Anti-NGF treatment can reduce chronic neuropathic pain by changing peripheral mediators and brain activity in rats

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Neuropathic pain is driven by abnormal peripheral and central processing, and treatments are insufficiently effective. Antibodies against nerve growth factor (anti-NGF) have been investigated as a potent analgesic treatment for numerous conditions. However, the peripheral and brain effects of anti-NGF in neuropathic pain remain unknown. We examined the effectiveness of anti-NGF in reducing chronic pain by local administration in a rat model of sciatic constriction injury (CCI). NGF and substance P in the dorsal root ganglion (DRG) and spinal cord were evaluated. Neuronal activation was measured using c-Fos in the anterior cingulate cortex and ventrolateral periaqueductal gray. At 14 days after CCI, anti-NGF promoted a significant dose-dependent improvement in mechanical threshold, thermal withdrawal latency, and cold sensitivity, lasting for 5 h. NGF upregulation in the DRG and spinal cord after CCI was decreased by anti-NGF, while substance P was increased only in the DRG, and the treatment reduced it. Anti-NGF induced a significant reduction of neuronal activation in the anterior cingulate cortex, but not in the ventrolateral periaqueductal gray. This study provides the first evidence of the anti-NGF effects on brain activity. Thus, our findings suggest that anti-NGF improves chronic neuropathic pain, acting directly on peripheral sensitization and indirectly on central sensitization. ***Behavioural Pharmacology*** **30:79–88**

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Introduction

The prevalence of chronic pain with neuropathic characteristics has been estimated to be in the range of 7–10% of the general population (Bouhassira *et al.*, 2008; van Hecke *et al.*, 2014). Neuropathic pain is caused by a lesion or disease of the somatosensory nervous system: common conditions include diabetic neuropathy, trigeminal neuralgia, and peripheral nerve injury (Colloca *et al.*, 2017). Consequently, the complexity of neuropathic symptoms is described by spontaneous burning pain and electrical-like sensations, accompanied by allodynia and hyperalgesia (Payne and Norfleet, 1986; Attal *et al.*, 2011; Colloca *et al.*, 2017). Additional symptoms of anxiety, depression, insomnia, and reduced quality of life are often seen in neuropathic patients because of suboptimal treatment options (Dworkin *et al.*, 2003; Finnerup *et al.*, 2015).

Trial outcomes are generally modest even for effective drugs in neuropathic pain control. Tricyclic antidepressants, serotonin–noradrenaline reuptake inhibitors, pregabalin, and gabapentin are still the first-line treatment even though they have side effects such as constipation, nausea, somnolence, and weight gain (Finnerup *et al.*, 2015). In an effort to develop therapeutic targets for conditions with associated pain, such as inflammation (Woolf *et al.*, 1994; McMahon *et al.*, 1995; Koltzenburg *et al.*, 1999; Banik *et al.*, 2005; Shinoda *et al.*, 2011), cancer (Sevcik *et al.*, 2005; Mantyh *et al.*, 2010; Jimenez-Andrade *et al.*, 2011; Guedon *et al.*, 2016), and arthritis (Tiseo *et al.*, 2014; Ishikawa *et al.*, 2015; Schnitzer and Marks, 2015; Xu *et al.*, 2016), blockage of nerve growth factor (anti-NGF) in animal models and humans, has been tested and shown to attenuate both allodynia and hyperalgesia. However, the mechanisms by which anti-NGF reduces neuropathic pain are not fully understood.

Currently, one of the mechanisms proposed for NGF involvement in neuropathic pain is the potential blockage of the tropomyosin receptor kinase (Trk) A cognate receptor, which is associated with upregulation of the expression of several genes involved in nociception, including sodium channels (Friedel *et al.*, 1997; Benn *et al.*, 2001), calcitonin gene-related peptide (CGRP) (Bowles *et al.*, 2004; Price *et al.*, 2005), substance P (SP) (Donnerer *et al.*, 1993; Skoff and Adler, 2006), and transient receptor potential vanilloid 1 receptors (Eskander *et al.*, 2015; Dos Reis *et al.*, 2016). However, studies examining neuropathic pain and anti-NGF have focused on preventive treatment based on collateral sprouting (Owolabi *et al.*, 1999; Ramer and Bisby, 1999; Ro *et al.*, 1999), but have not focused on potential mechanisms

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involving nociceptive mediators and their relationship to established neuropathic pain.

Pain relief is at least partially dependent on descending modulatory pathways, including those from anterior cingulate cortex (ACC) and ventrolateral periaqueductal gray (vlPAG), which exert inhibitory and facilitatory effects on pain perception (Heinricher *et al.*, 2009; Mason, 2012; Chen *et al.*, 2014; Samineni *et al.*, 2017a, 2017b; Tsuda *et al.*, 2017). Previous studies have shown that there are two pathways for descending output of the ACC: ACC–spinal dorsal horn and ACC–brainstem–spinal dorsal horn (Tsuda *et al.*, 2017). These two pathways work in parallel and have complementary regulatory effects in nociceptive sensory transmission at the spinal level, and chronic pain might be associated with the dysregulation of these systems (Chen *et al.*, 2014; Ossipov *et al.*, 2014; Chiou *et al.*, 2016; Tsuda *et al.*, 2017).

In the current study, we tested the hypothesis that anti-NGF alters nociception by modulation at the levels of the periphery, spinal cord, and brain. To our knowledge, this is the first study addressing the possible anti-NGF effects in all three levels of the nervous system. We assessed changes in NGF and SP in the dorsal root ganglion and spinal cord, neuronal activation in the ACC and vlPAG, as well as behavioral responses in chronic constriction injury (CCI). The present data suggest that anti-NGF might provide neuropathic pain relief by acting directly on peripheral sensitization and indirectly on central sensitization.

Methods

Subjects

Pathogen-free, adult male Wistar rats (*Rattus norvegicus*) weighing 190–210 g were housed five per cage on a bedding of wood shavings. They were maintained in a climate-controlled room on a 12-h light/dark cycle with free access to food and water. The rats were adapted to the experimental environment for 3 days before the experiments started. All procedures were approved by the Institutional Animal Care Committee of the University of Sao Paulo (protocol number 26 – book number 02/2010, Brazil) and performed in accordance with guidelines of the Committee for Research and Ethical Issues of the International Association for the Study of Pain (Zimmermann 1983). Efforts were made to improve welfare and minimize the number of animals used.

Sciatic nerve injury

CCI was performed using methods described by Bennett and Xie, 1988. Briefly, each rat was anesthetized with isoflurane (5% induction, 1–2% maintenance; Cristalia, Minas Gerais, Brazil), and four sutures (4.0 Ethicon chromic gut; Ethicon, New Brunswick, New Jersey, USA) were ligated around the sciatic nerve at ~1 mm apart. Sham animals had their sciatic nerves exposed as in CCI, but no further procedures were performed.

Anti-NGF treatment

Polyclonal rat β-NGF antibody (anti-NGF) was purchased from R&D Systems (Minneapolis, Minnesota, USA) and dissolved in 0.9% sodium chloride solution (saline). The drug and the vehicle were injected into the ventral surface of the right hind paw in a volume of 50 µl. Anti-NGF was administered only once at 14 days after surgery at 1 or 3 µg/50 µl doses (Dos Reis *et al.*, 2016). For behavioral analysis, rats were randomly placed into treatment groups of 15 animals that received either sterile saline (sham + vehicle, CCI + vehicle) or anti-NGF (CCI + 1 µg/50 µl anti-NGF, CCI + 3 µg/50 µl anti-NGF, and sham + 3 µg/50 µl anti-NGF).

Behavioral testing

The behavioral responses were evaluated before CCI surgery (baseline), 14 days after CCI (14 day), 1–7 h after anti-NGF or vehicle administration, and 15 days after CCI (24 h after treatment). The baseline and 14-day measurements were performed in each of the 15 rats/group. Thereafter, we divided all five experimental groups (CCI + 3 µg/50 µl anti-NGF, CCI + 1 µg/50 µl anti-NGF, sham + 3 µg/50 µl anti-NGF, sham + vehicle and CCI + vehicle) into two more groups to evaluate anti-NGF effects over time (1–24 h) to reduce stress and food deprivation. The first group was tested 1, 3, 5 and 7 h after anti-NGF or vehicle injection ($n = 7$), and the second group was tested 2, 4, 6 and 24 h after anti-NGF or vehicle injection ($n = 8$). The order of testing was thermal hyperalgesia, cold allodynia, and mechanical hyperalgesia. The animals had access to food and water between the measurements. Thermal paw withdrawal latency was tested using the Plantar test according to Hargreaves' method (Hargreaves *et al.*, 1988). The rats were placed in a clear plastic cage with glass floor and allowed to acclimate for about 30 min before the baseline and 14 day measurements. Withdrawal latencies to heat were assessed by applying a focused radiant heat source underneath the glass floor on the right hind paw. The latency to evoke a withdrawal was determined with a cutoff value of 30 s. Three trials 5 min apart were used to obtain average paw withdrawal latency. To evaluate cold allodynia using the acetone test (Choi *et al.*, 1994), the animals were placed in a clear plastic cage with wire grid floor and allowed to acclimatize for 30 min before the baseline and 14 day measurements. Acetone (50 µl) was applied in the ventral surface of the right hind paw and responses (paw elevation + flinching + biting + licking + scratching) over the course of 2 min were recorded for analysis. Mechanical hyperalgesia was assessed by the Randall and Selitto test (Randall and Selitto, 1957). An Ugo-Basile Analgesymeter (Ugo Basile SRL, Gemonio, Varese, Italy) applied a linearly increasing mechanical force to the dorsum of the right hind paw. The nociceptive threshold was defined by the intensity of pressure (g) causing a paw withdrawal behavior. Behavioral experiments were conducted by experimenters blinded to the treatment conditions.

Western blot

Ipsilateral dorsal root ganglia (DRG L4–L6) and spinal cord (lumbar portion) were removed 2 h after anti-NGF treatment and homogenized in extraction buffer containing 100 mmol/l Tris, pH 7.4, 10 mmol/l EDTA, 2 mmol/l phenylmethylsulfonyl fluoride, and 10 mg/ml aprotinin. The extracted proteins (75 μg/sample) were subjected to 12% acrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The membranes were treated for 2 h at room temperature with 5% blocking buffer solution containing full fat dry milk. They were incubated overnight at 4°C with rat monoclonal primary antibody against NGF (F30, 1 : 1000; Santa Cruz Biotechnology, Dallas, Texas, USA) and rabbit monoclonal primary antibody against SP (1 : 1000; Millipore, Burlington, Massachusetts, USA). A peroxidase-conjugated anti-rat antibody 1 : 5000 (Zymed Laboratories Inc., South San Francisco, California, USA) and anti-rabbit 1 : 5000 (GE Healthcare, Chicago, Illinois, USA) were used for signal amplification and detected with an enhanced chemiluminescence reagent kit (Amersham Biosciences, Little Chalfont, district of Buckinghamshire, England). For quantification, densitometry was performed using Image J (NIH, Bethesda, Maryland, USA). The bands were corrected, by the optical density of β-actin (1 : 10 000; Sigma-Aldrich, St. Louis, Missouri, USA), considering samples from control animals as the standard for normalization. Figures were prepared using Adobe Photoshop CS (Adobe Systems Incorporated, San Jose, California, USA). Western blot analysis was used to investigate anti-NGF effects after CCI or sham surgery from a separate group of rats consisting of 10 rats/group.

Immunohistochemistry

A noxious stimulus (0.6 mA three times at 30-s intervals – A360 WPI Stimulus Isolator; World Precision Instruments, Sarasota, Florida, USA) was applied on the injured paw 2 h after anti-NGF administration, and the rats were euthanized 90 min later (Baldi *et al.*, 2004; Luo *et al.*, 2009). For the perfusion procedure, the rats were deeply anesthetized with sodium pentobarbital (40 mg/kg, intraperitoneal; Cristalia, Minas Gerais, Brazil) and perfused transcardially with saline (0.9%), followed by 4% (mass/vol) paraformaldehyde in 0.1 mol/l phosphate buffer at pH 7.4; the brains were removed and left overnight in a solution of 20% sucrose in 0.1 mol/l phosphate buffer at 4°C. The brains were then frozen, and five series of 40 μm-thick sections were cut with a sliding microtome. One series was processed immunohistochemically to detect c-Fos (Harris 1998) using a rabbit anti-c-Fos antiserum (Ab-5; Calbiochem, Burlington, Massachusetts, USA) for 72 h at 4°C at a dilution of 1 : 20 000. The primary antiserum was localized using a variation of the avidin–biotin complex procedure. In brief, sections were incubated for 90 min at room temperature in a solution of biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, California, USA) and placed in the mixed avidin–biotin HRP complex solution (ABC Elite Kit; Vector Laboratories) for 90 min. The peroxidase complex was visualized by a 10 min exposure, to a chromogen solution containing 0.02% 3,3′-diaminobenzidine tetrahydrochloride (Sigma, St. Louis, Missouri, USA), with 0.3% nickel-ammonium sulfate in 0.05 mol/l Tris buffer (pH 7.6), followed by incubation for 10 min in chromogen solution with hydrogen peroxide (1 : 3000), to produce a blue-black product. The reaction was stopped by extensive washing in potassium PBS (pH 7.4). The sections were mounted on gelatin-coated slides, dehydrated, and cover slipped with DPX neutral mounting medium (Sigma). One adjacent series of sections was stained by the Nissl method with thionin for cytoarchitecture reference. Immunohistochemistry analysis investigated anti-NGF effects after CCI or sham surgery from a separate group of rats consisting of 10 rats/group.

The c-Fos immunoreactivity was quantified in part 1 of the ACC (Cg1: bregma 2.28 mm and interaural 11.28 mm) and vlPAG (bregma − 7.80 mm and interaural 1.20 mm). The analysis was bilateral, because we did not observe significant differences between the right and left sides (data not shown). The number of c-Fos immunoreactive neurons was evaluated by an observer without knowledge of the animal's experimental status and was quantified by using the ×10 objective of a Nikon Eclipse 80i (Nikon Corporation, Minato, Tokyo, Japan) microscope equipped with a Nikon Digital Camera DXM1200F (Nikon Corporation). We first delineated, in a given section, the borders of the region of interest, as defined in the adjoining Nissl-stained sections, and c-Fos-labeled cells were counted therein. Only darkly labeled oval nuclei that fell within the borders of the region of interest were counted. Measurements for c-Fos were taken from 10 different sections for each animal analyzed. The density of labeled cells was determined by dividing the number of c-Fos-labeled cells by the area of the region of interest. Both cell counting and area measurements were carried out using Image J program (NIH), transforming the images into 32 bits, and standardizing the values of the threshold function for all groups. Rat neuroanatomical parceling and mapping were carried out according to the Paxinos and Watson atlas (Paxinos and Watson, 2007).

Statistical analyses

Prism 5 (GraphPad Software Inc., San Diega, California, USA) was used to perform statistical analyses. Behavioral data were analyzed using two-way repeated measures analysis of variance with Bonferroni-corrected post-hoc test comparing groups (treatment × time). For western blot and immunohistochemical data, experimental groups were compared using one-way analysis of variance with Bonferroni-corrected post-hoc test. The results were considered statistically significant at P values less than 0.05, and they were shown as average ± SEM.

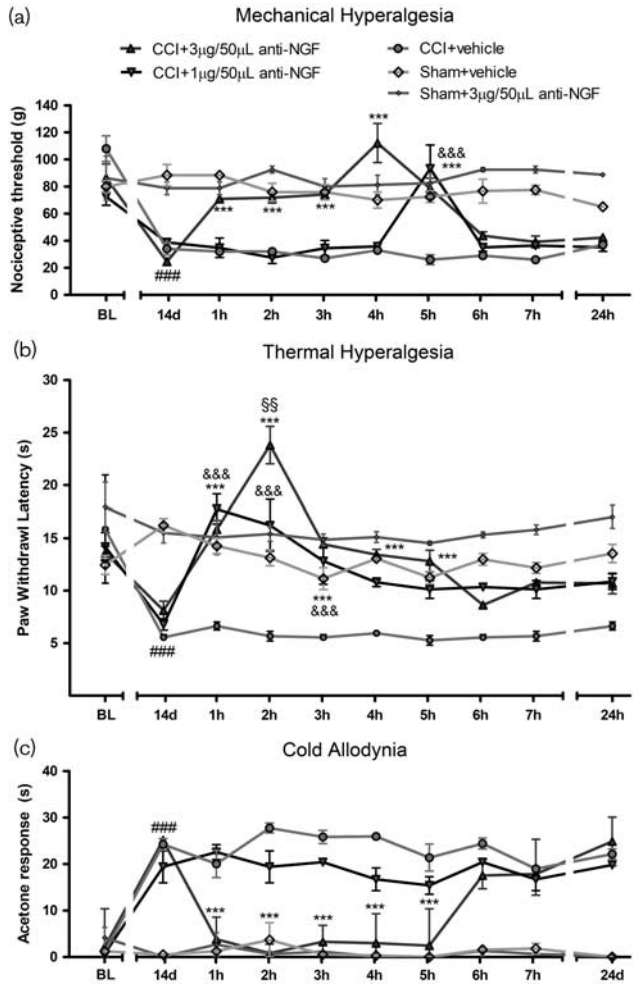
Results

Anti-NGF treatment reduces chronic neuropathic pain

The CCI group showed chronic neuropathic pain-related behaviors 14 days after surgery (Fig. 1a–c). Anti-NGF treatment (3 μg/50 μl) significantly decreased the mechanical hyperalgesia from the first to the fifth hour after injection in the CCI group [Fig. 1a – treatment: $F_{(4,225)} = 66.12$, $P < 0.001$; time: $F_{(9,225)} = 10.03$, $P < 0.001$; treatment × time interaction: $F_{(36,225)} = 7.36$, $P < 0.001$]. The lower dose was increased to the mechanical threshold only 5 h after anti-NGF injection. The two different doses of anti-NGF reduced the thermal hyperalgesia in the CCI groups but not with the same duration [Fig. 1b – treatment: $F_{(4,171)} = 45.48$, $P < 0.001$; time: $F_{(9,171)} = 15.51$, $P < 0.001$: treatment × time interaction: $F_{(36,171)} = 8.90$, $P < 0.001$]. The highest dose increased the paw withdrawal latency from the first to the fifth hour after injection in the CCI group ($P < 0.001$), whereas the CCI + 1 μg/50 μl anti-NGF was significantly different compared with CCI + vehicle from the first to the third hour after anti-NGF injection ($P < 0.001$). The improvement in thermal hyperalgesia using the anti-NGF (3 μg/50 μl) had larger effect 2 h after administration when compared with CCI + 1 μg/50 μl anti-NGF ($P < 0.01$). Accordingly, the anti-NGF treatment (3 μg/50 μl) also reduced cold allodynia from the first to the fifth hour after injection in CCI [Fig. 1c – treatment: $F_{(4,126)} = 104.25$, $P < 0.001$; time: $F_{(9,126)} = 21.02$, $P < 0.001$; treatment × time interaction: $F_{(36,126)} = 15.71$, $P < 0.001$]. However, there were no significant differences between CCI + vehicle and CCI + 1 μg/50 μl anti-NGF in cold allodynia test.

The anti-NGF (3 μg/50 μl) effects observed in all behavioral tests lasted for 5 h after administration. The sham + 3 μg/50 μl anti-NGF and sham + vehicle groups were not significantly different from one another in any assessment, in accordance with Dos Reis *et al.* (2016).

Fig. 1



Anti-NGF treatment reduces neuropathic pain (a–c). For (a) and (b): $^{***}P < 0.001$ CCI + 3 μg/50 μl anti-NGF vs. CCI + vehicle, $^{\#\#\#}P < 0.001$ CCI groups vs. sham groups, $^{\&\&\&}P < 0.001$ CCI + 1 μg/50 μl anti-NGF vs. CCI + vehicle, and $^{\S\S}P < 0.01$ CCI + 3 μg/50 μl anti-NGF vs. CCI + 1 μg/50 μl anti-NGF. For (c): $^{***}P < 0.001$ CCI + 3 μg/50 μl anti-NGF vs. CCI + vehicle and CCI + 1 μg/50 μl anti-NGF and $^{\#\#\#}P < 0.001$ CCI groups vs. sham groups. Anti-NGF, antibodies against nerve growth factor; CCI, chronic constriction injury.

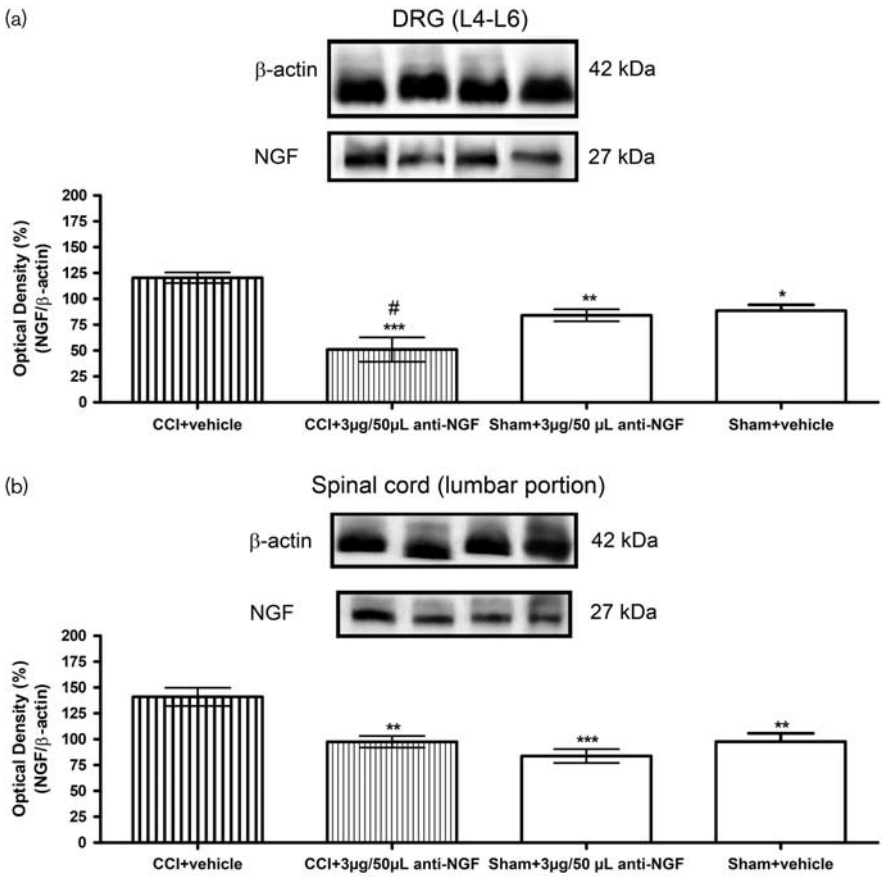
Anti-NGF treatment decreases NGF upregulation in the DRG and spinal cord following chronic neuropathic pain

Western blot showed an upregulation of NGF in the DRG and spinal cord after CCI compared with the control groups (Fig. 2a and b). The anti-NGF treatment reduced the NGF in both the DRG and the spinal cord after chronic pain when compared with CCI after vehicle treatment [Fig. 2a – treatment: $F_{(3,19)} = 17.73$, $P < 0.001$; Fig. 2b – treatment: $F_{(3,18)} = 10.27$, $P < 0.001$]. Moreover, the CCI + 3 μg/50 μl anti-NGF caused a larger decrease of NGF in the DRG, and it was significantly different from sham groups (Fig. 2a). There were no significant differences between CCI + 3 μg/50 μl anti-NGF and sham groups in the spinal cord (Fig. 2b). Sham rats treated with the higher dose of anti-NGF did not show significant alteration in NGF levels compared with sham with vehicle. β-Actin did not change under the experimental conditions.

Effects of anti-NGF treatment on substance P in the DRG and spinal cord following chronic neuropathic pain

SP protein was reduced in the DRG after anti-NGF treatment (3 μg/50 μl) compared with CCI + vehicle [Fig. 3a – treatment: $F_{(3,15)} = 18.82$, $P < 0.001$]. The CCI model did not show a significant difference in SP levels in the spinal cord compared with control groups, as reported in our previous paper (Da Silva *et al.*, 2017) (Fig. 3b). In addition, anti-NGF did not change the SP levels in the spinal cord of sham and CCI animals (Fig. 3b). Sham rats treated with the higher dose of anti-NGF did not show significant alteration in SP levels compared with sham with vehicle. β-actin did not change under the experimental conditions.

Fig. 2



Anti-NGF treatment decreases NGF in the DRG and spinal cord. Western blot analysis of the DRG (a) and lumbar level of the spinal cord (b) revealed a 42 kDa protein band specific for β-actin and a 27 kDa protein band specific for NGF. Each bar represents mean ± SEM of 10 rats. For (a): $^{*}P < 0.01$, $^{**}P < 0.001$, and $^{***}P < 0.0001$ vs. CCI + vehicle and $^{\#}P < 0.01$ vs. sham groups. For (b): $^{**}P < 0.001$ and $^{***}P < 0.0004$ vs. CCI + vehicle. Anti-NGF, antibodies against nerve growth factor; CCI, chronic constriction injury; DRG, dorsal root ganglion.

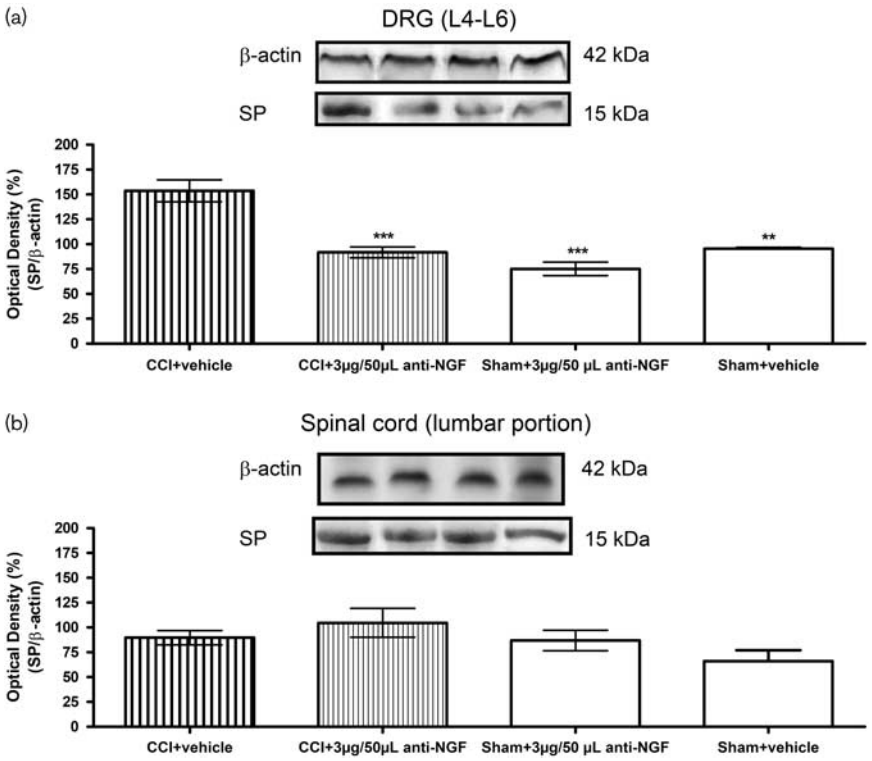
Anti-NGF therapy reduces the pain-induced upregulation of c-Fos in the ACC and has no effect in the vlPAG

Noxious stimulation induced a significant increase in c-Fos immunoreactive neurons in ACC of CCI + vehicle compared with control groups [Fig. 4c, e, and f – treatment: $F_{(3,12)} = 12.17$, $P < 0.001$]. The effect of the anti-NGF treatment in the ACC neuronal activity was detected by the reduced number of c-Fos immunoreactive neurons in CCI with treatment compared with saline (Fig. 4c and d). No significant differences in c-Fos were observed in ACC between the control groups and the CCI after anti-NGF. There was a significant increase in the number of c-Fos immunoreactive neurons in the vlPAG of control groups compared with the CCI + vehicle [Fig. 5c, e, and f – treatment: $F_{(3,16)} = 28.17$, $P < 0.001$]. However, CCI with anti-NGF treatment and CCI with vehicle were not significantly different in c-Fos expression in the vlPAG (Fig. 5c and d). CCI with anti-NGF caused a reduced number of c-Fos immunoreactive neurons in the vlPAG compared with control groups (Fig. 5d–f). Sham rats treated with the higher dose of anti-NGF did not show significant alteration in c-Fos compared with sham + vehicle in the ACC and in the vlPAG (Figs 4e, f and 5e, f).

Discussion

This is the first study to investigate how the blockade of endogenous NGF with a single administration of anti-NGF antibody can promote pain relief in a chronic neuropathic pain model. Anti-NGF applied in the hind paw significantly decreased mechanical and thermal hyperalgesia, as well as cold allodynia in rats with CCI. To date, there is only one published study using CCI and anti-NGF treatment for remediation of established pain in mice, which focused on effects of systemic administration, tactile allodynia and in-vitro activity of the antibody, thus not capturing the potential mechanisms related to the pain relief (Wild *et al.*, 2007). This current study performed analyses of behavior, NGF and SP in the DRG and spinal cord, and neuronal activity in the ACC and PAG, to demonstrate that anti-NGF can reduce chronic neuropathic pain.

Fig. 3

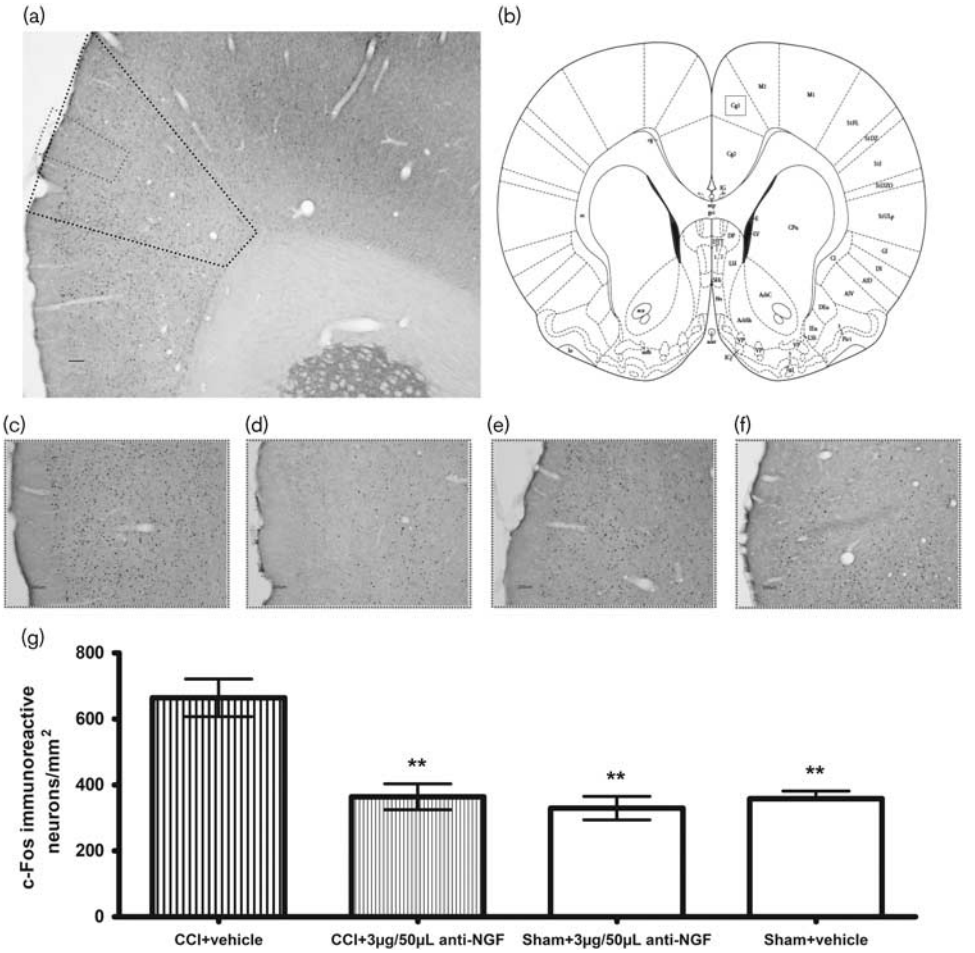


Anti-NGF treatment decreases the SP in the DRG but has no significant effect in the spinal cord. Western blot analysis of the DRG (a) and lumbar level of the spinal cord (b) revealed a 42 kDa protein band specific for β-actin and a 15 kDa protein band specific for SP. Each bar represents mean ± SEM of 10 rats. $^{**}P < 0.001$ and $^{***}P < 0.0001$ vs. CCI + vehicle. CCI, chronic constriction injury; DRG, dorsal root ganglion; SP, substance P.

The primary behavioral finding of this study was the decreased pain-related response after local anti-NGF administration in CCI animals. The continuous 5 h of improvement after the higher dose of anti-NGF treatment has been reported previously in thermal sensitivity of trigeminal neuropathic pain (Dos Reis *et al.*, 2016), and this duration was the same in terms of mechanical hyperalgesia and cold allodynia, as in our study. The lower dose did not robustly reverse mechanical hyperalgesia and cold allodynia, which could also be an effect of the high level of NGF produced in chronic nerve injury, and, consequently, the inability to neutralize it. Unfortunately, the literature does not resolve this issue – there was no effect of the lower dose in trigeminal neuropathic pain (Dos Reis *et al.*, 2016). Further studies will be required to determine anti-NGF responses in different types of chronic neuropathic pain. In addition, clinical trials showed significant efficacy of tanezumab, a humanized monoclonal anti-NGF, in diabetic peripheral neuropathy, but not in postherpetic neuralgia (Bramson *et al.*, 2013, 2015; Wang *et al.*, 2013). The inconsistent results may reflect the diverse mechanisms underlying neuropathic pain, which can include variations in the degree, type, and severity of neuropathy (Bramson *et al.*, 2015).

In view of the greater efficacy of the treatment in thermal hyperalgesia 2 h after injection, in the present report, NGF and SP levels, as well as brain activity were assessed at this time point. NGF and TrkA are transported in a retrograde direction to the DRG, resulting in increased synthesis of neuropeptides, such as SP and CGRP (Donnerer *et al.*, 1993; Mantyh *et al.*, 2011; Denk *et al.*, 2017). In this study, decreased NGF in the DRG and spinal cord after anti-NGF treatment of neuropathic pain appears to be integrally involved in the downregulation of SP in the DRG. SP seems to be involved in early stages of chronic pain development in the DRG and later stages in the spinal cord, as our previous data showed SP upregulation in the spinal cord 56 days after CCI (Da Silva *et al.*, 2017). Accordingly, early changes in SP immunoreactivity were not observed in the spinal cord using the same model and spinal cord region (Casals-Diaz *et al.*, 2009). The binding of NGF to TrkA on the peptidergic (TrkA-positive) fiber terminal activates intracellular signaling pathways, which results in an increased number of receptors at the membrane surface, including voltage-gated sodium (Nav) and calcium ion channels (Mantyh *et al.*, 2011). On the basis of previous findings that the Nav1.8 contributes to the release and/or synthesis of SP in adult DRG neurons (Tang *et al.*, 2008), we hypothesized

Fig. 4



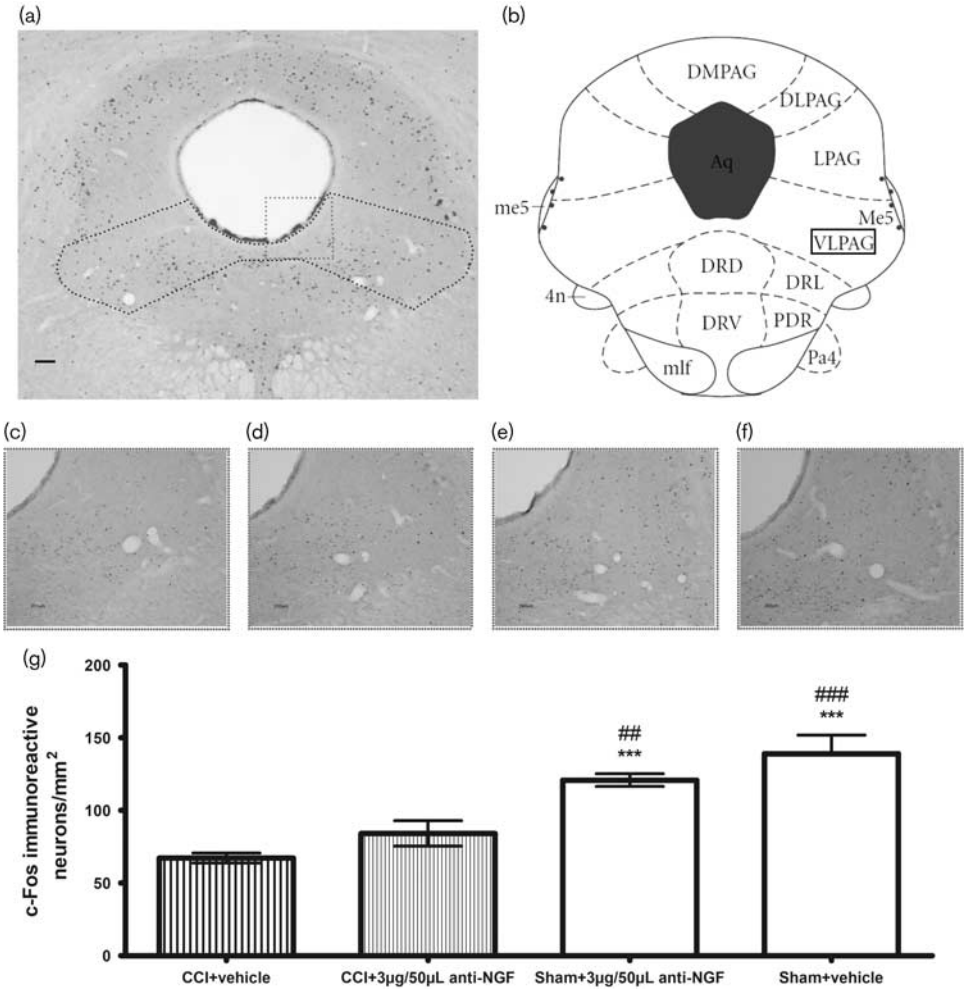
Anti-NGF treatment can decrease neuronal activation in the ACC. Photomicrograph of transverse c-Fos stained section of the ACC showing the correspondent Cg1 area, which is outlined in dotted lines (a), according to the atlas of Paxinos and Watson (b). Photomicrographs of c-Fos immunoreactive neurons in the ACC are represented by the smaller area (a) of the CCI + vehicle (c), CCI + 3 µg/50 µl anti-NGF (d), sham + 3 µg/50 µl anti-NGF (e), and sham + vehicle (f) groups. Quantification is shown in (g). Each bar represents mean ± SEM of 10 rats.**$P < 0.001$ vs. CCI + vehicle. Scale bars: 200 µm. ACC, anterior cingulate cortex; anti-NGF, antibodies against nerve growth factor; CCI, chronic constriction injury.

that SP upregulation in the DRG contributes to peripheral sensitization and central enhanced synaptic transmission of sensory information following neuropathy (Woolf, 1983; Gracely *et al.*, 1992; Chaban, 2010).

The International Association for the Study of Pain defines central sensitization as 'increased responsiveness of nociceptive neurons in the central nervous system to their normal or subthreshold afferent input' (Loeser and Treede, 2008). Jimenez-Andrade *et al.* (2007) reported that anti-NGF can reduce c-Fos upregulation in the spinal cord after femur fracture, and anti-hyperalgesic actions can result from the sensitization of CGRP/trkA-positive fibers. CGRP is a peptide also known to prolong the effects of SP in tissues by inhibiting SP degradation, thereby promoting central hyperexcitability (De Felipe *et al.*, 1998; Lee and Kim, 2007; Schlereth *et al.*, 2016). Taken together, those previous studies led us to investigate the possible anti-NGF effects on brain activity. We showed that rats with CCI have increased ACC activity following electrical stimulation, similar to previous studies in rats with sciatic nerve injury and different noxious stimulations (Takeda *et al.*, 2009; Pagano *et al.*, 2011). Moreover, a major finding in the current study was the reduced ACC activity in the CCI group after anti-NGF treatment, which could imply, at least in part, the indirect anti-NGF effect in the brain central sensitization.

As far as we know, no other pain study has investigated the indirect effects of anti-NGF in brain regions; most studies have been confined to addressing peripheral and spinal cord mechanisms (Ramer and Bisby, 1999; Ro *et al.*, 1999; Wild *et al.*, 2007; Dos Reis *et al.*, 2016). Recent evidence suggests that ACC can influence nociceptive processing in the spinal dorsal horn by descending pathways directly or indirectly through PAG (Tsuda *et al.*,

Fig. 5



Anti-NGF treatment has no significant effect on neuronal activation in the vlPAG after neuropathic pain. Photomicrograph of transverse c-Fos stained section of the PAG showing the correspondent vlPAG column, which is outlined in dotted lines (a), according to the atlas of Paxinos and Watson (b). Photomicrographs of the c-Fos immunoreactive neurons in the vlPAG are represented by the smaller area (a) of the CCI + vehicle (c), CCI + 3 µg/50 µl anti-NGF (d), sham + 3 µg/ 50 µl anti-NGF (e), and sham + vehicle (f) groups. Quantification is shown in (g). Each bar represents mean ± SEM of 10 rats. $^{***}P < 0.0001$ vs. CCI + vehicle, $^{\#\#}P < 0.001$ and $^{\#\#\#}P < 0.0001$ vs. CCI + 3 µg/50 µl anti-NGF. Scale bars: 200 µm. Anti-NGF, antibodies against nerve growth factor; CCI, chronic constriction injury; vlPAG, ventrolateral periaqueductal gray.

2017). The present results suggest that anti-NGF treatment does not influence the neuronal activation in vlPAG in chronic sciatic nerve injury. It has been recently documented that selective modulation of GABAergic or glutamatergic neurons in vlPAG shows an inverse regulation of nociceptive behaviors resulting in facilitation or suppression, and anti-NGF could not be involved in the modulation from these neuron subgroups (Samineni *et al.*, 2017a, 2017b). Our study showed an increased vlPAG activation in sham animals treated with anti-NGF compared with CCI, which could imply the lack of pain suppression exerted by vlPAG in chronic neuropathic pain. However, future research might be required to elucidate the participation of GABAergic and glutamatergic neurons in vlPAG following CCI.

The present study showed that anti-NGF treatment can attenuate pain-related behaviors in a rat model of chronic neuropathic pain. We also observed that a local injection of anti-NGF decreased the upregulated NGF in the DRG and spinal cord, as well as SP in the DRG, after neuropathic pain. The increased neuronal activity in the ACC was reduced after anti-NGF treatment in the CCI group. Therefore, the analgesic effect of anti-NGF in chronic neuropathic pain is thought to be due to its direct effect on peripheral sensitization through the NGF and SP reduction, and the indirect effect on central sensitization by decreased ACC activation. One possible mechanism for the brain effect seen in the current study might be that ACC sends projections to the spinal cord without synaptic transmission to the PAG to modulate

pain perception. ACC projecting fibers are distributed mainly in the lamina I–III of the lumbar spinal dorsal horn, which receives peptidergic (TrkA-positive) afferent projections (Mantyh *et al.*, 2011; Chen *et al.*, 2014). In addition, the ACC was shown to be involved in modulating the affective component of pain and the reward system (Johansen *et al.*, 2001; Navratilova *et al.*, 2015, 2016).

Conclusion

Our results are consistent with previous studies, which observed improvement in pain-related behaviors after anti-NGF treatment (Sevcik *et al.*, 2005; Mantyh *et al.*, 2010; Dos Reis *et al.*, 2016). The current study also adds novel findings to peripheral and central mechanisms associated with chronic neuropathic pain and anti-NGF. Further research is necessary to better understand anti-NGF mechanisms, as it might treat not only the sensory, but also the affective dimension of the pain experience.

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Conflicts of interest

There are no conflicts of interest.

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