Acupuncture and Herbal Medicine

Electroacupuncture participates in pain transition through the KCC2/GABAAR pathway in the spinal dorsal horn of male rats

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Abstract

Objective: Preventing the transition from acute to chronic pain (pain transition) is a new strategy for treating chronic pain. The present study aimed to investigate the role of K^+-Cl^- Cotransporter Isoform 2 (KCC2) and γ-aminobutyric acid receptor type A (GABAAR) in the spinal cord dorsal horn (SCDH) in pain transition and the intervention effect of electroacupuncture (EA), and to understand the mechanism of EA in preventing acute and chronic pain transition in the spinal center.

Methods: A rat model of hyperalgesic priming (HP) was established by injecting carrageenan (Car) into the plantar area of rats, followed by the injection of prostaglandin E_2 (PGE_2) into the dorsal foot 7 days later. The GABAAR agonist (muscimol) and KCC2 activator (CLP257) were intrathecally injected for three consecutive days after PGE_2 injection. EA was applied at a frequency of 2/100 Hz to the bilateral foot Zusanli (ST36) and Kunlun (BL60). A von Frey filament was used to detect the pain threshold in each group of rats. Western blotting (WB) and immunofluorescence (IF) were used to detect GABAAR and KCC2 expression in each rats group. By combining EA intervention with a KCC2 inhibitor (VU0240551), we explored the mechanism of pain transition of EA regulation of GABAAR and KCC2 expression in SCDH.

Results: The HP model was established by injecting mice with Car/PGE_2. Compared to the normal saline (NS) + NS and NS + PGE_2 groups, the pain threshold of the Car + PGE_2 group decreased significantly 48 hours after PGE_2 injection ($P < 0.01$). The WB results indicated that intrathecal injection of a GABAAR agonist upregulated GABAAR expression in the SCDH of HP model rats ($P < 0.05$). WB and IF results revealed that intrathecal injection of the KCC2 activator significantly increased GABAAR and KCC2 expression in the SCDH of HP model rats ($P < 0.01$) and that GABAAR and KCC2 were co-expressed in the same SCDH cells. Compared to the Car + PGE_2 group, EA intervention significantly increased MWTs from 48 to 72 hours after the first injection and 4, 24, and 48 hours after the second injection ($P < 0.01$). EA upregulated GABAAR and KCC2 expression in the SCDH of rats with HP ($P < 0.05$). Intrathecal injection of the KCC2 inhibitor blocked the analgesic effect of EA in HP model rats ($P < 0.01$).

Conclusions: In SCDH, KCC2 expression was downregulated, causing downregulation of GABAAR expression and resulting in pain transition. EA upregulates KCC2 and GABAAR expression and prevents pain transition.

Keywords: γ-Aminobutyric acid receptor type A, Electroacupuncture, Hyperalgesic priming, K^+-Cl^-cotransporter isoform 2, Spinal dorsal horn

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Introduction

Pain is not only one of the most common clinical symptoms but it is also identified by the World Health Organization (WHO) as the "fifth vital sign" of the human body. In June 2018, chronic pain was listed as an independent disease in the 11th revision of the International Classification of Diseases (ICD-11) by the WHO for the first time. Chronic pain is a difficult but important challenge in clinical treatment, owing to its serious side effects and complex pathogenesis. Modern studies have found that when pain transitions from acute to chronic (pain transition), its mechanism changes substantially, leading to a decrease in the efficacy of existing drugs and an increase in the difficulty of treatment[1–3]. Blocking the occurrence of pain transitions has recently become a new direction for the

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treatment of chronic pain and is a popular topic in pain treatment research[4–5].

γ-Aminobutyric acid receptor type A (GABAAR) is an important neuronal inhibitory receptor that plays a key role in pain regulation and transmission. Under physiological conditions, GABAAR activation can cause neuronal hyperpolarization and play a role in analgesia[6–7]. A close relationship has been found between GABAAR expression and pain[8]. A reduction in GABAAR inhibitory function in spinal cord dorsal horn (SCDH) neurons is an important low-center mechanism of pain transition[9]. GABAergic disinhibition also occurs after chronic morphine treatment[10]. Furthermore, gabapentin, a drug currently recommended as the first-line option for neuropathic pain, causes tolerance to analgesics in post-stroke pain after continuous use for 2 weeks[11]. It is widely accepted that impaired inhibitory control in pain-related neural circuits of the SCDH is responsible for various forms of chronic pain. Owing to impaired inhibitory transmission in SCDH after neuropathic injury, the feedforward disynaptic inhibition of Aβ-fiber is removed, causing mechanical allodynia[12]. In particular, GABAergic inhibition normally separates superficial laminae I and II, to which peripheral noxious information is transmitted from laminae III and IV by suppressing the activity of pre-existing excitatory synaptic circuits[13–15]. However, the mechanism by which nociceptive stimuli regulate GABAAR inhibitory function in SCDH remains unclear.

Neuroscientific studies have indicated that during the GABAAR-mediated inhibitory neurotransmission process, maintaining a low intracellular Cl^- concentration is necessary to exert a normal inhibitory effect[16]. Previous investigations have indicated that pizotifen alleviates neuropathic and inflammatory pain in mice by enhancing GABAergic inhibition[17]. Among them, K^+-Cl^-cotransporter isoform 2 (KCC2) plays a key role in maintaining neuronal chloride homeostasis and GABAAR-mediated neuronal inhibition[18]. Downregulation of KCC2 blocks Cl^- efflux from neurons, which weakens the neural inhibitory effect of GABAAR and induces excessive neuronal excitation[19]. These studies suggest that KCC2 is one of the important "switches" that determine the inhibitory effect of GABAAR[20–21]. Downregulation of KCC2 expression in SCDH has been observed in several chronic pain models[22–24]. KCC2 is responsible for maintaining the low intracellular chloride concentration, which is essential for the hyperpolarizing effect of GABAAR-mediated inhibition. Dysfunction of either KCC2 or GABAAR can lead to a decrease in inhibitory neurotransmission, resulting in enhanced pain perception. However, it remains unclear whether the downregulation of KCC2 expression in SCDH mediates changes in GABAAR expression, leading to the development of pain transition.

Electroacupuncture (EA) is one of the most widely used clinical applications of acupuncture. This has a certain degree of analgesic effect on various acute and chronic pain conditions[25–26], In clinical practice, 2/100 Hz EA has a good therapeutic effect on chronic pain[27]. Previous studies have focused on demonstrating the therapeutic effects of EA on acute or chronic pain; however, whether EA has an interventional effect on the transition from acute to chronic pain remains unclear. The hyperalgesic priming (HP) model is currently the main animal model used to study the mechanisms and interventions of pain transitions in pain research. Our previous study found that EA at Zusanli (ST36) and Kunlun (BL60) not only has an excellent analgesic effect but can also interfere with pain transition[28–30]. Our previous studies confirmed that EA regulated the expression of GABAAR and inhibited PKCε activation in the DRG[31] to exert analgesic effects[32–33]. Simultaneously, it was confirmed that EA could upregulate the expression of KCC2 in SCDH of rats with chronic pain[34]. These results suggest that EA upregulates the expression of KCC2 and GABAAR in SCDH, thus playing a regulatory role in pain transition in rats with chronic pain.

The present study aimed to establish an HP model and continuously observe the mechanical withdrawal thresholds (MWTs) of the affected foot withdrawal reflex to detect the expression of KCC2 and GABAAR in the SCDH of HP model rats. The study aimed to explore the therapeutic effects and mechanisms of EA on chronic pain from a new perspective to provide new research ideas for the clinical treatment of chronic pain using acupuncture.

Materials and methods

Experimental materials

Experiment with animals

All experimental procedures were approved by the Animal Care and Welfare Committee of Zhejiang University of Chinese Medicine, Zhejiang Province, China (approval number: IACUC-20180319-12). Male Sprague Dawley (SD) rats (weighing 200–230 g) used in this study were obtained from the Shanghai Laboratory Animal Center of the Chinese Academy of Sciences (SCXK [Hu] 2018-0006) and housed at the Laboratory Animal Center of Zhejiang Chinese Medical University (SYXK [Zhejiang] 2018-0012). All rats were fed a standard rodent diet (five per cage) and cycled regularly in the dark. To reduce the impact of individual differences on the experiment, the animals in the group were measured for the basic pain threshold before the experiment, and the rats with too high or too low pain thresholds were excluded to ensure that the basic pain threshold of the animals was close.

Main experimental reagents

Carrageenan (Car) and prostaglandin E_2 (PGE_2) were purchased from Sigma-Aldrich (USA). The GABAAR agonist muscimol (A13459), KCC2 activator CLP257 (94725), and KCC2 inhibitor VU0240551 (ab254523) were purchased from Adooq Bioscience (USA), Cell Signaling Technology (USA), and Abcam (UK), respectively. A stock solution of PGE_2 (1 μg/μL) was prepared in 10% ethanol and dissolved in normal saline (NS) at a concentration of 100 ng/25 μL immediately before injection. Car was dissolved in NS at a concentration of 2% and stored. The GABAAR agonist muscimol was dissolved in NS to prepare a solution of 0.1 μg/μL, the KCC2 activator CLP257 was dissolved in dimethyl sulfoxide (DMSO) to prepare a solution of 1 μg/μL, and the KCC2 inhibitor VU0240551 was dissolved in DMSO to prepare a solution of 0.1 μg/μL.

Experimental methods

Experiment grouping

The experiment was divided into five parts according to the content.

Part 1: To investigate changes in the pain threshold induced by hyperalgesia in rats and the expression of KCC2 and GABAAR in SCDH in a nociceptive HP model, SD rats were randomly assigned to blank (NS + NS), sham HP (NS + PGE_2), and HP (Car + PGE_2) groups.
Part 2: To determine whether GABAAR is involved in the induction nociceptive hyperalgesia in SCDH, SD rats were randomly assigned to blank (NS + NS), sham HP (NS + PGE_2), HP (Car + PGE_2), and GABAAR agonist (Car + PGE_2 + Mus) groups.
Part 3: To investigate whether KCC2 in the SCDH participates in nociceptive hyperalgesia and determine the relationship between KCC2 and GABAAR, SD rats were randomly assigned to blank (NS + NS), sham HP (NS + PGE_2), HP (Car + PGE_2), and KCC2 agonist (Car + PGE_2 + CLP257) groups.
Part 4: To investigate the effect of EA on the pain threshold and the expression of GABAAR and KCC2 in the SCDH of nociceptive HP model rats, SD rats were randomly assigned to four groups: sham HP (NS + PGE_2), HP (Car + PGE_2), EA (Car + PGE_2 + EA), and sham EA (Car + PGE_2 + sham EA).
Part 5: SD rats were randomly assigned to four groups: sham HP (NS + PGE_2), HP (Car + PGE_2), EA (Car + PGE_2 + EA), and KCC2 antagonist (Car + PGE_2 + EA + VU 0240551). We investigated whether VU0240551 antagonizes the analgesic effect of EA in an HP model.

Nociceptive hyperalgesia was induced in all rats in the HP, GABAAR, and KCC2 agonist groups. The sham HP group was injected with NS solution instead of Car and the other treatments were the same as those in the HP group. In the control group, two injections of NS were replaced with NS.

Establishment of the HP model

The HP model was prepared as described by Parada et al.[35]. Rats were subcutaneously injected with 100 μL of 1% Car (first injection) into the left plantar foot. When the pain threshold returned to baseline 7 days later, a low dose of 25 μL PGE_2 was injected into the ipsilateral dorsal foot (second injection). The pain threshold was observed in the left foot of rats, and the reduction in the pain threshold lasted for at least 48 hours, indicating that the HP model was successfully established. Otherwise, it represented a failure of the model and was excluded.

Behavioral test

The up-down method was used in this study[36]. The forces used were 0.4, 0.6, 1, 2, 4, 6, 8, 15, and 26 g of Von Frey filaments (Stoelting Co., Thermo, Gilroy, CA, USA). Rats were placed in clear plastic cages for 30 minutes each day before assessment for three consecutive days for acclimatization. A von Frey filament of 4 g force was first applied to the central surface of the hind paw (avoiding the footpads) until the filament bent in an "S" shape and held for 6 seconds. The filament was then selected with force, depending on whether the response was negative or positive. The response was recorded as X or O. Pain thresholds were calculated according to the following formula: MWT (g) = [10 (Xf + κδ)]/10,000, where "Xf" is the force from the last hair test, "κ" value is obtained from the κ-value table, and "δ" is the average difference between the logarithm of the hairs for each force, which is approximately 0.231.

EA intervention

After fixing all rats intervened by EA and sham EA, the bilateral ST36 and BL60 points of the rats were selected, and filiform needles with a size of 0.25 mm × 13 mm were used to stimulate the acupoints. The bilateral ST36 and BL60 were connected with the "HANS-200A acupoint nerve stimulator." EA was administered to the EA group 4 hours after Car injection (30 minutes before behavioral test) once a day until the end of the experiment. The stimulation parameters were as follows: 2/100 Hz (dispersed-dense wave) and intensities of 0.5, 1.0, and 1.5 mA (intensity was adjusted every 10 minutes) for 30 minutes[9]. These EA parameters have been proven to be effective[6–9].

In the sham EA group, the selection of acupoints and intervention time points was the same as that in the EA group. A HANS-200A acupoint nerve stimulator was connected to the needle handle without electrical stimulation (see the "Experimental Guide of Acupuncture and Moxibustion" 9th edition for acupoint location).

Intrathecal cannulation placement and drug administration

Rats were catheterized with a PE-10 intrathecal catheter 1 week before the establishment of the model. After anesthesia through an intrathecal catheter, the rats were kept in the prone position with the abdomen on an empty needle, and the waist was controlled to arch properly. A 0.5 cm longitudinal incision was made along the L4–5 lumbar spine, and after dissecting the subcutaneous fascia and ligament, the interspinous ligament was exposed, and a 5-gauge steel needle was inserted. During this process, the dura was punctured until a foot lift reaction occurred, and the needle was removed. In this process, the epidural catheter was inserted in the direction of the steel needle, and the progression was completed after the same stimulation reaction occurred. The left hand was slowly pushed into the catheter, and the right hand was pulled from the steel wire. If clear cerebrospinal fluid flows through the catheter, catheterization should be considered to meet these requirements. The back muscles were sutured, sterilized, and treated with antibacterial agents. Rats were housed in a single cage after the operation and were excluded if they were paralyzed or limped after a few days. Drugs were administered to each patient group.

In this study, the GABAAR agonist muscimol (1 μg/day, dissolved in 0.9% NS, 0.1 μg/μL), the KCC2 activator CLP257 (10 μg/day, dissolved in DMSO, 1 μg/μL), or the KCC2 inhibitor VU0240551 (1 μg/day, dissolved in DMSO, 0.1 μg/μL) was intrathecally injected three times once a day: before the PGE_2 injection, 23.5 and 47.5 hours after the PGE_2 injection.

Immunofluorescence (IF)

Rats were sacrificed 48 hours after the second injection, and the MWT assessment was completed. After anesthesia

with sodium pentobarbital (50 mg/kg, intraperitoneally), the rats were rapidly perfused with NS (4°C), followed by reperfusion with 4% paraformaldehyde in 0.1 M phosphate-buffered saline. The L4–6 spinal cord was removed and postfixed in 4% paraformaldehyde for 3 hours at 4°C, subsequently transferred to 15% and then 30% sucrose for dehydration and stored in –80°C. The spinal cord was transversely sectioned laterally (10 μm) using a cryostat and dried at 37°C for 20 minutes. The sections were blocked with 10% goat serum in Tris-buffered saline (TBST) (1% Tween-20) for 1 hour at 37°C. The sections were incubated overnight at 4°C with primary antibodies diluted in a blocking solution. The primary antibodies were mouse antiGABAARα2 (1:200, ab193311, Abcam) and rabbit anti-KCC2 (1:200, 94725, Cell Signaling Technology). The sections were washed with TBST and incubated with goat anti-rabbit (1:600, Alexa Fluor 488-labeled, 111-545-144; Jackson Immunoresearch, USA) or goat anti-mouse (1:600, Alexa Fluor 594 labeled, ab150120; Abcam) secondary antibodies at 37°C for 1 hour (TBST diluted with 10% goat serum). Images were captured using an M2 microscope (Zeiss, Germany). For the quantification of IF staining, three images per rat were randomly selected. The fluorescence intensity of the stained area in each of the selected images using ImageJ software, and then averaged and analyzed.

Western blot (WB)

To measure the protein expression of KCC2 and GABAAR, rats were deeply anesthetized after the last behavioral test, as previously described. The L4–6 dorsal horn of the spinal cord on the affected side was rapidly excised and stored at –80°C for further analysis. The samples were then lysed using radioimmunoprecipitation assay lysis buffer and centrifuged at 12,000 rpm for 12 minutes at 4°C. A bicinchoninic acid protein assay kit was used to measure the protein concentration in the supernatant. Equal amounts of protein samples (20 μg) were separated on 5% sodium dodecyl sulfate-polyacrylamide gels and transferred onto polyvinylidene difluoride membranes. After blocking with 5% non-fat milk at room temperature for 1 hour, the membranes were incubated overnight at 4°C with mouse anti-GABAAR α2 (1:1000, ab193311, Abcam), rabbit anti-KCC2 (1:1000, 94725, Cell Signaling Technology) as primary antibody, and mouse anti-β-actin (horseradish peroxidase coupled) (1:5000, ab20272, Abcam) as internal controls, and rabbit anti-p-TrkB (1:1000, NBP1-03499, NOVUS) as primary antibody and rabbit anti-TrkB (1:1000, 4603S, Cell Signaling Technology) as controls. The membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:500, 7074S, Abcam) or goat anti-mouse IgG (1:500, BK-M050, Bioker Biotechnology, China) for 1 hour at room temperature. We use monoclonal antibodies to avoid the potential for cross-reactivity and determine the optimal antibody dilution through pre-experiments to avoid non-specific binding caused by high antibody concentration. The signals were developed using an excellent chemiluminescent substrate kit (Pierce, Rockford, IL, USA), and the intensity of the bands was analyzed using ImageQuant TL 7.0 analysis software (GE, Marlborough, MA, USA).

Statistical analysis

The data used in this experiment were statistically processed using SPSS, and the data were described as mean ± standard ($\bar{x}$ ± SD) deviation. One-way analysis of variance (ANOVA) was used for comparisons between multiple groups. The least significant difference test was used for homogeneity of variance, and Dunnett's T3 test was used for heterogeneity of variance. Repeated measures ANOVA is used for data analysis at multiple time points. Statistical significance was set at $P < 0.05$.

Results

Changes in mechanical pain threshold in the model rats

The HP model was established by successive injections Car/PGE_2 into the left hind feet of rats. The rats were measured 1 day before (base) the first injection (indicated by the green arrow), 4, 24, 48, 72 hours, and 7 days after the first injection, and MWTs of the left foot at 1, 4, 24, and 48 hours after the second injection (blue arrow indicates the injection time) (Figure 1A).

The results (Figure 1B) did not show significant differences in the MWTs of rats in each group before modeling

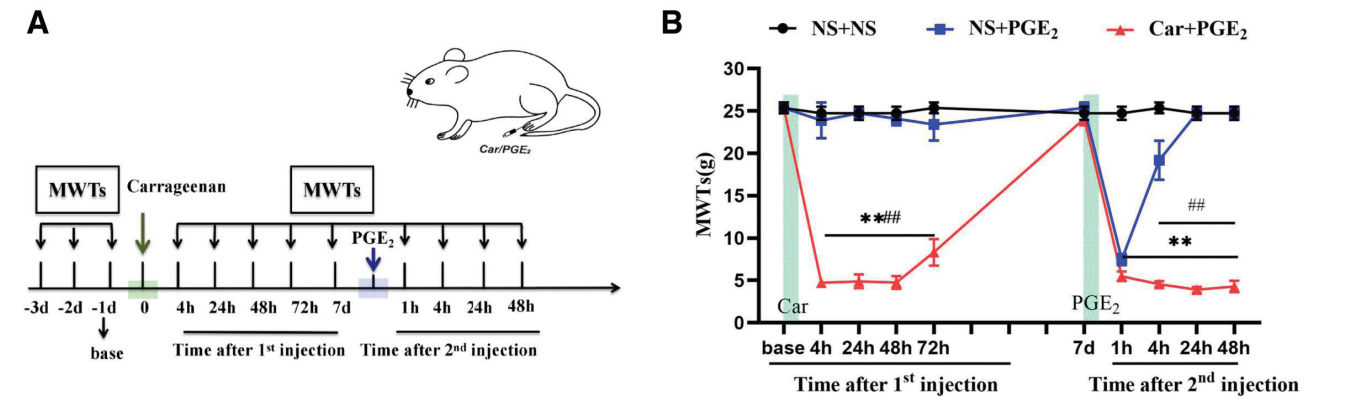


Figure 1. Establishment of the hyperalgesic priming rat model. (A) The time axis of the animal experiments and pain threshold are detected 1 day before the modeling and 4, 24, 48, 72 h, and 7 days after the first injection (green arrow). MWTs at 1, 4, 24, and 48 h after the second injection (blue arrow). (B) Changes in the MWTs at different time points in each group. Data are expressed as $\bar{x}$ ± SEM, $n = 6$; $^{**}P < 0.01$, *vs.* the NS + NS group; $^{\#\#}P < 0.01$ *vs.* the NS + PGE_2 group. MWTs: Mechanical withdrawal thresholds; NS: Normal saline; PGE_2: Prostaglandin E_2; SEM: Standard error of mean.

(Figure 1B, $P > 0.05$). Compared to the NS + NS and NS + PGE_2 groups, the MWTs measured 4, 24, 48, and 72 hours after the first subcutaneous injection of 100 μL of Car (the first injection) in the Car + PGE_2 group decreased significantly (Figure 1B, all $P < 0.01$). Subsequently, MWTs in the Car + PGE_2 group gradually recovered and returned to baseline levels on day 7, which were not statistically different from those in the NS + NS and NS + PGE_2 groups (Figure 1B, $P > 0.05$). Subsequently, a low dose of 100 ng/25 μL PGE_2 was injected into the ipsilateral dorsal foot. The results revealed that 1 hour after the second injection, the MWTs of the rats in the NS + PGE_2 and Car + PGE_2 groups were lower than those in the NS + NS group (Figure 1B, $P < 0.01$), and 4 hours after the second injection, no significant differences were observed between the NS + PGE_2 and NS + NS groups (Figure 1B, $P > 0.05$), indicating that the injection of PGE_2 into the NS + PGE_2 group only caused transient nociceptive hyperalgesia. However, the MWTs of the left hind foot in the Car + PGE_2 group at 4, 24, and 48 hours after the second injection were lower than those in the NS + NS and NS + PGE_2 groups (Figure 1B, all $P < 0.01$), and the induced nociceptive hyperalgesia lasted for at least 48 hours in the Car + PGE_2 group. These results indicate that the HP model was successfully established.

Low expression of GABAAR and KCC2 in SCDH of rats induced by hyperalgesia

To determine whether the expression of GABAAR and KCC2 in SCDH is involved in the pain transition, the SCDH of rats with lumbar enlargement was rapidly excised after the MWT test was completed 48 hours after the second injection. In this study, WB and IF were used to detect the expression of GABAAR and KCC2 in L4–6 SCDH on the affected side. WB results revealed that the expression level of GABAAR in the SCDH of the affected side in the Car + PGE_2 group was lower than that in the NS + NS and NS + PGE_2 groups (Figure 2A, $P < 0.01$ and $P < 0.05$). However, no significant differences were observed between the NS + NS and NS + PGE_2 groups (Figure 2A, $P > 0.05$). The expression of KCC2 in the SCDH of the affected side of rats in the Car + PGE_2 group was also lower than that in the NS + NS and NS + PGE_2 groups (Figure 2D, both $P < 0.05$), whereas no significant difference was observed in the expression of KCC2 in the SCDH of the affected side of rats in the NS + NS group compared to that in the NS + PGE_2 group (Figure 2D, $P > 0.05$). The IF results were similar to those of WB. White arrows represent immunopositive substances. The fluorescence intensity of GABAAR in the affected SCDH of the Car + PGE_2 group was significantly lower than that in the NS + NS and NS + PGE_2 groups (Figure 2C, $P < 0.05$ and $P < 0.01$, respectively). However, no significant differences were observed between NS + NS and NS + PGE_2 groups (Figure 2C, $P > 0.05$). The fluorescence intensity of KCC2 in the SCDH of the affected side in the Car + PGE_2 group was lower than that in the NS + NS and NS + PGE_2 groups (Figure 2F, $P < 0.05$ and $P < 0.01$, respectively), whereas that in the NS + NS group was significantly lower than that in the NS + PGE_2 group. No significant differences were observed in the fluorescence intensity of KCC2 in SCDH on the affected side between the two groups (Figure 2F, all $P > 0.05$). These results suggest that the decreased expression of GABAAR and KCC2 in SCDH of rats with hyperalgesia may be the main mechanism underlying their involvement in pain transition.

GABAAR in the SCDH is involved in the pain transition

To determine whether GABAAR is involved in pain transition, we further tested whether GABAAR agonists could block hyperalgesia induced by Car/PGE_2 injection. Muscimol was injected intrathecally three times a day to activate GABAAR. The results (Figure 3) did not show significant differences in the MWTs on the affected side of the rats in the various groups at any time point before Car injection (Figure 3B, $P > 0.05$). Compared to the NS + NS and NS + PGE_2 groups, the MWTs measured 4, 24, 48, and 72 hours after subcutaneous injection of 100 μL Car (the first injection) on the left plantar surface decreased significantly in the Car + PGE_2 and Car + PGE_2 + Mus groups (Figure 3B, all $P < 0.01$), which was consistent with the results observed in Figure 1B. The MWTs of the Car + PGE_2 and Car + PGE_2 + Mus groups gradually recovered and returned to baseline levels on day 7, and were not significantly different from those of the NS + NS and NS + PGE_2 groups (Figure 1B, $P > 0.05$). Next, a low dose of 100 ng/25 μL PGE_2 was injected into the ipsilateral dorsal foot, and the MWTs on the affected side of the rats were continuously measured at different time points. The results indicated that 1 hour after the second injection, MWTs in the NS + PGE_2, Car + PGE_2, and Car + PGE_2 + Mus groups were lower than those in the NS + NS group (Figure 3B, $P < 0.05$). However, 24 and 48 hours after the second injection, the MWTs in the Car + PGE_2 + Mus group were significantly higher than those in the Car + PGE_2 group (Figure 3B, $P < 0.01$, and $P < 0.05$, respectively), indicating that GABAAR activation in SCDH inhibited hyperalgesia and blocked the formation of hyperalgesia.

At 48 hours after the second injection, WB indicated that GABAAR expression in the SCDH of the affected side was downregulated in the Car + PGE_2 group compared to the NS + NS and NS + PGE_2 groups (Figure 3C, both $P < 0.01$), which was consistent with the results observed in Figure 2C. GABAAR expression in the SCDH of the affected side in the Car + $PGE_2$2 + Mus group was higher than that in the Car + PGE_2 group (Figure 3C, $P < 0.05$).

These results indicate that intrathecal injection of the GABAAR agonist muscimol successfully blocked the sustained reduction in pain threshold in hyperalgesia-primed rats and increased the expression of GABAAR in SCDH-blocked hyperalgesia priming.

KCC2 is involved in the pain transition and upregulates GABAAR expression in the SCDH

To determine whether KCC2 in SCDH is involved in pain transition, the KCC2 agonist CLP257 was administered intrathecally to test whether it could block hyperalgesia induced by the injection of Car/PGE_2. The

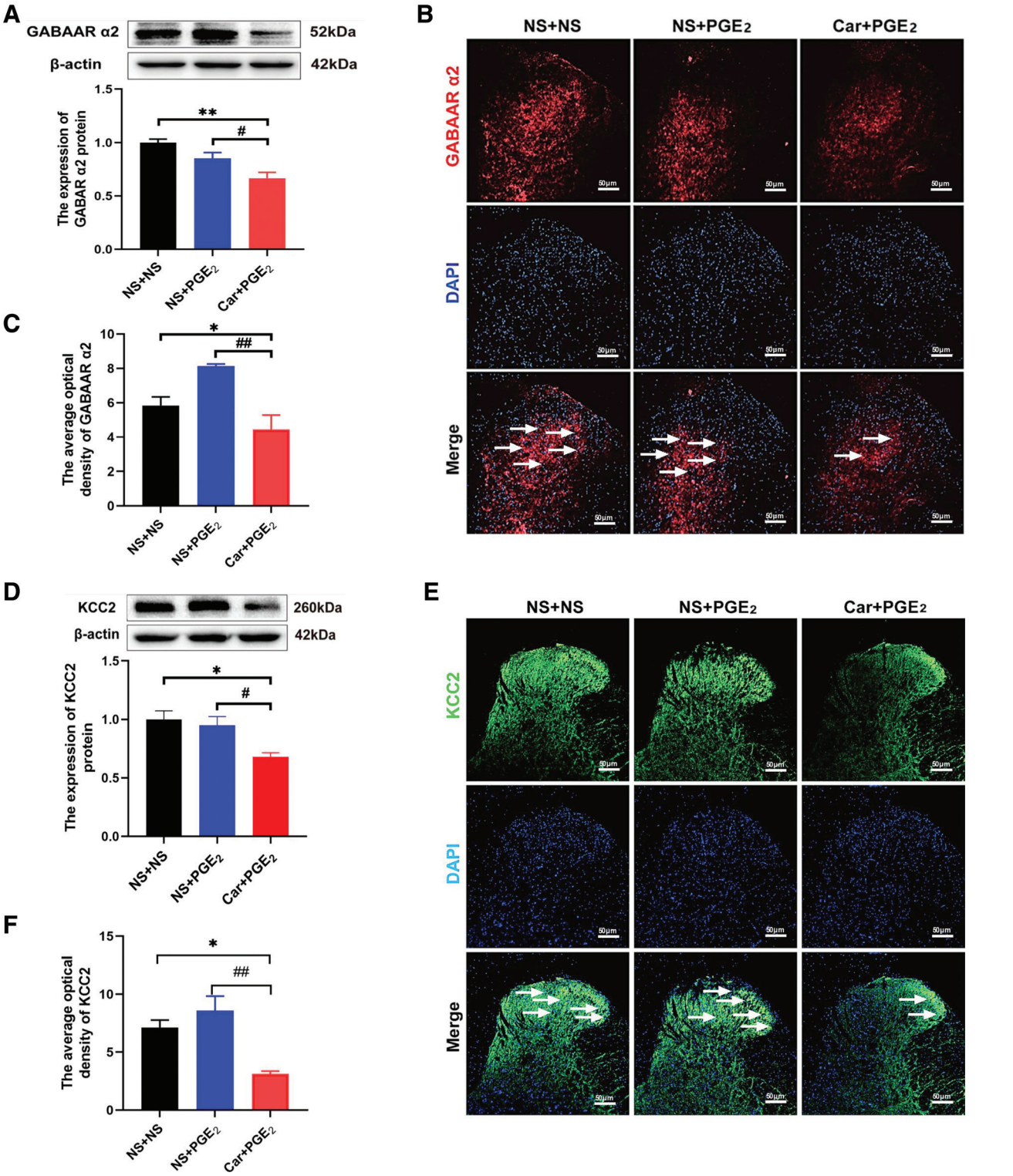


Figure 2. Changes in GABAAR and KCC2 protein expression in the SCDH induced by hyperalgesia in model rats. (A) Representative images and statistical graphs of changes in GABAAR protein expression in the SCDH of rats in each group detected by WB 48 h after the second injection. (B) Representative images of GABAAR fluorescence (red) and DAPI (blue) in the SCDH of rats in each group, 48 h after the second injection. (C) Quantification of GABAAR fluorescent expression in the SCDH 48 h after the second injection. (D) Representative images and statistical graphs of changes in KCC2 protein expression in the SCDH of rats in each group detected by WB 48 h after the second injection. (E) Representative images of KCC2 fluorescence expression (red) and DAPI (blue) in the SCDH of rats in each group 48 h after the second injection. White arrows represent immunopositive substances. (F) Quantification of KCC2 fluorescence intensity in SCDH 48 h after the second injection. Data are expressed as $\bar{x} \pm$ SEM, $n = 6$; $^{*}P < 0.05$, $^{**}P < 0.01$ *vs.* the NS + NS group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs.* the NS + PGE_2 group. DAPI: 4′,6-diamidino-2-phenylindole; GABAAR: γ-Aminobutyric acid receptor type A; KCC2: K^+-Cl^- cotransporter isoform 2; NS: Normal saline; PGE_2: Prostaglandin E_2; SCDH: Spinal cord dorsal horn; SEM: Standard error of mean; WB: Western blotting.

KCC2 agonist CLP257 was intrathecally injected three times a day to activate KCC2. The results (Figure 4) did not show significant differences in the MWTs on the affected side of the rats in the various groups at each time point before Car injection (Figure 4A, $P > 0.05$). Compared to the NS + NS and NS + PGE_2 groups, the

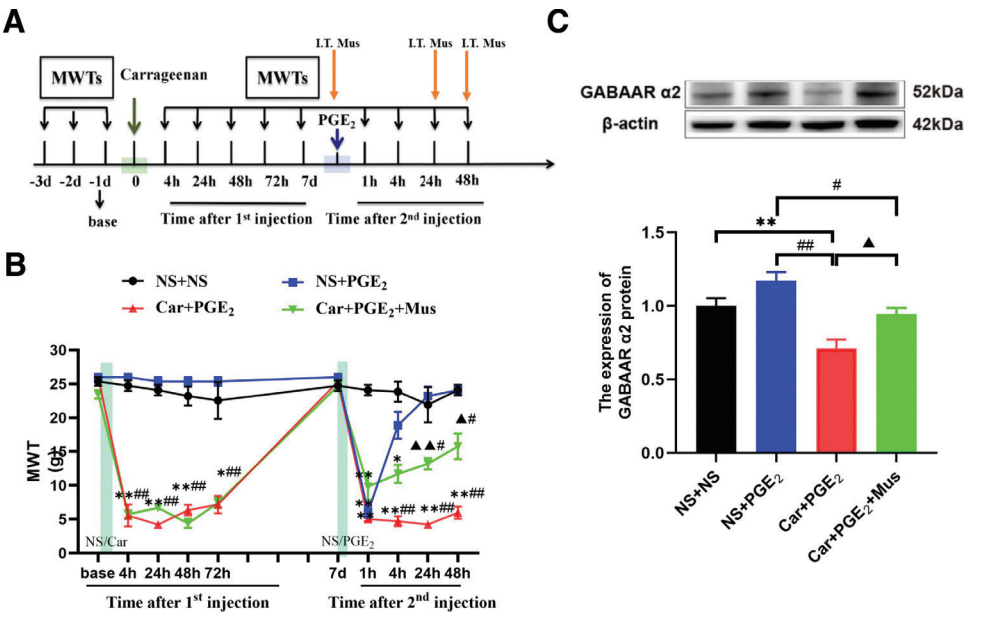


Figure 3. GABAAR participates in nociceptive hyperalgesia priming. (A) The time axis and time to detect the pain threshold in animal experiments were the same as shown in Figure 1. Muscimol is injected intrathecally before PGE_2 injection, 24 h after PGE_2 injection, 48 h after PGE_2 injection, and before MWT detection. (B) The MWTs of the rats at each time point were measured. (C) Representative pictures and statistical plots of changes in GABAAR protein expression in the SCDH of rats in each group are detected by WB 48 h after the second injection. All data are expressed as $\bar{x} \pm$ SEM, $n = 6$ rats in each group; $^{*}P < 0.05$, $^{**}P < 0.01$ *vs.* the NS + NS group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs.* the NS + PGE_2 group; $^{\blacktriangle}P < 0.05$, $^{\blacktriangle\blacktriangle}P < 0.01$ *vs.* the Car + PGE_2 group. Car: Carrageenan; GABAAR: γ-Aminobutyric acid receptor type A; MWTs: Mechanical withdrawal thresholds; NS: Normal saline; PGE_2: Prostaglandin E_2; SCDH: Spinal cord dorsal horn; SEM: Standard error of mean; WB: Western blotting.

MWTs measured 4, 24, 48, and 72 hours after subcutaneous injection of 100 μL Car (the first injection) on the left plantar surface were significantly decreased in the Car + PGE_2 and Car + PGE_2 + CLP257 groups (Figure 4A, all $P < 0.01$). The MWTs of the Car + PGE_2 and Car + PGE_2 + CLP257 groups gradually recovered and returned to baseline levels on day 7 and were not significantly different from those of the NS + NS and NS + PGE_2 groups (Figure 4A, $P > 0.05$). A low dose of PGE_2 (100 ng/25 μL) was injected into the ipsilateral dorsal foot of the rats, and MWTs on the affected side were continuously measured at different time points. The results revealed that 1, 4, 24, and 48 hours after the second injection, compared to the Car + PGE_2 group, the MWTS in the Car + PGE_2 + CLP257 group were significantly higher than those in the Car + PGE_2 group at each time point (Figure 4A, all $P < 0.01$), indicating that CLP257 successfully blocked the continuous reduction in the pain threshold in hyperalgesia-primed rats.

At 48 hours after the second injection, the WB results revealed that the expression of KCC2 in the SCDH of the affected side in the Car + PGE_2 group was lower than that in the NS + NS and NS + PGE_2 groups (Figure 4B, $P < 0.05$). However, KCC2 expression in the SCDH of the affected side of the rats in the Car + PGE_2 + CLP257 group was higher than that in the Car + PGE_2 group (Figure 4B, $P < 0.01$). These results suggest that low expression of KCC2 in SCDH plays a key role in hyperalgesia.

Furthermore, we investigated whether KCC2 activation upregulates GABAAR expression in SCDH. WB results indicated that the expression level of GABAAR in the SCDH on the affected side in the Car + PGE_2 + CLP257 group was higher than that in the Car + PGE_2 group (Figure 4C, $P < 0.01$), and no differences were observed between the Car + PGE_2, NS + NS, and NS + PGE_2 groups (Figure 4C, $P > 0.05$). The IF results suggested that GABAAR and KCC2 were co-localized in the rat SCDH (Figure 4D), suggesting that KCC2 regulated GABAAR expression. Moreover, IF results revealed that the expression of GABAAR and KCC2 in the SCDH of the Car + PGE_2 group was lower than that in the NS + NS and NS + PGE_2 groups (Figure 4E and F, $P < 0.05$). The expression levels of GABAAR and KCC2 in the SCDH of rats in the Car + PGE_2 + CLP257 group were higher than those in the Car + PGE_2 group (Figure 4E and F, both $P < 0.01$). These results suggest that KCC2 activation in the SCDH increases the pain threshold of the HP model rats and upregulates GABAAR expression.

EA effect on the pain threshold of HP model rats

The EA intervention process is illustrated in Figure 5A. EA was applied in bilateral ST 36 and BL60 once a day until the end of the experiment and 4 hours after the first injection and the MWT test was completed. At 4, 24, 48, 72 hours, and 7 days after the first injection and 1, 4, 24, and 48 hours after the second injection, the MWTs of the affected foot were measured to observe the intervention effect of EA on the pain threshold of the model rats. Before modeling, no significant differences were observed in the basal pain threshold of the rats in the three groups (Figure 5B, $P > 0.05$). Compared to the NS + PGE_2 group, the MWTs of the Car + PGE_2 and Car + PGE_2 + sham EA groups decreased significantly at 4, 24, 48, and 72 hours after the first injection (Figure 5B, all $P < 0.01$). Over time, the pain thresholds in the Car + PGE_2 and Car + PGE_2 + sham EA groups gradually increased. However, compared to the Car + PGE_2 and Car + PGE_2 + sham EA groups, the MWTs in the Car + PGE_2 + EA group increased significantly 48 and 72 hours after the first injection (Figure 5B, $P < 0.01$–0.05)

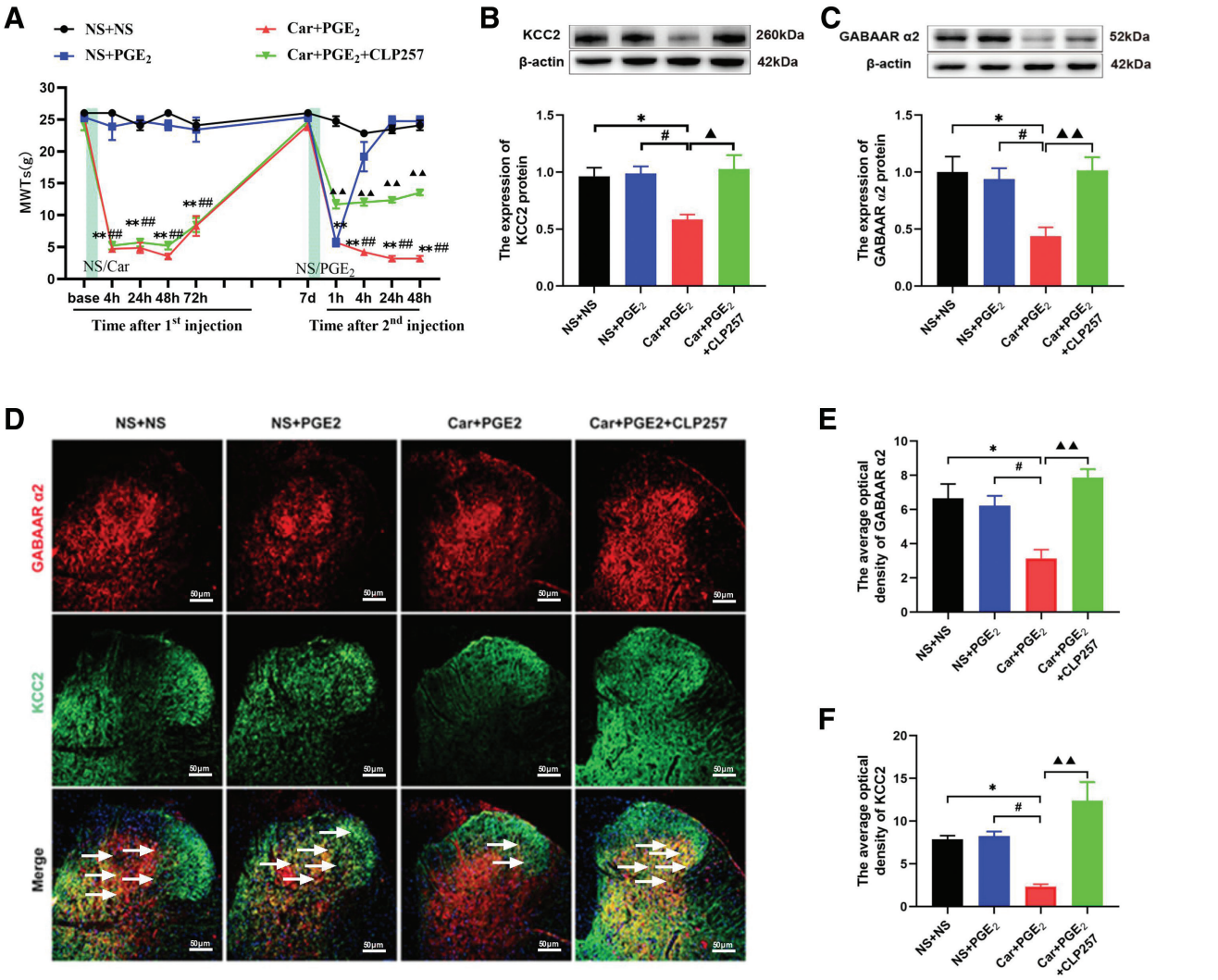


Figure 4. KCC2 in the SCDH participates in pain transition and its relationship with GABAAR. (A) CLP257 was administered intrathecally to rats before injection of PGE_2 and 24 and 48 h after, and the MWTs were measured at each time point. (B) Representative images and statistical graphs of changes in KCC2 protein expression in the SCDH of rats in each group detected by WB 48 h after the second injection. (C) Representative images and statistical graphs of changes in GABAAR protein expression in the SCDH of rats in each group detected by WB 48 h after the second injection. (D) Representative images of GABAAR (red) and KCC2 (green) expression in the SCDH of rats in each group 48 h after the second injection. The white arrows represent immunopositive substances. (E) Quantitative statistical plot of GABAAR fluorescence intensity in rats in each group. (F) Quantitative statistical graphs of the KCC2 fluorescence intensity in rats from each group. All data are expressed as ± SEM, $n = 6$ rats per group; $^{*}P < 0.05$, $^{**}P < 0.01$ *vs.* the NS + NS group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs.* the NS + PGE_2 group; $^{\blacktriangle}P < 0.05$, $^{\blacktriangle\blacktriangle}P < 0.01$ *vs.* the Car + PGE_2 group. Car: Carrageenan; GABAAR: γ-Aminobutyric acid receptor type A; KCC2: K^+-Cl^- cotransporter isoform 2; MWTs: Mechanical withdrawal thresholds; NS: Normal saline; PGE_2: Prostaglandin E_2; SCDH: Spinal cord dorsal horn; SEM: Standard error of mean; WB: Western blotting.

and returned to the baseline level on day 7. These results suggest that EA treats acute pain effectively. The MWTs in the Car + PGE_2 + EA group significantly increased at 4, 24, and 48 hours after the second injection. Compared to the Car + PGE_2 and Car + PGE_2 + sham EA groups at the same time point, the MWTs in the Car + PGE_2 + EA group were significantly higher (Figure 5B, all $P < 0.01$). No significant differences were observed in the MWTs between the Car + PGE_2 + sham EA and Car + PGE_2 groups at any time point ($P > 0.05$). These results suggest that EA treatment substantially prevented Car/PGE2 injection-induced prolonged hyperalgesia.

EA regulated the expression of GABAAR and KCC2 in SCDH

To clarify the mechanism of EA intervention on the hyperalgesia-induced pain threshold in rats, the protein expression of GABAAR and KCC2 in the SCDH of the affected side was detected by WB. Compared to the NS + PGE_2 group, the expression of KCC2 and GABAAR in the SCDH of the affected side was significantly downregulated in the Car + PGE_2 group 48 hours after the injection of PGE_2 (Figure 6A and B, $P < 0.05$ and $P < 0.01$, respectively). However, compared with the Car + PGE_2 and Car + PGE_2 + sham EA groups, the expression of KCC2 and GABAAR in the affected SCDH was significantly upregulated in the EA intervention group (Figure 6A and B, $P < 0.01$–0.05). In contrast, sham EA did not have a significant effect on the hyperalgesia-induced reduction in KCC2 and GABAAR expression (Figure 6A and B, $P > 0.05$).

EA intervenes in the pain transition by upregulating the expression of GABAAR and KCC2 in the SCDH

To confirm whether the expression of GABAAR and KCC2 was involved in the regulation of pain transition,

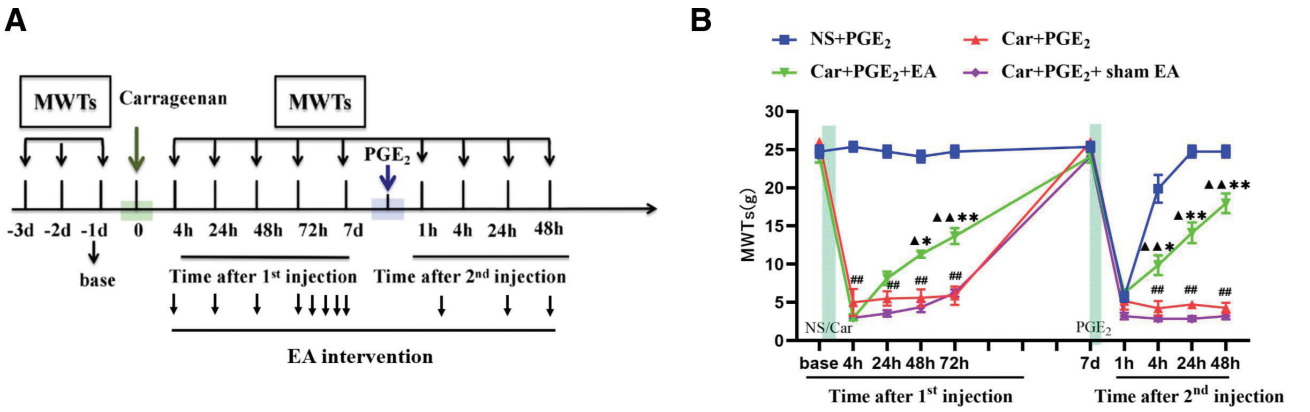


Figure 5. Acute to chronic pain transition process in EA intervention rats. (A) Time axis of EA treatment in hyperalgesia induced model rats. The pain threshold was detected 1 day before the first injection: 4, 24, 48, and 72 h; 7 days after the first injection; and 1, 4, 24, and 48 h after the second injection. EA was applied once a day from 4 h after the first injection to the end of the experiment. (B) MWT changes in each group at each test point. Compared to the Car + PGE_2 and Car + PGE_2 + shamEA groups, the postoperative MWTs of rats in the Car + PGE_2 + EA group increased significantly. Data are expressed as mean $\bar{x}$ ± SEM, $n = 6$; $^{\#\#}P < 0.01$ *vs.* the NS + PGE_2 group; $^{\blacktriangle}P < 0.05$, $^{\blacktriangle\blacktriangle}P < 0.01$, *vs.* the Car + PGE_2 group; $^{*}P < 0.05$, $^{**}P < 0.01$ *vs.* the Car + PGE_2 + sham EA group. Car: Carrageenan; EA: Electroacupuncture; MWTs: Mechanical withdrawal thresholds; NS: Normal saline; PGE_2: Prostaglandin E_2; SEM: Standard error of mean.

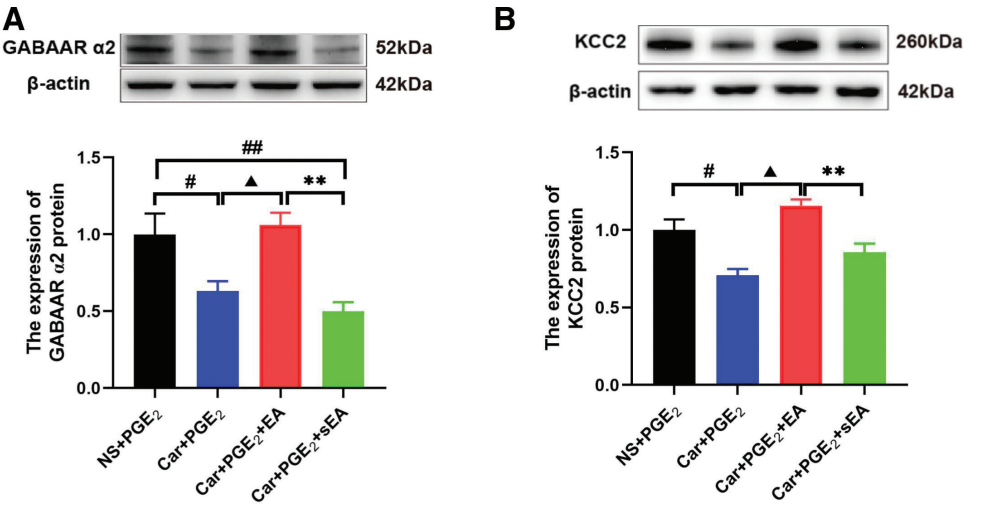


Figure 6. EA regulated the expression of GABAAR and KCC2 in SCDH. (A) Representative images and statistical graphs of the changes in GABAAR protein expression in the SCDH of rats in each group detected by WB. (B) Representative images and statistical graphs of changes in KCC2 protein expression in the SCDH of rats in each group detected by WB. All data are expressed as $\bar{x}$ ± SEM, $n = 6$ rats in each group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs.* the NS + PGE_2 group; $^{\blacktriangle}P < 0.05$, $^{\blacktriangle\blacktriangle}P < 0.01$ *vs.* the Car + PGE_2 group; $^{**}P < 0.01$ *vs.* the Car + PGE_2 + EA group. Car: Carrageenan; EA: Electroacupuncture; GABAAR: γ-Aminobutyric acid receptor type A; KCC2: K^+-Cl^- cotransporter isoform 2; NS: Normal saline; PGE_2: Prostaglandin E_2; SCDH: Spinal cord dorsal horn; SEM: Standard error of mean; WB: Western blotting.

the KCC2 inhibitor VU0240551 was injected intrathecally three times a day, and its effect on EA intervention was observed. These results are consistent with those of previous studies. EA significantly alleviated hyperalgesia induced by Car injection 72 hours after the first injection (Figure 7A, $P < 0.05$). However, at each time point after the second injection, no differences were observed between the Car + PGE_2 + VU0240551 and Car + PGE_2 groups (Figure 7A, $P < 0.05$). At 4, 24, and 48 hours after the second injection, the MWTs in the Car + PGE_2 + EA group were significantly higher than those in the Car + PGE_2 + VU0240551 group (Figure 7A, all $P < 0.01$), indicating that VU0240551 antagonized the analgesic effect of EA in HP model rats.

Compared to the NS + PGE_2 group, the expression levels of KCC2 and GABAAR in the SCDH of rats in the Car + PGE_2 group were significantly lower (Figure 7B and C, $P < 0.01$). However, the expression levels of KCC2 and GABAAR in the SCDH of the Car + PGE_2 + EA group were higher than those in the Car + PGE_2 and Car + PGE_2 + EA + VU0240551 groups (Figure 7B and C, $P < 0.05$). No significant differences were observed between the Car + PGE_2 + EA + VU0240551 and Car + PGE_2 groups (Figure 7B and C, $P > 0.05$), suggesting that VU0240551 blocked the regulatory effect of EA on KCC2 activation and GABAAR expression in SCDH.

These results suggest that EA regulates GABAAR expression by promoting KCC2 expression in SCDH and preventing the transition from acute to chronic pain.

Discussion

The incidence of chronic pain has increased. Chronic pain is not fatal but can seriously affect the quality

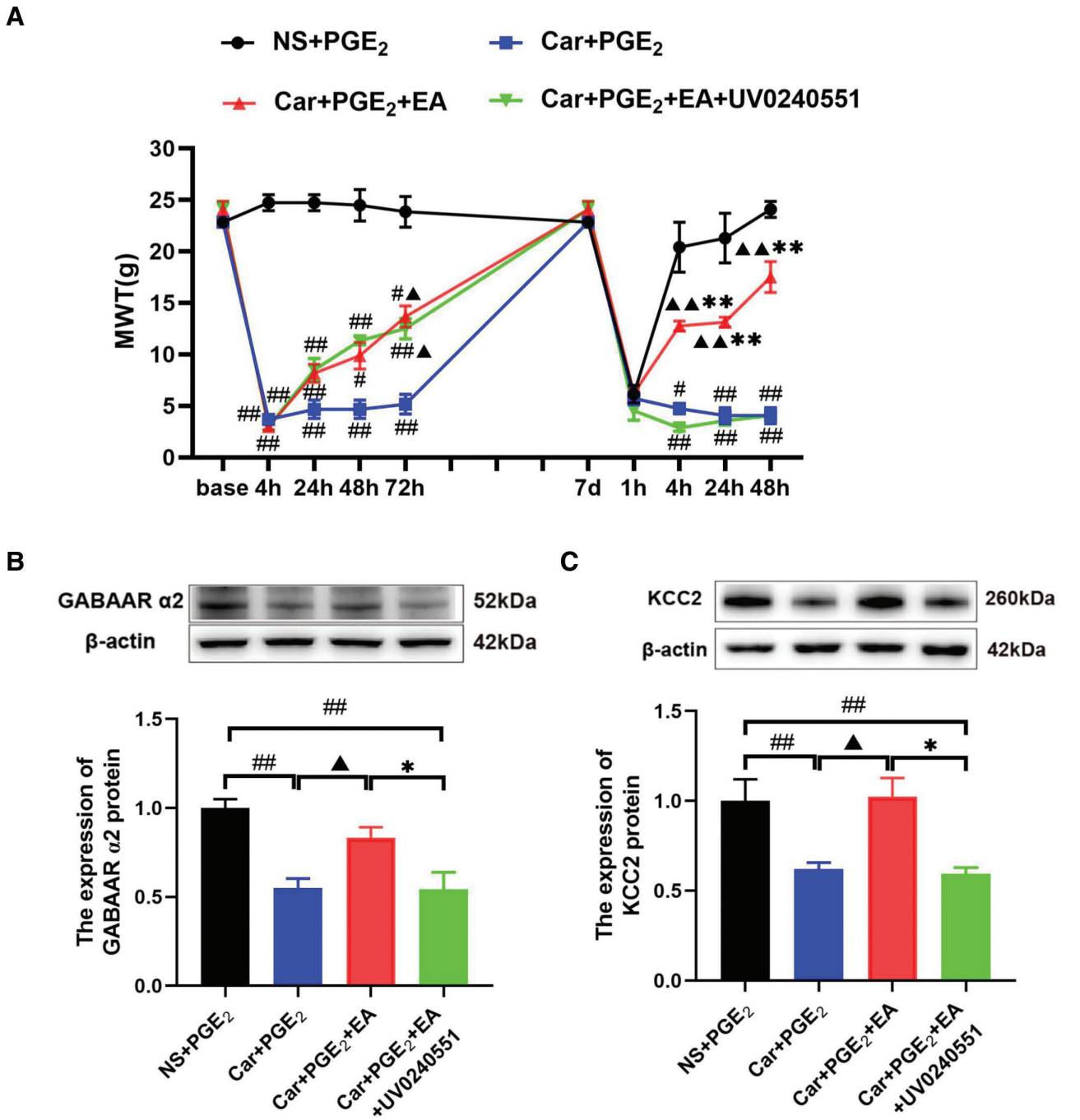


Figure 7. EA interferes with the process of pain transition by regulating the GABAAR/KCC2 pathway in the SCDH. (A) MWT changes in each group at each time point. Compared to that in the Car + PGE_2 + EA group, the MWTs in the Car + PGE_2 + EA + VU0240551 group decreased significantly after the second injection. (B) Representative image and statistical graphs of changes in GABAAR protein expression in the SCDH of rats in each group detected by WB. (C) Representative images and statistical graphs of changes in KCC2 protein expression in the SCDH of rats in each group detected by WB. All data are expressed as $\bar{x}$ ± SEM, $n = 6$ rats in each group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs.* the NS + PGE_2 group; $^{\blacktriangle}P < 0.05$, $^{\blacktriangle\blacktriangle}P < 0.01$ *vs.* the Car + PGE_2 group; $^{*}P < 0.05$, $^{**}P < 0.01$ *vs.* the Car + PGE_2 + EA group. Car: Carrageenan; EA: Electroacupuncture; GABAAR: γ-Aminobutyric acid receptor type A; KCC2: K^+-Cl^- cotransporter isoform 2; MWTs: Mechanical withdrawal thresholds; NS: Normal saline; PGE_2: Prostaglandin E_2; SCDH: Spinal cord dorsal horn; SEM: Standard error of mean; WB: Western blotting.

of life of patients. The prevalence of chronic pain varies from 8% to 55.2% in different countries[37–38]. However, drugs used to treat chronic pain have varying degrees of side effects and poor therapeutic effects. Chronic pain is a substantial challenge worldwide, owing to its high incidence, widespread impact, and considerable economic losses. SCDH is the primary center of sensory signals afferent to pain transmission[39]. GABAAR are widely distributed in SCDH[40–41] and are important inhibitory receptors closely related to the occurrence and development of pain[42] and the excitatory activity of neurons[43–44]. Previous studies have revealed that 25% to 31% of neurons in laminae I to II and approximately 40% of those in lamina III are GABA-immunoreactive and therefore presumably GABAergic inhibitory interneurons[45]. GABAergic neurons are concentrated in laminae I to III of the spinal dorsal horn, and present at lower densities

throughout the remaining gray matter[46]. KCC2 plays an important role in Cl^- co-transport in the central nervous system and is an important regulator of GABA-mediated hyperpolarization[47]. Dysfunction or downregulation of KCC2 is a common cause of neuropathic pain.

EA therapy is a new type of acupuncture that combines electrical stimulation of acupoints with acupuncture and has a good analgesic effect[48]. In modern studies, the induction of hyperalgesia is considered a sign of pain transition[24]. We used the HP model to explore the mechanism of EA during pain transition. Bilateral ST36 and BL60 were selected as acupoint formations, which is a common acupoint formation in the clinical treatment of chronic pain in the lower limbs. Different frequencies of EA have different analgesic effects[49]. The frequency of EA used in this study was 2/100 Hz. Previous animal experiments have indicated that the combination of EA parameters and acupoint formation can reduce mechanical pain threshold in HP rat models, which has a significant regulatory effect on SCDH[50]. Therefore, the EA parameters in this experiment were set as follows: disperse-dense wave, frequency 2/100 Hz, intensity 0.5 to 1.5 mA, increasing every 10 minutes, and lasting for 30 minutes.

In our study, we used HP model to investigate the mechanism of transition from acute to chronic pain. We found that HP model rats developed intense and persistent mechanically abnormal pain. KCC2 expression was decreased in the SCDH of HP model rats, whereas intrathecal injection of the KCC2 activator CLP257 upregulated KCC2 expression and effectively prevented Car/PGE_2-induced long-term hyperalgesia. Then we examined the association between GABAAR and KCC2 in SCDH using a series of pharmacological HP rat models. Results indicate that GABAAR and KCC2 are co-expressed in SCDH. Furthermore, CLP257 notably reversed persistently reduced pain threshold and upregulated the expression of GABAAR and KCC2 in the SCDH of HP model rats. These results suggest that KCC2 promotes pain transition in the SCDH by downregulating GABAAR expression. EA treatment substantially prevented Car/PGE_2 injection-induced prolonged hyperalgesia and upregulated GABAAR and KCC2 expression in the SCDH of lumbar expansion of the spinal cord in HP model rats. KCC2 antagonist reversed the analgesic effect of EA and downregulated the expression of KCC2 and GABAAR in SCDH. To summarize, our results suggest that EA prevents pain transition by upregulating the expression of KCC2 and GABAAR in the SCDH of HP model rats.

Our study aims to deepen the understanding of the neurobiological mechanisms underlying pain by regulating the KCC2/GABAAR pathway, providing new targets for pain treatment. EA alleviates pain effectively and reduces drug dependence and side effects by regulating the KCC2/GABAAR pathway. Our study promotes basic and clinical research in the field of pain, providing more scientific basis and effective means for pain treatment. However, our research has many limitations, as follows:1. The results of this study indicated that KCC2 and GABAAR are key proteins involved in pain transition in SCDH, but did not detect their specific cellular localization and structural stratification in the spinal cord. 2. Chloride ion homeostasis is strictly regulated during neuronal excitation, and activation of chloride ion channels is crucial for neuronal excitability. We detected the expression of KCC2 and GABAAR, but did not use electrophysiological techniques to study their activity and functional changes. 3. EA has regulatory effect on the activity of chloride ion channels. From existing research, the chloride channel receptor GABAAR and its regulatory protein KCC2 play a definite and effective role in the process of EA intervention for hyperalgesia. It is worth further studying whether other chloride channels are involved in the process of EA analgesia.

Conclusion

The results of this study indicated that EA prevents pain transition by upregulating the expression of KCC2 and GABAAR in the SCDH of HP model rats. Our study aims to deepen the understanding of the neurobiological mechanisms underlying pain transition by regulating the KCC2/GABAAR pathway, providing new targets for pain treatment. EA alleviates pain transition effectively by regulating the KCC2/GABAAR pathway.

Conflict of interest statement

The authors declare no conflict of interest.

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Author contributions

Mengting Shi and Yangkun Liu wrote the manuscript. Yin Jin revised the manuscript. Junfan Fang and Yi Liang completed the data analysis and image processing. Ruijie Ma and Jie Zhou designed this project. All authors shared the raw data of this experimental study. Furthermore, all authors contributed to and approved the final version of the manuscript accepted for publication.

Ethical approval of studies and informed consent

All experimental procedures were approved by the Animal Care and Welfare Committee of Zhejiang University of Chinese Medicine, Zhejiang Province, China (approval number IACUC-20180319-12).

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None.

Data availability

The data that support the findings of this study are available on request from the corresponding author Jie Zhou upon reasonable request.

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