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Cortistatin attenuates inflammatory pain via spinal and peripheral actions

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ABSTRACT

Clinical pain, as a consequence of inflammation or injury of peripheral organs (inflammatory pain) or nerve injury (neuropathic pain), represents a serious public health issue. Treatment of pain-related suffering requires knowledge of how pain signals are initially interpreted and subsequently transmitted and perpetuated. To limit duration and intensity of pain, inhibitory signals participate in pain perception. Cortistatin is a cyclic-neuropeptide that exerts potent inhibitory actions on cortical neurons and immune cells. Here, we found that cortistatin is a natural analgesic component of the peripheral nociceptive system produced by peptidergic nociceptive neurons of the dorsal root ganglia in response to inflammatory and noxious stimuli. Moreover, cortistatin is produced by GABAergic interneurons of deep layers of dorsal horn of spinal cord. By using cortistatin-deficient mice, we demonstrated that endogenous cortistatin critically tunes pain perception in physiological and pathological states. Furthermore, peripheral and spinal injection of cortistatin potently reduced nocifensive behavior, heat hyperalgesia and tactile allodynia in experimental models of clinical pain evoked by chronic inflammation, surgery and arthritis. The analgesic effects of cortistatin were independent of its anti-inflammatory activity and directly exerted on peripheral and central nociceptive terminals via Gαi-coupled somatostatin-receptors (mainly sstr2) and blocking intracellular signaling that drives neuronal plasticity including protein kinase A-, calcium- and Akt/ERK-mediated release of nociceptive peptides. Moreover, cortistatin could modulate, through its binding to ghrelin-receptor (GHSR1), pain-induced sensitization of secondary neurons in spinal cord. Therefore, cortistatin emerges as an anti-inflammatory factor with potent analgesic effects that offers a new approach to clinical pain therapy, especially in inflammatory states.

Introduction

Clinical persistent pain results from multiple changes in the peripheral and central nervous systems. Among them, changes in primary sensory neurons (nociceptors) are critical in sensing, initiating and perpetuating pain. Nociceptors are small-sized dorsal root ganglia (DRG) neurons that give rise to C- and Aδ-axons that can be activated by noxious mechanical, thermal, or chemical stimuli. Following tissue injury, local inflammatory mediators increase excitability and reduce thresholds of nociceptors, resulting in a facilitated afferent-evoked release of neurotransmitters (glutamate, aspartate) and neuropeptides (calcitonin gene-related peptide CGRP, substance P). The subsequent activation of spinal secondary neurons would further trigger cascades of ascending nociceptive pathways to the brain (Hucho and Levine, 2007; Julius and Basbaum, 2001; Todd, 2010).

The balance between excitation and inhibition is crucial for maintaining normal sensory function. Several inhibitory peptides (somatostatin, neuropeptide Y, enkephalins) produced by nociceptors in response to tissue injury or inflammation serve as key functions in regulating plasticity events that underlie chronic pain states (Pan et al., 2008). The identification of new endogenous antinociceptive peptides is critical to understand the process of nociception and to develop novel strategies for treating pathological pain. Cortistatin, a neuropeptide recently discovered in brain cortex based in its inhibitory neuronal activities (de Lecea et al., 1996), shows a remarkable sequential/structural resemblance with somatostatin. Due to its ability to bind/activate all the somatostatin-receptors (sstr) (Siehler et al., 2008), cortistatin shares many functions with somatostatin, especially concerning hormonal and neuronal regulation (Gahete et al., 2008). However, cortistatin exerts unique functions in brain and the vascular and immune systems (Duran-Prado et al., 2013; Gahete et al., 2008; Gonzalez-Rey et al., 2006a, 2006b, 2007; Souza-Moreira et al., 2013). These functions are related to its capacity to bind to receptors other than sstr, mainly ghrelin-receptor (GHSR1), Mas gene-related receptor X-2 and a still unidentified cortistatin-selective receptor (Deghenghi et al., 2001; Robas et al., 2003).

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Recent evidence supports a potential role of cortistatin modulating pathologic inflammatory pain. Cortistatin is a potent anti-inflammatory factor produced by immune cells in response to inflammation-mediated products and cytokines (Gonzalez-Rey et al., 2006a). Intracerebroventricular injection of cortistatin decreased the basal sensitivity to noxious heat in rats (Mendez-Diaz et al., 2004). Markovics et al. (2012) reported that peripheral injection of cortistatin reduced the severity of neurogenic skin inflammation induced by mustard oil and carrageenan. Moreover, we recently found that cortistatin alleviates hyperalgesia (an exaggerated response to subsequent noxious stimuli as heat and mechanical pressure) and allodynia (pain in response to normally innocuous tactile stimuli) in two models of inflammatory and arthritic pain (Morell et al., 2013). Thus, the aims of the present study are to investigate the occurrence of cortistatin in the peripheral nociceptive system and the role played by endogenous cortistatin in pain regulation under inflammatory conditions. We will also determine the sites of action of cortistatin at peripheral and spinal levels in various experimental models of clinical pain, as well as the receptors, signaling pathways and molecular mechanisms involved in such effects.

Materials and methods

Animals

We used male and female C57BL/6 mice (25–30 g, 12-wk-old, Charles River, Barcelona, Spain) throughout the experiments. We generated mice lacking the gene for cortistatin (CST-KO) in a C57BL/6 background at Stanford University and bred in-house (IPBLN-CSIC) as previously described (Cordoba-Chacon et al., 2011). We habituated all the animals to the experimental room conditions for at least 1 h before the behavioral tests and used once throughout the experiments. We did not observe significant differences between male and female mice in behavioral tests. The experiments reported in this study followed the current guidelines for the care of laboratory animals and the ethical guidelines for investigations of experimental pain in conscious animals, approved by the Animal Care Unit Committee IPBLN-CSIC (# protocol 202-11-3).

Reagents

We used mouse cortistatin-29 obtained from American Peptide (Sunnyvale, CA), mouse acylated-ghrelin and somatostatin-28 from Bachem AG (Bubendorf, Switzerland), cyclosomatostatin (cycloSOM), NS-398, GHRP6, CYN-154806, kaolin, capsaicin, nerve growth factor (NGF), octreotide, naloxone, pertussis toxin (PTX) and carrageenan from Sigma (St. Louis, MO), tumor necrosis factor-α (TNFα) from PreproTech (Rocky Hill, NJ) and brain-derived neurotrophic factor (BDNF) from Abnova (Heidelberg, Germany). BIM-28163, BIM-23627 and BIM-23867 were provided by Ipsen (Milford, MA). We dissolved all drugs in physiological saline (0.9% NaCl), except NS-398 and naloxone that were dissolved in 1% ethanol and capsaicin that was prepared in 1% DMSO.

Experimental models of pain

We induced visceral acute pain by intraperitoneal (i.p.) injection of acetic acid (0.6%, 200 μl, Sigma). Immediately after the injection of acetic acid, we placed the animals into a Perspex observation chamber and we recorded for 15 min the number of writhing, characterized by abdominal stretching combined with an extension of the hind limbs.

We induced chronic inflammatory pain by intraplantar (i.pl.) injection of Complete Freund's Adjuvant (CFA, 20 μg/20 μl, BD Difco, Detroit, MI) in the hind paw. We then monitored thermal and mechanical hypersensitivity in the inflamed paw at different time points starting 1 d (for mechanical allodynia), 2 d (for pressure hyperalgesia) or 3 d (for thermal hyperalgesia) after injecting CFA (which correspond to time points of maximal nocifensive responses).

We induced spontaneous and persistent pain with capsaicin and glutamate following two routes of administration: peripherally by i.pl. injection (1600 ng capsaicin or 30 μmol glutamate, 20 μl/paw), or alternatively, spinally by intrathecal (i.t.) injection (500 ng capsaicin or 0.5 μmol glutamate, in 10 μl). We immediately placed the mice into a Perspex observation chamber and recorded the time that spent on licking and biting the affected paw or flank during 5 min (for capsaicin) or 15 min (for glutamate). We then measured thermal and tactile nocifensive responses in the injected paw at different time points after i.pl. injection of capsaicin or at 45 min after i.pl. injection of glutamate. We also induced pain by i.t. injection of TNFα (10 ng/10 μl) or by i.pl. injection of TNFα (20 ng/20 μl) or NGF (100 ng/15 μl) and then assessed thermal and tactile hypersensitivity in the hind paws at different time points.

We evoked postoperative pain by incision in the hind paw according to a technique previously described for rats with minor modifications (Brennan et al., 1996). Briefly, we made a 5 mm longitudinal incision through skin and fascia of the plantar aspect of the foot in anesthetized mice (2% isoflurane), starting 2 mm from the proximal edge of the heel and extending toward the toes. We then elevated and incised longitudinally plantaris muscle, leaving the muscle origin and insertion intact. After hemostasis with gentle pressure, we apposed the skin with two 6–0 nylon sutures, covered the wound with a mixture of antibiotic ointment and assayed allodynia in the operated paw at different times after incision.

To induce knee arthritis (Bar et al., 2004a), we injected 4% kaolin (30 μl, Sigma) intra-articularly through the ligamentum patellae and manually bent and straightened the knee during 15 min in mice anesthetized with ketamine/xylazine. Subsequently, we injected 2% carrageenan (30 μl, Sigma) intra-articularly and moved again the knee for 5 min. This treatment causes inflammation and swelling of the knee within 1–3 h that lasts for about 2 wk without systemic spreading. We measured the resulting tactile allodynia at different times over 18 d in total and then assessed the severity of inflammatory edema by measuring the diameter of the knee joint with a caliper. We also assayed synovial neutrophil accumulation by measuring myeloperoxidase activity in the knee synovial fluids and histopathological signs in knees extracted 8 d after kaolin/carrageenan injection as previously described (Gangadharan et al., 2011).

Treatments

Mice received cortistatin, ghrelin and somatostatin through three routes: peripherally by i.pl. injection in the plantar surface of hind paw at 100 ng in 20 μl of saline (1.5 μM), spinally by i.t. injection in the lumbar region at 10 ng in 10 μl of saline (0.3 μM), and systemically by i.p. injection at 1000 ng in 200 μl of saline (1.5 μM) or by subcutaneous (s.c.) injection in right flank at 1000 ng in 20 μl of saline (1.5 μM). Mice received saline (same volume and route of injection as described for peptides) as vehicle control in most of the models throughout the study. When indicated, we assayed cortistatin at different doses for comparative effectiveness with the cycloxigenase-2 (COX-2) inhibitor NS-398 (i.t. or i.p., 100–1000 ng in 1% ethanol). We found that 1% ethanol did not alter heat hyperalgesia and allodynia when administered through systemic or spinal route. We used acetylsalicylic acid (ASA, 2 mg in 50 μl, i.p. injected 30 min before acetic acid) as reference drug in the model of visceral pain. In general, mice received neuropeptides and NS-398 15 min before the nociceptive stimuli or at different time points after pain induction (24, 48 or 72 h after i.pl. injection of CFA; 48 h after arthritis induction). In spontaneous and persistent pain induced by i.t. injection of capsaicin, glutamate or TNFα, we mixed cortistatin with these agents and injected them at the same time. To study the involvement of specific receptors, mice received i.t. or i.pl. injections of various cortistatin-receptor antagonists (BIM-28163, BIM-23627 and BIM-23867, CYN-

154806, cycloSOM and GHRP6, each at 500 μM) 1 h before cortistatin. The use of these antagonists was based in previous in vivo studies (Gonzalez-Rey et al., 2007; Inhof et al., 2011; Jerlhag et al., 2010; Takeda et al., 2007; Tulipano et al., 2002). To study the involvement of Gαi-coupled receptors, PTX was given in two i.t. injections (2 × 200 ng) 24 h and 12 h prior to nociceptive stimuli. To study the involvement of opioid receptors, mice received i.t. injections of naloxone (2 μg, 600 μM) 30 min before cortistatin.

Intrathecal administration of drugs by lumbar puncture

To avoid systemic effects of drugs and target spinal mechanisms, we delivered drugs into cerebral spinal fluid space around lumbosacral spinal cord via i.t. administration in some experiments. Spinal cord puncture was made with a 30-gauge needle between the L5 and L6 level to deliver the reagents (10 μl) to the cerebral spinal fluid. A successful spinal puncture was evidenced by a brisk tail-flick after the needle entry into subarachnoid space (change in resistance). >95% accuracy was achieved by a dye injection.

Measurement of nocifensive responses

All the nociceptive behavior assays were performed in a blinded manner by an independent observer. Thermal nociceptive responses were determined using Hargreaves's radiant heat apparatus (IITC Life Sciences, Woodland Hills, CA). Briefly, mice received an automatic heat source (50 W/10 V) onto the plantar surface of the hind paw and the paw withdrawal latency (PWL) was recorded. We adjusted the basal PWL to 9–12 s and set a cut-off of 20 s (defined as complete analgesia) to prevent tissue damage. The percentage of maximal possible antinociceptive effect (%MPE) was calculated with the equation $\%MPE = [(PL - BL2) / (BL1 - BL2)] \times 100$, where BL1 represents baseline latency before inflammation, BL2 represents baseline latency after inflammation but before drug injection, and PL represents latency after drug injection.

We evaluated mechanical pressure hypersensitivity in hind paw using a Randall–Selitto Pressure Analgesiometer (IITC Life Sciences).

We determined tactile allodynia by quantifying the withdrawal threshold of the hind paw in response to stimulation with flexible von Frey filaments (range 0.08–3.0 g; IITC Life Sciences). Mice were placed in Plexiglas boxes on a stainless steel mesh floor and allowed to adjust for at least 30 min. We then applied a series of calibrated von Frey hairs (six times each on the basis of the up-and-down method) perpendicularly to the plantar surface with sufficient force to bend the filament for 4–5 s. Brisk withdrawal or paw flinching were considered positive responses. We recorded the 50% withdrawal threshold (i.e., force of the von Frey hair to which an animal reacts in 50% of the presentations). %MPE to tactile hypersensitivity was calculated as described above.

Measurement of neuropeptide contents

The neuropeptide contents in DRGs, spinal cords and skin were determined in protein extracts isolated from DRGs (pulled from at least 4 mice) and spinal cord (sub-dissected dorsal halves) of the lumbar region (L4–L5 segment) and of plantar skin of hind paws. In each case, tissue (50 mg) was homogenized in 1 ml of lysis buffer (50 mM Tris–HCl, pH 7.4, with 0.5 mM DTT, and 10 μg/ml of a cocktail of proteinase inhibitors containing phenylmethylsulfonyl fluoride, pepstatin and leupeptin, all from Sigma) and centrifuged at 20,000 *g* for 15 min at 4 °C. The supernatants were collected and stored at −80 °C until use. In some experiments, the ipsilateral and contralateral DRGs and dorsal spinal cord halves were isolated separately. The contents of cortistatin, substance P, adrenomedullin and CGRP in the protein extracts were determined with specific ELISA kits from Phoenix Pharmaceuticals (Karlsruhe, Germany) following the manufacture's recommendations (according to the company information these ELISA kits were specific for the corresponding neuropeptide and did not cross-react with other related peptides).

To measure the contents of cortistatin in synovial fluid of arthritic mice, we excised the skin overlying the knee and then carefully dissected the patellar ligament to expose the synovial membrane. We inserted a 30-gauge needle through the membrane and washed twice the synovial cavity with 25 μl of heparinized saline (5 units/ml). The levels of cortistatin in the collected fluid were determined by ELISA as described above.

Moreover, we determined gene expression of cortistatin in total RNA isolated from lumbar L4–L6 DRGs (pooled from two mice), lumbar spinal cord segments or brain cortex by semiquantitative RT-PCR, using the following primers: cortistatin FW: 5′-AAGAGACCCTCGTCCACCAA-3′; cortistatin RV: 5′-ACCAGGCAAGGAAAGTCAGAAG-3′, β-actin FW: 5′-TGTTACCAACTGGGACGACA-3′; β-actin RV: 5′-GGGGTGTTGAAGGTCT CAAA-3′.

Primary DRG cultures

We performed primary DRGs cultures as previously described (Malin et al., 2007) with slight modifications. Briefly, DRGs (L3-L5) were aseptically removed from 4-wk-old mice and digested first with papain (40 U/ml) for 10 min and then with collagenase/dispase-II (800 U/ml and 157.4 U/ml, respectively) for 10 min at 37 °C. DRG cells were mechanically dissociated with a flame-polished Pasteur pipette and then plated onto poly-D-lysine and laminin-coated slide chambers. DRG cells were cultured in a DMEM-F12-defined medium (Invitrogen, Madrid, Spain) supplemented with 10% fetal calf serum and 1% penicillin/streptomycin for 24 h. DRG cells were cultured with medium alone or stimulated with capsaicin (1 μM), BDNF (20 ng/ml) or NGF (20 ng/ml) in the absence or presence of cortistatin (100 nM). After 24 h, supernatants were collected and assayed for cortistatin, adrenomedullin, substance P and CGRP contents by specific ELISA kits. DRG cells were fixed with 4% paraformaldehyde for 20 min and assayed for immunofluorescence detection of cortistatin, pAkt and pERK as described below.

Microfluorimetric calcium measurements

DRG cells were plated on poly-D-lysine/laminin-coated glass bottom dishes, cultured in a DMEM-F12-defined medium supplemented with 10% fetal calf serum and 1% penicillin/streptomycin for 24 h and then incubated with 3 μM of the Ca^{2+} sensitive dye Fura-2-acetoxymethyl ester (Molecular Probes, Madrid, Spain) in External Control Solution (ECS: 145 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM D-glucose and 10 mM HEPES, pH 7.4) for 30 min at 37 °C in 5% CO_2, in a humidified incubator. After two washes with EC, cells were pre-incubated for 3 min with 2 μM cortistatin, 500 nM somatostatin or 1 μM ghrelin before applying a pulse of 30 s with 500 nM capsaicin using a multibarreled gravity-driven, manually operated perfusion system. Coverslips were then continuously perfused (1 ml/min) with ECS at 20–22 °C. When indicated, cells were pre-treated with 1 μM cycloSOM for 5 min before adding cortistatin. Fluorescence measurements were performed with an Olympus IX81 inverted microscope through a 20× air objective. Fura-2 was excited consecutively at 340 nm and 380 nm using a computer controlled Lambda-10-2 filter wheel (Sutter Instruments, Novato, CA), and emitted fluorescence was filtered with a 510 nm long-pass filter and recorded with a CCD camera. Fluorescence from individual neurons was monitored as a function of time in a region of interest near to and within the cell boundary. The changes of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) are depicted as ratio of fluorescence intensities collected at 340 nm and 380 nm (ΔF340/380) at a time interval of 2 s (CellˆR 1.2 Olympus software). The threshold for capsaicin positive cells (ΔF340/380 = 0.04) was set

to 4 fold the standard deviation of the Ca^{2+} signal evoked by ethanol (vehicle).

Immunofluorescence staining of spinal cord and DRG cultures and sections

We perfused mice through the ascending aorta with cold-PBS and 4% paraformaldehyde/0.1 M phosphate buffer, pH 7.4. Spinal cord and DRGs from levels L3–L5 were removed, post-fixed in 4% paraformaldehyde for 4–8 h, cryoprotected in 30% sucrose/0.1 M phosphate buffer at 4 °C, embedded in OCT and cryosectioned (10 μm). Sections and DRG cells growth in slide chambers were blocked with 10% goat serum (30 min, 20 °C), incubated with rabbit anti-cortistatin (1:100, Phoenix Pharmaceuticals), guinea pig anti-CGRP (1:400, N20, Santa Cruz Biotechnology, Dallas, TX), mouse anti-GAD65 (1:400, ab26113, Abcam, Cambridge, MA), guinea pig anti-substance P (1:400, ab10353, Abcam), rabbit anti-phosphorylated Akt (1:200, anti-Thr308, Cell Signaling, Danvers, MA), mouse anti-TRPV1 (1:500, MAB5568, Merck Millipore, Billerica, MA) or rabbit anti-phosphorylated ERK (1:200, anti-p44/42 MAPK, Cell Signaling) antibodies (8 h, 4 °C) and detected with goat anti-rabbit, anti-mouse or anti-guinea pig secondary antibodies conjugated to Alexa Fluor-546 (1:1000, Invitrogen) or FITC (1:400, Jackson Immunoresearch, Suffolk, UK) or with Alexa Fluor-568-conjugated IB4 (1:500, I21412, Invitrogen) for 1 h at 20 °C. Slices were nuclei counterstained with Hoechst 33258 (1 μM for 5 min, Sigma), mounted in Prolong Gold antifade solution (Invitrogen) and examined in a Nikon fluorescence microscope. In some samples, we used Tyramide signal amplification system (TSA, PerkinElmer, Boston, MA) following the manufacturer's recommendations. Moreover, we analyzed cortistatin-staining in DRG sections by immunohistochemistry using the Dual-Link Horseradish Peroxidase System kit (Dako, Barcelona, Spain) for light microscopy. We captured images with a CCD spot camera and determined the diameter and staining intensity for each neuron using image analysis software (Sigma Scan, SPSS) as previously described (Ma et al., 2006). As controls, primary antibody omission resulted in negative staining in all tested sections. We also used normal rabbit serum (1:100) instead the primary rabbit anti-cortistatin antibody as negative control. Moreover, immunofluorescence and immunohistochemical analysis of cortistatin expression in DRGs and spinal cords of CST-KO mice resulted in the complete absence of staining.

Measurement of motor performance and locomotor activity

To evaluate the possible nonspecific muscle-relaxant or sedative effects of cortistatin, mice were submitted to the rotarod task and open-field test. To measure motor coordination, the rotarod apparatus (IITC Life Sciences) was set at a constant speed of 17 rpm, with alternative changes of direction every 25 s. The animals were selected 24 h previously by eliminating those mice that did not remain on the bar for two consecutive periods of 90 s. Animals were treated with cortistatin (1000 ng/i.p., 100 ng/i.pl. or 10 ng/i.t.) or saline (200 μl/i.p., 20 μl/i.pl. or 10 μl/i.t.) 15 min before being tested. The results are expressed as the length of time that each animal remained on the rotarod. The cut-off time used was 300 s.

The ambulatory behavior was assessed in an open-field test. The apparatus consisted of a wooden box measuring 40 × 60 × 50 cm. The floor of the arena was divided into 12 identical squares. Mice were treated with cortistatin (1000 ng/i.p., 100 ng/i.pl. or 10 ng/i.t.) or saline (200 μl/i.p., 20 μl/i.pl. or 10 μl/i.t.), and 15 min later mice were put in the center of the field and observed for 10 min. The number of times that the animals crossed the outlines of the squares (horizontal activity) was counted as well as the number of rearings (vertical activity).

Statistical analysis

All data are expressed as the mean ± SEM. Statistical analysis was performed by a statistical package (Graph-Pad Prism Software, San Diego, CA). We assumed significance at $p < 0.05$. For in vitro experiments, data were analyzed using an unpaired, two-tailed Student's *t*-test when two groups were compared, or one-way analysis of variance (ANOVA) for multiple group comparisons. For ex vivo determinations and behavioral pain tests, normality was tested with a D'Agostino-Pearson omnibus test to determine whether parametrical Student's *t*-test or nonparametrical Mann–Whitney *U*-test would be used when two groups were compared. For multiple group comparisons, we used a regular one-way ANOVA and post-hoc Bonferroni's tests, or the nonparametric equivalence Kruskal–Wallis test. For behavioral pain time courses and von Frey hair applications, we used two-way ANOVA with repeated measures and post-hoc Bonferroni's tests.

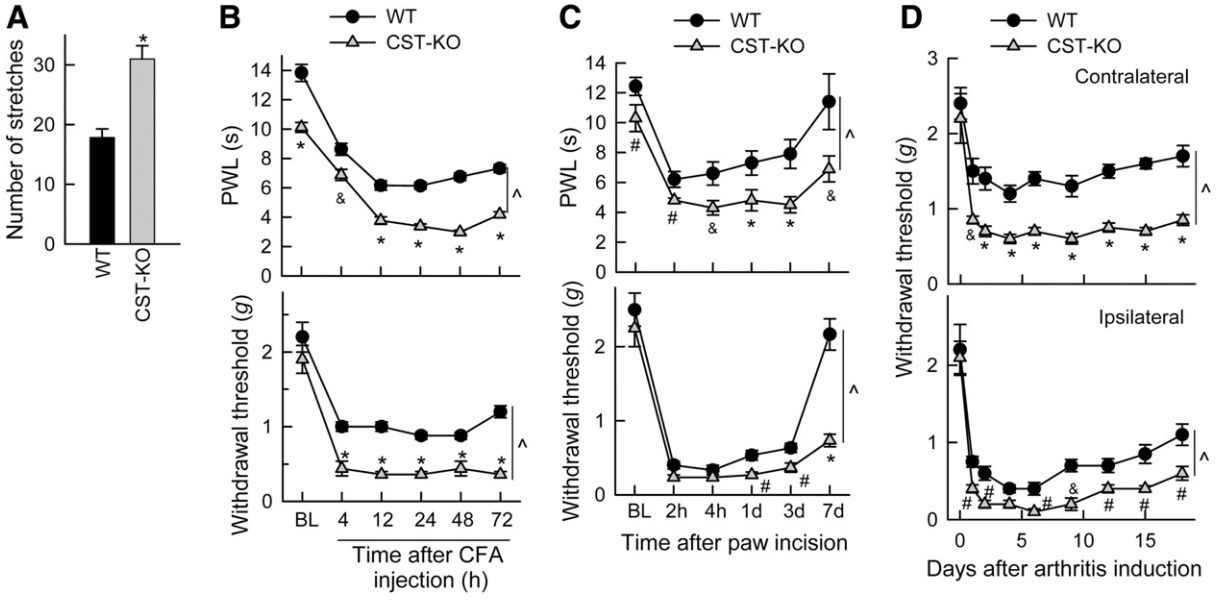

Fig. 1. Lack of cortistatin exacerbates nociceptive responses to inflammatory pain. Comparative nocifensive responses in wild-type (WT) and cortistatin-deficient (CST-KO) mice to acetic acid-induced visceral pain (A), to CFA-induced chronic inflammatory pain (B), to paw incision-induced allodynia and thermal hyperalgesia (C) and to arthritis-induced bilateral allodynia (D). Thermal hyperalgesia was determined by measuring the paw withdrawal latency (PWL) in response to radiant heat and tactile allodynia by determining the withdrawal threshold to plantar von Frey hair applications. BL: baseline. n = 6–8 mice/genotype. *$p < 0.0001$, &$p < 0.005$, #$p < 0.05$ vs. WT. ^$p < 0.001$ between groups.

Results

Lack of cortistatin exacerbates inflammatory pain responses

We first generated mice lacking the cortistatin gene to investigate the role played by this neuropeptide produced endogenously in pain regulation in physiological and pathological states. Cortistatin-deficient (CST-KO) mice showed increased basal sensitivity to heat, but not to mechanical stimulation, compared to wild-type mice (Supplementary Fig. 1). Furthermore, lack of cortistatin evoked higher visceral painful responses to i.p. injection of acetic acid (Fig. 1A) and exacerbated heat hyperalgesia and tactile allodynia elicited by CFA-induced chronic inflammation in the paw (Fig. 1B). Similarly, CST-KO mice showed increased and longer thermal and mechanical hypersensitivity than wild-type mice in a preclinical model of post-operative pain (Fig. 1C). Interestingly, when all wild-type mice fully recovered (7 d after the surgery), CST-KO mice still suffered allodynia (Fig. 1C). Finally, lack of cortistatin resulted in an exacerbated bilateral secondary allodynia (especially in the contralateral side) in mice suffering monoarticular arthritis caused by a combination of irritant agents (carrageenan and kaolin) and mechanical damage (Fig. 1D). These findings support a potential role of this neuropeptide in nociception.

The nociceptive system produces cortistatin in response to noxious stimuli

Although the expression of cortistatin gene was reported in human DRGs (Robas et al., 2003), the occurrence of cortistatin in the nociceptive pathways is largely unknown. We found high levels of cortistatin in mouse DRGs and spinal cord segments of lumbar region, comparable to those found in brain cortex (Fig. 2A). Immunohistochemistry analysis of DRGs demonstrated that cortistatin is mainly expressed by small-to-

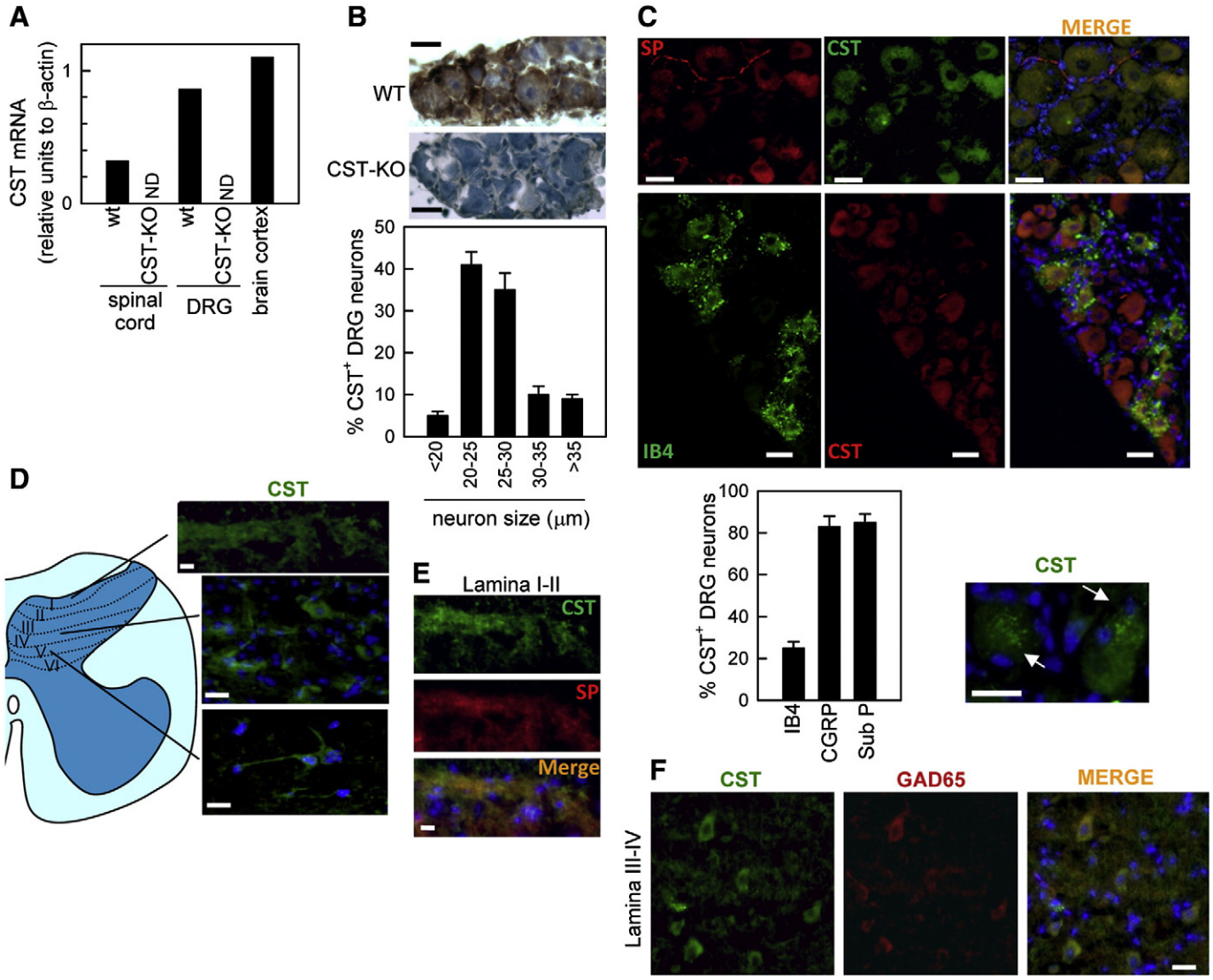

Fig. 2. Expression of cortistatin by the peripheral nociceptive system of mouse. A: Cortistatin (CST) content in DRGs and spinal cord of lumbar region (L4-L6) and in brain cortex (positive control). We used samples from cortistatin-deficient (CST-KO) mice as negative controls (ND, not detected). n = 3 mice, in duplicate. B: Quantitative analysis of size frequency of cortistatin-positive neurons based on immunohistochemical analysis in lumbar DRGs from wild-type (WT) and CST-KO mice (negative controls). Rabbit serum instead of primary anti-cortistatin antibody was also used as negative control (not shown). Samples were counterstained with hematoxylin. Scale bar: 25 μm. n = 5 mice. C: Quantitative analysis of cortistatin-positive neurons and its co-localization with markers of peptidergic (CGRP and substance P) and nonpeptidergic (isolectin B4) nociceptors in lumbar DRGs. Note the preferential localization of cortistatin-staining in intracellular perinuclear vesicles in cell bodies (white arrows). Dual immunofluorescence of cortistatin and substance P in DRG sections from CST-KO mice were used as negative controls (see Supplementary Fig. 2A). Scale bars: 30 μm. Nuclei were counterstained with Hoechst. n = 5–7 mice. D: Immunodetection of cortistatin in different laminas of lumbar spinal cord dorsal horn. n = 5 mice. E: Co-localization of cortistatin and substance P (SP) in laminas I and II of spinal cord dorsal horn. Scale bar: 40 μm. F: Co-localization of cortistatin and the GABA-processing enzyme (GAD65) in neurons of laminas III and IV of spinal cord dorsal horn analyzed by fluorescence. Scale bar: 25 μm.

medium neurons, generally recognized as nociceptive neurons, and only occasionally by large sensitive neurons (Fig. 2B). According to this observation, cortistatin mostly (>80%) co-localized with peptidergic CGRP/substance P-expressing nociceptors, resulting a total of 96% of peptidergic neurons expressing cortistatin (Fig. 2C, Supplementary Fig. 2A). Moreover, around 20% of neurons that express cortistatin corresponded to non-peptidergic isolectin-B4^{+} nociceptors and 60% of neurons that bind IB4 expressed cortistatin (Fig. 2C). In some cases, cortistatin accumulated in perinuclear vesicles in cell bodies of DRG neurons (pointed by arrows in lower panel Fig. 2C). In spinal cord, cortistatin-staining was predominantly enriched in axonal-terminals in the superficial dorsal horn (LI–LII, which correspond to the entrance of nociceptive central terminals) and in cell profiles of LIII–LV (Fig. 2D). As expected, cortistatin-staining co-localized with substance P in axonal-terminals of LI–LII of dorsal horn (Fig. 2E). Interestingly, most of the cortistatin-expressing cells of deep layers of spinal dorsal horn were GABA-ergic interneurons with short axonal-terminals that co-express glutamic acid decarboxylase GAD65 (Fig. 2F) and GABA (not shown).

Next, we asked whether cortistatin expression in nociceptive pathways changes with noxious stimuli. Direct activation of peptidergic nociceptors with an inflammatory mediator such as NGF increased the release of cortistatin in dissociated DRG neuron cultures (Fig. 3A). In contrast, BDNF, which specifically activates non-peptidergic nociceptors, failed to increase significantly the production of cortistatin by DRG neurons (Fig. 3A). Immunofluorescence analysis of DRG cultures confirmed the expression of cortistatin in neuronal soma and axons/terminals and showed that the cortistatin-specific staining increased in neurons activated with NGF (Supplementary Fig. 2B). Moreover, we found that cortistatin localized in DRG neurons expressing the vanilloid sensitive receptor TRPV1 (Supplementary Fig. 2C), a nonselective cation channel that integrates nociceptive thermal and chemical stimuli and it is crucial for central sensitization to inflammatory pain (Caterina et al., 2000; Shu and Mendell, 1999). According to this finding, the production of cortistatin by DRG neurons increased in the presence of the TRPV1 agonist capsaicin (Fig. 3A). Next, we tried to corroborate these findings in vivo. Thus, acute pain evoked by plantar or spinal injection of capsaicin increased the levels of cortistatin in lumbar DRGs and plantar skin (Fig. 3B). Similarly, chronic inflammatory pain elicited by plantar injection of CFA increased the contents of cortistatin in lumbar DRGs and spinal cord in the ipsilateral, but not the contralateral side (Fig. 3C). Moreover, cortistatin was elevated in synovial fluid of knees with arthritis (Fig. 3D). These results indicate that inflammatory and noxious stimuli induce the release of cortistatin by peripheral and spinal terminals of peptidergic nociceptors.

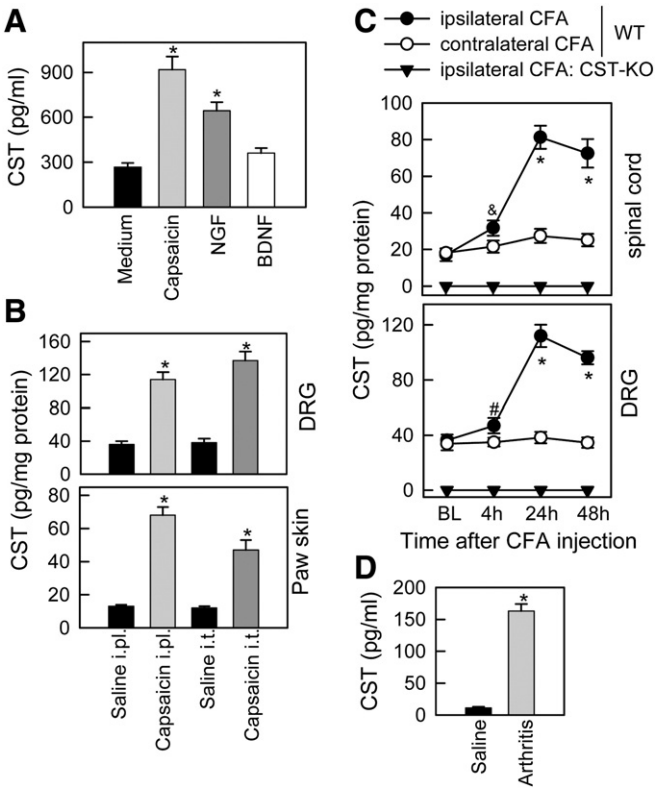

Fig. 3. The nociceptive system produces cortistatin in response to inflammatory and noxious stimuli. A: Noxious stimuli, capsaicin and NGF, but not BDNF, stimulated cortistatin release by DRG nociceptors in vitro (24 h of culture). n = 3 mice, in duplicate. B: Contents of cortistatin in protein extracts isolated from lumbar DRGs and plantar skin of paws isolated 1 h after the injection i.pl. or i.t. of saline or capsaicin. n = 3–4 mice/group. C: Levels of cortistatin in lumbar DRGs and dorsal spinal cord (ipsilateral and contralateral to the injected paw) isolated before (BL) or at different time points after i.pl. injection of CFA to wild-type (WT) and cortistatin-deficient (CST-KO) mice. n = 5 mice/group. D: Inflammatory stimuli induced the release of cortistatin in periphery. Contents of cortistatin in synovial fluid isolated from knees 24 h after the induction of arthritis. Contralateral knees injected with saline were used as controls. n = 4 mice/group. $^{*}p < 0.0001$, $^{\&}p < 0.005$, $^{\#}p < 0.05$ vs. BL or medium.

Cortistatin ameliorates acute and chronic inflammatory pain

We next evaluated the actions of cortistatin in different models of pathologic pain. To discriminate its site of action, we used three routes of administration: systemic (i.p.), peripheral (i.pl.) and spinal (i.t. by lumbar puncture). First, we found that cortistatin reduced the nocifensive response in a model of acute visceral pain when administered i.p. and i.t. (Fig. 4A). Notably, cortistatin showed higher analgesic effects than the related neuropeptides ghrelin and somatostatin (or its analog octreotide) and it was as effective as the treatment with drugs of reference such as acetyl salicylic acid and the COX-2 inhibitor NS-398 (Fig. 4A).

In a model of CFA-induced chronic inflammatory pain, mice initially suffered tactile and mechanical hypersensitivity followed by persistent thermal hyperalgesia (Fig. 4B). The injection of cortistatin through any of the three routes attenuated allodynia as well as thermal and mechanical hyperalgesia (Fig. 4B). To discriminate its analgesic action from its anti-inflammatory effect (Gonzalez-Rey et al., 2006a, 2007), we injected cortistatin 1–3 d after CFA, when hyperalgesia and allodynia were fully developed. Spinal delivery showed the highest efficiency and cortistatin was again more efficient than somatostatin, ghrelin and NS-398 (Figs. 4C and D).

Notably, from a clinical point of view, cortistatin markedly reduced mechanical allodynia in more relevant preclinical models of pain, such as plantar incision-induced postoperative pain (Fig. 5A) and knee arthritis (Figs. 5B and C). The model of unilateral knee arthritis is particularly useful for studying central changes elicited by enhanced excitatory drive from the periphery into the spinal cord, because the primary hyperalgesia evoked in the injured knee is accompanied by secondary long-lasting hyperalgesia in the ipsilateral and contralateral hind paws. Systemic and repetitive treatment with cortistatin markedly reduced intensity and duration of bilateral allodynia (Fig. 5B) and ongoing knee inflammation (Supplementary Fig. 3A). Even more important, spinal and systemic injection of cortistatin reduced fully developed bilateral allodynia (Fig. 5C), in the absence of significant effects in inflammation during this short time period of treatment (Supplementary Fig. 3B). These findings suggest that cortistatin attenuates injury-evoked spinal changes in the processing of nociceptive inputs independently of its effects in the peripheral inflammatory response.

Importantly, we found that cortistatin did not alter significantly baseline sensory (thermal and tactile) thresholds in naive mice (Supplementary Fig. 4A; $p > 0.05$ between groups). Moreover, the analgesia evoked by cortistatin was not due to impairment in motor performance (Supplementary Figs. 4B and C, $p > 0.05$ between groups).

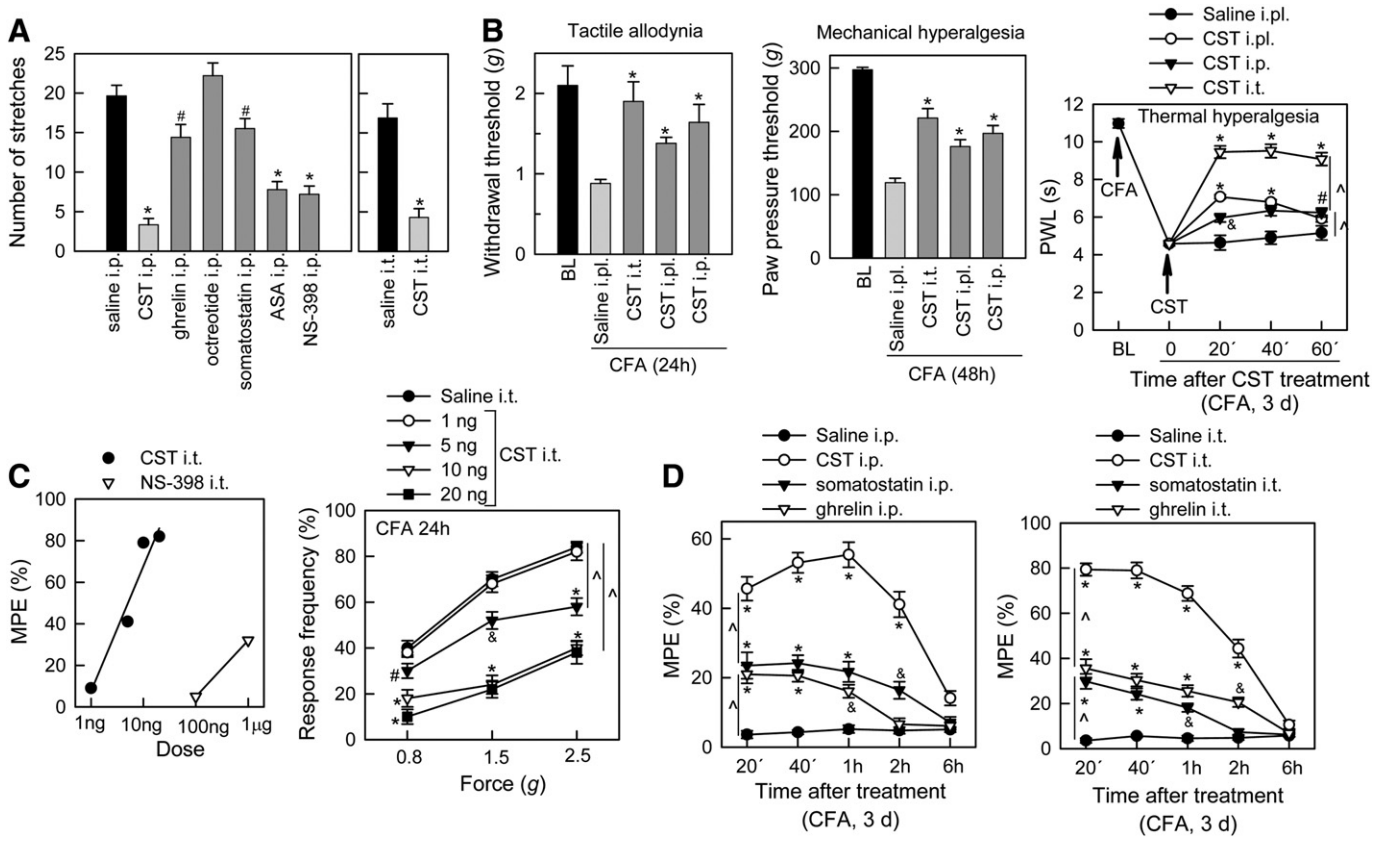

Fig. 4. Analgesic actions of cortistatin in acute visceral pain and chronic inflammatory pain. A: Cortistatin reduced acetic acid-induced acute abdominal pain. Left panel: comparative effect of i.p. administration of cortistatin, ghrelin, octreotide, somatostatin (each at 1 μg), acetyl salicylic acid (ASA, 2 mg) and NS-398 (5 μg). Right panel: effect of i.t. injection of cortistatin (10 ng). n = 6–8/group. B: Spinal, peripheral and systemic cortistatin alleviated CFA-induced chronic thermal hyperalgesia, mechanical hypersensitivity and allodynia. Withdrawal threshold to plantar von Frey hair applications (left panel), mechanical hypersensitivity represented as the paw pressure threshold (middle panel) and paw withdrawal latency (PWL) in response to radiant heat (right panel) assayed before CFA (BL) and at various time points (15 min in middle and left panels) after the injection of cortistatin (i.t. at 10 ng; i.pl. at 100 ng; i.p. at 1 μg; starting 1–3 d after CFA). n = 6–7/group. C: Dose–response curve of cortistatin-mediated analgesic action on chronic inflammatory pain. Left panel: Comparative effect of cortistatin and NS-398 (expressed as maximum possible analgesic effect, MPE) on CFA-induced heat hyperalgesia (determined 30 min after treatment). Right panel: Effect of spinal injection of cortistatin (administered at different doses 24 h after CFA) on the response frequencies to plantar von Frey hair applications (determined 15 min after CST injection). n = 5–6/group. D: Comparative MPE of cortistatin, ghrelin and somatostatin on CFA-evoked heat hyperalgesia. Neuropeptides were injected i.p. (1 μg) or i.t. (10 ng) 3 d after the injection of CFA. The response to radiant heat was determined at different time points after injecting each neuropeptide. n = 6–8/group. $^{*}p < 0.0001$, $^{\&}p < 0.005$, $^{\#}p < 0.05$ vs. saline. $^{\wedge}p < 0.05$ between groups.

Mechanisms involved in the analgesic effect of cortistatin

To determine the peripheral and spinal mechanisms through which cortistatin attenuates inflammatory pain, we examined its impact on TNFα and NGF signaling, because both factors are key contributors to the genesis of inflammation and pain via both peripheral and central mechanisms (Hucho and Levine, 2007; Julius and Basbaum, 2001). Spinal and peripheral treatment with cortistatin abolished heat hyperalgesia and allodynia evoked by intraplantar injection of NGF (Fig. 6A) and TNFα (Supplementary Fig. 5A). Because CFA, TNFα and NGF partially exert their effects in inflammatory pain by increasing the activity of TRPV1 in primary nociceptors (Caterina et al., 2000; Shu and Mendell, 1999), we evaluated the effect of cortistatin in the nociceptive response elicited by the TRPV1 agonist capsaicin. Intraplantar injection of capsaicin evoked acute spontaneous pain that was followed by persistent allodynia (Fig. 6B). Preemptive and delayed injection of cortistatin by either spinal or peripheral routes reduced these nocifensive responses (Fig. 6B). Interestingly, peripheral and spinal injection of cortistatin alleviated the nociceptive responses elicited centrally by TNFα (Fig. 6C) and capsaicin (Fig. 6D). These findings indicate that cortistatin inhibits direct sensitization of nociceptors by inflammatory and algogenic mediators at both peripheral and spinal levels.

We further observed similar antinociceptive effects of cortistatin in pain elicited peripherally (Supplementary Fig. 5B) and spinally (Fig. 6D) by glutamate, the main neurotransmitter involved in peripheral and central pain sensitization. This later finding suggests that, besides its action on presynaptic nociceptive terminals, cortistatin could impair glutamate-induced spinal sensitization in secondary postsynaptic neurons.

To sensitize/maintain pain in DRG neurons, numerous inflammatory and noxious stimuli require signaling through the kinases ERK and Akt. Activation of Akt and ERK initiates a cascade of events that facilitate the sensitization of nociceptors, involving activation and expression of TRPV1, synthesis and release of CGRP and substance P by peripheral and central terminals, and activation of tetrodotoxin-resistant sodium channels Nav1.8/1.9, subsequent membrane depolarization and release of excitatory neurotransmitters such as glutamate (Hucho and Levine, 2007). Cortistatin inhibited the activation of ERK and Akt by nociceptive neurons stimulated with inflammatory and algogenic factors in vitro (Fig. 7A, Supplementary Fig. 6A). Moreover, cortistatin impaired the activation of TRPV1 by capsaicin, because it reduced the changes in $[Ca^{2+}]_i$ induced by this algogen in DRG neurons (Fig. 7B). Interestingly, somatostatin, but not ghrelin, mimicked the inhibitory effect of cortistatin in capsaicin-induced $[Ca^{2+}]_i$ (Fig. 7B). As a consequence of its effects on ERK/Akt and $[Ca^{2+}]_i$, cortistatin decreased the release of pronociceptive peptides (CGRP, substance P and adrenomedullin) by DRG neurons (Fig. 7A, Supplementary Fig. 6B). Importantly, we confirmed these effects in vivo. Thus, we observed that cortistatin decreased, at both peripheral and spinal levels, the activation of Akt (Fig. 7C, Supplementary Fig. 6C) and ERK (not shown) and the synthesis

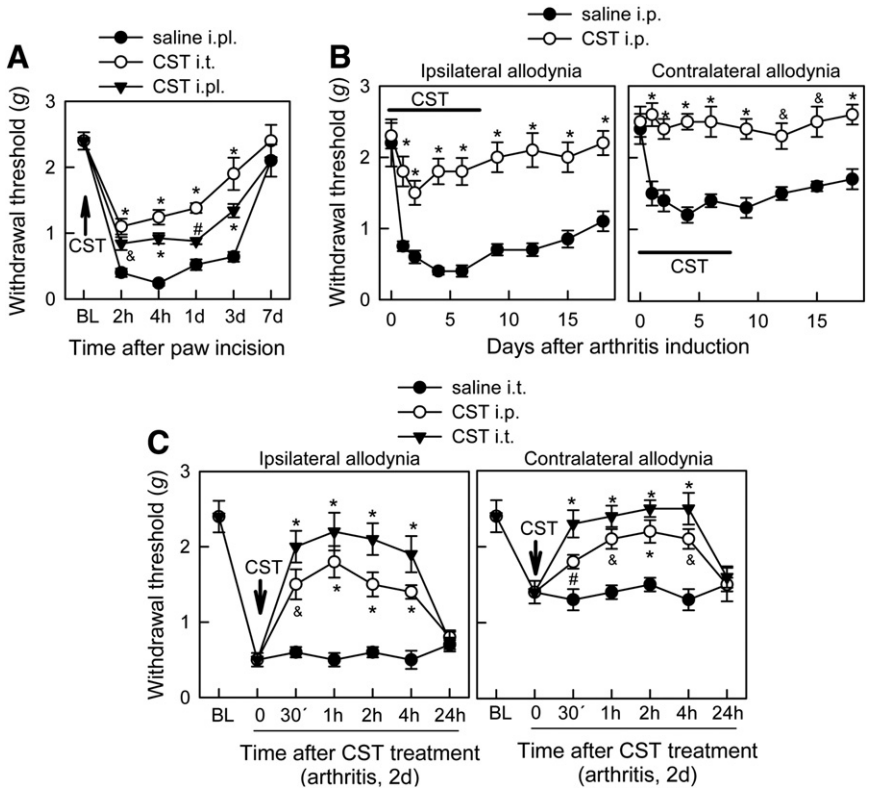

Fig. 5. Analgesic effects of cortistatin in postoperative and arthritic inflammatory pain. A: Inhibitory effects of cortistatin (injected i.t. at 10 ng or i.pl. at 100 ng, immediately before surgery) in paw incision-induced postoperative allodynia. n = 6–7/group. $p < 0.05$ between groups. B and C: Cortistatin ameliorated bilateral allodynia evoked by monoarticular knee arthritis. Cortistatin was administered i.p. at 1 μg or i.t. at 10 ng once daily from day 1 to day 8 after the induction of arthritis (B) or at day 2 on mice with established arthritis (C). The withdrawal threshold to plantar von Frey hair applications to hind paws ipsilateral and contralateral to the arthritic knee was determined at baseline (BL) prior to induction of arthritis and at different time points after inducing arthritis (B) or after the injection of cortistatin (C). n = 6–8/group. $p < 0.05$ between groups. $^{*}p < 0.0001$, $^{\&}p < 0.005$, $^{\#}p < 0.05$ vs. saline at the indicated time points.

and/or release of pronociceptive peptides (Fig. 7D, Supplementary Fig. 6D) by DRG neurons and their central terminals in response to inflammatory and algogenic stimuli.

Moreover, endogenous cortistatin seems to play a major role in the regulation of the molecular events that tune peripheral pain perception. Thus, direct activation of nociceptors with capsaicin or glutamate elicited higher nocifensive responses and allodynia in CST-KO mice than in wild-type mice (Figs. 8A and B). This observation correlated with the fact that CST-KO mice showed increased production of nociceptive mediators in lumbar DRGs and spinal cords in comparison to wild-type animals (Fig. 8C). Cortistatin-deficiency in primary nociceptors could be the responsible of this altered nocifensive behavior, since DRG neurons lacking cortistatin expressed higher levels of phosphorylated-ERK and produced more CGRP than wild-type DRG neurons (Fig. 8D).

All these findings indicate that cortistatin might attenuate pain and modulate synaptic plasticity by interfering with ERK/Akt-mediated release of nociceptive peptides by primary nociceptors in response to inflammatory stimulation and TRPV1 activation.

Differential involvement of somatostatin- and ghrelin-receptors in the peripheral and spinal actions of cortistatin in pain regulation

We next investigated the involvement of specific receptors in the analgesic actions of cortistatin. Because cortistatin binds to all somatostatin-receptors (sstr1-5) and ghrelin-receptor (GHSR1) and some of these receptors, including sstr1, sstr2 and GHSR1, are differentially expressed in murine DRG and spinal cord neurons (Bar et al., 2004b; Bencivinni et al., 2011; Inhof et al., 2011; Segond von Banchet et al., 1999; Vergnano et al., 2008), they emerge as potential mediators of the analgesic effects of cortistatin. Indeed, spinal and peripheral injection of a nonselective sstr-antagonist (cyclosomatostatin) and of two specific sstr2-antagonists (Bar et al., 2004b; Tulipano et al., 2002) partially reversed the analgesic effects of cortistatin in CFA-induced chronic pain (Fig. 9A). However, we observed no reversal effect by an antagonist for sstr5 (Fig. 9A), which is not expressed in the nociceptive system of mice (Inhof et al., 2011). Moreover, two different specific GHSR1-antagonists (Jerlhag et al., 2010) partially abrogated the analgesia evoked by cortistatin in chronic inflammatory pain solely when they were administered i.t., but not i.pl. (Fig. 9A). Importantly, co-injection of sstr/sstr2- and GHSR1-antagonists at the spinal level, but not at the periphery, showed a significant additive effect and almost completely abrogated the analgesia caused by cortistatin in chronic pain (Fig. 9A). Interestingly, blockade of peripheral and central sstr/sstr2, but not of GHSR1, reversed the inhibitory effect of cortistatin in CFA-induced production of CGRP (Fig. 9B) and substance P (not shown) by DRG and spinal cord neurons. Moreover, the sstr antagonist fully reversed the inhibitory action of cortistatin in capsaicin-induced increases of $[Ca^{2+}]_i$ in DRG neurons (Supplementary Fig. 7A). These results indicate that cortistatin mediates its effects in chronic inflammation-induced hyperalgesia and allodynia via GHSR1 and sstr (probably sstr2) at the spinal level, and through its binding to sstr/sstr2 at the periphery. The specificity of site of action of the antagonists used in this study was confirmed by the fact that they did not affect the cortistatin effects when administered in a place other than the route of cortistatin injection (Supplementary Fig. 7B). Moreover, spinal and peripheral injection of PTX, an inhibitor of the Gαi-protein subunit, abolished cortistatin actions in CFA- and capsaicin-induced nocifensive responses and substance P production by DRGs and spinal cord (Figs. 9C and D, Supplementary Fig. 7C). In contrast, naloxone, an opioid-receptor antagonist, did not affect the analgesic effect of cortistatin (Fig. 9C). The effects of

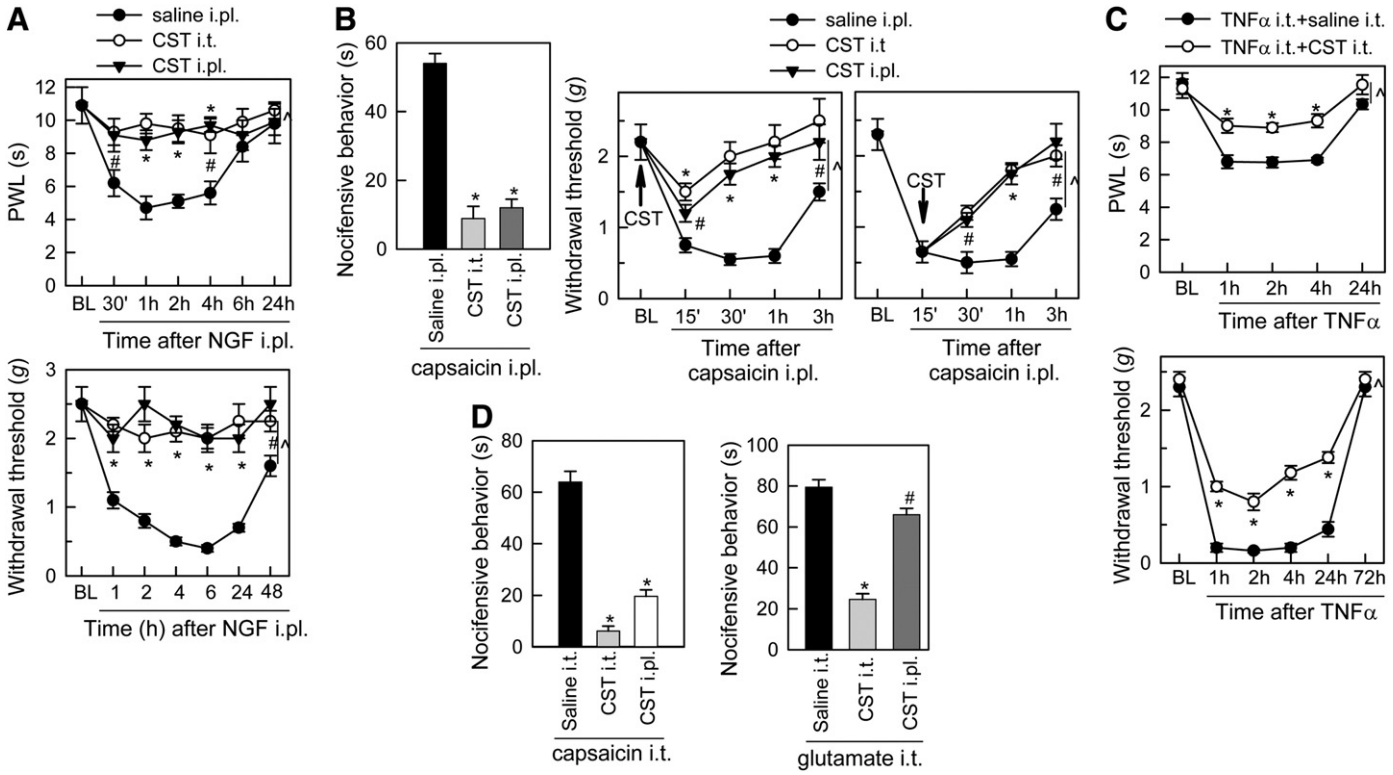

Fig. 6. Analgesic effect of cortistatin in inflammatory pain evoked by TNF, NGF, capsaicin and glutamate. A: Spinal (i.t.) and peripheral (i.pl.) effects of cortistatin in heat hyperalgesia (PWL, paw withdrawal latency in response to radiant heat) and tactile allodynia (withdrawal thresholds to plantar von Frey hair applications) induced by i.pl. injection of NGF (20 ng, immediately after injecting saline or cortistatin). BL, baseline before the injection of NGF. n = 6–7/group. B: Spinal and peripheral actions of cortistatin in acute spontaneous pain and persistent mechanical allodynia evoked by i.pl. injection of capsaicin. Cortistatin was injected immediately before (BL, in left and middle panels) or 15 min after (right panel) injecting capsaicin. n = 6–7/group. C: Spinal and peripheral actions of cortistatin in persistent thermal hyperalgesia and allodynia induced by spinal injection of TNFα. n = 6/group. D: Spinal and peripheral injection of cortistatin attenuated acute spontaneous pain evoked by spinal injection of capsaicin and glutamate. n = 6–7/group. *$p < 0.0001$, #$p < 0.05$ vs. saline at the indicate time points. ^$p < 0.05$ between groups.

PTX could be mediated at the level of peripheral nociceptors since it abolished the inhibitory action of cortistatin on the production of nociceptive peptides and the activation of ERK in vitro (Fig. 9E). These findings support the involvement of Gαi-protein-coupled receptors, like sstr2 and GHSR1 (Dezaki et al., 2011; Pinter et al., 2006) and the inhibition of cAMP/PKA, which is generally related to nociceptive signaling (Aley and Levine, 1999; Hucho and Levine, 2007; Julius and Basbaum, 2001).

Discussion

This study identifies cortistatin as a new neuropeptide produced by the peripheral arm of the pain pathway involved in tuning nociception in physiological and inflammatory states and provides proof of principle for a therapeutic relevance of cortistatin in clinical pain. We demonstrated that cortistatin is mainly produced by peptidergic nociceptive neurons, although it is also secreted by non-peptidergic nociceptors. In response to peripheral tissue injury or local inflammation, peripheral and central terminals of nociceptors release cortistatin locally and at spinal level, presumably concomitantly with pronociceptive excitatory neuropeptides and neurotransmitters, to counterbalance pain sensitization, swelling and other long-term deleterious changes that accompany inflammation. Our data also indicate that nociceptors respond to central sensitization with inflammatory and algogenic factors. The fact that, in basal conditions, cortistatin appears stored in intracellular peptidergic vesicles in cell bodies of DRG neurons and in axons and nociceptive terminals, suggests that this neuropeptide could be rapidly released in acute pain states. Moreover, persistent stimulation of the nociceptor in chronic inflammatory conditions probably induces the sustained synthesis of new cortistatin in an attempt to modulate pain sensitization.

Our data clearly indicate that the endogenous production of cortistatin is important to define the final nociceptive response to inflammation, since lack of cortistatin resulted in exacerbated and longer thermal and tactile hyperalgesic responses in inflammatory states. Because we generated mice lacking cortistatin in all body tissues/cells, we cannot rule out that the higher thermal hyperalgesia observed in these animals is not a consequence of lack of control by cortistatin of supraspinal pain regulatory centers in brain. In fact, intracerebral infusion of cortistatin increased basal thermal sensitivity in rats (Mendez-Diaz et al., 2004). However, we found that DRG neurons deficient in cortistatin responded to pain sensitization with increased nociceptive signaling in vitro and in vivo, pointing out to a relevant role of cortistatin in the control of the peripheral nociceptive system.

Importantly from a therapeutic point of view is that, besides its effect on acute pain and heat hyperalgesia, cortistatin ameliorated hypersensitivity to mechanical stimuli in models of inflammatory pain. Tactile allodynia represents one of the most debilitating manifestations of pathological pain in clinical relevant conditions such as arthritis, nerve injury and postoperative conditions, often difficult to treat effectively with conventional drugs. Interestingly, the effect of cortistatin in inflammatory allodynia pertained not only to primary hyperalgesia in the injured tissue. Cortistatin also alleviated in magnitude and duration secondary mechanical hyperalgesia in places located far away from the affected member, a centrally maintained process (Hucho and Levine, 2007; Julius and Basbaum, 2001; Todd, 2010). This suggests that the cortistatin-induced impairment in activity of peripheral nociceptors is causally linked to an inhibition of excitatory drive into the spinal cord, which would thereby interferes central plasticity processes involved in secondary hyperalgesia. In this sense, our study

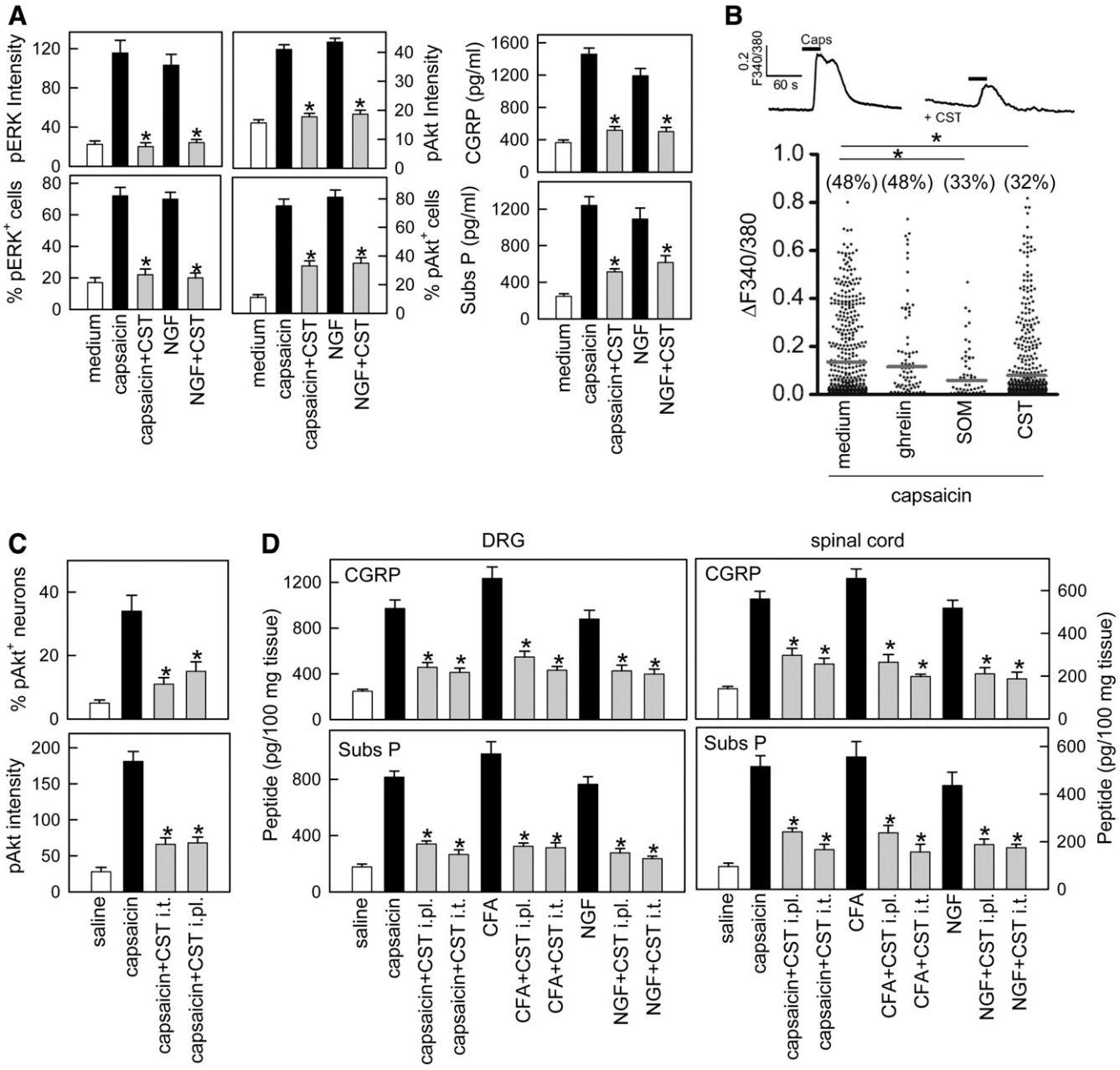

Fig. 7. Cortistatin impairs the activation of peptidergic nociceptors by inflammatory and noxious stimuli in vitro and in vivo. A: Cortistatin inhibits capsaicin- and NGF-induced activation of ERK and Akt (measured as percentage of phosphorylated-ERK$^+$ and phosphorylated-Akt$^+$ cells and mean fluorescence intensity per cell) in lumbar DRG neurons after 10 min of culture (see related Supplementary Fig. 6A for representative images of immunofluorescence). The levels of CGRP and substance P in culture supernatants were determined after 1 h of culture. n = 4/group, in duplicate. B: Effects of cortistatin, ghrelin and somatostatin (SOM) in the changes of $[Ca^{2+}]_i$ in DRG neurons stimulated with capsaicin. Results show the ratio of fluorescence intensities of Fura-2 collected at 340 nm and 380 nm (ΔF340/380) in each individual neuron. Gray horizontal lines correspond to mean of ΔF340/380 values for each experimental group. Numbers in parenthesis represent the percentage of cells in the culture that responded to capsaicin. Upper panels show representative profiles of response to capsaicin (caps, horizontal black bars) in untreated (left profile) and cortistatin-treated (right profile) neurons. C: Spinal and peripheral actions of cortistatin in the expression of phosphorylated-Akt in ipsilateral lumbar DRGs induced by i.pl. injection of capsaicin (at 15 min). See related Supplementary Fig. 6C for representative images of immunofluorescence. n = 4/group, in duplicate. D: Effects of cortistatin administered i.t. or i.pl. immediately before i.pl. injection of capsaicin or NGF or 24 h after i.pl. injection of CFA, on the expression of substance P and CGRP determined by ELISA in ipsilateral lumbar DRGs and spinal cords (isolated 1 h after injecting cortistatin). n = 4/group. *p < 0.0001, #p < 0.05 vs. capsaicin, NGF or CFA.

demonstrates that the analgesic effects of cortistatin are exerted at both peripheral and spinal level.

At the periphery, there were clear dissociations between the anti-inflammatory and analgesic activity of cortistatin which, via local interactions with specific receptors expressed in peripheral terminals of nociceptive neurons, directly inhibited ERK/Akt-mediated pain signaling and release of nociceptive peptides evoked by inflammatory factors and algogens. At spinal level, the most plausible mechanism for cortistatin actions is the inhibition of pain sensitization through receptors expressed in central terminals of presynaptic nociceptors, although our findings could also support its action in spinal secondary neurons (as discussed below). By using various specific antagonists for sstr and GHSR1, we demonstrated the differential participation of these receptors in the analgesic action of cortistatin in persistent and chronic inflammatory pain, with a predominant involvement of sstr2 at the periphery and of both sstr2 and GHSR1 at the spinal level. However, besides sstr and GHSR1, we cannot rule out the involvement of a still unidentified cortistatin-specific receptor that mediates its unique functions in nervous system, which are not shared with somatostatin or ghrelin (Gahete et al., 2008). The capacity of cortistatin to activate

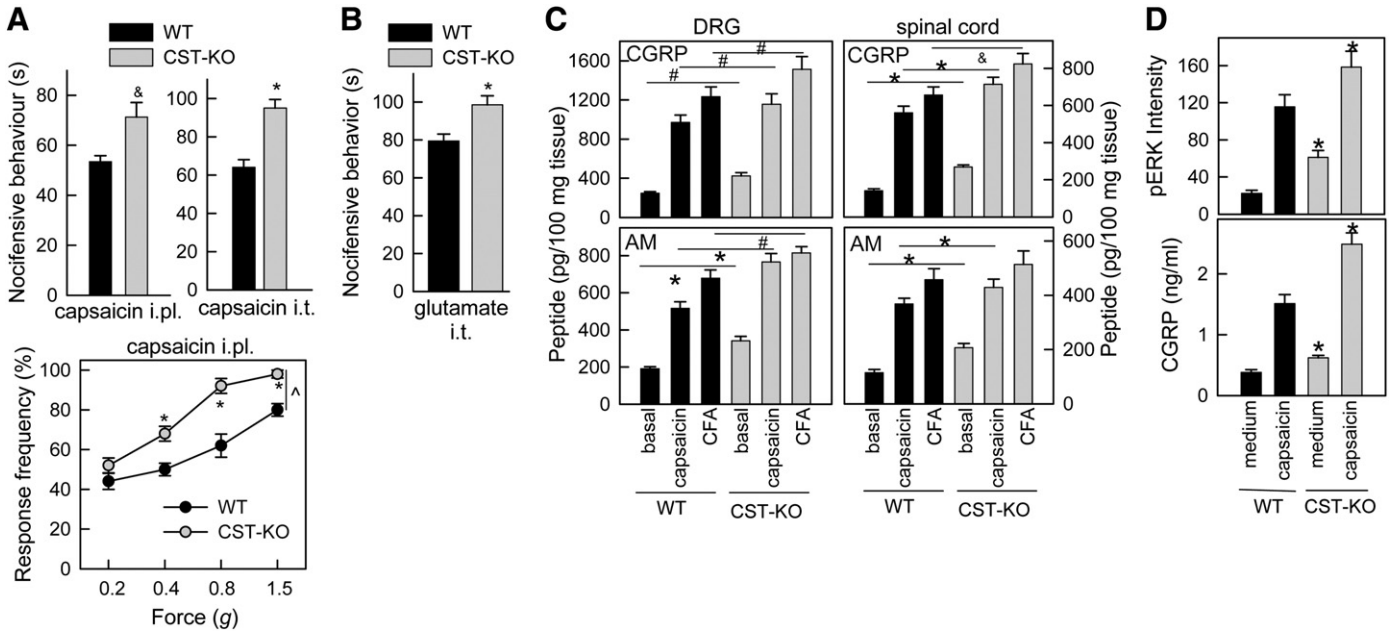

Fig. 8. Lack of cortistatin increases the expression of nociceptive mediators. A and B: Spontaneous nocifensive behavior and response frequencies to plantar application of various von Frey hairs in wild-type (WT) and cortistatin-deficient (CST-KO) mice after peripheral (i.pl.) and spinal (i.t.) injection of capsaicin and glutamate. n = 5–8 mice/genotype. C: Lack of cortistatin increased CGRP and adrenomedullin (AM) in lumbar DRG and spinal dorsal horn in basal conditions and in inflammatory pain evoked by i.pl. injection of capsaicin or CFA. n = 4–6 mice/genotype. D: Expression of phosphorylated-ERK (determined by immunofluorescence) and CGRP in unstimulated (medium) or capsaicin-stimulated lumbar DRG cell cultures isolated from WT and CST-KO mice. n = 3–5, in duplicate. $^{*}p < 0.0001$, $^{\&}p < 0.005$, $^{\#}p < 0.05$ vs. WT mice at the indicated time points. $^{\wedge}p < 0.05$ between groups.

both types of receptors (Deghenghi et al., 2001; Siehler et al., 2008) explains its higher analgesic effects in comparison to ghrelin, somatostatin or analogs (Markovics et al., 2012; Pan et al., 2008; Pinter et al., 2006; Sibilia et al., 2006). The expression of sstr2 and sstr1 in DRG nociceptors and peripheral and central terminals (Bar et al., 2004b; Bencivinni et al., 2011; Capuano et al., 2011; Inhof et al., 2011) would support the actions of cortistatin at the presynaptic level. Although the involvement of sstr1 has not been addressed in this study, a major role of sstr2 is supported by the fact that two different specific sstr2-antagonists had similar reversal effects on cortistatin analgesic action to that showed by a nonselective sstr-antagonist. This is relevant from a mechanistic point of view, because our data indicate that the analgesic effects of cortistatin are significantly linked to the activation of Gαi. It is widely reported that the sstr2 expressed in nociceptive neurons are coupled to Gαi and sstr2-agonists inhibit PKA and $[Ca^{2+}]_i$ and impair excitatory activity of nociceptors (Pinter et al., 2006). Moreover, the activation of PKA and the increase of $[Ca^{2+}]_i$ in primary nociceptive neurons activated TRPV1 and ERK/Akt signaling and enhanced pain sensitivity (Aley et al., 2001; Hucho and Levine, 2007; Julius and Basbaum, 2001). In fact, we found that Gαi-coupled sstr/sstr2 play a major role in the inhibitory effect of cortistatin in the activation of Akt/ERK and the release pronociceptive peptides in primary nociceptors sensitized by inflammatory mediators. Moreover, cortistatin significantly diminished the capsaicin-evoked Ca^{2+} transients of TRPV1 in DRG neurons mainly through sstr. Therefore, it is plausible that cortistatin activation Gαi-coupled sstrs (mainly sstr2) in primary nociceptors is linked to inhibition of synthesis of nociceptive peptides (such as CGRP, substance P and adrenomedullin) and/or their release by peripheral and central terminals. In agreement, cortistatin was recently reported to impair CGRP release by trigeminal neurons and skin peripheral terminals stimulated with capsaicin (Capuano et al., 2011; Markovics et al., 2012).

Regarding GHSR1, this receptor is not expressed by DRGs or by cells and terminals of the superficial dorsal horn of spinal cord (Vergnano et al., 2008). In fact, neither GHSR1 participated in the peripheral action of cortistatin in pain regulation nor ghrelin affected capsaicin-induced $[Ca^{2+}]_i$ in DRG neurons. However, GHSR1 is expressed by inhibitory projection neurons of deep dorsal horn (laminas IV–VI) (Vergnano et al., 2008). These neurons are involved in the transmission of noxious stimuli to supraspinal centers that regulate plastic changes in chronic painful states. This finding supports a role of cortistatin at this spinal level via direct regulation of secondary postsynaptic neurons. Indeed, spinal injection of cortistatin showed the highest analgesic effect in all models tested, mainly in established painful states. Moreover, administration of cortistatin at the spinal level prevented nociceptive responses evoked by intrathecal injection of TNF, capsaicin and glutamate, which are factors that facilitate postsynaptic NMDA-signaling via ERK activation in spinal secondary neurons (Todd, 2010). In any case, electrophysiological studies on isolated secondary neurons activated with NMDA-agonists in the presence of cortistatin will further confirm this hypothesis. Although the coupling of GHSR1 to Gαi in these secondary neurons is still unknown, it has been reported that GHSR1 activation in pancreatic β-cells resulted in a Gαi-dependent inhibition of PKA and Ca^{2+} increases (Dezaki et al., 2011). Moreover, cortistatin inhibited Ca^{2+} increases in smooth muscle cells through a GHSR1-dependent mechanism (Duran-Prado et al., 2013). On the other hand, whereas ghrelin is not expressed by spinal cord neurons (Furness et al., 2011), we demonstrated the expression of the cortistatin mRNA in spinal cord and cortistatin-producing interneurons in deep layers (LIII–LV) of dorsal horn proximal to $GHSR1^+$ neurons. Noteworthy is that, as occurs in cortex and hippocampus where cortistatin exerted inhibitory actions in neuronal transmission (de Lecea et al., 1997), we found that cortistatin is almost solely expressed in GABAergic interneurons in deep dorsal horn. Therefore, cortistatin might be the endogenous inhibitory ligand of these GHSR1-expressing neurons and may play a role in central regulation of tonic pain in physiological conditions.

Finally, because cortistatin is able to deactivate microglia and astrocytes in an inflammatory milieu (Souza-Moreira et al., 2013), and activated glial cells play critical roles in the development and maintenance of nociceptive responses, specially at the spinal level (Mika et al., 2013; Schomberg and Olson, 2012), we cannot rule out that cortistatin could partially exert its analgesic effect through the impairment of glial cells, especially in preventing the development of

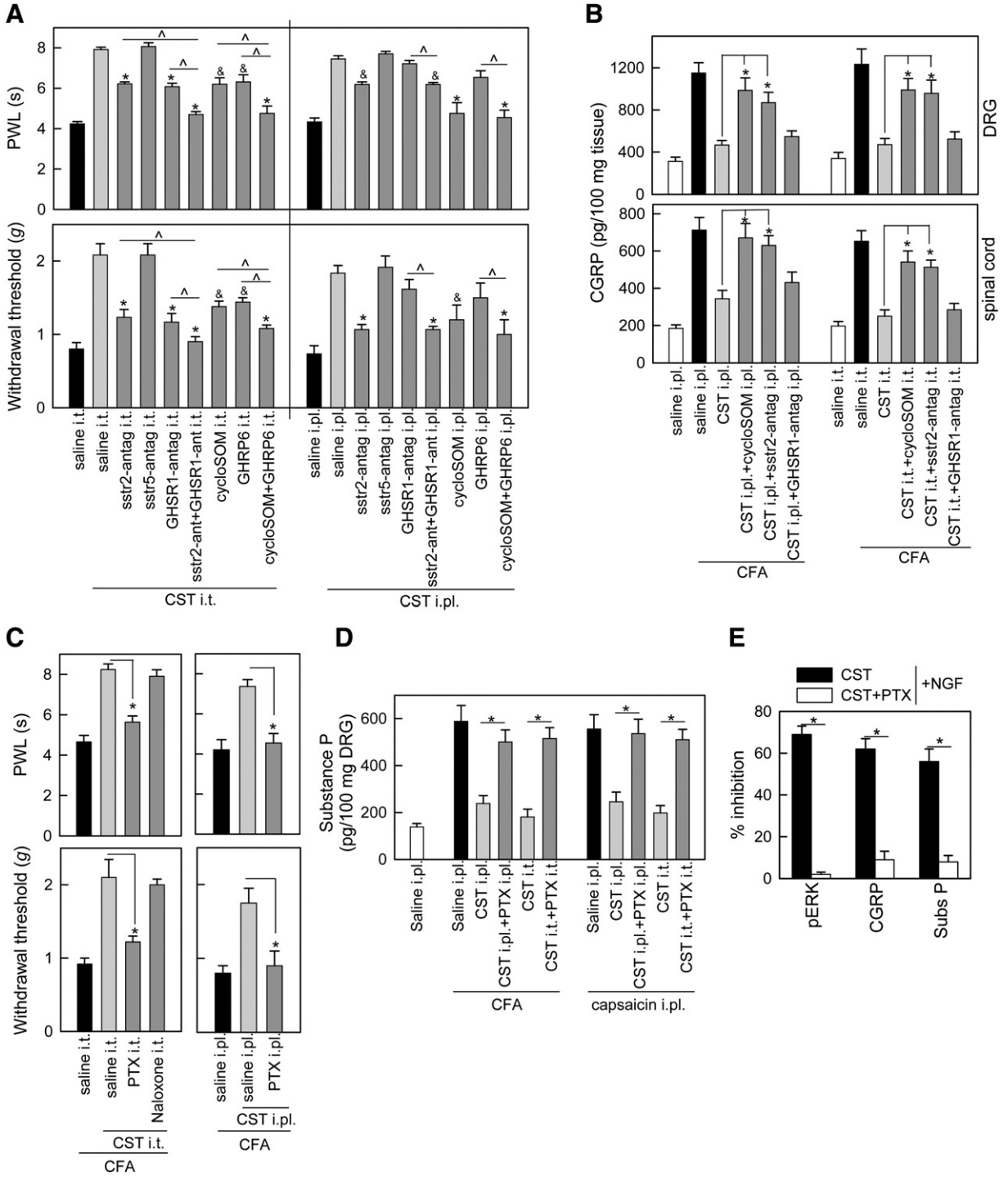

Fig. 9. Receptors involved in the analgesic action of cortistatin. A and B: Reversal of cortistatin-mediated attenuation of CFA-induced heat hyperalgesia (in response to radiant heat) and allodynia (paw withdrawal in response to von Frey hair applications) and of CGRP expression by lumbar DRG and spinal cords by antagonists for all sstr (cycloSOM), GHSR1 (GHRP6 and BIM-28163), sstr2 (BIM-23627, similar results were observed by using the sstr2-antagonist CYN-154806) and sstr5 (BIM-23867) administered centrally (i.t.) or peripherally (i.pl.). n = 6–7/group. *$p < 0.0001$, &$p < 0.005$ vs. cortistatin-treated mice. ^$p < 0.005$ between the indicated groups. C and D: Effect of pertussis toxin (PTX) and naloxone (opioid-receptor antagonist) in cortistatin-mediated attenuation of capsaicin- and CFA-induced heat hyperalgesia and allodynia (C) and of substance P expression by lumbar DRGs (D). n = 6–7/group. *$p < 0.0001$ vs. cortistatin-treated mice. E: Reversal by PTX of the inhibitory effect of cortistatin (expressed as percentage of inhibition) in ERK activation (measured by immunofluorescence of phosphorylated-ERK) and in the production of substance P and CGRP by NGF-activated DRG cultures. n = 3 mice, in duplicate. *$p < 0.0001$ vs. cortistatin-treated mice.

chronic pain states. However, this mechanism should be unlikely involved in the rapid analgesic actions of cortistatin in established pain states or in its effect in acute nocifensive responses. On the other hand, the fact that isolated brain microglia and astrocytes did not produce cortistatin (M.D., unpublished data) argues against the contribution of these cells as endogenous sources of cortistatin in the peripheral nociceptive system.

Conclusions

In summary, our results demonstrate an important contribution of cortistatin produced by nociceptive pathways in modulating the activation properties of nociceptive neurons in response to algogens or in an inflammatory milieu impacting both peripheral and spinal hyperexcitability processes (see Fig. 10 detailing a model). From a therapeutic

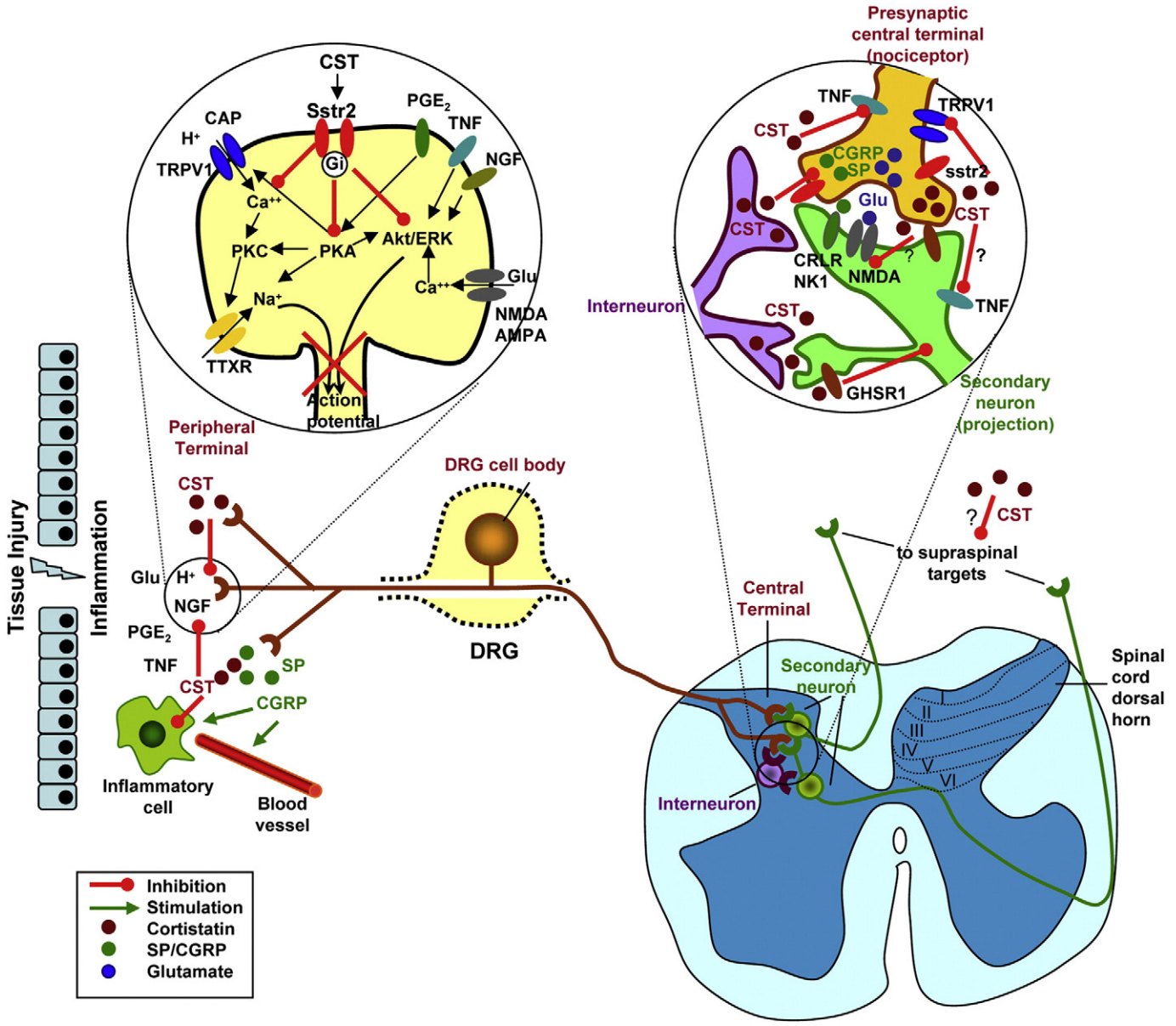

Fig. 10. Model illustrating the role played by cortistatin in the murine nociceptive system. Tissue injury is normally associated with inflammation and inflammatory pain. Inflammatory pain is induced by inflammatory mediators released in injured tissue, such as lipids (PGE2), peptides (NGF) and cytokines (TNF) acting on nociceptors in the peripheral nerve terminals, via interaction to cell-surface receptors. Each of these mediators initiate a cascade of intracellular signaling pathways, including protein kinase A (PKA), protein kinase C (PKC) and Ca^{2+}-dependent kinases (Akt and ERK) that sensitize (decrease the threshold) or excite the terminals of the nociceptors by activation of cation channels, such as the non-selective vanilloid receptor (TRPV1) and the tetrodotoxin-resistant (TTX-R) voltage-gated sodium channels (i.e., $Nav_{1.8/1.9}$). Moreover, responses of TRPV1 to heat can be enhanced by direct interaction of the channel with extracellular protons (H^+, acidic inflammatory milieu). Alteration in excitability of nociceptor transmits afferent messages to the spinal cord dorsal horn via release of excitatory neurotransmitters (glutamate) and neuropeptides (substance P and CGRP) by the central terminal, subsequent activation of secondary spinal neurons and from there to supraspinal centers. Concomitant to release of excitatory peptides, nociceptors respond to noxious and inflammatory stimuli secreting cortistatin (CST) in peripheral and central terminals. By activating Gαi-coupled receptors (sstr, mainly sstr2) and blocking PKA-, calcium- and Akt/ERK-mediated signaling, cortistatin can counteract the increases in excitability of the nociceptor, inhibit the release of nociceptive peptides and produce analgesia at both peripheral and spinal levels. At the spinal level, cortistatin is also produced by inhibitory interneurons and can exert inhibitory responses in secondary projecting neurons in superficial (laminas II–III, via sstr) and deep (laminas IV–VI, via GHSR1) levels of spinal dorsal horn. Finally, cortistatin released by efferent peripheral terminals can counteract local neurogenic inflammation (and persistent inflammatory pain) induced by substance P and CGRP by deactivating inflammatory cells, presumably via GHSR1 and sstr. CAP: capsaicin; Glu: glutamate; DRG: dorsal root ganglia.

point of view, beside as a potent anti-inflammatory factor, cortistatin emerges as a novel analgesic agent enable to alleviate inflammatory pain in various clinical situations. Noteworthy is also that treatment with cortistatin neither causes sedation nor alters the peripheral mechanisms of baseline sensory nociception, supporting that its anti-hyperalgesic actions depend on the presence of noxious/inflammatory stimuli and that the safety of using cortistatin has been proven with other experimental settings (Duran-Prado et al., 2013; Gonzalez-Rey et al., 2006a, 2006b, 2007; Souza-Moreira et al., 2013) and in human clinical trials (Giordano et al., 2007).

Conflict of interest statement

M.D. Culler is employed by IPSEN Group.

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Appendix A. Supplementary data

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