Spinal 5-HT$_{2A}$ receptor is involved in electroacupuncture inhibition of chronic pain

Xiao-Cui Yuan[1,2], Yu-Ying Wang[1,2], Li-Xia Tian[1,2], Xiang-Ji Yan[1,2], Yi-Xiao Guo[1,2], Yu-Long Zhao[1,2], Sani S Baba[1,2] ⓘ, Hong Jia[1,2], Hui-Sheng Wang[1,2], Man Li[3] ⓘ, and Fu-Quan Huo[1,2] ⓘ

Abstract

Knee osteoarthritis (KOA) is a highly prevalent, chronic joint disorder, and it is a typical disease which can develop chronic pain. Our previous study has proved that endocannabinoid (2-AG)-CB1R-GABA-5-HT pathway is involved in electroacupuncture (EA) mediated inhibition of chronic pain. However, it is still unclear which among the 5-HT receptor subtype is involved in EA evoked 5-HT mediated inhibition of chronic pain in the dorsal spinal cord. 5-HT$_{2A}$ is a G protein-coupled receptor and it is involved in 5-HT descending pain modulation system. We found that EA treatment at frequency of 2 Hz +1 mA significantly increased the expression of 5-HT$_{2A}$ receptor in the dorsal spinal cord and intrathecal injection of 5-HT$_{2A}$ receptor antagonist or agonist reversed or mimicked the analgesic effect of EA in each case respectively. Intrathecal injection of a selective GABA$_A$ receptor antagonist Bicuculline also reversed the EA effect on pain hypersensitivity. Additionally, EA treatment reversed the reduced expression of GABA$_A$ receptor and KCC2 in the dorsal spinal cord of KOA mice. Furthermore, we demonstrated that intrathecal 5-HT$_{2A}$ receptor antagonist/agonist reversed or mimicked the effect of EA up-regulate of KCC2 expression, respectively. Similarly, intrathecal injection of PLC and PKC inhibitors prevented both anti-allodynic effect and up-regulation of KCC2 expression by EA treatment. Our data suggest that EA treatment up-regulated KCC2 expression through activating 5-HT$_{2A}$-Gq-PLC-PKC pathway and enhanced the inhibitory function of GABA$_A$ receptor, thereby inhibiting chronic pain in a mouse model of KOA.

Keywords

knee osteoarthritis (KOA), electroacupuncture analgesia, 5-HT$_{2A}$ receptor, GABA$_A$ receptor, KCC2, chronic pain

Introduction

Knee osteoarthritis (KOA) is a chronic degenerative bone metabolic disease that primarily affects elderly adults, leading to chronic pain and disability which affects patients' daily activities.[1] Our previous study has proved that electroacupuncture (EA) treatment is effective in relieving chronic pain in patients with KOA.[2] In the same vein, previous animal studies have found that EA treatment may potentiate the endogenous cannabinoid system and the expression of CB1 receptors on GABAergic neurons in the periaqueductal gray (PAG) to enhance the 5-hydroxytryptamine (5-HT) mediated descending inhibitory control of chronic pain.[3] However, it is unclear which of the specific 5-HT receptor subtype is involved in EA evoked 5-HT mediated inhibition of chronic pain in the dorsal spinal cord.

[1]Department of Physiology and Pathophysiology, School of Basic Medical Sciences, Institute of Neuroscience, Translational Medicine Institute, Xi'an Jiaotong University Health Science Center, Xi'an, China
[2]Ministry of Education, Key Laboratory of Environment and Genes Related to Diseases (Xi'an Jiaotong University), Xi'an, China
[3]Department of Neurobiology and Key Laboratory of Neurological Diseases of Ministry of Education, The Institute of Brain Research, School of Basic Medicine, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China
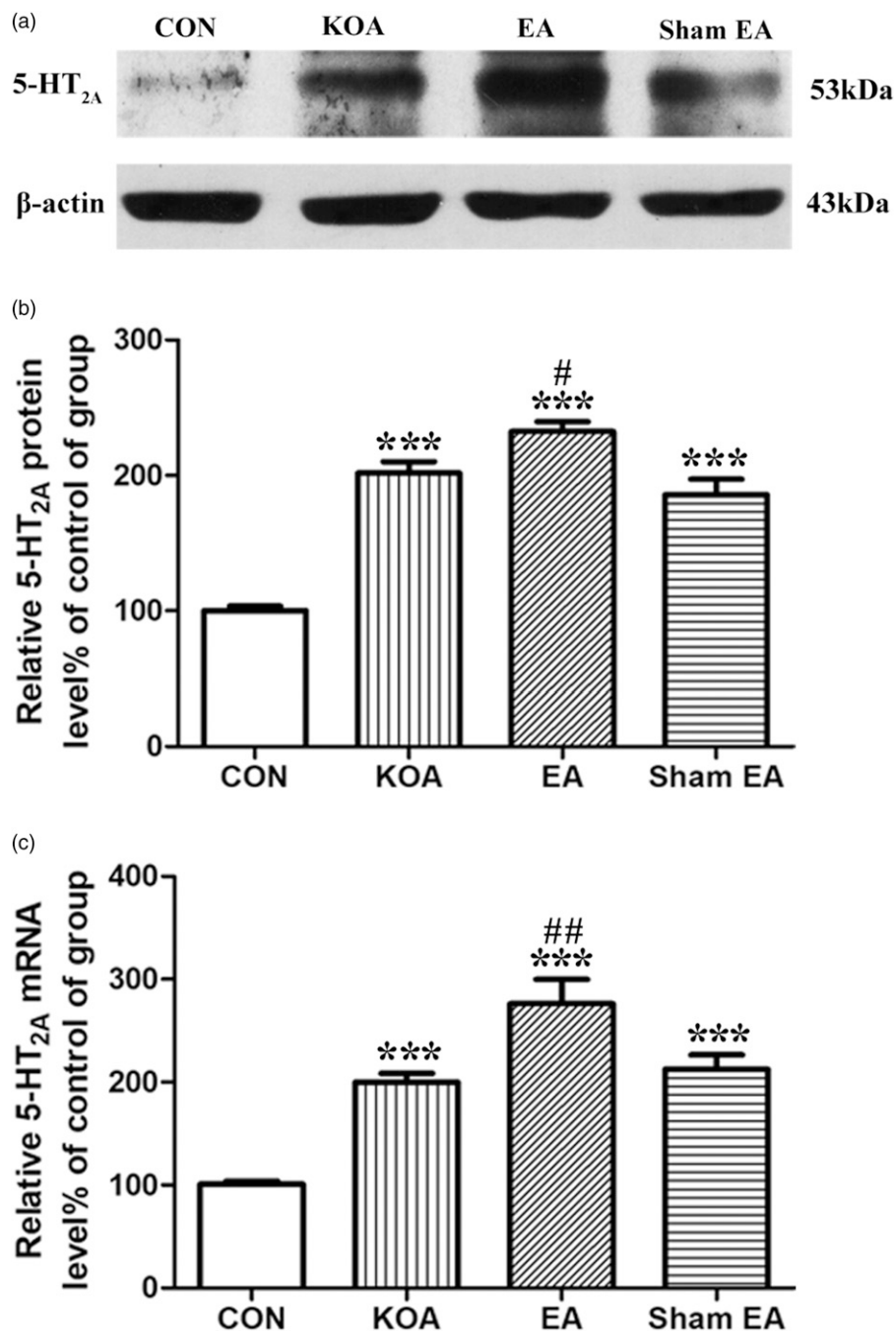
Corresponding Author:
Fu-Quan Huo, Department of Physiology and Pathophysiology, School of Basic Medical Sciences, Xi'an Jiaotong University Health Science Center, 76 Yan-Ta West Rd, Xi'an 710061, China.
Email: huofq@xjtu.edu.cn

Figure 1. Quantitative analysis of protein and mRNA levels of 5-HT$_{2A}$ receptor in the dorsal spinal cord tissues. (a) Representative gel image shows the protein level of 5-HT$_{2A}$ receptor in dorsal spinal cord tissues obtained from control (CON), KOA, KOA treated with EA and KOA treated with Sham EA. β-actin was used as a loading control. The protein band at 53 kDa corresponds to the 5-HT$_{2A}$ receptor. (b) Summary data show the effect of KOA, EA and Sham EA on the protein level of 5-HT$_{2A}$ receptor in dorsal spinal cord tissues. (c) Effects of KOA, EA and Sham EA on the mRNA level of 5-HT$_{2A}$ receptor in dorsal spinal cord tissues. Data are expressed as means ± SME ($n = 4$ mice in each group). *** $p < .001$ compared with the control group; # $p < .05$; ## $p < .01$, compared with the KOA group. KOA: knee osteoarthritis; EA: electroacupuncture.

The 5-HT receptor family is divided into seven subfamilies (5-HT$_1$, 5-HT$_2$, 5-HT$_3$, 5-HT$_4$, 5-HT$_5$, 5-HT$_6$, 5-HT$_7$), comprising of 14 receptor subtypes, among which 5-HT$_{2A}$ receptor was reported to be important in the perception of pain.[4] The expression of 5-HT$_{2A}$ receptor in the descending pain modulation pathway of rat brainstem was detected following an increase in inflammatory pain induced by carrageenan.[5] Previous studies have also indicated that administration of 5-HT$_{2A}$ receptor agonist mediated antinociception in both inflammatory and neuropathic pain

models in rats,[6,7] suggesting that 5-HT$_{2A}$ receptor is involved in 5-HT mediated descending pain modulation system.

5-HT$_{2A}$ receptor is a G protein-coupled receptor which upon coupling to Gq subunit could activate phospholipase C (PLC) with resultant increase in the intracellular Ca^{2+} concentration and activation of the protein kinase C (PKC).[8] Previous studies have found that 5-HT$_{2A}$ receptor activity can up-regulate the expression of the potassium-chloride cotransporter 2 (KCC2) through a mechanism mediated by PKC and improve hyperalgesia in a rat spinal cord injury model.[9,10] Expression of KCC2, which is neuron specific, pumps out intracellular Cl$^-$ to maintain the inhibitory function of GABA$_A$ receptor.[8,11]

Large body of evidence have also proved that 5-HT$_{2A}$ and GABA$_A$ receptors are co-expressed in neuron, and 5-HT$_{2A}$ receptor activity can enhance the inhibitory function of GABA$_A$ receptor.[12,13] Therefore, in the present study, we first determined whether 5-HT$_{2A}$ receptor is involved in the EA mediated inhibition of chronic pain in a mouse model of KOA. We also determined whether spinal GABA$_A$ receptor and KCC2 were involved in the mechanism of EA mediated inhibition of chronic pain. Furthermore, we were curious to also elucidate whether EA increase the expression of KCC2 via activating the 5-HT$_{2A}$-Gq-PLC-PKC pathway, thus enhancing the inhibitory function of GABA$_A$ receptor and consequent chronic pain inhibition.

Results

EA increases 5-HT$_{2A}$ receptor expression in dorsal spinal cord of KOA mice

The 5-HT$_{2A}$ receptor protein bands were presented in the dorsal spinal cord tissues (Figure 1(a)). The 5-HT$_{2A}$ receptor protein level in the dorsal spinal cord of KOA mice was significantly higher than that in the control group 4 weeks after the KOA induction ($p <.001$; Figure 1(b)). EA treatment at 2 Hz + 1 mA significantly increased the 5-HT$_{2A}$ receptor level in the dorsal spinal cord compared with that in the KOA group ($p <.05$; Figure 1(b)). Moreover, consistent result was obtained with regards to the mRNA level of 5-HT$_{2A}$ receptor measured in the dorsal spinal cord, being significantly higher in the KOA compared to that in the control group 4 weeks after KOA induction ($p <.001$; Figure 1(c)). Furthermore, same dosage of EA treatment also significantly increased the mRNA level of 5-HT$_{2A}$ receptor in the dorsal spinal cord compared to that in KOA mice ($p <.01$; Figure 1(c)). However, sham EA had no significant effect on 5-HT$_{2A}$ receptor protein and mRNA levels of KOA mice ($p >.05$; Figures 1(b) and (c)).

Intrathecal administration of the 5-HT$_{2A}$ receptor antagonist Ketanserin can reverse the EA effect on mechanical allodynia

Our previous studies have reported establishment of chronic pain stage to have been on the 17 days post-KOA induction.[3] In line with this, we therefore administered *i.t.* selective 5-

HT$_{2A}$ receptor antagonist Ketanserin 30 min before EA on the 18th day after KOA induction. There was no difference in the baseline withdrawal threshold between different groups before MIA injection. KOA induction significantly reduced mechanical withdrawal threshold, consistent with our previous results.[3,14] Intrathecal administration of 10% DMSO did not influence the effect of EA treatment on the mechanical thresholds (Figure 2(a)).

However, intrathecal administration of Ketanserin (10 µg), a selective 5-HT$_{2A}$ receptor antagonist, significantly reversed the EA effect on the tactile withdrawal thresholds. As shown in Figure 2(a), the time course curves (i.e., CON, KOA, 10% DMSO plus EA, Ketanserin plus EA and Sham EA treated groups) were significantly different between treatments ($F_{(4, 525)} = 1752.88; p <.0001$), across times ($F_{(14, 525)} = 136.36; p <.0001$), and for their interactions ($F_{(56, 525)} = 14.64; p <.0001$). Further analyses indicated that the mechanical thresholds in the Ketanserin plus EA treated group was significantly less than that in the 10% DMSO plus EA group from 21st to 27th day after KOA induction ($p <.001$; Figure 2(a)). However, no significant difference was observed between the Ketanserin plus EA group and the KOA group from 21st to 27th day after KOA induction ($p >.05$; Figure 2(a)).

Intrathecal application of the 5-HT$_{2A}$ receptor agonist DOI can mimic the EA effect on mechanical allodynia

The 5-HT$_{2A}$ receptor agonist DOI (10 µg) was administered *i.t.* and EA stimulation started from the 18th day following KOA induction, once every other day for 5 times. There was marked significant increase in the mechanical thresholds observed in the DOI group ($p <.05$), as well as the Saline plus EA group ($p <.001$).

As shown in Figure 2(b), the time course curves (i.e., DOI, KOA and Saline plus EA treated groups) were significantly different between treatments ($F_{(2, 315)} = 9.82; p <.0001$), across times ($F_{(14, 315)} = 228.22; p <.0001$), and for their interactions ($F_{(28, 315)} = 3.78; p <.0001$). Further analyses indicated that the mechanical thresholds in the DOI treated group was significantly higher than that in the KOA group ($p <.05$), and so also in the Saline plus EA group ($p <.001$) from 23rd to 27th day after KOA induction. Moreover, no significant difference was observed between the DOI group and the Saline plus EA group from 23rd to 27th day after KOA induction ($p >.05$; Figure 2(b)).

Intrathecal application of GABA$_A$ receptor antagonist Bicuculline can reverse the EA effect on mechanical allodynia

In order to explore whether GABA$_A$ receptor is involved in the analgesic effect of EA, the GABA$_A$ receptor antagonist Bicuculline (0.1 µg) was administered *i.t.* 30 min before EA starting from the 18th day after KOA induction, once every
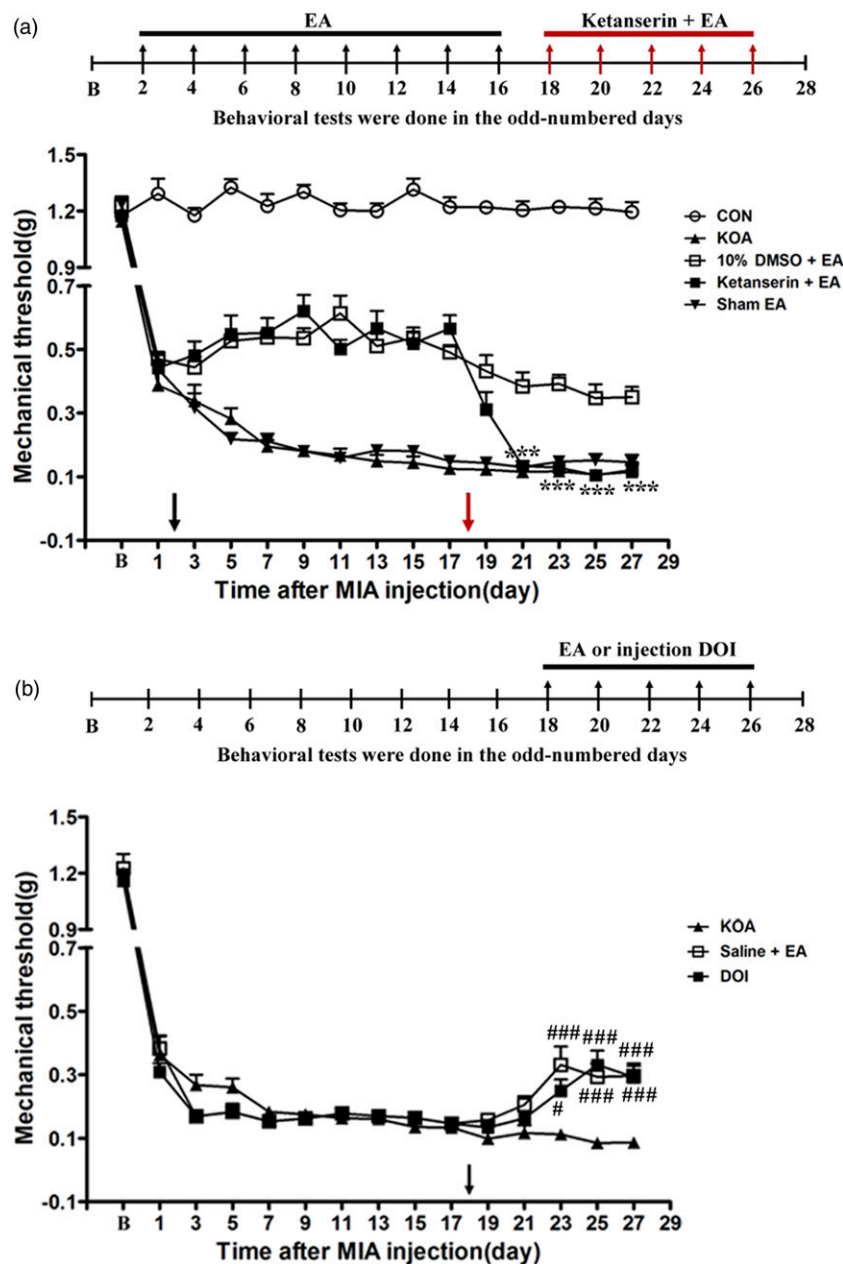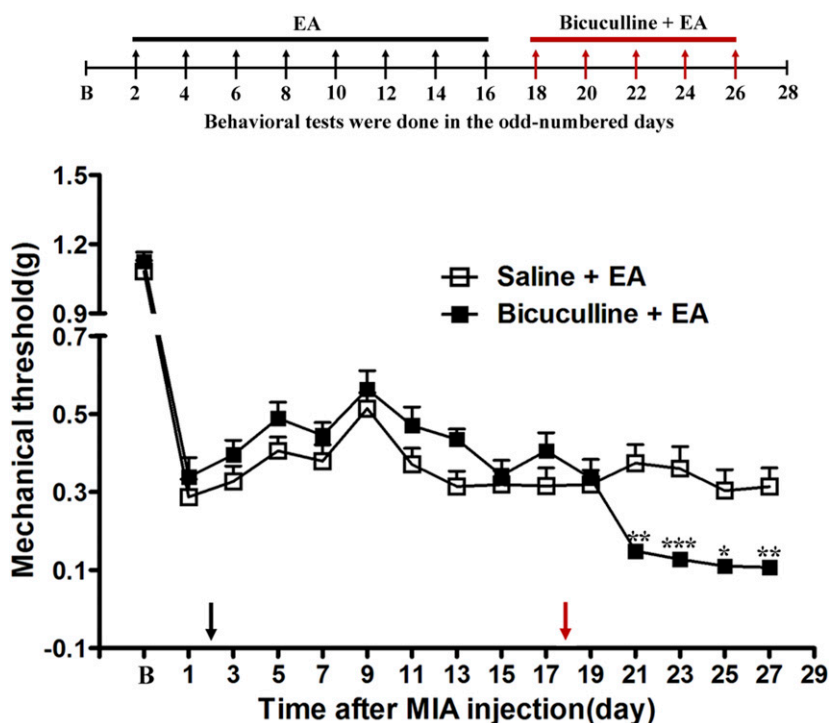
Figure 2. Time course of the effect of 5-HT$_{2A}$ receptor antagonist/agonist on pain hypersensitivity. (a) Schematic show the timeline of EA and Ketanserin administered. Time course of tactile threshold in response to von Frey filaments in control (CON), KOA, 10% DMSO + EA, Ketanserin + EA and Sham EA mice. EA was administered for 30 min, once every other day for 4 weeks, starting from 2 days after MIA injection, as indicated by black arrow. 5-HT$_{2A}$ receptor antagonist Ketanserin (10 μg) was administered *i.t* 30 min before EA starting from the 18 days after MIA injection, once every other day for 5 times, as indicated by red arrow. (b) Schematic show the timeline of EA and DOI administered. Time course of the effect of EA or 5-HT$_{2A}$ receptor agonist DOI on tactile withdrawal threshold of KOA mice. 5-HT$_{2A}$ receptor agonist DOI (10 μg) was administered *i.t* or EA stimulation starting from the 18 days after MIA injection, once every other day for 5 times, as indicated by black arrow. Data are expressed as means ± SME (*n* = 8 mice in each group). *** *p* < .001, compared with the 10% DMSO + EA group; # *p* < .05; ### *p* < .001, compared with the KOA group. KOA: knee osteoarthritis; EA: electroacupuncture.

other day for 5 times. We found that the GABA$_A$ receptor antagonist Bicuculline significantly reversed the EA effect on the tactile withdrawal thresholds in KOA mice ($p < .05$).

As shown in Figure 3, the time course curves (i.e., Bi-cuculline plus EA and Saline plus EA treated groups) were

significantly different between treatments ($F_{(1, 210)} = 97.92$; $p < .05$), across times ($F_{(14, 210)} = 57.11$; $p < .0001$), and for their interactions ($F_{(14, 210)} = 5.24$; $p < .0001$). Further analyses indicated that the mechanical thresholds in the Bicuculline treated group was significantly less than that in

Figure 3. The effect of GABA$_A$ receptor antagonist Bicuculline on EA analgesia. Schematic show the timeline of EA and Bicuculline administered. Time course of the effect of GABA$_A$ receptor antagonist Bicuculline on tactile withdrawal threshold of EA mice. EA was administered for 30 min, once every other day for 4 weeks, starting from 2 days after MIA injection, as indicated by black arrow. The GABA$_A$ receptor antagonist Bicuculline (0.1 μg) was administered *i.t* 30 min before EA starting from the 18 days after MIA injection, once every other day for 5 times, as indicated by red arrow. Data are expressed as means ± SME (n = 8 mice in each group). * p < .05; ** p < .01; *** p < .001, compared with the Saline + EA group. EA: electroacupuncture.

the Saline plus EA treated group from 21st to 27th day after KOA induction (p < .05; Figure 3).

EA increased the GABA$_A$ receptor and KCC2 expression in dorsal spinal cord of KOA mice

We have demonstrated that the GABA$_A$ receptor is involved in the analgesic effect of EA, and KCC2 is the primary protein in control of the inhibitory function of the GABA$_A$ receptor.[8,11] Thus, we used the western blotting technique to evaluate the effect of EA on the GABA$_A$ receptor and KCC2 protein levels. The GABA$_A$ receptor protein bands were presented in the dorsal spinal cord tissues (Figure 4(a)). It was discovered that, GABA$_A$ receptor protein level was significantly lower than that in the control group 4 weeks after KOA induction (p < .001; Figure 4(c)). However, EA treatment at 2 Hz + 1 mA significantly increased GABA$_A$ receptor level compared with that in the KOA group (p < .001; Figure 4(c)). Sham EA had no significant effect on GABA$_A$ receptor protein level of KOA mice (p > .05; Figure 4(c)).

The KCC2 protein bands were presented in the dorsal spinal cord tissues (Figure 4(b)), and it was elucidated that, KCC2 protein level was significantly lower than that in the control group 4 weeks after KOA induction (p < .001; Figure 4(d)). However, EA treatment at 2 Hz + 1 mA significantly increased KCC2 protein level compared with that in the KOA group (p < .001; Figure 4(d)).

Similarly, Sham EA had no significant effect on KCC2 protein level of KOA mice (p > .05; Figure 4(d)) as observed in the case of sham EA on GABA$_A$ receptor level in the same group.

Intrathecal application of 5-HT$_{2A}$ receptor antagonist/agonist can reverse or mimic the effect of EA in up-regulation of KCC2 expression

Previous studies have proved that 5-HT$_{2A}$ receptor activity can up-regulate the expression of KCC2.[10] Thus, we used the western blotting technique to observe the KCC2 protein level dynamics after intrathecal administration of 5-HT$_{2A}$ receptor antagonist or agonist. The KCC2 protein bands were presented in the dorsal spinal cord tissues (Figure 5(a)). Compared to the 10% DMSO plus EA group, the KCC2 protein level was significantly decreased in the Ketanserin plus EA group (p < .05; Figure. 5(c)), indicating that the 5-HT$_{2A}$ receptor antagonist Ketanserin can reverse the effect of EA mediated increase KCC2 expression.

We also used the western blotting to study whether intrathecal 5-HT$_{2A}$ receptor agonist DOI can mimic the effect of EA in up-regulating the KCC2 protein level in the dorsal spinal cord. We have found that the KCC2 protein level was significantly increased in the DOI group compared to that in KOA group, as well as the Saline plus EA group (p < .001; Figure 5(b) and (d)). Moreover, no significant difference was
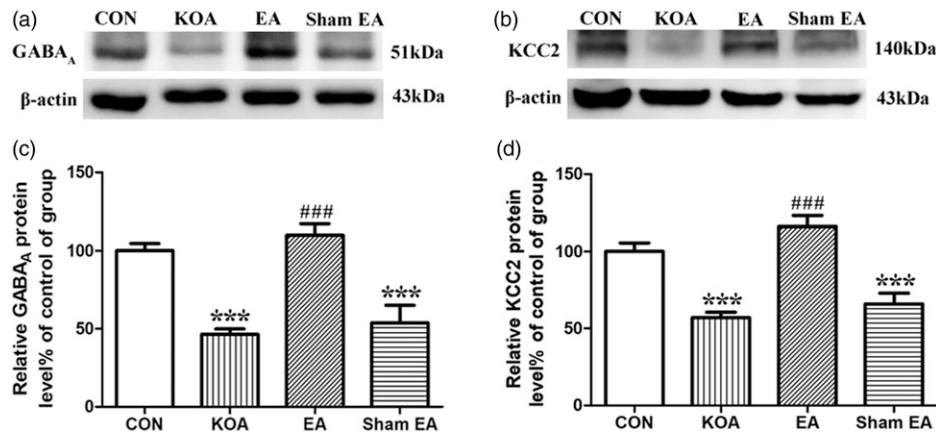
Figure 4. Quantitative analysis of protein levels of GABA$_A$ receptor and KCC2 in the dorsal spinal cord tissues. (a) and (b) Representative gel images show the protein level of GABA$_A$ receptor and KCC2 in dorsal spinal cord tissues obtained from control (CON), KOA, KOA treated with EA and KOA treated with Sham EA. β-actin was used as a loading control. The protein band at 51 kDa and 140 kDa corresponds to the GABA$_A$ receptor and KCC2, respectively. (c) Summary data show the effect of KOA, EA and Sham EA on the protein level of GABA$_A$ receptor in dorsal spinal cord tissues. (d) Effects of KOA, EA and Sham EA on the protein level of KCC2 in dorsal spinal cord tissues. Data are expressed as means ± SME (n = 4 mice in each group). *** $p < .001$ versus the control group; ### $p < .001$, compared with the KOA group. KOA: knee osteoarthritis; EA: electroacupuncture.
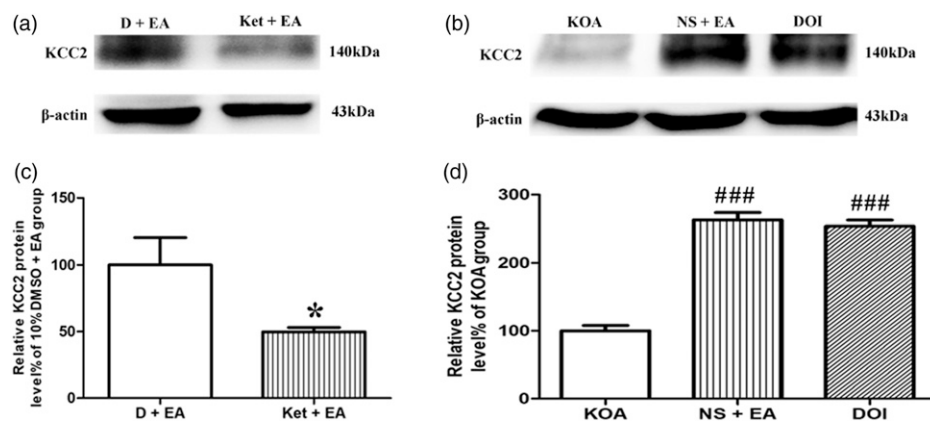
Figure 5. Effects of 5-HT$_{2A}$ receptor antagonist/agonist on KCC2 expression in the dorsal spinal cord tissues. (a) Representative gel image shows the protein level of KCC2 in dorsal spinal cord tissues obtained from 10% DMSO + EA (D + EA), Ketanserin + EA (Ket + EA). (b) Representative gel image shows the protein level of KCC2 in dorsal spinal cord tissues obtained from KOA, Saline + EA (NS + EA), DOI. β-actin was used as a loading control. The protein band at 140 kDa corresponds to KCC2. (c) Summary data show the effect of 5-HT$_{2A}$ receptor antagonist Ketanserin on the protein level of KCC2 in dorsal spinal cord tissues. (d) Effects of 5-HT$_{2A}$ receptor agonist DOI and EA on the protein level of KCC2 in dorsal spinal cord tissues. Data are expressed as means ± SME (n = 4 mice in each group). * $p < .05$ versus the 10% DMSO + EA (D + EA) group; ### $p < .001$, compared with the KOA group. KOA: knee osteoarthritis; EA: electroacupuncture.

measured between the DOI group and the Saline plus EA group ($p > .05$; Figure 5(b) and (d)).

Intrathecal application of the PLC inhibitor U73122 can reverse the EA effect on mechanical allodynia and the expression of KCC2

5-HT$_{2A}$ is a G protein-coupled receptor which mediates a series of functions by activation of PKC after coupling with Gq. The activation of 5-HT$_{2A}$ receptor was reported to induce the expression of KCC2 through the mediation of PKC.[9]

Therefore, we explored whether the Gq-PLC-PKC pathway mediated EA up-regulation of KCC2 expression through intrathecal injection of PLC and PKC inhibitors.

Phospholipase C inhibitor U73122 (5 nmol) was administered *i.t.* 30 min before EA starting from the 18 days after KOA induction, once every other day for 5 times. The U73122 was found to significantly reverse the EA effect on the tactile withdrawal thresholds ($p < .001$). As shown in Figure 6(a), the time course curves (i.e., U73122 plus EA and 10% DMSO plus EA treated groups) were significantly different between treatments (F $_{(1, 210)}$ = 103.41; $p < .0001$),
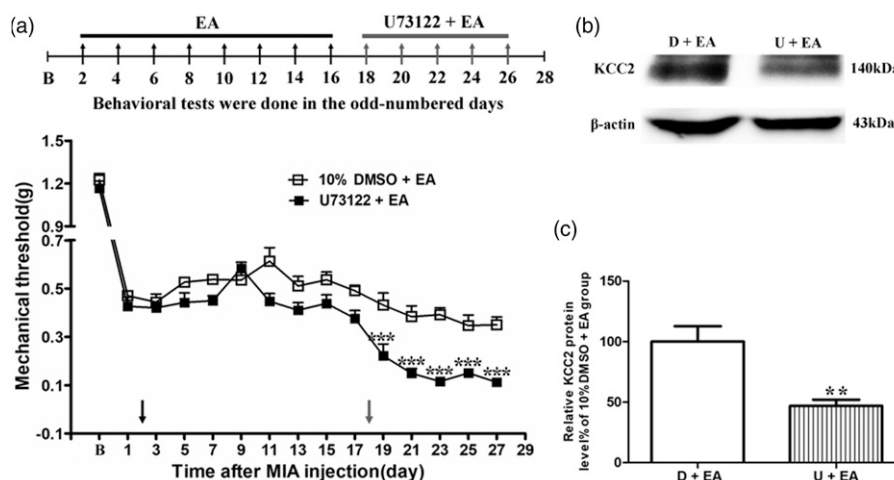
Figure 6. Effects of PLC inhibitor U73122 on EA analgesia and KCC2 expression in the dorsal spinal cord tissues. (a) Schematic show the timeline of EA and U73122 administered. Time course of the effect of PLC antagonist U73122 on tactile withdrawal threshold of EA mice. (b) Representative gel image shows the protein level of KCC2 in dorsal spinal cord tissues obtained from 10% DMSO + EA (D + EA) and U73122 + EA (U + EA). β-actin was used as a loading control. The protein band at 140 kDa corresponds to KCC2. (c) Summary data show the effect of PLC antagonist U73122 on the protein level of KCC2 in dorsal spinal cord tissues. EA was administered for 30 min, once every other day for 4 weeks, starting from 2 days after MIA injection, as indicated by black arrow. The PLC antagonist U73122 (5 nmol) was administered *i.t* 30 min before EA starting from the 18 days after MIA injection, once every other day for 5 times, as indicated by red arrow. Data are expressed as means ± SME ($n = 8$ mice in each group (behavioral test); $n = 4$ mice in each group (western blotting)). $** p < .01$; $*** p < .001$, compared with the 10% DMSO + EA (D + EA) group. KOA: knee osteoarthritis; EA: electroacupuncture; PLC: phospholipase C.

across times ($F_{(14, 210)} = 94.21$; $p < .0001$), and for their interactions ($F_{(14, 210)} = 3.66$; $p < .0001$). Further analyses indicated that the mechanical thresholds in the U73122 plus EA treated group was significantly less than the 10% DMSO plus EA treated group from 19th to 27th day after KOA induction ($p < .001$; Figure 6(a)).

In addition, we used western blotting technique to examine the protein level of KCC2 after intrathecal U73122 before EA treatment. The KCC2 protein bands were presented in the dorsal spinal cord tissues (Figure 6(b)). Compared with the 10% DMSO plus EA group, the KCC2 protein level was significantly decreased in the U73122 plus EA group ($p < .01$; Figure 6(c)). These results indicated that the PLC antagonist U73122 can reverse the analgesic effect of EA and the EA mediated up-regulation of KCC2 expression.

Intrathecal application of the PKC inhibitor Chelerythrine can reverse the EA effect on mechanical allodynia and the expression of KCC2

Protein kinase C inhibitor Chelerythrine (1 nmol) was administered *i.t.* 30 min before EA treatment starting from the 18th day after KOA induction, once every other day for 5 times. The Chelerythrine significantly reversed the EA effect on the tactile withdrawal thresholds ($p < .01$). As shown in Figure 7(a), the time course curves (i.e., Chelerythrine plus EA and 10% DMSO plus EA treated groups) were significantly different between treatments ($F_{(1, 210)} = 51.21$; $p < .0001$), across times ($F_{(14, 210)} = 86.04$; $p < .0001$), and for

their interactions ($F_{(14, 210)} = 4.38$; $p < .0001$). Further analyses indicated that the mechanical thresholds in the Chelerythrine plus EA treated group was significantly less than the 10% DMSO plus EA treated group from 21st to 27th day after KOA induction ($p < .01$; Figure 7(a)).

Moreover, western blotting technique was also employed to study the protein level of KCC2 after intrathecal application of Chelerythrine before EA treatment on to the dorsal spinal cord. It was consequently found that, there was significant reduction in KCC2 protein level after intrathecal injection of Chelerythrine before EA compared to that in 10% DMSO plus EA group ($p < .05$; Figure 7(b) and (c)). These results indicated that the PKC antagonist Chelerythrine can reverse the analgesic effect of EA and the up-regulation of KCC2 expression by EA.

Discussion

In this study, we have demonstrated that EA treatment of 2 Hz+1 mA significantly increased the expression of 5-HT$_{2A}$ receptor in the dorsal spinal cord and intrathecal administration of 5-HT$_{2A}$ receptor antagonist/agonist reversed or mimicked the analgesic effect of EA, respectively. Antagonist of GABA$_A$ receptor, Bicuculline administered *i.t.* 30 min before EA treatment was found to have blocked the EA effect on pain hypersensitivity. Moreover, EA was also found to reversed the reduced expression of GABA$_A$ receptor and KCC2 in the dorsal spinal cord of KOA mice. Furthermore, we have also proved that intrathecal application of 5-HT$_{2A}$
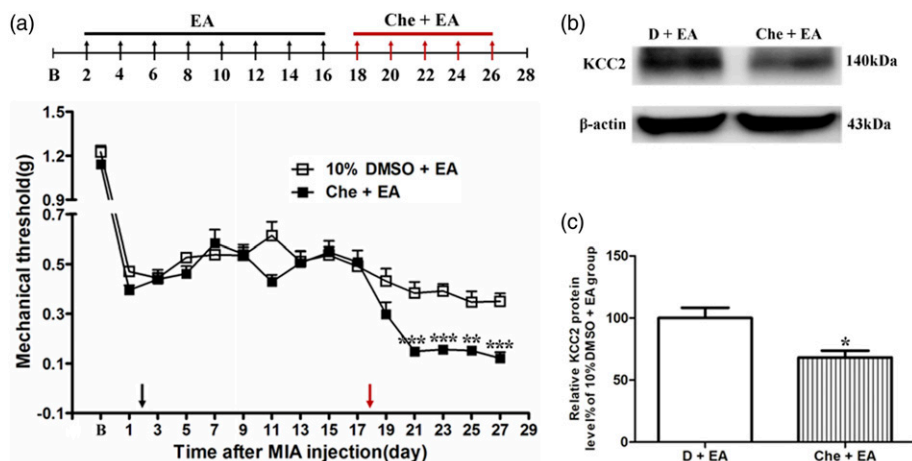
Figure 7. Effects of PKC inhibitor Chelerythrine on EA analgesia and KCC2 expression in the dorsal spinal cord tissues. (a) Schematic show the timeline of EA and Chelerythrine administered. Time course of tactile threshold in response to von Frey filaments in 10% DMSO + EA and Chelerythrine + EA mice. (b) Representative gel image shows the protein level of KCC2 in dorsal spinal cord tissues obtained from 10% DMSO + EA (D + EA) and Chelerythrine + EA (Che+ EA). β-actin was used as a loading control. The protein band at 140 kDa corresponds to KCC2. (c) Summary data show the effect of PKC antagonist Chelerythrine on the protein level of KCC2 in dorsal spinal cord tissues. EA was administered for 30 min, once every other day for 4 weeks, starting from 2 days after MIA injection, as indicated by black arrow. The PKC antagonist Chelerythrine (1 nmol) was administered *i.t* 30 min before EA starting from the 18 days after MIA injection, once every other day for 5 times, as indicated by red arrow. Data are expressed as means ± SME ($n = 8$ mice in each group (behavioral test); $n = 4$ mice in each group (western blotting)). * $p < .05$; ** $p < .01$; *** $p < .001$, compared with the 10% DMSO + EA (D + EA) group. PKC: protein kinase C; KOA: knee osteoarthritis; EA: electroacupuncture.

receptor antagonist/agonist reversed or mimicked the effect of EA mediated up-regulation of KCC2 expression in the dorsal spinal cord. Additionally, intrathecal injection of PLC and PKC inhibitors prevented anti-allodynia effect of EA and up-regulation of KCC2 expression by EA treatment. Our study provided clear evidences that EA up-regulated KCC2 expression through activation of 5-HT$_{2A}$-Gq-PLC-PKC pathway which enhanced the inhibitory function of GABA$_A$ receptor, thereby exerting chronic pain inhibition in a mouse model of KOA.

The present study has demonstrated that intrathecal injection of 5-HT$_{2A}$ receptor antagonist Ketanserin 30 min before EA, significantly reduced mechanical withdrawal threshold compared to 10% DMSO plus EA group. This result suggests that the 5-HT$_{2A}$ receptor antagonist blocked the analgesic effect of EA in KOA model. This data corroborates the findings of Li et al. (2011)[15] who reported that same 5-HT$_{2A}$ receptor antagonist Ketanserin was able to blocked the analgesic effect of EA in rat model of osteoarthritis. On the other hand, we have also proved that intrathecal injection of the 5-HT$_{2A}$ receptor agonist DOI mimicked the analgesic effect of EA. Furthermore, EA treatment was also found to have significantly increased the expression of 5-HT$_{2A}$ receptor in the dorsal spinal cord. Our data suggest that EA treatment induces the release of 5-HT, which activates 5-HT$_{2A}$ receptor in the spinal cord to inhibit chronic pain, hence, supporting the normal physiologic mechanism through which serotonin might bring about pain inhibition and its involvement in spinal analgesia. Previous study by

Lee et al. (2016)[16] has demonstrated that spinal 5-HT$_3$ receptor antagonist, but not the 5-HT$_{2A}$ receptor antagonist Ketanserin, blocked EA-produced inhibition of cold allodynia in a rat model of neuropathic pain, which is contrary to the findings of the present study. It is therefore reasonable to suggest that the antiallodynia effect of EA may involve different or multiple mechanisms depending on the nature of the noxious stimulus (e.g., cold allodynia versus mechanical allodynia).

Large body of literatures have documented the fact that GABA$_A$ receptor is the primary synaptic target of the major inhibitory neurotransmitter GABA in the CNS, and that this neurotransmitter may largely be involved in pain. In support of this, numerous researches has strongly reported significant down-regulation in the inhibitory function of GABA$_A$ receptor during neuropathic pain state, and intrathecal GABA$_A$ receptor agonist blocked the pain induced by spinal cord injury.[11,17] Results of western blotting from the present study has observed significant increase in the protein level of GABA$_A$ receptor in the dorsal spinal cord following repeated EA treatments, and intrathecal injection of GABA$_A$ receptor antagonist Bicuculline prevented the anti-allodynia effect of EA. This observation is in line with the findings of a previous study,[18] and also suggests that GABA$_A$ receptor in the spinal cord is involved in EA mediated inhibition of chronic pain in KOA model.

Activation of the GABA$_A$ receptor causes opening of chloride ion channels leading to Cl$^-$ influx by transferring extracellular Cl$^-$ intracellularly, thereby hyperpolarizing

neurons with consequent generation of Inhibitory Postsynaptic Potentials.[19] In addition, internal flow of Cl^- depends on the concentration gradient across the cellular membrane which itself depends on the KCC2 as the primary protein in control of the inhibitory function of $GABA_A$ receptor. In line with this fundamental fact, we have found significant increase in the protein level of KCC2 in the dorsal spinal cord following repeated EA treatments. It was also demonstrated by earlier studies that activation of $5\text{-}HT_{2A}$ receptor induced the expression of KCC2 through the mediation of PKC to enhance the inhibitory function of $GABA_A$ receptor.[9,12] In this study, we have shown that the level of KCC2 was significantly decreased after intrathecal injection of $5\text{-}HT_{2A}$ receptor antagonist Ketanserin before EA, and intrathecal injection $5\text{-}HT_{2A}$ receptor agonist DOI mimicked the effect of EA induced up-regulation of KCC2 protein level.

$5\text{-}HT_{2A}$ receptor is a G protein-coupled receptor which could couple with Gq and activate PLC leading to increase in intracellular Ca^{2+} concentration and activation of PKC.[8] Previous study has demonstrated that activation $5\text{-}HT_{2A}$ receptor could increase the expression of KCC2 through a mechanism mediated by the protein kinase C (PKC).[9] Our study further revealed that intrathecal injection of PLC and PKC inhibitors prevented the anti-allodynia effect of EA and up-regulation of KCC2 expression by EA. Moreover, reduced KCC2 function on the cell membrane causes opening of more chloride ion channels leading to a decreased concentration of intracellular Cl^-, which in turn activate and depolarizes the $GABA_A$ receptors, leading to the declined in their inhibitory function.[12] Therefore, these data suggest that EA up-regulated KCC2 expression through activation of $5\text{-}HT_{2A}$-Gq-PLC-PKC pathway, which mediate the enhancement of the inhibitory function of $GABA_A$ receptor.

In conclusion, our study have demonstrated that EA may potentiate the expression of $5\text{-}HT_{2A}$ receptor in the dorsal spinal cord to enhance the KCC2 expression via the activation of the $5\text{-}HT_{2A}$-Gq-PLC-PKC pathway thereby enhancing the inhibitory function of $GABA_A$ receptor, which could in turn lead to inhibition of chronic pain in a mouse model of KOA. Therefore, our results provide new insight into the mechanisms through which EA mediated activation of $5\text{-}HT_{2A}$ receptor could reduce chronic pain in KOA mouse model.

Materials and methods

Animals

112 with Eight-weeks-old male C57BL/6J mice were obtained from the Medical Experimental Animal Center of Xi'an Jiaotong University, Shaanxi Province, China. Animals were housed in a colony room with controlled ambient temperature ($25 \pm 2°C$), humidity ($50 \pm 10\%$) and under a 12/12 h light-dark cycle (lights on at 08:00) with access to food and water ad libitum. The experimental protocol was in accordance with the ethical guidelines of the International Association for the Study of Pain[20] and approved by the Institutional Animal Care Committee of Xi'an Jiaotong University. All efforts were made to minimize the number of animals used, as well as distress to the animals during the experimental procedures. Mice were randomly divided into different groups.

Induction of knee osteoarthritis (KOA)

KOA was induced by intra-articular injection of monosodium iodoacetate (MIA; Sigma, UK) into the left knee joint after mice were briefly anesthetized with isoflurane. The knee joint was shaved and flexed at a 90 angle. Five microliters of 5 mg/mL MIA in sterile saline (0.9%) were injected through the infrapatellar ligament into the joint space of the left knee with a 30-gauge needle.[21] The chosen MIA concentration was believed to have been sufficient enough to cause histological changes in the cartilage[14] and also induces joint pain[22] in mice. Mice in the control group received an intra-articular injection of vehicle (5 μl of sterile saline, 0.9%) as placebo.

EA treatment

The animals were habituated to the restricting bag for 30 min each day for three consecutive days before KOA induction. In the EA treatment group, mice received EA administration on the left "Neixiyan" (Ex-LE4) and "Dubi" (ST35) once every other day for 4 weeks, starting from 2 or 18 days after MIA injection. EA dosage (1 mA and 0.1 ms) was administered at the 2 Hz frequency for 30 min. The current was delivered with a Han's Acupoint Nerve Stimulator (LH202; Huawei Co., Ltd., Beijing, China). Two acupuncture needles were inserted into two acupoints corresponding to Ex-LE4 and ST35 in humans. Ex-LE4 is located at the medial cavity of the patella and the patellar ligament, and ST35 lies on the lateral cavity of the patella and patellar ligament. Ex-LE4 and ST35 were chosen by virtue of their specificity as targeted acupoints for treating knee problems, and also yield results in KOA at highest frequency.[23,24]

For Sham treatment control, acupuncture needles were inserted bilaterally into Ex-LE4 and ST35 without electrical stimulation or manual manipulation. This procedure mimicked the actual treatment but produces little therapeutic effect. Our previous data has revealed that needles inserted into active acupoints without administering any electrical or manual stimulation, do not produce analgesic effect.[3]

Mechanical paw withdrawal threshold measurement

Mechanical allodynia was demonstrated in the hind paw of animals with KOA, using von Frey filaments.[21,25] The behavioral test was performed three times before KOA induction and once every other day, starting from the first day to

4 weeks after KOA induction. The animals were habituated to the testing environment for 30 min.

Mechanical allodynia was assessed by placing mice on an elevated mesh floor, and the tactile threshold was measured by using the "up-down" method.[26] After an acclimation period of 30 min, a series of calibrated von Frey filaments (Stoelting, Kiel, WI, USA) were applied perpendicularly to the plantar surface of the left hind paw with sufficient force to bend the filament for 6 s. Any brief withdrawal or paw flinching due to this procedure was considered as a positive response. The test was repeated two times after which the mean value was calculated.

Quantitative polymerase chain reaction

The dorsal spinal cord tissues were removed 4 weeks after vehicle or MIA injection. The tissues were excised from the animals immediately following decapitation under 10% chloralic hydras anesthesia. Total RNA was isolated from the spinal cord specimens by using RNAeasy^{TM} Animal RNA Isolation Kit with Spin Column (Beyotime Biotechnology, Nanjing, China). Aliquots of 2 μg total RNA were reverse transcribed into cDNA using ReverTra Ace-a-TM (Toyobo, Japan). The PCR mixture (10 μL) consisted of 1 μL diluted cDNA, 5 μL SYBR quantitative polymerase chain reaction (qPCR) mix (Takara, Japan), 1 μL of each primer, and 3 μL water. qPCR was performed using CFX96 system (Bio-Rad, UK), and the relative expression levels were quantified with CFX Manager software. The expression level of each gene was determined by the threshold cycle (CT). The amount of target mRNAs, normalized to the endogenous control (GAPDH), was obtained by $2^{-\triangle\triangle CT}$. Results of independent experiments were expressed as the percentage change over mRNA level of the control group. Specific sequence primers are listed in the follower: Mouse-5-HT_{2A} forward 5′-AACCCCATTCACCATAGCCG-3′; reverse 5′-GGA-TATGGTCCACACCGCAA-3′. Mouse-GAPDH forward 5′-AGGTCGGTGTGAACGGATTTG-3′; reverse 5′-TGTA-GACCATGTAGTTGAGGTCA-3′.

Western blotting

The dorsal spinal cord tissue was removed as described above, minced with scissors, and homogenized in RIPA lysis buffer with 200 μL (Beyotime Biotechnology, China) and 2 mM phenylmethylsulfonyl fluoride and centrifuged at 12,000 g for 15 min. The pellet was discarded, and protein concentrations of the supernatant were determined by using the BCA Protein Assay Kit (Pioneer Biotechnology, China). Proteins were denatured with sodium dodecyl sulfatepolya-crylamide gel electrophoresis (SDS-PAGE) loading buffer at 98°C for 5 min and separated on glycine-SDS-PAGE gel. The proteins were transferred onto a polyvinylidene fluoride membrane, blocked for 1 h by 5% non-fat dry milk in Tris-buffered saline (TBS) containing 0.1% Tween-20. The membrane was incubated with rabbit anti-5-HT_{2A} antibody (1:300, Bioss, China); mouse anti-$GABA_A$ antibody (1:1000, Santa Cruz, Dallas, TX, USA); rabbit anti-KCC2 antibody (1:1000, Abcam, Hong Kong); or mouse anti-β-actin antibody (1:5000; Proteintech, USA) at 4°C overnight. The membranes were washed 3 times in 0.1% TBS-Tween 20 (pH 7.6), and then incubated with horseradish peroxidase-conjugated secondary antibodies from Merck Milipore: goat anti-rabbit secondary antibody (1:5000) or goat anti-mouse secondary antibody (1:5000) for 2 h at room temperature and washed three times. The enhanced chemiluminescence method (ECL Plus Western blotting detection reagents, Merck Milipore, USA) was used to reveal the protein bands according to the manufacturer's protocol. The optical density of each band was then measured with a computer-assisted imaging analysis system (Quantity One, Bio-Rad, UK) and normalized with β-actin. Results of independent experiments were expressed as the percentage change over the protein amount in the control group.

Drug dosing and administration

Drugs were administered intrathecally (i.t., 10 μL) 30 min prior to EA treatment under isoflurane anesthesia applied by lumbar puncture. Needle was first inserted through the vertebral column into the L5 to L6 intervertebral space, tail flick response following the insertion into the i.t. space indicated a successful insertion.[27] Each drug was then slowly injected over a period of 10 s, after which the needle was carefully removed from the spinal cord. The drug control groups received an identical injection of vehicle without drug as placebo.

Drugs used in this study, including the 5-HT_{2A} receptor agonist DOI ((2,5-dimethoxy-4-idophenyl)-2-aminopropane hydrochloride, 10 μg), 5-HT_{2A} receptor antagonist Ketanserin (3-[2-[4-(fluorobenzoyl)piperidin-1-yl]ethyl]-1H-quinazoline-2,4-dione, 10 μg), PLC inhibitor U73122 (1-(6-((8R,9S,13S,14S,17S)-3-methoxy-13-methyl-7,8,9,11,12, 13,14,15,16,17-decahydro-6H-cyclopenta[a]phenanthren-17-ylamino)hexyl)-1H-pyrrole-2,5-dione, 5 nmol), PKC inhibitor Chelerythrine (1,2-dimethoxy-12-methyl-[1,3] benzodioxolo[5,6-c]phenanthridine-12-ium, 1 nmol), and $GABA_A$ receptor antagonist (+)-Bicuculline ([R-(R*,S*)]-6-(5,6,7,8-Tetrahydro-6-methyl-1,3-dioxolo[4,5-g]isoquinolin-5-yl)furo[3,4-e]-1,3-benzodioxol-8(6H)-one, 0.1 μg), were purchased from RBI/Sigma, St Louis, MO, USA. The drugs were freshly prepared in saline or 10% DMSO. The doses of each drug were chosen according to previous studies, where they were reported to have been effective.[28–31] Equal volumes of saline or 10% DMSO were injected into the spinal cord as vehicle in the control mice as placebo.

Data Analysis

Data are presented as means ± SEM. Two-way repeated-measures ANOVA were used to determine the statistical

difference in the withdrawal thresholds, where each factor as a "between subjects" (group) or "repeated measures factor" (time points), followed by a Bonferroni post-hoc analysis of multiple comparisons were conducted. Other data were analyzed using *t*-test or one-way ANOVA followed by Newman–Keuls post-hoc test with two-tailed hypothesis. GraphPad Prism software 5.0 were used to perform the data analysis. *p*-value less than 0.05 was considered significant.

Authors' Contribution

X. C. Y. and F. Q. H. conceived and designed the experiments. X. C. Y. did most of the experiments and analyzed the data. X. J. Y. and L. X. T. helped with the western blotting experiment. Y. Y. W., Y. X. G. H. J. and H. S. W. helped with mouse model and behavior test experiments. Y. Y. W., Y. L. Z., and M. L. helped with the data collection and analyzed the data. X. C. Y., S. S. B. and F. Q. H. wrote the manuscript. All authors reviewed the manuscript.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

ORCID iDs

Sani S Baba https://orcid.org/0000-0002-6597-4602
Man Li https://orcid.org/0000-0003-4041-0437
Fu-Quan Huo https://orcid.org/0000-0002-4329-5502

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