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ORIGINAL ARTICLE

Dynorphinergic system alterations in the corticostriatal circuitry of neuropathic mice support its role in the negative affective component of pain

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The dynorphinergic system is involved in pain transmission at spinal level, where dynorphin exerts antinociceptive or pronociceptive effects, based on its opioid or non-opioid actions. Surprisingly, little evidence is currently available concerning the supraspinal role of the dynorphinergic system in pain conditions. The present study aimed to investigate whether neuropathic pain is accompanied by prodynorphin (Pdyn) and κ-opioid receptor (Oprk1) gene expression alterations in selected mouse brain areas. To this end, mice were subjected to chronic constriction injury of the right sciatic nerve and neuropathic pain behavioral signs were ascertained after 14 days. At this interval, a marked increase in Pdyn mRNA in the anterior cingulate cortex (ACC) and prefrontal cortex (PFC) was observed. Oprk1 gene expression was increased in the PFC, and decreased in the ACC and nucleus accumbens (NAc). No changes were observed in the other investigated regions. Because of the relationship between dynorphin and the brain-derived neurotrophic factor, and the role of this neurotrophin in chronic pain-related neuroplasticity, we investigated brain-derived neurotrophic factor gene (Bdnf) expression in the areas showing Pdyn or Oprk1 mRNAs changes. Bdnf mRNA levels were increased in both the ACC and PFC, whereas no changes were assessed in the NAc. Present data indicate that the dynorphinergic system undergoes quite selective alterations involving the corticostriatal circuitry during neuropathic pain, suggesting a contribution to the negative affective component of pain. Moreover, parallel increases in Pdyn and Bdnf mRNA at cortical level suggest the occurrence of likely interactions between these systems in neuropathic pain maladaptive neuroplasticity.

KEYWORDS

anterior cingulate cortex, Bdnf, corticostriatal circuitry, gene expression, neuropathic pain, nucleus accumbens, Oprk1, pain aversion, Pdyn, prefrontal cortex

1 | INTRODUCTION

Neuropathic pain is a complex disease characterized by maladaptive plasticity within the nociceptive system, leading to a pathological amplification of noxious and innocuous stimuli.[1] During chronic pain conditions multiple alterations occur, such as microglia activation,[2,3] the release of many immune modulators[4,5] and massive transcriptional changes, including ion channel subunits[6,7] and neuropeptides.[8–10]

Dynorphin is an opioid neuropeptide deeply involved in the modulation of pain. Much evidence suggests that increases in prodynorphin (Pdyn) mRNA and dynorphin peptide levels in the spinal cord are responsible for the development of allodynia and hyperalgesia in rats.[8,10–13] Notably, dynorphin seems to be required for the maintenance of neuropathic pain, rather than for its initiation. In this regard, Pdyn knock-out (KO) mice do not show any difference in pain

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threshold immediately after spinal nerve ligation (SNL) when compared with wild type mice. However, at post-SNL day 10 only, the Pdyn KO animals showed a return to nociceptive baseline values, suggesting that dynorphin could play a pronociceptive role through the maintenance of central sensitization.[14]

Nevertheless, it is also known that the administration of κ-opioid receptor antagonists enhanced mechanical and thermal allodynia[15] and, similarly, κ-opioid receptor gene (Oprk1) KO mice exhibited enhanced thermal hyperalgesia and increased allodynic response.[16,17] These different roles exerted by dynorphin (ie, pronociceptive and antinociceptive) can be mediated through opioid and non-opioid spinal mechanisms.[18] In fact, when injected at relatively low doses, dynorphin exhibits antinociceptive properties mediated by κ-opioid receptor activation. Conversely, the intrathecal administration of a high dose seems to activate *N*-methyl-D-aspartate (NMDA) receptors, producing non-opioid receptor-mediated neurotoxicity and hyperalgesia.[19–21]

The brain-derived neurotrophic factor (BDNF) is an important modulator of neuropathic pain.[9,22,23] In fact, an increase in this neurotrophin release was detected in the dorsal horn of animals exhibiting thermal hyperalgesia.[24,25] Moreover, rats subjected to chronic constriction injury (CCI) showed typical neuropathic pain signs, together with a higher BDNF immunoreactivity in the ipsilateral dorsal horn.[26] Interestingly, it has been demonstrated that BDNF significantly increases Pdyn mRNA when applied to cultured spinal neurons, thus highlighting an important correlation between BDNF and dynorphin.[27]

Neuroimaging studies revealed that morphological and functional changes are induced by neuropathic pain in several brain regions.[28–30] Plastic alterations developing after nerve injury involve cortical and subcortical structures, such as the thalamus (TH) and the brainstem (BS).[31] Moreover, the reorganization of the anterior cingulate cortex (ACC) and prefrontal cortex (PFC) was detected in patients suffering from chronic pain[32–34] and changes in the nucleus accumbens (NAc) have been documented. This evidence suggests that alterations of the functional connectivity in the corticostriatal circuitry are implicated in the transition from acute to chronic pain.[35] Finally, morphological and neuroanatomical alterations of the limbic regions, such as the amygdala (AMY) and the hippocampus (HIPPO), have been detected as a consequence of chronic pain conditions.[36]

Since limited information on the dynorphinergic system at supraspinal level during neuropathic pain conditions is currently available, the present study aimed to investigate whether a persistent pain condition could evoke alterations of Pdyn and Oprk1 gene expression in the BS, TH, AMY, HIPPO, caudate putamen (CPu), NAc, ACC and PFC, 14 days after the CCI surgery. Because of the relationship between BDNF and dynorphin in spinal neurons,[27] the expression levels of the brain-derived neurotrophic factor gene (Bdnf) were also measured in the areas where Pdyn and Oprk1 gene expression was found to be altered.

2 | MATERIAL AND METHODS

2.1 | Animals

Adult male Swiss mice (ICR/CD-1®, Harlan Lab, Udine, Italy) weighing 28.36 ± 2.26 g (about 5 weeks of age at the time of arrival) were housed 4 per cage in temperature- and humidity-controlled conditions under a constant 12 hour light/dark cycle (lights on at 7 AM). Standard lab chow and tap water were available ad libitum. The mice were allowed to acclimatize for at least 1 week before any experimental procedure. All experiments were carried out in accordance with the European Communities Council Directive of November 24, 1986 (86/609/EEC), National (Ministry of Health, Italy) laws and policies (authorization number 139/2012-B) and with the International Association for the Study of Pain guidelines. Care was taken to minimize the number of animals and to avoid animal stress and discomfort during handling and procedures. This study was approved by the "Ethical Scientific Committee for Animal Experiments" at the University of Bologna.

2.2 | Surgical procedures

The mice were subjected to sciatic nerve lesion, according to the CCI model[37] adapted for mouse.[38] Briefly, the mice were anesthetized with a mixture of sodium pentobarbital and chloral hydrate (30 and 130 mg/kg intraperitoneal injection) and 3 loose ligatures were tied around the right sciatic nerve exposed at the level of the mid-thigh, proximal to the trifurcation of the nerve. Sham-operated mice, subjected to sciatic nerve exposure without ligature, were used as controls. A total of 12 mice (6 injured and 6 sham-operated, previously randomly assigned to groups) were used for behavioral tests and, subsequently, for the gene expression analysis.

2.3 | Behavioral tests

The presence of neuropathic pain behavioral signs was assessed using the plantar test (heat hyperalgesia)[39] and the acetone test (cold allodynia),[40] before (day 0) and 14 days after sciatic nerve lesion or sham surgical procedure (n = 6 for each group). For both behavioral tests, the thermal latency was determined in triplicate for each animal by experimenters who were blind to group assignment, with 5-minute intervals to prevent thermal sensitization and behavioral disturbances. No significant differences among the 3 independent measurements were observed.

In the plantar test,[39] the animals were placed in the test chambers (Ugo Basile, Varese, Italy) 15 minutes prior to measurements, to enable acclimation. Then, right paw withdrawal latencies to low intensity heat stimuli were measured automatically. Infrared beam intensity was set at 20 to produce a baseline threshold of approximately 10 seconds; a 30-second cut-off time was adopted.[41]

Two hours after the plantar test assessment, the acetone test was used to investigate the presence of thermal allodynia conditions. Briefly, the mice were placed in plexiglas cages and, after about 15 minutes of acclimation, acetone (40 μL) was applied to the dorsal hind paw ipsilateral to the injury. The evoked behavioral response was recorded by means of an arbitrary score as follows: 0, no response; .5, licking response; 1, flinching and brushing of the paw; 2, strong flinching; 3, strong flinching and licking.[40] The observation was carried out during the 30 seconds following acetone application.

2.4 | Tissue collection

On day 14, 4 hours after the behavioral tests, the animals were sacrificed and the brain areas of interest were removed and quickly frozen

on dry ice. The brains were dissected under a stereomicroscope and the areas were collected in accordance with mouse brain atlas.[42] The brains were placed onto an ice-cold matrix with 1 mm coronal section slice intervals. The AMY was punched from the slice taken from −1 mm to 0 mm (bregma). The ACC was punched from slice 0 to +1 mm, the NAc from slice +1 mm to +2 mm, and the CPu was collected from both slices (0 to +2 mm). PFC was obtained from the +2 mm to +3 mm slice. Finally, HIPPO, TH and BS were obtained by gross dissection. The tissues were stored at −80°C until gene expression analysis.

2.5 | Quantitative real-time RT-PCR

Total RNA was extracted according to the method of Chomczynski and Sacchi.[43] Each sample (n = 6 per group) was subjected to DNase treatment and converted to cDNA with the GeneAmp RNA PCR kit (Life Technologies Italia, Monza, Italy), according to the manufacturer's protocol. Quantitative real-time (RT) PCR analysis was performed on a StepOne Real-Time PCR System (Life Technologies) using the SYBR Green PCR MasterMix (Life Technologies). Each sample was run in triplicate. Relative expression of different gene transcripts was calculated by the Delta-Delta Ct (DDCt) method and converted to relative expression ratio (2^{-DDCt}) for statistical analysis.[44] All data were normalized to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (Gapdh). The specificity of each PCR product was determined by melting curve analysis, constructed in the range of 60°C to 95°C.[45]

The primers used for PCR amplification were designed using Primer 3, and are here reported: Gapdh Forward 5′-AACTTTGGCATTGTGGAAGG-3′; Gapdh Reverse 5′-ACACATTGGGGGTAGGAACA-3′; Pdyn Forward 5′-CCCTCTAATGTTATGGCGGA-3′; Pdyn Reverse 5′-AGAGACCGTCAGGGTGAGAA-3′; Oprk1 Forward 5′-GGTGACTTGGAAAGCTGACG-3′; Oprk1 Reverse 5′-AAGCACTGGGAGAGCAGGTA-3′; Bdnf Forward 5′-GCGGCAGATAAAAAGACTGC-3′; Bdnf Reverse 5′-CCTATGAATCGCCAGCCAAT-3′.

2.6 | Statistical analysis

Data from the plantar test were statistically analyzed by Student's t test, and results from the acetone test by Mann-Whitney U-test. Gene expression data were analyzed by Student's t-test. For the statistical analysis, the GraphPad Prism 7 software (San Diego, California) was used. Results are expressed as mean ± SEM of 6 animals per group. The threshold of statistical significance was set at a P value <.05.

3 | RESULTS

3.1 | Hyperalgesia and allodynia development

In the plantar test, the withdrawal latencies of the CCI mice right paw were significantly lower at day 14 compared with pre-surgery values (2.30 ± .37 vs 11.04 ± .71; P < .001, t = 1.99, DF = 10) (Figure 1A).

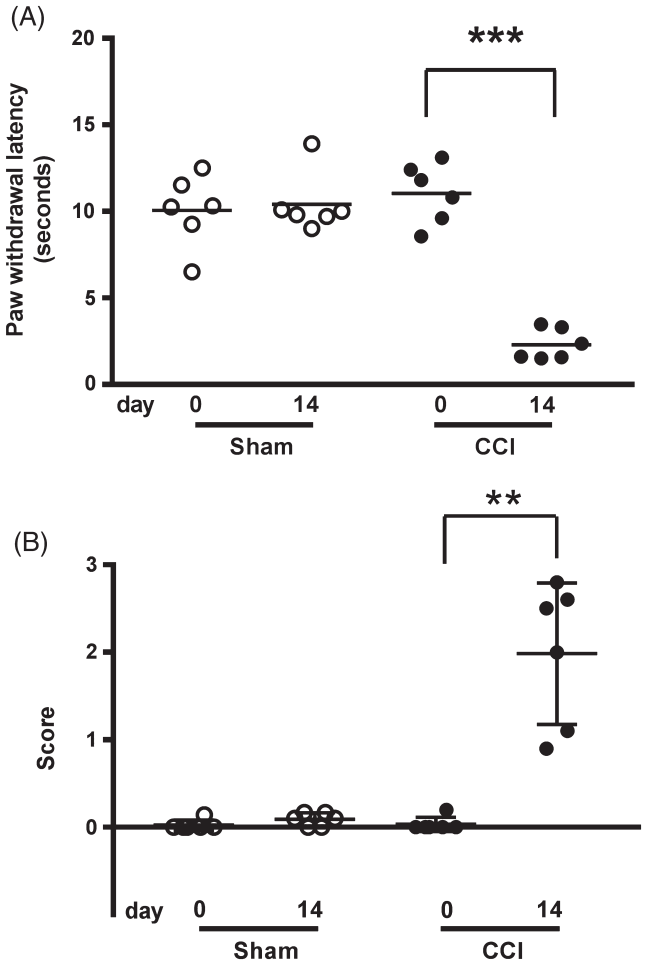

FIGURE 1 Behavioral signs of neuropathic pain (hyperalgesia and allodynia) assessed before surgery (day 0) and 14 days after CCI or sham ligature (Sham). (A) Plantar test: hyperalgesia was assessed by recording paw withdrawal latency. (B) Acetone test: cold allodynia was measured by a response score (0 = no response, .5 = licking, 1 = flinching and brushing the paw, 2 = strong flinching, 3 = strong flinching and licking). Data from the plantar test were analyzed by Student's t-test, whereas acetone test results were analyzed by Mann–Whitney U-test (**P < .01; ***P < .001). All values are expressed as mean ± SEM

In addition, the CCI animals also exhibited thermal allodynia in the acetone test, showing significantly higher scores at day 14 in comparison with pre-surgery values (1.98 ± .33 vs .03 ± .03; P < .01, sum of ranks = 21, 57, U = 0) (Figure 1B).

3.2 | Pdyn gene expression

Fourteen days after surgery, no significant alterations of Pdyn gene expression were observed in the BS, TH, AMY and HIPPO (Figure 2A). A significant increase was observed in the ACC of the sciatic nerve-injured mice compared with the sham animals (CCI group = 3.98 ± .27 vs sham mice = 1.00 ± .04, t = 10.71, DF = 10; P < .001) (Figure 2B). Similarly, an up-regulation of Pdyn mRNA was detected in the PFC of the CCI mice (CCI group = 1.85 ± .17 vs sham mice = 1.00 ± .14, t = 3.96, DF = 10; P < .01) (Figure 2B). Finally, no changes in Pdyn mRNA levels were detected in the CPu and NAc areas (Figure 2B).

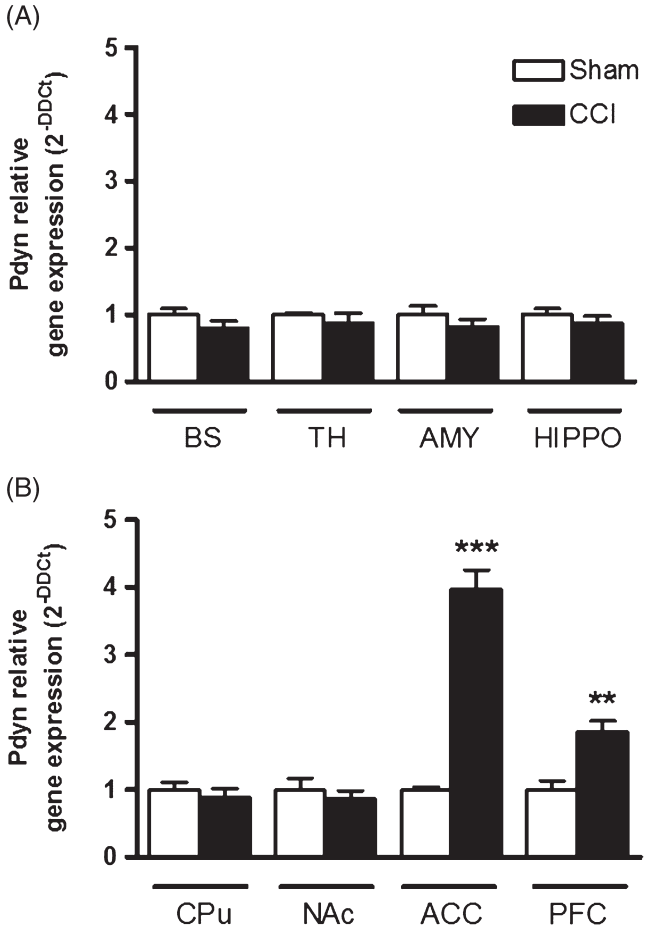

FIGURE 2 Pdyn mRNA changes in the (A) BS, TH, AMY, HIPPO and (B) CPu, NAc, ACC, PFC of mice subjected to CCI compared with sham-operated (Sham) animals (n = 6 animals per group), 14 days after surgery. Relative gene expression was calculated by the Delta-Delta Ct (DDCt) method and normalized to the reference gene Gapdh. Data were analyzed by t-test (**P < .01, ***P < .001 vs Sham) and are expressed as mean ± SEM

3.3 | Oprk1 gene expression

Similarly to the observations for Pdyn gene expression, no significant changes in Oprk1 mRNA levels were detected in the BS, TH, AMY and HIPPO (Figure 3A). Instead, a significant decrease in Oprk1 gene expression was observed in the NAc of the CCI mice compared with the sham group (CCI group = .41 ± .12 vs sham mice = 1.00 ± .17, t = 2.75, DF = 10; P < .05) (Figure 3B). Moreover, a similar trend in Oprk1 gene expression was detected in the ACC, with a significant decrease in its mRNA levels (CCI group = .58 ± .16 vs sham mice = 1.00 ± .03, t = 2.52, DF = 10; P < .05) (Figure 3B). On the contrary, a marked up-regulation in Oprk1 mRNA was observed in the PFC of the CCI mice (CCI group = 2.40 ± .13 vs sham mice = 1.00 ± .14, t = 7.16, DF = 10; P < .001, Figure 3B). No significant Oprk1 gene expression alteration was observed in the CPu (Figure 3B).

3.4 | Bdnf gene expression

In areas showing a significant change in Pdyn/Oprk1 gene expression, we also investigated the Bdnf mRNA levels. In particular, nerve-injured mice showed a marked increase in total Bdnf mRNA in the ACC (CCI group = 1.53 ± .10 vs sham mice = 1.00 ± .10, t = 3.72, DF = 10; P < .01) (Figure 4). Similarly, a significant up-regulation of Bdnf gene expression was observed in the PFC (CCI group = 2.54 ± .16 vs sham mice = 1.00 ± .08, t = 8.68, DF = 10; P < .001) (Figure 4). Finally, no significant alteration was observed in the NAc (Figure 4).

4 | DISCUSSION

Dynorphinergic system involvement in neuropathic pain has mainly been investigated at spinal level,[10,18,46] and the lack of adequate information at supraspinal level makes it difficult to understand its role in this pathological condition.

In the present study, Pdyn and Oprk1 gene expression were assessed in the BS, TH, AMY, HIPPO, CPu, NAc, ACC and PFC of sciatic nerve injured mice, at 14 days after the lesion. At this time point, the presence of neuropathic pain was ascertained by the detection of heat hyperalgesia and thermal allodynia.

The RT-PCR analysis indicated a significant up-regulation of Pdyn gene expression in the ACC and PFC of the CCI mice. ACC and PFC

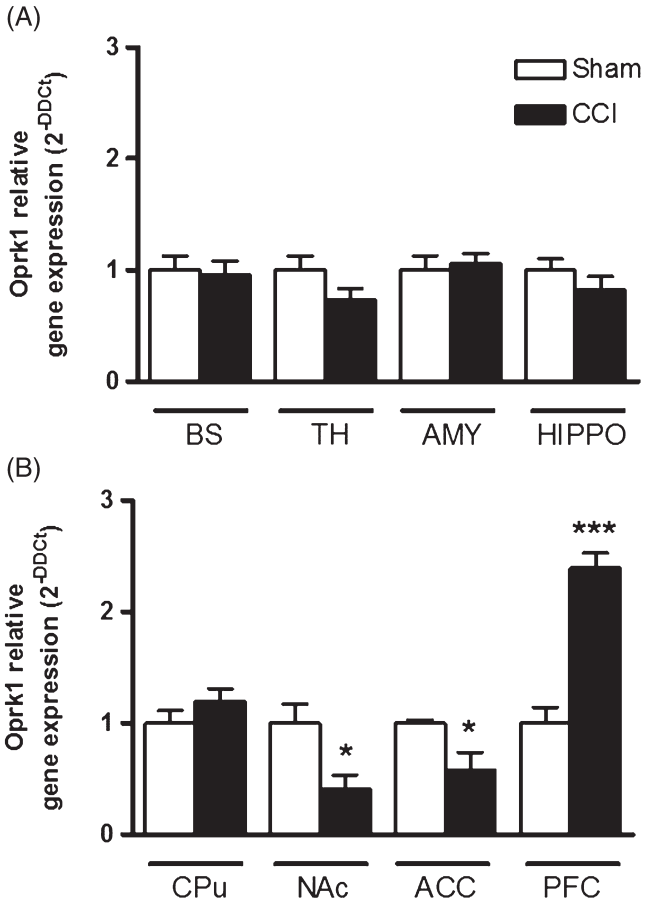

FIGURE 3 Oprk1 mRNA changes in the (A) BS, TH, AMY, HIPPO and (B) CPu, NAc, ACC, PFC of mice subjected to CCI compared with sham-operated (Sham) animals (n = 6 animals per group), 14 days after surgery. Relative gene expression was calculated by the Delta-Delta Ct (DDCt) method and normalized to the reference gene Gapdh. Data were analyzed by Student's t-test (*P < .05, ***P < .001 vs Sham) and are expressed as mean ± SEM

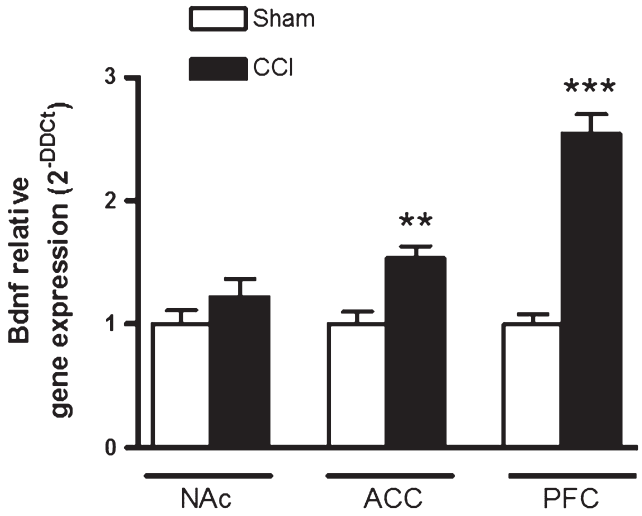

FIGURE 4 Bdnf mRNA changes in the NAc, ACC and PFC of mice subjected to CCI compared with sham-operated (Sham) animals (n = 6 animals per group), 14 days after surgery. Relative gene expression was calculated by the Delta-Delta Ct (DDCt) method and normalized to the reference gene Gapdh. Data were analyzed by Student's t-test (**P < .01, ***P < .001 vs Sham) and are expressed as mean ± SEM

are sequentially activated along the pathway conveying nociceptive information from the TH. Together with the AMY, ACC and PFC project to the periaqueductal gray (PAG) matter, which in turn, modulates the pain control descending system.[29] Thus, our results indicate that after peripheral nerve injury Pdyn mRNA alterations, so far mainly reported at spinal level,[11,47,48] also occur in the cortical regions. In this regard, it has been observed that excitotoxic spinal cord injury causes an increase in Pdyn mRNA in the parietal cortex.[49,50]

A parallel gene expression increase was observed for Pdyn in the ACC and PFC, whereas the changes in Oprk1 gene expression displayed different directions in these 2 areas. In particular, Oprk1 mRNA levels in the CCI animals were significantly lower compared with the sham mice in the ACC, while they were significantly higher than the sham controls in the PFC. This peculiar alteration pattern emphasizes the involvement of the dynorphinergic system in the altered cortical function observed in chronic pain conditions.[29,32]

It is well known that the dynorphin release at spinal level can affect nociceptive behavior[51] in different ways. In addition to the antinociceptive effects due to the activation of κ-opioid receptors, pronociceptive actions are also reported following the interaction of high dynorphin concentrations with non-opioid receptors, such as the NMDA receptor.[19,21] Based on our results, it is conceivable that the up-regulation of both Pdyn and Oprk1 gene expression in the PFC could be related to a κ receptor-mediated action of dynorphin. On the other hand, the ACC marked increase (approximately 4-fold) in Pdyn mRNA levels accompanied by a decrease in Oprk1 gene expression, might suggest a non-opioid receptor-mediated action in this specific cortical region. In this regard, it is worth noting that the NMDA receptor blockade in the ACC prevents the development of neuropathic pain.[52]

In addition, the reported Pdyn and Oprk1 expression increase in the PFC could be related to the concomitant development of a negative emotional state, frequently associated with a neuropathic pain condition.[53–55]

Besides cortical regions, altered Oprk1 gene expression was observed in the NAc with lower levels of Oprk1 mRNA in the CCI mice compared with the sham animals. These selective supraspinal changes (ACC, PFC, NAc) suggest that the dynorphinergic system could participate in alterations to the corticostriatal circuitry, observed in persistent pain conditions.[35,56]

In particular, the κ-opioid receptor modulates the dopaminergic tone in the NAc,[57,58] and alterations in the accumbal dopamine-dynorphin interplay during neuropathic pain can lead to an enhanced aversive learning state.[59] It has been observed that 28 days later, but not after 5 days, the spared nerve injury animals exhibited a decrease in Oprk1 gene expression in the NAc, together with a similar decrease in dopamine receptors.[59] Data here reported indicated that the down-regulation in Oprk1 gene expression is already present 14 days after surgery in our CCI model. Accordingly, a reduced expression of dopamine receptor 2 was observed 2 weeks after peripheral nerve injury in the same area.[60]

Differently from what we observed in the NAc, we did not report any gene expression change for the dynorphinergic system in the CPu of the CCI animals. These selective striatal alterations observed during neuropathic pain underline the specific involvement of this system at accumbal level, where close dopamine-dynorphin interaction is relevant in modulating negative emotional states.[54,59,61]

The other investigated areas, the BS and TH, are essential relays for the rostral transmission of the nociceptive input. Their interconnection with cortical and limbic structures, such as the AMY, is crucial in pain modulation.[62,63] Functional and neurochemical alterations occurring in these areas during neuropathic pain can be responsible for the dysregulation of the descending pain modulatory system.[64–66] In the present study, no alterations in Pdyn and Oprk1 mRNA levels were observed in the BS, TH and AMY 14 days after surgery. As previously mentioned, few data are available about the dynorphinergic system during chronic pain at supraspinal level. In this regard, our results are in agreement with previous studies showing the absence of dynorphin peptide changes in BS nuclei,[67] and the lack of Pdyn mRNA alterations in the TH,[50] in distinct models of persistent pain.

Our results did not show any significant alteration in Pdyn and Oprk1 mRNAs in the HIPPO, suggesting that the dynorphinergic system might not be implicated in the morphological and functional changes to this region, observed in neuropathic pain conditions.[68,69]

In addition to the dynorphinergic system, we also investigated Bdnf gene expression in the brain areas showing changes in Pdyn and/or Oprk1 mRNA levels. In particular, changes in the Bdnf total transcript were observed in the ACC and PFC, and not in the NAc 14 days after nerve injury. The pronociceptive role of BDNF has been reported at spinal level,[9] together with an increase in its immunoreactivity in the dorsal horn of CCI animals.[26] Data here reported indicate that a significant increase in Bdnf mRNA also occurs at the cortical level in neuropathic pain conditions.

According with our results, the up-regulation of the BDNF mature protein has been observed in the ACC during inflammatory pain.[70] However, another study reported that a decrease in Bdnf mRNA and protein takes place in rat ACC 21 days after the sciatic

nerve injury.[71] The only study focusing on BDNF and PFC in neuropathic pain did not observe significant changes in the amount of BDNF protein .[72] Considering that few and contrasting data are currently available, further studies are required to clarify BDNF alterations at cortical level in neuropathic pain conditions.

It is worth noting that we observed a simultaneous increase in Bdnf and Pdyn mRNAs in both the ACC and PFC. in vitro experiments demonstrated that neurotrophins, such as BDNF or neurotrophin-3, can modulate the expression of several neurotransmitters, including neuropeptides. In particular, BDNF induces a significant increase in Pdyn mRNA in cultured spinal neurons.[27] In this view, our results suggest that the interplay between BDNF and dynorphin may also occur in an in vivo model of neuropathic pain, precisely at cortical level.

In conclusion, our data indicated that CCI animals show significant alterations in Pdyn and Oprk1 mRNA levels in selected brain areas (ACC, PFC and NAc) 14 days after surgery. Taking into account the dynorphinergic role in driving dysphoric and aversive behavior at corticostriatal level, these changes might contribute to the negative affective component of pain. Cortical changes in Pdyn mRNA are accompanied by an increase in Bdnf gene expression, suggesting an interaction between these 2 systems in the maladaptive neuroplasticity that characterizes neuropathic pain conditions.

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Conflict of interest

Authors declare that they have no conflicts of interest.

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