Reversal of Monoarthritis-induced Affective Disorders by Diclofenac in Rats

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ABSTRACT

Background: Nonsteroidal anti-inflammatory drugs are effective for arthritic pain, but it is unknown whether they also benefit anxiety and depression that frequently coexist with pain. Using the monoarthritis model, the authors evaluated the activation of extracellular signal–regulated kinases 1 and 2 ($ERK_{1/2}$) in structures implicated in both sensorial and emotional pain spheres, and it was verified whether analgesia can reverse monoarthritis-mediated affective responses.

Methods: Monoarthritis was induced in male rats by complete Freund's adjuvant injection. Allodynia (ankle-bend test), mechanical hyperalgesia (paw-pinch test), anxiety- and depression-like behaviors (elevated zero maze and forced swimming tests, respectively), and $ERK_{1/2}$ phosphorylation (Western blot) in the spinal cord, paragigantocellularis nucleus, locus coeruleus, and prefrontal cortex were evaluated at 4, 14, and 28 days postinoculation (n = 6 per group). Changes in these parameters were evaluated after induction of analgesia by topical diclofenac (n = 5 to 6 per group).

Results: Despite the pain hypersensitivity and inflammation throughout the testing period, chronic monoarthritis (28 days) also resulted in depressive- (control [mean ± SEM]: 38.3 ± 3.7 *vs.* monoarthritis: 51.3 ± 2.0; $P < 0.05$) and anxiogenic-like behaviors (control: 36.8 ± 3.7 *vs.* monoarthritis: 13.2 ± 2.9; $P < 0.001$). These changes coincided with increased $ERK_{1/2}$ activation in the spinal cord, paragigantocellularis, locus coeruleus, and prefrontal cortex (control *vs.* monoarthritis: 1.0 ± 0.0 *vs.* 5.1 ± 20.8, $P < 0.001$; 0.9 ± 0.0 *vs.* 1.9 ± 0.4, $P < 0.05$; 1.0 ± 0.3 *vs.* 2.9 ± 0.6, $P < 0.01$; and 1.0 ± 0.0 *vs.* 1.8 ± 0.1, $P < 0.05$, respectively). Diclofenac decreased the pain threshold of the inflamed paw and reversed the anxio-depressive state, restoring $ERK_{1/2}$ activation levels in the regions analyzed.

Conclusion: Chronic monoarthritis induces affective disorders associated with $ERK_{1/2}$ phosphorylation in paragigantocellularis, locus coeruleus, and prefrontal cortex which are reversed by diclofenac analgesia. **(ANESTHESIOLOGY 2014; 120:1476-90)**

PATIENTS with arthritis primarily seek medical assistance due to persistent pain caused by the inflammation and destruction of joints.[1] This pain results from a complex interaction between the peripheral and central nervous systems. In general consensus, chronic pain provokes neuroplastic alterations that contribute to the maintenance and amplification of painful sensation and to the onset of related disorders.[2] One of the most common consequences of chronic pain is the development of anxio-depressive disorders, manifested as helplessness, pessimism, rumination, and catastrophism.[3] These affective alterations originate due to the disruption of central mechanisms and should be carefully monitored, because their presence is directly related with pain severity.[4] Indeed, such modifications establish a vicious circle whereby chronic pain triggers profound emotional changes, which in turn enhance pain perception.[4] It is therefore critical to determine whether these plastic changes are reversible, because this could strongly condition the treatment strategies used. One potentially effective approach is to explore the effect of topically administering nonsteroidal anti-inflammatory drug in animal models which are among the first-line treatments to alleviate joint pain[5] and are frequently administered topically to avoid the side effects associated with their oral administration.[6]

What We Already Know about This Topic

- Many types of chronic pain including pain related to arthritis are associated with depressed mood and anxiety

What This Article Tells Us That Is New

- Using a rat model of arthritis, diclofenac reduced nociceptive sensitization and reduced additional behaviors suggesting anxiety- and depression-like changes in the animals
- The activation of extracellular signaling-related kinase in several brain regions was implicated in these changes

We focused on the pathway formed by the spinal cord (SC), paragigantocellularis nucleus (PGi), locus coeruleus (LC), and prefrontal cortex (PFC). The LC is involved in

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 Anesthesiology 2014; 120:1476-90

ascending and descending pain pathways and constitutes the main source of noradrenaline in the central nervous system, a neurotransmitter implicated in pain, emotion, stress, depression, anxiety, and other disorders.[7] The LC receives ascending nociceptive inputs from the SC through the PGi[8] and it projects to forebrain structures similar to the anterior cingulate cortex in the PFC, which is implicated in cognition and pain-related emotion.[9–13] Although it is clear that the anatomical and modulatory link between these structures may mediate the integration of pain processing at the emotional level, the underlying molecular and cellular mechanisms remain poorly understood. The extracellular signal–regulated kinases 1 and 2 ($ERK_{1/2}$), members of the mitogen-activated protein kinase superfamily, have been used as markers of activity in the SC after noxious stimulation and tissue injury.[14] Beneficial results were demonstrated in a clinical trial using a mitogen-activated protein kinase inhibitor to treat neuropathic pain,[15] suggesting this kinase as a target in the treatment of pathological pain.[16] Interestingly, $ERK_{1/2}$ activation is also strongly implicated in the regulation of pain-associated disorders. Indeed, recent studies indicate that $ERK_{1/2}$ is activated in the anterior cingulate cortex of the PFC after tissue or nerve injury,[9,10,13] suggesting that $ERK_{1/2}$ is involved in both the sensorial and emotional aspects of pain.

We hypothesized that an effective treatment of the inflammatory condition is able to reverse the emotional and molecular responses produced by this condition. Thus, we used an experimental rat model of rheumatoid arthritis (monoarthritis) and we assessed the subsequent effects of diclofenac treatment on pain-induced anxiety, depression, and $ERK_{1/2}$ activation in the SC–PGi–LC–PFC pathway.

Materials and Methods

Animals

Adult male Harlan Sprague–Dawley rats (n = 116) weighing 250 to 300 g were provided by the Experimental Unit of the University of Cádiz, Cádiz, Spain (registration number: ES110120000210). The animals were housed 2 to 4 per cage, with an *ad libitum* access to food and water, and they were maintained on a 12-h light–dark cycle at 22°C and with 45 to 60% humidity. All experimental procedures were carried out in accordance with the European Communities Council Directive of September 22, 2010 (2010/63/EC), Spanish Law (RD 1201/2005), and ethical guidelines for the study of experimental pain in animals.[17] The experimental protocols were reviewed and approved by the Institutional Ethical Committee for animal care and use (Cádiz, Andalucía, Spain).

Inflammatory Pain Model: Monoarthritis

Monoarthritis was induced as described previously.[18] In brief, anesthesia was induced and maintained with isoflurane (4 and 2%, respectively; Abbott, Madrid, Spain), and the rats were injected in the left tibiotarsal joint with 50 μl of complete Freund's adjuvant (CFA) solution containing 30 mg of desiccated *Mycobacterium butyricum* (Difco Laboratories, Detroit, MI) diluted in the vehicle solution (3 ml paraffin oil, 2 ml saline, and 500 μl Tween-80). Control rats were injected with the vehicle solution alone and any animal exhibiting sign of polyarthritis was excluded from the study.

Inflammation Assessment

After CFA or vehicle injection, all the animals were monitored weekly for 4 weeks, measuring their body weight, rectal temperature (Chy 580BR Thermometer; Bioseb, Vitrolles, France), paw volume (using a plethysmometer apparatus; Ugo Basile, Comerio, Italy), and inflammation score. The inflammation score was a subjective scoring based on the signs of inflammation at the injection site and locomotion, whereby: 0 corresponds to the absence of inflammation and 4 to severe inflammation with persistent flexion of the affected limb and motor activity effects.[19]

Nociceptive Behavioral Assessment

Each week, nociceptive behavior was assessed in both the ipsi- and contralateral paws. Physiological evaluation of movement-induced nociception (allodynia) was performed using the ankle-bend test,[20] which involves the assessment of squeak and/or struggle reactions in response to five alternate flexions and extensions of the ankle joint. Higher scores (score 2) indicate squeak responses to moderate manipulations of the inflamed joint, whereas lower scores (score 0) indicate the absence of a response to manipulation. The reactions recorded in response to each extension or flexion are summed to obtain the ankle-bend score, giving a maximum value of 20, and a high ankle-bend score is indicative of allodynia. In addition, the presence of mechanical secondary hyperalgesia was determined using the paw-pinch test.[21] In brief, increasing pressure was gradually applied to the dorsal side of the paw using a graded motor-driven device (Ugo Basile) and beginning at 30 g of pressure. Three measurements were taken for each paw at 5-min intervals and the average value determined, with a 250 g cutoff applied to prevent damage to the paw. Secondary hyperalgesia is indicated by a reduction in the pressure-provoking withdrawal.

Anxiety- and Depression-like Behavior

Pain-induced emotional and affective changes were assessed in a separate set of animals at 4 (monoarthritis [MA]4D), 14 (MA14D), and 28 days (MA28D) after inducing monoarthritis. Anxiety-like behavior was evaluated by using the marble-burying and elevated zero maze (EZM) tests.

In the marble-burying test,[22] after a 30-min acclimatization, the rats were placed individually in a transparent plastic cage (51 × 22 × 15 cm) illuminated by a 100-W light and containing bedding (5 cm deep) in which 20 marbles were arranged in four columns and five rows. After 30 min,

the rats were removed and the number of buried marbles was counted, considering the marbles to be buried if they were at least two thirds covered with bedding. A larger number of buried marbles are taken as an indicator of increased anxiety-like behavior.

The EZM consisted of a black circular platform 105 cm in diameter that was elevated 65 cm above the floor. This maze was divided into four equal length quadrants: the two opposing open quadrants had 1-cm-high clear lips to prevent falls, whereas the two opposing closed quadrants were enclosed by 27-cm-high black walls. Each 5-min trial was carried out under the same lighting conditions and commenced with the animal being placed in the center of a closed quadrant. Spontaneous Motor Activity Recording and Tracking software (Panlab S.L.U., Barcelona, Spain) was used to analyze the percentage time spent in the open arms and the total distance travelled by each rat. An increase in the amount of time spent in the closed areas is correlated with anxiety-like behavior.

Depression-like behavior was evaluated by using a modified version of the forced swimming test (FST), as described previously.[23] When confined to a limited space, rodents engage in vigorous escape behavior. When it becomes clear that escape is impossible, these animals adopt an immobile posture, performing only the necessary movements required to keep their head above the water. Accordingly, in the FST, rats were placed for 15 min (pretest) in a clear cylindrical plastic container (46 cm high and 20 cm in diameter) filled with 30 cm of water (25° ± 1°C). After 24 h, the rats were once again exposed to the same conditions for 5 min (test) and they were videotaped to score their immobility (floating without struggling, using small movements to maintain the head above water), swimming (actively moving limbs more than is required to maintain the head above water), and climbing (active forepaw movements in and out of the water, often directed at the wall of the tank).[23] Customized software (Red Mice, Cádiz, Spain) was used to analyze the videos and to determine the predominant behavior at 5-s intervals. The total counts for each behavior during a 5-min test were averaged and although longer periods of immobility are taken as an indication of depression-like behavior, changes in the time spent climbing or swimming have been correlated with alterations in the availability of noradrenaline and serotonin, respectively.[23] As a positive control of antidepressant activity in the FST, the antidepressant desipramine (20 mg/kg; Sigma-Aldrich, St Louis, MO) was administered intraperitoneally to naive rats at 23.5, 5, and 1 h before testing. The behavioral tests were separated by a 3-day interval.

Western Blotting

Fresh tissue samples from the ipsilateral SC segments L3–L6, PGi, LC, and PFC regions were collected at 4, 14, and 28 days after injection of the CFA or vehicle. All the samples were processed for Western blotting and after the tissue was lysed, an aliquot (50 μg) was separated on a 10% polyacrylamide gel and then transferred to a polyvinylidene difluoride membrane (BioRad, Hercules, CA). After washing in Tris-buffered saline containing 0.1% Tween-20 (TBST), the blots were blocked with 5% bovine serum albumin (Sigma) in TBST and probed overnight at 4°C with rabbit anti-phospho-$ERK_{1/2}$ (1:5,000; Acris Antibodies, Herford, Germany), mouse-anti $ERK_{1/2}$ (1:2,000; Cell Signaling Technology, Danvers, MA), and mouse anti-tubulin (1:250,000; Sigma) antibodies diluted in 5% bovine

Table 1. Descriptive Statistics and Exact *P* Values for the Repeated-measures ANOVA

	Control				
	Basal (a)	First Week (b)	Second Week (c)	Third Week (d)	Fourth Week (e)
Weight (g)	303.7 ± 13.3 (6)	329.8 ± 10.7 (6)	352.3 ± 9.7 (6)	374.8 ± 9.3 (6)	388.9 ± 9.1 (6)
Temperature (°C)	37.5 ± 0.1 (6)	37.5 ± 0.1 (6)	37.1 ± 0.3 (6)	37.3 ± 0.1 (6)	36.9 ± 0.2 (6)
Paw volume Ip (ml)	1.7 ± 0.0 (6)	1.8 ± 0.0 (6)	1.8 ± 0.0 (6)	1.8 ± 0.0 (6)	1.8 ± 0.0 (6)
Paw volume Ctr (ml)	1.7 ± 0.0 (6)	1.8 ± 0.0 (6)	1.8 ± 0.0 (6)	1.8 ± 0.0 (6)	1.8 ± 0.0 (6)
Inflammation score	0.0 ± 0.0 (6)	0.0 ± 0.0 (6)	0.0 ± 0.0 (6)	0.0 ± 0.0 (6)	0.0 ± 0.0 (6)
Ankle-bend score	0.0 ± 0.0 (6)	0.0 ± 0.0 (6)	0.0 ± 0.0 (6)	0.0 ± 0.0 (6)	0.0 ± 0.0 (6)
Paw withdrawal Ip (g)	223.8 ± 17.0 (6)	196.3 ± 4.5 (6)	222.5 ± 6.0 (6)	223.8 ± 7.1 (6)	218.8 ± 7.9 (6)
Paw withdrawal Ctr (g)	248.8 ± 17.9 (6)	218.8 ± 9.6 (6)	225.0 ± 13.3 (6)	252.5 ± 17.8 (6)	238.8 ± 9.0 (6)

Values expressed as mean ± SEM for each experimental group followed by the sample size (n). In the *post hoc* column, c/h ($P = 0.0008$) means that at the 2nd week, controls were statistically different from monoarthritic rats with a *P* value of 0.0008.

*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

Ctr = contralateral; Ip = ipsilateral; MA = monoarthritis; ns = no significancies.

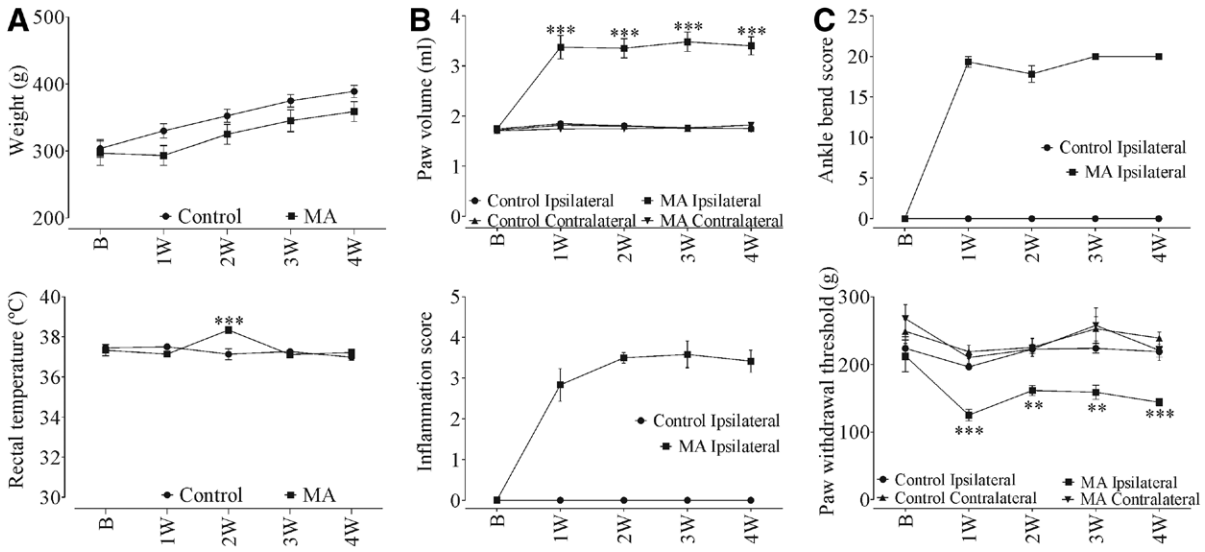

Fig. 1. Time course of the development of inflammation and nociceptive behavior in control and monoarthritic rats. (*A*) General parameters of health: weight and temperature. (*B*) Parameters of inflammation: paw volume and inflammation score. (*C*) Parameters of nociception: allodynia (ankle-bend score) and hyperalgesia (paw withdrawal threshold). All values are expressed as the mean ± SEM: $^{**}P < 0.01$, $^{***}P < 0.001$ monoarthritis (MA) *versus* corresponding controls, repeated-measures ANOVA followed by Student–Neuman–Keuls *post hoc* test for each time point. n = 6 per experimental group. B = baseline; W = week.

serum albumin–TBST. After thorough washing, these primary antibodies were detected by incubating for 1 h at room temperature with IRDye 800CW goat anti-rabbit (green) or IRDye 680LT goat anti-mouse (red) secondary antibodies (1:10,000; LI-COR®, Lincoln, NE). After three final washes with TBST, the antibody binding was detected by using a LI-COR Odyssey® two-channel quantitative fluorescence imaging system (LI-COR®). Digital images of Western blots were analyzed by densitometry using the ImageJ free access software (National Institutes of Health, Bethesda, MD). The data were expressed as $pERK_{1/2}$ expression levels relative to those of total $ERK_{1/2}$, as no significant differences in the loading control (tubulin) were observed. As no differences in $pERK_{1/2}$ expression were observed between the ipsilateral and contralateral sides for the PGi, LC, or PFC, these values were combined and averaged.

Topical Diclofenac Administration

To induce analgesia, 10 mg of sodium diclofenac (equivalent to 1 g of commercial Voltaren Gel®; Novartis, Basel,

MA					*P* Value (Repetead-measures ANOVA)			
Basal (f)	First Week (g)	Second Week (h)	Third Week (i)	Foruth Week (j)	Arthritis	Time	Interaction	Student–Newman–Keuls *Post Hoc* Test
296.7 ± 18.1 (6)	293.2 ± 14.9 (6)	324.8 ± 14.8 (6)	345.2 ± 16.3 (6)	358.7 ± 14.9 (6)	0.1812	0.0000***	0.0178*	ns
37.3 ± 0.3 (6)	37.1 ± 0.1 (6)	38.3 ± 0.2 (6)	37.1 ± 0.2 (6)	37.2 ± 0.2 (6)	0.1562	0.0191*	0.0014**	c/h (*P* = 0.0008)
1.7 ± 0.0 (6)	3.4 ± 0.2 (6)	3.4 ± 0.2 (6)	3.5 ± 0.2 (6)	3.4 ± 0.2 (6)	0.0000***	0.0000***	0.0000***	b/g (*P* = 0.0001); c/h (*P* = 0.0001); d/i (*P* = 0.0001); e/j (*P* = 0.0001)
1.7 ± 0.0 (6)	1.7 ± 0.0 (6)	1.7 ± 0.0 (6)	1.8 ± 0.0 (6)	1.8 ± 0.0 (6)	0.6992	0.0994	0.1439	ns
0.0 ± 0.0 (6)	2.8 ± 0.4 (6)	3.5 ± 0.1 (6)	3.6 ± 0.3 (6)	3.4 ± 0.3 (6)	—	—	—	—
0.0 ± 0.0 (6)	19.3 ± 0.7 (6)	17.8 ± 1.0 (6)	20.0 ± 0.0 (6)	20.0 ± 0.0 (6)	—	—	—	—
212.5 ± 23.4 (6)	125.0 ± 8.6 (6)	161.3 ± 7.4 (6)	158.8 ± 10.5 (6)	143.8 ± 5.3 (6)	0.0000***	0.0004***	0.5470	b/g (*P* = 0.0005); c/h (*P* = 0.0032); d/i (*P* = 0.0039); e/j (*P* = 0.0004)
267.5 ± 20.7 (6)	210.0 ± 9.3 (6)	222.5 ± 6.6 (6)	257.5 ± 26.0 (6)	220.8 ± 15.0 (6)	0.9179	0.0244*	0.8047	ns

Switzerland) was applied topically twice daily for 3 to 5 days to the ipsilateral paw of control and monoarthritic rats until analgesia was achieved, beginning 21 days after CFA injection. Pure Vaseline (Acofarmaderm, Acofarma S.A., Barcelona, Spain) was applied to the control and monoarthritic rats and served as a control of diclofenac application. A plastic Elizabeth collar was fixed around the neck of each animal to prevent ingestion of the cream/vaseline.[24] The effect of diclofenac on the signs of inflammation and nociceptive behavior was analyzed after the last topical application (see Inflammation Assessment and Nociceptive Behavioral Assessment sections). As indicators of paw inflammation, photographs and footprints of the hind paw were taken to evaluate the shape and area of paw. At the end of the experiment, Western blot procedures were performed to evaluate the effect of diclofenac in the pattern of $ERK_{1/2}$ activation in the SC, PGi, LC, and PFC. Next, topical application of diclofenac/vaseline was repeated in another set of animals to evaluate the effect of nonsteroidal anti-inflammatory drug analgesia on pain-induced affective changes following the protocols described in the Anxiety- and Depression-like Behavior section.

To study the possible site of action of diclofenac, an additional group of animals was organized to receive local topical treatment of diclofenac in the contralateral paw (right paw) following the same protocol as described in the first paragraph of this section. Thus, the following extra experimental groups consisted of control and monoarthritic animals receiving vaseline (Cont + VAS and MA + VAS) and a group of monoarthritic animals receiving diclofenac treatment (MA + DIC). Before and after vaseline/diclofenac administration, baseline measures were taken for the assessment of the paw volume, ankle-bend score, and paw-pinch test values. Afterwards, Western blot procedures were performed to evaluate the effect of contralateral paw administration in the pattern of $ERK_{1/2}$ activation in the SC, PGi, LC, and PFC.

Statistical Analysis

All data are presented as the means ± SEM and all the results were analyzed using STATISTICA 10.0 (StatSoft, Tulsa, OK) or GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA) using either a Student *t* test (unpaired or paired, two-tailed) or a one-way or two-way ANOVA with or without repeated measures, followed by the appropriate *post hoc* tests (Student–Newman–Keuls or Dunnett tests). The independent variables were monoarthritis (between-groups) and treatment (between-groups). The level of significance was set at a *P* value of less than 0.05. Detailed information regarding statistical results is shown in tables 1-4.

Results

Monoarthritis as a Model of Chronic Inflammatory Pain

The injection of the vehicle alone (control group) did not provoke any inflammatory reaction in rats and these animals exhibited a normal behavior. By contrast, stable monoarthritis was induced by CFA injection, with marked inflammatory signs within several hours of induction that persisted for 4 weeks. Monoarthritic rats displayed: (1) normal body weight gain; (2) normal body temperature, except in the second week when a significant increase was observed ($P < 0.001$; monoarthritis *vs.* control for week 2 by ANOVA followed by Student–Newman–Keuls *post hoc* test; fig. 1A); (3) a significant increase in the ipsilateral paw volume evident each week ($P < 0.001$ for each time point; monoarthritis *vs.* control by ANOVA followed by Student–Newman–Keuls *post hoc* test; fig. 1B); and (4) a higher inflammation score in the ipsilateral paw that persisted until the fourth week (fig. 1B). Significantly, none of these features were observed in the contralateral paw of these monoarthritic rats. When the movement-induced nociceptive behavior was evaluated, CFA injection provoked higher ankle-bend scores (allodynia) throughout the experimental period (fig. 1C) and monoarthritic rats exhibited significantly stronger mechanical hyperalgesia in the ipsilateral paw ($P < 0.001$ for weeks 1 and 4, $P < 0.01$ for weeks 2 and 3; monoarthritis *vs.* control by ANOVA followed by Student–Newman–Keuls *post hoc* test; fig. 1C). No painful reactions were observed in the control rats or in the contralateral paw of monoarthritic rats. Descriptive statistics is shown in table 1.

Monoarthritis-induced Anxiety- and Depression-like Behavior

To determine whether chronic pain associated with joint inflammation induced anxiogenic-like behavior, the rats were subjected to the marble-burying and EZM tests at several time points during the development of monoarthritis (fig. 2A). Unlike MA4D rats, more marbles were buried by MA14D ($P < 0.05$ by one-way ANOVA followed by Dunnett *post hoc* test) and MA28D ($P < 0.01$ by one-way ANOVA followed by Dunnett *post hoc* test) rats compared with the marbles buried by their corresponding controls, indicative of anxiety-like behavior.

In the EZM maze test, there was no difference in the percentage of time spent in the open arms between control rats and MA4D or MA14D rats. However, MA28D rats spent significantly lesser in the open arms than by their corresponding controls ($P < 0.001$ by one-way ANOVA followed by Dunnett *post hoc* test), again indicative of the development of anxiety-like behavior. No changes in locomotor activity were detected between groups in the EZM, ruling out a motor component in the effects observed.

The FST was performed to evaluate the possible development of depressive-like behaviors (fig. 2B), in which MA28D but not MA4D or MA14D rats exhibited significantly higher immobility scores compared with the scores of their corresponding controls ($P < 0.05$ by one-way ANOVA followed by Dunnett *post hoc* test). Moreover, this effect was accompanied by a significant decrease in climbing behavior ($P < 0.05$ by one-way ANOVA followed by Dunnett *post hoc* test), but there were no significant changes in swimming

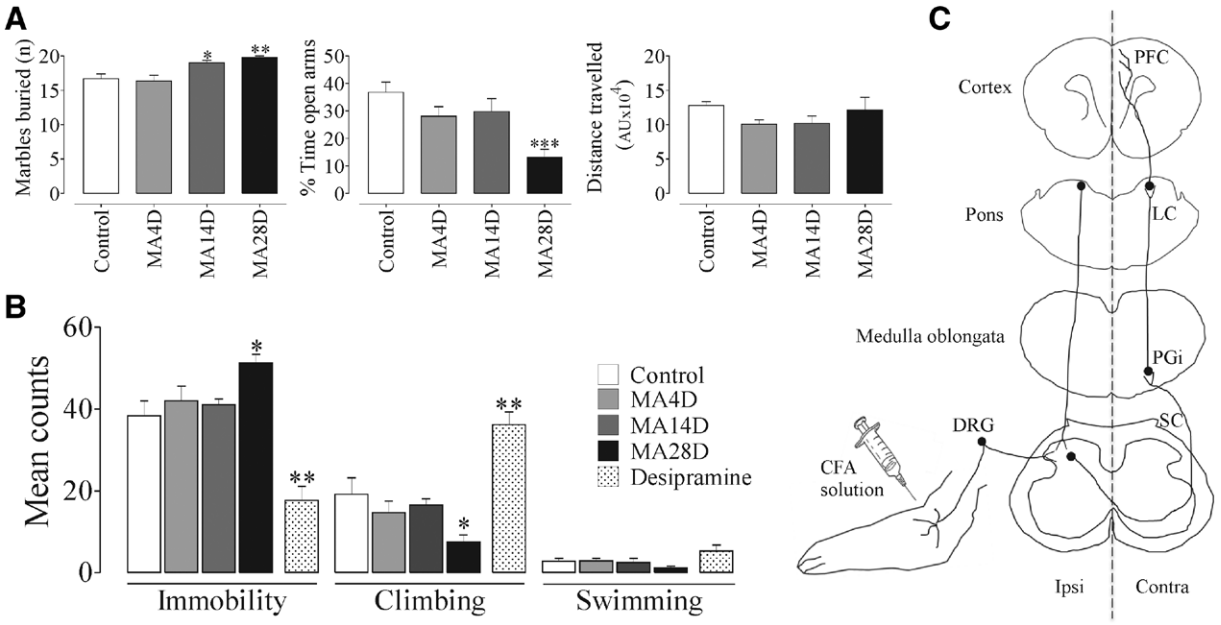

Fig. 2. Anxiety- (*A*) and depressive- (*B*) like behavioral responses to monoarthritis and schematic representation (*C*) of the paragigantocellularis (PGi)–locus coeruleus (LC)–prefrontal cortex (PFC) pathway. (*A*) Increased anxiety was observed in MA28D rats in the marble-burying test (*left*), as indicated by an increase in burying behavior relative to the controls. Similarly, MA28D rats spent more time in closed areas of an elevated zero maze (*center*), instead of exploring the open areas, a hallmark of anxiety-like behavior. Measurement of the total distance travelled indicated that there were no changes in locomotor activity in the elevated zero maze (*right panel*). (*B*) The modified forced swimming test was used as an index of depressive-like behavior/pro-depressive activity, indicated by an increase in the immobility time of MA28D rats with respect to controls. Naive rats treated with the antidepressant desipramine were used as positive controls of antidepressant activity in all the tests. Values are expressed as the mean ± SEM: **P* < 0.05, ***P* < 0.01, and ****P* < 0.001 monoarthritis *versus* control, one-way ANOVA followed by Dunnett *post hoc* test. n = 5 to 6 per experimental group; ***P* < 0.01, desipramine *versus* control; unpaired Student *t* test (two-tailed). (*C*) Scheme illustrating the PGi–LC–PFC nociceptive pathway, originating in the inflamed paw, passing through the dorsal root ganglion neurons (DRGs) to spinal cord (SC) neurons, where it crosses the *medial line* and finally ascends to the supraspinal structures. AU = arbitrary units; CFA = complete Freund's adjuvant; MA = monoarthritis.

behavior. As expected, desipramine treatment diminished the rat's immobility ($P < 0.01$ *vs.* corresponding control, Student *t* test) and significantly increased their climbing behavior ($P < 0.01$ *vs.* corresponding control, Student *t* test). Descriptive statistics is shown in table 2.

Monoarthritis-induced $ERK_{1/2}$ Activation in the SC–PGi–LC–PFC Pathway

The expression of $pERK_{1/2}$ was evaluated as an indicator of neuronal activity in the SC, PGi, LC, and PFC in the early, middle, and late phases of monoarthritis development (fig. 3). In the ipsilateral side of the SC, significant increases in the expression of $pERK_{1/2}$ were observed at 14 ($P < 0.05$ by one-way ANOVA followed by Dunnett *post hoc* test) and 28 ($P < 0.001$ by one-way ANOVA followed by Dunnett *post hoc* test) days after monoarthritis induction when comparing with that in control groups. No significant changes were observed at 4 days of monoarthritis disease. In addition, no significant changes in $pERK_{1/2}$ were observed at 4 or 14 days after monoarthritis induction in any of the brain areas analyzed. By contrast, significantly more $pERK_{1/2}$ was observed in the PGi ($P < 0.05$ by one-way ANOVA followed by Dunnett *post hoc* test), LC ($P < 0.01$ by one-way ANOVA followed by Dunnett *post hoc* test), and PFC ($P < 0.05$ by one-way ANOVA followed by Dunnett *post hoc* test) at 28 days after monoarthritis induction compared with that in the corresponding controls. Descriptive statistics is shown in table 2.

Effect of Topical Administration of Diclofenac on Nociceptive Behavior

As expected, topical application of diclofenac to the ipsilateral paw decreased the inflammation and increased the pain threshold of that paw, without producing any significant change in body weight or body temperature (fig. 4). Although a small edema was observed in monoarthritic rats that received diclofenac ($P < 0.01$, control + diclofenac *vs.* monoarthritis + diclofenac by two-way ANOVA followed by Student–Newman–Keuls *post hoc* test), this was substantially smaller than that seen in those that received vaseline ($P < 0.001$, monoarthritis + vaseline *vs.* monoarthritis + diclofenac by two-way ANOVA followed by Student–Newman–Keuls *post hoc* test: fig. 4B). A similar effect on the inflammation score was observed (fig. 4B) and the ankle-bend score of monoarthritic rats that received a topical application of diclofenac was significantly lower ($P < 0.001$, monoarthritis + vaseline *vs.* monoarthritis + diclofenac by two-way ANOVA followed by Student–Newman–Keuls

Table 2. Descriptive Statistics and Exact *P* Values for the One-way ANOVA

	Control (a)	MA4D (b)	MA14D (c)	MA28D (d)	Desipramine (e)	*P* Value (One-way ANOVA) Arthritis	*P* Value (Student *t* Test, Unpaired, Two-tailed)	Dunnett *Post hoc* Test
Marbles buried (n)	16.7 ± 0.7 (6)	16.3 ± 0.8 (6)	19.0 ± 0.4 (6)	19.8 ± 0.2 (5)	—	0.0016**	—	a/c (*P* = 0.0344); a/d (*P* = 0.0065)
Time in open arms (%)	36.8 ± 3.7 (6)	28.1 ± 3.4 (6)	29.8 ± 4.7 (6)	13.2 ± 2.9 (6)	—	0.0020**	—	a/d (*P* = 0.0007)
Distance travelled (AU × 10^4)	12.8 ± 0.6 (6)	10.1 ± 1.1 (6)	10.1 ± 1.1 (6)	12.1 ± 1.9 (6)	—	0.2732	—	ns
Immobility (mean counts)	38.3 ± 3.7 (6)	42.0 ± 3.6 (6)	41.0 ± 1.4 (6)	51.3 ± 2.0 (6)	17.7 + 3.5 (6)	0.0228*	a/e (*P* = 0.0021)	a/d (*P* = 0.0112)
Climbing (mean counts)	19.2 ± 4.0 (6)	14.7 ± 2.8 (6)	16.5 ± 1.5 (6)	7.5 ± 1.7 (6)	36.2 + 3.1 (6)	0.0371*	a/e (*P* = 0.0072)	a/d (*P* = 0.0167)
Swimming (mean counts)	3.5 ± 0.8 (6)	3.3 ± 0.8 (6)	2.5 ± 1.1 (6)	1.2 ± 0.4 (6)	6.2 + 1.3 (6)	0.1858	ns	ns
$pERK_{1/2}$ (SC)	1.0 ± 0.0 (8)	2.3 ± 0.5 (12)	3.1 ± 0.7 (11)	5.1 ± 0.8 (8)	—	0.0009***	—	a/c (*P* = 0.0314); a/d (*P* = 0.0003)
$pERK_{1/2}$ (PGi)	0.9 ± 0.0 (6)	1.3 ± 0.3 (6)	0.8 ± 0.3 (6)	1.9 ± 0.4 (6)	—	0.0328*	—	a/d (*P* = 0.0386)
$pERK_{1/2}$ (LC)	1.0 ± 0.3 (6)	1.6 ± 0.3 (6)	1.6 ± 0.8 (6)	2.9 ± 0.6 (6)	—	0.0114*	—	a/d (*P* = 0.0043)
$pERK_{1/2}$ (PFC)	1.0 ± 0.0 (6)	0.9 ± 0.2 (5)	1.0 ± 0.3 (6)	1.8 ± 0.1 (6)	—	0.0092**	—	a/d (*P* = 0.0207)

Values expressed as mean ± SEM for each experimental group followed by the sample size (n). For desipramine-positive control, a Student test was performed to directly compare with the control group. In the *post hoc* column, a/c (*P* = 0.0344) means that MA14D was statistically different from control rats with a *P* value of 0.0344.

*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

AU = arbitrary units; LC = locus coeruleus; MA = monoarthritis; ns = no significancies; pERK1/2 = phosphorylated extracellular signal–regulated kinases 1 and 2; PFC = prefrontal cortex; PGi = paragigantocellularis; SC = spinal cord.

post hoc test), reflecting a higher nociceptive threshold, although this score remained significantly higher than that observed in control animals ($P < 0.001$ for the inflammation score and $P < 0.01$ for the ankle-bend score, control + diclofenac *vs.* monoarthritis + diclofenac by two-way ANOVA followed by Student–Newman–Keuls *post hoc* test: fig. 4C). Diclofenac treatment also increased the force supported by the ipsilateral paw of monoarthritic rats ($P < 0.001$, monoarthritis + vaseline *vs.* monoarthritis + diclofenac by two-way ANOVA followed by Student–Newman–Keuls *post hoc* test), completely abolishing mechanical hyperalgesia (fig. 4C). Although not quantified, photographs and footprints revealed a clear improvement in paw posture and weight loading in the affected paw after diclofenac treatment (fig. 5). Descriptive statistics is shown in table 3.

Effect of Diclofenac on Anxiety- and Depression-like Behaviors

Diclofenac administration had significant effects on monoarthritis-induced anxiety- and depression-like behaviors. In the EZM, monoarthritis-induced anxiety-like behavior was reversed by diclofenac treatment ($P < 0.05$, monoarthritis + vaseline *vs.* monoarthritis + diclofenac by two-way ANOVA followed by Student–Newman–Keuls *post hoc* test; fig. 5A), yet no changes in locomotor activity were produced in this paradigm (fig. 5A). Similarly, monoarthritis-induced depression-like behavior was successfully reversed by topical diclofenac administration, as revealed by the significant differences in immobility time between groups ($P < 0.05$, monoarthritis + vaseline *vs.* monoarthritis + diclofenac by two-way ANOVA followed by Student–Newman–Keuls *post hoc* test; fig. 5B). The climbing behavior displayed by the monoarthritic rats receiving vaseline showed a tendency to decrease compared with the respective control group ($P = 0.056$ control + vaseline *vs.* monoarthritis + vaseline, by two-way ANOVA followed by Student–Newman–Keuls *post hoc* test; fig. 5B). Descriptive statistics is shown in table 3.

Effect of Diclofenac on $pERK_{1/2}$ Expression in the SC, PGi, LC, and PFC

Local inflammation produced a significant increase in the expression of $pERK_{1/2}$ levels in the ipsilateral SC as witnessed in monoarthritic rats treated with vaseline as compared with that in the corresponding controls ($P < 0.001$, monoarthritis + vaseline *vs.* control + vaseline by two-way ANOVA followed by Student–Newman–Keuls *post hoc* test). Analgesia through application of diclofenac to the inflamed paw significantly reduced $pERK_{1/2}$ levels ($P < 0.001$, monoarthritis + vaseline *vs.* monoarthritis + diclofenac by two-way ANOVA followed by Student–Newman–Keuls *post hoc*

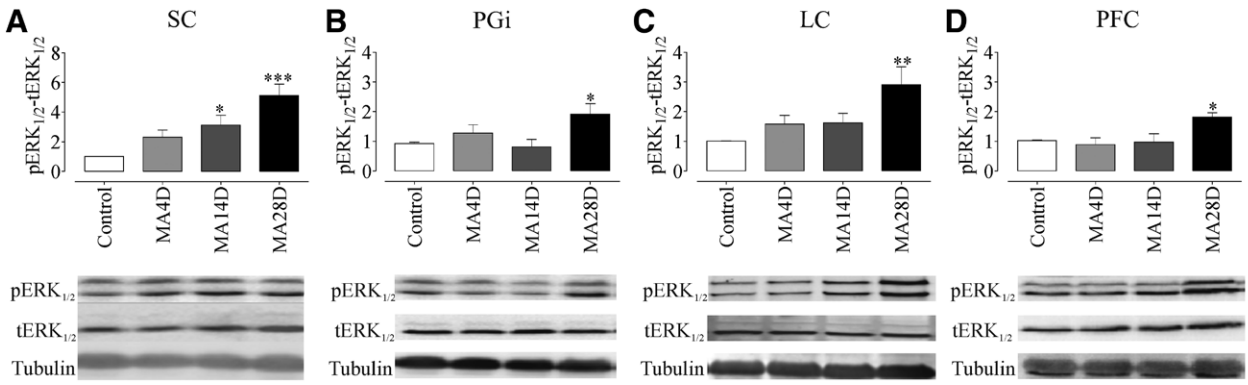

Fig. 3. pERK$_{1/2}$ expression in the spinal cord (SC)–paragigantocellularis (PGi)–locus coeruleus (LC)–prefrontal cortex (PFC) pathway in response to chronic monoarthritis (MA). (*A*) In the SC, significant changes were observed in MA14D and MA28D rats. In addition, increased ERK$_{1/2}$ phosphorylation was observed in MA28D rats in the PGi (*B*), LC (*C*), and PFC (*D*). Below the graphs are images of the blots showing pERK$_{1/2}$ (44–42 kDa), tERK$_{1/2}$ (44–42 kDa), and tubulin (50 kDa) expression for each structure from each experimental group. Values are expressed as mean ± SEM: **P* < 0.05, ***P* < 0.01, and ****P* < 0.001, MA *versus* corresponding controls, one-way ANOVA followed by Dunnett test. *Each column* represents the mean pERK$_{1/2}$ levels from three assays performed on samples from independent groups of 2–4 animals. These levels were normalized to the corresponding total ERK$_{1/2}$ values, as no significant changes in tubulin levels were observed. pERK$_{1/2}$/tERK$_{1/2}$ = phosphorylated/total extracellular signal–regulated kinases 1 and 2, respectively.

test; fig. 6, A and B) and the significant increase in pERK$_{1/2}$ levels in the PGi–LC–PFC pathway of monoarthritic rats ($P < 0.05$, monoarthritis + vaseline *vs.* control + vaseline in the PGi; $P < 0.01$ monoarthritis + vaseline *vs.* control + vaseline in the LC and PFC by two-way ANOVA followed by Student–Newman–Keuls *post hoc* test; fig. 6, A and B) was also successfully restored to control levels after topical treatment with diclofenac ($P < 0.05$, monoarthritis + vaseline *vs.* monoarthritis + diclofenac in the PGi and PFC; $P < 0.01$, monoarthritis + vaseline *vs.* monoarthritis + diclofenac in the LC by two-way ANOVA followed by Student–Newman–Keuls *post hoc* test; fig. 6, A and B). Descriptive statistics is shown in table 3.

Effect of Diclofenac Administration in the Contralateral Paw

The results point to the absence of an effect of the contralateral administration of diclofenac (fig. 7) on the paw volume

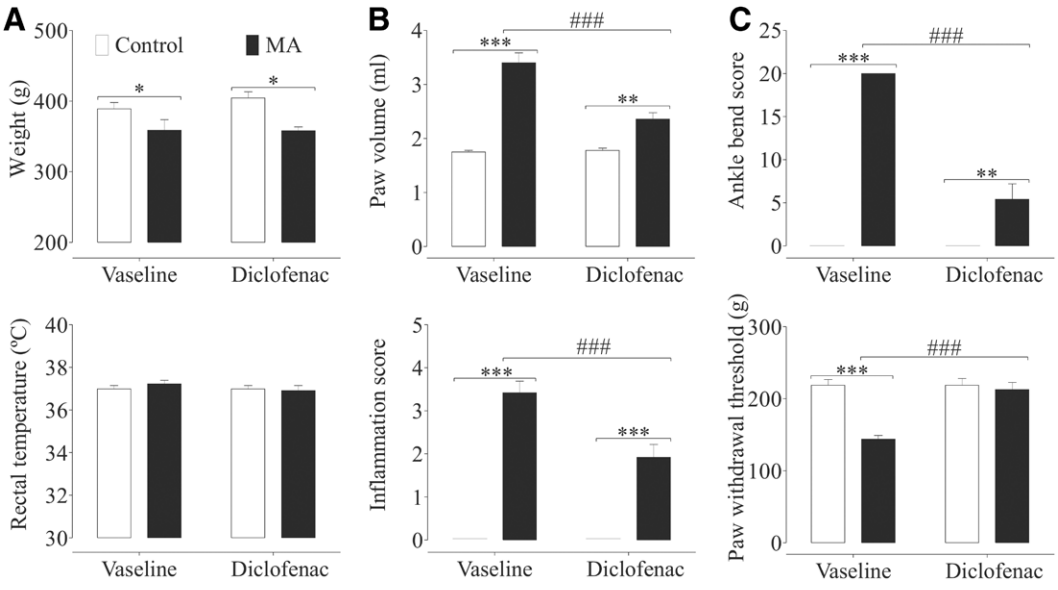

Fig. 4. Effect of topical diclofenac application to the ipsilateral paw on inflammation and nociceptive behavior. (*A*) General parameters of health (weight and temperature) were not significantly altered by diclofenac treatment. (*B*) Paw volume and the inflammation score in monoarthritic rats were significantly attenuated by diclofenac treatment. (*C*) The ankle-bend score was significantly lower in diclofenac-treated monoarthritic rats and the paw withdrawal threshold was restored to control levels. Values are expressed as the mean ± SEM: **P* < 0.05, ***P* < 0.01, and ****P* < 0.001, monoarthritis (MA) *versus* corresponding controls; ###*P* < 0.001, MA + vaseline *versus* MA + diclofenac, two-way ANOVA followed by Student–Neuman–Keuls *post hoc* test. n = 6 animals per experimental group.

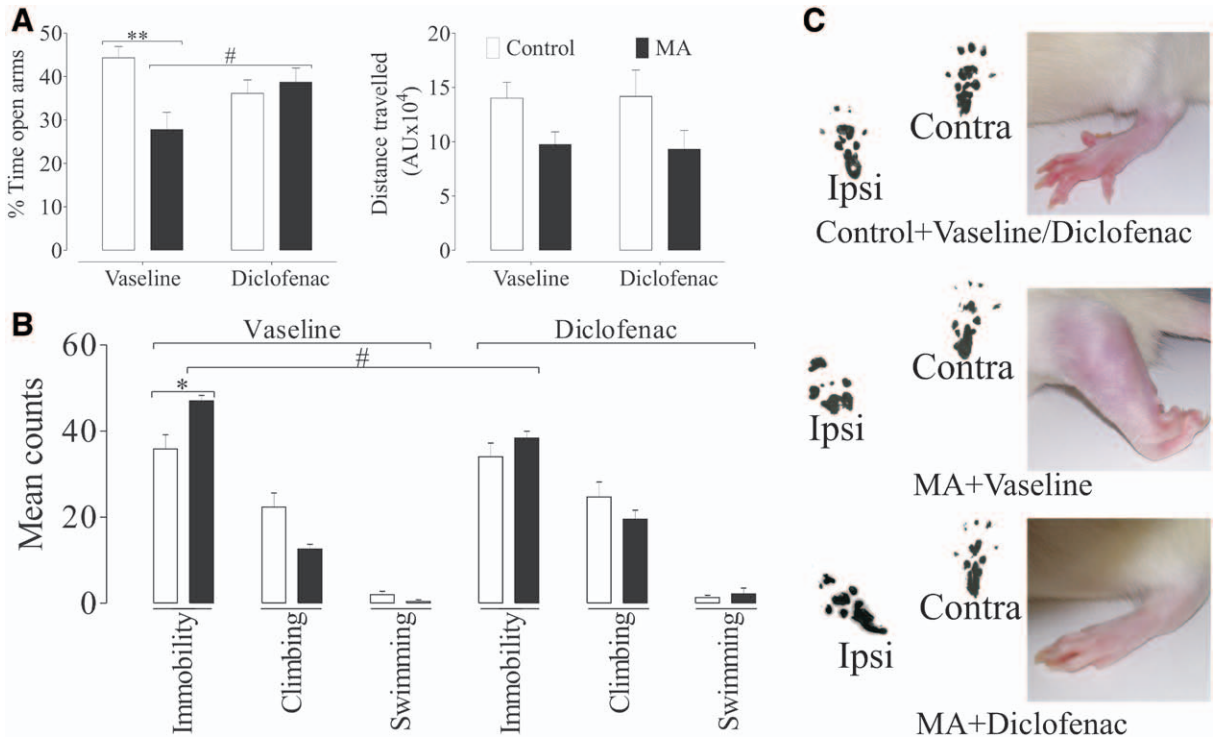

Fig. 5. Effect of topical diclofenac application to the ipsilateral paw on anxiety- and depression-like behaviors induced by chronic monoarthritis (MA). (*A*) Diclofenac treatment abolished the anxiety-like behavior produced by chronic MA, as witnessed by an increase in the time spent in the open areas of the elevated zero maze. There were no significant differences between experimental groups in the total distance travelled in the elevated zero maze. (*B*) Topical diclofenac application also eliminated MA-induced depressive-like behavior, as witnessed by the decreased immobility in the forced swimming test. (*C*) Hind paw photographs and footprints revealed a clear improvement in the guarding posture in diclofenac-treated monoarthritic rats (Ipsi = ipsilateral paw; Contra = contralateral paw). Note the paw position of monoarthritic rats treated with vaseline or diclofenac. Values are expressed as mean ± SEM: **P* < 0.05 and ***P* < 0.01, MA *vs.* corresponding control; #*P* < 0.05, MA + vaseline *versus* MA + diclofenac; two-way ANOVA followed by Student–Neuman–Keuls *post hoc* test. n = 5 to 6 animals per experimental group. AU = arbitrary units.

(fig. 7A), and paw-pinch threshold (fig. 7C) of the inflamed paw (ipsilateral paw), as can be observed by the lack of significant changes between the values obtained before the treatment and after the treatment, in the group of monoarthritic animals receiving diclofenac (paired Student *t* test). However, in the ankle-bend score (fig. 7B), contralateral treatment with diclofenac caused a decrease in the values obtained after the treatment when comparing with those obtained before the treatment ($P < 0.01$; paired Student *t* test; fig. 7B). Descriptive statistics is shown in table 4.

In addition, quantification of the immunoblots (fig. 8) showed that the increased $ERK_{1/2}$ activation observed in MA + VAS group was maintained in the MA + DIC group in the SC ($P < 0.01$ for MA + VAS and MA + DIC *vs.* control, unpaired Student *t* test), PGi ($P < 0.01$ for MA + VAS *vs.* control and $P < 0.05$ for MA + DIC *vs.* control unpaired Student *t* test), LC ($P < 0.05$ for MA + VAS *vs.* control and $P < 0.01$ for MA + DIC *vs.* control, unpaired Student *t* test), and PFC ($P < 0.05$ for MA + VAS and MA + DIC *vs.* control, unpaired Student *t* test). Descriptive statistics is shown in table 4.

Discussion

The current findings demonstrate that chronic joint inflammation interferes with sensitivity to noxious stimulation and with physiological movement of the inflamed joint, as well as profoundly affecting emotional states when the painful condition is prolonged. Furthermore, we demonstrated that chronic pain enhances $ERK_{1/2}$ activation in certain central nervous system regions, specifically in the SC, PGi, LC, and PFC. Promoting analgesia through the topical application of anti-inflammatory cream successfully attenuated nociception, as well the anxiety- and depressive-like behaviors observed. Interestingly, this effect was accompanied by the normalization $ERK_{1/2}$ activation in the SC, PGi, LC, and PFC.

We initially analyzed here the nociceptive behavior produced by joint inflammatory pain with the use of the monoarthritis model. As expected, unilateral arthritic inflammation induced constant allodynia and hyperalgesia in the ipsilateral paw at all time points studied. The induction of monoarthritis was associated with the development of anxiety-like symptoms in the EZM and marble-burying tests. The EZM is based on the natural aversion of rodents to bright

Table 3. Descriptive Statistics and Exact *P* Values for the Two-way Analysis

	Control		MA		*P* Value (Two-way ANOVA)			Student–Newman–Keuls *Post hoc* Test	
	Vaseline (a)	Diclofenac (b)	Vaseline (c)	Diclofenac (d)	Arthritis	Treatment	Interaction		
Weight (g)	388.8 ± 9.1 (6)	404.3 ± 8.8 (6)	358.7 ± 14.9 (6)	358.0 ± 5.5 (6)	0.0012**	0.4736	0.4353	a/c (*P* = 0.0486); b/d (*P* = 0.0200)	
Temperature (°C)	36.9 ± 0.2 (6)	36.9 ± 0.2 (6)	37.2 ± 0.2 (6)	36.9 ± 0.2 (6)	0.6943	0.4098	0.4098	ns	
Paw volume Ip (ml)	1.8 ± 0.0 (6)	1.8 ± 0.0 (6)	3.4 ± 0.2 (6)	2.4 ± 0.1 (6)	0.0000***	0.0002***	0.0001***	a/c (*P* = 0.0002); b/d (*P* = 0.0016); c/d (*P* = 0.0002)	
Inflammation score Ip	0.0 ± 0.0 (6)	0.0 ± 0.0 (6)	3.4 ± 0.3 (6)	1.9 ± 0.3 (6)	0.0000***	0.0014**	0.0014**	a/c (*P* = 0.0001); b/d (*P* = 0.0001); c/d (*P* = 0.0002)	
Ankle bend score Ip	0.0 ± 0.0 (6)	0.0 ± 0.0 (6)	20.0 ± 0.0 (6)	5.4 ± 1.8 (6)	0.0000***	0.0000***	0.0000***	a/c (*P* = 0.0001); b/d (*P* = 0.0011); c/d (*P* = 0.0002)	
Paw withdrawal Ip (g)	218.6 ± 7.8 (6)	218.6 ± 9.4 (6)	143.8 ± 5.3 (6)	212.6 ± 10.0 (6)	0.0001***	0.0005***	0.0005***	a/c (*P* = 0.0002); c/d (*P* = 0.0002)	Ipsilateral treatment
Time in open arms (%)	44.3 ± 2.6 (6)	36.1 ± 3.1 (6)	27.8 ± 4.0 (6)	38.7 ± 3.2 (6)	0.0465*	0.6783	0.0084**	a/c (*P* = 0.0017); c/d (*P* = 0.0132)	
Distance travelled (AU × 10^4)	14.0 ± 1.5 (6)	14.2 ± 2.4 (6)	9.8 ± 1.2 (6)	9.3 ± 1.7 (6)	0.0170*	0.9356	0.8669	ns	
Immobility (mean counts)	35.8 ± 3.3 (6)	34.0 ± 3.2 (6)	47.0 ± 1.3 (5)	38.3 ± 1.6 (6)	0.0077**	0.0578	0.2045	a/c (*P* = 0.0180); c/d (*P* = 0.0294)	
Climbing (mean counts)	22.3 ± 3.2 (6)	24.5 ± 3.5 (6)	12.6 ± 1.1 (5)	19.5 ± 2.1 (6)	0.0145	0.1120	0.4201	ns	
Swimming (mean counts)	2.0 ± 0.8 (6)	1.3 ± 0.5 (6)	0.4 ± 0.4 (5)	2.2 ± 1.3 (6)	0.6629	0.5328	0.1760	ns	
$pERK_{1/2}$ (SC)	1.0 ± 0.0 (6)	1.7 ± 0.4 (5)	5.0 ± 0.5 (5)	2.5 ± 0.5 (5)	0.0000***	0.0444*	0.0010**	a/c (*P* = 0.0002); c/d (*P* = 0.0006)	
$pERK_{1/2}$ (PGi)	1.1 ± 0.1 (6)	1.2 ± 0.4 (6)	3.0 ± 0.9 (6)	1.0 ± 0.1 (6)	0.0730	0.0984	0.0472*	a/c (*P* = 0.0338); c/d (*P* = 0.0466)	
$pERK_{1/2}$ (LC)	1.0 ± 0.0 (6)	1.0 ± 0.1 (6)	1.9 ± 0.3 (6)	1.2 ± 0.1 (6)	0.0083**	0.0287*	0.0800	a/c (*P* = 0.0082); c/d (*P* = 0.0096)	
$pERK_{1/2}$ (PFC)	1.0 ± 0.0 (6)	1.6 ± 0.3 (6)	3.1 ± 0.6 (6)	1.4 ± 0.3 (6)	0.0225*	0.1730	0.0076**	a/c (*P* = 0.0052); c/d (*P* = 0.0150)	

Values expressed as mean ± SEM for each experimental group followed by the sample size (n). In the *post hoc* column, a/c (*P* = 0.0486) means that MA + vaseline group was statistically different from control + vaseline group with a *P* value of 0.0486.

*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

AU = arbitrary units; Ip = ipsilateral; LC = locus coeruleus; MA = monoarthritis; ns = no significancies; $pERK_{1/2}$ = phosphorylated extracellular signal–regulated kinases 1 and 2; PFC = prefrontal cortex; PGi = paragigantocellularis; SC = spinal cord.

and exposed places, whereas marble-burying behavior gauges the level of anxiety-like behavior of a rodent on encountering unfamiliar and bright objects. In both tests, anxiety-like behavior was evident at late disease stages, and in the case of the marble-burying test, the anxiety-like behavior was already evident at 14 days after monoarthritis induction. We also evaluated the time course of depression-like behavior by using the FST. This test induces "behavioral despair" in animals and abandoning the struggle to escape a stressful environment may resemble the psychological concept of "entrapment" described in clinical populations.[23] MA28D rats displayed greater immobility and milder escape behavior (climbing) in the FST test. This reduction in climbing behavior was not accompanied by changes in swimming behavior, suggesting noradrenergic dysfunction. This is mainly due to the fact that noradrenaline-selective reuptake inhibitors produce significant increases in this behavior in the FST[23] indicating that a decrease in the climbing behavior suggests a dysfunction in the noradrenergic system. More studies are underway to clarify whether this impairment is really occurring. These data are in consistent with previous findings,[25–27] and with clinical data obtained from patients with osteoarthritis,[28] indicating that chronic inflammatory pain conditions profoundly affect emotional states, as also described previously in other rheumatic diseases.[29] Moreover, the time course for development of anxiety and then depression may indicate a certain chronological development of pain-related mood disorders, which was already observed in other pain models.[30] Overall, this implies that the affective consequences of chronic inflammatory pain evolve over time, probably mediated by long-term molecular and neural plastic changes at different brain areas.

Because $ERK_{1/2}$ have been proposed as promising target molecules in the regulation of both pain[31,32] and affective disorders,[33–35] we investigated whether monoarthritis-induced affective disorders were associated with altered $ERK_{1/2}$ activation. In the SC, $pERK_{1/2}$ was not significantly increased

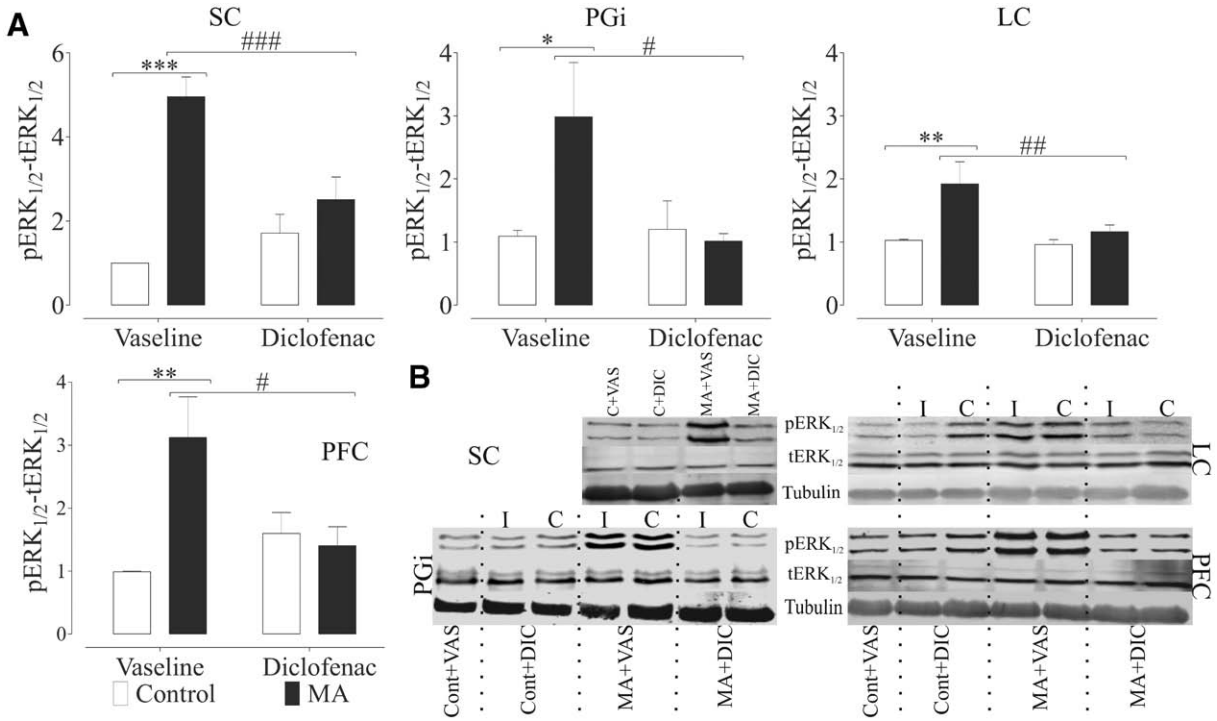

Fig. 6. Effect of topical diclofenac application to the ipsilateral paw on the $pERK_{1/2}$ in the spinal cord (SC)–paragigantocellularis (PGi)–locus coeruleus (LC)–prefrontal cortex (PFC) pathway. (*A*) Graphs depict the changes in the $pERK_{1/2}$ expression in response to chronic monoarthritis (MA). Diclofenac administration significantly reduced $pERK_{1/2}$ levels on the ipsilateral side of the SC and it reversed the increase in $pERK_{1/2}$ observed in the PGi, LC, and PFC. (*B*) Images of the blots showing $pERK_{1/2}$ (44–42 kDa), $tERK_{1/2}$ (44–42 kDa), and tubulin (50 kDa) expression for each structure from each experimental group. Values are expressed as the mean ± SEM: **P* < 0.05, ***P* < 0.01, and ****P* < 0.001, control *versus* MA; #*P* < 0.05, ##*P* < 0.01, ###*P* < 0.001, MA + vaseline *versus* MA + diclofenac; two-way ANOVA followed by Student–Neuman–Keuls *post hoc* test. *Each column* represents the mean $pERK_{1/2}$ levels of three assays performed on samples from independent groups of 2–4 animals. These levels were normalized to the corresponding total $ERK_{1/2}$ values, as no significant changes in tubulin levels were observed. C = contralateral; Cont = control; DIC = diclofenac; I = ipsilateral; $pERK_{1/2}$/$tERK_{1/2}$ = phosphorylated/total extracellular signal–regulated kinases 1 and 2, respectively; VAS = vaseline.

at 4 days after monoarthritis induction but was significantly increased at both 14 and 28 days after monoarthritis. Indeed, other authors showed that 2 and 4 days of inflammation were not accompanied by increased $ERK_{1/2}$ activation in the SC.[36] The significant increase of $pERK_{1/2}$ observed at 14 and 28 days may be related with increased metabolic activity which was already demonstrated by Schadrack *et al.*,[37] at least for the 14 days of monoarthritic time point. The onset of affective symptoms was accompanied by increased $pERK_{1/2}$ levels in the PGi, LC, and PFC of monoarthritic rats, in agreement with previous reports of increased $pERK_{1/2}$ expression in the rat PGi 7 days after the induction of neuropathic

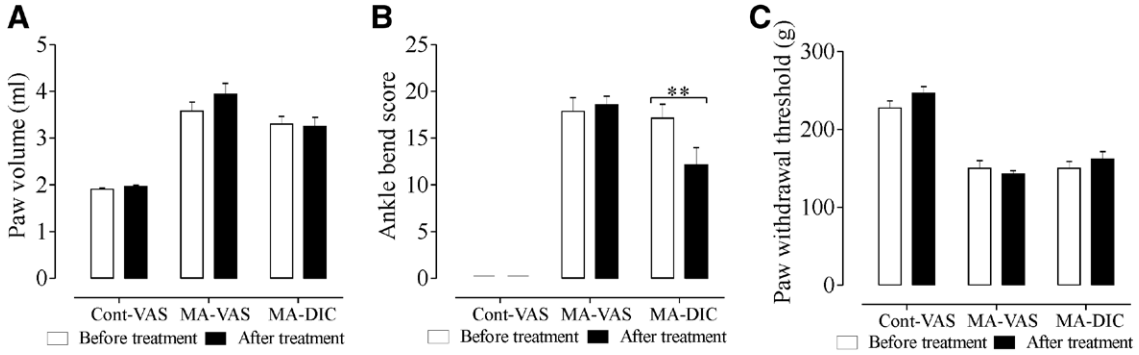

Fig. 7. Effect of the topical administration of vaseline/diclofenac to the contralateral paw on the paw volume (*A*), ankle-bend score (*B*), and paw withdrawal threshold (*C*). Valued expressed as mean ± SEM. Comparisons between the values obtained before and after the treatment, for each experimental group, were performed by using a paired Student *t* test. ***P* < 0.01. Cont = control; DIC = diclofenac; MA = monoarthritis; VAS = vaseline.

Table 4. Descriptive Statistics and Exact *P* Values for the Student *t* Test

	Before Treatment			After Treatment				
	Control	MA		Control	MA			
	Vaseline (a)	Vaseline (c)	Diclofenac (e)	Vaseline (b)	Vaseline (d)	Diclofenac (f)	*P* Value (Paired Student *t* Test)	
Paw volume Ip (ml)	1.9 ± 0.0 (6)	3.6 ± 0.2 (7)	3.3 ± 0.2 (7)	2.0 ± 0.0 (6)	3.9 ± 0.2 (7)	3.2 ± 0.2 (7)	ns	
Ankle-bend score Ip	0.0 ± 0.0 (6)	17.9 ± 1.5 (7)	17.1 ± 1.5 (7)	0.0 ± 0.0 (6)	18.6 ± 0.9 (7)	12.1 ± 1.8 (7)	e/f (*P* = 0.0038)	
Paw withdrawal Ip (g)	227.5 ± 8.8 (6)	150.0 ± 9.8 (7)	150.0 ± 8.7 (7)	246.3 ± 8.8 (7)	142.5 ± 4.3 (7)	161.8 ± 9.4 (7)	ns	
							P Value (Unpaired Student *t* Test)	Contralateral treatment
$pERK_{1/2}$ (SC)		—		1.0 ± 0.0 (6)	3.9 ± 0.9 (6)	2.9 ± 0.5 (6)	b/d (*P* = 0.0072); b/f (*P* = 0.0023)	
$pERK_{1/2}$ (PGi)		—		1.0 ± 0.0 (6)	1.3 ± 0.1 (6)	1.9 ± 0.3 (6)	b/d (*P* = 0.0039); b/f (*P* = 0.0198)	
$pERK_{1/2}$ (LC)		—		1.0 ± 0.0 (6)	1.4 ± 0.1 (6)	2.2 ± 0.3 (6)	b/d (*P* = 0.0174); b/f (*P* = 0.0013)	
$pERK_{1/2}$ (PFC)		—		1.0 ± 0.0 (6)	3.6 ± 1.1 (6)	2.5 ± 0.5 (6)	b/d (*P* = 0.0157); b/f (*P* = 0.0427)	

Values expressed as mean ± SEM for each experimental group followed by the sample size (n). e/f (*P* = 0.0038) means that MA diclofenac before treatment was statistically different from MA diclofenac after treatment with a *P* value of 0.0038.

Ip = ipsilateral; LC = locus coeruleus; MA = monoarthritis; ns = no significancies; pERK1/2 = phosphorylated extracellular signal–regulated kinases 1 and 2; PFC = prefrontal cortex; PGi = paragigantocellularis; SC = spinal cord.

pain.[38,39] We observed increased $ERK_{1/2}$ activation in the LC after chronic monoarthritis, possibly due to increased excitatory input from the PGi. Interestingly, CFA injection has been shown to induce a sharp increase in LC $pERK_{1/2}$ after 5 min that disappears 7 h later,[40] and indeed, we observed no changes in $pERK_{1/2}$ in the early stages after CFA administration. By contrast, $pERK_{1/2}$ expression was consistently increased 28 days postadministration, coinciding with altered nociceptive behavior and the onset of anxiety and, especially, depressive states. These findings suggest that the LC is involved in both acute pain and the subsequent development of pain-induced affective disorders. Finally, $ERK_{1/2}$ activation was also enhanced in the PFC region 28 days after CFA administration. The PFC is one of the most important projection targets of LC noradrenergic terminals, and increased $pERK_{1/2}$ expression in this area has been correlated with anxiety- and depressive-like behaviors.[9,10,13,35] Another interesting observation was that the pattern of $ERK_{1/2}$ activation at supraspinal level was bilateral, which means that unilateral inflammation of the ankle produced both ipsilateral and contralateral phosphorylation of $ERK_{1/2}$ in the PGi–LC–PFC pathway. This was already shown in other pain studies,[38,39] and particularly, such lack of lateralization of $ERK_{1/2}$ phosphorylation was common in studies regarding

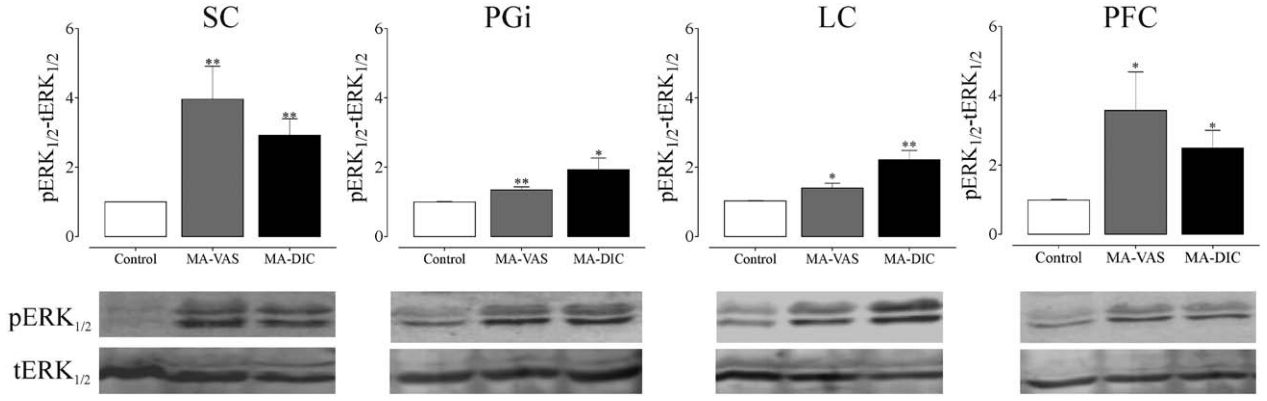

Fig. 8. Effect of the topical administration of vaseline/diclofenac to the contralateral paw in the pattern of $ERK_{1/2}$ activation in the spinal cord (SC)–paragigantocellularis (PGi)–locus coeruleus (LC)–prefrontal cortex (PFC) pathway. Below the graphs, there is a representation of the immunoblots showing the differences between experimental groups. Values expressed as mean ± SEM. **P* < 0.05 and ***P* < 0.01 by unpaired Student *t* test *versus* the control group. Cont = control; DIC = diclofenac; MA = monoarthritis; $pERK_{1/2}$/$tERK_{1/2}$ = phosphorylated/total extracellular signal–regulated kinases 1 and 2, respectively; VAS = vaseline.

pain-related anxiety paradigms.[9,10,13] Taken together, these findings suggest that alterations in the levels of $pERK_{1/2}$ may be one of the molecular mechanisms that underlie the onset of chronic pain–induced affective disorders.

One of the most remarkable findings of the current study was that the blockade of nociceptive inputs affected monoarthritis-induced anxiety- and depression-like behavior. As indicated elsewhere, administration of sodium diclofenac ointment attenuated inflammation and pain,[41,42] as well as reducing $pERK_{1/2}$ levels in the lumbar SC. Significantly, the induction of analgesia was accompanied by the disappearance of anxiety- and depression-like behaviors, suggesting that sensorial pain inputs are the source of affective alterations in chronic monoarthritis. Importantly, the increased $ERK_{1/2}$ activation in the PGi–LC–PFC pathway on monoarthritis induction was successfully restored to control levels by diclofenac treatment, indicating that the increased $ERK_{1/2}$ activity in these regions is a consequence of the nociceptive inputs. Based on its increased expression only when anxiety- and depression-like behaviors are present, we propose that sustained altered pERK1/2 expression in the PGi–LC–PFC pathway in chronic monoarthritis is probably more closely related to the development of pain-related affective disorders than with nociception itself. However, further studies in other areas widely involved in facilitating or inhibiting nociception, such as the periaqueductal gray, rostral ventromedial medulla, and dorsal reticular nucleus, will be necessary to confirm this hypothesis.

To study the possible site of action of diclofenac, we evaluated the effect of administering vaseline/diclofenac in the contralateral paw. Behavioral studies showed that diclofenac administered into the contralateral paw modify neither the paw volume nor the hyperalgesia level, but a slight reduction was observed in the ankle-bend score. Nevertheless, when studying the effect of contralateral application of diclofenac on the expression of $pERK_{1/2}$ in the PGi, LC, and PFC, no significant changes were observed with respect to its control group of monoarthritic rats receiving vaseline on the contralateral paw. Hence, these data suggest that topical application has a low systemic effect that would explain the small effect in the ankle-bend test. Indeed, previous data have shown that local administration (cream, gel, or dermal patch) produces a very low systemic effect when compared with that in oral administration.[43–45] However and of relevance for our study, $pERK_{1/2}$ expression is not modified in any of the brain areas studied; so, it seems unlikely that a central effect produced may be involved in all the pain-related features restored by diclofenac treatment. Overall data suggest that diclofenac might be peripherally acting and that the blockade of the nociceptive inputs originated in the inflamed paw is able to restore $pERK_{1/2}$ expression levels in the brain areas studied. However, it is important to note that further studies will be necessary to discern the peripheral and/or central effect of diclofenac in the reversal of all the monoarthritis-related features.

Although the translation of findings from animal models to humans must always be approached with caution, we believe that our findings have interesting parallels in the clinical setting. Thus, we propose that achieving effective pain relief reverses the molecular changes induced by chronic pain. This hypothesis is consistent with the data from patients with osteoarthritis in whom successful arthroplasty reverses thalamic atrophy.[46] Furthermore, the current findings suggest that effective analgesia also benefits other symptoms (anxio-depressive behaviors) that, although not directly related to sensorial pain, have been identified as major contributors to a worse patient outcome. Finally, it is important to note that neuroelasticity (*i.e.*, reversal of an effect on removal of the stimulus) is observed at the onset of anxio-depressive symptoms. It is possible that a critical window exists after which inducing such reversal will be more difficult, or no longer feasible, due to additional changes in neuronal architecture. These findings have important implications for the ongoing debate regarding the optimal therapeutic approaches to treating patients with arthritis (pharmacological *vs.* surgical strategies).

The delayed onset of monoarthritis-induced affective pathologies suggests that peripheral pain inputs induce some reorganization in the central nervous system. Our study demonstrates that affective behavioral changes are accompanied by $ERK_{1/2}$ activation in the PGi–LC–PFC pathway. Moreover, these findings indicate that successful analgesia can reverse sensorial and affective pain-induced changes.

Acknowledgments

The authors thank Raquel Rey-Brea, A.S. (Department of Neuroscience, University of Cádiz, Cádiz, Spain), José Antonio García Partida, B.S. (Department of Neuroscience, University of Cádiz, Cádiz, Spain), Jesus Gallego-Gamo, A.S. (Department of Neuroscience, University of Cádiz, Cádiz, Spain), and Elisa Galvão, A.S. (Departamento de Biologia Experimental, Universidade do Porto, Porto, Portugal), for assistance throughout this study. The authors also thank Joaquin Berrocoso Domínguez, Diploma in Computer Science (Consejería de Economía, Innovación, Ciencia y Empleo de la Junta de Andalucía, Seville, Spain), for the Red Mice software.

The main funding sources of this study are: "Cátedra Externa del Dolor Fundación Grünenthal-Universidad de Cádiz," Cádiz, Spain (to Borges); "Cátedra em Medicina da Dor Fundação Grünenthal-Faculdade de Medicina da Universidade do Porto," Porto, Portugal; "Fondo de Investigación Sanitaria," Madrid, Spain (PI13/02659, PI12/00915); "Centro de Investigación Biomédica en Red de Salud Mental," Madrid, Spain (G18); and "Consejería de Economía, Innovación, Ciencia y Empleo de la Junta de Andalucía," Seville, Spain (CTS-510, CTS-4303 and CTS-7748).

Competing Interests

The authors declare no competing interests.

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References

1. Phillips K, Clauw DJ: Central pain mechanisms in the rheumatic diseases: Future directions. Arthritis Rheum 2013; 65:291–302
2. Price DD: Psychological and neural mechanisms of the affective dimension of pain. Science 2000; 288:1769–72
3. Dersh J, Polatin PB, Gatchel RJ: Chronic pain and psychopathology: Research findings and theoretical considerations. Psychosom Med 2002; 64:773–86
4. Holzberg AD, Robinson ME, Geisser ME, Gremillion HA: The effects of depression and chronic pain on psychosocial and physical functioning. Clin J Pain 1996; 12:118–25
5. Pisetsky DS, St Clair EW: Progress in the treatment of rheumatoid arthritis. JAMA 2001; 286:2787–90
6. Stanos SP: Topical agents for the management of musculoskeletal pain. J Pain Symptom Manage 2007; 33:342–55
7. Itoi K, Sugimoto N: The brainstem noradrenergic systems in stress, anxiety and depression. J Neuroendocrinol 2010; 22:355–61
8. Ennis M, Aston-Jones G, Shiekhattar R: Activation of locus coeruleus neurons by nucleus paragigantocellularis or noxious sensory stimulation is mediated by intracoerulear excitatory amino acid neurotransmission. Brain Res 1992; 598:185–95
9. Cao H, Gao YJ, Ren WH, Li TT, Duan KZ, Cui YH, Cao XH, Zhao ZQ, Ji RR, Zhang YQ: Activation of extracellular signal-regulated kinase in the anterior cingulate cortex contributes to the induction and expression of affective pain. J Neurosci 2009; 29:3307–21
10. Dai RP, Li CQ, Zhang JW, Li F, Shi XD, Zhang JY, Zhou XF: Biphasic activation of extracellular signal-regulated kinase in anterior cingulate cortex distinctly regulates the development of pain-related anxiety and mechanical hypersensitivity in rats after incision. ANESTHESIOLOGY 2011; 115:604–13
11. Rainville P, Duncan GH, Price DD, Carrier B, Bushnell MC: Pain affect encoded in human anterior cingulate but not somatosensory cortex. Science 1997; 277:968–71
12. Wager TD, Rilling JK, Smith EE, Sokolik A, Casey KL, Davidson RJ, Kosslyn SM, Rose RM, Cohen JD: Placebo-induced changes in FMRI in the anticipation and experience of pain. Science 2004; 303:1162–7
13. Wei F, Zhuo M: Activation of Erk in the anterior cingulate cortex during the induction and expression of chronic pain. Mol Pain 2008; 4:28
14. Gao YJ, Ji RR: c-Fos and pERK, which is a better marker for neuronal activation and central sensitization after noxious stimulation and tissue injury? Open Pain J 2009; 2:11–7
15. Kingwell K: Pain: MAPK inhibitor shows promise in clinical trial for neuropathic pain. Nat Rev Neurol 2011; 7:360
16. Ji RR, Kawasaki Y, Zhuang ZY, Wen YR, Zhang YQ: Protein kinases as potential targets for the treatment of pathological pain. Handb Exp Pharmacol 2007:359–89
17. Zimmermann M: Ethical guidelines for investigations of experimental pain in conscious animals. Pain 1983; 16:109–10
18. Butler SH, Godefroy F, Besson JM, Weil-Fugazza J: A limited arthritic model for chronic pain studies in the rat. Pain 1992; 48:73–81
19. Castro-Lopes JM, Tavares I, Tölle TR, Coito A, Coimbra A: Increase in GABAergic cells and GABA levels in the spinal cord in unilateral inflammation of the hindlimb in the rat. Eur J Neurosci 1992; 4:296–301
20. Butler SH, Weil-Fugazza J: The foot-bend procedure as test of nociception for chronic studies in a model of monoarthritis in the rat. Pharmacol Commun 1994; 4:327–34
21. Randall LO, Selitto JJ: A method for measurement of analgesic activity on inflamed tissue. Arch Int Pharmacodyn Ther 1957; 111:409–19
22. Njung'e K, Handley SL: Evaluation of marble-burying behavior as a model of anxiety. Pharmacol Biochem Behav 1991; 38:63–7
23. Detke MJ, Rickels M, Lucki I: Active behaviors in the rat forced swimming test differentially produced by serotonergic and noradrenergic antidepressants. Psychopharmacology (Berl) 1995; 121:66–72
24. Sekiguchi M, Shirasaka M, Konno S, Kikuchi S: Analgesic effect of percutaneously absorbed non-steroidal anti-inflammatory drugs: An experimental study in a rat acute inflammation model. BMC Musculoskelet Disord 2008; 9:15
25. Narita M, Kaneko C, Miyoshi K, Nagumo Y, Kuzumaki N, Nakajima M, Nanjo K, Matsuzawa K, Yamazaki M, Suzuki T: Chronic pain induces anxiety with concomitant changes in opioidergic function in the amygdala. Neuropsychopharmacology 2006; 31:739–50
26. Alba-Delgado C, Llorca-Torralba M, Horrillo I, Ortega JE, Mico JA, Sánchez-Blázquez P, Meana JJ, Berrocoso E: Chronic pain leads to concomitant noradrenergic impairment and mood disorders. Biol Psychiatry 2013; 73:54–62
27. Parent AJ, Beaudet N, Beaudry H, Bergeron J, Bérubé P, Drolet G, Sarret P, Gendron L: Increased anxiety-like behaviors in rats experiencing chronic inflammatory pain. Behav Brain Res 2012; 229:160–7
28. Axford J, Butt A, Heron C, Hammond J, Morgan J, Alavi A, Bolton J, Bland M: Prevalence of anxiety and depression in osteoarthritis: Use of the Hospital Anxiety and Depression Scale as a screening tool. Clin Rheumatol 2010; 29:1277–83
29. Goldenberg DL: The interface of pain and mood disturbances in the rheumatic diseases. Semin Arthritis Rheum 2010; 40:15–31
30. Yalcin I, Bohren Y, Waltisperger E, Sage-Ciocca D, Yin JC, Freund-Mercier MJ, Barrot M: A time-dependent history of mood disorders in a murine model of neuropathic pain. Biol Psychiatry 2011; 70:946–53
31. Imbe H, Senba E, Kimura A, Donishi T, Yokoi I, Kaneoke Y: Activation of mitogen-activated protein kinase in descending pain modulatory system. J Signal Transduct 2011; 2011:468061
32. Ji RR, Gereau RW IV, Malcangio M, Strichartz GR: MAP kinase and pain. Brain Res Rev 2009; 60:135–48
33. Einat H, Yuan P, Gould TD, Li J, Du J, Zhang L, Manji HK, Chen G: The role of the extracellular signal-regulated kinase signaling pathway in mood modulation. J Neurosci 2003; 23:7311–6
34. Qi X, Lin W, Wang D, Pan Y, Wang W, Sun M: A role for the extracellular signal-regulated kinase signal pathway in depressive-like behavior. Behav Brain Res 2009; 199:203–9
35. Zhong XL, Wei R, Zhou P, Luo YW, Wang XQ, Duan J, Bi FF, Zhang JY, Li CQ, Dai RP, Li F: Activation of anterior cingulate cortex extracellular signal-regulated kinase-1 and -2 (ERK1/2) regulates acetic acid-induced, pain-related anxiety in adult female mice. Acta Histochem Cytochem 2012; 45:219–25
36. Cruz CD, Neto FL, Castro-Lopes J, McMahon SB, Cruz F: Inhibition of ERK phosphorylation decreases nociceptive behaviour in monoarthritic rats. Pain 2005; 116:411–9
37. Schadrack J, Neto FL, Ableitner A, Castro-Lopes JM, Willoch F, Bartenstein P, Zieglgänsberger W, Tölle TR: Metabolic activity changes in the rat spinal cord during adjuvant monoarthritis. Neuroscience 1999; 94:595–605
38. Alba-Delgado C, Borges G, Sánchez-Blázquez P, Ortega JE, Horrillo I, Mico JA, Meana JJ, Neto F, Berrocoso E: The function of alpha-2-adrenoceptors in the rat locus coeruleus is preserved in the chronic constriction injury model of neuropathic pain. Psychopharmacology (Berl) 2012; 221:53–65

39. Borges GS, Berrocoso E, Ortega-Alvaro A, Mico JA, Neto FL: Extracellular signal-regulated kinase activation in the chronic constriction injury model of neuropathic pain in anaesthetized rats. Eur J Pain 2013; 17:35–45
40. Imbe H, Okamoto K, Donishi T, Kawai S, Enoki K, Senba E, Kimura A: Activation of ERK in the locus coeruleus following acute noxious stimulation. Brain Res 2009; 1263:50–7
41. Dawane JS, Pandit VA, Rajopadhye BD: Experimental evaluation of anti-inflammatory effect of topical application of entada phaseoloides seeds as paste and ointment. N Am J Med Sci 2011; 3:513–7
42. Dong XD, Svensson P, Cairns BE: The analgesic action of topical diclofenac may be mediated through peripheral NMDA receptor antagonism. Pain 2009; 147:36–45
43. McCarberg BH, Argoff CE: Topical diclofenac epolamine patch 1.3% for treatment of acute pain caused by soft tissue injury. Int J Clin Pract 2010; 64:1546–53
44. Lionberger DR, Brennan MJ: Topical nonsteroidal anti-inflammatory drugs for the treatment of pain due to soft tissue injury: Diclofenac epolamine topical patch. J Pain Res 2010; 3:223–33
45. Tse S, Powell KD, Maclennan SJ, Moorman AR, Paterson C, Bell RR: Skin permeability and pharmacokinetics of diclofenac epolamine administered by dermal patch in Yorkshire-Landrace pigs. J Pain Res 2012; 5:401–8
46. Gwilym SE, Filippini N, Douaud G, Carr AJ, Tracey I: Thalamic atrophy associated with painful osteoarthritis of the hip is reversible after arthroplasty: A longitudinal voxel-based morphometric study. Arthritis Rheum 2010; 62:2930–40

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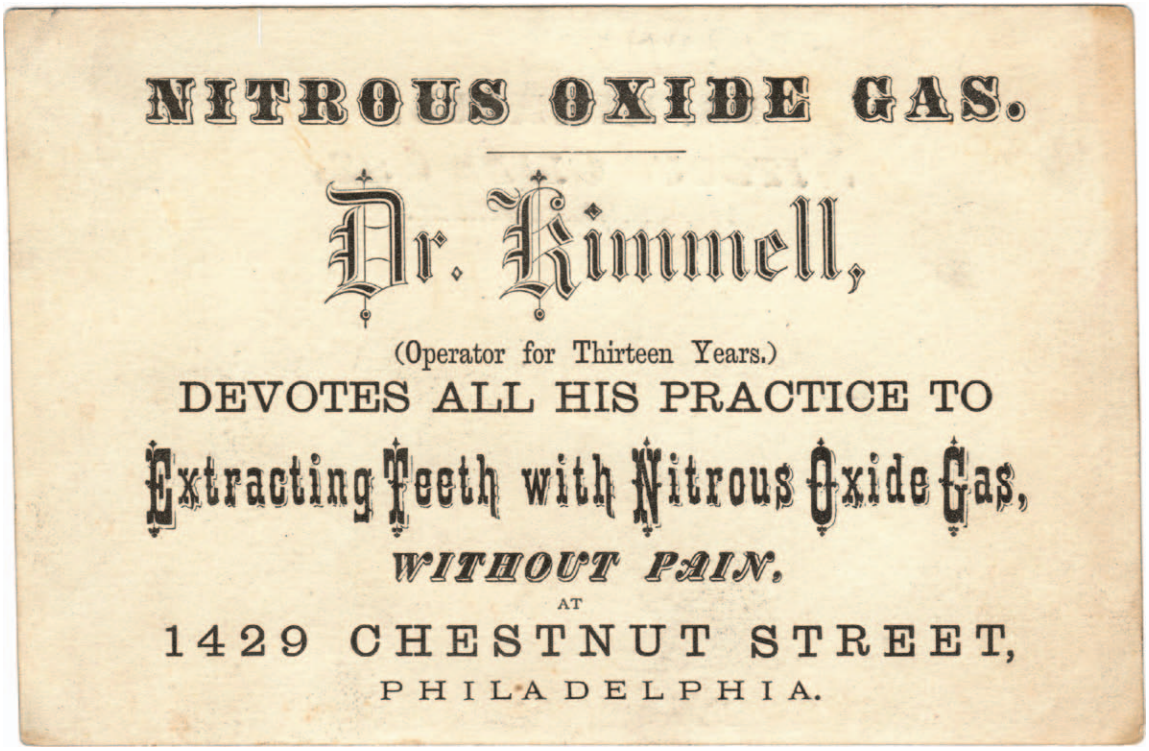
Kimmell's Practice Devoted to Extracting Teeth Using Nitrous Oxide



Unlike the obverse (see Anesthesiology Reflections, this issue, p. 1369), the reverse of dentist Samuel Kimmell's trade card made no mention of the Centennial Exposition of 1876. However, Dr. Kimmell advertised that he devoted "ALL HIS PRACTICE TO Extracting Teeth with Nitrous Oxide Gas, Without Pain…." Because he also noted that he had worked as an "Operator for Thirteen Years," Dr. Kimmell may have begun using nitrous oxide fairly shortly after Gardner Q. Colton revived dental use of nitrous oxide anesthesia in 1863. This trade card is part of the Wood Library-Museum's Ben Z. Swanson Collection. (Copyright © the American Society of Anesthesiologists, Inc.)

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