2007).

3. Berta, T., Liu, T., Liu, Y.C., Xu, Z.Z. & Ji, R.R. Acute morphine activates satellite glial cells and up-regulates IL-1beta in dorsal root ganglia in mice via matrix metalloprotease-9. *Molecular pain* **8**, 18 (2012).

Supplementary table legends

1. **Supplementary Table 1**: Complete set of data obtained by genome-wide screening of genes potentially differentially regulated in High pain versus Low pain strains of rats following nerve injury. Excel file contains two sheets: The first is the raw data un-analyzed; the second gives average fold differences between Sham and 3 day SNL for each strain (HP and LP). Probe set expression values determined using Affymetrix methods. Please see supplementary methods for further details.

2. **Supplementary Table 2**: Results of the primary screen given in two expression formats (RMA and Affymetrix). Eight outlier transcripts were identified by graphical analysis in a plot of chi statistic versus interaction effect magnitude shown in **Figure 1b**. Primary data for those primary screen positive transcripts given in first sheet of this Excel file (RMA). Primary expression data for these genes given in second sheet

(Affymetrix). Please see supplementary methods for further details of the primary screen.

Supplementary figure legends:

Supplementary Figure 1. Northern slot blot mRNA expression analysis of the genes identified in the primary array-based screen as potentially differentially regulated between High pain and Low pain strain DRGs, before and after nerve injury. For each gene, left panels represents differential transcript regulation data in DRG between rats with high and low neuropathic pain, sham and 3 days post-SNL, as revealed by Northern slot blotting (n = 3). Right panels correspond to the comparison of the quantification of transcript regulation determined by Northern blot (for the secondary screen) vs. the Affymetrix expression array data for those transcripts (from the primary screen). Each transcript was normalized to its respective *Cyclophilin A* transcript, which was used as house-keeping gene. In cases where the Northern data obtained a significant interaction effect the P-value is given, n = 3, *P < 0.05 as compared to sham, post hoc. † P < 0.05 between High pain and Low pain groups post-SNI, post hoc. Two-way ANOVA followed by Tukey's post-hoc test. Error bars: standard error of the mean.

Supplementary Figure 2. Real-Time quantitative PCR (qPCR) analysis of changes in expression of *Serpina3n* and several other members of the *Serpina3* cluster following peripheral nerve injury using GAPDH as housekeeping (reference) gene. (**a**) qPCR analysis of changes in *Serpina3n* mRNA expression in ipsilateral thoracic DRGs at various time points post-SNI. n = 4, *P < 0.05 as compared to sham, 1-way ANOVA followed by Tukey's post-hoc test. (**b**) qPCR analysis showing relative mRNA expression levels of *Serpina3n*, *-a3g*, *-a3h*, *-a3k* and *–a3f* in L3–L5 DRGs

from naïve and 1 day post-SNI mice (n = 4; *P < 0.05 as compared to naïve mice; Two-tailed unpaired T-test). (**c**) qPCR analysis showing time-course of changes in expression of *Serpina3n* and other members of the *Serpina3* cluster in ipsilateral L3–L5 DRGs post-SNI using GAPDH as a house-keeping, reference gene (n = 4 mice per time point; *P < 0.05 compared to naive DRG; 1-way ANOVA followed by Tukey's post-hoc test). Error bars: standard error of the mean.

Supplementary Figure 3. Immunohistochemical characterization of SerpinA3-like immunoreactive cells following nerve injury. (**a**) Cell diameter-frequency histogram of SerpinA3-like immunoreactive cells and non SerpinA3-like cells in L4 DRG neurons 3 days after sham surgery. Approximately 800 cells were analyzed. (**b**) Percentage of SerpinA3-like immunoreactive cells from total number of cells in L4 DRGs 3 days after SNI compared to sham treatment (n = 3 independent experiments with pairs of SNI and sham mice; *P < 0.05 as compared to sham; Two-tailed unpaired T-test). (**c**) Cell diameter-frequency histogram of SerpinA3-like immunoreactive cells and non SerpinA3-like cells in L4 DRG neurons 3 days after SNI or sham surgery. Approximately 700-800 cells were analyzed in each condition. (**d**) Typical examples and quantitative analysis of experiments on *in situ* mRNA hybridization (ISH) with antisense riboprobes specific for *Serpina3n* followed by immunohistochemical staining (IHC) using an anti-ATF3 antibody performed on L4 DRG sections on day 1 post-SNI. White arrows represent *Serpina3n*-positive/ATF3-positive neurons, white arrowheads represent *Serpina3n*-positive/ATF3-negative neurons and yellow arrowheads represent *Serpina3n*-negative/ATF3-positive neurons. Scale bar represents 50 µm**.** (**e**) Quantitative analyses on L4 DRG neurons on day 3 post-SNI

immunostained for ATF3 expression and SerpinA3-like immunoreactivity. Error bars: standard error of the mean.

Supplementary Figure 4. (**a**) Western blot showing that secretion of SerpinA3N into the culture medium of cultured DRG neurons does not vary upon 20 min incubation with 50 mM KCl or vehicle. Panel on the right shows quantitative estimation of SerpinA3N expression in supernatants normalized to tubulin expression in cellular lysates (input) from 3 independent culture experiments. (**b**) Comparison of *Serpina3n* expression between the DRG and spinal cord in naïve mice and 1 day post-SNI, as revealed by qPCR using the gene encoding GAPDH as an internal reference (n = 4; *P < 0.05 as compared to naive; † P < 0.05 as compared to DRG, Two-tailed unpaired T-test). (**c**) Immunoblot using an antibody against SerpinA3N in L3–L4 DRG and L3–L4 spinal cord lysates revealed abundant expression of SerpinA3N in DRG but low expression levels in spinal cord. SerpinA3N corresponds to the 55 kDa band. Mouse recombinant SerpinA3N (rSerpinA3N) was used as positive control and a-tubulin as loading control. Error bars: standard error of the mean.

Supplementary Figure 5. Generation of mice lacking *Serpina3n*. (**a**) Schematic representation of the strategy used for the generation of *Serpina3n*$^{-/-}$ mice (for details see Methods section). (**b**) Verification of deletion of targeted regions of *Serpina3* gene via *PCR* performed on genomic DNA and cDNA derived from hypothalamus.

Supplementary Figure 6. Comparison of nociception and neuropathic pain-related behavior in *Serpina3n*$^{-/-}$ mice and WT littermates. (**a**) Under basal conditions,

Serpina3n$^{-/-}$ mice show comparable paw withdrawal latency to radiant heat at 3 different temperatures (Hot plate test) in comparison with WT animals. (**b**) *Serpina3n*$^{-/-}$ mice show no significant differences in the frequency of paw withdrawal to plantar stimulation with von Frey filaments of graded forces in comparison with WT littermates under basal conditions. (**c, d**) Time spent by the mice performing nocifensive behaviors (licking, flinching or biting their paws) did not differ between *Serpina3n*$^{-/-}$ and WT mice following plantar application of acetone (**c**) or pinprick (**d**) under basal conditions (n = 6-8 mice per group; *P > 0.05 as compared to WT controls; repeated measures 2-way ANOVA followed by Tukey's post-hoc test or Two-tailed unpaired T-test). (**e–i**) Comparison of SNI-induced changes in nocifensive behavioral responses to plantar stimulation with acetone (**e**) and pinprick (**f**), withdrawal latency to a radiant heat ramp (Hargreaves test, **g**), upon contact with a hot metal plate at 52°C (Hot plate test, **h**) or in response to a brush stimulus (**i**) between *Serpina3n*$^{-/-}$ and WT mice following SNI (n = 6–16 animals per group; *P < 0.05 as compared to basal values, † P < 0.05 as compared to control littermates at the same time point; 2-way ANOVA of repeated measures followed by Tukey's post-hoc test). (**j**) Analysis of mechanical hypersensitivity to plantar application of graded von Frey hair in WT mice receiving a single i.t. injection of 10 pmol rSerpinA3N or BSA at 18 days post-SNI (n = 9 mice per group; P > 0.05 as compared to BSA controls at the same time point; 2-way ANOVA of repeated measures followed by Tukey's post-hoc test). Error bars: standard error of the mean.

Supplementary Figure 7. Assessment of the integrity of the spinal cord and its main cell populations after i.t. injections of rSerpinA3N via immunohistochemical analysis of expression of NeuN, Iba1 and GFAP in lumbar spinal cord segments L3–L4

isolated 24 h after i.t. injections of 30 pmol rSerpinA3N or BSA to mice. Scale bars represent 100 mm. Error bars: standard error of the mean.

Supplementary Figure 8. Effects of intrathecal administration of rSerpinA3N on glial reaction and neuroinflammation in the DRG and spinal cord during peripheral neuropathy. Images are derived from mice 7 days post-SNI (except in panel (**b**): mice were treated at day 2 post-SNI) that received single i.t. injections of rSerpinA3N or BSA (10 pmol) every day (starting on the day of the operations) compared to sham mice receiving similar i.t. injections of BSA. Mice receiving i.p. injections of minocycline (twice every day, 30 mg/kg) were used as controls for microglial activation. (**a**) Typical examples (left panel) and quantification (right panel) of immunolabeling of microglia with Anti-Iba-1 in spinal cord from mice treated as described above. Immunolabelled cells from 3 representative regions of interest (ROIs), 100 sq. microns each, were counted for each section. (**b**) Typical examples of Anti-GFAP-immunolabeled astrocytes from the same set of mice as described under (**a**). (**c**) Analysis of glial reaction satellite cells in the DRG post-injury by costaining of Anti-GFAP with a neuronal marker protein, ß-tubulin III. (**d–f**) Immunohistochemical characterization of immune cell infiltration into L3–L4 ipsilateral DRG using antibodies against Iba-1 (macrophages) (**d**), CD3 (T-cells) (**e**) and Gr-1 (neutrophils, represented by arrows) (**f**) in the above-described mice. In all panels, *n* = 3–6 mice per treatment group; **P* < 0.05 as compared to sham-treated mice receiving vehicle with BSA: two-tailed unpaired T test. Scale bars represent 100 µm. Error bars: standard error of the mean.

Supplementary Figure 9. Analysis of effects of SerpinA3N on the catalytic activity of 2 serine proteases and of Sivelestat on LE activity *in vitro*. (**a**) Fluorometric activity of

purified mouse thrombin (1.1 nM) preincubated either alone, with increasing concentrations of rSerpinA3N or with BSA (equivalent to the higher dose) as a control. (**b**) Fluorometric activity of purified mouse recombinant pro-MMP-9 (1 nM), which was chemically activated with APMA (1 mM) and then incubated with increasing concentrations of rSerpinA3N. Inactive pro-MMP-9 was used as an additional negative control for MMP-9 activity. (**c**) Dose-dependent inhibitory effect of Sivelestat on rLE activity. rLE (1 nM) was preincubated with increasing concentrations of sivelestat and then its flurometric activity was measured over time. AFU: arbitrary fluorescence units. Panels at the right show the quantification of enzyme activity (**a,b**) or the percentage of rLE activity compared to 1 nM rLE at indicated time points (**c**). $n = 3$; $^*P < 0.05$ as compared to activity of enzyme alone at the corresponding time point; 2-way ANOVA of repeated measures followed by Tukey's post-hoc test). Error bars: standard error of the mean.

Supplementary Figure 10. (**a–e**) Immunohistochemical characterization of infiltration of T-cells into the DRG after SNI using an anti-CD3 antibody in naïve mice (**a**), at day 1 (**b**), day 3 (**c**) and day 8 post-SNI (**d**) and in paw skin punches isolated 8 days post-SNI (**e**). DAPI (blue) was used as counterstain for cell nuclei. (**f**) Quantification of CD3-positive T-cells in L3–L4 DRGs of naïve and 1, 3 and 8 days post-SNI mice compared to sham controls ($^*P < 0.05$ as compared to sham control; $n = 3$-6 mice; 2-way ANOVA followed by Tukey's post-hoc test). (**g**) Immunohistochemical characterization of neutrophil infiltration into L4 DRG using an anti-Gr-1 antibody, at day 3 and day 8 after SNI or sham treatment. Arrows indicate positive immunoreactivity for Gr-1. Paw skin punches isolated 8 days after SNI operations were used as positive control for stainings. Scale bars represent 100 mm. Error bars: standard error of the mean.

Supplementary Figure 11. T cell viability in vivo at 13 days post-transfer. T cells were isolated from spleens of LE deficient mice and littermate controls. $1x10^7$ T cells from the respective donors were transferred i.v. into *Rag2*$^{-/-}$ mice one week prior to nerve injury. Frequency of circulating $CD3^+$ cells was determined via flow cytometry on blood derived from mice 13 days after transfer (i.e. 1 week post-SNI). (**a**) FACS analysis of T cells from individual mice of the respective groups. (**b**) The efficiency of T-cell transfer and T cell viability from $LE^{-/-}$ and WT mice was comparable ($p = 0.0908$, $n = 4$, two tailed unpaired T-test). Error bars: standard error of the mean.

Supplementary Figure 12. Graphic summary of the salient steps and findings in the study pertaining to identification of SerpinA3N and LE as novel players in neuropathic pain (**a**) and the temporal course of events involving endogenously expressed SerpinA3N and LE as well as therapeutic windows for exogenously administered SerpinA3N and LE inhibitors in neuropathic pain (**b**).