Inhibition of spinal microglial cathepsin S for the reversal of neuropathic pain

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Edited by David Julius, University of California, San Francisco, CA, and approved April 25, 2007 (received for review December 6, 2006)

A recent major conceptual advance has been the recognition of the importance of immune system–neuronal interactions in the modulation of brain function, one example of which is spinal pain processing in neuropathic states. Here, we report that in peripheral nerve-injured rats, the lysosomal cysteine protease cathepsin S (CatS) is critical for the maintenance of neuropathic pain and spinal microglia activation. After injury, CatS was exclusively expressed by activated microglia in the ipsilateral dorsal horn, where expression peaked at day 7, remaining high on day 14. Intrathecal delivery of an irreversible CatS inhibitor, morpholinurea-leucine-homophenylalanine-vinyl phenyl sulfone (LHVS), was antihyperalgesic and antiallodynic in neuropathic rats and attenuated spinal microglia activation. Consistent with a pronociceptive role of endogenous CatS, spinal intrathecal delivery of rat recombinant CatS (rrCatS) induced hyperalgesia and allodynia in naïve rats and activated p38 mitogen-activated protein kinase (MAPK) in spinal cord microglia. A bioinformatics approach revealed that the transmembrane chemokine fractalkine (FKN) is a potential substrate for CatS cleavage. We show that rrCatS incubation reduced the levels of cell-associated FKN in cultured sensory neurons and that a neutralizing antibody against FKN prevented both FKN- and CatS-induced allodynia, hyperalgesia, and p38 MAPK activation. Furthermore, rrCatS induced allodynia in wild-type but not CX3CR1-knockout mice. We suggest that under conditions of increased nociception, microglial CatS is responsible for the liberation of neuronal FKN, which stimulates p38 MAPK phosphorylation in microglia, thereby activating neurons via the release of pronociceptive mediators.

chemokines | microglia | proteases | allodynia | fractalkine

Neuropathic pain is a significant clinical problem for which current treatments are inadequate (1, 2). This is due in large part to the fact that the mechanisms underlying neuropathic allodynia and hyperalgesia are insufficiently understood. Growing preclinical evidence suggests that immune cells contribute to neuropathic pain after peripheral nerve trauma. Immune cells intervene peripherally at the site of injury and in the dorsal root ganglia (DRG) as well as centrally, in the dorsal horn of the spinal cord (3, 4). However, our understanding of immune–neuronal nociceptive processing that leads to persistent pain is still limited.

We have recently found that after peripheral nerve injury, the mRNA for the lysosomal cysteine protease cathepsin S (CatS) was up-regulated in the DRG because of CatS expression in infiltrating macrophages (5). Using pharmacological inhibition, we have shown that CatS expressed and released by macrophages contributed to the maintenance of neuropathic hyperalgesia, whereas allodynia remained unaffected (5). In the brain, the mRNA for CatS has been found to be expressed in microglia, where the enzyme is essential for antigen presentation and turnover of intracellular and extracellular proteins in tissue remodeling (6, 7). Microglial cells are receiving growing interest as modulators of neuronal plasticity in the spinal cord under conditions of persistent pain by producing surface proteins and secreting signaling molecules (8).

Here, we identified CatS protein expression in spinal cord microglia and then tested for the involvement of centrally located CatS in hypersensitivity associated with the partial ligation of the sciatic nerve (PNL) model of neuropathic pain.

Results

In naïve rat dorsal horn, CatS immunoreactivity (ir) had a punctate appearance (Fig. 1*A*) in cells that were neither neurons nor astrocytes because they failed to colabel with antibodies to MAP-2 (a dendritic marker), NeuN (a neuronal marker), or GFAP (an astrocytic marker) (data not shown). After PNL, an increase of CatS-ir was observed in the mediolateral part of the ipsilateral dorsal horn (Fig. 1*B*). By the absence of staining with the sensory neuron marker isolectin B_4, the mediolateral ipsilateral dorsal horn was identified as the area where peripherally injured nerve fibers terminate (Fig. 1*C*). In this region, the appearance of OX42 staining indicated the occurrence of microglial activation (Fig. 1*D*). All CatS-expressing cells in this area costained with OX42 (Fig. 1*E*) and lacked colabeling with astrocytic or neuronal markers (Fig. 1 *F–H*). A temporal profile of CatS expression showed that the number of positive cells in the ipsilateral dorsal horn 1 day after PNL was similar to the naïve and the contralateral dorsal horn. However, there was a significant increase in the ipsilateral dorsal horn within 3 days, which peaked at 7 days and was maintained at a high level 14 days after injury (Fig. 1*I*). No significant change in CatS expression was noted in the contralateral dorsal horn throughout the time course (Fig. 1*I*). These data suggest that CatS is likely to be constitutively expressed by spinal microglia and that the number of CatS-expressing microglia is increased after peripheral nerve damage in the area innervated by injured sensory fibers.

To investigate the possible function of endogenous CatS in the development and/or establishment of neuropathic pain, we examined the effects of morpholinurea-leucine-homophenylalanine-vinyl phenyl sulfone (LHVS), an irreversible, synthetic CatS inhibitor (9) on mechanical hypersensitivity in the PNL model.

Author contributions: A.K.C., C.G., J.W., and M.M. designed research; A.K.C., P.K.Y., J.G., C.G., A.A.S., F.M., M.D., G.W., and J.U. performed research; A.K.C., P.K.Y., J.G., C.G., A.A.S., M.D., J.W., and M.M. analyzed data; and S.B. and M.M. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Abbreviations: CatS, cathepsin S; rrCatS, rat recombinant CatS; LHVS, morpholinurea-leucine-homophenylalanine-vinyl phenyl sulfone; PNL, partial nerve ligation; DRG, dorsal root ganglia; FKN, fractalkine; ir, immunoreactivity; PWT, paw withdrawal threshold.

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This article contains supporting information online at www.pnas.org/cgi/content/full/0610811104/DC1.

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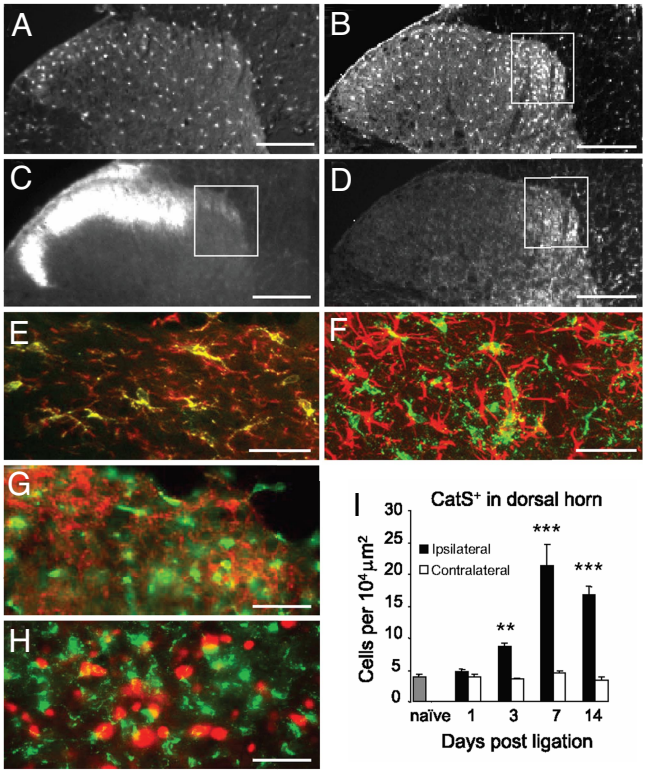

Fig. 1. CatS is expressed by activated microglia in the dorsal horn of neuropathic rats. (*A*) CatS-ir is found in the dorsal horn of naïve rat lumbar spinal cord. (*B*) Increased CatS-ir in the lumbar dorsal horn, especially in the mediolateral part of the ipsilateral dorsal horn (outlined area) 14 days after PNL. (*C*) Lack of isolectin B_4-ir in the mediolateral dorsal horn ipsilateral to PNL. (*D*) Ipsilateral OX42 staining. (*E*) Confocal image of CatS and OX42 in the dorsal horn. CatS (green) is coexpressed (yellow) with OX42 (red). (*F–H*) Confocal images of CatS (green) and GFAP (*F*, red), MAP2 (*G*, red), or NeuN (*H*, red). (Scale bars: 250 μm in *A–D*; 20 μm in *E–G*; and 10 μm in *H*.) (*I*) Temporal profile of CatS-ir in the dorsal horn after PNL. **, $P < 0.01$; ***, $P < 0.001$ versus naïve tissues, four rats per group.

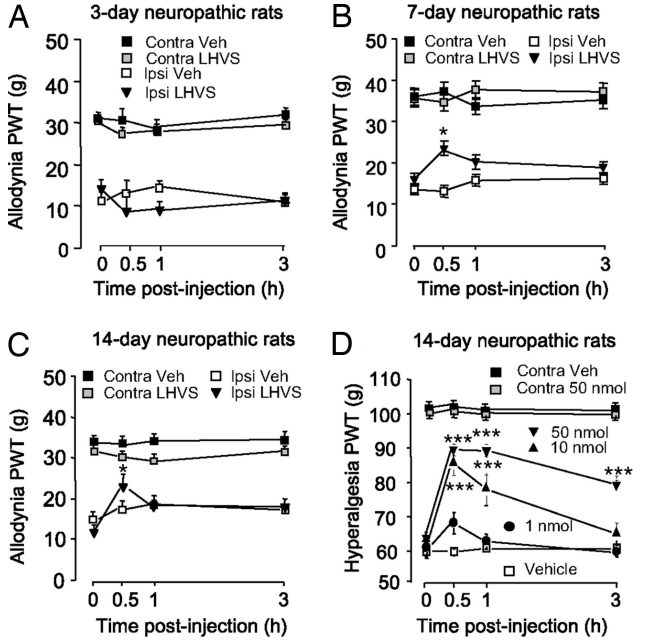

Fig. 2. Spinal delivery of the CatS inhibitor LHVS is antinociceptive in neuropathic rats. Acute injection of LHVS (50 nmol per rat) is not antiallodynic in 3-day neuropathic rats (*A*) but is analgesic in 7-day (*B*) and 14-day (*C*) neuropathic rats. Intrathecal injection of LHVS (10 or 50 nmol per rat) reverses established neuropathic mechanical hyperalgesia in 14-day neuropathic rats (*D*). *, $P < 0.05$, ***, $P < 0.001$ versus vehicle (Veh) group, six to eight rats per group. PWT, paw withdrawal threshold.

Both mechanical allodynia and hyperalgesia are known to be well established 3 days after injury and are maintained for several weeks (5, 10). Spinal intrathecal delivery of LHVS (50 nmol per rat) in the lumbar spinal cord did not alter mechanical allodynia in 3-day neuropathic rats (Fig. 2*A*) at a time when CatS expression in activated microglia had not peaked (Fig. 1*I*). However, 7 and 14 days after nerve injury, when high CatS expression was found in the dorsal horn (Fig. 1*I*), established mechanical allodynia was attenuated by LHVS (Fig. 2 *B* and *C*). Accordingly, LHVS reduced established mechanical hyperalgesia. This effect was dose-dependent and remained significant until 3 h after administration of the highest dose (Fig. 2*D*), suggesting that LHVS preferentially reversed hyperalgesia rather than allodynia. Consistent with the pattern of CatS expression in the dorsal horn, continuous delivery of LHVS (30 nmol per rat per day) from day 0 to day 7 post-PNL failed to prevent the development of allodynia but significantly reversed allodynia on day 7 post-PNL (Fig. 3*A*). In addition, the delivery of LHVS from day 7 to day 14 post-PNL significantly reversed established mechanical allodynia from day 8 (Fig. 3*B*). Intrathecal administration of LHVS had no effect on contralateral mechanical thresholds (Fig. 2 *A–D*) or motor behavior (rotarod test) in naïve rats (data not shown), which excludes possible nonspecific effects of CatS inhibitors. To evaluate whether the 7- to 14-day LHVS treatment affected PNL-induced microglia activation, we quantified the number of $OX42^+$ cells in the dorsal horn and found them significantly reduced by LHVS (Fig. 3 *C–E*). These data show that endogenous CatS in the dorsal horn of the spinal cord contributes to the maintenance of neuropathic allodynia and hyperalgesia and that inhibition of endogenous CatS attenuates microglia activation.

CatS enzymatic activity is optimal at the acidic pH found in lysosomes as well as at extracellular neutral pH (11), and activated microglia are known to secrete CatS (12, 13). Therefore, to test the possibility that extracellular CatS was pronociceptive, we evaluated the effect of rat recombinant CatS (rrCatS) on pain behavior in naïve rats. Spinal delivery of rrCatS, but not nonactivated rrCatS, produced a significant and dose-dependent mechanical hypersensitivity that developed within 30 min, lasted until hour 3 (Fig. 3*F*), and returned to baseline 24 h after injection (data not shown). In contrast to the effects of rrCatS, administration of rat recombinant cathepsin B and rat recombinant cathepsin L at the same or higher doses than rrCatS had no effect on nociceptive thresholds (Fig. 3*G*). The hyperalgesic effect of rrCatS was completely inhibited by pretreatment with LHVS (Fig. 3*H*). The inhibitory effects of LHVS were specific for CatS-induced pain behaviors: prior administration of LHVS had no effect on the hyperalgesia induced by injections of either [Sar^9,$Met(O_2)^{11}$]substance P [Sar, sarcosine; $Met(O_2)$, methionine *S*,*S*-dioxide] or NMDA (data not shown). These data indicate that exogenous CatS, and not other closely related cathepsins, can exert a pronociceptive extracellular effect in the lumbar spinal cord.

To identify possible substrates for the CatS pronociceptive effect, we used the bioinformatics program Pat_Scan to search predefined cleavage signatures in the target sequences. This approach revealed a number of potential substrates that may be susceptible to CatS cleavage, including kininogen (precursor of bradykinin) and the membrane-bound chemokine, fractalkine (FKN). Intrathecal administration of either des-Arg^{10}-HOE140 (B1 receptor antagonist, 50 μg per rat) or HOE140 (B2 receptor

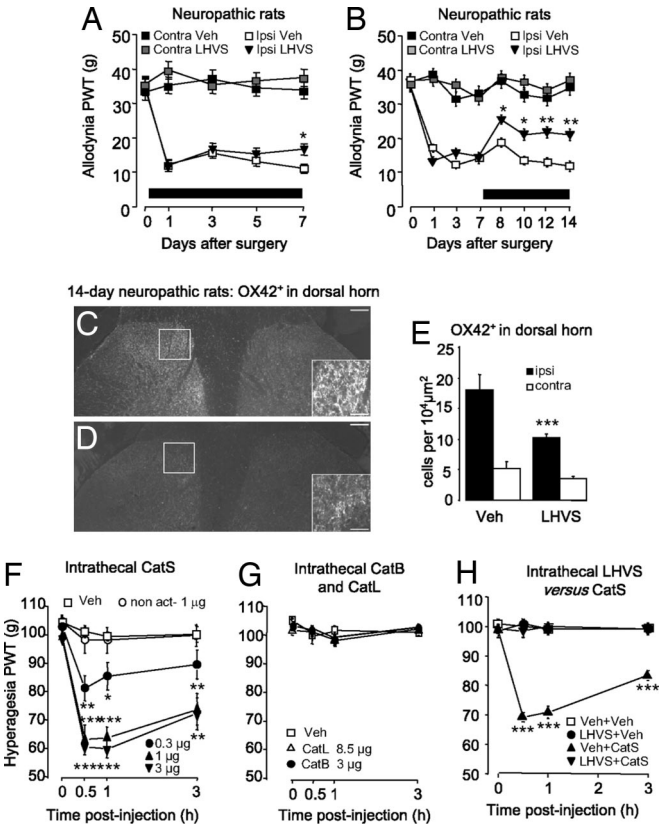

Fig. 3. Prolonged spinal delivery of LHVS reverses tactile allodynia and attenuates microglia activation in neuropathic rats. rrCatS is pronociceptive in naïve rats. LHVS (30 nmol per rat per day) intrathecally delivered from day 0 to day 7 after injury (horizontal black bar) does not modify allodynia after 3 and 5 days but reverses allodynia after 7 days of treatment (*A*). LHVS delivered from day 7 to day 14 after injury (horizontal black bar) reverses established mechanical allodynia (*B*) and attenuates microglia activation (OX42-ir) (*D*) as compared with vehicle (*C*). (*C* and *D*) Ipsilateral dorsal horn (left) and contralateral dorsal horn (right) and high-power images (*Insets*: 20× magnification; scale bars: 50 μm) of the ipsilateral dorsal horn. (Scale bars: 100 μm.) (*E*) Quantitative analysis of OX42-ir. ***, $P < 0.001$ versus vehicle group, four rats per group. (*F*) Intrathecal injection of activated rrCatS- (0.3–3 μg per rat) induced mechanical hypersensitivity in naïve rat hind paws, whereas nonactivated rrCatS (1 μg per rat) is ineffective. (*G*) Intrathecal activated CatB and CatL do not alter mechanical thresholds. (*H*) Intrathecal LHVS (50 nmol per rat, 30 min before CatS) prevents the hyperalgesia evoked by intrathecal activated rrCatS (1 μg per rat). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ versus vehicle group in *A*, *B*, and *F*; versus LHVS + rrCatS group in *H*, six to eight rats per group.

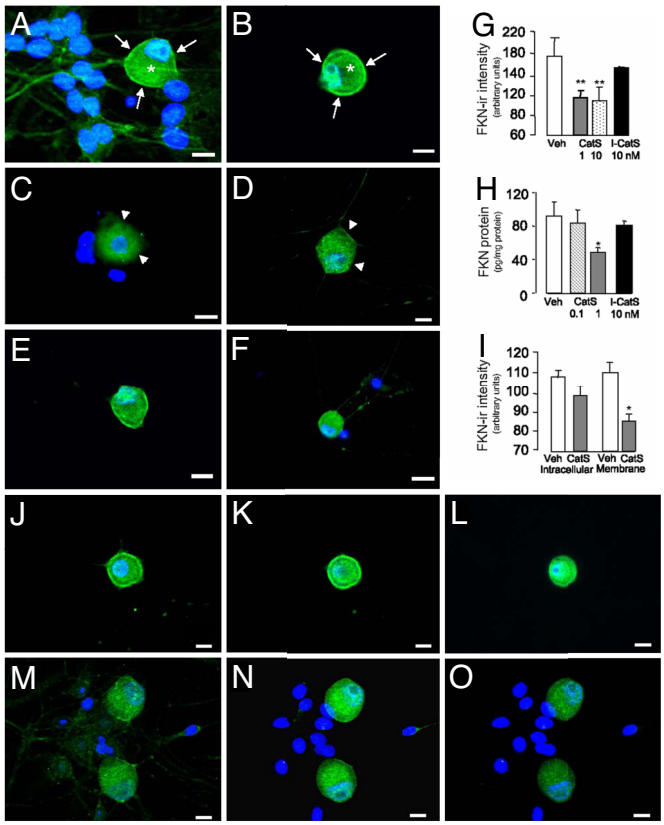

Fig. 4. CatS incubation reduces the levels of sensory neuron-associated FKN. (*A* and *B*) DRG neurons in culture express FKN immunoreactivity (FKN-ir) (green) on the cell membrane (arrows) as well as intracellularly (asterisks). (*C* and *D*) Incubation with rrCatS (10 nM) for 30 min reduces FKN-ir (green) (arrowheads). (*E* and *F*) Inactivated (heat-denatured) CatS (10 nM) does not significantly reduce FKN-ir (green). (*G*) Quantitative analysis of FKN-ir in cultured DRG, three independent experiments. **, $P < 0.01$ versus vehicle. (*H*) Quantitative analysis of FKN protein in cultured DRG extracts by using ELISA. Activated rrCatS was incubated for 30 min. Data represent three independent experiments (two rats per experiment). *, $P < 0.05$ versus vehicle. (*I*) Quantitative analysis of membrane and intracellular FKN-ir at the midplane of cultured DRG (e.g., *K* and *N*). Data represent three independent experiments. *, $P < 0.05$. (*J–O*) A series of *z*-stack images of DRG neurons incubated without rrCatS (*J–L*) or in the presence of activated rrCatS (*M–O*). Images were taken with the Zeiss Axioplan 2 fluorescence microscope via a Zeiss ApoTome system. DAPI (blue) was used as a nuclear marker for all of the panels. (Scale bars: 10 μm.) I-CatS, inactivated CatS.

antagonist, 50 μg per rat) did not modify rrCatS (1 μg per rat)-induced hyperalgesia (data not shown), suggesting that CatS does not produce pronociceptive bradykinin (14) in the dorsal horn. However, we noted that the predicted cleavage site for FKN would liberate the soluble, biologically active extracellular domain of this chemokine that is believed to mediate communication in nociceptive processing between neurons and microglia expressing the CX3CR1 receptor (15–17). The protease responsible for the formation of soluble FKN in the spinal cord has not yet been identified. Therefore, we tested the possibility that the pronociceptive effects and/or activation of microglia by CatS in the spinal cord were due to enzymatic release of neuronal FKN.

We found that incubation with rrCatS reduced the amount of FKN associated with cultured DRG neurons, which are known to express the chemokine (16). FKN staining was found mostly at the cell membrane, but was also present intracellularly in sensory neurons (Fig. 4 *A*, *B*, *J–L*). The existence of FKN in at least two distinct cellular compartments has been shown in endothelial cell lines (18). Notably, we have been able to detect the subcellular distribution of the endogenous chemokine in primary cells. A 30-min incubation of cultured DRG neurons with activated rrCatS significantly decreased both the intensity of FKN staining (Fig. 4 *C*, *D*, *G*, *M–O*) and FKN protein content (Fig. 4*H*) in comparison with control groups (Fig. 4 *A*, *B*, *G*, *J–L*). Inactivated (heat-denatured) rrCatS did not alter the intensity of FKN-ir or FKN protein content as compared with controls (Fig. 4 *E–H*). However, because we presumed that extracellular CatS would not penetrate DRG cells and affect FKN intracellular pools, we examined the effect of this protease on FKN staining at the cell surface and in the cytoplasm. The incubation of cultured DRG with activated rrCatS significantly decreased the FKN-ir at the cell membrane (Fig. 4 *I*, *K*, *N*) but no significant changes in the intracellular FKN-ir were observed (Fig. 4 *I*, *J–O*). Furthermore, the application of activated rrCatS (0.1 pM for 1 min) enhanced FKN content in the DRG culture media to

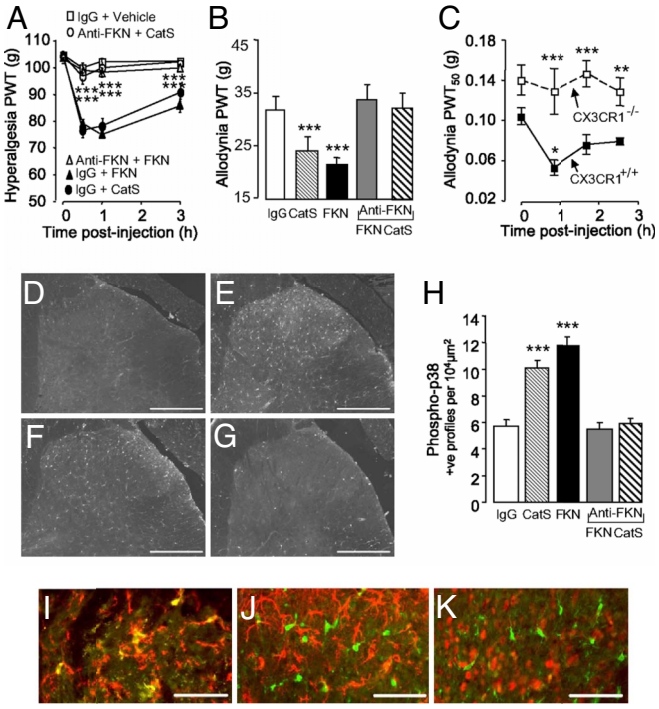

Fig. 5. FKN-neutralizing antibody prevents CatS-induced hyperalgesia, allodynia, and activation of p38 MAPK in the dorsal horn. CatS-induced allodynia does not develop in CX3CR1-knockout mice. (*A*) Intrathecal injection of a rat FKN-neutralizing antibody (anti-FKN, 1 μg per rat) 1 h before intrathecal administration of either activated rrCatS (1 μg per rat) or FKN (amino acids 25–100; 30 ng per rat) prevents both CatS- and FKN-induced mechanical hyperalgesia. One microgram of IgG per rat was used as a control. ***, $P <$ 0.001 versus IgG + FKN or IgG + CatS. (*B*) Intrathecal injection of anti-FKN (1 μg per rat) 1 h before intrathecal administration of activated rrCatS (1 μg per rat, twice at 4-h intervals) or FKN (amino acids 25–100; 30 ng per rat) prevents CatS- and FKN-induced mechanical allodynia measured 1 h after CatS or FKN injections. ***, $P < 0.001$ versus relevant control group (CatS, FKN, or Control IgG), six rats per group. (*C*) Intrathecal rrCatS (1.5 μg per mouse, twice at 4-h intervals) induces allodynia in wild-type but not CX3CR1-knockout mice. *, $P <$ 0.05 versus wild-type mouse values at time 0. **, $P < 0.01$; ***, $P < 0.001$ versus wild-type mice, six mice per group. (*D–G*) Activation of p38 MAPK after intrathecal delivery of control IgG (1 μg per rat) (*D*), FKN (30 ng per rat) (*E*), or activated rrCatS (1 μg per rat) (*F*) 15 min after injection. Anti-FKN (1 μg per rat) prevented rrCatS-induced p38 activation (*G*). (Scale bars: *D–G*, 100 μm.) (*H*) Quantitative analysis of p38 phosphorylation in the dorsal horn. ***, $P <$ 0.001, four rats per group. (*I*) CatS-activated p38 (green) colocalizes with OX42 (red) in the dorsal horn, but not with either GFAP (*J*, red) or NeuN (*K*, red). (Scale bars: *I–K*, 50 μm.)

107.8 ± 15.8 pg/ml ($n = 6$ rats) compared with 66.4 ± 2.5 pg/ml in control cultures ($n = 6$ rats) ($P < 0.05$ Student's t test). Altogether, these data indicate cleavage of cell membrane-bound FKN by CatS.

We then performed two sets of experiments showing that CatS pronociceptive effects in the spinal cord are prevented by an FKN-neutralizing antibody and fail to develop in CX3CR1-knockout mice. First, preadministration of a rat FKN-neutralizing antibody (19), but not a control IgG antibody, significantly inhibited the mechanical hyperalgesia and allodynia induced with either rrCatS or FKN (Fig. 5 *A* and *B*). Incidentally, both CatS- and FKN-induced hyperalgesia were more pronounced than allodynia. The inhibitory effects of the FKN-neutralizing antibody were specific for CatS-induced hyperalgesia because spinal administration of the antibody had no effect on the hyperalgesia induced by injections of either [Sar^9,Met(O$_2$)^{11}]substance P (1 μg per rat, intrathecally) or NMDA (1 nmol per rat, intrathecally) (data not shown). In addition, intrathecal delivery of FKN-neutralizing antibody reversed mechanical hyperalgesia and allodynia in 14-day neuropathic rats by 56.3 ± 9.0% and 54.5 ± 7.5%, respectively.

Additionally, we found that intrathecal administration of rrCatS (1.5 μg per mouse) was proallodynic in wild-type mice but not in CX3CR1-knockout mice (Fig. 5*C*). Similarly, intrathecal injection of FKN (30 ng per mouse) produced allodynia in wild-type mice {PWT$_{50}$ [PWT mean response (g) from 0.10 ± 0.007 to 0.07 ± 0.008]} but not in knockout mice [PWT$_{50}$ (g) from 0.11 ± 0.006 to 0.11 ± 0.006 1 h after injection] ($P < 0.01$, wild-type versus knockout, eight mice per group) [supporting information (SI) Fig. 6]. The CX3CR1 receptor for FKN is mainly found on microglial cells (16, 17), in which p38 mitogen-activated protein kinase (MAPK) is rapidly phosphorylated upon cell activation (20). Therefore, we tested whether extracellular CatS indirectly activated microglial p38 MAPK by proteolytically liberating the soluble form of neuronal FKN. Injections of either FKN (Fig. 5 *E* and *H*) or rrCatS (Fig. 5 *F* and *H*) activated p38 MAPK in the dorsal horn of the spinal cord, and these effects were reduced by prior administration of the FKN-neutralizing antibody (Fig. 5 *G* and *H*). rrCatS-induced activation of p38 MAPK occurred in microglial cells in the dorsal horn, as shown by colabeling with OX42 (Fig. 5*I*), but not in astrocytic or neuronal cells as judged by lack of colabeling with GFAP or NeuN (Fig. 5 *J* and *K*). These data suggest that in the dorsal horn, extracellular CatS indirectly activates microglial p38 MAPK by proteolytically liberating soluble FKN.

Discussion

We provide evidence that the cysteine protease CatS from spinal microglial cells contributes to the maintenance of neuropathic hypersensitivity and microglia activation. First, we show that spinally administered CatS inhibitors are analgesics and attenuate microglia activation in the dorsal horn. Second, we show that CatS pronociceptive effects and CatS-induced microglia activation are mediated by the enzymatic release of soluble FKN from neurons.

Two pieces of evidence indicate that activated microglia in the spinal cord are an important source of CatS. First, the increased expression of CatS within the dorsal horn after peripheral nerve injury is associated with cells that costain with the microglial marker OX42, with no clear astrocytic or neuronal staining. Second, microglial cells are established secretory cells (21) that release CatS upon activation by inflammatory mediators (12, 13).

The temporal profile for CatS expression in the dorsal horn shows few CatS$^+$ cells in the spinal cord 1 day after injury when mechanical hyperalgesia and allodynia are known to already be well established (5, 10). Over the next 2 days, however, CatS expression increased significantly, peaked at day 7, and remained elevated until day 14. The time course of CatS expression is consistent with published data on microglia activation in the ipsilateral dorsal horn after PNL (10). Microglia activation is evident by postoperative day 2 within the somatotopic localization of sciatic nerve primary afferents. This activation is maximal by day 14 and remains high for several weeks in association with neuropathic pain (10). Consistent with the temporal profile in spinal microglia, we found that CatS expression in infiltrating macrophages at the site of injury and in the DRG peaked 7–14 days after PNL (5).

These data suggest that CatS contained in microglial cells is a major contributor to the maintenance of neuropathic pain rather than to the initial development of mechanical hypersensitivity. This conclusion is reinforced by the observation that the CatS inhibitor LHVS, delivered acutely or over several days, reversed pain behavior in 7- and 14-day neuropathic rats, but did not inhibit the development of neuropathic pain. Importantly, the central microglial source of the enzyme is essential for the

persistence of neuropathic allodynia, to which macrophage-derived peripheral CatS does not contribute (5). Many other mediators and receptors have been suggested to play a role in microglia activation after peripheral nerve injury, including ATP, which releases brain-derived neurotrophic factor via activation of microglial $P2X_4$ receptors (22, 23). However, as opposed to $P2X_4$ inhibition, which did not modify microglia activation, inhibition of CatS reduced OX42 labeling, suggesting that this enzyme is essential for the microglial cells to remain in their hyperactive phenotype.

Several lines of evidence, from experiments in which we administered either exogenous CatS enzyme or a specific CatS inhibitor, support the view that CatS is pronociceptive. Mechanical hyperalgesia and allodynia were evoked, although to different extents, by injections of activated CatS into the spinal cord. The evoked hyperalgesia required active enzyme and was specific to CatS; the related enzymes CatB and CatL had no pronociceptive actions. The effects of spinally administered CatS were blocked by intrathecal administration of LHVS, indicating a local, spinal site of action for the enzyme.

The finding that exogenous CatS is pronociceptive clearly points to an extracellular site of action and is consistent with the observations that the behavioral effects were rapid and maximal at the first time point studied, 30 min after enzyme injection. Our *in silico* analysis and experimental results provide evidence that the molecular substrate for CatS is neuronal FKN and that the mechanism responsible for the pronociceptive effects of CatS is FKN shedding from neuronal membranes. Specifically, we show that CatS promotes FKN shedding from sensory neuron membranes and that CatS produces biologically active FKN in the dorsal horn, which induces nociception and microglia activation via CX3CR1 receptors.

Our data are consistent with the scheme shown in SI Fig. 7. After peripheral nerve injury, CatS-expressing spinal cord microglia in the area of the dorsal horn innervated by damaged fibers release CatS, which then liberates soluble FKN. The released FKN feeds back onto the microglial cells via the CX3CR1 receptor to activate the p38 MAPK pathway. Activation of this intracellular pathway is thought to contribute to neuropathic pain (24) by modulating the synthesis and release of pronociceptive mediators such as cytokines (3, 4). Our results establish a role for the CatS/FKN pair in the maintenance of neuropathic hypersensitivity and suggest that CatS inhibition constitutes a therapeutic approach for the treatment of chronic pain.

Methods

Animals. Adult male Wistar rats and C57BL/6 mice were purchased from Harlan (Bicester, Oxon, U.K.). $CX3CR1^{-/-}$ C57BL/6 mice were obtained from Taconic (Germantown, NY), where they had been generated as described (25) and backcrossed onto the C57BL/6 background for 10 generations.

Surgical Procedures. Male Wistar rats received a partial ligation of the left sciatic nerve (PNL) (26). For chronic delivery of LHVS (NeoMPS, San Diego, CA) an intrathecal cannula was inserted and attached to a s.c. osmotic minipump (27).

Drug Administration. Rats or mice were injected intrathecally with rrCatS, rrCatB, or rrCatL [purified at Novartis Genomic Foundation, San Diego, CA, 95% pure as determined by SDS/PAGE; activity was measured by the active enzyme's ability to cleave a fluorogenic peptide substrate, either Z-Leu-Arg-AMC or Z-Val-Val-Arg-AMC (Norvatis Genomics Foundation), in which Z is benzyloxycarbonyl and AMC is 7-amino-4-methylcoumarin, while incubated for 30 min at room temperature with the fluorescence measured at 360/460 nm]. Enzymes were solubilized in 25 mM sodium acetate and 1 mg/ml rat serum albumin and activated by incubation with DTT (0.5 mM); LHVS was solubilized in 20% Cremophor EL/saline; recombinant FKN (chemokine domain, amino acids 25–100; R&D Systems, Abingdon, Oxfordshire, U.K.), anti-rat FKN-neutralizing antibody (AF537; R&D Systems), normal goat IgG (R&D Systems) and $[Sar^9,Met(O_2)^{11}]$substance P were dissolved in saline and rat serum albumin 1 mg/ml. NMDA was dissolved in saline. Intrathecal injections (10 μl per rat, 5 μl per mouse) were performed by lumbar puncture (20). Briefly, rats or mice were immobilized under enflurane/O_2 and the injections were made into the lumbar region (between the L5 and L6 vertebrae) of the spine by using a 26G 3/8-inch needle.

Behavioral Studies. Mechanical hyperalgesia was assessed by measuring PWT to an increasing pressure stimulus placed on the dorsal surface of the hind paw by using an Analgesymeter (Ugo Basile, Comerio, Italy) (20, 26). Tactile allodynia in rats was assessed by measuring PWT by using an automated von Frey hair applicator (Dynamic Plantar Aesthesiometer; Ugo Basile) calibrated to apply a pressure ramp of 2.5 g/sec for 20 sec (50-g cut-off) (27). Tactile allodynia in mice was assessed by applying a series of von Frey filaments to the plantar surface of the hind paw (range 0.01–1.66 g) in ascending order of force. Each filament was applied five times (28). The percentage withdrawal was calculated for each filament and the PWT mean response was fitted with a standard sigmoidal dose–response curve: $y = \text{minimum} + (\text{maximum} - \text{minimum})/[1 + (x/EC_{50})]^n$, in which n is the Hill coefficient, to produce a 50% PWT.

Immunohistochemistry. Tissue processing was performed as described in refs. 20 and 26. Lumbar spinal cords were cut (20–30 μm thick) with a cryostat, and transverse L5 spinal cord sections were processed for "antigen retrieval" by microwaving in Antigen Unmasking solution (Vector Laboratories, Burlingame, CA) at a dilution of 1:100. Sections were then incubated with antibodies for CatS (goat anti-cathepsin S M-19; Santa Cruz Biotechnology, Santa Cruz, CA), GFAP (rabbit anti-glial fibrillary acidic protein; DakoCytomation, Glostrup, Denmark), OX42 (mouse anti-CD11b; Serotec, Oxford, U.K.), NeuN (mouse anti-neuronal nuclei; Abcam, Cambridge, U.K.), MAP2 (rabbit anti-microtubule-associated protein 2, Chemicon International, Temecula, CA), or phospho-p38 MAPK (rabbit anti-phospho-p38 MAPK; Cell Signaling Technology, Danvers, MA), followed by incubation with secondary antibody solution (Alexa Fluor 488- or 546-conjugated antibody to the appropriate IgG, 1:1000, Molecular Probes, Eugene, OR). For visualization of isolectin B_4, sections were incubated with an isolectin B_4 antibody conjugated to biotin (BSI-BS_4, biotinylated; catalog no. L2140 from Sigma Chemicals, Poole, Dorset, U.K.), followed by incubation with ExtrAvidin conjugated to fluorescein isothiocyanate (ExtrAvidin FITC; 1:400; Sigma Chemicals). CatS staining was abolished by prior absorption with the peptide immunogen. Images were captured by using a Zeiss Pascal confocal microscope or a Zeiss Axioplan 2 fluorescence microscope (Zeiss, Welwyn Garden City, U.K.). CatS, phospho-p38, and OX42-ir were quantitatively determined by counting the number of profiles in the spinal cord in a 10^4-μm^2 box placed onto areas of the lateral, central, and medial dorsal horn. All samples were scored blind until analysis was complete.

***In Silico* Prediction of Potential CatS Substrates.** As described in ref. 29, to predict potentially novel substrates, sequence information from endogneous and synthetic CatS substrates were used to predict potentially novel substrates, sequence information from known endogenous and synthetic CatS substrates were used to generate a cleavage signature motif extending from P4 to P2′ positions (the substrate is cleaved at the peptide bond between P1 and P1′ according to the Schechter and Berger nomenclature;

see Table 1). The position-specific scoring matrix was derived by aligning substrate sequences corresponding to P4 to P2′ and calculating normalized amino acid frequencies at each position (30) (SI Table 1).

DRG in Culture. Adult rat DRG neurons were dissected out, dissociated, and grown on coverslips (3,000 cells per well) for 48 h (27, 31). After incubation (30 min, 37°C) with medium containing either 0.5 mM DTT or rrCatS and DTT, the neurons were fixed and underwent immunocytochemistry using goat anti-CX3CL1/FKN (AF537, R&D Systems (1:100), mouse anti-β-III tubulin (neuronal marker, Promega) (1:1,000) and DAPI (nuclei marker, 10 μg/ml). FKN-ir was quantified (using Axiovision LE 4.2 software; Carl Zeiss Imaging Solutions, Welwyn Garden City, U.K.) as average pixel intensity on at least 50 neurons per group (three independent experiments) within each β-III tubulin-expressing cell (15- to 35-μm diameters). To ensure consistency between groups, the staining was carried out at the same time within each independent experiment and the capturing of images and analysis were carried out using the same settings. In addition, the intensities of intracellular and cell membrane-associated FKN-ir were determined. *z*-stack images (1.2-μm serial section) of DRG neurons (at least 50 neurons, from three independent experiments) were taken with a Zeiss Axioplan 2 fluorescence microscope (Zeiss) with an ApoTome imaging system under the same exposure settings, so that the midplane section of the DRG neuron (a single plane in which cell membrane and intracellular FKN levels were viewed simultaneously) could be acquired for analysis. The average pixel intensities of the cell membrane (around the rim of the DRG) and the cytoplasm (within the cell) were determined.

FKN ELISA. Cultured DRG neurons were incubated at 37°C with medium containing either 0.5 mM DTT or rrCatS and DTT, for 1 or 30 min. CatS activity was stopped by the addition of the site-directed inhibitor E64 (10 μM). DRG extracts were obtained by homogenizing the cultured neurons in 20 mM Tris·HCl/1% Nonidet P-40/10% glycerol/0.1% BSA/10 μg of aprotinin/0.5 mM sodium vanadate. FKN contents in the DRG media or extracts from three independent experiments (two rats per experiment) were determined by 96-well ELISA plates (RayBio Rat Fractalkine ELISA kit, sensitivity 2.74 pg; RayBiotech, Wembly, Middlesex, U.K.).

Statistical Analysis. Two-way ANOVA followed by Tukey's test was used for behavioral data. One-way ANOVA followed by Tukey's test or Student's *t* test was used for immunohistochemistry. ANOVA followed by Dunnett's test or Student's *t* test was used for ELISA analysis (SigmaStat 3.1; Systat Software, San Jose, CA).

We thank Jennifer Harris (Novartis Genomic Foundation, San Diego, CA) for the recombinant cathepsins and fluorogenic substrates; Philip Murphy (National Institutes of Health, Bethesda, MD) for the kind gift of CX3CR1-knockout mice; Sami Naeem, Jodie Hall, Marta D'Auria, and Dongwook Kim for technical assistance; and Jane Barclay and Pam Ganju for valuable discussion. A.K.C. is supported by a Biotechnology and Biological Sciences Research Council Cooperative Awards in Science and Engineering studentship sponsored by the Novartis Institutes for Biomedical Research. King's College London provided financial support (start-up sum to M.M.).

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