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Chronic stress induces transient spinal neuroinflammation, triggering sensory hypersensitivity and long-lasting anxiety-induced hyperalgesia

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ABSTRACT

Chronic stressful events induce biochemical, physiological and psychological changes, resulting in stress-related neuropsychiatric disorders, such as anxiety or depression. Using repeated social defeat as a stressful event model, we show that this preclinical paradigm induces a transient increase in the expression of the genes encoding the pro-inflammatory molecules iNOS and COX-2. We provide the first demonstration that chronic stress affects spinal plasticity through a mechanism involving local neuroinflammation. The functional consequences of such neuroinflammation are associated with a transient decrease in the mechanical nociceptive threshold. Administration of the cholecystokinin(CCK)-2 receptor antagonist, CI-988, directly into the Rostral Ventromedial Medulla reverses the chronic stress-induced decrease in the nociceptive threshold. These data strongly suggest that chronic stress induces a spinal neuroinflammation associated with transient sensory hypersensitivity involving the activation of CCK-dependent nociceptive descending facilitatory pathways. Pharmacological data show that chronic social stress-induced long-lasting state of anxiety is not responsible for maintaining the spinal neuroinflammation and, therefore, for the associated sensory hypersensitivity. Conversely, an evaluation of pain-related behavior in the formalin model indicates that anxiety is directly related to prolonged hyperalgesia prevented by systemic benzodiazepine or CCK-2 receptor antagonist treatments. The present study highlights the adverse effects of chronic stress on spinal neuroinflammation triggering sensory hypersensitivity. Exploration of this phenomenon points out the divergence between pain sensitivity and anxiety-induced hyperalgesia, which is in agreement with clinical observations. Altogether, these data open up new perspectives for clinical research devoted to the evaluation and treatment of pain in anxio-depressive patients.

1. Introduction

The stress response engendered by an acute stressor event is often beneficial, protecting the individual from injury, and is essential for surviving adaptation to such situations [27]. However, if the stress becomes repetitive and sustained, adaptation is impaired and pathological changes occur as a result of hypercortisolism, hypertension and psychological changes [11]. Although stress is not a disease by itself, continuous exposure to stressful stimuli has been directly related to onset, progression or outcome of many pathological processes. Indeed, chronic stress leads to neuropsychiatric disorders, including anxious or depressive disorders in particular.

Emerging data showed that chronic stress affects the immune responses in various areas of the brain and, under certain conditions, stress may potentiate inflammatory responses to subsequent peripheral immune stimulation [15,30,31,33,39]. In particular, an excess of pro-inflammatory molecules in various areas of the brain may lead to both neuronal functional impairment and structural damage [31]. Cyclooxygenase-2 (COX-2), for example, may be responsible for stress-induced brain damage [16,24]. However, only a few studies have suggested that psychological stress may produce spinal neuroinflammation. Bradesi et al. [7,8] demonstrated involvement of the spinal neurokinin-1 receptor (NK-1) and microglial phosphorylated p38 levels in the model of water avoidance-induced visceral hyperalgesia.

Social defeat, such as that resulting from the exposure of a male rat to social agonistic encounters, occurs frequently in natural environment [20,28]. The social-defeat procedure is a

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psychological chronic stress protocol allowing the studies of the physiological alterations induced by the chronic stress stimulus itself and the long-term consequences of this stressful situation [28]. We have previously shown that 5 days after the end of the social-defeat procedure, animals displayed anxio-depressive behaviors similar to those observed in humans with anxiety/depression disorders [2]. This procedure may therefore be considered an appropriate model for the analysis of changes associated with social stress in rats. Using this preclinical model, we demonstrated that chronic social defeat leads to anxiety-related symptoms associated with exacerbated pain score in the formalin model involving cortical cholecystokinin-(CCK)ergic systems [2].

In this study, our objectives are as follows. Objective 1 is to test the hypothesis that chronic stress induces spinal neuroinflammation. For this, we study the effects of an appropriate preclinical model of social defeat on spinal pro-inflammatory gene expression. Objective 2 is to evaluate the functional consequences of chronic stress-induced spinal neuroinflammation, focusing on spinal functions, such as nociception, through classical mechanical nociceptive tests. Objective 3 is to better understand by which pathways chronic stress modulates the nociceptive test responses. Given that recent studies were consistent with direct modulation of descending pathways by CCK in the rostroventromedial medulla (RVM) [6,18,29,37], an area involved in nociception modulation (see for review [18]), we examine the potential role of CCK activity in the RVM to activate descending pain facilitatory mechanisms.

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats (Centre d'Elevage R. Janvier, 53940 Le Genest-St-Isle, France), weighing 300 to 325 g (8 weeks old), were used as intruder rats. On their arrival at the laboratory, they were housed in chronobiologic animal facilities (*Enceinte Autonome d'Animalerie*, A110SP, Thermo Electron Corporation, Saint Herblain, France). They were housed in groups of 4 rats per cage for 3 days and were then transferred to individual cages (l: 45 cm; w: 25 cm; h: 17 cm) before the start of the experiments. The chronobiologic facility is equipped with equidistant, sound-proofed, temperature-controlled compartments, each supplied with filtered air. Each compartment had its own light–dark cycle control. Long Evans (LE) rats (Centre d'Elevage R. Janvier, 53940 Le Genest-St-Isle, France), weighing 700–800 g, were used as resident rats in confrontation encounters. All animals are housed in controlled environments (22 ± 1 °C, 60% relative humidity, 12/12 h light–dark cycle with lights on at 7:00 a.m., food and water available *ad libitum)*. Procedures involving animals and their care were carried out in conformity with the institutional guidelines which are in compliance with national and international laws and policies (Council directive #87–848, October 19, 1987, Ministère de l'Agriculture et de la Forêt, Service Vétérinaire de la Santé et de la Protection Animale, permission #75–1178 to J.J.B.) and with the guidelines published in a Guest Editorial in Pain on ethical standards for the investigation of experimental pain in animals (Committee for Research and Ethical issues of the I.A.S.P., 1980). To observe the effects of social stress, rats were randomly divided into two groups: the first group corresponded to rats not subjected to chronic stress procedure. The second group corresponded to rats submitted to the social stress. To observe the effects of pharmacological treatment on social stress-induced effects, rats were randomly divided into four groups: the first one corresponded to rats not submitted to the social receiving vehicle treatment; the second group corresponded to rats not submitted to the social receiving drug treatment; the third group corresponded to rats submitted to the social receiving vehicle treatment; the fourth group corresponded to rats submitted to the social defeat receiving drug treatment.

2.2. Experimental design

Animals were handled and accustomed to the equipment used for assessing mechanical nociceptive threshold for 12 days before the beginning of the social defeat. The social-defeat procedure was carried out as previously described [2,4]. Briefly, this method involves subjecting pairs of residents and intruders to four daily conditioning sessions (the same pair in each of the four sessions, from D_{-3} to D_0). The 45-min conditioning sessions were divided into two consecutive periods. During period I (30 min), intruders were placed individually in a protective cage inside the resident home cage. The protective cage allowed unrestricted visual, auditory, and olfactory contact with the resident but prevented close physical contact. During period II (15 min), the protective cage was removed, either with the resident remaining present, allowing physical confrontation with the intruder (3–4 confrontations of ~10 s during each of which the intruding (defeated) animal was always dominated by the resident rat) or with the resident removed, giving the intruder access to the entire resident home cage (non-defeated intruders). Therefore, the non-defeated intruders were never physically attacked and defeated by the resident. All animals submitted to the social defeat were included in the study. The basal nociceptive threshold (von Frey filament application and Randall–Selitto test) was determined on the left hindpaw the day before the social defeat experiment was started (i.e., D_{-4}) and just before the first period (period I; D_{-3}). Experiments were begun only if no statistical change in basal nociceptive threshold was observed between D_{-4} and D_{-3} (ANOVA, $P > 0.05$). Thereafter, nociceptive threshold was measured once daily until it returned to basal values.

Five days (D_5) or 15 days (D_{15}) after completion of the social-defeat procedure (D_0), the experimental animals underwent treatment with formalin. Briefly, animals were subjected to intraplantar injections of sterile 2.5% formalin (0.05 ml) just under the skin on the dorsal surface of the hindpaw. Pain responses were recorded for a period of 70 min. Pain scores were calculated as previously described [2]. Briefly, the pain responses were recorded for a period of 70 min. This injection resulted in a pain-induced behavior that can be assessed on a five-level scale in relation to posture: 0, normal posture of the paw; 1, injected paw remaining on the ground but not supporting the animal; 2, injected paw lifted without contact with any surface; 3, injected paw completely raised; 4, injected paw licked, nibbed, or shaken. These pain behaviors were scored using a method based on the scoring method of Dubuisson and Dennis [14]. Behaviors were measured by two experienced observers who were blind to the treatment conditions. The results were expressed as follows. First, pain scores in the different figures were expressed with the following formula: pain score = $[(0 \times T0) + (1 \times T1) + (2 \times T2) + (3 \times T3) + (4 \times T4)]/T$, in which $T0$, $T1$, $T2$, $T3$, and $T4$ were the duration (in seconds) spent in levels 0, 1, 2, 3, and 4, respectively, and T was the total duration of the measure intervals (i.e., $T0 + T1 + T2 + T3 + T4$). Measures were made over 180 s spans during the first 6 min, over 240 s spans for 4 min, and then over 300 s spans until the end of the observation period. All behavioral measurements were taken in conscious, unrestrained, male rats by individuals blind to the pharmacological treatments received by the rats and to the stress procedure (non-defeated or defeated rats).

2.2.1. Elevated plus-maze test (EPM)

The EPM test was used to evaluate anxiety-related behavior in animals on D_5 and D_{15}. The plus-maze consisted of a weakly

illuminated plain wood structure with two open arms (50 × 10 cm) and two enclosed arms (50 × 10 × 40 cm), placed at a height of 50 cm. The walls of the enclosed arms were painted an intermediate shade of gray and the entire floor was black. A 0.5 cm transparent Plexiglas hedge surrounded the open arms. Experimental drug-naive rats were placed at the center of the plus-maze and allowed to explore freely for 4 min. The rats' behavior was videotaped with an LCD camera connected to control and recording equipment located in the adjacent room. The time spent in the various arms and the numbers of entries into the open and closed arms of the plus-maze were recorded with custom-made software. At the time of behavioral assessment, the experimenter was blind to the treatments of individual rats. The elevated plus-maze was cleaned after each rat. Animals were tested between 9:00 and 11:00 a.m. The time spent in the open arms is expressed as a percentage of total test time. The total number (open + closed) of arm entries was taken as an indicator of general activity.

2.2.2. Real-time polymerase chain reaction (RT-PCR)

Experimental animals were killed on D_5 or D_{15} by decapitation and the dorsal lumbar spinal cord of the rat was dissected, immediately frozen in liquid nitrogen and stored at −80 °C. RNA was extracted and quantitative RT-PCR was carried, with probes for the target genes *iNOS* and *COX-2*. Briefly, total RNA was extracted from frozen pieces of tissue with the NucleoSpin_RNA II Purification Kit (Macherey–Nagel, Hoerdt, France). RNA quality and concentration were determined by measuring absorbance with the NanoDrop system (Nyxor Biotech, France). First-strand cDNA synthesis (0.5 μg total RNA per 20 μl reaction) was carried out with Superscript III reverse-transcriptase and random primers (0.25 μg ribosomal phospho-protein per reaction) (Invitrogen, Cergy-Pontoise, France). Real-time PCR was carried out in triplicate on the ABI Prism 7300 apparatus (Applied Biosystems, Courtaboeuf, France) with ABgene Absolute QPCR ROX Mix (ABgene, Courtaboeuf, France). The Assay-on-Demand Gene TaqMan PCR probes (Applied Biosystems, Courtaboeuf, France) were used for the target genes: iNOS (Rn00561646_m1), COX-2 (Rn00568225_m1) and glyceraldehyde-3-phosphate dehydrogenase (Rn99999916_s1). For semi-quantitative assays, glyceraldehyde-3-phosphate dehydrogenase was used as the control housekeeping gene.

2.2.3. RVM drug administration

One week before the social-defeat procedure, the rats were anesthetized with isofluorane. Stainless-steel guide cannulae (26-gauge, Phymep, Paris, France) were inserted bilaterally at coordinates placing the tip just above the lateral portion of the RVM (anteroposterior: −11.0 mm from bregma; lateral: ± 0.6 mm from midline; dorsoventral: −8.5 mm from the skull, Paxinos and Watson [36]). The cannulae were secured to the skull with dental cement, and the skin was sutured. CCK2 receptor antagonist (CI-988, 50 ng in each side) was injected into the RVM on D_5 or D_{15}. Antagonist solution (0.5 μl/side) was slowly administered through injection cannulae inserted into the guide cannulae and protruding by 1 mm. At the termination of the experiments, methylene blue was injected into the site of the RVM injection. After decapitation, the brain was removed from the skull and immediately frozen in isopentane for cryostat sections. The correct placement of the injection cannulae was checked by histological examination.

Pharmacological treatments: The benzodiazepine receptor agonist chlordiazepoxide (10 mg/kg/day), the CCK2 receptor antagonist CI-988 (1 mg/kg/day) and the anti-inflammatory compound aspirin (2 mg/kg/day) were dissolved in saline (chlordiazepoxide, aspirin) or DMSO (CI-988) and the resulting solution was used to fill ALZET osmotic mini-pumps (Charles River Laboratories, L'Arbresle, France). Pumps filled with chlordiazepoxide (ALZET 2ML1), CI-988 (ALZET 2002), aspirin (ALZET 2001), DMSO or saline were implanted subcutaneously on the back of the intruder rats under light isoflurane anesthesia, the day after completion of the social defeat study (D_1). A small incision was made in the skin between the scapulae. Using a hemostat, a small pocket was formed by spreading the subcutaneous connective tissues apart. The pump was inserted into the pocket. The skin incision was closed with absorbable sutures. Aspirin (lysine–acetylsalicylate, Pharmacie Centrale des Hôpitaux, Paris, France) was administered through an intrathecal catheter (Alzet intrathecal catheter No. 0007740; 8.5 cm long) implanted 1 week before the social defeat experiments, as described by Yaksh and Rudy [46]. Rats with severe motor impairments were discarded from the study (<5%). On D_1, the aspirin-filled ALZET osmotic mini-pump was connected to the ALZET intrathecal catheter.

2.2.4. Data analysis and statistics

All data presented are means ± SEM. The effect of social defeat (group effect) was analyzed by one-way analysis of variance (ANOVA). The group effect and treatment effect were validated by two-way ANOVAs. The time effect was analyzed by ANOVA for repeated measurements. When ANOVAs showed a significant effect of treatment, social defeat and/or time, Bonferroni post hoc test was used to determine the significance of differences. P value < 0.05 was considered statistically significant.

3. Results

3.1. Effects of chronic social defeat on anxiety-like behavior and spinal neuroinflammation (Fig. 1)

The elevated plus-maze test was used to evaluate anxiety-related behavior in animals. The percentage of time spent in the open arm was significantly lower in defeated rats than in non-defeated rats, on both D_5 ($F_{(1,10)} = 8.95$, $P = 0.01$) and D_{15} ($F_{(1,10)} = 12.92$, $P = 0.004$) (Fig. 1a). We found no statistically significant difference in the total number of visits between non-defeated and defeated rats (D_5: 24.0 ± 2.3 vs. 23.0 ± 1.0, respectively; D_{15}: 26.0 ± 3.6 vs. 23.0 ± 2.0, respectively; $n = 6$ for each group). Thus, the observed difference in time spent in the open arm was not due to a change in general activity.

We examined the effects of chronic social defeat on pro-inflammatory gene expression in the dorsal spinal cord, on D_1, D_5 or D_{15}. Rats subjected to chronic social defeat showed markedly higher levels of iNOS and COX-2 gene expression than non-defeated rats on both D_1 (iNOS: $F_{(1,7)} = 12.85$, $P = 0.008$; COX-2: $F_{(1,7)} = 10.32$, $P = 0.01$) and D_5 (iNOS $F_{(1,7)} = 14.64$, $P = 0.006$; COX-2: $F_{(1,7)} = 14.72$, $P = 0.006$) (Fig. 1b). By contrast, no difference in iNOS and COX-2 gene expression between the two groups of animals was observed on D_{15} (iNOS $F_{(1,6)} = 0.62$, $P = 0.46$; COX-2: $F_{(1,6)} = 3.30$, $P = 0.11$) (Fig. 1b). Overall these findings show that chronic stress induces a long-lasting anxious profile and a transient inflammatory response in the dorsal spinal cord. These observations suggest that spinal neuroinflammation is not related to the anxious state of the animals.

3.2. Effects on chronic social defeat-induced anxiety and spinal neuroinflammation of curative chronic treatment with the anxiolytic compound chlordiazepoxide (Fig. 2)

As expected, on D_5, chlordiazepoxide (10 mg/kg/day) had a marked anxiolytic effect in defeated rats in the elevated plus-maze, with chlordiazepoxide-treated rats spending more time in the open arm than saline-treated rats (social-defeat effect: $F_{(1,20)} = 2.96$, $P = 0.10$; chlordiazepoxide effect: $F_{(1,20)} = 11.67$, $P = 0.002$; social defeat × chlordiazepoxide effect: $F_{(1,20)} = 8.81$, $P = 0.007$) (Fig. 2a). The total number of visits did not differ

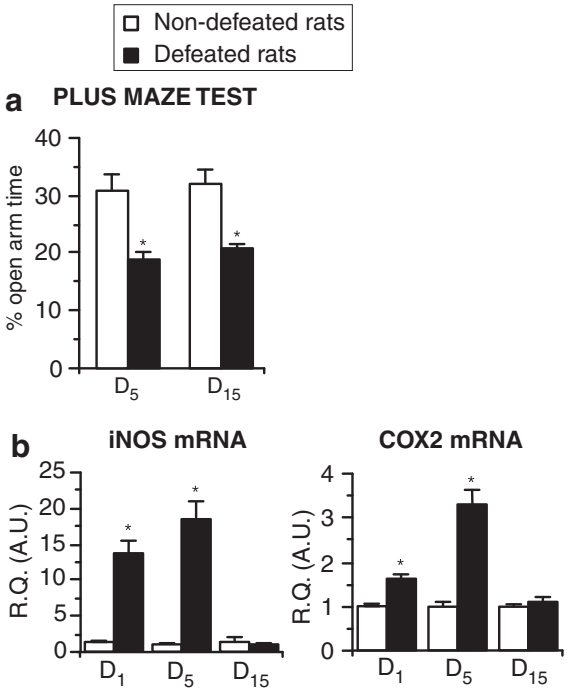

Fig. 1. Anxiety-like behavior and expression of the genes encoding the spinal pro-inflammatory mediators iNOS and COX-2, in the dorsal horn of the spinal cord of animals subjected, or not subjected to social defeat. (a) Evaluation of the anxious profile of rats subjected to social defeat (defeated rats, $n = 6$) and rats not subjected to social defeat (non-defeated rats, $n = 6$), in the elevated plus-maze on D_5 and D_{15}. (b) Time-course of expression of the genes encoding the spinal pro-inflammatory mediators iNOS and COX-2, in defeated and non-defeated animals. Semi-quantitative analyses of iNOS and COX-2 mRNA levels, to assess the expression of genes in rats subjected to the chronic social stress paradigm, on D_1 ($n = 5$), D_5 ($n = 5$) and D_{15} ($n = 4$), and that in non-defeated rats ($n = 4$ for each set of experimental conditions). All data points are means ± SEM. $^{*}P < 0.05$, vs. non-defeated rats.

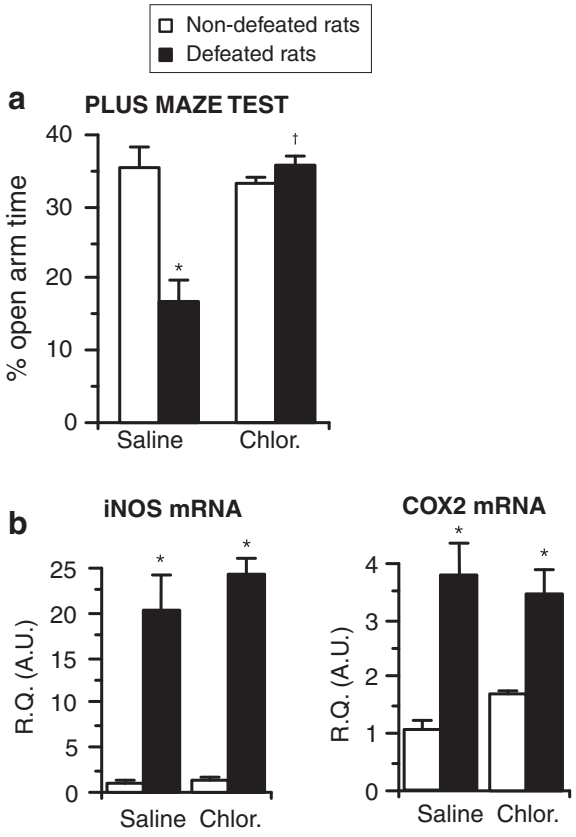

Fig. 2. Effects of systemic chlordiazepoxide treatment on anxiety-like behavior and spinal iNOS and COX-2 gene expression in rats subjected, or not subjected to social defeat. Chronic anxiolytic treatment (chlordiazepoxide [Chlor.], 10 mg/kg/day) was administered through subcutaneous osmotic mini-pumps from the day after the end of the social-defeat procedure until the end of the experiment (D_5) in rats subjected to chronic stress and rats not subjected to such stress. (a) Evaluation of the effect of chronic chlordiazepoxide treatment on anxious profile of treated defeated and non-defeated rats ($n = 6$), compared with saline-treated defeated or non-defeated animals ($n = 6$), based on the elevated plus-maze. (b) Effects of chronic chlordiazepoxide treatment on expression of the genes encoding the spinal pro-inflammatory mediators iNOS and COX-2 in treated defeated rats ($n = 6$), or treated non-defeated rats ($n = 4$), compared with saline-treated defeated rats ($n = 5$), or saline-treated non-defeated rats ($n = 4$). All data points are means ± SEM. $^{*}P < 0.05$, vs. non-defeated rats. $^{\dagger}P < 0.05$ for the comparison between chlordiazepoxide-treated defeated rats and saline-treated defeated rats.

significantly between non-defeated and defeated rats (saline: 23.83 ± 1.0 vs. 23.1 ± 1.9, respectively; chlordiazepoxide: 18.6 ± 2.6 vs. 21.3 ± 1.5, respectively; $n = 6$ for each group). In defeated animals, chlordiazepoxide treatment had no effect on the up-regulated expression of genes encoding iNOS (social-defeat effect: $F_{(1,15)} = 47.33$, $P < 0.0001$; chlordiazepoxide effect: $F_{(1,15)} = 0.85$, $P = 0.37$; social defeat x chlordiazepoxide effect: $F_{(1,15)} = 0.72$, $P = 0.40$) and COX-2 (social-defeat effect: $F_{(1,15)} = 22.89$, $P = 0.0002$; chlordiazepoxide effect: $F_{(1,15)} = 0.081$, $P = 0.77$; social defeat × chlordiazepoxide effect: $F_{(1,15)} = 1.03$, $P = 0.32$) in the dorsal horn of the spinal cord (Fig. 2b). Spinal iNOS – COX-2 gene expression in chlordiazepoxide-treated non-defeated animals was not significantly different from that of saline-treated non-defeated rats (Fig. 2b). Thus, the increase in expression of the pro-inflammatory gene related to spinal neuroinflammation is not linked to the anxious profile of the animal.

3.3. Functional consequences of spinal neuroinflammation on mechanical pain sensitivity (Fig. 3)

Up to day 12 (D_{12}), rats subjected to social defeat had a significantly lower mechanical nociceptive threshold – estimated using the Randall–Selitto test and the von Frey filament application – than non-defeated rats, demonstrating a state of sensory hypersensitivity in rats subjected to chronic social stress (Paw pressure test: social-defeat effect: $F_{(1,12)} = 29.94$, $P < 0.0001$; time effect: $F_{(19,228)} = 15.51$, $P < 0.0001$; social defeat × time effect: $F_{(19,228)} = 9.11$, $P < 0.0001$; Von Frey test: social-defeat effect: $F_{(1,12)} = 167.83$, $P < 0.0001$; time effect: $F_{(19,228)} = 20.57$, $P < 0.0001$; social defeat × time effect: $F_{(19,228)} = 18.77$, $P < 0.0001$) (Fig. 3a,b). After D_{12}, mechanical nociceptive thresholds returned to baseline values. Thus, there is a transient increase in sensory sensitivity defeated animals. The significant changes observed on both iNOS and COX-2 gene expression and sensory sensitivity on D_5 but not on D_{15} suggest a relationship between these two phenomena. This possible direct link was investigated in rats receiving curative intrathecal anti-inflammatory treatment. We used aspirin (NSAID) that blocks the formation of pro-inflammatory prostaglandins at the level of COX activity by the acetylation of serine residue at the active site of COX, especially of COX-2, which is involved in the inflammatory process [22]. Since a positive feedback loop has been reported between prostaglandins and COX-2 gene expression [23], aspirin administration can then block COX-2 over-expression observed in defeated rats. On D_5, in rats subjected to chronic social defeat, chronic treatment by aspirin (1 mg/day/rat) administration to the spinal cord did not show anxiolytic-like effects (social-defeat effect: $F_{(1,22)} = 20.28$, $P = 0.0002$; aspirin effect: $F_{(1,22)} = 0.92$, $P = 0.34$; social defeat x aspirin effect: $F_{(1,22)} = 0.29$, $P = 0.59$) (Fig. 3c). The total number of visits did not differ significantly between non-defeated and defeated rats (saline: 22.0 ± 1.77 vs.

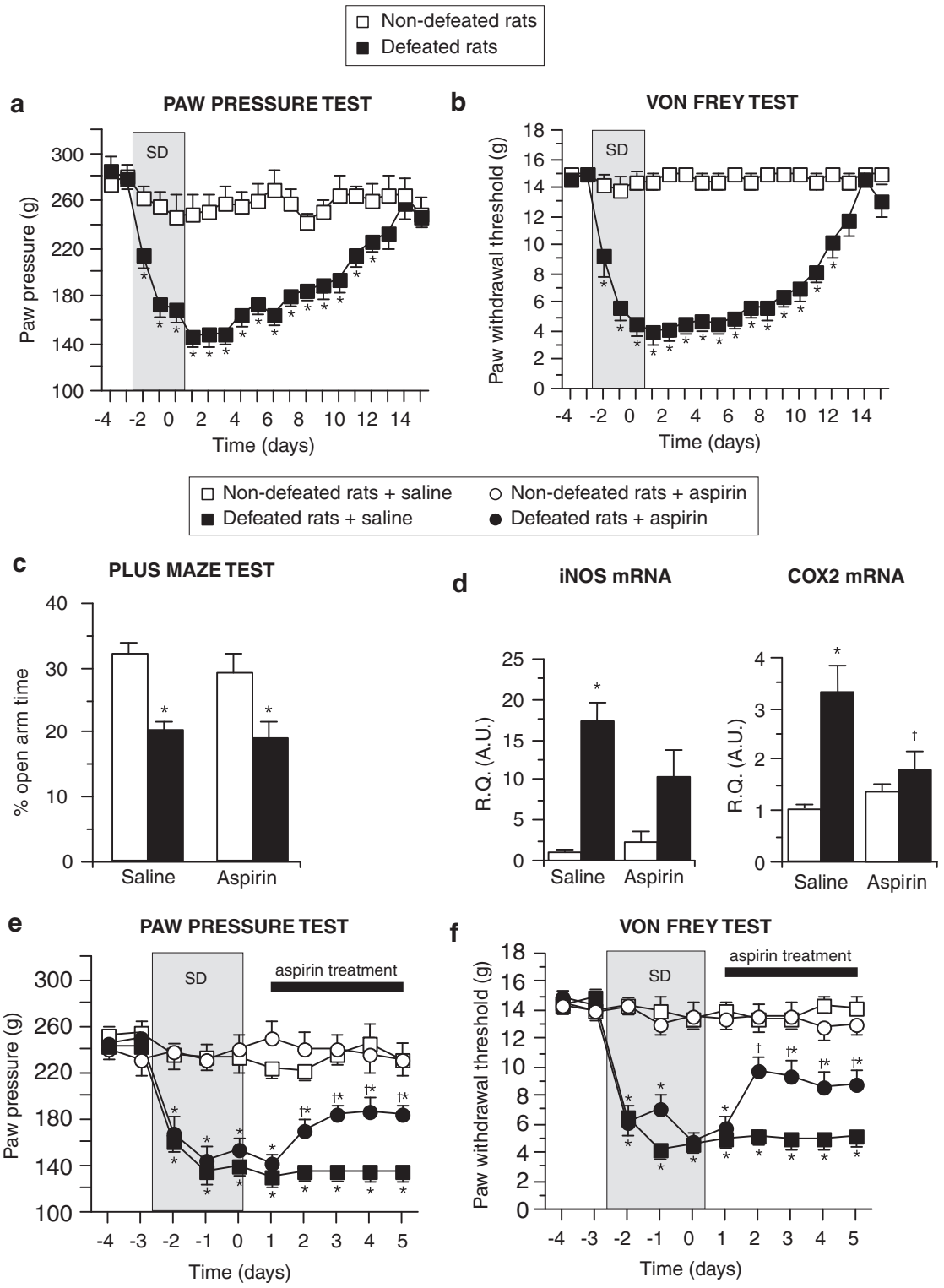

Fig. 3. Effects of intrathecal aspirin treatment on mechanical sensory sensitivity and pro-inflammatory gene expression, in rats subjected, or not subjected to social defeat. (a and b) Comparison of nociceptive responses to von Frey hairs (a) or paw pressure vocalization tests (Randall–Selitto) (b) in rats subjected ($n = 8$), or not subjected ($n = 6$) to the social defeat (SD) procedure. Mechanical sensory sensitivity was measured in animals 2 days before (D_{-4}, D_{-3}), during (D_{-2}, D_0) and after (D_1, D_{15}) chronic social defeat, until nociceptive values returned to baseline. (c–f) Chronic intrathecal anti-inflammatory treatment (aspirin, 1 mg/day/rat) was administered through subcutaneous osmotic mini-pumps from the day after the end of the social-defeat procedure until the end of the experiment in rats subjected, or not subjected to chronic stress. (c) Evaluation of the effect of chronic aspirin treatment on anxious profile of treated defeated ($n = 8$) and non-defeated rats ($n = 6$), compared with saline-treated defeated ($n = 6$) or non-defeated animals ($n = 6$), based on the elevated plus-maze. (d) Effects of chronic curative aspirin treatment on expression of the genes encoding the spinal pro-inflammatory mediators iNOS and COX-2 in treated defeated rats ($n = 6$), or treated non-defeated animals ($n = 4$), compared with saline-treated defeated animals ($n = 5$), or saline-treated non-defeated animals ($n = 4$), on D_5. (e and f) Effects of chronic aspirin treatment (black bar) on mechanical sensory hypersensitivity – evaluated by the paw pressure vocalization test (d) or the von Frey test (e) – in treated defeated or non-defeated rats ($n = 6$) compared with saline-treated defeated or non-defeated animals ($n = 6$). All data points are means ± SEM. $^{*}P < 0.05$, vs. non-defeated rats. $^{\dagger}P < 0.05$ for the comparison between aspirin-treated defeated rats and saline-treated defeated rats.

21.5 ± 2.02, respectively; aspirin: 22.66 ± 0.49 vs. 21.0 ± 1.5, respectively; $n = 6–8$). In defeated animals, intrathecal aspirin treatment decreased iNOS gene expression and completely abolished COX-2 gene expression (Fig. 3d). This treatment also significantly decreased mechanical sensory hypersensitivity, from D_2. From D_3, nociceptive threshold values reached a steady state significantly lower than that in aspirin-treated non-defeated rats (Paw pressure test: social-defeat effect: $F_{(1,20)} = 49.87$, $P < 0.0001$; time effect: $F_{(9,180)} = 15.25$, $P < 0.0001$; time × aspirin effect: $F_{(9,180)} = 2.05$, $P = 0.036$; Von Frey test: social-defeat effect: $F_{(1,20)} = 106.75$, $P < 0.0001$; time effect: $F_{(9,180)} = 37.34$, $P < 0.0001$; time x aspirin effect: $F_{(9,180)} = 2.42$, $P = 0.012$) (Fig. 3e and f). These data suggest that the mechanical hypersensitivity is related to the spinal cord neuroinflammation induced by chronic stress. Complementary statistical analyses by two-way ANOVA for repeated measures are shown in Table 1 (see Supplementary data).

3.4. Effects on mechanical pain hypersensitivity of curative chronic treatment with an anxiolytic compound, chlordiazepoxide, in chronically stressed animals (Fig. 4)

As spinal neuroinflammation is not related to the anxious profile of the animal and pain hypersensitivity is supported by neuroinflammation, we investigated the possible link between pain hypersensitivity and anxious state.

As expected, curative chronic treatment with the anxiolytic compound chlordiazepoxide (10 mg/kg/day) did not reverse the mechanical sensory hypersensitivity observed in animals subjected to chronic defeat (comparison with saline-treated defeated rats; Fig. 4a and b) (Paw pressure test: social-defeat effect: $F_{(1,20)} = 122.87$, $P < 0.0001$; time effect: $F_{(19,380)} = 28.57$, $P < 0.0001$; time × chlordiazepoxide effect: $F_{(19,380)} = 0.63$, $P = 0.87$; Von Frey test: social-defeat effect: $F_{(1,20)} = 919.84$, $P < 0.0001$; time effect: $F_{(19,380)} = 52.09$, $P < 0.0001$; time × chlordiazepoxide effect: $F_{(19,380)} = 2.07$, $P = 0.005$). No difference in chronic mechanical sensory sensitivity was observed between chlordiazepoxide-treated non-defeated animals and saline-treated non-defeated animals (Fig. 4a and b). Complementary statistical analyses by two-way ANOVA for repeated measures are shown in Table 1 (see Supplementary data).

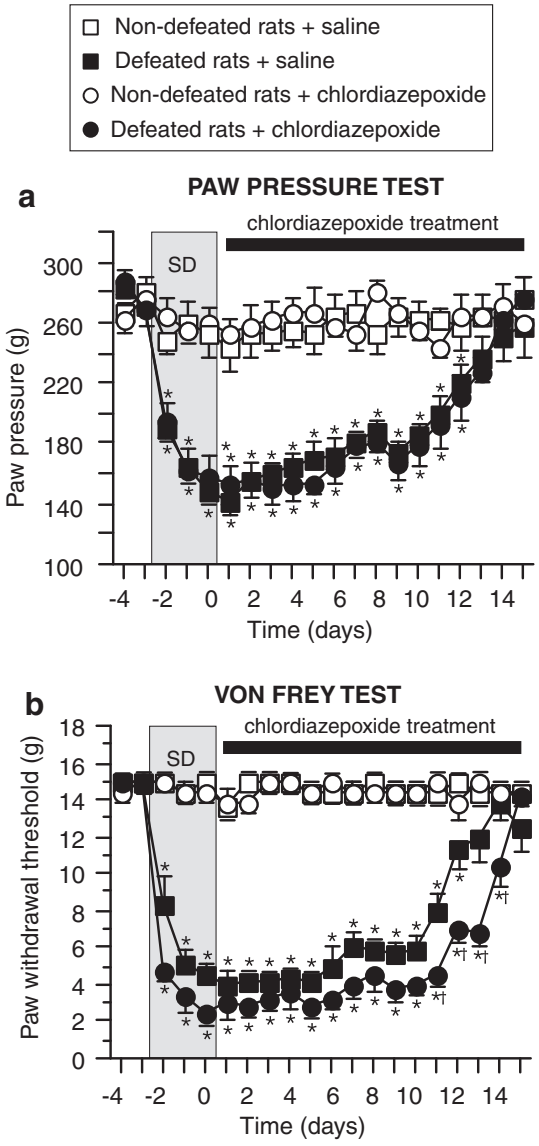

Fig. 4. Effects of systemic chlordiazepoxide treatment on mechanical nociceptive thresholds in rats subjected, or not subjected to social defeat. Chronic anxiolytic treatment (chlordiazepoxide, 10 mg/kg/day) was administered via subcutaneous osmotic mini-pumps from the day after the end of the social-defeat procedure (D_1) until the end of the experiment (D_{15}) in rats subjected, or not subjected to chronic stress (black bar). (a and b) Effects of chronic chlordiazepoxide treatment on mechanical sensory hypersensitivity, as evaluated by the paw pressure vocalization test (a) or the von Frey test (b), in treated defeated or non-defeated rats ($n = 6$), compared with saline-treated defeated or non-defeated animals ($n = 6$). All data points are means ± SEM. $^{*}P < 0.05$, vs. non-defeated rats. $^{\dagger}P < 0.05$ for the comparison between chlordiazepoxide-treated defeated rats and saline-treated defeated rats.

3.5. Effects of blockade of the CCKergic neurotransmission on anxiety-related behavior profile, spinal neuroinflammation and mechanical pain hypersensitivity (Fig. 5)

We tested the involvement of CCKergic neurotransmission by administering the CCK2 receptor antagonist, CI-988 (1 mg/kg/day), from D_1 until the end of the experiment, in curative manner. In the elevated plus-maze, defeated rats treated with CI-988 and non-defeated rats spent similar amounts of time in the open arms (social-defeat effect: $F_{(1,20)} = 2.20$, $P = 0.15$; CI-988 effect: $F_{(1,20)} = 12.15$, $P = 0.002$; social defeat × CI-988 effect: $F_{(1,20)} = 1.79$, $P = 0.19$) (Fig. 5a). The total number of visits did not differ significantly between non-defeated and defeated rats (saline: 21.83 ± 1.3 vs. 24.1 ± 1.5, respectively; CI-988: 19.3 ± 1.0 vs. 19.8 ± 1.2, respectively; $n = 6$ for each group). Thus, chronic CI-988 treatment had an anxiolytic-like effect. In opposition to chlordiazepoxide effects, the upregulation of iNOS (social-defeat effect: $F_{(1,15)} = 11.87$, $P = 0.003$; CI-988 effect: $F_{(1,15)} = 6.95$, $P = 0.018$; social defeat × CI-988 effect: $F_{(1,15)} = 11.50$, $P = 0.004$) and COX-2 (social-defeat effect: $F_{(1,15)} = 11.01$, $P = 0.004$; CI-988 effect: $F_{(1,15)} = 6.28$, $P = 0.02$; social defeat × CI-988 effect: $F_{(1,15)} = 10.05$, $P = 0.006$) gene expression and sensory hypersensitivity (Paw pressure test: social-defeat effect: $F_{(1,22)} = 36.88$, $P < 0.0001$; time effect: $F_{(19,418)} = 25.03$, $P < 0.0001$; time × CI-988 effect: $F_{(19,418)} = 5.46$, $P < 0.0001$; Von Frey test: social-defeat effect: $F_{(1,22)} = 462.77$, $P < 0.0001$; time effect: $F_{(19,418)} = 59.13$, $P < 0.0001$; time - CI-988 effect: $F_{(19,418)} = 18.50$, $P < 0.0001$) were completely reversed by CI-988 treatment in animals subjected to chronic social stress (Fig. 5b–d). This effect on sensory hypersensitivity was observed from 1 day after the implantation of osmotic pumps (D_2), peaking on D_4 (Fig. 5c and d). Spinal iNOS - COX-2 gene expression and sensory sensitivity in CI-988-treated defeated animals did not differ significantly from those in DMSO-treated non-defeated or CI-988-treated non-defeated rats (Fig. 5b–d). Thus, the beneficial effects of CI-988 treatment on sensory hypersensitivity coincide with the reversal of iNOS and COX-2 upregulation in animals subjected

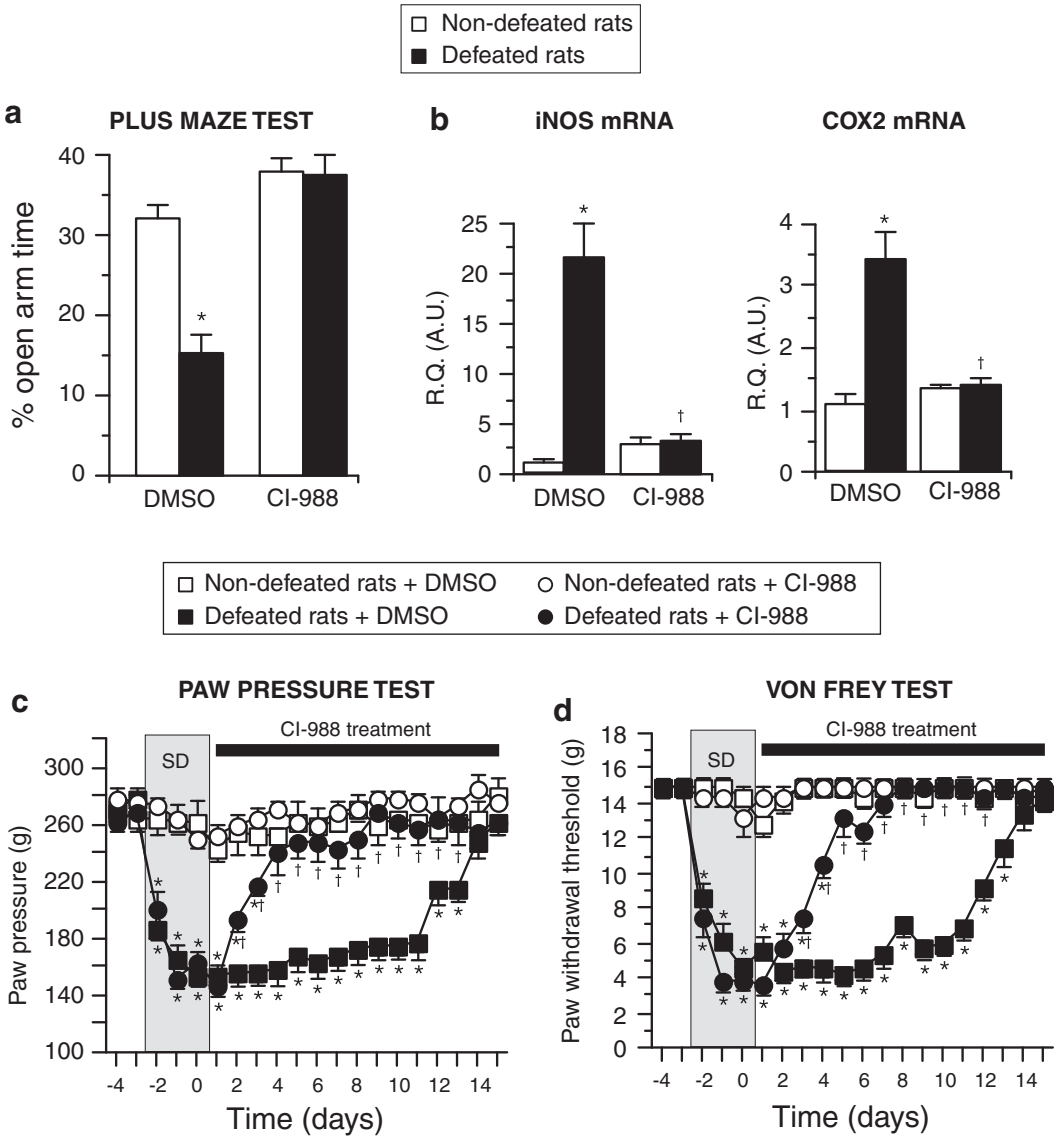

Fig. 5. Effects of systemic CI-988 treatment on anxiety-like behavior, spinal iNOS and COX-2 gene expression and mechanical nociceptive thresholds in rats subjected, or not subjected to social defeat. Defeated and non-defeated rats received chronic systemic treatment with CI-988 (1 mg/kg/day) through subcutaneous osmotic mini-pumps, from the day after the end of the social-defeat procedure until the end of the experiment. (a) Evaluation of the effects of chronic CI-988 treatment on anxious profile of treated defeated or non-defeated rats ($n = 6$) compared with DMSO-treated defeated or non-defeated animals ($n = 6$), in the elevated plus-maze on D_5. (b) Effects of chronic CI-988 treatment on expression of the genes encoding the spinal pro-inflammatory mediators iNOS and COX-2, in treated defeated rats ($n = 6$), or treated non-defeated rats ($n = 4$), compared with DMSO-treated defeated rats ($n = 5$), or DMSO-treated non-defeated rats ($n = 4$) on D_5. (c and d) Effects of chronic CI-988 treatment on mechanical sensory hypersensitivity, as evaluated by the paw pressure vocalization test (c) or the von Frey test (d), in treated defeated or non-defeated rats ($n = 6$) compared with DMSO-treated defeated animals ($n = 8$) or DMSO-treated non-defeated animals ($n = 6$). All data points are means ± SEM. $^{*}P < 0.05$, vs. non-defeated rats. $^{\dagger}P < 0.05$ for comparisons between CI-988-treated defeated rats and DMSO-treated defeated rats.

to chronic stress. Complementary statistical analyses by two-way ANOVA for repeated measures are shown in Table 1 (see Supplementary data).

3.6. Effects of chronic social defeat on nociception: role of CCK-dependent descending facilitatory pathways (Fig. 6)

As CCKergic neurotransmission is involved in both spinal neuroinflammation and mechanical pain hypersensitivity, we hypothesized that chronic stress might promote mechanical hypersensitivity through CCK-dependent descending facilitatory pathways. Mechanical sensory sensitivity in CI-988-treated non-defeated animals was not significantly different from that recorded for DMSO-treated non-defeated rats (Fig. 6a and b). Defeated rats receiving the CCK2 receptor antagonist, CI-988, by intra-RVM injection displayed lower mechanical sensory hypersensitivity – as estimated using paw pressure (social-defeat effect: $F_{(1,24)} = 94.89$, $P < 0.0001$; time effect: $F_{(5,120)} = 16.87$, $P < 0.0001$; time - CI-988 effect: $F_{(5,120)} = 13.36$, $P < 0.0001$) and von Frey tests (social-defeat effect: $F_{(1,24)} = 178.88$, $P < 0.0001$; time effect: $F_{(5,120)} = 13.79$, $P < 0.0001$; time × CI-988 effect: $F_{(5,120)} = 12.36$, $P < 0.0001$) – for a period of 1 h, than did defeated rats microinjected with DMSO (Fig. 6a and b). Thus, the microinjection of CI-988 into the RVM of rats subjected to chronic social defeat transiently blocks mechanical sensory hypersensitivity. By contrast, DMSO has no effect (Fig. 6a and b). Complementary statistical analyses by two-way ANOVA for repeated measures are shown in Table 1 (see Supplementary data).

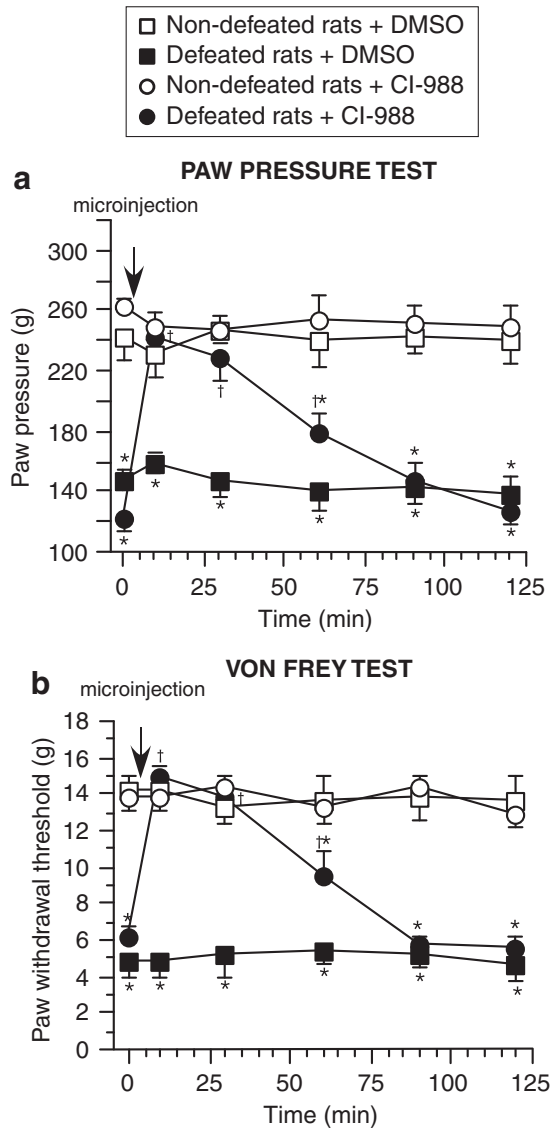

Fig. 6. Effects of intra-RVM CI-988 injection on mechanical nociceptive thresholds in rats subjected, or not subjected to social defeat. (a and b) Effects of the blockade of CCK receptors with CI-988 administration in the RVM on mechanical sensory hypersensitivity on D_5, in rats subjected to the chronic stress procedure ($n = 8$), or not subjected to this procedure ($n = 8$). Comparison with DMSO-microinjected defeated or non-defeated animals ($n = 6$). All data points are means ± SEM. $^{*}P < 0.05$, vs. non-defeated rats. $^{\dagger}P < 0.05$ for comparisons between CI-988-treated defeated rats and DMSO-treated defeated rats.

3.7. Effects of chronic social defeat on formalin-related behavior (Fig. 7)

As the results presented above clearly indicate that sensory hypersensitivity is related to spinal neuroinflammation, we investigated whether the pain-related behavior induced by formalin is also dependent on spinal neuroinflammation. Formalin-treated rats subjected to social defeat had significantly higher behavioral pain scores than non-defeated rats, on D_5 (social-defeat effect: $F_{(1,13)} = 30.26$, $P < 0.0001$; time effect: $F_{(14,182)} = 35.00$, $P < 0.0001$; social defeat × time effect: $F_{(14,182)} = 3.62$, $P < 0.0001$) and D_{15} (social-defeat effect: $F_{(1,12)} = 16.82$, $P = 0.0015$; time effect: $F_{(14,168)} = 56.74$, $P < 0.0001$; social defeat × time effect: $F_{(14,168)} = 2.43$, $P = 0.0039$) (Fig. 7a and b), reflecting excess of pain induced by anxiety (anxiety-induced hyperalgesia [2]). On D_5, the chronic intrathecal administration of aspirin had no significant effect on the pain scores observed in the formalin model (social-defeat effect: $F_{(1,20)} = 50.60$, $P < 0.0001$; aspirin effect: $F_{(1,20)} = 0.75$, $P = 0.39$; time effect: $F_{(14,280)} = 70.88$, $P < 0.0001$) (Fig. 7c). Furthermore, the blockade of CCK-dependent descending facilitatory pathways by microinjection of the CCK2 receptor antagonist CI-988 into the RVM of rats subjected to chronic social defeat had no effect on increased pain scores on D_5 (social-defeat effect: $F_{(1,24)} = 38.85$, $P < 0.0001$; CI-988 effect: $F_{(1,24)} = 0.021$, $P = 0.88$; time effect: $F_{(14,336)} = 98.37$, $P < 0.0001$) and D_{15} (social-defeat effect: $F_{(1,22)} = 41.33$, $P < 0.0001$; CI-988 effect: $F_{(1,22)} = 0.11$, $P = 0.73$; time effect: $F_{(14,308)} = 127.45$, $P < 0.0001$) (Fig. 7d and e). However, chronic systemic chlordiazepoxide or CI-988 completely abolished the increase in pain scores in animals subjected to social defeat (chlordiazepoxide: social-defeat effect: $F_{(1,20)} = 16.07$, $P = 0.0007$; chlordiazepoxide effect: $F_{(1,20)} = 8.59$, $P = 0.0082$; time effect: $F_{(14,280)} = 93.91$, $P < 0.0001$; CI-988: social-defeat effect: $F_{(1,22)} = 16.63$, $P = 0.0002$; CI-988 effect: $F_{(1,22)} = 6.13$, $P = 0.021$; time effect: $F_{(14,308)} = 93.07$, $P < 0.0001$) (Fig. 7f and g). The pain scores obtained for chlordiazepoxide- or CI-988-treated defeated animals did not differ significantly from those obtained for saline- or DMSO-treated non-defeated animals or for chlordiazepoxide- or CI-988-treated non-defeated rats (Fig. 7f and g). Complementary statistical analyses by two-way ANOVA for repeated measures are shown in Table 1 (see Supplementary data).

4. Discussion

The present data describe, for the first time, the induction of a transient plastic change in the spinal cord by a psychological chronic stress procedure, as demonstrated by a change in the expression of the pro-inflammatory genes encoding iNOS and COX-2. The functional consequences of this induction are a close correlation between spinal inflammatory state and mechanical sensory hypersensitivity, through the activation of RVM-CCK-dependent descending pathways. No such correlation is observed for the pain-related behavior induced by formalin injection. These original observations suggest that distinct mechanisms are involved in sensory hypersensitivity and formalin-induced hyperalgesia.

We provide here the direct demonstration that psychological chronic stress activates spinal expression of the pro-inflammatory genes encoding iNOS and COX-2 in a time-dependent manner. These data are consistent with a few studies showing the possible influence of stress on spinal cord [7,8,42]. Our findings also show that the increase in pro-inflammatory gene expression induced by chronic stress gives rise to dorsal horn plasticity. This raises questions about the functional consequences of spinal neuroinflammation. Among the diseases that involved spinal neuroinflammation, pain is one of the best known. It is also widely accepted among clinicians that stress-related disorders may trigger or worsen pain complaints [6,12,40]. We therefore focus on the effects of psychological chronic stress on nociception in relation to spinal neuroinflammation. We assess nociceptive sensitivity with the von Frey and Randall-Selitto test in rats subjected to chronic social defeat. We observed sensory hypersensitivity – a transient decrease in the nociceptive threshold – in defeated animals. The time-course of mechanical sensory hypersensitivity is closely correlated with up-regulated expression of genes encoding iNOS and COX-2. Both the nociceptive threshold and the levels of expression of the pro-inflammatory genes encoding iNOS and COX-2 return to baseline levels by D_{15}. Spinal pronociceptive systems may therefore be responsible for the stress-induced sensory hypersensitivity. However, this observation alone is clearly not sufficient to demonstrate a link between the two phenomena. We therefore give rats local intrathecal injections of aspirin to block the activity of spinal

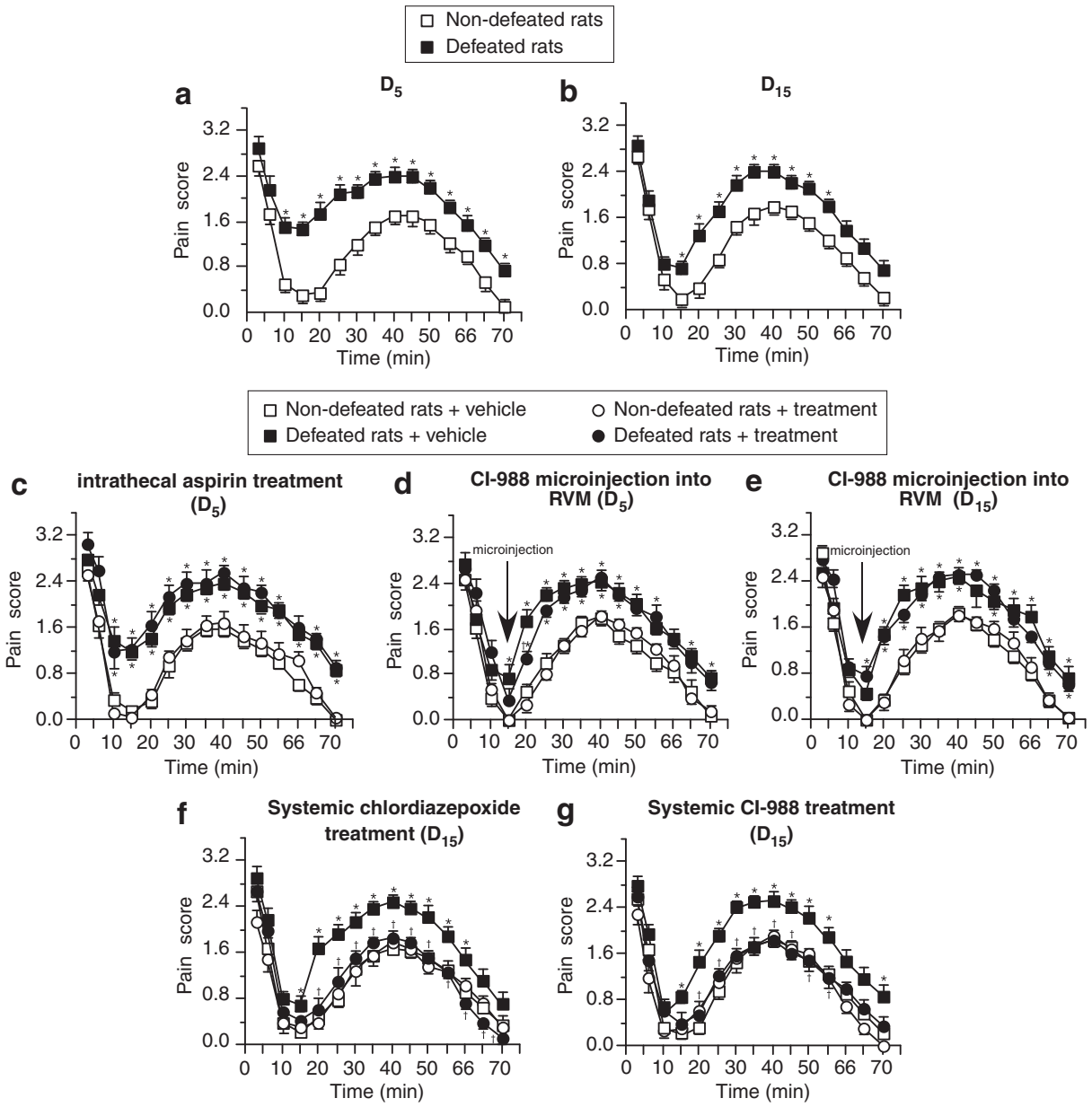

Fig. 7. Effects of aspirin, chlordiazepoxide, systemic CI-988 treatment or intra-RVM CI-988 microinjection on formalin-evoked pain behavior in animals subjected, or not subjected to social defeat. (a and b) Time-course of the response to formalin injection (2.5%, 50 μl) in defeated and non-defeated rats on D_5 or on D_{15}. Pain scores were measured for 70 min in rats subjected to the chronic social stress paradigm ($n = 8$ [D_5], $n = 8$ [D_{15}]) and in non-defeated rats ($n = 7$ [D_5], $n = 6$ [D_{15}]). (c) Effects of chronic intrathecal treatment with aspirin on pain scores following formalin injection in treated defeated or non-defeated animals ($n = 6$) compared with saline-treated defeated or non-defeated rats ($n = 6$). (d and e) Effects on formalin-induced pain scores of CI-988 injection into the RVM 15 min after formalin administration, in rats subjected to the chronic stress procedure ($n = 8$ [D_5]; $n = 6$ [D_{15}]), or not subjected to this procedure ($n = 8$ [D_5]; $n = 6$ [D_{15}]), compared with DMSO-microinjected defeated rats ($n = 6$ [D_5]; $n = 6$ [D_{15}]) or DMSO-microinjected non-defeated rats ($n = 6$ [D_5]; $n = 8$ [D_{15}]). (f and g) Effects of chronic curative chlordiazepoxide or CI-988 treatment in treated defeated rats ($n = 6$ [Chlor.]; $n = 6$ [CI-988]), or in treated non-defeated rats ($n = 6$ [Chlor.]; $n = 6$ [CI-988]), compared with saline-treated, DMSO-treated defeated rats ($n = 6$ [Chlor.]; $n = 8$ [CI-988]), or saline-treated, DMSO-treated non-defeated rats ($n = 6$ [Chlor.]; $n = 6$ [CI-988]). All data points are means ± SEM. $^{*}P < 0.05$, vs. non-defeated rats. $^{\dagger}P < 0.05$ for the comparison between aspirin-treated, chlordiazepoxide-treated or CI-988-treated defeated rats and saline-treated or DMSO-treated defeated rats.

pro-inflammatory molecules. This treatment reduces sensory hypersensitivity and prevents the increase in pro-inflammatory gene expression without affecting anxiety-like behavior. These results clearly demonstrate that the spinal neuroinflammation induced by psychological chronic stress is related to sensory hypersensitivity mediated by the pronociceptive properties of iNOS and COX-2 [9,13] but not to the anxiety state.

We previously showed that the social-defeat procedure induced biochemical, physiological and behavioral changes (HPA axis hyperactivity, anhedonia, absence of body weight gain) 5 days after its completion [2]. In this study, we find that defeated animals also display anxiety-like behavior, as assessed by their performance in the elevated plus-maze, on both D_5 and D_{15}. The psychological chronic stress procedure induces a transient spinal neuroinflammation associated with a sensory hypersensitivity and a long-lasting anxious state that persists while neuroinflammation and sensory hypersensitivity return to basal level. These observations strongly suggest that spinal neuroinflammation and its functional consequences are linked to the stress itself rather than to the anxious state of the animals induced by the psychological chronic stress procedure. Consistent with this observation, chronic chlordiazepoxide treatment prevents anxiety-like behavior without affecting the upregulation of iNOS and COX-2 gene expression and sensory hypersensitivity. The lack of chlordiazepoxide effects

in reducing sensory hypersensitivity in defeated rats seems a little bit surprising since $GABA_A$ receptors through the activation of α2 and α3 subunits mediate the anti-nociceptive effects of benzodiazepine agonists [19,32]. Nevertheless, beneficial effects of benzodiazepine treatment on sensory hypersensitivity were observed only in models of inflammatory and neuropathic pain but never in chronic stress conditions. This suggests that psychological chronic stress-induced sensory hypersensitivity could involve different $GABA_A$ receptor subunits. Whatever the mechanisms, these data confirm the close relationship between psychological chronic stress and spinal neuroinflammation, whereas anxiety does not maintain these spinal changes.

Blockade of the CCK receptor with CI-988 completely abolished anxiety-induced hyperalgesia [2]. We therefore investigate the effects of this compound in the present experimental paradigm. As expected, chronic CI-988 treatment has an anxiolytic-like effect [2,4,41]. Unlike chlordiazepoxide, chronic CI-988 treatment also prevents the increase in iNOS and COX-2 gene expression and sensory hypersensitivity. Thus, CCK is clearly involved in the stress-induced sensory hypersensitivity related to the plasticity of the spinal region. These findings raise questions about the pathways involved in the effects of CCKergic neurotransmission on the spinal cord. The activation of stress-related circuitry in supraspinal areas may activate pain-facilitating neurons, leading to sensory hypersensitivity. To our knowledge, only one study has investigated the consequences of a stress-like event (stimulation of the dorsomedial nucleus of the hypothalamus), a region involved in autonomic aspects of the response to psychological stress, on sensory sensitivity. In this previous study, the authors mimicked the effect of stress events in anesthetized rats. This led to a decrease in sensory sensitivity through the activation of descending pathways directly or indirectly connected to the RVM [25]. In our study, microinjection of the CCK-2 receptor antagonist CI-988 into the RVM abolishes mechanical sensory hypersensitivity in animals previously subjected to social defeat. Thus, in conscious animals subjected to psychological chronic stress, the decrease in the mechanical nociceptive threshold is related to the activation of CCK-dependent descending facilitatory nociceptive pathways. This hypothesis is consistent with studies showing that the local application of CCK to the RVM promotes behavioral manifestation and the long-term maintenance of sensory hypersensitivity through the activation of descending pathways [3,17,34,35,37,45,47].

Thus far, we focus specifically on the effects of psychological chronic stress on the spinal cord and its functional consequences. As the results presented above clearly indicate that sensory hypersensitivity is related to spinal neuroinflammation, we explored the effect of psychological chronic stress on a model of pain generating a complex behavioral response to determine whether anxiety-induced hyperalgesia is also dependent on spinal neuroinflammation. We use the formalin model, in which brain structures involved in motivational, affective, and cognitive processes are known to be activated [38,44]. It is also possible to score behavior in this model, facilitating the description of pain-related behavior [43]. As previously reported [2], animals subjected to the social-defeat procedure have exaggerated pain scores following formalin injection on D_5. This effect persists until day D_{15} when the animals are in an anxious state. Anxiety-induced hyperalgesia is prevented by chronic anxiolytic treatments (chlordiazepoxide, CI-988) but not by blocking spinal neuroinflammation with an NSAID or by blocking CCK-dependent descending facilitatory pathways by intra-RVM CI-988 injection. Thus, the pathways underlying the changes in dorsal horn plasticity may be activated directly by the initial stressful confrontations. Stress thus induces spinal neuroinflammation, whereas social defeat-induced hyperalgesia is related to the effects of anxiety on supraspinal areas. The discrepancy between the beneficial effect of CCK microinjection into the RVM on sensory hypersensitivity and the absence of an effect on anxiety-induced hyperalgesia are comparable to the findings of Carrasquillo and Gereau [10]. These authors suggested that ERK-induced tactile hypersensitivity, detected in the absence of injury, involved descending pain pathways, such as the CeA-PAG-RVM pathway, which may mediate sensory hypersensitivity but not formalin-related pain behavior. These findings suggest a role for descending nociceptive pathways in the sensory component of pain but not in complex algesic behavior. Thus, CCK is involved in stress-induced sensory hypersensitivity, through neuroinflammation of the dorsal horn of the spinal cord. Moreover, as shown in a previous study in rats, but also in human using a nocebo protocol, CCK acts through supraspinal pathways underlying anxiety-induced hyperalgesia [2,5]. As CCK is involved in both these stress-induced phenomena, a CCK2 receptor antagonist may be a good candidate pain-killer in the context of anxiety-induced hyperalgesia. Clinical studies reported the absence of severe side effects indicating that CCK2 receptor antagonists are well tolerated in humans [1,26].

With respect to polymorphism of pain as described in terms of sensation and emotion inseparably bound, our data point out the need to take these two components into account for efficient relief of the pain associated with stress-related neuropsychiatric disorders. The long-lasting anxiety inducing excess of pain as well as the fact that this exaggerated pain is not necessarily related to sensory hypersensitivity are close to the features of pain described in patients with anxio-depressive disorders. Lautenbacher et al. (1999) reported divergence between pain complaints and pain sensitivity in anxio-depressive patients [21]. Thus, the clinical pain complaints of patients with anxiety/depression cannot simply be explained by changes in pain sensitivity. Until now, only speculations were possible to explain this lack of relationship. In this context, our study shows that the divergence between pain complaint and pain sensitivity is based on real biochemical correlates opening up new clinical research for evaluation and treatment of pain in anxio-depressive patients.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.pain.2010.05.031.

References

[1] Adams JB, Pyke RE, Costa J, Cutler NR, Schweizer E, Wilcox CS, Wisselink PG, Greiner M, Pierce MW, Pande AC. A double-blind, placebo-controlled study of a CCK-B receptor antagonist, CI-988, in patients with generalized anxiety disorder. J Clin Psychopharmacol 1995;15:428–34.

[2] Andre J, Zeau B, Pohl M, Cesselin F, Benoliel JJ, Becker C. Involvement of cholecystokininergic systems in anxiety-induced hyperalgesia in male rats: behavioral and biochemical studies. J Neurosci 2005;25:7896–904.

[3] Basbaum AI, Fields HL. The origin of descending pathways in the dorsolateral funiculus of the spinal cord of the cat and rat: further studies on the anatomy of pain modulation. J Comp Neurol 1979;187:513–31.

[4] Becker C, Thiebot MH, Touitou Y, Hamon M, Cesselin F, Benoliel JJ. Enhanced cortical extracellular levels of cholecystokinin-like material in a model of anticipation of social defeat in the rat. J Neurosci 2001;21:262–9.

[5] Benedetti F, Mayberg HS, Wager TD, Stohler CS, Zubieta JK. Neurobiological mechanisms of the placebo effect. J Neurosci 2005;25:10390–402.
[6] Blackburn-Munro G, Blackburn-Munro RE. Chronic pain, chronic stress and depression: coincidence or consequence? J Neuroendocrinol 2001;13: 1009–23.
[7] Bradesi S, Kokkotou E, Simeonidis S, Patierno S, Ennes HS, Mittal Y, McRoberts JA, Ohning G, McLean P, Marvizon JC, Sternini C, Pothoulakis C, Mayer EA. The role of neurokinin 1 receptors in the maintenance of visceral hyperalgesia induced by repeated stress in rats. Gastroenterology 2006;130:1729–42.
[8] Bradesi S, Svensson CI, Steinauer J, Pothoulakis C, Yaksh TL, Mayer EA. Role of spinal microglia in visceral hyperalgesia and NK1R up-regulation in a rat model of chronic stress. Gastroenterology 2009;136:e1331–1332.
[9] Bujalska M, Tatarkiewicz J, de Corde A, Gumulka SW. Effect of cyclooxygenase and nitric oxide synthase inhibitors on streptozotocin-induced hyperalgesia in rats. Pharmacology 2008;81:151–7.
[10] Carrasquillo Y, Gereau RWt. Activation of the extracellular signal-regulated kinase in the amygdala modulates pain perception. J Neurosci 2007;27:1543–51.
[11] Chrousos GP. Stress and disorders of the stress system. Nat Rev Endocrinol 2009;5:374–81.
[12] Currie SR, Wang J. More data on major depression as an antecedent risk factor for first onset of chronic back pain. Psychol Med 2005;35:1275–82.
[13] Dolan S, Field LC, Nolan AM. The role of nitric oxide and prostaglandin signaling pathways in spinal nociceptive processing in chronic inflammation. Pain 2000;86:311–20.
[14] Dubuisson D, Dennis SG. The formalin test: a quantitative study of the analgesic effects of morphine, meperidine, and brain stem stimulation in rats and cats. Pain 1977;4:161–74.
[15] Frank MG, Baratta MV, Sprunger DB, Watkins LR, Maier SF. Microglia serve as a neuroimmune substrate for stress-induced potentiation of CNS pro-inflammatory cytokine responses. Brain Behav Immun 2007;21:47–59.
[16] Guo JY, Li CY, Ruan YP, Sun M, Qi XL, Zhao BS, Luo F. Chronic treatment with celecoxib reverses chronic unpredictable stress-induced depressive-like behavior via reducing cyclooxygenase-2 expression in rat brain. Eur J Pharmacol 2009;612:54–60.
[17] Heinricher MM, Neubert MJ. Neural basis for the hyperalgesic action of cholecystokinin in the rostral ventromedial medulla. J Neurophysiol 2004;92:1982–9.
[18] Heinricher MM, Tavares I, Leith JL, Lumb BM. Descending control of nociception: specificity, recruitment and plasticity. Brain Res Rev 2009;60:214–25.
[19] Knabl J, Witschi R, Hosl K, Reinold H, Zeilhofer UB, Ahmadi S, Brockhaus J, Sergejeva M, Hess A, Brune K, Fritschy JM, Rudolph U, Mohler H, Zeilhofer HU. Reversal of pathological pain through specific spinal GABAA receptor subtypes. Nature 2008;451:330–4.
[20] Koolhaas JM, Meerlo P, De Boer SF, Strubbe JH, Bohus B. The temporal dynamics of the stress response. Neurosci Biobehav Rev 1997;21:775–82.
[21] Lautenbacher S, Spernal J, Schreiber W, Krieg JC. Relationship between clinical pain complaints and pain sensitivity in patients with depression and panic disorder. Psychosom Med 1999;61:822–7.
[22] Lecomte M, Laneuville O, Ji C, DeWitt DL, Smith WL. Acetylation of human prostaglandin endoperoxide synthase-2 (cyclooxygenase-2) by aspirin. J Biol Chem 1994;269:13207–15.
[23] Li Z, Jansen M, Ogburn K, Salvatierra L, Hunter L, Mathew S, Figueiredo-Pereira ME. Neurotoxic prostaglandin J2 enhances cyclooxygenase-2 expression in neuronal cells through the p38MAPK pathway: a death wish? J Neurosci Res 2004;78:824–36.
[24] Madrigal JL, Moro MA, Lizasoain I, Lorenzo P, Fernandez AP, Rodrigo J, Bosca L, Leza JC. Induction of cyclooxygenase-2 accounts for restraint stress-induced oxidative status in rat brain. Neuropsychopharmacology 2003;28:1579–88.
[25] Martenson ME, Cetas JS, Heinricher MM. A possible neural basis for stress-induced hyperalgesia. Pain 2009;142:236–44.
[26] McCleane GJ. A phase 1 study of the cholecystokinin (CCK) B antagonist L-365, 260 in human subjects taking morphine for intractable non-cancer pain. Neurosci Lett 2002;332:210–2.
[27] McEwen BS. Protective and damaging effects of stress mediators: central role of the brain. Prog Brain Res 2000;122:25–34.
[28] Miczek KA, Yap JJ, Covington 3rd HE. Social stress, therapeutics and drug abuse: preclinical models of escalated and depressed intake. Pharmacol Ther 2008;120:102–28.
[29] Millan MJ. Descending control of pain. Prog Neurobiol 2002;66:355–474.
[30] Minami M, Kuraishi Y, Yamaguchi T, Nakai S, Hirai Y, Satoh M. Immobilization stress induces interleukin-1 beta mRNA in the rat hypothalamus. Neurosci Lett 1991;123:254–6.
[31] Munhoz CD, Garcia-Bueno B, Madrigal JL, Lepsch LB, Scavone C, Leza JC. Stress-induced neuroinflammation: mechanisms and new pharmacological targets. Braz J Med Biol Res 2008;41:1037–46.
[32] Munro G, Lopez-Garcia JA, Rivera-Arconada I, Erichsen HK, Nielsen EO, Larsen JS, Ahring PK, Mirza NR. Comparison of the novel subtype-selective GABAA receptor-positive allosteric modulator NS11394 [3'-[5-(1-hydroxy-1-methyl-ethyl)-benzoimidazol-1-yl]-biphenyl-2-carbonitr ile] with diazepam, zolpidem, bretazenil, and gaboxadol in rat models of inflammatory and neuropathic pain. J Pharmacol Exp Ther 2008;327:969–81.
[33] Nguyen H, Lim J, Dresner ML, Nixon B. Effect of local corticosteroids on early inflammatory function in surgical wound of rats. J Foot Ankle Surg 1998;37:313–8.
[34] Ossipov MH, Lai J, Malan Jr TP, Porreca F. Spinal and supraspinal mechanisms of neuropathic pain. Ann N Y Acad Sci 2000;909:12–24.
[35] Ossipov MH, Lai J, Vanderah TW, Porreca F. Induction of pain facilitation by sustained opioid exposure: relationship to opioid antinociceptive tolerance. Life Sci 2003;73:783–800.
[36] Paxinos G, Watson C. The rat brain in stereotaxic coordinates. San Diego: Academic Press; 1986.
[37] Porreca F, Ossipov MH, Gebhart GF. Chronic pain and medullary descending facilitation. Trends Neurosci 2002;25:319–25.
[38] Porro CA, Cavazzuti M. Spatial and temporal aspects of spinal cord and brainstem activation in the formalin pain model. Prog Neurobiol 1993;41:565–607.
[39] Pugh CR, Nguyen KT, Gonyea JL, Fleshner M, Wakins LR, Maier SF, Rudy JW. Role of interleukin-1 beta in impairment of contextual fear conditioning caused by social isolation. Behav Brain Res 1999;106:109–18.
[40] Salovey P, Birnbaum D. Influence of mood on health-relevant cognitions. J Pers Soc Psychol 1989;57:539–51.
[41] Shlik J, Vasar E, Bradwejn J. Cholecystokinin and psychiatric disorders. CNS Drugs 1997;8:134–52.
[42] Suarez-Roca H, Leal L, Silva JA, Pinerua-Shuhaibar L, Quintero L. Reduced GABA neurotransmission underlies hyperalgesia induced by repeated forced swimming stress. Behav Brain Res 2008;189:159–69.
[43] Tjolsen A, Berge OG, Hunskaar S, Rosland JH, Hole K. The formalin test: an evaluation of the method. Pain 1992;51:5–17.
[44] Vaccarino AL, Melzack R. Analgesia produced by injection of lidocaine into the anterior cingulum bundle of the rat. Pain 1989;39:213–9.
[45] Xie JY, Herman DS, Stiller CO, Gardell LR, Ossipov MH, Lai J, Porreca F, Vanderah TW. Cholecystokinin in the rostral ventromedial medulla mediates opioid-induced hyperalgesia and antinociceptive tolerance. J Neurosci 2005;25:409–16.
[46] Yaksh TL, Rudy TA. Analgesia mediated by a direct spinal action of narcotics. Science 1976;192:1357–8.
[47] Zhang W, Gardell S, Zhang D, Xie JY, Agnes RS, Badghisi H, Hruby VJ, Rance N, Ossipov MH, Vanderah TW, Porreca F, Lai J. Neuropathic pain is maintained by brainstem neurons co-expressing opioid and cholecystokinin receptors. Brain 2009;132:778–87.