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ORIGINAL ARTICLE

Substance P and Calcitonin Gene Related Peptide Mediate Pain in Chronic Pancreatitis and Their Expression is Driven by Nerve Growth Factor

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ABSTRACT

Context Calcitonin gene-related peptide (CGRP), substance P and nerve growth factor play an important role in inflammatory pain in various somatic pain models but their role in chronic pancreatitis has not been well studied. **Objectives** The aim of this study was to investigate the effects of intrathecal administration of calcitonin gene-related peptide antagonist and substance P receptor antagonist on pain behavior in a rat model of chronic pancreatitis and to determine whether nerve growth factor drives the up-regulation of expression of these neuropeptides in sensory neurons. **Methods** Pancreatitis was induced by retrograde infusion of trinitobenzene sulfonic acid into the pancreatic duct of adult rats. Three weeks post infusion continuous intrathecal infusion of the calcitonin gene-related peptide antagonist alpha CGRP8-37 or neurokinin-1 receptor antagonist CP-96345 or its inactive enantiomer CP-96344 was administered for seven days. The effects of treatment on pancreatic hyperalgesia were assessed by sensitivity of the abdominal wall to von Frey filament probing as well as by the nocifensive response to electrical stimulation of the pancreas. In a separate experiment chronic pancreatitis was induced and pancreas specific dorsal root ganglion neurons labeled with DiI were assessed for calcitonin gene-related peptide and substance P immunoreactivity. **Results** Intrathecal infusion of calcitonin gene-related peptide and neurokinin-1 receptor antagonists significantly attenuated behavioral pain responses in rats with chronic pancreatitis. Further, treatment of chronic pancreatitis rats with nerve growth factor antibody significantly reduced pancreas specific neurons expressing calcitonin gene-related peptide and substance P in thoracic dorsal root ganglion. **Conclusions** Calcitonin gene-related peptide and substance P mediate pancreatic hyperalgesia in chronic pancreatitis and nerve growth factor in turn sustains the up-regulation of these neuropeptides in pancreatic sensory neurons.

INTRODUCTION

Pain is the most challenging symptom in chronic pancreatitis. In a recent prospective study, nearly 77% of patients self-reported pain [1]. Patients with pain had significant impairment of their quality of life and more than 25% were on disability benefits. Further, there was no association between the duration of chronic pancreatitis and the quality or frequency of pain, suggesting that the earlier concept of pain "burn-out" was not necessarily valid [2]. In the absence of truly effective and safe therapy, such patients are at risk of iatrogenic complications, continued suffering and dependency on narcotics.

Lack of progress in the treatment of pain in chronic pancreatitis is at least in part due to our imperfect understanding of the underlying pathophysiology. Although "mechanical" factors such as increased pancreatic duct and intraparenchymal pressure have traditionally been implicated, more recent studies strongly indicate a major role for alterations in pancreatic sensory nerves [3]. Neuropathic and peri-neural inflammatory changes are commonly seen in patients with chronic pancreatitis and their severity correlates with the intensity of pain [4, 5, 6]. Sustained or intense stimulation of sensory nerves results in the release of several neuropeptides along with glutamate at their spinal terminals. The most well studied peptide neurotransmitters are substance P, calcitonin gene-related peptide (CGRP) and brain-derived neurotrophic factor acting via their specific receptors (principally neurokinin-1, calcitonin receptor-like/receptor activity-modifying proteins and TrkB, respectively). These

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Abbreviations CGRP: calcitonin gene-related peptide; DiI: 1,1'-dioleyl-3,3,3',3-tetramethylindocarbocyanine methanesulfonate; NGF: nerve growth factor; TNBS: trinitrobenzene sulfonic acid; TRPV1: transient receptor potential vanilloid 1

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neurotransmitters facilitate nociceptive signaling to second order neurons in the spinal cord and blocking their action is expected to produce an analgesic effect. We have previously shown up-regulation of these peptides in nociceptive neurons in rats with chronic pancreatitis [7, 8]. Further, we have previously shown that intrathecal administration of a neutralizing brain-derived neurotrophic factor antibody attenuates hypersensitivity of the pancreas in chronic pancreatitis [8]. We now show that robust analgesia can also be produced by blocking the effects of substance P and CGRP in the spinal cord and that up-regulation of their expression is mediated by nerve growth factor (NGF).

METHODS

Animals

Adult male Sprague-Dawley (Harlan, Indianapolis, IN, USA) rats were used in all the experiments.

Induction of Chronic Pancreatitis, Retrograde Labeling and Implantation of Electrodes

The induction of chronic pancreatitis in rats is described in detail elsewhere [7]. Briefly, under anesthesia common bile duct was temporarily occluded near the hepatic end and 0.5 mL of 6 mg/mL trinitrobenzene sulfonic acid (TNBS) in 10% ethanol in PBS, pH 8.0 was retrogradely infused into the pancreatic duct using a 30 gauge needle connected to the PE-10 tubing via the duodenal papilla. For experiments involving immunohistochemistry the pancreas was injected with the lipid soluble fluorescence dye, DiI (1,1'-dioleyl-3,3,3',3-tetra-methylindocarbocyanine methanesulfonate; 25 mg in 0.5 mL methanol; Molecular Probes, Eugene, OR, USA) at 8-10 sites on the exposed pancreas in 2 µL volumes, prior to TNBS infusion. For experiments involving electrical stimulation testing a pair of electrodes (Myo-Wires; A&E Medical, Farmingdale, NJ, USA) were sutured into the pancreas soon after the TNBS infusion and the open ends were subcutaneously tunneled and externalized at the dorsal neck region.

Intrathecal Placement of Catheters and Sub-cutaneous Implantation of Osmotic Pumps for Continuous Delivery of Antagonists

Three weeks post TNBS treatment, under ketamine/ xylazine anesthesia, an intrathecal catheter (6 cm long) was inserted through the rat atlanto-occipital membrane to spinal cord level T9-T10. The tubing was secured to the muscle and an osmotic mini pump (Durect Corporation, Cupertino, CA, USA) implanted subcutaneously and connected to the intrathecal tubing. The pump was filled with about 200 µL (1 mg/mL) of the CGRP antagonist CGRP8-37 (Bachem, Torrance, CA, USA), 250 nM of the neurokinin-1 receptor antagonist CP-96345 or an equal concentration of the inactive enantiomer CP-96344 as control (both from Pfizer Central Research, Groton, CT, USA). The pump was designed to constantly deliver the antagonist or vehicle at 1 µL/h for 7 days after an initial activation period of 4 hours inside the animal.

Behavioral Testing

Von Frey Filament Testing

Von Frey filament testing was performed at three weeks post TNBS infusion and repeated one week after initiation of intrathecal antagonist/vehicle treatment as described in detail previously [7]. Briefly, the belly was shaved and rats were placed in a plastic cage with a mesh floor. After about 30 minutes of acclimatization, von Frey filament (Stoelting, Wood Dale, IL, USA) of various caliber/strengths were applied to the rat's abdomen in ascending order 10 times each for 1-2 seconds with 10 second interval between applications. A positive response consisted of lifting the belly and/or scratching and licking the abdomen. The data was expressed as number of responses during the 10 applications of the filaments. Once the highest level of 10 was reached further testing was not done and for analysis purposes, it was assumed that higher filament strength would also result in the same score. All tests were performed in a blinded manner.

Electrical Stimulation of the Pancreas and Measurement of Nocifensive Responses

The nocifensive response was performed after the von Frey filament test. The rats were placed in individual plastic restrainers in a quiet environment and allowed to adapt for an hour. The previously implanted electrodes were connected to an electrical stimulator (A310 Accupulser, WPI, Sarasota, FL, USA). Animals received successive applications of current at 2, 5, and 10 mA for 5 minutes with 20 minutes rest between stimulations. The number of nocifensive behaviors consisting of stretching, licking of the abdomen, contraction of abdominal wall muscles and extension of the hind limbs were counted during 5 minute stimulation period as previously described [7].

At the termination of the experiment sections from paraffin embedded, formalin fixed pancreatic specimens of rats from both the treatment groups were stained with hematoxylin and eosin. The severity of pancreatitis was assessed and graded by a pathologist blinded for the treatment groups.

Administration of Anti-NGF and Staining of Dorsal Root Ganglion Sections for Substance P and CGRP Immunoreactivity

Chronic pancreatitis was induced in a separate group of rats with intraductal TNBS, followed by intrapancreatic injection of DiI. Three weeks later, rats were injected either with 0.5 mL of neutralizing polyclonal NGF antibody (16 µg/kg BW) in PBS (R&D Systems, Minneapolis, MN, USA) or an equal volume of non-immune normal goat serum (MP Biomedicals, Solon, OH, USA) by the intra-peritoneal route daily for seven days. Subsequently, rats were transcardially perfused

with normal saline followed by 4% paraformaldehyde in 0.1 M PBS (pH 7.4). DRGs (T9-T10) were removed and post fixed in the same fixative solution over night and cryoprotected in 30% sucrose in PBS for 24 hours at 4ºC. Tissue was embedded in optimal cutting temperature and frozen sections (10 μm) were prepared. To ensure that a neuron was counted only once, serial sections were placed on consecutive slides with at least 50 microns between sections on the same slide. The sections were washed in PBS, placed in blocking buffer containing 5% NGS/2%BSA in PBS for 1 hour. Then sections were incubated with a mixture of primary antibodies (anti-CGRP, Bachem America Inc, Torrance, CA, USA, Cat#T-4032, 1:200 and anti-substance P, Neuromics, Edina, MN, USA, Cat#MO15094, 1:100 in PBS containing 5% NGS, 0.05% triton X-100, 1%BSA) at 4°C overnight. After washing in PBS, sections were incubated for 2 hours with secondary antibody mixture consisting of Alexa Fluor 355 (anti-rabbit for CGRP) and 488 (anti-mouse for substance P). Slides were visualized on an Eclipse Ti-s microscope (Nikon, Tokyo, Japan) equipped with filters appropriate for DiI (in red), CGRP (in blue) and substance P (in green). Images were captured with NIS-Elements imaging software (Nikon, Tokyo, Japan) and analyzed using the Image J (NIH). The cells that stained for DiI and overlapped with cells stained for CGRP and/or substance P were counted from 3 sections per individual dorsal root ganglion (T9-T10) per animal and 6 rats per group in a blinded manner.

ETHICS

The animal protocol was approved by the Institutional Animal Care and Use Committee of the Stanford University Medical Center.

DATA ANALYSIS

All values were presented as means±SE. Behavior data from von Frey filament and electrical stimulation were analyzed by repeated-measures analysis of variance with treatment as the between factor and filament strength (for von Frey filament) or current strength (for electrical stimulation) as the within-group factor. The Bonferroni post-test analysis was also applied. For immunofluorescent staining the average of three sections/individual dorsal root ganglion/rat were counted and the average from T9-T10 were analyzed by Fisher's exact test. The software StatView (Abacus Concepts Inc., Berkley, CA, USA) was used for the statistical analyses. Two-tailed P values less than 0.05 were considered statistically significant.

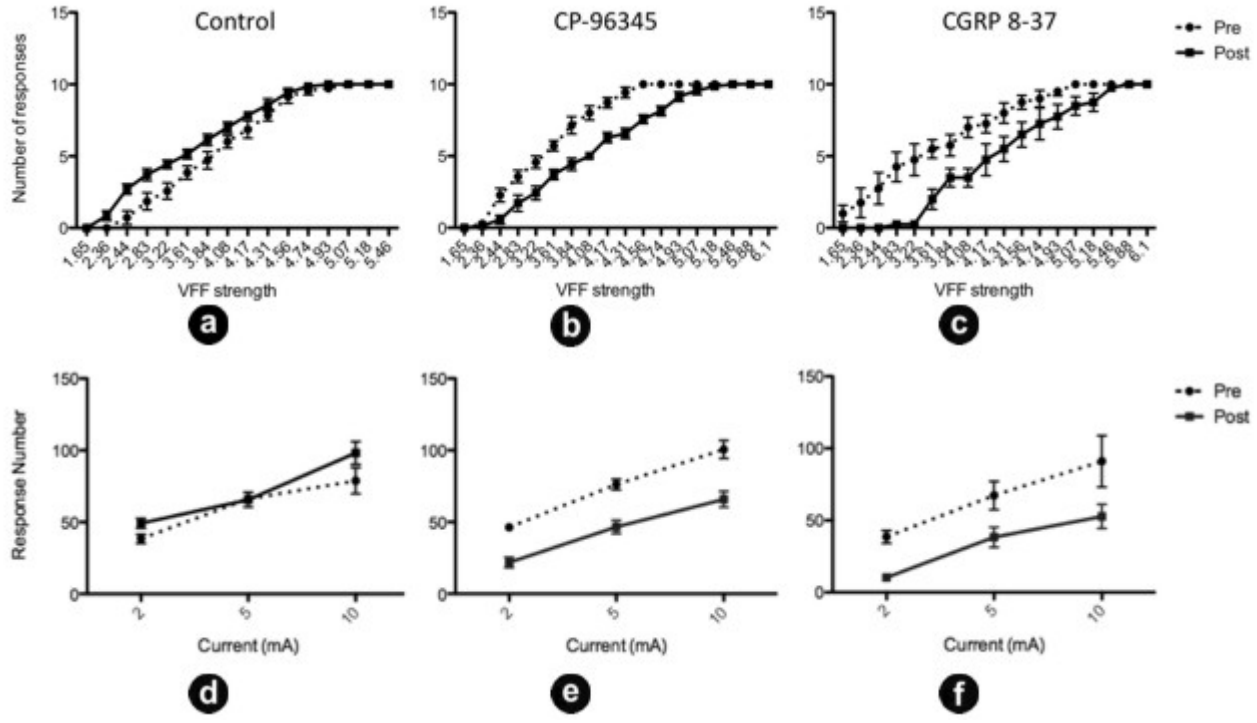

Figure 1. Intrathecal neurokinin-1 receptor and CGRP antagonists attenuate nocifensive behavior response to von Frey filament and electrical stimulation in rats with chronic pancreatitis. **a. b. c.**: Von Frey filament (VFF) responses. Intrathecal infusion of the neurokinin-1 receptor antagonist CP-96345 and the CGRP antagonist CGRP8-37 significantly reduced von Frey filament mean response frequencies compared with pre-treatment (Figures 1b and 1c, respectively). The von Frey filament response curve was shifted to the left in animals treated with the inactive control peptide (Figure 1a), suggesting worsening of the response. See text for details. (Figure 1a; two-way ANOVA: stimulus effect, P<0.001; treatment effect, P<0.001; n=7. Figure 1b; two-way ANOVA: stimulus effect, P<0.001; treatment effect, P<0.001; n=7; Figure 1c; two-way ANOVA: stimulus effect, P<0.001; treatment effect, P<0.001; n=4). **d. e. f.**: Responses to electrical stimulation. As with von Frey filament, infusion of both antagonists resulted in a significant decrease in the behavioral response to electrical stimulation (Figures 1e and 1f) whereas infusion of the inactive control peptide was associated with an increase in the behavioral responses after one week (Figure 1d). See text for details. (Figure 1d; two-way ANOVA: stimulus effect, P<0.001; treatment effect, P=0.003; n=5. Figure 1e; two-way ANOVA: stimulus effect, P<0.001; treatment effect, P<0.001; n=7. Figure 1f; two-way ANOVA: stimulus effect, P=0.006; treatment effect, P<0.001; n=4).

RESULTS

Intrathecal CP-96345 and CGRP8-37 Diminish Referred Somatic Sensitization in Rats with Chronic Pancreatitis

We first measured the sensitivity of the abdomen to mechanical stimulation (an assay for referred somatic hyperalgesia, a characteristic of painful visceral conditions) before and after administration of intrathecal agents. Overall, the response frequencies of rats treated with the neurokinin-1 receptor antagonist, CP-96345 were significantly lower compared to pretreatment baseline, with the stimulus-response curve shifting downwards (Figure 1b; two-way ANOVA: stimulus effect, $P<0.001$; treatment effect, $P<0.001$; n=7). Similarly, the response frequencies of rats treated with the CGRP antagonist, CGRP8-37 were significantly lower compared to pretreatment baseline, with the stimulus-response curve shifting to the right (Figure 1c; two-way ANOVA: stimulus effect, $P<0.001$; treatment effect, $P<0.001$; n=4). In contrast, rats treated with inactive control showed an increase in the response frequency compared to pretreatment baseline (Figure 1a; two-way ANOVA: stimulus effect, $P<0.001$; treatment effect, $P<0.001$; n=7).

Intrathecal CP-96345 and CGRP8-37 Diminish Pancreatic Hyperalgesia in Rats with Chronic Pancreatitis

To measure pancreatic hyperalgesia directly, we used our previously validated assay that measures behavioral responses to electrical stimulation of the organ [7]. Our results suggest that overall, the response curve to graded electrical stimulation was significantly shifted to the left after intrathecal infusion of CP-96345 in rats compared to pretreatment responses (Figure 1e; two-way ANOVA: stimulus effect, $P<0.001$; treatment effect, $P<0.001$; n=7). Bonferroni post-test analysis showed that this effect is significant at all three levels of current strength. Similarly, infusion of CGRP8-37 resulted in significant improvement in the behavioral response to electrical stimulation (Figure 1f; two-way ANOVA: stimulus effect, $P=0.006$; treatment effect, $P<0.001$; n=4). On the contrary, infusion of inactive serum caused an increase in the responsiveness (Figure 1d, two-way ANOVA: stimulus effect, $P<0.001$; treatment effect, $P=0.003$; n=5) with post test analysis revealing that this difference was only significant at the highest level of stimulation (10 mA). Histological examination of H&E stained pancreas specimens from intrathecal antagonists (CGRP and neurokinin-1 receptor) treated groups revealed no difference in pathology when compared with vehicle treated groups (data not shown).

Anti-NGF Treatment Results in Downregulation of Substance P and CGRP in Sensory Afferents in Rats with Chronic Pancreatitis

One week after treatment with anti-NGF or control serum, the number of DiI labeled neurons expressing CGRP and substance P immunoreactivity were counted in thoracic T9-T10 segments. The results showed that anti-NGF treatment group had significantly lower proportion of DiI labeled cells that stained positive for CGRP and substance P as compared with vehicle treatment group (51.6±2.2% and 61.8±1.3% for CGRP *vs*. 37.2±2.1% and 44.4±2.6% for substance P; $P=0.001$ and $P=0.045$, respectively; Figure 2).

DISCUSSION

We have previously shown that chronic pancreatitis, in keeping with other painful inflammatory disorders, is associated with nociceptive sensitization manifested by an increase in behavioral responses to pancreatic stimulation as well as excitability of sensory neurons and up-regulation of key molecules for noxious transduction such as transient receptor potential vanilloid 1 (TRPV1) [7, 9, 10]. Such peripheral sensitization leads to an increase in excitatory signaling to second order neurons via neurotransmitter release. The early response to relatively mild stimuli is transmitted via glutamate but if the stimulus is sustained or intense enough, it causes release of substance P, which acts on the neurokinin-1 receptor to produce a correspondingly more intense post-synaptic response [11, 12]. However, CGRP, co-expressed with substance P, augments its effects but also has independent effects on sensitization, possibly mediated by protein kinase A and protein kinase C [13].

Although the pro-nociceptive effects of these neuropeptides have been thoroughly established in somatic pain models, they have not been well studied in chronic visceral pain syndromes. Both substance P and CGRP have been shown to contribute to the pain response in acute pancreatitis [14]. We have also previously shown an increase in the neuronal expression of both these peptides in the model of TNBS-induced chronic pancreatitis described in this paper [7]. This is in keeping with human data from patients with chronic pancreatitis where investigators have shown both an increase in the expression of substance P and CGRP in pancreatic nerves as well as correlation between pain levels and the expression of the neurokinin-1 receptor [15, 16]. We now show conclusively that pain behavior can be significantly attenuated by blocking the spinal effects of either of these neurotransmitters in chronic pancreatitis. Although this finding was expected given our knowledge of the role of these peptides in pain signaling, our study differs importantly in some aspects from most of the other reports on the use of these antagonists in somatic pain models. Such models have assessed the short-term effects of single administrations and this may not be predictive of the effects of more long-term blockade on sensitization mechanisms, many of which are dependent on new protein synthesis. The use of a "chronic" infusion model such as that used in our study produces a steady state of the drug and avoids the problems associated with the fluctuations caused by bolus delivery,

particularly if the troughs fall below the therapeutically effective levels. Further, continuous infusion models avoid repetitive handling of the animals, which by itself can create confounding problems associated with a classical conditioning model [17].

In this study we also addressed the question of what is driving the up-regulation of these changes in neuropeptide expression in pancreatic sensory neurons. Our previous studies have shown a significant increase in the expression of pancreatic NGF in our model of chronic pancreatitis [10], in keeping once again with human studies that have shown the same [18]. We now show that NGF is a likely mediator of these changes as anti-NGF treatment is associated with a lower expression of both neurotransmitters in sensory neurons. This is in accordance with the known role of NGF in producing significant changes in sensory neuronal expression as well as release of neurotransmitters in chronic persistent pain states [19, 20, 21]. Retrograde transport of the NGF-trkA complex to the cell body in the dorsal root ganglion leads to up-regulation of genes including those that encode spinal neurotransmitters. NGF induces increase in substance P and CGRP levels in sensory neurons *in vitro* and *in vivo* [22], as well as increases basal and stimulus-evoked substance P and CGRP release in rat spinal cords [23]. We have previously shown that treatment with anti-NGF antibody at these doses reverses pancreatic hyperalgesia in this model of chronic pancreatitis, associated with a decrease in the expression of TRPV1 in sensory neurons [24]. The present study adds another mechanism by which NGF drives pain behavior in chronic pancreatitis and provides the possibility of additional therapeutic targets.

In summary, we have demonstrated that the neurotransmitters substance P and CGRP play a role in pancreatic hyperalgesia and that their expression is driven by NGF. Along with previous publications, NGF thus emerges as a "master regulator" of the pain

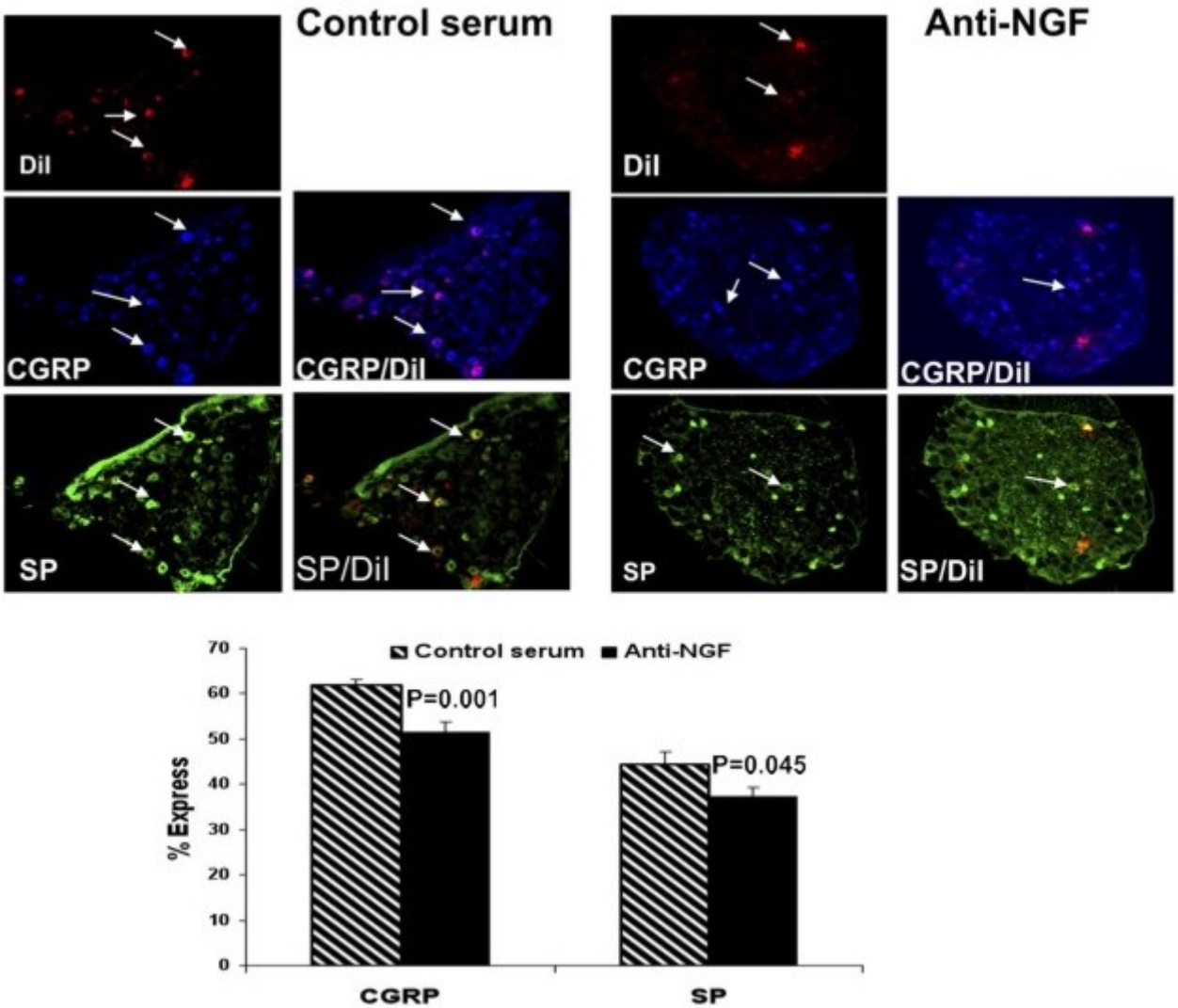

Figure 2. Anti-NGF treatment results in downregulation of substance P and CGRP in sensory afferents in chronic pancreatitis rats. **Top**. Representative photographs of dorsal root ganglion sections stained for DiI (previously injected into the pancreas), CGRP and substance P (SP) in anti-NGF or control serum treated-chronic pancreatitis rats; 10x magnification. **Bottom**. When compared with the serum treatment, anti-NGF resulted in a significantly lower proportion of DiI-labeled cells (pancreatic neurons) in dorsal root ganglions T9 and T10 that also stained positive for CGRP and substance P.

response in this condition. Our results should stimulate research into targeting this axis for new treatments for painful chronic pancreatitis.

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Conflict of interest The authors have no potential conflict of interest

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