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Spinal CCK contributes to somatic hyperalgesia induced by orofacial inflammation combined with stress in adult female rats

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Abstract

In some chronic primary pain conditions such as temporomandibular disorder (TMD) and fibromyalgia syndrome (FMS), mild or chronic stress enhances pain. TMD and FMS often occur together, but the underlying mechanisms are unclear. The purpose of this study was to investigate the role of cholecystikinin (CCK) in the spinal cord in somatic hyperalgesia induced by orofacial inflammation combined with stress. Somatic hyperalgesia was detected by the thermal withdrawal latency and mechanical withdrawal threshold. The expression of CCK₁ receptors, CCK₂ receptors, ERK1/2 and p-ERK1/2 in the spinal cord was examined by Western blot. After the stimulation of orofacial inflammation combined with 3 day forced swim, the expression of CCK₂ receptors and p-ERK1/2 protein in the L4-L5 spinal dorsal horn increased significantly, while the expression of CCK₁ receptors and ERK1/2 protein remained unchanged. Intrathecal injection of the CCK₂ receptor antagonist YM-022 or mitogen-activated protein kinase (MAPK) kinase (MEK) inhibitor PD98059 blocked somatic hyperalgesia induced by orofacial inflammation combined with stress. Intrathecal administration of the MEK inhibitor blocked somatic sensitization caused by the CCK receptor agonist CCK8. The CCK₂ receptor antagonist YM-022 significantly reduced the expression of p-ERK1/2. These data indicate that upregulation of CCK₂ receptors through the MAPK pathway contributes to somatic hyperalgesia in this comorbid pain model. Thus, CCK₂ receptors and MAPK pathway may be potential targets for the treatment of TMD comorbid with FMS.

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Declaration of competing interest

None of the authors has any financial interest or conflict of interest related to the manuscript.

Keywords

Hyperalgesia; Cholecystokinin receptor; ERK1/2; Temporomandibular disorder; Fibromyalgia syndrome; Comorbidity

1. Introduction

Chronic primary pain (CPP) is a chronic clinical condition manifesting a variety of unexplained somatic symptoms with females at higher risk, which has been proposed as an inclusive nosology encompassing individual syndromes, such as temporomandibular disorder (TMD) and fibromyalgia syndrome (FMS). TMD has a series of manifestations characterized by pain and functional disorder in the temporomandibular joint and masticatory muscles (Scrivani et al., 2008), and ranks fourth in oral epidemiology (Landi et al., 2004, 2005). FMS is a disease characterized chiefly by diffuse pain and clinical features including wide-spreading pain, hyperalgesia, mood disorders and anxiety (Sugerman, 2014). TMD and FMS have a high degree of similarity and co-occurrence, but the mechanisms of comorbidity are unclear (Rabhi et al., 2010). It has been shown that the occurrence and development of TMD and FMS are closely associated with stress such as mental and psychological factors (Furness et al., 2018; Giannakopoulos et al., 2010). Our previous studies have shown that 3 day forced swim (FS) stress induces mechanical allodynia and thermal hyperalgesia in hindpaws (Li et al., 2019; Xu et al., 2020). Central sensitization in the spinal dorsal horn and the descending facilitation contributed to the development of wide-spreading hyperalgesia in the model of comorbid TMD and FMS (Li et al., 2020). In the study, injection of estradiol (E2) to ovariectomized rats, Complete Freund's Adjuvant (CFA) into the masseter muscles or application of FS stress alone could not induce somatic hyperalgesia of the hindpaw. All of three factors were essential and necessary for the development of long-lasting and wide-spreading hyperalgesia (Li et al., 2020).

Cholecystokinin (CCK) is a gut-brain peptide that participates in the pain facilitation system (Noble et al., 1999). The roles of CCK closely related to digestion, pain, stress and depression are mediated by CCK₁ and CCK₂ receptor subtypes. A few studies have shown that CCK contributes to the descending pain facilitatory system from the periaqueductal gray (PAG) and rostral ventromedial medulla (RVM). CCK mediates anxiety-induced hyperalgesia and hyperalgesia associated with peripheral neuropathy by activating the descending pro-nociceptive facilitatory pathways (Lovick, 2008a). CCK activates ON-cells in the RVM selectively and produces behavioral hyperalgesia (Heinricher and Neubert, 2004). Microinjection of CCK into the RVM produces hyperalgesia via activation of CCK₂ receptors, which can be reversed by CCK₂ receptor antagonists (Marshall et al., 2012). Blockade of CCK₂ receptors reduces anxiety-induced hyperalgesia in humans (Benedetti et al., 2006). It has been demonstrated that CCK expressing cells in the dorsal horn of the spinal cord have important roles in mechanical allodynia in neuropathic pain states (Gutierrez-Mecinas et al., 2019). However, whether spinal CCK contributes to the development of the comorbidity of TMD and FMS remains unclear.

A few studies have shown that CCK₂ receptors can activate the extracellular signal-regulated kinase 1/2 (ERK1/2) signal transduction pathway in the digestive system (Grossini et al., 2012; Mao et al., 2014). ERK1/2 is activated in neurons in the dorsal horn of spinal cord in response to stimulation of nociceptors and is involved in nociceptive plasticity (Ji et al., 1999; Liao et al., 2020). However, whether the upregulation of CCK₂ receptors contributes to hyperalgesia via the activation of ERK1/2 is unknown. In our previous study, we demonstrated that ERK activation contributed to visceral hypersensitivity evoked by orofacial inflammation combined with stress (Zhao et al., 2018). Thus, the purpose of this study was to explore the independent and joint contributions of CCK₂ receptors and ERK1/2 in the spinal cord in the somatic hyperalgesia induced by orofacial inflammation combined with stress using an established animal model for comorbidity of TMD and FMS.

2. Materials and methods

2.1. Animals

Female Sprague-Dawley rats weighing 200–250 g (about 10 weeks of age) were used in the present study. The animals were provided by Xi'an Jiaotong University Laboratory Animal Center (Xi'an, Shaanxi, China) and housed in a climate-controlled room on a 12-h light/dark cycle (lights on at 7 a.m.), with food and water available ad libitum. All experiments were approved by the Institutional Animal Care and Use Committees of Xi'an Jiaotong University Health Science Center, China (approved number: 2019-950), and adhered to guidelines for experimental pain in animals published by the International Association for the Study of Pain.

2.2. Experimental protocol

The experimental scheme is shown in Fig. 1. Rats were anesthetized with isoflurane (3%) and ovariectomized (OVx) by a dorsolateral approach (Traub et al., 2014). Ten days after OVx, rats were given a subcutaneous injection of 17- β -estradiol (E2, 50 μ g in 100 μ L safflower oil, Sigma, St Louis, MO, USA) or safflower oil (vehicle, 100 μ L). The E2 injection was repeated every 4 days to mimic the estrous cycle of the rats so that the E2 levels of OVx rats were similar at the same time and the effect of the natural estrous cycle of the rats on the experimental results was excluded (Ji et al., 2003; Traub et al., 2014). Complete Freund's adjuvant (CFA, 150 μ L, 1:1 in saline) or saline (control for CFA injection) was injected into the bilateral masseter muscles. Stress was induced by a 3 day FS paradigm starting the day following CFA injection (Li et al., 2020; Traub et al., 2014). Rats were forced to swim (10 min on the first day, 20 min on the next 2 days) in a cylindrical container (30 cm diameter) filled to 20 cm with 25–26 °C water (Imbe and Kimura, 2015; Imbe et al., 2014; Li et al., 2019; Quintero et al., 2000). After each swim session, the rats were dried with a towel and placed in a cage partially heated with a fan before being returned to their home cage. The control rats for FS remained undisturbed in their home cages. The day after the last FS was designated Day 1 post-stress. All studies employed randomized and vehicle-controlled designs with observers blind to treatment allocation.

2.3. Experimental groups

The expression of CCK receptors and ERK1/2 was investigated in E2 replaced rats having orofacial inflammation combined with stress (E2 + CFA + FS group, i.e. comorbid pain condition, n = 4) and the control group (oil + saline + non-FS group, n = 6). The effects of CCK₂ receptor antagonist YM-022 (E2 + CFA + FS + YM-022 vs. E2 + CFA + FS + saline, n = 7 for each group) and MEK inhibitor PD98059 (E2 + CFA + FS + PD98059 vs. E2 + CFA + FS + DMSO, n = 9 for each group) on the somatic hyperalgesia in the comorbid pain condition were examined. Whether blocking CCK₂ receptor affected the expression of p-ERK1/2 in the spinal cord was also investigated in the comorbid pain condition (E2 + CFA + FS + YM-022 vs. E2 + CFA + FS + saline, n = 6 and 4, respectively). Lastly, whether inhibiting ERK1/2 activation blocked somatic hyperalgesia induced by intrathecal injection of CCK receptor agonist CCK8 was examined (E2 + CCK8 + PD98059 vs. E2 + CCK8 + DMSO, n = 8 and 10, respectively).

2.4. Behavioral tests

Thermal withdrawal latency and mechanical withdrawal threshold were tested 1 and 2 days, respectively, following each E2 or oil injection. This corresponded to days 2–19 after the end of FS (see Fig. 1). Thermal sensitivity was measured as the latency to withdraw from radiant heat directed at the hindpaw (Hargreaves et al., 1988). Rats were placed on a glass plate, individually covered with an inverted clear plastic cage and allowed to acclimate for 30 min before testing. Radiant heat was directed to the plantar surface of the right hindpaw and the stimulus ceased when the rat withdrew its hindpaw and the latency was recorded using a plantar thermal test device (Ugo Basile, Gemonio, Italy). Rats were tested three times with an inter-stimulus interval of 5 min, and the mean value was determined. A cutoff of 20 s was set to avoid tissue damage.

The mechanical withdrawal threshold was measured with a series of calibrated von Frey filaments. Rats were individually placed in a plastic cage with a wire mesh bottom which allowed full access to the hindpaw. After 30 min of adaptation, the von Frey filaments (Stoelting, Wood Dale, IL, USA) with logarithmically incremental stiffness (0.41–26 g) were applied from underneath the wire mesh floor to the plantar surface of the left hindpaw. The cutoff force was 26 g to prevent tissue damage. The mechanical withdrawal threshold was determined by sequentially increasing and decreasing the stimulus strength (“up and down” method) (Chaplan et al., 1994; Marshall et al., 2012).

2.5. Intrathecal injection

Rats were anesthetized with isoflurane and placed in a prone position with a round tube underneath the abdomen. A portion of the caudal half of the rat’s back was shaved and scrubbed with povidone iodine solution. Then a disposable 30-gauge needle connected to a 25- μ l Hamilton syringe was slowly inserted into the intervertebral space between the L4-L5 spinous process and the needle was allowed to penetrate the dura. A quick flick of the tail or hindlimb was a sign that the needle entered the intrathecal space. The CCK₂ receptor antagonist YM-022 (30 μ g/10 μ l), MEK inhibitor PD98059 (2 μ g/10 μ l) or their vehicle was injected in the E2 + CFA + FS rats. The intrathecal injection was administered 30 min before the CFA injection and each FS. In the CCK-induced hyperalgesia experiments, the MEK

inhibitor PD98059 (2 µg/10 µl) or its vehicle was intrathecally injected 30 min before the administration of a CCK receptor agonist (CCK-8, 3 µg/10 µl) daily for 5 days. Drug doses were chosen based on the literature (Fukazawa et al., 2007; Yamamoto and Nozaki-Taguchi, 1996; Zhao et al., 2018) and our preliminary studies.

2.6. Western blot

Two days after the last FS, rats in the Western blot experiments group were anesthetized with isoflurane (5%) and quickly decapitated. The spinal cord was flushed out with ice-cold saline (Cao et al., 2015). The L4 to L5 section of the spinal cord was rapidly isolated and immediately snap frozen on dry ice. The dorsal part of the spinal cord was separated and stored at -80 °C until use. Tissues were homogenized in solubilization buffer (1% NP-40, 1% Sodium deoxycholate, 0.1% SDS) and protease inhibitor cocktail. The homogenates were centrifuged at 10,000 rpm for 10 min at 4 °C. The supernatant was collected and protein concentration was determined using the bicinchoninic acid (BCA) method. Protein samples (18 µg) were separated on 10% SDS-PAGE and blotted onto PVDF membranes after denaturing. The blots were blocked with 5% non-fat milk in Tris-buffered saline (TBS) and incubated with primary antibody directed against CCK₁ receptor (1:1000, Bioss, bs-11514R, Beijing, China), CCK₂ receptor (1:1000, Bioss, bs-1777R), p-ERK1/2 (1:1000, Cell Signaling Technology, Danvers, MA, USA) and GAPDH (1:4000, Boster, BA2913, Wuhan, China) at 4 °C overnight. The membranes were washed with TBST and incubated with goat anti-rabbit secondary antibody (Boster, BA1054) at 1:4000 dilution. The immunoreactivity was detected using an enhanced chemiluminescence (ECL) detection system (Thermo Scientific, Waltham, MA, USA). The immunoreactive band densities were analyzed using Image J software. The blot of p-ERK1/2 was stripped for 30 min and reprobed with antibodies against ERK1/2 (1:1000, Cell Signaling Technology, Danvers, MA, USA) which served as the internal control for p-ERK1/2 and then GAPDH for ERK1/2.

2.7. Drugs

All drugs were dissolved to a final concentration in 10 µL vehicle. The CCK₂ receptor antagonist YM-022 (30 µg, Santa Cruz Biotechnology, Santa Cruz, US) was dissolved in saline. The CCK receptor agonist cholecystokinin octapeptide (CCK-8, 3 µg, Med Chem Express, Monmouth Junction, NJ, USA) and the MEK inhibitor PD98059 (2 µg, Sigma, St Louis, MO, USA) were dissolved in 4% DMSO. Saline and DMSO have no significant effect on the nociceptive information transmission in the spinal cord.

2.8. Data analysis

All data are presented as mean ± SEM. Statistical and figure analyses were performed using GraphPad Prism 6 software. The data of protein expression of CCK receptors, ERK1/2, and p-ERK1/2 between two groups were compared using unpaired *t*-test. Two-way repeated measures analysis of variance (ANOVA) followed by Sidak post hoc test was used for thermal withdrawal latency and mechanical withdrawal threshold. *P* < 0.05 was considered significant.

3. Results

3.1. CCK₂ receptors are involved in the somatic hyperalgesia in the comorbid pain condition

3.1.1. The comorbid pain condition significantly increases the expression of CCK₂ receptors—We previously reported that E2, CFA or FS alone had no effect on somatic hyperalgesia relative to baseline, but E2 + CFA + FS induced thermal and mechanical hyperalgesia in the hindpaws (Li et al., 2020). Therefore, the control group for comparison was the oil + saline + non-FS group.

To determine whether spinal CCK receptors mediate somatic hyperalgesia in the comorbid pain condition, we examined the protein expression of CCK₁ and CCK₂ receptors in the L4-L5 spinal dorsal horn. There were no significant differences in the expression of CCK₁ receptors in the E2 + CFA + FS group compared with the oil + saline + non-FS group ($P = 0.7161$, Fig. 2A). In contrast, the expression of CCK₂ receptors significantly increased in the E2 + CFA + FS group ($P = 0.0279$ vs. the oil + saline + non-FS group, $n = 4$ and 6 , respectively, Fig. 2B), suggesting that CCK₂ receptors, but not CCK₁ receptors, are upregulated in the spinal cord in the comorbid pain condition.

3.1.2. CCK₂ receptor antagonist YM-022 blocks the hyperalgesia in the comorbid pain condition—Next, it was determined if a CCK₂ receptor antagonist could prevent the somatic hyperalgesia in the comorbid pain condition. YM-022 or saline was injected intrathecally for 5 consecutive days (before CFA injection, before each FS and before injection of E2). Two way ANOVA revealed a significant effect of YM-022 on the thermal withdrawal latency compared with saline treated rats ($F_{5,60} = 8.311$, $P < 0.0001$ for interaction; $F_{5,60} = 6.611$, $P < 0.0001$ for time factor; $F_{1,12} = 15.15$, $P = 0.0021$ for group factor, $n = 7$ for each group, Fig. 3A) and mechanical withdrawal threshold ($F_{5,60} = 4.427$, $P = 0.0017$ for interaction; $F_{5,60} = 12.16$, $P < 0.0001$ for time factor; $F_{1,12} = 3.83$, $P = 0.0740$ for group factor, Fig. 3B). In the E2 + CFA + FS + saline group, thermal hyperalgesia persisted from Day 2 to Day 6 post FS ($P < 0.0001$ and $P = 0.0204$ for Day 2 and Day 6 vs. Baseline, respectively, Fig. 3A), and mechanical hyperalgesia lasted from Day 3 to Day 11 post FS ($P < 0.0001$ for Day 3 and Day 7 vs. Baseline, $P = 0.0045$ for Day 11 vs. Baseline, Fig. 3B).

In contrast, YM-022 blocked the reduction in the thermal withdrawal latency in the E2 + CFA + FS + YM-022 group compared with the E2 + CFA + FS + saline group ($P > 0.05$ for each time point vs. Baseline). Comparing the YM-022 group vs. saline at each time point, the thermal withdrawal latency ($P < 0.0001$ and $P = 0.0089$ for Day 2 and Day 6, respectively, Fig. 3A) and mechanical withdrawal threshold ($P = 0.0147$ and 0.0286 for Day 3 and Day 7, respectively, Fig. 3B) in hindpaws in the E2 + CFA + FS + YM-022 group were significantly different from that of the E2 + CFA + FS + saline group from Day 1 to Day 7 after FS stress. These results showed that CCK₂ receptor antagonist YM-022 prevented the hyperalgesia in the comorbid pain condition, suggesting that CCK₂ receptors in the spinal cord contribute to the hyperalgesia.

3.2. Activation of ERK1/2 is involved in the somatic hyperalgesia in the comorbid pain condition

3.2.1. The comorbid pain condition significantly increases the expression of p-ERK1/2—To explore the role of ERK1/2 in the somatic hyperalgesia, we examined the protein expression of ERK1/2, p-ERK1/2 in the L4-L5 spinal dorsal horn in the comorbid pain condition. There were no significant differences in the expression of ERK1 ($P = 0.7450$) and ERK2 ($P = 0.6870$) between the E2 + CFA + FS and oil + saline + non-FS groups ($n = 4$ and 6 respectively, Fig. 4A and B). However, E2 + CFA + FS significantly increased the expression of p-ERK1 ($P = 0.0081$, Fig. 4C) and p-ERK2 ($P = 0.0017$, Fig. 4D) in the spinal cord compared with the oil + saline + non-FS group.

3.2.2. MEK inhibitor PD98059 prevents somatic hyperalgesia in the comorbid pain condition—To further verify the role of ERK1/2 in the somatic hyperalgesia, we explored whether MEK inhibitor PD98059 could prevent somatic hyperalgesia in the comorbid pain condition through blocking the activation of ERK1/2. There were significant differences between the E2 + CFA + FS + PD98059 group and E2 + CFA + FS + DMSO group in the thermal withdrawal latency ($F_{5,80} = 3.219$, $P = 0.0107$ for interaction; $F_{5,80} = 1.122$, $P = 0.3553$ for time factor; $F_{1,16} = 3.617$, $P = 0.0754$ for group factor, $n = 9$ for each group, Fig. 5A) and mechanical withdrawal threshold ($F_{5,75} = 5.319$, $P = 0.0003$ for interaction; $F_{5,75} = 4.255$, $P = 0.0018$ for time factor; $F_{1,15} = 25.2$, $P = 0.0002$ for group factor, Fig. 5B). Post-hoc tests showed that the thermal hyperalgesia persisted over 6 days post FS compare with Baseline ($P = 0.0031$ for Day 2, $P = 0.0398$ for Day 6, Fig. 5A) and the mechanical allodynia persisted over 15 days ($P = 0.0001$ for Day 3, $P < 0.0001$ for Day 7, $P = 0.0004$ for Day 11, $P = 0.0003$ for Day 15, Fig. 5B) in the E2 + CFA + FS + DMSO group. No thermal hypersensitivity or mechanical allodynia was observed compared with Baseline in the E2 + CFA + FS + PD98059 group ($P > 0.05$ for each time point compared with Baseline). In addition, when comparing the values in each time point between the two groups, the mechanical withdrawal threshold in the E2 + CFA + FS + DMSO group was significantly lower than that in the E2 + CFA + FS + PD98059 group from day 3 to day 15 post FS ($P < 0.0001$ for all time points, Fig. 5B). These results indicate that ERK1/2 activation participates in the somatic hyperalgesia in the comorbid pain condition.

3.3. ERK1/2 signaling pathway mediates CCK-induced somatic hyperalgesia in the spinal cord

3.3.1. CCK receptor agonist CCK8 induces somatic hyperalgesia and the hyperalgesia is blocked by inhibiting ERK1/2 activation—Since the expressions of CCK₂ receptors and p-ERK1/2 in the L4-L5 spinal dorsal horn were significantly up-regulated in the E2 + CFA + FS group compared with the oil + saline + non-FS group, we hypothesized that there existed a CCK-ERK signaling pathway in which CCK₂ receptors might activate ERK1/2 to produce somatic hyperalgesia in the comorbid pain condition. We examined whether the MEK inhibitor PD98059 could block ERK1/2 signaling cascade induced by the CCK receptor agonist CCK8. There were significant differences in the thermal withdrawal latency ($F_{5,80} = 26.42$, $P < 0.0001$ for interaction; $F_{5,80} = 35.6$, $P < 0.0001$ for time factor; $F_{1,16} = 112.7$, $P < 0.0001$ for group factor, Fig. 6A) and mechanical withdrawal threshold ($F_{5,80} = 4.508$, $P = 0.0011$ for interaction; $F_{5,80} = 2.283$, $P = 0.0541$

for time factor; $F_{1,16} = 0.1008$, $P = 0.7550$ for group factor, Fig. 6B) between the E2 + CCK8 + DMSO ($n = 10$) and E2 + CCK8 + PD98059 groups ($n = 8$). Intrathecal injection of CCK8 daily for 5 days produced thermal hyperalgesia starting from Day 2 to Day 10 ($P < 0.0001$ for Day 2 and Day 6 vs. Baseline, $P = 0.0423$ for Day 10 vs. Baseline, Fig. 6A) and mechanical allodynia appeared on day 3 ($P < 0.0001$ vs. Baseline, Fig. 6B) in the E2 + CCK8 + DMSO group. No thermal hyperalgesia or mechanical allodynia was observed in the E2 + CCK8 + PD98059 group in which PD98059 was injected 30 min before each CCK8 injection ($P > 0.05$ for all time points vs. Baseline). In addition, when comparing the values in each time point between the two groups, the thermal withdrawal latency ($P < 0.0001$ for Day 2 and Day 6, $P = 0.0024$ for Day 10 and $P = 0.0197$ for Day 14, Fig. 6A) and mechanical withdrawal threshold ($P < 0.0001$ for Day 2, Fig. 6B) in the E2 + CCK8 + PD98059 group significantly increased compared with the E2 + CCK8 + DMSO group from Day 2 to Day 14. These findings suggest that spinal administration of the CCK receptor agonist CCK8 induces somatic hyperalgesia and the hyperalgesia can be blocked by inhibiting ERK1/2 activation.

3.3.2. Intrathecal injection of CCK₂ receptor antagonist YM-022 decreases p-ERK1/2 expression in the spinal cord—The effects of intrathecal injection of CCK₂ receptor antagonist on the activation of ERK1/2 in the L4-L5 spinal cord were examined. The Western blot data showed that there were no significant differences in the expression of ERK1 ($P = 0.2060$) and ERK2 ($P = 0.0988$) in the E2 + CFA + FS + YM-022 group ($n = 4$) compared with the E2 + CFA + FS + saline group ($n = 6$, Fig. 7A and B). However, intrathecal injection of CCK₂ receptor antagonist YM-022 caused a significant reduction of p-ERK1 and p-ERK2 expression ($P = 0.0089$ and 0.0298 , respectively, Fig. 7C and D) in the L4-L5 spinal cord in the E2 + CFA + FS + YM-022 group compared with the E2 + CFA + FS + saline group. The behavioral results combined with the Western blot data indicate that CCK-induced somatic hyperalgesia is mediated by the ERK1/2 signaling pathway in the spinal cord.

4. Discussion

In the present study, we aimed to analyze the independent and joint contributions of spinal CCK₂ receptors and ERK1/2 activation to the somatic hyperalgesia in the comorbid pain condition. We demonstrated that CCK₂ receptors in the spinal cord were involved in the regulation of central sensitization in the comorbid pain condition by activating the ERK1/2 pathway.

In our previous study, we found that stress induced visceral hypersensitivity and mechanical allodynia in the lower back of female rats with preexisting orofacial inflammation (Traub et al., 2014). We used the same inflammation plus stress paradigm in the current study to simulate TMD and FMS comorbidity (Li et al., 2020). The perception of pain is a dynamic process that is subject to ongoing modulation by descending inhibition and descending facilitation. Accumulating evidence shows that descending facilitation of spinal nociception is the main factor in central sensitization and secondary hyperalgesia (Baron et al., 2013; Liu et al., 2018). Descending facilitation has been studied and characterized in animal models of pain and hyperalgesia/allodynia. Therefore, the somatic hyperalgesia caused by orofacial

inflammation combined with repeated FS stress may be associated with a dysregulation in descending pain modulation, both inhibitory and facilitatory, from the midbrain and brainstem. Disrupting the balance of descending modulatory circuits to favor facilitation and impair inhibition may promote and maintain the spreading pain (Li et al., 2020; Xue et al., 2020; Xu et al., 2020). Our results are agreement with the previous studies. For example, repeated stimulation of meningeal nociceptors by injecting an inflammatory soup in rats, an animal model of chronic migraine, leads to facial and hindpaw mechanical allodynia. Such wide-spreading central sensitization have been demonstrated to require both the activation of descending facilitation from the RVM (Edelmeyer et al., 2009) and impairment of diffuse noxious inhibitory controls (Boyer et al., 2014).

The descending facilitation system consists of the PAG, RVM, anterior cingulate cortex (ACC), hypothalamus, amygdala, and nucleus of the solitary tract and there are reports indicating that CCK plays an important role in the descending facilitatory pathway. There is a close relationship between CCK and a few brain nuclei involved in the composition and regulation of the descending facilitation pathway. For example, pain-related behaviors and CCK release in the ACC significantly increased in rats with carrageenan-induced arthritis compared with the control animals (Heilborn et al., 2007). In addition, the PAG of both rodents and primates is rich in CCK₂ receptors and CCK-containing neuronal cell bodies (Mercer et al., 2000). CCK₂ receptor agonists excite PAG neurons by inhibiting K⁺ channels and increasing calcium influx, which in turn leads to increased glutamate release and eventually hyperalgesia (Yang et al., 2006). Similar to the PAG, the nociceptive facilitatory system may modulate tactile allodynia and thermal hyperalgesia, through increased activity of CCK within the RVM (Kovelowski et al., 2000). Both direct injection into the RVM and intrathecal injection of CCK₂ receptor antagonists produce anti-nociception in neuropathic pain rats, presumably by interrupting transmission in the pain facilitatory pathway (Coudoré-Civiale et al., 2000; Inoue et al., 2017; Kovelowski et al., 2000; Xie et al., 2005). In the spinal dorsal horn, CCK is found mainly in interneurons and terminals of descending fibers, and receptor binding sites for CCK are located throughout the spinal dorsal horn with the highest density in the superficial laminae (Ghilardi et al., 1992). After spinal hemisection at the T13 level, CCK mRNA expression increased on both sides of the L4-L5 segments of spinal cord and intrathecal injection of a CCK₂ receptor antagonist dose-dependently increased the paw withdrawal threshold in both paws (Kim et al., 2009). The present study showed that the expression of CCK₂ receptors, but not CCK1 receptors, significantly increased in the L4-L5 spinal dorsal horn following orofacial inflammation plus stress. Intrathecal administration of the selective CCK₂ receptor antagonist significantly reduced the thermal hyperalgesia and mechanical allodynia in the hindpaws, suggesting that CCK₂ receptors mediate the somatic hyperalgesia.

It has been shown that activation of CCK₂ receptor related signaling leads to ERK phosphorylation (Grossini et al., 2012; Mao et al., 2014). ERK1/2 mediates intracellular signal transduction in response to various stimuli. Some evidence suggests that ERK1/2 is activated in neurons in the spinal dorsal horn in response to the stimulation of nociceptors and that ERK signaling is involved in nociceptive plasticity (Pezet et al., 2002a, 2002b). Acute noxious stimuli, such as formalin or capsaicin, induce ERK phosphorylation in spinal dorsal horn neurons (Ji et al., 1999). Intrathecal injection of the MEK inhibitor PD98059

reduces acute pain behavior after subcutaneous injection of formalin (Karim et al., 2001). In the previous study, we demonstrated that orofacial inflammation followed by 3 day FS stress induced ERK1/2 activation in the lumbosacral spinal segments in female rats, and intrathecal administration of the MEK inhibitor PD98059 blocked the visceral hypersensitivity induced by orofacial inflammation followed by stress, indicating that ERK1/2 activation contributes to the visceral hypersensitivity (Zhao et al., 2018). Therefore, we investigated whether ERK1/2 activation in the spinal cord contributed to the somatic hyperalgesia in this comorbid pain condition. We found that the expression of p-ERK1/2 in the spinal dorsal horn of rats in the E2 + CFA + FS group was significantly higher than that in the oil + saline + non-FS group. Intrathecal injection of a MEK inhibitor blocked the somatic hyperalgesia in the comorbid pain condition, suggesting that spinal ERK1/2 activation plays an important role in the development of somatic hyperalgesia in the comorbidity of TMD and FMS.

Since both CCK₂ receptors and ERK1/2 activation in the spinal cord are involved in the regulation of somatic hyperalgesia in the comorbid pain condition, we speculate whether there is a close relationship between CCK and ERK1/2 in the spinal cord. To determine the effect of ERK1/2 activation on the function of CCK₂ receptors, rats were intrathecally injected with the CCK receptor agonist CCK8 in the E2 + CCK8 + DMSO group. The resulting thermal hyperalgesia and mechanical allodynia in the hindpaws were similar to the model of TMD and FMS comorbidity. The CCK-8-induced hyperalgesia was blocked by intrathecal injection of the MEK inhibitor PD98059, inhibiting the activation of ERK1/2. These results are consistent with the fact that pharmacological inhibition of ERK1/2 reverses the increase of BDNF expression evoked by CCK-8 (Hwang et al., 2013), suggesting that CCK exerts pro-nociceptive properties by binding to the CCK₂ receptors and ERK1/2 activation may participate in the reaction as a downstream signaling molecule of CCK. To investigate the effect of CCK₂ receptors on ERK1/2 activation, rats in the comorbid pain group were intrathecally injected with the CCK₂ receptor antagonist YM-022. It was found that the expression of p-ERK1/2 in the L4-L5 segments of the spinal cord was significantly reduced compared with the E2 + CFA + FS + saline group, which further confirmed that CCK₂ receptors enhanced the activation of ERK1/2 and participated in the process of somatic hyperalgesia in the comorbid pain condition. Thus, in the spinal cord, there is a CCK-ERK1/2 signaling pathway leading to hyperalgesia.

Taken together, we hypothesize that orofacial inflammation combined with FS stress activates the descending facilitation system. Complex networks of pathways projected from various brain structures integrate to affect the spinal processing of nociceptive input. The descending facilitatory system from the midbrain and brainstem promotes CCK binding to CCK₂ receptors on the membrane of neurons in the spinal cord, and further stimulates the activation of ERK1/2 through Ras-Raf-MEK1/2-ERK1/2 pathway, increasing spinal excitability causing somatic hyperalgesia.

5. Conclusion

In the current study, we demonstrated that orofacial inflammation combined with stress affected spinal plasticity and led to a decrease in the thermal withdrawal latency and mechanical nociceptive threshold by activating CCK₂ receptors. Intrathecal injection of

CCK₂ receptor antagonist prevented the somatic hyperalgesia by affecting the activation of ERK1/2. Therefore, these data may provide a therapeutic target to treat TMD and FMS comorbidity in the clinic.

Acknowledgments

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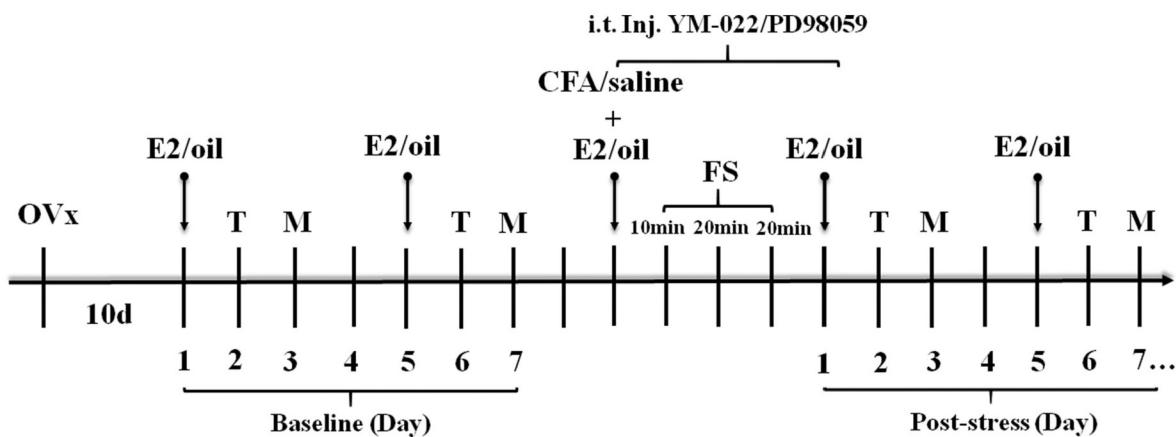


Fig. 1. Experimental design. All rats were ovariectomized (Ovx). After 10 days of recovery, 17- β -estradiol (E2) or oil was injected subcutaneously every 4 days. The baselines of thermal withdrawal latency (T) and mechanical withdrawal threshold (M) were tested twice to ensure the reproducibility. The average of two baseline values was used for analysis. The same day as an E2/oil injection the bilateral masseter muscles were injected with complete Freund’s adjuvant (CFA) and a 3 day forced swim (FS) protocol was initiated the following day. E2/oil injections continued every 4th day starting one day after the last FS. The thermal withdrawal latency and mechanical withdrawal threshold were measured 1 and 2 days following each E2/oil injection, respectively. The measurements were continued until the thermal withdrawal latency or mechanical withdrawal threshold returning to the baseline level.

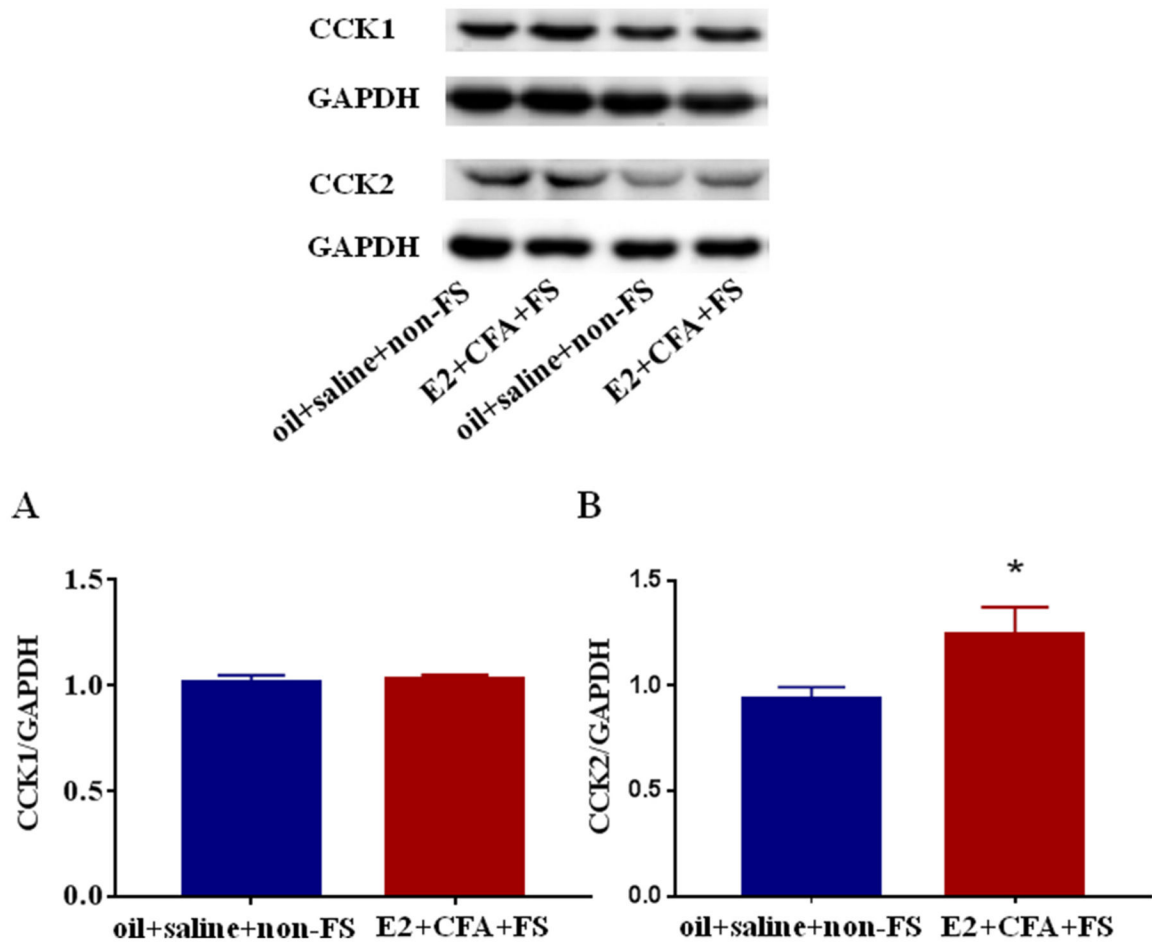


Fig. 2. The expression of CCK₁ receptors (A) and CCK₂ receptors (B) in the L4-L5 spinal dorsal horn in the comorbid pain condition. The expression of CCK₂ receptors in the E2 + CFA + FS group (n = 4) was significantly increased compared with the oil + saline + non-FS group (n = 6). The expression of CCK₁ receptors in the E2 + CFA + FS group did not significantly change. * $P < 0.05$ vs. the oil + saline + non-FS group.

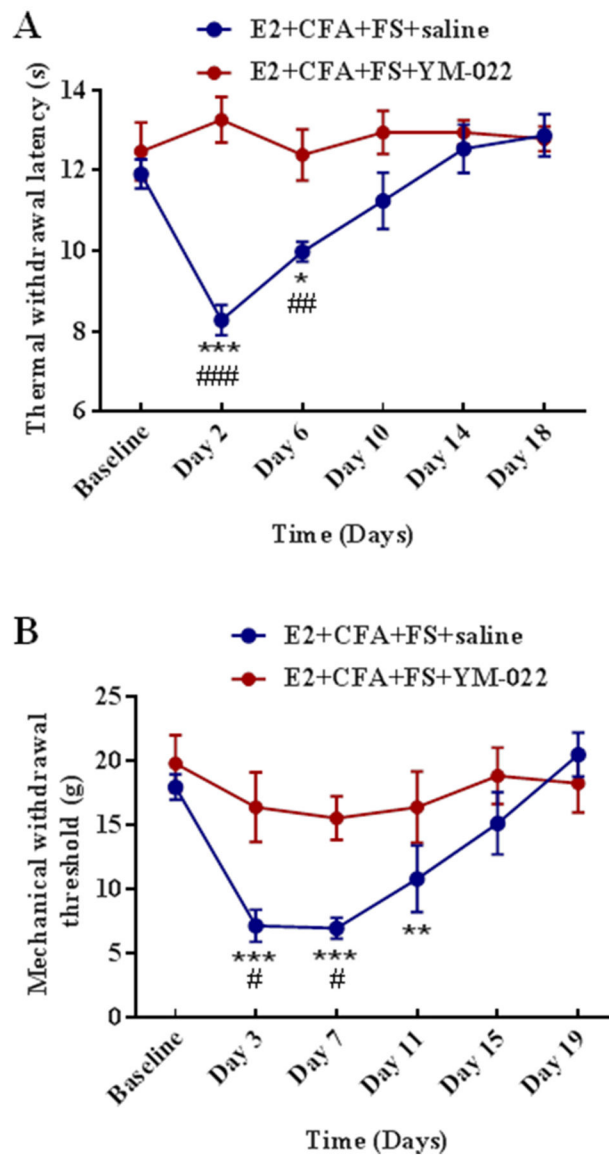


Fig. 3. CCK₂ receptors are involved in the somatic hyperalgesia in the comorbid pain condition. (A) YM-022 inhibited the thermal hyperalgesia. The thermal withdrawal latency significantly decreased at Day 2 and Day 6 post FS in saline treated rats. *, *** $P < 0.05, 0.001$ vs. Baseline. YM-022 blocked the thermal hyperalgesia compared with saline. ##, ### $P < 0.01, 0.001$ vs. the E2 + CFA + FS + YM-022 group at the same time point. $n = 7$ for each group. (B) YM-022 inhibited mechanical hyperalgesia. The mechanical withdrawal threshold significantly decreased at Day 3, 7 and 11 post FS in saline treated rats. **, *** $P < 0.01, 0.001$ vs. Baseline. YM-022 blocked the mechanical hyperalgesia compared with saline. # $P < 0.05$ vs. the E2 + CFA + FS + YM-022 group at the same time point.

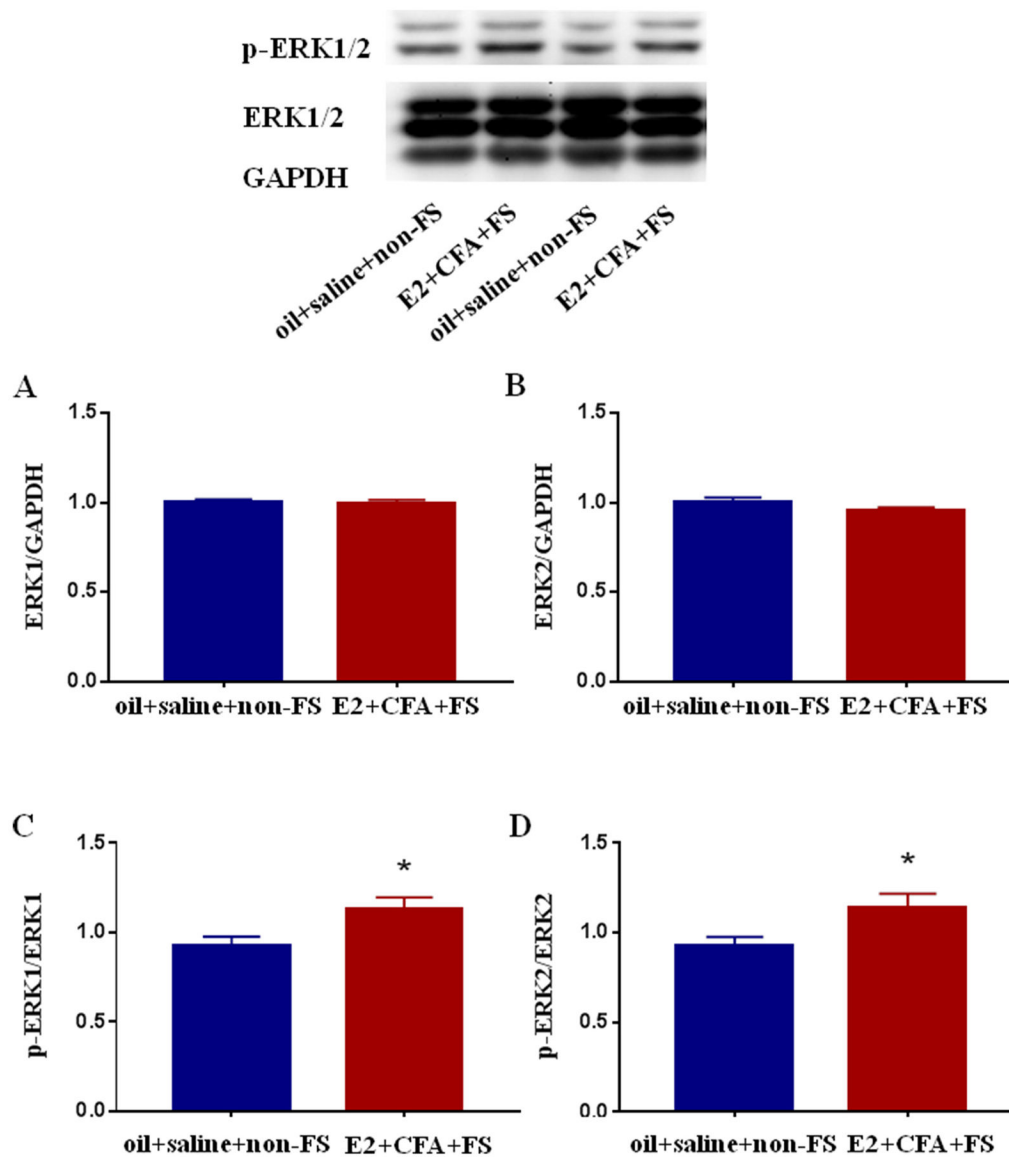


Fig. 4.

The expression of ERK1/2 (A, B) and p-ERK1/2 (C, D) in the L4-L5 spinal dorsal horn in the comorbid pain condition. The expression of ERK1/2 in the E2 + CFA + FS group (n = 4) did not significantly change compared with the oil + saline + non-FS group (n = 6). The expression of p-ERK1/2 in the E2 + CFA + FS group significantly increased compared with the oil + saline + non-FS group. Data were normalized to the oil + saline + non-FS group. * $P < 0.05$ vs. the oil + saline + non-FS group.

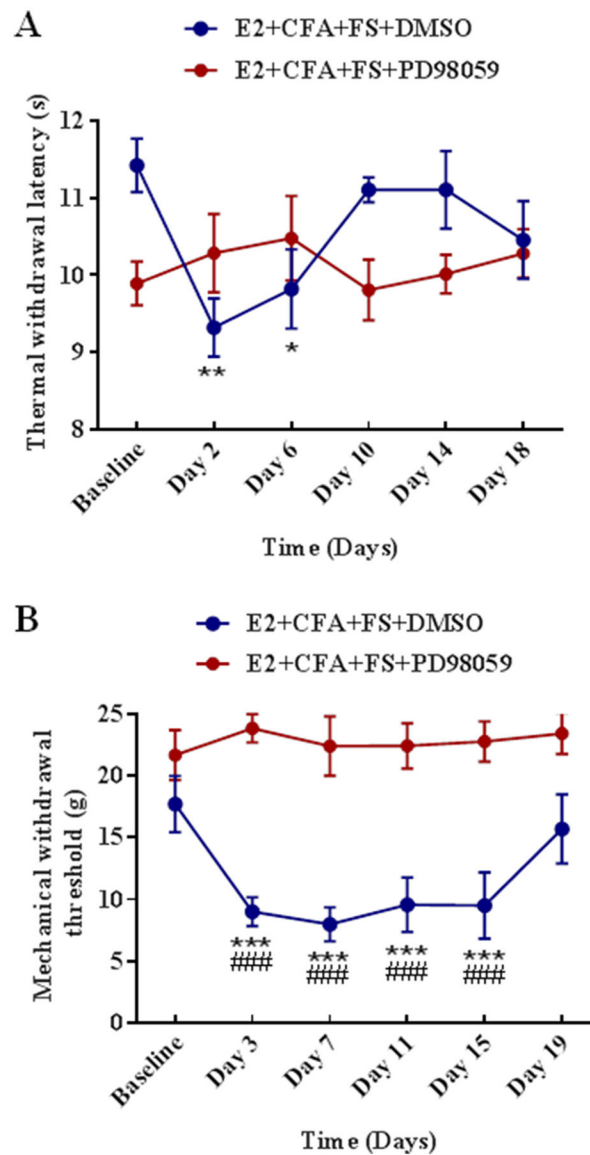


Fig. 5. The activation of ERK1/2 is involved in the somatic hyperalgesia in the comorbid pain condition. (A) PD98059 inhibited the thermal hyperalgesia. $n = 9$ for each group. *, ** $P < 0.05, 0.01$ vs. Baseline. (B) PD98059 inhibited the mechanical hyperalgesia. *** $P < 0.001$ vs Baseline. The mechanical withdrawal threshold significantly decreased at Day 3, 7, 11, 15 post stress in DMSO treated rats compared with PD98059 treated. #### $P < 0.001$ vs. the E2 + CFA + FS + PD98059 group at the same time point.

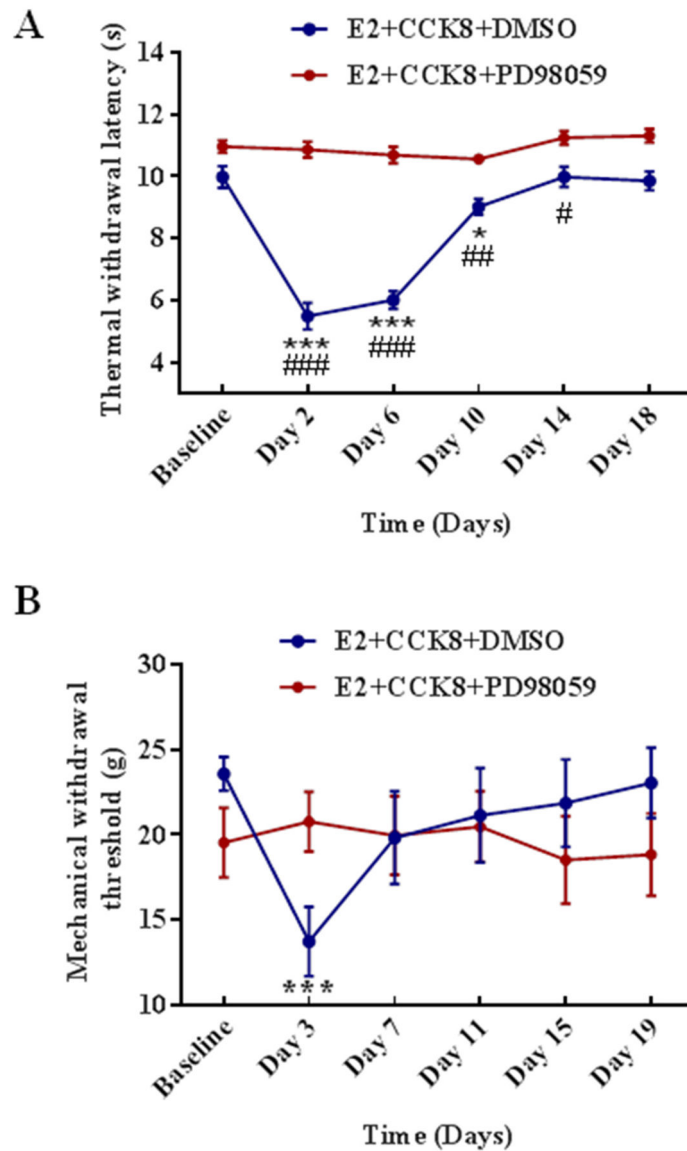


Fig. 6. The effect of ERK1/2 signaling cascade blocking on the function of CCK receptor agonist. (A) PD98059 inhibited thermal hyperalgesia induced by CCK8. CCK8 decreased the thermal withdrawal latency for 10 days in the E2 + CCK8 + DMSO rats ($n = 10$) compared with Baseline, and PD98059 ($n = 8$) blocked the decrease in the thermal withdrawal latency. *, *** $P < 0.05, 0.001$ vs. Baseline. The thermal withdrawal latency significantly decreased at Day 2, 6, 10, 14 post injection of CCK8 in the E2 + CCK8 + DMSO rats compared with PD98059 treated. #, ##, ### $P < 0.05, 0.01, 0.001$ vs. the E2 + CCK8 + PD98059 group at the same time point. (B) PD98059 inhibited mechanical hyperalgesia induced by CCK8. CCK8 decreased the mechanical withdrawal threshold for 3 days in the E2 + CCK8 + DMSO group compared with Baseline, and PD98059 blocked the decrease in the mechanical withdrawal threshold. *** $P < 0.001$ vs. Baseline.

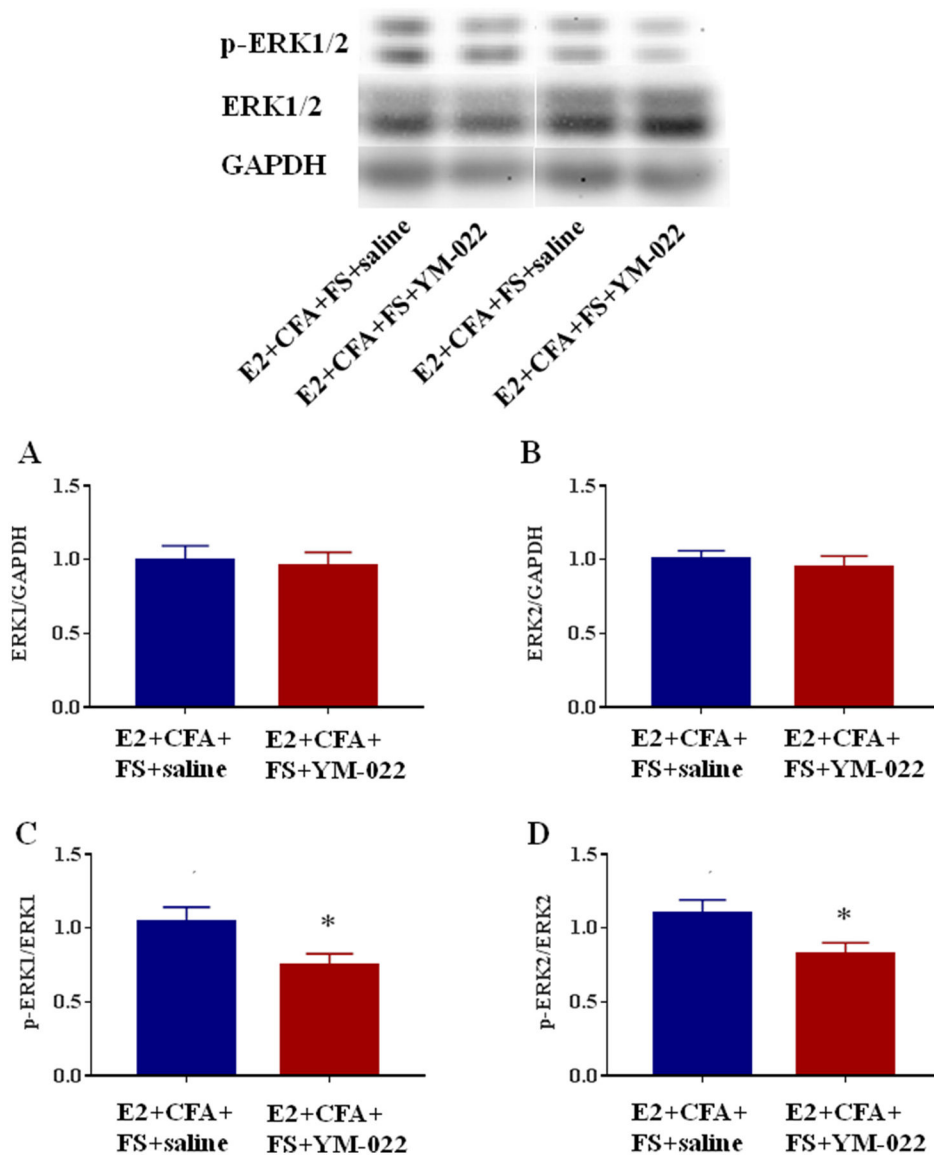


Fig. 7. The effect of intrathecal injection of CCK₂ receptor antagonist on the activation of ERK1/2 in the spinal cord. The expression of ERK1/2 in the L4-L5 spinal dorsal horn in the E2 + CFA + FS + YM-022 group (n = 4) did not significantly change compared with the E2 + CFA + FS + saline group (n = 6) (A, B), but the expression of p-ERK1/2 in the E2 + CFA + FS + YM-022 group significantly decreased than that in the E2 + CFA + FS + saline group (C, D). * $P < 0.05$ vs. the E2 + CFA + FS + saline group.