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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-