Neurochemical Research (2019) 44:2068–2080
https://doi.org/10.1007/s11064-019-02843-z

ORIGINAL PAPER

N-methyl-D-aspartate Receptors in the Prelimbic Cortex are Critical for the Maintenance of Neuropathic Pain

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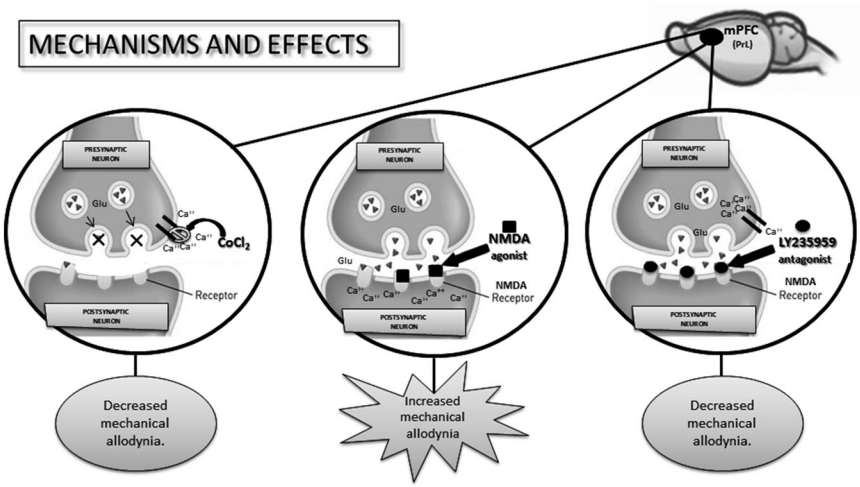
Received: 14 September 2018 / Revised: 27 June 2019 / Accepted: 4 July 2019 / Published online: 17 July 2019

Abstract

The mechanisms underlying chronic and neuropathic pain pathology involve peripheral and central sensitisation. The medial prefrontal cortex (mPFC) seems to participate in pain chronification, and glutamatergic neurotransmission may be involved in this process. Thus, the aim of the present work was to investigate the participation of the prelimbic (PrL) area of the mPFC in neuropathic pain as well as the role of *N*-methyl D-aspartate (NMDA) glutamate receptors in neuropathic pain induced by a modified sciatic nerve chronic constriction injury (CCI) protocol in Wistar rats. Neural inputs to the PrL cortex were inactivated by intracortical treatment with the synapse blocker cobalt chloride ($CoCl_2$, 1.0 mM/200 nL) 7, 14, 21, or 28 days after the CCI or sham procedure. The glutamatergic agonist NMDA (0.25, 1 or 4 nmol) or the selective NMDA receptor antagonist LY235959 (2, 4 or 8 nmol) was microinjected into the PrL cortex 21 days after surgery. $CoCl_2$ administration in the PrL cortex decreased allodynia 21 and 28 days after CCI. NMDA at 1 and 4 nmol increased allodynia, whereas LY235959 decreased mechanical allodynia at the highest dose (8 nmol) microinjected into the PrL cortex. These findings suggest that NMDA receptors in the PrL cortex participate in enhancing the late phase of mechanical allodynia after NMDA-induced increases and LY235959-induced decreases in allodynia 21 days after CCI. The glutamatergic system potentiates chronic neuropathic pain by NMDA receptor activation in the PrL cortex.

Graphic Abstract



Mechanism of neuropathic pain. The infusion of $CoCl_2$, a synapse activity blocker, into the prelimbic (PrL) division of the medial prefrontal cortex (mPFC) decreased the severity of mechanical allodynia, showing the late participation of the limbic cortex. The glutamatergic system potentiates chronic neuropathic pain via NMDA receptor activation in the PrL cortex.

Sérgio Henrique Ferreira—In memoriam.

Extended author information available on the last page of the article

Keywords Sciatic nerve chronic constriction injury · Neuropathic pain · Prelimbic cortex · Medial prefrontal cortex · Cobalt chloride · NMDA glutamatergic receptor

Introduction

The global incidence rate of chronic pain is approximately 8% [1–4]. The estimated prevalence of neuropathic pain is 23% [4]. According to the United States Institute of Medicine, 100 million Americans suffer from chronic pain at a cost of U$600 billion. For this estimate, the incremental cost of health care and the cost of lost productivity attributable to pain are combined [5].

Neuropathic pain is related to the response of nervous system tissue to neural damage. Neuropathic pain can be induced by lesions of the peripheral nervous system (PNS) caused by tumour invasion, metabolic diseases, infection, neurotoxic chemicals or mechanical trauma leading to pathophysiological changes in either the PNS or the central nervous system (CNS) [6, 7]. Pain is an important signal for preventing further lesions or avoiding tissue injury. The genesis and duration of chronic pain are critically different from those of acute pain, and the underlying mechanisms of chronic pain need to be more thoroughly studied [8].

In fact, neuropathic pain develops through neurochemical changes in the PNS and CNS, affecting peripheral nerves and spinal and cortical structures [9]. In the periphery, neuropathic lesions trigger peripheral sensitisation that induces inflammatory processes and abnormal long-term neural activity (long-term potentiation, LTP) along primary afferent pathways [10]. In the spinal cord, dorsal horn neurons exhibit enhanced excitatory responses and decreased firing thresholds in response to pain [11, 12]. Concerning supraspinal/cortical regions, there is evidence of changes in excitatory transmission in animal models of neuropathic pain [9].

Currently, there is increased attention focused on the identification of cortical regions that mediate chronic neuropathic pain [9]. For that purpose, different experimental models have been used to reproduce neuropathic pain in rodents. Animal models of sciatic nerve injuries have been used to investigate the mechanisms underlying neuropathic pain and to test effective pharmacological treatments [13–17]. In both human and laboratory animal models, peripheral nerve injury manifests as spontaneous pain that can be induced by innocuous stimuli (allodynia) [13].

Cortical brain regions such as the rostral divisions of the frontal lobe have also been implicated in the amplification of neuropathic pain. In fact, the medial prefrontal cortex (mPFC) is involved in signalling the unpleasantness of pain [18], and the anterior cingulate cortex (ACC) is implicated in the affective component of pain [19]. The ACC and mPFC are also involved in the anticipation of pain [20], and the prelimbic (PrL) division of the mPFC contributes to innate fear-induced antinociception [21, 22], a robust phenomenon capable of reducing both pain and inflammation [23]. In addition, Falconi-Sobrinho et al. [24] showed that pretreatment of the ACC with lidocaine decreased the panic-like and antinociceptive effects evoked by inhibitory γ-aminobutyric acid A ($GABA_A$) receptor blockade in the hypothalamus, which indicates that there are descending excitatory pathways from the former region to the latter. In addition, the mPFC has different functions that can result in different types of behavioural outputs. Wang and collaborators [25] have shown that bilateral or contralateral lesions of the PrL of the mPFC increase paw withdrawal thresholds in animals with chronic inflammatory lesions. In fact, studies have consistently shown that glutamatergic synapses not only play a significant role in sensory transmission, including pain and itch transmission, but also contribute to nociceptive sensitisation at different levels of the brain [26]. Current pharmacological treatments focused on neurochemical modifications at the spinal cord level that fail to decrease chronic pain are often followed by adverse side effects and decrease in efficacy over time. However, recent studies have focused on molecular and functional changes within brain regions associated with pain modulation and the development of neuropathic pain. In fact, synaptic plasticity within key cortical areas is involved in pain facilitation in the context of neuropathic pain [26].

Considering that evidence suggests the participation of neocortical regions in neuropathic pain, our hypothesis in the present study is that the PrL cortex is recruited during the chronic phase of pain to potentiate neuropathic pain through *N*-methyl D-aspartate (NMDA) glutamatergic receptor signalling. Then, we investigated the involvement of the PrL cortex in the potentiation and maintenance of neuropathic pain in a modified chronic constriction injury (CCI) model of neuropathic pain generated by placing a single loose ligature around a peripheral nerve. For this purpose, we microinjected the synapse transmission blocker cobalt chloride ($CoCl_2$) into the PrL cortex of Wistar rats and monitored mechanical allodynia from 7 to 28 days after CCI surgery. In addition, we investigated the involvement of NMDA glutamate receptors localised to the PrL division of the mPFC in the potentiation and maintenance of mechanical allodynia 21 days after CCI in an experimental model of chronic neuropathic pain.

Materials and Methods

Animals and Housing Conditions

Male Wistar rats from the animal care facility of the University of São Paulo (USP; Campus of Ribeirão Preto) were

used. The animals were housed in groups of four with free access to food and water. The housing room was kept at a constant room temperature (22 ± 1 °C) and illuminated on a fixed light–dark cycle (lights on 07:00–19:00 h). All protocols were in compliance with the recommendations of the Committee for Ethics in Animal Experimentation (CETEA) of the Ribeirão Preto Medical School of the University of São Paulo (FMRP-USP) (Processes 015/2005 and 036/2017), which are in accordance with the animal research ethics adopted by the National Council for Animal Experimentation Control (CONCEA) and with the International Association for the Study of Pain (IASP) guidelines for pain research on animals [27]. Each animal was used in a single experimental group, and all efforts were made to minimise the discomfort of the animals.

Neuropathic Pain Induction

A modified model of CCI of the sciatic nerve was used to induce neuropathic pain. A single ligature consisting of a chromic catgut suture was loosely placed around the right sciatic nerve. Each animal was anaesthetised in turn with a mixture of ketamine (União Química Farmacêutica Nacional, Embu-Guaçu, São Paulo, Brazil) and xylazine (Hertape/Calier, Juatuba, Minas Gerais, Brazil) (92 mg/kg and 9.2 mg/kg, respectively, i.p.). The right hind paw was shaved and moistened with a disinfectant, and the common sciatic nerve was exposed at the mid-thigh level by blunt dissection through the biceps femoris. Then, proximal to the sciatic nerve trifurcation, approximately 7 mm of the nerve was freed of the adhering tissues, and one loose ligature (4.0 chromic gut) was placed around the peripheral nerve. The wound was then closed by suturing the muscle with chromic catgut sutures in a continuous suture pattern. Finally, the skin was closed with 3–0 black braided silk sutures in a horizontal mattress suture pattern. Sham surgery was performed by exposing the sciatic nerve as described above but without performing ligation. The animals were then transferred to their home cages and allowed to recover.

Measurement of Mechanical Allodynia

Mechanical allodynia was assessed by von Frey test filaments (Stoelting, Chicago, IL, USA). Each animal was placed in a transparent acrylic cage (22 × 16.5 × 14 cm) with a wire-grid floor for approximately 20 min to allow behavioural acclimation to the novel environment before testing. Then, von Frey filaments in ascending order from 10 to 100 g were applied through the mesh floor to the plantar surface of the centre of the paw or to the base of the third or fourth toe of the injured and uninjured hind paws for approximately 3–4 s per filament to induce the withdrawal reflex. The interval before the application of the next filament was at least 10 s. The data are expressed as mechanical withdrawal thresholds (MWTs) in grams. MWTs were evaluated before and after CCI or sham surgery and before and after pharmacological treatment. The test was repeated three times for both hind paws (the ipsilateral and contralateral paws were tested in alternation) with an interval of at least 2 min between measurements. Before surgery, no differences in the basal responses of the right and left paws were confirmed [28, 29].

Stereotaxic Surgery and Intra-PrL Cortex Drug Administration

For the stereotaxic procedure, a stainless-steel guide cannula (outer diameter of 0.6 mm, inner diameter of 0.4 mm) was implanted in the PrL division of the mPFC in the CCI and sham groups 7, 14 or 21 days after the CCI or sham procedure. Immediately after baseline, MWTs were recorded; the animals were anaesthetised as previously described (2.2) and fixed in a stereotaxic frame (David Kopf, Tujunga, California, USA). Subsequently, a guide cannula was fixed to the skull above the PrL cortex (AP +1.2 mm from bregma; L, ± 0.5 mm from bregma; V, 2.6 mm from the skull) with acrylic resin and two stainless-steel screws [29]. At the end of the surgery, each guide cannula was sealed with a stainless-steel wire to protect it from obstruction.

A neurophysiological approach involving the pretreatment of the mPFC with 200 nL of artificial cerebrospinal fluid (aCSF) vehicle or $CoCl_2$ (1.0 mM) [21, 30–33] was carried out 7, 14, 21 or 28 days after the CCI or sham procedure to evaluate the involvement of the PrL cortex in the acute and chronic stages of neuropathic pain development. Other groups of CCI and sham rats received microinjections of aCSF or $CoCl_2$ into the ipsilateral or contralateral PrL cortex relative to the paw submitted to surgery (the right side). The microinjection was made with a thin dental needle (Mizzy, outer diameter of 0.3 mm) linked by a polyethylene tube to a 1.0-µL hand-driven syringe (Hamilton, Reno, Nevada, USA) and inserted through the guide cannula until its lower end was 1 mm below the tip. The needle was left in place for an additional 1 min after the injection. $CoCl_2$ or vehicle was administered over the course of 5 min, and mechanical allodynia was measured 0, 15, 30, 45 and 60 min after the infusion was finished.

The role of NMDA receptors located in the PrL cortex was investigated by the treatment of the PrL cortex with a selective NMDA receptor agonist and antagonist. Volumes of 200 nL aCSF; NMDA (0.25, 1 or 4 nmol); a selective NMDA receptor agonist; or LY235959 (2, 4 and 8 nmol), an NMDA receptor antagonist, were microinjected into the PrL

cortex 21 days after the CCI or sham procedure. The drugs or vehicle were infused into the PrL cortex contralateral to the surgery (the right side). Each drug or vehicle was administered over the course of 5 min, and mechanical allodynia was measured 0, 15, 30, 45 and 60 min after the infusion was finished. The 0 min time point represents the end of the 5-min PrL cortex pretreatment.

Histological Analysis

After testing, the rats were anaesthetised as previously described (2.2) and perfused through the left ventricle with cold, oxygen-enriched, Ca^{++}-free Tyrode's buffer (40 mL at 4 °C) and ice-cold paraformaldehyde (200 mL, 4% (w/v) in 0.1 M sodium phosphate buffer, pH 7.3) for 15 min at a pressure of 50 mmHg with a perfusion pump (Master Flex L/S peristaltic tubing pump, East Bunker Court Vernon Hills, Illinois, USA). The brainstem was quickly removed, sectioned, and immersed in fresh fixative for 4 h at 4 °C. Then, the brainstem was rinsed for at least 12 h each in 10 and 20% sucrose dissolved in 0.1 M sodium phosphate buffer (pH 7.4) at 4 °C. The tissue pieces were immersed in 2-methylbutane (Sigma-Aldrich, St. Louis, Missouri, USA), frozen on dry ice, embedded in Tissue-Tek optimal cutting temperature (OCT), and cut with a cryostat (CM 1950 Leica, Wetzlar, Germany) at − 22 °C. The slices were subsequently mounted on glass slides (coated with chrome alum-gelatine to prevent detachment) and stained with haematoxylin–eosin using an Autostainer (CV 5030 Autostainer XL, Leica). The positions of the guide cannula tips were determined according to the Paxinos and Watson atlas [34] under a motorised photomicroscope (AxioImager Z1; Zeiss, Oberkochen, Germany). Statistical analysis was performed exclusively with the data from the animals in which the evidence showed that the microinjection into the PrL cortex had been successfully carried out.

Drugs

$CoCl_2$ (1.0 mM/0.2 nL, Sigma) was administered into the right or left hemisphere of the PrL cortex, and aCSF was microinjected into the PrL cortex as a control. NMDA (an NMDA receptor agonist; Sigma-Aldrich, St. Louis, MO, USA) and the NMDA receptor antagonist receptor LY235959 (Tocris; Bristol, United Kingdom) were diluted in aCSF and administered into the left hemisphere.

Analysis of Results

The data are expressed as the mean ± standard error of the mean (S.E.M.). The mechanical allodynia results from the von Frey test were analysed by analysis of variance (ANOVA) for repeated measures. In the case of a significant treatment-by-time interaction, one-way ANOVA was performed, followed by Duncan's post hoc test at each time interval. ANOVA for repeated measures uses the same conceptual framework as classical ANOVA followed by Duncan's post hoc test [20, 21, 30–32, 34].

Results

Effect of Synaptic Blockade in the PrL Cortex on Mechanical Allodynia

The microinjection sites were mainly situated in the prelimbic division of the mPFC, as shown in Fig. 1a–c, e.

To evaluate the involvement of the ipsilateral PrL cortex in the *potentiation* and maintenance of neuropathic pain induced in our modified model of CCI, we microinjected $CoCl_2$ (1.0 mM/200 nL) into the PrL cortex (right hemisphere) 7, 14, 21 or 28 days after the CCI or sham procedure (right hind paw). No changes were observed in the MWTs of the contralateral paws of the rats exposed to the CCI or sham procedure (data not shown).

There were significant effects of treatment [$F_{(3,22)} = 20{,}92$; $p < 0.0001$], time [$F_{(6,17)} = 3.91$; $p < 0.05$] and the treatment-by-time interaction [$F_{(18,47)} = 2.00$; $p < 0.05$] on mechanical allodynia on day 7 (n = 6–7 per group) after CCI induction. Significant effects of treatment [$F_{(3,22)} = 13{,}24$; $p < 0{,}0001$], time [($F_{(6,17)} = 5.97$; $p < 0.01$] and the treatment-by-time interaction [$F_{(18,47)} = 2.26$; $p < 0.05$] on mechanical allodynia were also observed on day 14 (n = 6–7 per group) after CCI, according to repeated-measures ANOVA. The microinjection of $CoCl_2$ into the right PrL cortex did not significantly alter mechanical allodynia in the right hind limb 7 or 14 days after CCI induction (Duncan's post hoc test, $P > 0.05$) (Fig. 2a, b).

On day 21 (n = 6 per group) after CCI, there were significant effects of treatment [$F_{(3,20)} = 21.41$; $p < 0{,}0001$], time [($F_{(6,15)} = 3.94$; $p < 0.05$], and the treatment-by-time interaction [$F_{(18,41)} = 4.44$; $p < 0.0001$], according to repeated-measures ANOVA. Compared with CCI rats that received aCSF (200 nL) in the PrL cortex, rats that received the microinjection of $CoCl_2$ (1.0 mM/200 nL) into the right PrL cortex 21 days after CCI induction exhibited decreased mechanical allodynia between 5 and 30 min after infusion in the PrL cortex (Duncan's post hoc test; $P < 0.05$) (Fig. 2c).

On day 28 (n = 6 per group) after CCI induction, there were significant effects of treatment [$F_{(3,20)} = 119.97$; $p < 0.0001$], time [($F_{(6,15)} = 3.16$; $p < 0.05$], and the treatment-by-time interaction [$F_{(18,41)} = 5.84$; $p < 0.0001$] on mechanical allodynia, according to repeated-measures ANOVA. Compared with CCI rats that received aCSF (200 nL) in the PrL cortex, rats that experienced synaptic

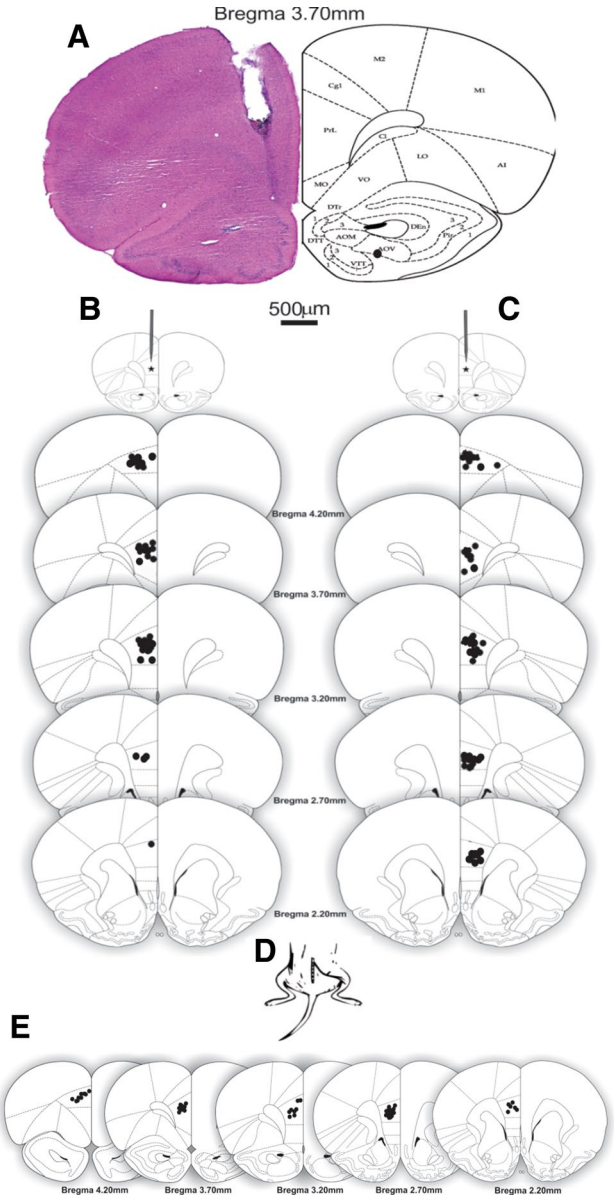


Fig. 1 Histologically confirmed sites of cobalt chloride or aCSF microinjections into the prelimbic (PrL) division of the medial prefrontal cortex in accordance with Paxinos and Watson's stereotaxic atlas (1997). **a** Representative photomicrographs of a transverse brain section showing a microinjection site in the PrL cortex. **b** Microinjections performed in the *contralateral* brain hemisphere in relation to the side on which the CCI or sham procedure was performed. **c** Microinjections performed in the *ipsilateral* brain hemisphere in relation to the side on which the CCI or sham procedure was performed. **d** A representation of the CCI or sham procedure on the right paw. **e** Microinjections performed in the contralateral brain hemisphere in relation to the side on which the CCI or sham procedure was performed in glutamatergic studies

blockade induced by $CoCl_2$ (1.0 mM/200 nL) administration into the right PrL cortex also exhibited decreased mechanical allodynia 21 days after CCI induction, with increased MWT observed between 5 and 30 min after infusion (Duncan's post hoc test; $P < 0.05$) (Fig. 2d).

Effect of Synaptic Blockade in the Contralateral PrL Cortex on Mechanical Allodynia

We also evaluated the involvement of the contralateral PrL cortex in the potentiation and maintenance of neuropathic pain. For that purpose, we microinjected $CoCl_2$ (1.0 mM/200 nL) into the PrL cortex of the left hemisphere 7, 14, 21 and 28 days after the CCI or sham procedure (right hind paw). No changes were observed in the MWTs of the contralateral paws of rats exposed to the CCI or sham procedure (data not shown).

On day 7 ($n = 6$ per group) after CCI, there were significant effects of treatment [$F_{(3,20)} = 37.62$; $p < 0.0001$], time [$F_{(6,16)} = 4.73$; $p < 0.01$], and the treatment-by-time interaction [$F_{(18,41)} = 4.32$; $p < 0.001$], whereas on day 14 ($n = 6$ per group) after CCI induction, there were significant effects of treatment [$F_{(3,20)} = 64.40$; $p < 0.0001$] and the treatment-by-time interaction [$F_{(15,44)} = 2.95$; $p < 0.05$] but not of time [$F_{(5,16)} = 2.73$; $p > 0.05$] on mechanical allodynia, according to repeated-measures ANOVA. The microinjection of $CoCl_2$ (1.0 mM/200 nL) into the left PrL did not modify mechanical allodynia on days 7 or 14 after CCI induction (ipsilateral hind paw) (Duncan's post hoc test; $P > 0.05$) (Fig. 3a, b).

On day 21 ($n = 6$–8 per group) after the sham or CCI procedure, there were significant effects of treatment [$F_{(3,26)} = 14.32$; $P < 0.0001$], time [$F_{(6,21)} = 4.44$; $p < 0.001$], and the treatment-by-time interaction [$F_{(18,59)} = 3.02$; $P < 0.0001$], according to repeated-measures ANOVA. Compared with CCI rats that received aCSF in the contralateral PrL cortex, rats that received the microinjection of $CoCl_2$ (1.0 mM/200 nL) into the contralateral PrL cortex 21 days after CCI induction exhibited decreased mechanical allodynia between 5 and 30 min after infusion (Duncan's post hoc test; $P < 0.05$) (Fig. 3c).

On day 28 ($n = 6$ per group) after CCI induction, there were significant effects of treatment [$F_{(3,20)} = 8.81$; $p < 0.001$], time [$F_{(6,15)} = 5.01$; $p < 0.001$] and the interaction between treatment and time [$F_{(18,41)} = 3.68$; $p < 0.001$] on mechanical allodynia, according to repeated-measures ANOVA. However, compared with CCI rats that received aCSF in the contralateral PrL cortex, rats that experienced synaptic blockade in the contralateral PrL cortex induced by the local administration of $CoCl_2$ (1.0 mM/200 nL) 28 days after CCI induction exhibited decreased mechanical allodynia between 15 and 30 min after infusion, according to Duncan's post hoc test (Fig. 3d).

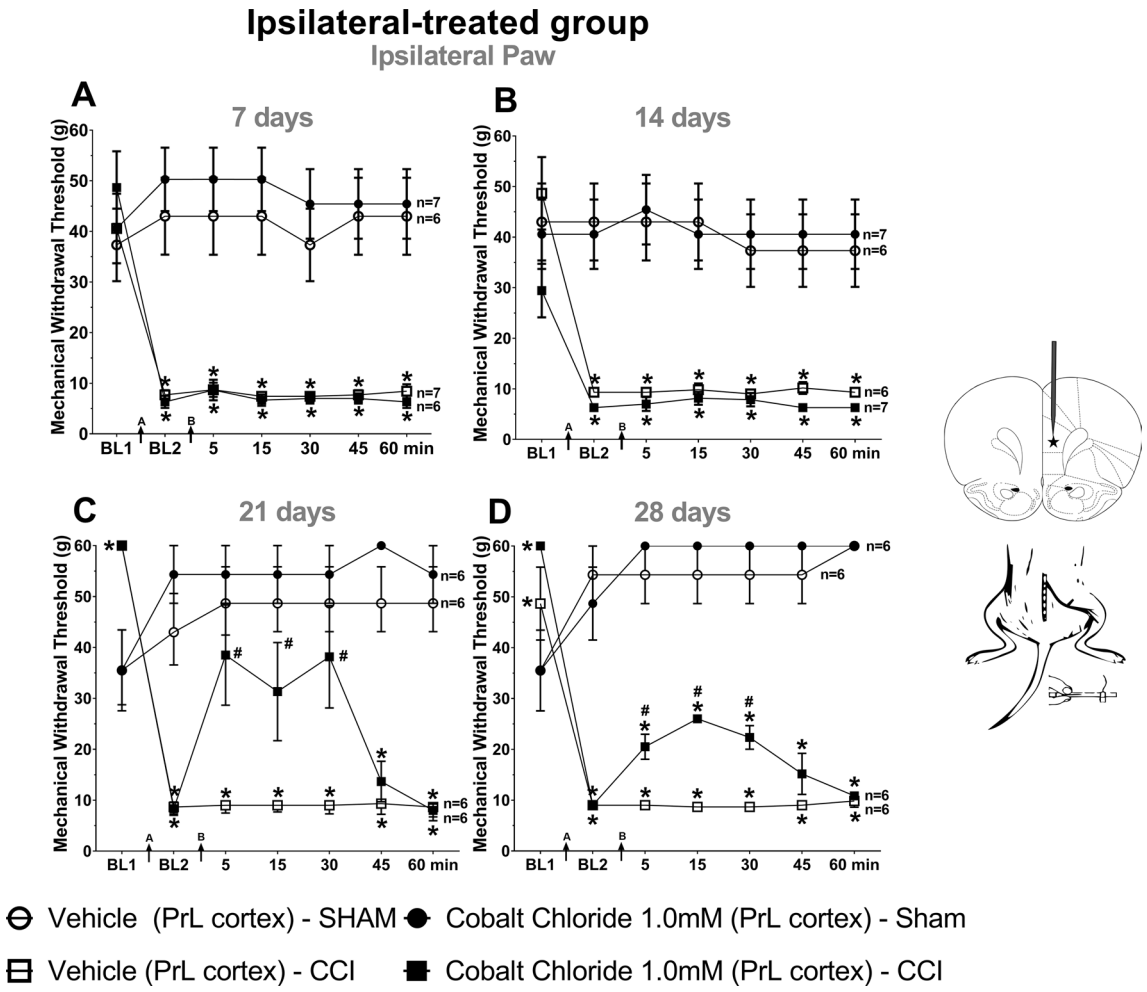


Fig. 2 **a**–**d** The effect of synaptic activity blockade in the *right prelimbic cortex* (PrL) on the genesis and maintenance of neuropathic pain in the *ipsilateral paw* induced by our adapted model of CCI of the right sciatic nerve. *BL1* baseline von Frey test results recorded before each procedure. *Arrow A* the sciatic nerve CCI or sham procedure. *BL2* new baselines recorded 7, 14, 21 and 28 days after the sham or CCI procedure. *Arrow B* treatment with cobalt chloride or aCSF in the PL cortex of sham or CCI rats after BL2 recordings was followed by the von Frey test until 60 min after injection. The microinjection was administered in the *ipsilateral* hemisphere of 6–7 animals per group in relation to the side on which the CCI or sham procedure was performed. *Indicates significant differences compared with the sham + vehicle group; #indicates differences compared with the CCI + vehicle group

Effect of NMDA Microinjection into the PrL Cortex on Mechanical Allodynia

The animals that underwent our modified CCI procedure involving one loose ligature of the sciatic nerve showed mechanical allodynia 21 days after the CCI procedure. The treatment of the PrL cortex increased the MWT 21 days after CCI induction.

On day 21 (n = 6–8 per group) after CCI, there were significant effects of treatment [$F_{(3,24)} = 14.21$; $p < 0.0001$] and time [$F_{(6,19)} = 32.30$; $p < 0.0001$] on the MWT, as well as a significant interaction between those factors [$F_{(18,53)} = 3.66$; $p < 0.0001$], according to repeated-measures ANOVA. Compared with the vehicle-treated group, the group administered the highest dose of NMDA (4 nmol) in the PrL exhibited a significant increase in mechanical allodynia in the ipsilateral paws at the 5 min to 30 min time points ($P < 0.05$), according to repeated-measures ANOVA followed by Duncan's post hoc test. The microinjection of NMDA (1 nmol) significantly changed the MWT but only at 15 min ($P < 0.05$). Compared with aCSF microinjection, the microinjection of the lowest dose of NMDA (0.25 nmol) did not change the MWT ($P > 0.05$) (Fig. 4a). On day 21 after CCI induction or the sham procedure, there were no changes in the MWT of the contralateral hind paw (data not shown).

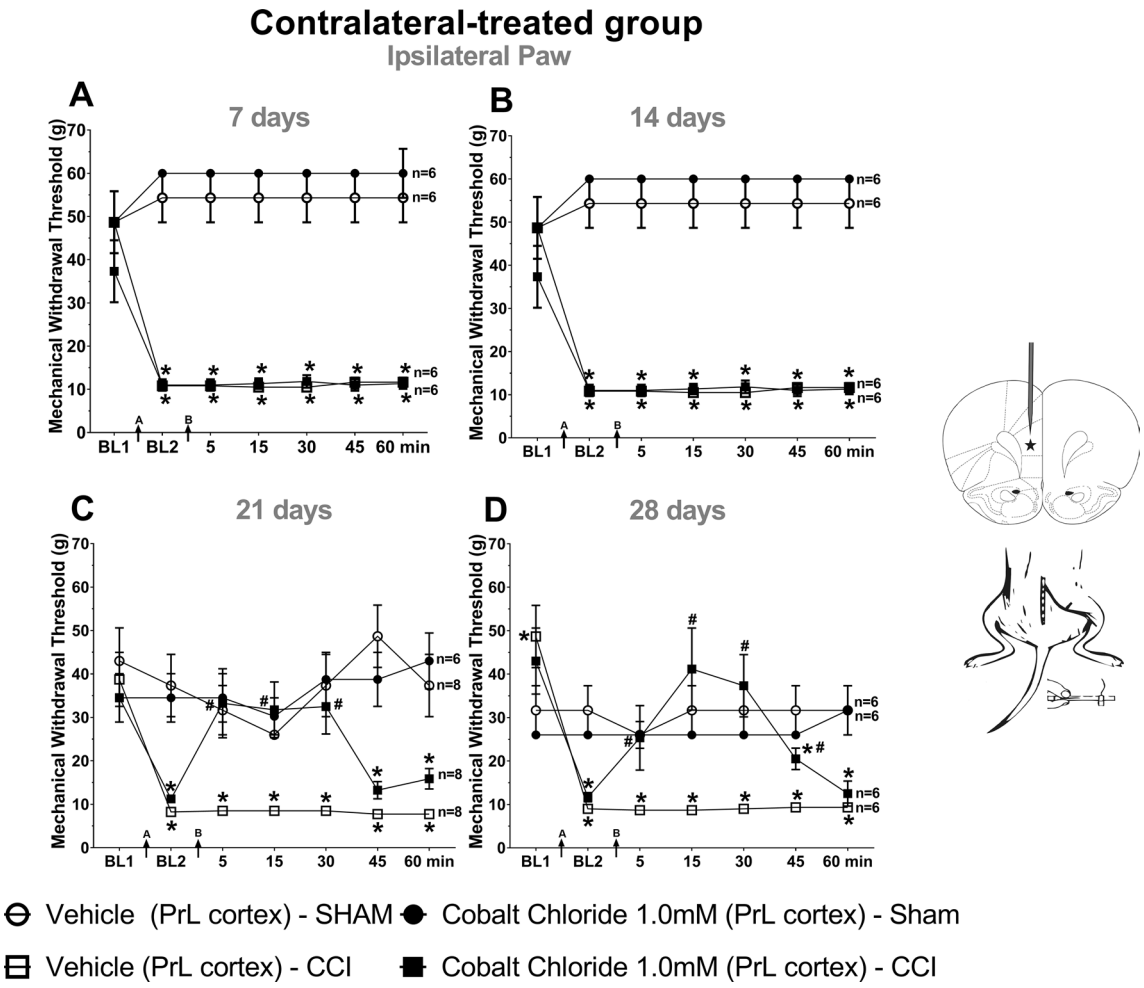


Fig. 3 **a**–**d** The effect of synaptic blockade in the *left prelimbic cortex* (PrL) on the genesis and maintenance of neuropathic pain in the *ipsilateral paw* induced by our adapted model of CCI of the right sciatic nerve. *BL1* baseline von Frey test results recorded before each procedure. *Arrow A* the sciatic nerve CCI or sham procedure. *BL2* new baselines recorded 7, 14, 21 and 28 days after the sham or CCI procedure. *Arrow B* treatment with cobalt chloride or aCSF in the PrL cortex of sham or CCI rats after BL2 recordings was followed by the von Frey test until 60 min after injection. The microinjection was administered in the *contralateral* hemisphere of 6–8 animals per group in relation to the side on which the CCI or sham procedure was performed. *Indicates significant differences compared with the sham + vehicle group; #indicates differences compared to the CCI + vehicle group

Effect of the Microinjection of an NMDA Receptor Antagonist into the PrL Cortex on Mechanical Allodynia

To evaluate the effect of NMDA receptor blockade, we treated the PrL cortex with LY235959 (2, 4 and 8 nmol) 21 days (n = 6–7 per group) after the sham or CCI procedure and tested mechanical allodynia. PrL cortex microinjection decreased the MWT 21 days after CCI induction.

There were significant effects of treatment [$F_{(3,21)} = 6.01$; $p < 0.0001$] and time [$F_{(6,16)} = 110.61$; $p < 0.0001$] on the MWT, as well as a treatment-by-time interaction [$F_{(18,44)} = 31.18$; $p < 0.0001$]. Compared with the vehicle-treated group, the group treated with the highest dose of LY235959 (8 nmol) in the PrL cortex exhibited a significant decrease in mechanical allodynia in the ipsilateral paws between 0 and 30 min ($P < 0.05$), according to repeated-measures ANOVA followed by Duncan's post hoc test. The intermediate and lowest doses of LY235959 (4 and 2 nmol, respectively) produced no significant differences compared to the control at any of the analysed time points ($P > 0.05$) (Fig. 4b). There were no changes observed in the MWTs in the contralateral paws of the rats that underwent the CCI or sham procedure (data not shown).

Discussion

The present modified model of CCI using only one ligature around the sciatic nerve produced mechanical allodynia in rats. In accordance with the results of Xu and collaborators

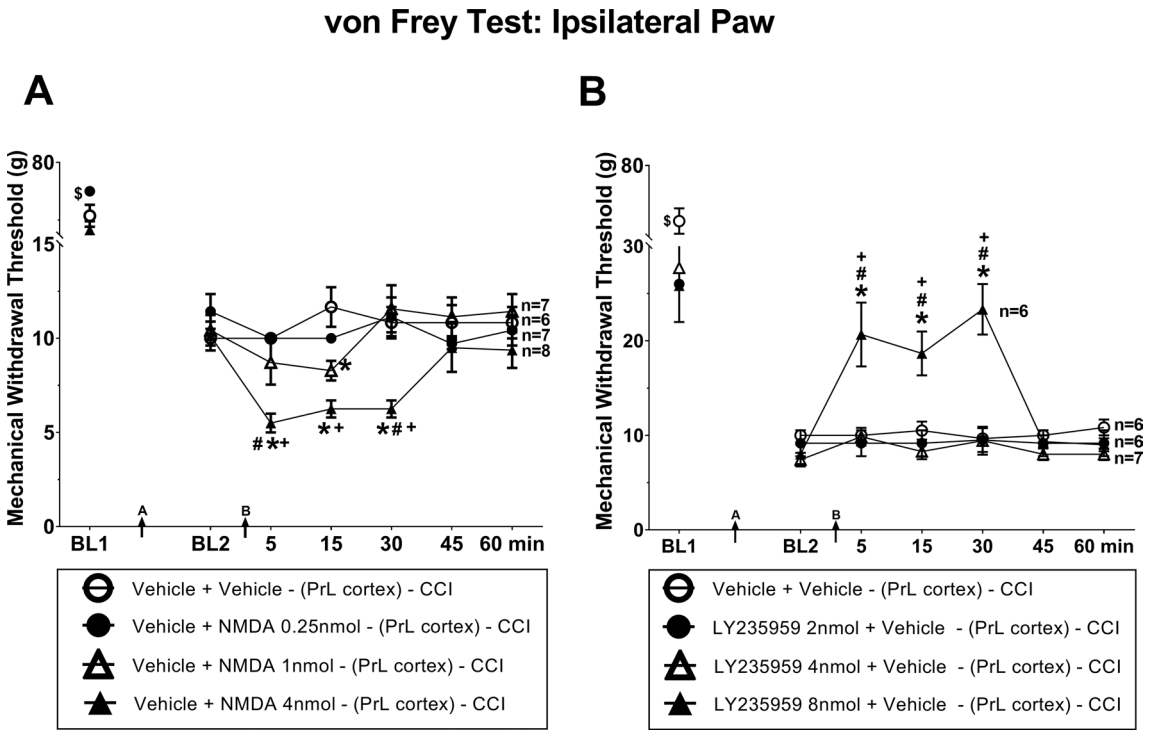


Fig. 4 **a**, **b**—**a** The effect of an NMDA receptor agonist in the left *prelimbic cortex* (PrL) on mechanical allodynia in rats with neuropathic pain induced by our modified model of CCI of the *ipsilateral* right sciatic nerve. *BL1* baselines before the procedures. *Arrow A* the sciatic nerve CCI or sham procedure. *BL2* new baselines recorded 21 days after the sham or CCI procedure. *Arrow B* treatment with NMDA (0.25, 1 or 4 nmol) or vehicle in the PrL cortex of sham or CCI rats after BL2 recordings was followed by the von Frey test until 60 min after injection. The microinjection was administered in the *contralateral* hemisphere of 6–8 animals per group in relation to the side on which the CCI or sham procedure was performed. *Indicates significant differences compared with the CCI + vehicle group; #indicates significant differences compared with the CCI + NMDA 1 nmol group; +indicates significant differences compared with the CCI + NMDA 0.25 nmol group; $indicates significant differences compared with all groups. **b** The effect of an NMDA receptor antagonist in the left PrL on mechanical allodynia in neuropathic pain induced by CCI of the *contralateral* right sciatic nerves. *Indicates significant differences compared with the CCI + vehicle group; #indicates significant differences compared with the CCI + LY235959 4 nmol group; +indicates significant differences compared with the CCI + LY235959 2 nmol group; $indicates significant differences compared with all groups

[9, 35], the mechanisms of the genesis and potentiation of neuropathic pain occur in three stages: (a) injury and release of inflammatory mediators in the peripheral nervous system; (b) spinal neurons activation; (c) supraspinal or cortical potentiation. The majority of investigations have mainly focused on the peripheral and spinal mechanisms of neuropathic pain, and less is known about cortical changes in neuropathic pain. The mPFC efferent neurons consist mainly of excitatory pyramidal cells (80–90% of the total local cortical neuronal population), which, in turn, are modulated by local inhibitory GABAergic afferent interneurons (10–20% of the total local cortical neuronal population).

In the current study, we evaluated the involvement of the PrL division of the mPFC in the induction and maintenance of neuropathic pain through unilateral microinjections of $CoCl_2$, a synaptic blocker, into either the ipsilateral or contralateral PrL cortex [21, 23, 30–33]. The synaptic activity blockade in either the contralateral or ipsilateral PrL cortex of CCI rats attenuated mechanical allodynia in the late stage of neuropathic pain, specifically on days 21 and 28 after CCI.

The role of the PrL cortex in the maintenance of neuropathic pain, independent of the side into which it was administered, was evidenced in the present investigation. The anterolateral spinothalamic system is a standard component of the ascending pain pathway and processes temperature and protopathic sensations. Through these ascending projections, impulses originating in nociceptors located in the trunk and limbs travel through the contralateral spinothalamic tract and reach the sensory cortex. In addition to the connections between the periphery and the CNS, there are commissural fibres that connect the corresponding areas of the two cerebral hemispheres [36]. In the present study, the CCI procedure was performed on the right hind paw, and considering that the ascending sensory pain pathways cross the median plane before the nociceptive information reaches the somatosensory cortex, it is of great importance to study the level of participation of the PrL division of the mPFC in both the

right and left hemispheres in the modulation of neuropathic pain. The corpus callosum allows the seamless transfer of information from one hemisphere to the other [36].

Additionally, there is evidence for a neural mechanism by which chronic pain disrupts the descending pain modulatory system via functional changes in cerebral neural circuits, for example, the specific changes in mPFC-periaqueductal grey (PAG) neuronal pathways in mice displaying chronic pain behaviour after CCI. Indeed, as demonstrated by a recent study, a reduction in the excitability of the PrL and infralimbic (IFL) cortices, primarily from external granular and pyramidal cortical layers, interferes with the activity of the connections between the mPFC and PAG neurons ipsilateral to the CCI procedure [37].

The mPFC plays a major role in both sensory and affective aspects of pain. Based on recent studies, persistent pain facilitates the response to morphine reward, and corticotropin-releasing factor in the mPFC is involved in sensitisation, which facilitates behavioural responses to morphine reward in neuropathic pain conditions induced by CCI [38]. Barcelon et al. [39] showed that brain microglial activation plays a role in chronic pain-associated affective disorders. These authors showed increases in both soma size and microglial cell number in the mPFC, hippocampus, and amygdaloid complex 8 weeks after CCI.

In addition, the dynorphinergic system undergoes selective alterations involving the ACC, mPFC and nucleus accumbens (NAc) circuitry during neuropathic pain induced by CCI surgeries, suggesting a contribution to the negative affective component of pain. Furthermore, parallel increases in prodynorphin (Pdyn) and κ-opioid receptor (Oprk1) gene expression at the cortical level suggest the occurrence of likely interactions between these systems in the maladaptive neuroplasticity underlying neuropathic pain [40].

Here, we hypothesised that the PrL cortex of the mPFC plays a role in supraspinal/cortical sensitisation and that NMDA glutamate receptors are critical for neuromodulation during pain chronification. Regarding the mechanisms underlying neuropathic pain, molecular, cellular and functional alterations have been observed at many sites in the pain matrix after peripheral nerve injury. In fact, spontaneous ectopic activity in dorsal root ganglion (DRG) cell bodies and injured afferent sensory axonal fibres is substantially increased [41], and abnormal contacts between the sympathetic and sensory nervous systems [42], as well as changes in the neuronal substrates of pain in the spinal cord and brain, are observed. At the peripheral level, nerve injuries that drive neuropathic pain trigger sensitisation and may evoke long-term abnormal neural activity along primary afferent pathways [43, 44]. At the spinal cord level, dorsal horn neurons display potentiated excitatory responses and a reduced firing threshold in neuropathic pain conditions [11, 12, 45].

Our data demonstrated the involvement of the PrL cortex in the peripheral neuropathy and the activation of its glutamatergic receptors by NMDA agonist administration (1 and 4 nmol) decreased the mechanical allodynia threshold, as assessed by the von Frey test. In other words, NMDA activation worsened neuropathic pain in rodents after 21 days of CCI. Interestingly, the blockade of NMDA receptors in the neocortex caused an analgesic effect; the treatment of the PrL cortex with the antagonist LY235959 at the highest dose (8 nmol) decreased mechanical allodynia in animals with peripheral neuropathy.

In addition to the research on peripheral alterations, extensive research has been conducted on the role of the cortex in chronic pain. Based on electrophysiological studies, observing changes in cortical function one hour after the induction of a spinal cord lesion is possible, and this reorganisation of the cerebral cortex can lead to pathological consequences, such as phantom pain and neuropathic pain [46]. Additionally, in neuroimaging studies, patients with neuropathic pain have modifications in areas related to the sensory and affective perception of pain, such as the thalamus, prefrontal cortex, amygdaloid complex, insula, and posterior parietal lobe [47, 48].

Latremoliere and Woolf [49] showed that NMDA receptors and other subtypes of glutamatergic receptors are involved in the induction and maintenance of persistent pain and the induced sensitisation of the nociceptive pathway during inflammation and neuropathic pain. Moreover, Wang and collaborators [25] showed that bilateral and contralateral lesions of the PrL region of the mPFC increase the paw withdrawal threshold in animals with chronic inflammatory lesions.

In fact, the CNS contains synapses with high plasticity, and long-term changes in synaptic transmission contribute to different functions of the neural system. In the spinal cord dorsal horn where the first sensory synapses are located, the LTP of sensory synaptic transmission can be induced by peripheral injury [11]. Potentiated excitatory synaptic transmission contributes to spinal sensitisation and consequently evokes hyperalgesia and allodynia in chronic pain. The activation of NMDA receptors is a crucial requirement for the induction of LTP, and the key mechanism of the involvement of NMDA receptors in the induction of LTP is their voltage dependence. At resting membrane potentials, NMDA receptors are inactive, even in the presence of glutamate, due to pore blockade by extracellular Mg^{2+}. Thus, for activation of NMDA receptors at synapses, two events must occur simultaneously. First, glutamate needs to be released and to bind to NMDA receptors; second, the postsynaptic membrane needs to be depolarised so that the extracellular Mg^{2+} blockade can be removed. In the ACC, bath application of an NMDA receptor antagonist, AP-5, completely abolishes the

induction of LTP in pyramidal cells by different induction protocols [50], indicating that the induction of ACC LTP is completely dependent on the postsynaptic activation of NMDA receptors.

The activation of NMDA receptors causes an intracellular cascade that triggers a series of biochemical reactions, resulting in alterations in the structure and function of the synapse, and these synaptic changes greatly enhance excitatory synaptic transmission and thus contribute to chronic pain [51]. In some of studies of the brain, the N-methyl-D-aspartate receptor subtype 2B (GluNR2B) subunit is the most important tyrosine-phosphorylated protein, and the phosphorylation of GluNR2B receptor subunits by the tyrosine kinase Fyn [52, 53] has been proposed to lead to increased Ca^{2+} entry through the receptor during both central sensitisation and NMDA-dependent synaptic plasticity [54, 55].

Our findings demonstrate that NMDA glutamatergic receptors in the PrL of the mPFC mediate cortical sensitisation during chronic neuropathic pain 21 days after CCI surgery in rats. There is further evidence that glutamatergic neurotransmission is involved in nociception and thus mediates chronic pain. According to clinical data, NMDA receptor antagonists can reduce both hyperalgesia and allodynia in neuropathic pain patients [54–58]. Wu and colleagues [59] have suggested that neuronal reorganisation associated with pain occurs in animal models of chronic pain. Indeed, functional changes have been described after peripheral inflammation in the primary somatosensory cortex and the anterior cortical area of the cingulate gyrus in the form of NMDA receptor subunit upregulation [59].

The activation of NMDA receptors in the PrL cortex increased mechanical allodynia, and the blockade of NMDA receptors produced the opposite effect. Nevertheless, the involvement of the mPFC in chronic pain may still seem paradoxical. Millecamps et al. [60] showed that the mPFC is responsible for the analgesic effect induced by the administration of D-cycloserine, a partial NMDA receptor agonist, in a model of neuropathic pain; they also showed that the cortical area is responsible for the modulation of pain [60]. However, although these authors performed microinjections in different regions of the mPFC, PrL and IFL divisions, they considered the effect of the intracortical treatment as the mPFC is a unique cortical area. These different divisions of the prefrontal cortex can be anatomically and functionally distinguished in rodents [61]. In the present study, we specifically evaluated the critical role played by the NMDA signalling of the mPFC-PrL division in pain perception.

In addition, the microinjection of an NMDA antagonist and a metabotropic glutamate receptor (mGluR) antagonist into the PrL and IFL cortices increases mechanical allodynia [62]. Palazzo and colleagues [63] also showed that the microinjection of an allosteric modulator of metabotropic receptors into the limbic cortex reverses neuropathic pain, as well as the cognitive deficits caused by it. Although these data contradict the data obtained in the present study, importantly, both the abovementioned groups examined the mPFC in its entirety and did not specifically target the PrL cortex, as we did in the present study. Moreover, the cited studies used different models of neuropathic pain in their experimental approaches. This inconsistency reinforces the importance of our efforts to investigate the glutamatergic participation of the PrL region in our CCI model, which was adapted from Bennett and Xie [13].

Furthermore, genetic and pharmacological studies have demonstrated the involvement of NMDA receptors containing the GluNR2B subunit in the ACC and insular cortex (IC). The overexpression of GluNR2B in the mouse forebrain causes an increase in behavioural responses to pain [64]. Additionally, the microinjection of an NMDA-GluNR2B antagonist into the cortex, especially the ACC or IC, produces an analgesic effect in rodents [59, 65, 66]. According to biochemical and electrophysiological analyses, animals with peripheral inflammation show increases in the expression of the NMDA GluNR2B subunit and NMDA GluNR2B-mediated synaptic currents in the ACC [35].

Caveolin-1 is a modulator of NMDA receptor upregulation in response to injury [67]. In freely moving mice, the increase in GluNR2B-containing receptors after inflammation contributes to enhanced NMDA receptor-mediated responses in the ACC [66, 67]. Similar findings have been made in the IC [65]. Peripheral nerve injury leads to the development of neuropathic pain, and the IC shows a long-term increase in the abundance of synaptic NMDA receptors. These increases do not occur in extrasynaptic NMDA receptors [65].

Furthermore, the increase in NMDA receptor expression relies on the phosphorylation of the GluN2B subunit at Tyr1472 by a pathway involving adenylyl cyclase subtype 1 (AC1), protein kinase A (PKA), and Src family kinases. Changes in NMDA receptor-mediated responses have also been reported in animals with cancer pain [68, 69], and the functional inhibition of these receptors has an analgesic effect. In fact, excitatory synaptic transmission in the CNS is mainly mediated by glutamate.

At the level of the cerebral cortex where pain perception is processed, glutamatergic synapses also play a major role in the storage and generation of the long-term memory of pain. Interestingly, ionotropic and metabotropic glutamate receptors appear to have different functions in the mPFC, which may explain the conflicting results in the literature.

In conclusion, considering that mechanical allodynia is a typical sensory symptom of neuropathic pain and that pain signals are processed by pathways ascending contralaterally from the spinal cord to the cerebral cortex, these findings suggest that the morphological reorganisation of the PrL division of the mPFC occurs, leading to the potentiation and

maintenance of chronic pain. In an animal model of chronic pain, the blockade of NMDA receptors in the neocortex decreases chronic neuropathic pain, and glutamatergic activation through NMDA receptors potentiates neuropathic pain.

Acknowledgements This research was supported by *Fundação de Amparo à Pesquisa do Estado de São Paulo* (FAPESP) (Research Grant 2013/12916-0 and Multi-user Equipment Grant 2014/11869-0) and *Conselho Nacional de Pesquisa e Desenvolvimento Tecnológico* (CNPq) (Grants 483763/2010-1, 474853/2013-6, and 427397/2018-9). Neither of these funding sources had any role in the study design; in the collection, analysis, and interpretation of the data; in the writing of the report; or in the decision to submit the paper for publication. Renato Leonardo de Freitas was supported by FAPESP (Scientific Initiation Scholarship Grant 2001/03752-6, M.Sc. Fellowship Grant 2003/05256-1, Post-doctoral Fellowship Grant 2009/17258-5, and Researcher Fellowship Grant 2014/07902-2) and CAPES (Sc.D. Fellowship Grant 001). FAPESP also supported Priscila de Medeiros (Sc.D. Fellowship Grant 2012/25167-2; Post-doctoral Fellowship Grant 2017/13560-5). Norberto Cysne Coimbra is a researcher (level 1A) from CNPq (Processes 301905/2010-0 and 301341/2015-0). Sérgio Henrique Ferreira was supported by FAPESP (Thematic Project Processes 2011/19670-0 and 2013/08216-2; Centre for Research in Inflammatory Diseases—CRID), the University of São Paulo Pro-Rectory (Process NAP-DIN 11.1.21625.01.0), the European Union Seventh Framework Programme (FP7-2007-2013; HEALTH-F4-2011-281608), and the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, Brazil). Dr. Sabatino Maione was supported by Regione Campania (FarmaBioNet process FESR 2007-2013—00 2.1). The authors thank Daoud Hibrahim Elias-Filho, Ieda R. dos Santos, and Maria Rossatto for their expert technical assistance. D.H. Elias-Filho was the recipient of technician scholarships from FAPESP (TT-2, Grant. 2002/01497-1), CNPq (Grants 501858/2005-9, 372654/2006-1, 372810/2008-0, 372877/2010-9, and 372838/2018-9), and FAEPA (Grants 345/2009 and 185/2010).

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflicts of interest concerning the work presented herein.

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