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Formalin-induced long-term secondary allodynia and hyperalgesia are maintained by descending facilitation

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ABSTRACT

This work analyzes the role of cholecystokinin (CCK) receptors, dynorphin A_{1-17} and descending facilitation originated in the rostral ventromedial medulla (RVM) on secondary allodynia and hyperalgesia in formalin-injected rats. Formalin injection (50 μL, 1%, s.c.) produced acute nociception (lasting 1 h) and long-term secondary allodynia and hyperalgesia in ipsilateral and contralateral hind paws (lasting 1–12 days). Once established, intra-RVM administration of lidocaine at day 6, but not at 2, reversed secondary allodynia and hyperalgesia in rats. The injection of YM022 (CCK_2 receptor antagonist), but not lorglumide (CCK_1 receptor antagonist), into the RVM or spinal cord reversed both nociceptive behaviors. Pre-treatment with lidocaine, lorglumide or YM022 did not prevent the development of secondary allodynia or hyperalgesia regardless of the administration route. Formalin injection increased dynorphin content in the dorsal, but not the ventral, spinal cord sections at day 6. Moreover, intrathecal administration of dynorphin antiserum reversed, but was unable to prevent, secondary allodynia and hyperalgesia in both hind paws. These results suggest that formalin-induced secondary allodynia and hyperalgesia are maintained by activation of descending facilitatory mechanisms which are dependent on CCK_2 receptors located in the RVM and spinal cord. In addition, data suggest that spinal dynorphin A_{1-17} and CCK play an important role in formalin-induced secondary allodynia and hyperalgesia.

1. Introduction

The brain stem pain modulating centers, including the rostral ventromedial medulla (RVM), were initially identified with a descending inhibitory role in the control of nociceptive input to the central nervous system (Basbaum and Fields, 1978; Urban and Gebhart, 1999; Vanegas and Schaible, 2004). Further investigations demonstrated that the RVM is also involved in descending facilitation of nociceptive input. This facilitatory effect is based upon the behavior of two groups of neurons that increase (ON) or decrease (OFF) their firing at the initiation of the tail flick reflex in anesthetized rats (Fields et al., 1983; Kaplan and Fields, 1991). Activation of this descending facilitatory mechanism plays an important role in the initiation and perpetuation of neuropathic pain. For instance, lidocaine injection into the RVM or transection of spinal cord reduces the hyperalgesia induced by tissue damage (Morgan and Fields, 1994; Mansikka and Pertovaara, 1997; Wiertelak et al., 1997; Pertovaara, 1998; Calejesan et al., 1998; Urban and Gebhart, 1999; Kincaid et al., 2006) and the tactile allodynia that results from nerve injury in rats (Pertovaara et al., 1996; Ossipov et al., 2000; Kovelowski et al., 2000; Burgess et al., 2002).

Facilitatory influences from RVM, produced by electrical stimulation, glutamate or neurotensin injection, involve descending projections which once activated release serotonin and cholecystokinin (CCK) at the spinal cord (Heinricher and Neubert, 2004). Regarding the later, functional studies show that local application of CCK into RVM activates ON cells which in turn produce behavioral allodynia and hyperalgesia (Kovelowski et al., 2000; Heinricher and Neubert, 2004; Xie et al., 2005). It is believed that activation of ON cells by CCK leads to a time-dependent dynorphin and CCK release in the spinal cord. Accordingly, tissue damage (Iadarola et al., 1988; Ruda et al., 1988; Weihe et al., 1989; Noguchi et al., 1991; Parra et al., 2002; Luo et al., 2008; Taketa et al., 2010) or nerve injury (Kajander et al., 1990; Wagner et al., 1993; Laughlin et al., 1997; Malan et al., 2000; Burgess et al., 2002; Labombarda et al., 2008) produces a significant regional elevation of dynorphin A levels in the spinal cord. Likewise, nerve injury significantly increases CCK concentration in the spinal cord (Gustafsson et al., 1998; Xu et al., 2001; Kim et al., 2009). Interestingly, there are no data about the effect of inflammatory pain on CCK levels in the spinal cord.

Classically, the formalin test is considered as a model of acute inflammatory pain which produces two well-identified phases of nociceptive behavior (Wheeler-Aceto and Cowan, 1991; Rocha-

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González et al., 2005). However, if enough time is given, formalin induces secondary mechanical hyperalgesia after being injected into either the hind paw or tail of rodents (Wiertelak et al., 1994; Fu et al., 2000, 2001; Lin et al., 2007; Vierck et al., 2008). Our group has recently published that formalin also produces secondary allodynia (Ambriz-Tututi et al., 2009). The finding that spinal transection prevents facilitation of the tail-flick reflex after formalin injection suggests that a supraspinal site is involved in formalin-induced secondary hyperalgesia (Wiertelak et al., 1994). Further support to this idea comes from a study showing that lesion of the RVM prevents the appearance of secondary hyperalgesia induced by formalin (Wiertelak et al., 1997). To date, the sites and mechanisms involved in the secondary allodynia and hyperalgesia induced by formalin are unknown. Thus, in the present study we analyzed the role of the RVM and the spinal cord on these processes. Specifically, we tested the hypothesis that CCK receptors located in the RVM and spinal cord, and spinal dynorphin are involved in the maintenance of formalin-induced secondary allodynia and hyperalgesia.

2. Material and methods

2.1. Animals

Experiments were carried out in 198 adult female Wistar rats (180–200 g) of 8 to 10 weeks of age. Female rats were used because in previous experiments performed under the same conditions (Wistar rats, 1% formalin and weight range 180–200 g) we found no significant differences between males and females (unpublished data). Other authors have found differences, but only with other rat strains, animals of greater weight, or using different formalin concentrations (Aloisi et al., 1994; Gaumond et al., 2002).

Animals were obtained from our own breeding facilities and had free access to food and water before experiments. All experiments are in compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985) and Guidelines on Ethical Standards for Investigation of Experimental Pain in Animals (Zimmermann, 1983) and were approved by our local Ethics Committee.

2.2. Induction of secondary allodynia and hyperalgesia

Rats were briefly immobilized to get open access to the right hind limb. Then, they received a s.c. injection of saline solution or formalin (1%, 50 μL) into the dorsal surface of the hind paw using a 30-gage needle (Fu et al., 2000, 2001). The sensitization induced by formalin was tested at 0, 1, 3, 6, 9 and 12 days after injection. The sixth day was chosen to evaluate nociceptive behaviors for subsequent experiments because secondary allodynia and hyperalgesia were already established at this time without evidence of tissue damage and on previous evidence from literature (Burgess et al., 2002). Rats were sacrificed in a CO_2 chamber at the end of the experiment.

2.3. Behavioral testing of formalin-induced secondary allodynia and hyperalgesia

Secondary mechanical allodynia and hyperalgesia were assessed as previously reported (Fu et al., 2000, 2009; Valencia-de Ita et al., 2006; Ambriz-Tututi et al., 2009). Briefly, rats were placed in testing cages with a wire mesh bottom and allowed to acclimate for 30 min. Baseline measurements were recorded first. Two von Frey filaments (Stoelting Co, Wood Dale, IL, USA, bending forces of 10 mN [1 g] and 250 mN [26 g]) were applied ten times in each testing set at the base of the third toe on the plantar surface on both paws. Three trials were completed to get an average of the number of paw withdrawal responses. Under normal conditions, a force of 10 mN neither activates cutaneous nociceptors (Leem et al., 1993) nor causes paw withdrawal in normal animals. Accordingly, the occurrence of responses to the 10 mN filament was indicative of allodynia. On the other hand, a force of 250 mN or higher is considered a noxious stimulus, and hyperalgesia occurred when there was an increased response to the 250 mN filament. Allodynia and hyperalgesia were considered secondary as stimulation with the von Frey filaments was applied in a site different from that of the formalin injection.

2.4. Spinal surgery

Rats underwent surgery for insertion of a spinal catheter for drug administration five days prior to formalin injection. Animals were anesthetized with a ketamine/xylazine mixture (45/12 mg/kg, i.p.), placed in a stereotaxic head holder, and the atlantooccipital membrane was exposed (Yaksh and Rudy, 1976). The membrane was pierced, and a PE-10 catheter (8 cm) was introduced intrathecally to the level of the thoracolumbar junction after which the wound was sutured. Rats were allowed to recover from surgery for at least five days in individualized cages before use. Animals showing any sign of motor impairment were euthanized in a CO_2 chamber.

2.5. Placement of guide cannula in RVM

Intracerebral guide cannula was placed in the RVM four days prior to formalin injection. Rats were anesthetized with a mixture of ketamine/xylazine (45/12 mg/kg, respectively, i.p.) and positioned in a stereotaxic head holder. The skull was exposed and a small hole was drilled for placement of guide cannula. The cannula was placed 2 mm above the RVM using the following coordinates: −8.5 mm anterior to bregma, 0.0 mm lateral from the midline and −8.4 mm ventral to dura with a flat skull (Paxinos and Watson, 1997). The cannula was secured to the skull with dental cement and rats were allowed to recover for four days.

Drug administration into the RVM was performed by slowly expelling 0.5 μL of drug solution through a 33-gage injection cannula inserted through the guide cannula and protruding an additional 1 mm into fresh brain tissue to prevent backflow of drug into the guide cannula. To examine placement of the cannula, an equivalent volume of methylene blue was injected through the cannula at the end of the experiment. Rats were then euthanized in a CO_2 chamber, and the brain removed and postfixed in 10% formalin at room temperature for at least one week. Marks were identified with reference to a stereotaxic atlas.

2.6. Dynorphin determination

Content of spinal dynorphin was assessed as previously described (Malan et al., 2000). Six days after formalin or saline injection, rats were decapitated and spinal cords were removed and rapidly dissected. The lumbar spinal cord was divided into left and right segments by a sagittal cut and the dorsal and ventral quadrants by a cut in a plane slightly dorsal to the central canal. Dynorphin content was quantitated with a commercial enzyme immunoassay system using an antibody specific for dynorphin A_{1-17} (Peninsula Laboratories, Belmont, CA). Protein concentrations were determined by the Lowry method.

2.7. Drugs

Lorglumide (Sigma, St. Louis, MO, USA) and YM022 (Tocris Bioscience, Ellisville, MO, USA) were dissolved in saline. Anti-dynorphin antiserum (Peninsula Laboratories, Belmont, CA, USA) was injected diluted with bovine non-immune serum in a 1:1 proportion. Lidocaine (Sigma, St. Louis, MO, USA) was dissolved in saline.

2.8. Experimental design

2.8.1. Controls

In order to determine acute (flinching behavior, 1 h) and long-term (secondary allodynia and hyperalgesia, 1–12 days) nociceptive effects of formalin, animals received s.c. formalin (1%, 50 μL) or saline (50 μL) and the following hour (for acute nociception) or 1–12 days (for long-term nociception) later they were tested.

In order to exclude the possibility that the effect of different receptor antagonists on paw withdrawal responses was affected by the vehicle, a group of animals was injected with saline (50 μL) into the formalin-treated paw 10 min before or 6 days after s.c. formalin injection.

2.8.2. Pre-treatment study

In order to determine if lidocaine, CCK receptor antagonists or the anti-dynorphin antiserum was able to prevent the occurrence of formalin-induced secondary mechanical allodynia and hyperalgesia, a dose of each drug was administered through an intrathecal or intra-RVM cannula 10 min before formalin injection. In all cases, animals were tested before drug administration, to register baseline, and six days later after formalin injection. The doses of these drugs were selected based on literature reports (Malan et al., 2000; Xie et al., 2005; Juárez-Rojop et al., 2006) and pilot experiments in our laboratory.

2.8.3. Post-treatment study

In order to establish if treatments were able to reverse formalin-induced secondary mechanical allodynia and hyperalgesia, at least one dose of each drug was administered either intrathecally or intra-RVM two (for lidocaine) or six (for lidocaine, CCK receptor antagonists or anti-dynorphin antiserum) days after formalin injection. Animals were tested before drug administration, to register baseline, and 1 h after drug administration. This schedule was chosen based on pilot studies showing maximal antinociceptive response at that time.

2.8.4. Dynorphin enzyme immunoassay

In order to determine the effect of formalin on spinal dynorphin content, rats were injected with either saline or 1% formalin. Six days later, animals were sacrificed and spinal cords removed and dissected. Dynorphin A_{1-17} content was expressed in pg/mg protein.

2.9. Data and statistical analysis

Data are expressed as the mean ($n=6$) ± S.E.M. The mean responses of three trials with ten applications of the von Frey filaments in each testing set were obtained from ipsilateral and contralateral paws for each treatment.

Statistical differences between groups were determined by one- or two-way analysis of variance followed by the Student–Newman–Keuls' test. A $P<0.05$ was considered statistically significant.

3. Results

3.1. Formalin induced-secondary allodynia and hyperalgesia

As previously reported (Ambriz-Tututi et al., 2009), injection of 1% formalin into the dorsal surface of the right hind paw resulted in acute nociception lasting approximately 1 h (Fig. 1A) and secondary mechanical allodynia and hyperalgesia lasting from 1 to 12 days (Fig. 1B–C). These effects were observed not only in the injected paw but also in the contralateral one. This was seen as a bilateral increase in number of paw withdrawal responses to the application of von Frey filaments (10 and 250 mN) which was significantly different from baseline one day after formalin injection and lasted for at least 12 days (Fig. 1B–C). The sixth day was chosen to evaluate nociception because at this time nociceptive behaviors were completely established (Burgess et al., 2002).

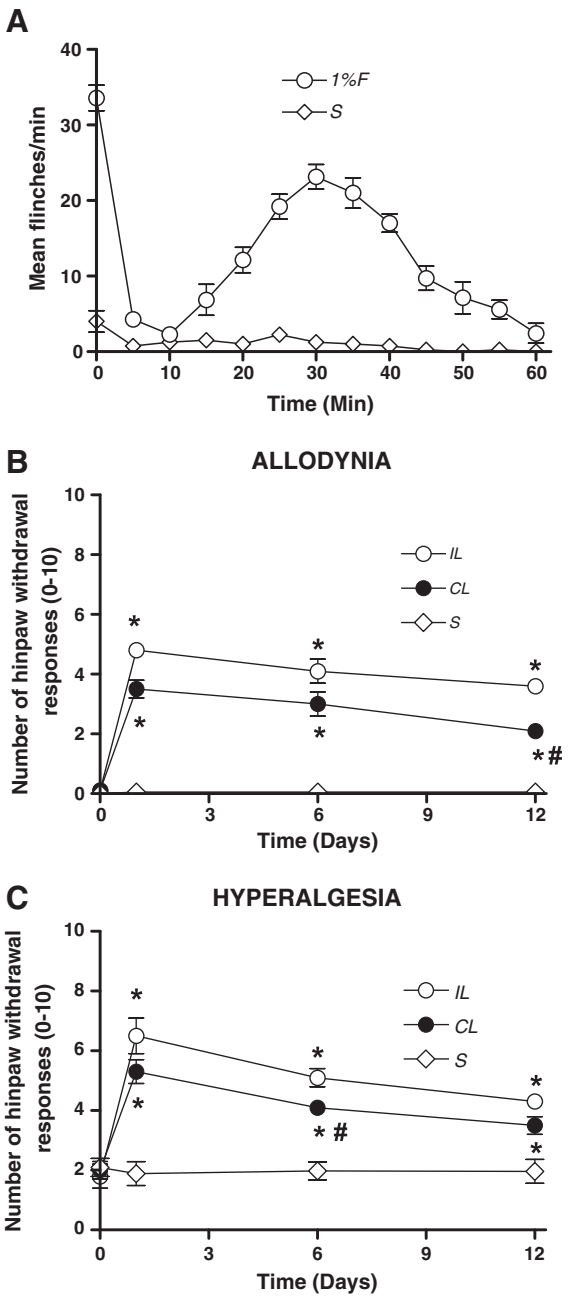

Fig. 1. Time course of acute nociception (A) and long-term secondary mechanical allodynia (B) and hyperalgesia (C) in rats submitted to either 1% formalin or saline. Data are expressed as mean of the number of flinches per min (A) or hind paw withdrawal responses (B and C) to the applications of von Frey filaments (10 or 250 mN) to the plantar surface of rat paws before (baseline) and after formalin injection. IL: ipsilateral; CL: contralateral; S: saline. *$P<0.05$ vs baseline (time 0) in the same rats and $^{\#}P<0.05$ vs IL paws by two-way ANOVA followed by the Student–Newman–Keuls' test.

3.2. Effect of lidocaine into RVM on formalin-induced secondary allodynia and hyperalgesia

Administration of lidocaine, as pre-treatment, into the RVM did not modify formalin-induced acute (flinching behavior, data not shown) or long-term nociception. Contrariwise, intra-RVM lidocaine significantly ($P<0.05$) reversed mechanical secondary allodynia and hyperalgesia in both hind paws when it was given as post-treatment

six days after formalin injection (Fig. 2B–C). However, two days post-treatment with intra-RVM lidocaine did not alter the development of nociceptive behaviors (Fig. 2). Fig. 2A shows the sites of injection for lidocaine into RVM. These injections were effective after post-treatment (day 6) but not pre-treatment. In marked contrast, injections of lidocaine outside but surrounding the RVM were ineffective at post- and pre-treatment (data not shown). Since lidocaine was ineffective at 2 days post-treatment, no further experiments were done at this post-treatment day.

3.3. Effect of intrathecal and intra-RVM administration of CCK receptor antagonists on formalin-induced secondary allodynia and hyperalgesia

Intra-RVM (2.5–25 ng) or intrathecal (3–30 μg) post-treatment (day 6), but not pre-treatment, with the selective CCK_2 receptor antagonist YM022 significantly ($P<0.05$) reversed secondary mechanical allodynia and hyperalgesia in both paws (Fig. 3). In marked contrast, neither intra-RVM (5–50 ng) nor intrathecal (3–30 μg) post-treatment (day 6) or pre-treatment with lorglumide (selective CCK_1 receptor antagonist) had effect on paw withdrawal frequency (Fig. 4).

3.4. Determination of dynorphin in spinal cord

Formalin, but not saline, significantly ($P<0.05$) increased the spinal content of dynorphin A_{1-17} in rats at day 6 (Fig. 5). This increase was observed in the ipsilateral and contralateral dorsal sections. In contrast, formalin injection did not modify dynorphin A_{1-17} content in the ipsilateral and contralateral ventral sections (Fig. 5).

3.5. Effect of intrathecal anti-dynorphin antiserum on formalin-induced secondary allodynia and hyperalgesia

Spinal post-treatment (day 6) with anti-dynorphin antiserum significantly ($P<0.05$) reversed secondary mechanical allodynia and hyperalgesia in both hind paws of rats (Fig. 6). On the contrary, spinal pre-treatment with anti-dynorphin antiserum did not significantly modify formalin-induced secondary allodynia and hyperalgesia. Spinal pre-treatment or post-treatment (day 6) with control serum did not alter formalin-induced secondary allodynia and hyperalgesia.

4. Discussion

4.1. Formalin-induced secondary allodynia and hyperalgesia

Formalin (1%) injection produced acute nociceptive behaviors (~1 h) as well as long-term (1–12 days) secondary mechanical allodynia and hyperalgesia in the ipsilateral and contralateral paws. Previous observations have reported similar long-term nociceptive behaviors using 10% (Cadet et al., 1993), 5% (Fu et al., 2000, 2001; Wu et al., 2001; Vierck et al., 2008), 1% (Ambriz-Tututi et al., 2009) and 0.5% formalin (Jolivalt et al., 2006). In our study, secondary mechanical allodynia and hyperalgesia were observed from day 1 to day 12. These effects were maximal at day 1 and declined afterwards. Thus, our study confirms evidence that secondary allodynia and hyperalgesia develop after a relatively low formalin concentration injected into the dorsal surface of a rat hind paw.

The mechanisms leading to the long-term nociceptive behaviors induced by formalin are unknown. However, there is growing

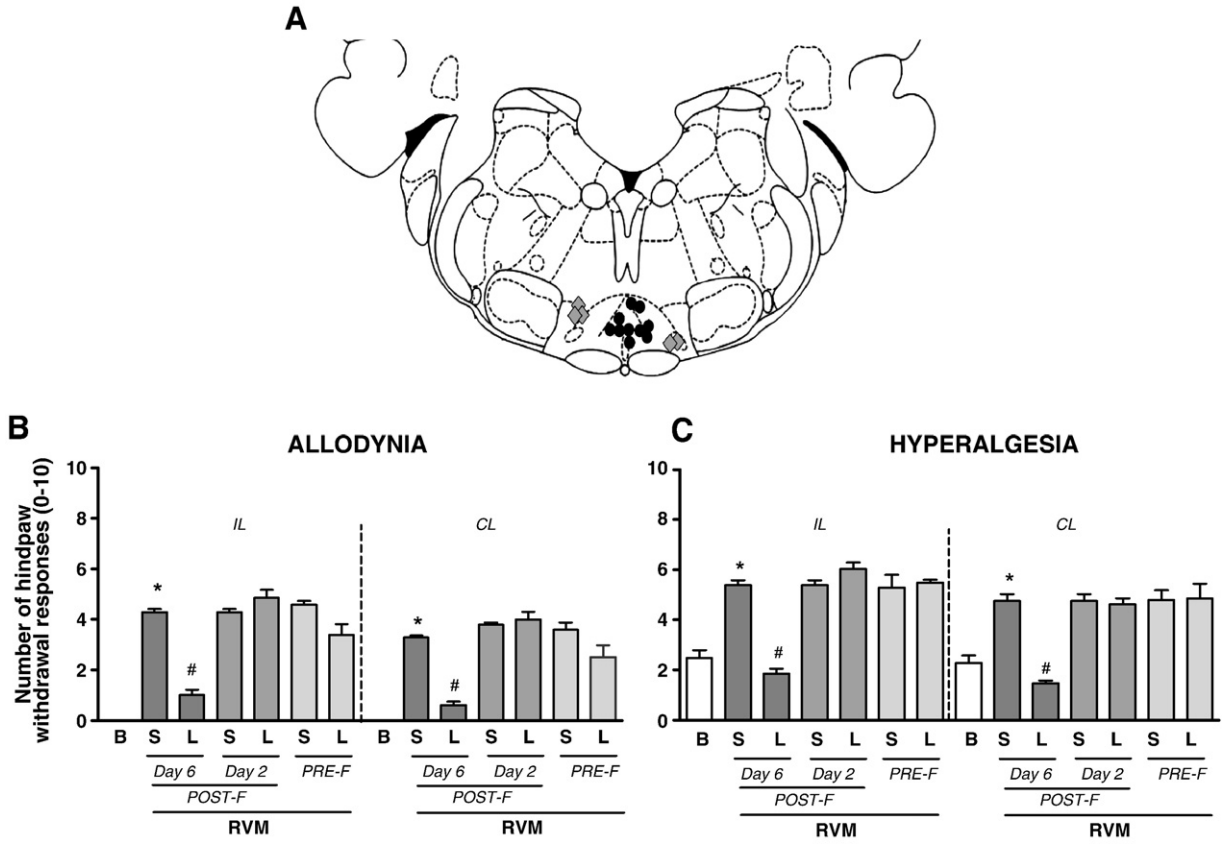

Fig. 2. Site of injection of lidocaine into the rostral ventromedial medulla (RVM) and surrounding sites (A). Effect of intra-RVM saline (S) or 4% lidocaine (L) administration on formalin-induced secondary mechanical allodynia (B) and hyperalgesia (C). Data are expressed as mean of the number of hind paw withdrawal responses to the applications of von Frey filaments (10 or 250 mN) to the plantar surface of rat paws before (baseline) and after 1% formalin. Post-F: treatment received 6 or 2 days after formalin injection; Pre-F: treatment received 10 min before formalin injection; IL: ipsilateral; CL: contralateral; RVM: rostral ventromedial medulla. *$P<0.05$ vs baseline (B) in the same rats and $^{\#}P<0.05$ vs saline groups, by one-way ANOVA followed by the Student–Newman–Keuls' test.

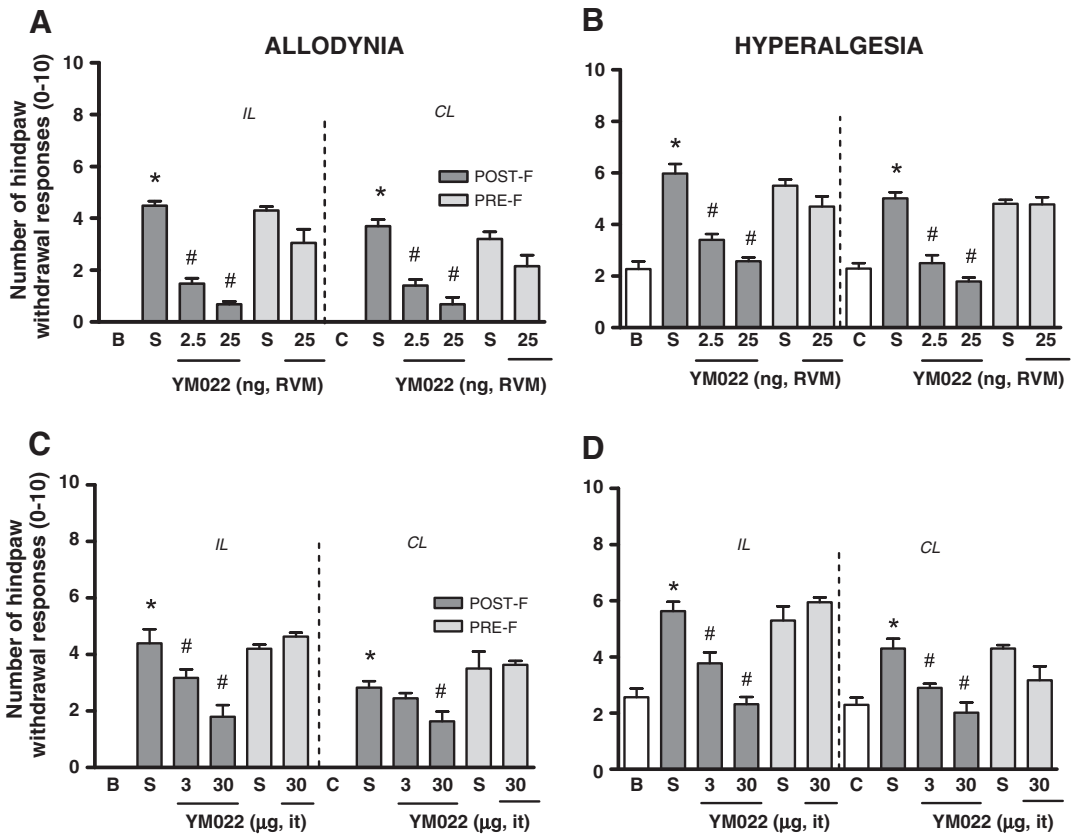

Fig. 3. Effect of intra-RVM (upper panel, A and B) and intrathecal (lower panel, C and D) administration with the selective CCK_2 receptor antagonist YM022 on formalin induced-secondary mechanical allodynia and hyperalgesia. Data are expressed as mean of the number of hind paw withdrawal responses to the applications of von Frey filaments (10 or 250 mN) to the plantar surface of rat paws before (baseline) and after 1% formalin (1%F). Post-F, treatment received 6 days after 1% formalin injection; Pre-F, treatment received 10 min before formalin injection. IL: ipsilateral; CL: contralateral; RVM: rostral ventromedial medulla; it: intrathecal. *$P<0.05$ vs baseline (B) in the same rats and $^{\#}P<0.05$ vs saline groups, by one-way ANOVA followed by the Student–Newman–Keuls' test.

evidence that descending modulatory projections from RVM play an important role in persistent pain states (Porreca et al., 2002) and prompted our study on the role of RVM and spinal cord in formalin-induced long-term secondary allodynia and hyperalgesia.

4.2. Role of RVM on formalin-induced secondary allodynia and hyperalgesia

Post-treatment (day 6), but not pre-treatment or post-treatment (day 2), with lidocaine into the RVM significantly reduced formalin-induced secondary allodynia and hyperalgesia in rats. Our results agree with previous reports showing that lidocaine administered into the RVM reverses but does not prevent tactile allodynia in neuropathic rats (Pertovaara et al., 1996; Ossipov et al., 2000; Kovelowski et al., 2000; Burgess et al., 2002). In addition, our results agree with evidence showing that spinal transection or RVM lesion prevents facilitation of the tail-flick reflex or secondary hyperalgesia after formalin injection, respectively (Wiertelak et al., 1994, 1997). Taken together, these data suggest that the RVM plays an important role in maintenance but not in the development of neuropathic pain or in long-term secondary allodynia and hyperalgesia induced by formalin (this study). Other reports have shown that intra-RVM lidocaine also reduces short-term secondary allodynia and hyperalgesia in rats subjected to an inflammatory insult (Mansikka and Pertovaara, 1997; Pertovaara, 1998; Calejesan et al., 1998). This evidence has been used to support the hypothesis that a descending pain facilitation system from the RVM to the spinal cord is necessary for the maintenance of neuropathic pain or chronic inflammatory pain (Ossipov et al., 2000; Kovelowski et al., 2000; Porreca et al., 2001; Burgess et al., 2002; Gardell et al., 2003; Sanoja et al., 2008; Bee and Dickenson, 2008). In support of this, selective ablation of dorso lateral funiculus, which includes the spinopetal projections from RVM to the spinal cord, also prevented neuropathic pain when assessed at post-injury day 7 (Porreca et al., 2001). Thus the present study provides evidence that formalin-induced long-term mechanical secondary allodynia and hyperalgesia are maintained by descending facilitation from the RVM.

4.3. Role of CCK receptors on formalin-induced secondary allodynia and hyperalgesia

Our results show that the selective CCK_2 receptor antagonist YM022 (Foucaud et al., 2006) significantly reversed but did not prevent secondary allodynia and hyperalgesia when injected into the RVM. Interestingly, the CCK_1 receptor antagonist lorglumide (Makovec et al., 1987) had no effect. These data suggest that CCK_2 receptors located at the RVM play a key role in the maintenance of formalin-induced long-term secondary allodynia and hyperalgesia. Our data agree with a previous report showing that the CCK_2 receptor antagonist L365,260 injected into the RVM reverses established neuropathic pain (Kovelowski et al., 2000). Thus, it is likely that both nerve- and formalin-induced injury may lead to CCK release in the RVM which in turn would activate CCK_2 receptors located therein that would then facilitate secondary allodynia and hyperalgesia. In line with this suggestion, there is evidence that CCK-like immunoreactivity is present in the RVM (Skinner et al., 1997). In this site, CCK activates ON cells selectively to produce behavioral allodynia and hyperalgesia (Kovelowski et al., 2000; Heinricher and Neubert, 2004; Xie et al., 2005; Carlson et al., 2007) via CCK_2 receptors (Zhang et al., 2009).

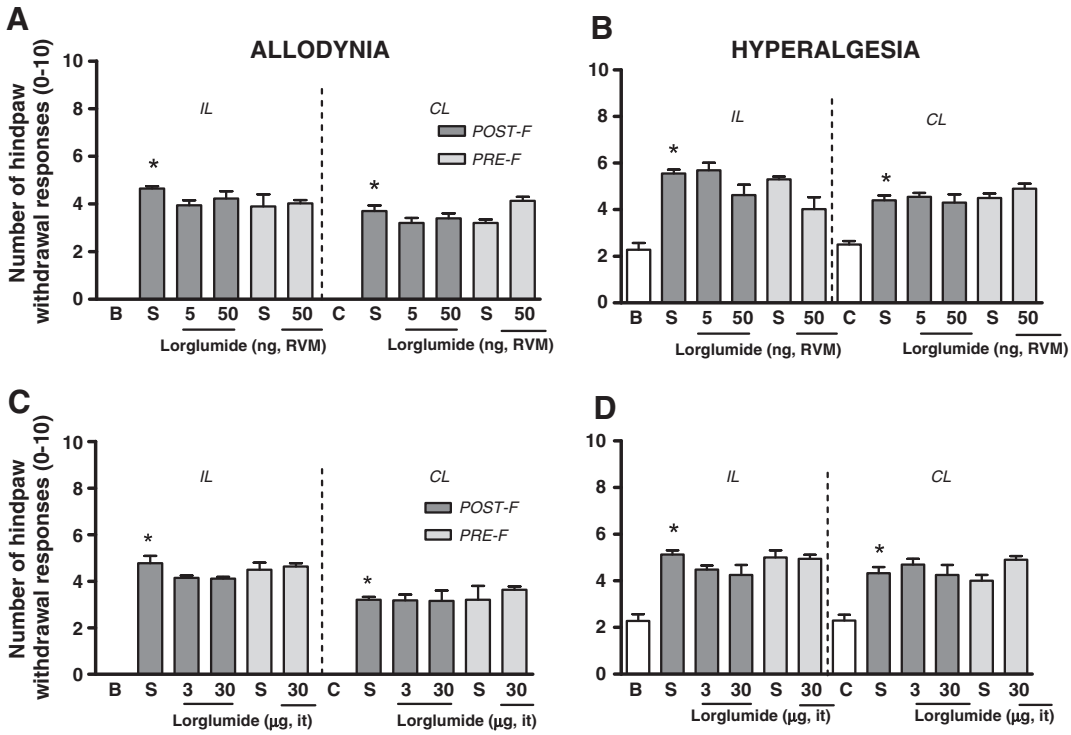

Fig. 4. Effect of intra-RVM (upper panel, A and B) and intrathecal (lower panel, C and D) administration with the selective CCK_1 receptor antagonist lorglumide on formalin induced-secondary mechanical allodynia (A and C) and hyperalgesia (B and D). Data are expressed as mean of the number of hind paw withdrawal responses to the applications of von Frey filaments (10 or 250 mN) to the plantar surface of rat paws before (Control, C) and after 1% formalin (1%F). Post-F: treatment received 6 days after 1% formalin injection; Pre-F: treatment received 10 min before formalin injection; IL: ipsilateral; CL: contralateral; RVM: rostral ventromedial medulla; it: intrathecal. *P<0.05 vs baseline (B) in the same rats, by one-way ANOVA followed by the Student–Newman–Keuls' test.

In addition to its effect in RVM, intrathecal post-treatment (day 6), but not pre-treatment, with the CCK_2 receptor antagonist YM022 reversed formalin-induced long-term secondary nociceptive behaviors. Our data agree with published reports showing that intrathecal administration of CCK_2 receptor antagonists reverses diabetes- or nerve injury-induced hyperalgesia and allodynia (Kamei and Zushida, 2001; Coudoré-Civiale et al., 2000; Kim et al., 2009) as well as mustard oil-induced secondary hyperalgesia (Urban et al., 1996). Thus, this study confirms previous observations showing that spinal CCK_2 receptors are also important in long-term pain processes and extends these findings to formalin-induced long-term secondary allodynia and hyperalgesia.

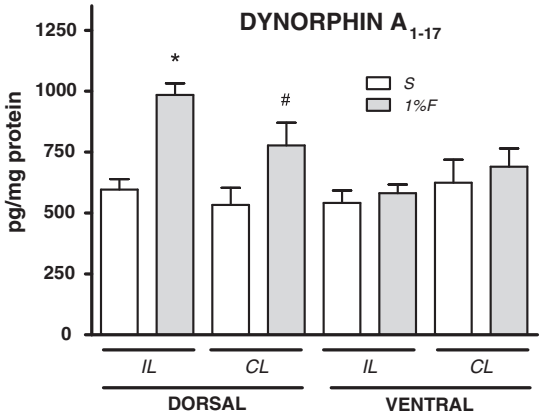

Fig. 5. Effect of formalin on the spinal dynorphin content in rats injected with saline or 1% formalin. Dynorphin levels were quantified 6 days after treatment. Data are expressed as dynorphin A_{1-17} per milligram of total protein. IL: ipsilateral; CL: contralateral; 1%F: 1% formalin. *P<0.05 vs saline group and #P<0.05 vs saline group, by the Student's *t*-test.

There is evidence that spinally injured rats undergoing chronic pain-like behaviors have elevated levels of CCK-like immunoreactivity in the dorsal horn spinal cord (Gustafsson et al., 1998; Xu et al., 1994, 2001; Kim et al., 2009). Moreover, nerve injury leads to a marked ipsilateral increase in both CCK_2 receptor mRNA and protein expression in the superficial layers of the lumbar dorsal horn (Antunes Bras et al., 1999). Accordingly, deletion of the CCK_2 receptor gene reduces mechanical sensitivity and abolishes the development of hyperalgesia in neuropathic mice (Kurrikoff et al., 2004). Based on this evidence, it is tempting to suggest that formalin-induced secondary allodynia and hyperalgesia is maintained but not originated not only by neurons in the RVM but also in the spinal cord which are sensitive to CCK via CCK_2 but not CCK_1 receptors.

4.4. Role of dynorphin in the spinal cord

Formalin injection not only induces secondary allodynia and hyperalgesia but also increases dynorphin A_{1-17} content in the dorsal (ipsilateral and contralateral) section of the spinal cord six days after its injection. Our results agree with previous reports indicating that nerve injury (Wagner et al., 1993; Laughlin et al., 1997; Bian et al., 1999; Malan et al., 2000; Burgess et al., 2002; Labombarda et al., 2008) or chronic inflammatory pain (Ruda et al., 1988; Parra et al., 2002; Luo et al., 2008; Taketa et al., 2010) augments the spinal dynorphin content. At least in some reports, this increase has been associated with long-term allodynia (Vanderah et al., 1996; Laughlin et al., 1997). Accordingly, spinal post-treatment (day 6), but not pre-treatment, with the anti-dynorphin antiserum reversed formalin-induced secondary allodynia and hyperalgesia. Our study is in line with previous reports showing that intrathecal post-treatment with anti-dynorphin antiserum reverses complete Freund's adjuvant-induced thermal hyperalgesia (Luo et al., 2008) and neuropathic pain in rodents (Malan et al., 2000; Wang et al., 2001). Further

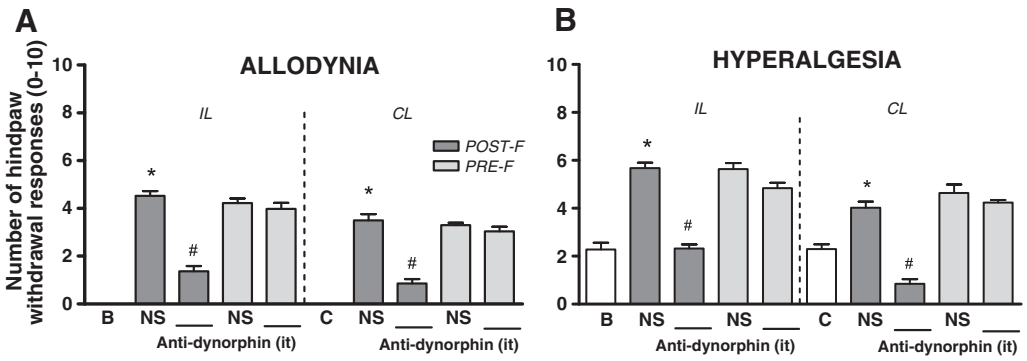

Fig. 6. Effect of intrathecal administration with the anti-dynorphin antiserum on formalin induced-secondary mechanical allodynia (A) and hyperalgesia (B). Data are expressed as mean of the number of hind paw withdrawal responses to the applications of von Frey filaments (10 or 250 mN) to the plantar surface of rat paws before (baseline) and after 1% formalin (1%F). Post-F: treatment received 6 days after 1% formalin injection; Pre-F: treatment received 10 min before formalin injection; IL: ipsilateral; CL: contralateral; NS: non-immune serum; it: intrathecal. *P<0.05 vs baseline (B) in the same rats and #P<0.05 vs NS group, by one-way ANOVA followed by the Student–Newman–Keuls' test.

evidence for the role of dynorphin in chronic pain comes from the finding that a modified mouse strain lacking prodynorphin does not exhibit sustained neuropathic pain after nerve injury (Wang et al., 2001; Gardell et al., 2004). Taken together, these data suggest that spinal dynorphin up-regulation is a critical step for chronic pain occurrence in the rat.

The mechanisms by which dynorphin exerts its actions at the spinal cord are not completely understood. However, it has been suggested that this opioid peptide has either a direct or an indirect interaction with spinal NMDA receptors because MK-801 pre-treatment, but not post-treatment, blocks the pronociceptive effect of dynorphin (Vanderah et al., 1996; Laughlin et al., 1997; Wang et al., 2001). Moreover, the nerve injury-induced increase in spinal CGRP release is blocked with an anti-dynorphin antiserum when it is administered at day 10 but not at day 2 post-injury (Gardell et al., 2003). In addition, dynorphin activates spinal B_2 bradykinin receptors to maintain either inflammatory (Luo et al., 2008) or neuropathic (Lai et al., 2006) pain. These findings, together with the time (6 days) at which we saw an increase in spinal dynorphin levels after formalin injection suggest that spinal dynorphin up-regulation depends on the time-related development of descending modulatory influences from RVM to maintain secondary allodynia and hyperalgesia, as previously reported for neuropathic pain (Burgess et al., 2002; Gardell et al., 2003).

In summary, our results provide evidence for the presence of time related descending facilitatory influences arising from the RVM and dependent on CCK release and CCK_2 receptor activation in RVM and spinal cord in formalin-induced secondary allodynia and hyperalgesia in rats. In addition, our data suggest that formalin-induced increase in spinal dynorphin also plays a role in the maintenance of secondary allodynia and hyperalgesia. These events appear to be critical for maintenance but not the initiation of secondary allodynia and hyperalgesia induced by formalin as is the case for neuropathic pain.

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