International Journal of Neuropsychopharmacology (2017) 20(6): 463–475

doi:10.1093/ijnp/pyx005
Advance Access Publication: February 1, 2017
Regular Research Article

REGULAR RESEARCH ARTICLE

Activation of Extracellular Signal-Regulated Kinases (ERK 1/2) in the Locus Coeruleus Contributes to Pain-Related Anxiety in Arthritic Male Rats

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Abstract

Background: There is increasing evidence suggesting that the Locus Coeruleus plays a role in pain-related anxiety. Indeed, we previously found that prolonged arthritis produces anxiety-like behavior in rats, along with enhanced expression of phosphorylated extracellular signal-regulated kinase 1/2 (a marker of plasticity) in the Locus Coeruleus. However, it is unknown how this effect correlates with the electrophysiological activity of Locus Coeruleus neurons or pain-related anxiety.
Methods: Using the complete Freund's adjuvant model of monoarthritis in male Sprague-Dawley rats, we studied the behavioral attributes of pain and anxiety as well as Locus Coeruleus electrophysiology in vivo 1 (MA1W) and 4 weeks (MA4W) after disease induction.
Results: The manifestation of anxiety in MA4W was accompanied by dampened tonic Locus Coeruleus activity, which was coupled to an exacerbated evoked Locus Coeruleus response to noxious stimulation of the inflamed and healthy paw. When a mitogen-activating extracellular kinase inhibitor was administered to the contralateral Locus Coeruleus of MA4W, the phosphorylated extracellular signal-regulated kinase 1/2 levels in the Locus Coeruleus were restored and the exaggerated evoked response was blocked, reversing the anxiogenic-like behavior while pain hypersensitivity remained unaltered.

Received: October 27, 2016; **Revised:** December 24, 2016; **Accepted:** January 13, 2017

Conclusion: As phosphorylated extracellular signal-regulated kinase 1/2 blockade in the Locus Coeruleus relieved anxiety and counteracted altered LC function, we propose that phosphorylated extracellular signal-regulated kinase 1/2 activation in the Locus Coeruleus plays a crucial role in pain-related anxiety.

Keywords: Locus Coeruleus, anxiety, pain, arthritis, ERK1/2.

Significance Statement

This work provides strong evidence of the prominence of the Locus Coeruleus (LC)-noradrenergic system in the development of anxiety in chronic pain, a widely known occurrence in clinical practice. We show that this phenomenon is accompanied by severe changes in the activity of the LC neurons. Indeed, we propose a molecular pathway that is crucial for the development of anxiety in such conditions, involving the activation of extracellular signal-regulated kinase 1/2 (pERK1/2). The manipulation of LC activity by blocking the overexpression of pERK1/2 produced a clear reversion of the anxiety behavior produced by prolonged exposure to a painful condition, without interfering with the pain threshold. This outcome supports the dissociation of the mechanisms underlying the sensorial-affective component of pain.

Introduction

Pain is a disease with repercussions that reach far beyond hypersensitivity. For example, chronic pain is associated with an alarming risk of suffering emotional disturbances, such as those characterized by persistent anxiety, establishing a vicious circle that magnifies the painful experience (Price, 2000; Sharp and Keefe, 2005; Edwards et al., 2011). In the case of painful arthritis, it is estimated that more than 30% of the arthritic population in the US suffers anxiety (Murphy et al., 2012). However, while this comorbidity is clinically well established, the underlying mechanisms remain poorly studied. We previously reported that prolonged experimental arthritis produces anxiety-like behavior in rats (Borges et al., 2014), as also observed elsewhere (Narita et al., 2006; Amorim et al., 2014). This provides us with a reliable experimental tool to assess the mechanisms underlying the development of anxiety in association with arthritic pain. By studying phosphorylated isoforms of extracellular signal-regulated kinase 1/2 (pERK1/2) as a marker of plasticity, we found that long-term arthritis enhances pERK1/2 expression in the Locus Coeruleus (LC) and its projection area, the prefrontal cortex (Borges et al., 2014), strongly suggesting that this pathway is implicated in the effects of arthritis.

The noradrenergic-LC system is a bilateral pontine nucleus that modulates pain perception through an intricate network of projections. It is widely known that acute noxious or inflammatory stimuli activate LC descending innervations to the spinal cord to promote feedback inhibition of pain (Kwiat and Basbaum, 1992; Ossipov et al., 2010; Kwon et al., 2013). However, in more complex pain conditions, where anxiety and other features also manifest, there is little information available regarding the regulation of the LC. This is particularly true from an electrophysiological perspective and with regards to possible repercussions for its ascending projections to the prefrontal areas where the emotional consequences of chronic pain might be modulated (Berridge and Waterhouse, 2003; Sara, 2009) (Figure 1A). Indeed, it is known that LC function is compromised in stressful and anxiogenic conditions (Bravo et al., 2013; George et al., 2013; McCall et al., 2015) and that experimental arthritis engages a stress component involving the corticotropin-releasing factor (CRF) (Hummel et al., 2010; Borges et al., 2015). Therefore, it is possible that the LC may modulate the sensorial and affective features of chronic pain in different ways (Alba-Delgado et al., 2013; Bravo et al., 2013; Borges et al., 2014).

The aim of the present work was to study the role and activity of LC neurons in pain-related anxiety. Furthermore, as ERK1/2 activity has been implicated in long-lasting plasticity in several areas involved in pain processing (e.g., the spinal cord and anterior cingulate cortex) (Ji et al., 2009; Zhong et al., 2012), we also set out to investigate the implication of the ERK1/2 cascade on the electrophysiological activity of the LC in painful states. As such, we have used monoarthritis (MA) as a model of inflammatory pain, studying both the early (anxiety-free) and late (anxiety associated) phase of the disease. In this system, we evaluated the characteristics of LC discharge at baseline and following noxious stimulation, and we also studied the behavioral and electrophysiological effects of pharmacologically inhibiting ERK1/2 activation at the LC level.

Methods

Animals and Experimental Design

Adult male Harlan Sprague-Dawley rats (200–300 g, 2–4/cage: University of Cádiz ES110120000210) were housed under controlled conditions: food and water supplied ad libitum; a 12-hour-light/12-hour-dark cycle; constant temperature (22°C) and humidity (45–60%). All experiments were carried out in accordance with the European Communities Council Directive of September 22, 2010 (2010/63/EC), Spanish Law (RD 53/2013), and the ethical guidelines governing the investigation of experimental pain in animals (Zimmermann, 1983). All the experimental protocols were approved by The Animal Research Ethics Committee at the Faculty of Medicine of the University of Cadiz.

Behavior (Cohort 1) and electrophysiological LC activity (Cohort 2) were evaluated in control rats, and in rats 1 (MA1W) or 4 weeks (MA4W) after inducing MA. The effect of intra-LC administration of SL327 on LC electrophysiological activity and behavior was evaluated in control and MA4W rats (Cohort 3 and 4, respectively). In addition, immunohistochemistry was performed on parallel groups (Cohort 5; Figure 1B).

MA Induction and Behavioral Assessment

MA was induced under isoflurane anesthesia (4% to induce anesthesia and 2% to maintain it: Abbott, Madrid, Spain) by injecting a complete Freund's adjuvant solution (CFA, 50 µL) into the left

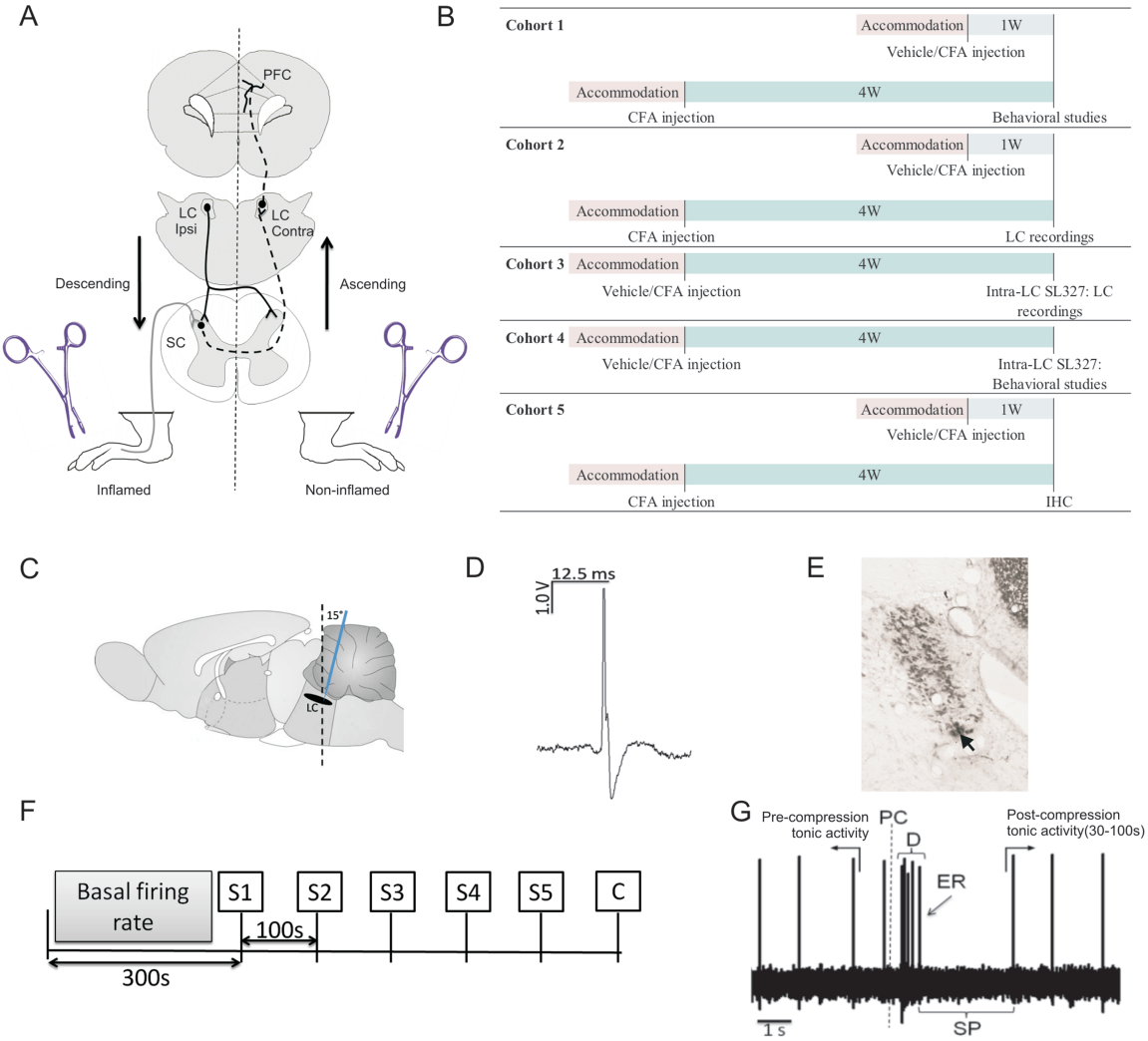

Figure 1. Summary of the methodological approach. (A) Proposed pathway involved in pain transmission from the paw, passing through the spinal cord (SC), the Locus Coeruleus (LC), and reaching the prefrontal cortex (PFC: ascending dashed lines) to trigger the descending LC projections to the SC (descending solid line). (B) Representation of the experimental design. (C) Representation of the recording electrode's position. (D) Typical LC spike shape. (E) Pontamine spot certifying the correct position of the recording electrode. (F) Electrophysiological protocol used for mechanical stimulation of the paw. (G) Oscillography trace representing the evoked-response (ER) of paw compression (PC), duration (D) of the response, suppression period (SP) and postcompression tonic activity of a LC neuron. C, contralateral; CFA, complete Freund's adjuvant; Contra, contralateral; IHC, immunohistochemistry; Ipsi, ipsilateral; S, stimulation; W, week.

tibiotarsal joint (ipsilateral) as described previously (Butler et al., 1992; Borges et al., 2014). The CFA-injected rats were studied 1 and 4 weeks after the induction of MA, and the animals that developed polyarthritis were excluded from the study. Control rats were injected with the vehicle solution and were studied 1 or 4 weeks later in function of the experiment (Figure 1B).

The paw pressure test was used to evaluate secondary mechanical hypersensitivity (Randall and Selitto, 1957). Briefly, increasing pressure was gradually applied to the dorsal side of the paw using a graded motor-driven device (Ugo Basile, Varese, Italy), beginning at 30 g of pressure. A reduction in the pressure that provoked withdrawal indicates hyperalgesic-like hypersensitivity. Two measurements were taken in both the ipsi- and contralateral paws.

Anxiety-like behavior was evaluated using the elevated zero maze (EZM; Borges et al., 2014), which consisted of a black circular platform divided into 4, equal-sized quadrants: 2 opposing open quadrants with 1-cm high clear borders to prevent falls; and 2 opposing closed quadrants with black walls. A 5-minute trial was carried out under the same lighting conditions, which began by placing the animal in the center of one of the closed quadrants. Spontaneous Motor Activity Recording and Tracking (Panlab S.L., Barcelona, Spain) software was used to analyze the time the rat spent in the open arms and the total distance each rat traveled. A decrease in the time spent in the open area is related to anxiety-like behavior.

LC Electrophysiology

Details of the electrophysiological procedures can be found in the Supplementary Information. As the ascending pain pathway is mainly contralateral (Pertovaara, 2006), single-unit extracellular recordings of LC neurons were obtained from the brain hemisphere contralateral to the site of injection (Berrocoso and Mico, 2007; Alba-Delgado et al., 2013) (Figure 1A). When a single LC unit was isolated, the spontaneous basal discharge (baseline) was recorded for 2 to 5 minutes, and the following parameters were calculated: firing rate (Hz), the incidence of burst activity, and the number of spikes per burst. A LC cell was considered to exhibit burst firing when it displayed at least 2 spikes with

an initial interspike interval <80 milliseconds and with subsequent interspike intervals of at least 160 milliseconds (Grace and Bunney, 1984). The sensory-evoked response of LC neurons was studied after stable basal spontaneous firing by manually applying mechanical pressure for 1 second to the ipsilateral or contralateral paw (paw compression [PC]) using surgical forceps (Figure 1E). This sensory-evoked discharge was followed by a suppression period (SP) (Grant and Weiss, 2001; West et al., 2009; Bravo et al., 2013; Torres-Sanchez et al., 2013), although importantly, repeated trials using this type of PC produces neither sensitization nor habituation in naive animals (West et al., 2010). For each neuron recorded, PC was applied 5 times with a 100-second interval, quantifying the sensory evoked-response, the duration of the response, and the SP after PC. Finally, a similar PC was applied to the contralateral paw (Figure 1F). To prevent paw damage, no more than 4 neurons were studied per rat. The postcompression tonic activity was measured over an interval of 70 seconds, starting 30 seconds after each PC. Changes in the postcompression discharge were expressed relative to the baseline for each neuron and averaged across recordings. At the end of the experiment, a Pontamine Sky Blue mark was deposited by applying a 5-μA cathodic current through the recording electrode and subsequently, the site of recording was verified histologically (Figure 1G). Only measurements from cells within the LC area were included in this study.

For the electrophysiological experiments to assess the effect of SL327, a mitogen-activating extracellular kinase (MEK) inhibitor that has a negative impact on ERK1/2 phosphorylation (S4069: Sigma-Aldrich, Madrid, Spain) was used in another set of rats. In these experiments, a calibrated pipette was glued adjacent to a recording micropipette (Ruiz-Ortega and Ugedo, 1997), and it contained SL327 dissolved in the vehicle solution of 50% DMSO and 50% of Dulbecco's buffered saline containing: NaCl, 136.9 mM; KCl, 2.7 mM; NaH_2PO_4, 8.1 mM; KH_2PO_4, 1.5 mM; $MgCl_2$, 0.5 mM; and $CaCl_2$, 0.9 (pH 7.40). Pressure pulses (50–150 milliseconds) were applied with a Picospritzer II (General Valve Corporation, Fairfield, NJ) to inject the compound. The volume injected was measured by monitoring the movement of the meniscus in the pipette, having previously calibrated the device to deliver 2 nL of the solution with each pulse. This procedure was performed during the recording of the neuron. Injecting the vehicle solution alone did not affect LC electrical activity in control or MA rats.

Intra-LC Drug Administration

Five days before completing the 4 weeks of MA, rats were anaesthetized with an i.p. injection of ketamine (100 mg/kg) and xylazine (20 mg/kg), and they were placed in a stereotaxic frame with their head tilted at an angle of 15° to the horizontal plane (nose down). A guide cannula (22 gauge, 15-mm length) was implanted in the contralateral (right side) LC at the same coordinates relative to lambda as those used in the electrophysiological studies (Paxinos and Watson, 2009). After positioning, the guide cannula was fixed to the skull and anchored to stainless screws with polyacrylic cement. Microinjection was performed by lowering into the brain a removable injector 1 mm longer than the guide tip, which was connected to a 10-μL Hamilton syringe (Bravo et al., 2013). Animals received a 0.5-μL injection administering 4.17 mM of SL327 in DMSO as the vehicle. The behavioral tests (paw pressure and EZM tests) were performed within 10 to 25 minutes of drug administration. Random animals from each group were selected for histological confirmation of the localization of the implanted cannula, and any animal in which it was misplaced was excluded.

Immunohistochemistry

Control, MA1W, and MA4W rats were anaesthetized with 8% chloral hydrate (400 mg/kg), and they were immediately perfused through the ascending aorta with 250 mL of oxygenated Tyrode's solution followed by 750 mL of 4% paraformaldehyde in phosphate buffer (0.1 M, pH 7.2). The rat's brain was removed and processed for free-floating immunohistochemistry as described previously (Nascimento et al., 2011; Borges et al., 2013). Transverse sections of the LC region were washed, blocked (Cruz et al., 2005), and then incubated with antibodies against tyrosine hydroxylase (TH, raised in sheep, 1:2000; 48 hours, Abcam), the phosphorylated ERK1/2 isoforms (raised in rabbit, pERK1/2, 1:500, 48 hours; Acris Antibodies, Herford, Germany), or both to confirm the nature and localization of pERK1/2 positive cells in the LC, as described elsewhere (Borges et al., 2013). Antibody binding to pERK1/2 was detected with a biotinylated donkey anti-rabbit secondary antibody (1:200, 1 hour; Jackson Immunoresearch Laboratories), and it was visualized with an ABC kit (1:200, 1 hour; ABC Elite kit, Vector Laboratories, Peterborough, UK) and a 3,3'-diaminobenzidine solution (10 minutes). In the case of TH, an alexa 568 conjugated donkey anti-sheep antibody was used (1:1000, 1 hour; Invitrogen, Life Technologies S.A., Madrid, Spain). For dual pERK1/2 and TH visualization, the secondary antibodies used were a biotinylated donkey anti-rabbit (1:200, 1 hour) followed by incubation with Streptavidin conjugated alexa 488 (1:200, 2 hours; Invitrogen), and an alexa 568 conjugated donkey anti-sheep antibody (1:500, 2 hours).

TH positive cells were quantified by counting the number of labeled cell bodies per animal in at least 6 sections containing the LC region (caudal or the rostral portions) under an Olympus BX60 microscope coupled to a digital camera (Olympus DP71) and image software (Cell F 2.4). The average number of immunoreactive (IR) cells/section/side was used for statistical purposes. The expression of pERK1/2 in the LC was quantified by densitometry, as described previously (Borges et al., 2013).

Western Blotting

Detailed information can be found in the supplementary Information.

Statistical Analysis

All the data are presented as the means ± SEM, and the results were all analyzed using STATISTICA 10.0 (StatSoft) or GraphPad Prism 5 software (GraphPad Software), applying either a Student's *t* test (unpaired or paired, 2-tailed), or a 1-way or 2-way ANOVA, followed by the appropriate posthoc tests (Bonferroni or Dunnett's tests). The independent variables were MA (between groups), paw, or drug treatment (between groups) (supplementary Table 1). A Fisher's exact test was used to evaluate the incidence of burst activity. Significance was accepted at $P < .05$.

Results

Nociceptive and Anxiety-Like Behavior

The paw withdrawal threshold was evaluated in the ipsilateral (inflamed) paw of MA rats, and it was significantly lower than that of control rats ($P < .001$ for MA1W and MA4W), indicative of mechanical hypersensitivity (Figure 2A). By contrast, no differences were observed when the withdrawal threshold was evaluated in the contralateral paw. In addition, MA4W rats spent

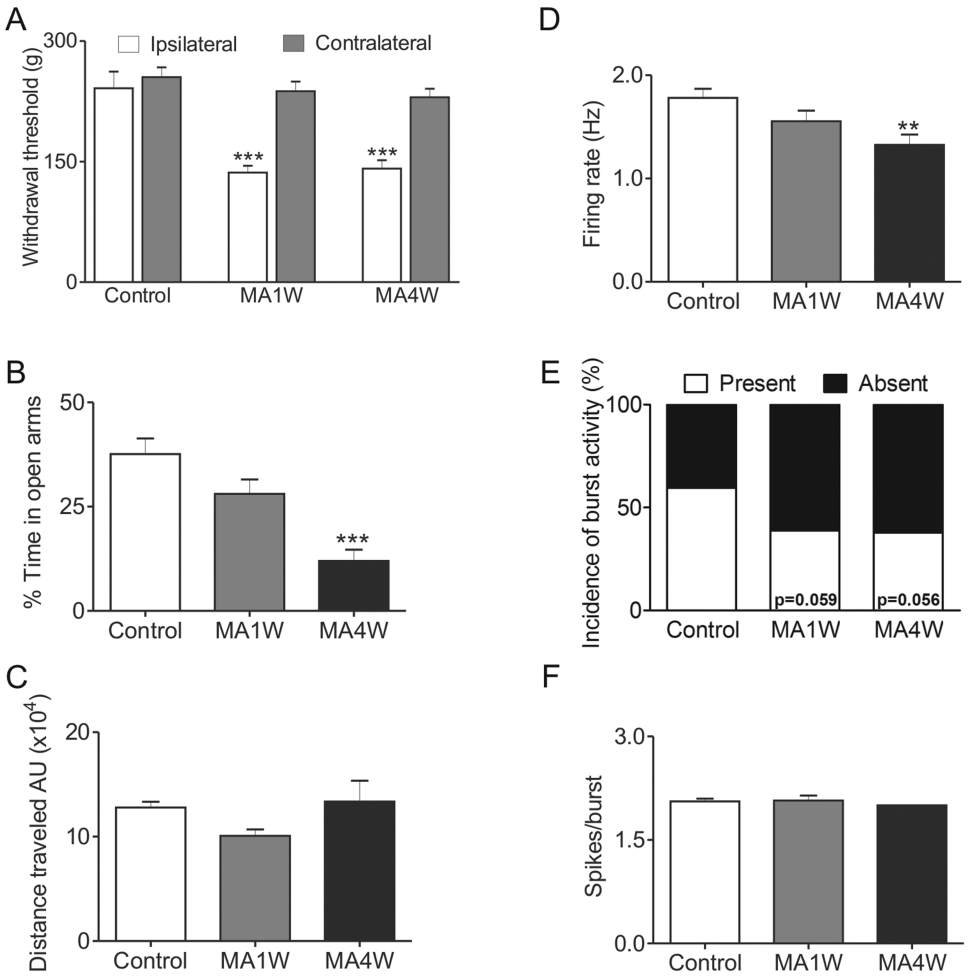

Figure 2. Nociception, anxiety, and basal/spontaneous electrophysiological Locus Coeruleus (LC) properties in control and 1- and 4-week MA rats (MA1W and MA4W). (A) Mechanical hypersensitivity was confirmed by a lower withdrawal threshold of the ipsilateral paw of MA rats: 2-way ANOVA and Bonferroni posthoc, control vs MA1W/MA4W for each paw (n = 6). (B) The anxiety-like behavior was reflected by a significant decrease in the time spent in the open arms of the elevated zero maze (EZM): 1-way ANOVA and Dunnett's posthoc, control vs MA4W (n = 6/7). (C) The total distance traveled in the EZM was unaffected: 1-way ANOVA and Dunnett's posthoc (n = 6/7). (D) The spontaneous firing discharge of LC neurons diminished significantly in MA4W rats: 1-way ANOVA and Dunnett's posthoc, control vs MA4W. (E) The incidence of spontaneous burst activity in MA rats tended to decrease: Fisher's exact test. (F) The number of spikes/burst was maintained. AU, arbitrary units. **$P < .01$ and *** $P < .001$.

significantly less time in the open arms of the EZM than control rats, indicative of anxiety-like behavior ($P < .001$), although no such changes were observed in MA1W rats (Figure 2B). Notably, no significant changes were observed in the total distance traveled in the EZM, indicating that locomotor activity was not impaired in the MA rats (Figure 2C).

Spontaneous Activity of Contralateral LC Neurons

Data from 57, 36, and 37 neurons were recorded from 19 control, 11 MA1W, and 17 MA4W rats, respectively, and to maintain the same experimental and recording conditions, whenever possible at least one control and one MA rat (MA1W or MA4W) were evaluated on the same day. The basal spontaneous LC activity was significantly lower in MA4W rats than in the controls ($P < .01$: Figure 2D), although no significant differences were observed when the MA1W rats were compared with the controls. While the burst activity tended to be weaker in the MA1W and MA4W rats ($P = .059$ and $P = .056$ vs control, respectively: Figure 2E), the number of spikes per burst did not differ among the 3 experimental groups (Figure 2F).

Activity of LC Neurons Evoked by Ipsilateral PC

When the electrophysiological profile of the sensory-evoked response was assessed during ipsilateral PC (Figure 1F-G), the basal firing rate in each experimental group followed the same pattern as that described in Figure 2D (data not shown). The application of 5 sequential stimulations did not significantly alter any parameters of the sensory responses studied, irrespective of the treatment (S1 vs S5 for control, MA1W and MA4W, $P > .05$). However, the activity evoked by the last stimulation in MA4W rats was significantly stronger than that in the control and MA1W rats ($P < .05$ vs control) (Figure 3A). This reinforcement was particularly evident when the averaged data of all 5 stimulations was analyzed, reflecting the significant increase in this parameter in MA4W rats ($P < .001$ vs control) (Figure 3A, right bar graph). Indeed, this analysis also revealed a significantly weaker evoked response in MA1W rats than in the control rats ($P < .05$) (Figure 3A, right bar graph). Furthermore, the response also lasted longer in MA4W, particularly following stimulations S3 ($P < .01$), S4 ($P < .05$), and S5 ($P < .001$ vs control) (Figure 3B). Likewise, a significant increase in duration was observed in

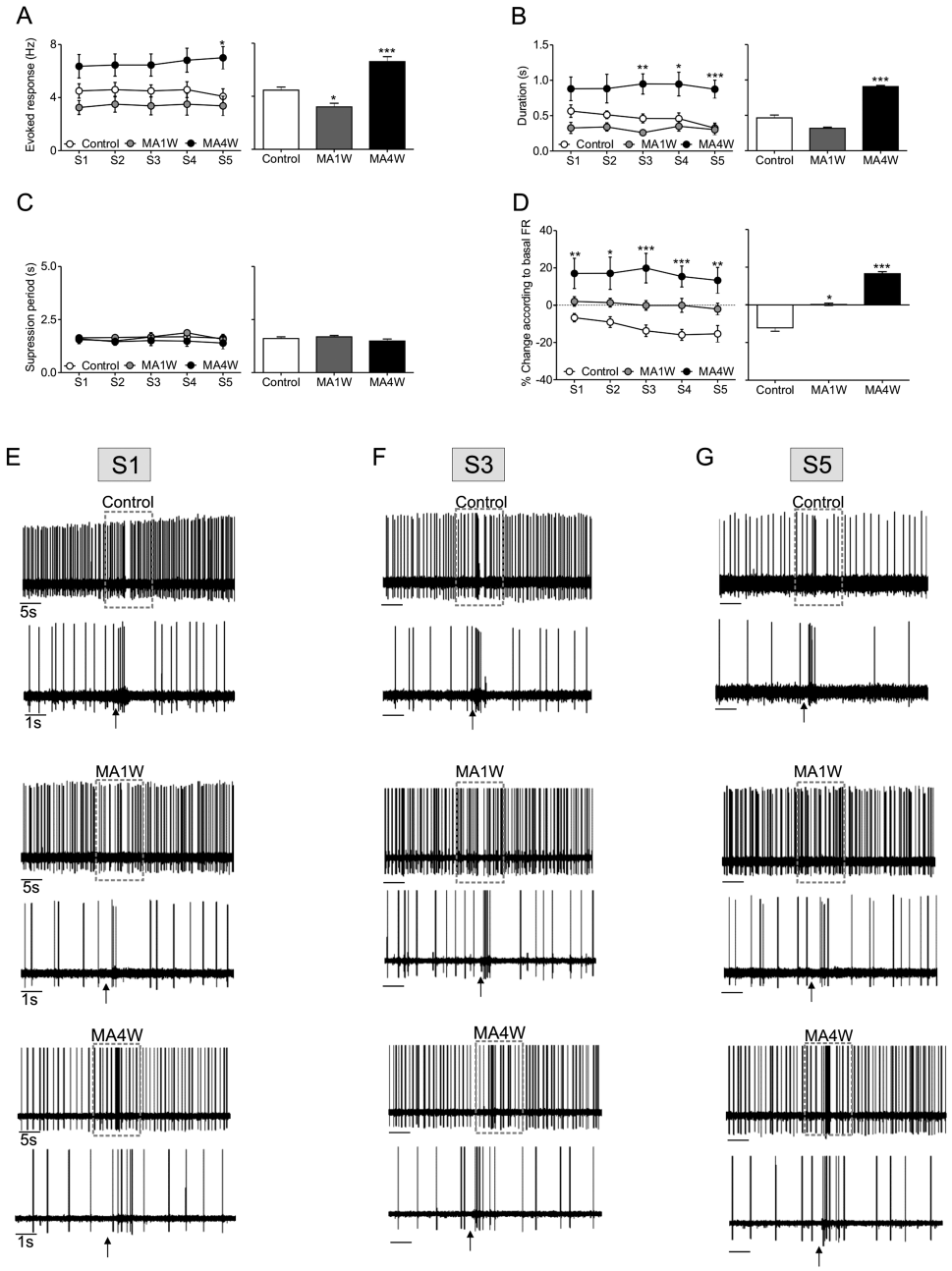

Figure 3. Effect of sequential ipsilateral paw compressions (PCs) on the electrophysiological response of Locus Coeruleus (LC) neurons in control and 1- and 4-week MA rats (MA1W and MA4W). (A-D) Graphs depicting the effect of sequential ipsilateral PC on the phasic activity of LC neurons (n = 10, 8 and 11 neurons in control, MA1W and MA4W, respectively) and the overall data for all stimulations. (A) The evoked-response decreased in MA1W and increased in MA4W rats: 1-way ANOVA and Dunnett's posthoc, MA1W/MA4W vs control. (B) The duration of the response increased in MA4W rats: 1-way ANOVA and Dunnett's posthoc, MA4W vs control. (C) The SP did not change: 1-way ANOVA and Dunnett's posthoc. (D) The postcompression tonic activity was altered in MA1W and MA4W rats: 1-way ANOVA and Dunnett's posthoc, MA1W/MA4W vs control. (E-G) Oscillography traces representing the LC responses to ipsilateral stimulation S1 (E), S3 (F), and S5 (G) in control, MA1W, and MA4W rats. FR, firing rate; S, stimulation. *$P<.05$, **$P<.01$, and ***$P<.001$.

MA4W rats when the averaged data of all stimulations was compared with the responses of the control ($P<.001$) (Figure 3B, right bar graph). No significant differences were observed regarding the duration of the evoked response in MA1W rats, and no significant differences were observed in the suppression period between each of the experimental groups (Figure 3C).

We also explored the tonic LC rate after each noxious stimulus, expressed relative to the basal firing rate (postcompression tonic activity) (Figure 1E). In the control group, a mild decrease (12.2%) in tonic activity was observed during the application of mechanical stimulation, while the MA1W rats hardly changed their spontaneous firing pattern in response to sequential stimulation. By contrast, sequential PCs in MA4W rats induced an increase in tonic activity of approximately 16.5%, significantly higher than in the MA4W and control rats following each PC ($P<.05$ for S2; $P<.01$ for S1 and S5; $P<.001$ for S3 and S4) (Figure 3D). Moreover, there was a significant increase in the averaged postcompression tonic activity in MA1W and MA4W rats compared with the control ($P<.05$ and $P<.001$, respectively) (Figure 3D, bar graph on the right). The LC response following PC applied

to the ipsilateral paw in control, MA1W, and MA4W rats to S1 (Figure 3E), S3 (Figure 3F), and S5 (Figure 3G) is also shown in representative histograms.

Activity of LC Neurons Evoked by Contralateral PC

After 5 successive ipsilateral PCs, one compression was applied to the noninflamed contralateral paw (Figure 1F), which failed to evoke a response from the LC neurons of control rats (Figure 4A). However, when the same procedure was applied to monoarthritic rats after 1 or 4 weeks of inflammation, a sensory-evoked response was observed that was particularly evident in MA4W rats (Figure 4A). Indeed, burst activity appeared to be stronger in MA1W rats than in control rats ($P = .052$), and in MA4W rats it was significantly higher than in the control ($P < .05$) (Figure 4A). No significant differences were detected between the MA1W and MA4W rats (see representative histograms of the LC response to PC applied to the contralateral paw in control, MA1W, and MA4W rats) (Figure 4B).

TH and pERK1/2 Expression in the LC

Since increased use of noradrenaline is accompanied by upregulation of TH, the rate-limiting enzyme for noradrenaline synthesis (Smith et al., 1991), we assessed TH expression in the LC. As expected, TH was detected in the cytoplasm and dendrites of noradrenergic neurons. Moreover, at the caudal level of the LC there were significantly more neurons expressing TH in MA4W rats than in control animals ($P < .01$ and $P < .05$ for the ipsi- and contralateral sides, respectively) (Figure 5A,C). Similar results were obtained when considering the entire rostro-caudal extension (total portion, $P < .05$ for ipsi- and contralateral sides), although no differences were observed in the rostral domain of MA4W rats or when comparing the ipsi- and contralateral sides (Figure 5A). TH labeling may be so dense that it may become difficult to count the number of TH IR cells (Figure 5C), although this problem can be overcome by also determining the amount of TH in the LC in western blots, which confirmed the increased TH expression in the LC of MA4W animals (Figure 5D,Da).

Similar results were obtained when pERK1/2 was evaluated in the LC, which was detected in the cytoplasm, nucleus, and dendrites of neurons, as well as in a large number of fibers surrounding the LC neurons (Borges et al., 2013). Again, no differences were observed between the ipsi- and contralateral LC. The density of pERK1/2 labeling in MA4W rats increased significantly in the caudal and rostral domain of the LC compared with control rats (ipsilateral, $P < .001$ caudal or $P < .01$ rostral; contralateral, $P < .001$: Figure 5B,E) as well as along the entire rostro-caudal extension (ipsilateral, $P < .001$; contralateral, $P < .001$) (Figure 5B). These data suggest that prolonged inflammatory pain produces stronger activation of the ERK cascade in the LC (MA4W). Moreover, a slight increase in pERK1/2 expression was detected in the contralateral LC of MA1W rats when the entire rostro-caudal extension was considered ($P < .05$) (Figure 5B). It appears that most of the pERK1/2 is found in noradrenergic neurons in the LC (Figure 5F).

Effect of Intra-LC SL327 Microinjection on the pERK1/2 Expression

To find an appropriate concentration of SL327 to use in the behavioral and electrophysiological experiments (the MEK inhibitor that affects ERK1/2 phosphorylation; Papadeas et al., 2008), 2 doses were evaluated based on the literature: 4.17 mM and 8.35 mM. In a pilot study to evaluate the inhibition of pERK1/2 in MA4W upon microinjection of SL327, both 4.17 mM and 8.35 mM produced appropriate inhibition when the accumulation of pERK1/2 was assessed in MA4W rats that received SL327 or the vehicle solution alone ($P < .001$) (Figure 6A). Thus, the lower dose of 4.17 mM was used in all the following studies.

Electrophysiological Effect of Intra-LC SL327 Microinjection

As the group of MA4W rats presented the more prominent changes in spontaneous LC activity and pERK1/2 accumulation, the effect of SL327 on the LC discharge evoked by ipsilateral PC was studied in control and MA4W rats. After noxious stimulations S1, S2, S3, and S4, a dose of SL327 (8.35–33.39 pmol) was administered to the LC and the evoked response was measured (Figure 6B). SL327 administration did not significantly modify the responses provoked by the stimulation of the LC in control animals (evoked response, duration, suppression period, and postcompression tonic activity: S1 vs S2-S5, $P > .05$: Figure 6C-F), yet SL327 produced a dose-dependent decrease in the evoked discharge after each noxious stimulus in MA4W rats, as was particularly evident at S5 (S1 vs S2-S4, $P > .05$; S1 vs S5, $P < .01$) (Figure 6C). Moreover, we found that the discharge evoked in MA4W and control rats receiving SL327 differed significantly after S4 and S5 (control vs MA4W for S4 and S5, $P < .05$ and $P < .01$, respectively). However, the duration of the response in control and MA4W rats was not modified by microinjection of SL327 ($P > .05$). Overall, these results suggested that SL327 decreased the duration of the response that had been significantly increased in MA4W rats (Figure 3B). In MA4W rats, SL327 induced a dose-dependent increase in the period of suppression that was statistically different from the basal S1 value following the administration of 33.39 pmol of SL327 (MA4W: S1 vs S5, $P < .01$) (Figure 6E) as well as compared with the response in control animals receiving the same dose (control vs MA4W, $P < .001$) (Figure 6E). Finally, microinjection of SL327 produced a significant decrease in the postcompression tonic activity in MA4W animals relative to the basal S1 value (S1 vs S3-S5, $P < .001$) (Figure 6F) and to the control animals receiving the same dose (control vs MA4W, $P < .05$ for S3,

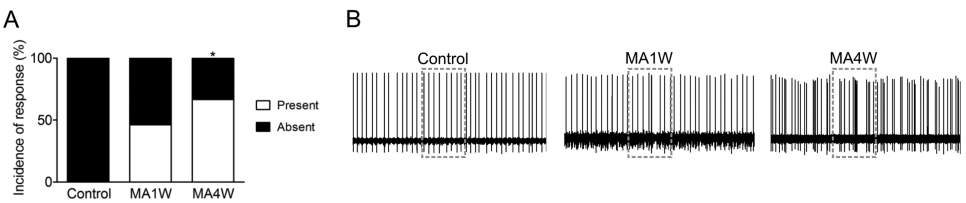

Figure 4. Effect of contralateral stimulation on the incidence of evoked burst activity in control and 1- and 4-week MA rats (MA1W and MA4W). (A) In MAW rats, there is an increase in the incidence of evoked burst activity: *$P < .05$ (Fisher's exact test). (B) Representation of the oscillography traces for the Locus Coeruleus (LC) response to contralateral stimulation.

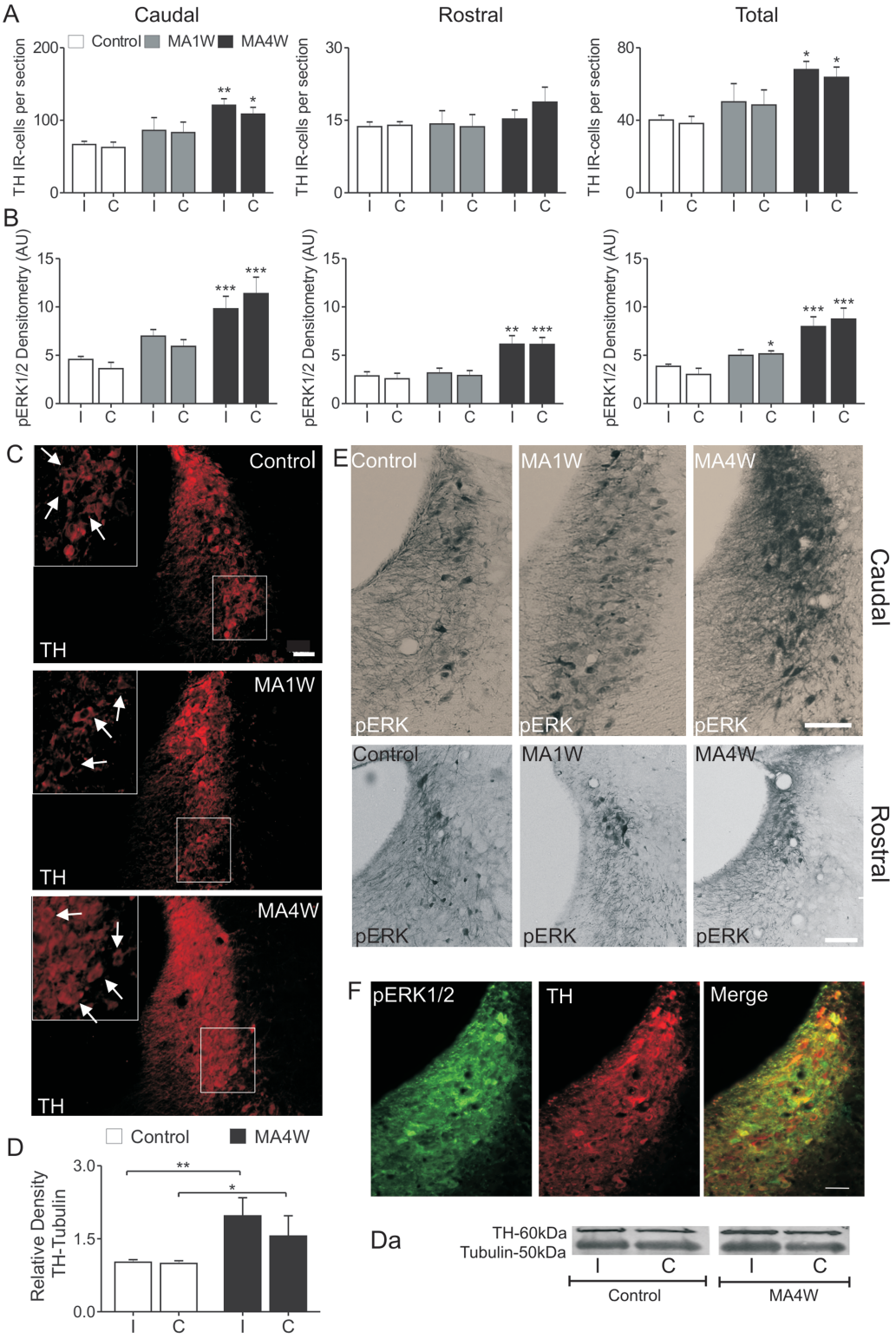

Figure 5. Phosphorylated extracellular signal-regulated kinase 1/2 (pERK1/2) and tyrosine hydroxylase (TH) levels in the Locus Coeruleus (LC) of control and 1- and 4-week MA rats (MA1W and MA4W). (A) There were significantly more TH immunoreactive (IR) cells in the caudal and total LC of MA4W rats: 2-way ANOVA and Bonferroni posthoc, control vs MA4W for each side (n = 5–7). (B) The pERK1/2 mean gray values were significantly higher in the entire rostro-caudal extension of the LC: 2-way ANOVA and Bonferroni posthoc control vs MA1W/MA4W for each side (n = 5–7). (C) Representative photomicrographs showing TH and detailing the immunolabeling in the amplified inset. Scale bar = 100 µm. (D) The increase in TH expression in MA4W rats was also observed in western blots. (Da) Representative regions of the immunoblots showing the bands for TH and Tubulin proteins. (E) Representative photomicrographs showing pERK1/2 in the caudal and rostral domains. Scale bar = 100 µm. (F) Photomicrographs showing the pERK1/2 immunolabeling in TH positive neurons in the LC. Scale bar = 50 µm. AU, arbitrary units; C, contralateral; I, ipsilateral. *P < .05, ** P < .01, and ***P < .001.

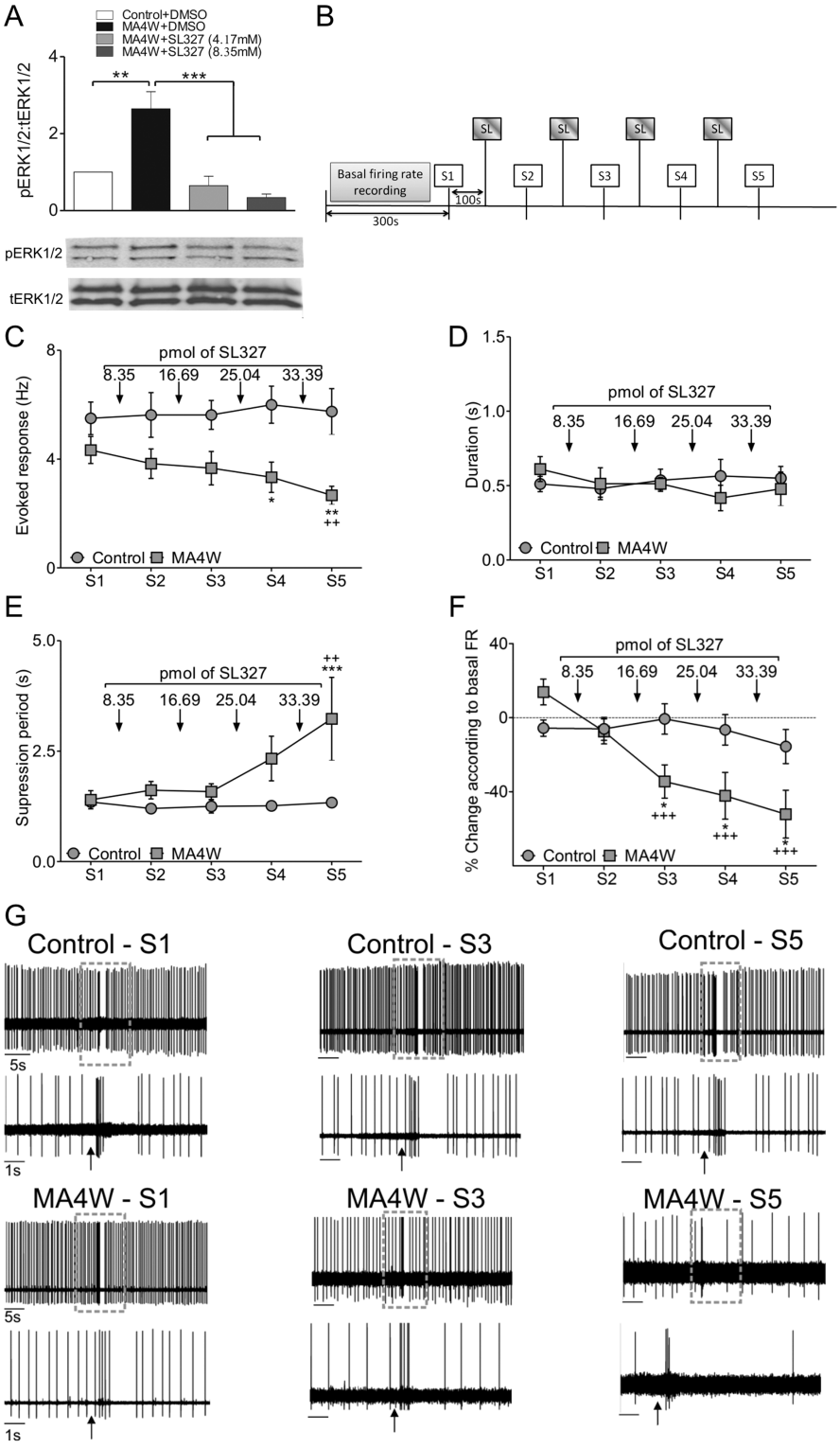

Figure 6. Effect of microinjecting the MEK inhibitor SL327 into the Locus Coeruleus (LC) on the electrophysiological parameters in control and 4-week MA rats (MA4W). (A) Expression of phosphorylated extracellular signal-regulated kinase 1/2 (pERK1/2; 42–44 kDa) in the LC in response to SL327 microinjection (4.17 mM and 8.35 mM) and the corresponding images of western blots (relevant regions of the original blot presented below the graph): 1-way ANOVA and Dunnett's posthoc, control, or MA + SL327 vs MA4W + DMSO (n = 4 assays). (B) Electrophysiological protocol used to study the effects of SL327 in the LC response to mechanical stimulation of the paw. (C) The sensory-evoked response of MA4W rats was significantly weaker: repeated measures and Bonferroni posthoc test comparing control vs MA4W; 1-way ANOVA and Dunnett's posthoc test comparing S2-S5 vs S1 (n = 6–8 neurons/experimental group). (D) No significant effects were observed on the duration of the response. (E) The SP of MA4W rats increased significantly following SL327 microinjection: repeated measures and Bonferroni posthoc test comparing control vs MA4W; 1-way ANOVA and Dunnett's posthoc test comparing S2-S5 vs S1 (n = 6–8 neurons/experimental group). (F) SL327 microinjection significantly dampened the change in tonic activity relative to the basal values in MA4W rats: 1-way ANOVA and Dunnett's posthoc test comparing S2-S5 vs S1; repeated measures and Bonferroni posthoc test comparing control vs MA4W (n = 6–8 neurons/experimental group). (G) Representative oscillography traces for S1, S3, and S5 in control and MA4W rats: *$P < .05$, **$P < .01$, and ***$P < .001$ (repeated measures); ++$P < .01$ and +++$P < .001$ (1-way ANOVA).

S4, and S5) (Figure 6F). Representative histograms were drawn up of the LC response in control and MA4W rats to S1, S3, and S5 PC applied to the ipsilateral paw in the presence of SL327 (Figure 6G).

Effect of Intra-LC SL327 Microinjection on Nociception and Anxiety

Having verified that SL327 (4.17 mM) reversed the accumulation of pERK1/2 in the LC to control levels (Figure 6A), we assessed the behavioral effects of this MEK inhibitor after micro-injection into the LC (Figure 7A). A significant decrease in the pain threshold was observed in the ipsilateral paw of MA4W rats before contralateral microinjection of SL327 (predrug, $P<.05$ vs contralateral paw), which persisted after administering the inhibitor (SL327, $P<.01$ vs contralateral paw) (Figure 7B). Hence, pERK1/2 inhibition appeared to have no effect on the ipsilateral and contralateral paw withdrawal threshold in MA4W rats. However, when their anxiety-like behavior was assessed, microinjection of SL327 reverted the shorter times spent by MA4W rats in the open arms that were indicative of an anxiolytic-like effect ($P<.05$, MA4W saline vs MA4W SL327) (Figure 7C). No changes were observed in control animals receiving SL327 and moreover, no significant changes were observed in the total distance traveled (Figure 7D).

Discussion

The present study provides new evidence and further confirmation of the role played by ERK1/2 signaling in the contralateral/ascending pathway of the LC, a crucial element in the manifestation of anxiety associated with MA pain. Indeed, in addition to our previous reports of enhanced ERK1/2 signaling activity in the LC associated with prolonged MA (Borges et al., 2014, 2015), we explored here the electrophysiological activity of the LC neurons in such painful conditions, also evaluating their tonic and phasic activities. After finding that when MA provokes anxiety it is accompanied by low tonic and high phasic activity of LC neurons, we pharmacologically blocked the activation/phosphorylation of ERK1/2 on the side contralateral to the inflamed joint, assessing the impact on LC electrophysiology and behavior. Such manipulation reversed the electrophysiological parameters and anxiety-like behavior, without interfering with the withdrawal threshold in MA4W rats.

In accordance with our previous data, we show that the withdrawal threshold in MA rats decreases significantly in the first week of inflammation, although these animals do not display emotional changes (Borges et al., 2014). Hence, MA1W rats serve as a control for pain in the absence of emotional changes given that the anxiogenic phenotype was not evident until 4 weeks of MA. Electrophysiological studies highlighted the lower firing rate in the contralateral LC of MA4W rats and a tendency towards weaker burst activity in MA1W and MA4W rats. In addition, increases in TH expression were only evident in MA4W rats, suggesting an increase in the demand for this neurotransmitter. These data are consistent with the altered climbing behavior in the forced swimming test observed previously in MA4W rats (Borges et al., 2014), possibly reflecting diminished noradrenaline availability in the face of a stressful situation (Detke et al., 1995; Cryan et al., 2005). Interestingly, we

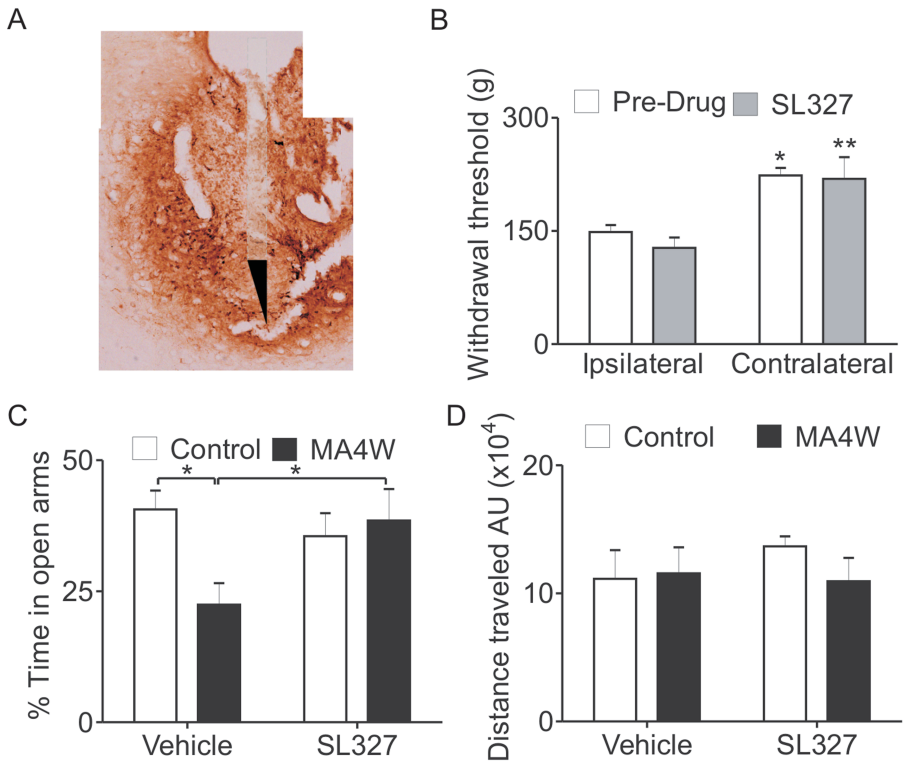

Figure 7. Effect of microinjecting the mitogen-activating extracellular kinase (MEK) inhibitor SL327 into the Locus Coeruleus (LC) on the nociceptive and anxiety behavior of control and 4-week MA rats (MA4W). (A) Histological representation of the unilateral injection sites and cannula placement within the LC. (B) SL327 microinjection into the LC had no effect on the paw withdrawal threshold: 2-way ANOVA and Bonferroni posthoc (n = 5). (C) SL327 microinjection into the LC reversed the anxiety-like behavior in MA4W rats, as reflected by the increase in the time spent in the open arms: 2-way ANOVA and Bonferroni posthoc (n = 6). (D) The locomotor activity in the elevated zero maze test was not affected by microinjection of SL327: *$P<.05$ and **$P<.01$.

also reported an increase in TH expression 4 weeks after chronic constriction injury, a model of neuropathic pain caused by nerve trauma. No significant changes in the spontaneous firing rate were detected after nerve constriction, although an increase in burst activity was evident (Alba-Delgado et al., 2013). These differences indicate that the origin of pain is decisive, suggesting that different and specific neuroplastic mechanisms are at play.

We also evaluated the sensory-evoked activity of LC neurons by applying a noxious stimulation to the paw. As expected and in agreement with previous studies, contralateral LC neurons are strongly activated by noxious stimulation of the hind paw in control and MA animals, consistent with the activation of the contralateral ascending pain pathway (Millan, 1999). However, an enhanced evoked discharge rate was seen in prolonged MA (MA4W), while this discharge was significantly weaker earlier in MA (MA1W). This might indicate a dampening in the transmission of ascending information during the early phase of MA due to the activity of inhibitory descending circuits, whereas the late-phase response to nociceptive inputs is exaggerated, perhaps due to the disruption of the same inhibitory circuits. The increased ratio of evoked/tonic activity in MA4W might result in hyperarousal towards the noxious stimulation, a phenomenon that has been related to stress-related disorders like anxiety (George et al., 2013). An analogous increase in the evoked response and in TH expression was observed in chronic neuropathic rats under social stress (Bravo et al., 2013) as well as in a rat model of posttraumatic stress (George et al., 2013). Hence, prolonged MA may produce similar changes in the LC as those observed in stressful conditions. Thus, the increased responsiveness in prolonged MA might reflect the enhanced pain transmission following noxious events as well as repercussions on signaling to the corticolimbic areas.

LC activation releases moderate amounts of noradrenaline to coordinate arousal, attention, and vigilance (Berridge and Foote, 1991; Berridge and Abercrombie, 1999). However, hyperactivation of LC circuits may lead to excessive noradrenaline release, an effect that could provoke anxiogenic-like behavior (Berridge and Waterhouse, 2003). Interestingly, PC of the healthy paw (contralateral) evoked an LC response that was associated with prolonged painful states in spine or brain areas and that might fully or partially reflect disturbances to the ascending sensory pathways. Nevertheless, as the contralateral paw was stimulated after several stimulations of the injured paw, this effect may be secondary to the stimulation of the injured paw.

Consistent with our previous studies (Borges et al., 2014), we also detected here an enhancement of pERK1/2 in MA4W by immunohistochemistry. We also explored the effect of the pharmacological blockade of ERK1/2 phosphorylation (SL327, MEK inhibitor) on contralateral LC activity, which reverted the alterations to sensory-evoked parameters and postcompression activity after prolonged inflammation. Importantly, this effect only occurred in pathological conditions and SL327 did not produce any significant change in the control rats. We also assessed whether SL327 administration to the contralateral LC had a repercussion on the manifestation of anxiety and pain in MA4W rats. Interestingly, while anxiety behavior reverted to control levels following SL327 administration, mechanical hypersensitivity remained unaffected. A similar effect was observed in a model of visceral pain (acetic acid-induced pain) after subcutaneous injection of SL327, where anxiety-like behavior was attenuated but the nocifensive responses remained unchanged (Zhong et al., 2012). However, in the present study, the lack of effect of this compound on pain hypersensitivity after blocking the phosphorylation of ERK1/2 may be due to the LC side to which SL327 is administered (contralateral to the inflamed joint). The spinal projections of the LC are thought to be bilateral in the rat strain studied here (Herbert and Proudfit, 2002). Therefore, while we did not exclude the possibility of observing an effect on pain hypersensitivity produced by contralateral injection of the MEK inhibitor, this was not the result obtained. The injection of histamine contralateral to nerve injury has been shown to decrease mechanical hypersensitivity (Wei et al., 2014), although this effect was less potent than its ipsilateral injection. Since these experiments were performed in a time window of 2 to 3 weeks, one could argue that persistent pain would lead to the total loss of nociceptive intervention on the contralateral side. This could explain why the MEK1/2 inhibitor had no effect on pain hypersensitivity on the contralateral side. Another possible explanation is that exacerbated ERK1/2 phosphorylation in the LC is not pain related. This is corroborated by the fact that pERK1/2 expression in the LC peaks a few hours after injection in the CFA model (Imbe et al., 2009) and then again after 4 weeks (Borges et al., 2014), while pain hypersensitivity persists throughout this period. Thus, further studies involving the ipsilateral administration of the MEK inhibitor will help to clarify the role of the ERK pathway in the control of pain hypersensitivity.

CRF is a well-known anxiety-related neuropeptide that modulates LC activity (Weiss et al., 1994), and it is a key compound in initiating responses to stress in the body. We recently showed that microinjection of a CRF antagonist into the contralateral LC reverted anxiogenic-like behavior and the pERK1/2 levels in the LC, without modifying the withdrawal threshold (Borges et al., 2015). This suggests that enhanced ERK1/2 phosphorylation may be linked to CRF signaling in the contralateral LC after prolonged MA and that it is related to the emergence of "affective-behaviors." This also accounts for the possibility that a stress component participates in MA, as indicated above.

These data also demonstrate that pain-related anxiety is dissociated from the perception of the sensory component of pain, at least in the contralateral/ascending pathway of the LC. This result sheds light on the link between pain hypersensitivity and the secondary mental effects. In this sense, it was recently shown that gabapentin administration produces analgesia, but it does not rescue the impaired attention produced by neuropathic pain (Berrocoso, 2014; Suto et al., 2014). Hence, pain-related affective and cognitive impairment does not appear to be due to the inherent stress provoked by the painful experience but rather, it may be attributed to a permanent alteration to a specific circuit.

Our results suggest that a brain region involved in the processing of both pain and affective information (the LC) may be disrupted by prolonged arthritis, and that this may lead to an alteration in the processing of affective information, thereby triggering a secondary effect. In an animal model of inflammatory pain (MA), our data show that rats display a sharp decrease in their sensorial threshold within 1 week of induction, although anxiety-like behavior does not appear until much later (4 weeks). This later phase temporally coincides with a decrease in tonic LC activity but also with an exacerbated evoked LC response to noxious stimulation of the inflamed paw. These changes were accompanied by enhanced TH and pERK1/2 expression in the LC. Moreover, when ERK1/2 activation was blocked pharmacologically in the contralateral LC, the exaggerated electrophysiological evoked LC response of MA rats was impaired. Importantly, ERK1/2 blockade did not modify pain hypersensitivity but rather, it reversed the anxiogenic-like behavior. Therefore, we provide direct evidence of how restoring ERK1/2 activation in the contralateral LC provides relief from pain-related anxiety without affecting nociception.

Supplementary Material

Supplementary data are available at *International Journal of Neuropsychopharmacology* online.

Acknowledgments

We are very grateful to Ms Raquel Rey-Brea, Mr José Antonio García Partida, Mr Jesus Gallego-Gamo, Ms Paula Reyes Perez, Mr Santiago Muñoz and Ms Elisa Galvão for their excellent technical assistance. We thank the UPV/EHU Advanced Research Facilities SGIker for their collaboration.

This work was supported by the "Cátedra Externa del Dolor Fundación Grünenthal/Universidad de Cádiz," which paid a grant to the first author; the "Cátedra em Medicina da Dor from Fundação Grünenthal-Portugal and Faculdade de Medicina da Universidade do Porto"; co-financed by the Fondo Europeo de Desarrollo Regional "Una manera de hacer Europa" (PI13/02659); the "Ministerio de Economía y Competitividad", co-financed byFEDER (SAF2015-68647-R (MINECO/FEDER)); CIBERSAM G18; the "Consejería de Economía, Innovación, Ciencia y Empleo de la Junta de Andalucía", Seville, Spain (CTS-510 and Proyecto de Excelencia: CTS-7748); 2015 NARSAD Young Investigator Grant from the Brain & Behavior Research Foundation; Fundación Española de Dolor (travel fellowship granted to Gisela Borges – 1536); and Gobierno Vasco (IT747-13).

Statement of Interest

None.

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