Effect of Electroacupuncture on AMPA Receptor GluR2 Subunit in Complete Freund's Adjuvant-induced Inflammatory Pain Model

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AMPA receptor (AMPAR)s are heterotetrameric structures made up from 4 units (GluR1-4) and are thought to underlie perception of persistent inflammatory pain. Complete Freund's adjuvant (CFA)-evoked inflammation induces synaptic GluR2 internalization, which is initiated by GluR2 phosphorylation, in dorsal horn neurons during the maintenance of CFA-induced hypersensitivity. The present study investigated whether electroacupuncture (EA) stimulation has any effect on GluR2 trafficking by using immunoblot and immunohistochemistry. We examined that CFA-induced dorsal horn GluR2 internalization was attenuated by EA treatment. EA treatment could also decrease the level of pGluR2 regardless of whether CFA injection was administrated or not. In addition, previous studies suggest that microglial cells are increased without morphological change in CFA injected animal. In our study, increases in microglial cells in CFA group were observed, whereas EA with or without CFA-injected group showed similar aspects with normal group. In conclusion, our results indicate that EA might blunt CFA-evoked inflammation by coordinating mechanisms at the upstream step of neuron activation and GluR2 phosphorylation.

Key words : electroacupuncture, AMPA receptor, GluR2, CFA, glia cell

Introduction

Acupuncture has been used as a healing art in traditional Korean Medicine by stimulating specific acupoints on the skin of the patient's body[1]. Noxious stimuli such as complete Freund's adjuvant (CFA), carrageenan, formalin and capsaicin cause tissue inflammation[2-4]. These stimuli cause peripheral pain sensitization at the tissue damage site and are transferred through Aδ or C nerve fiber to spinal cord where central sensitization is formid. In the spinal cord dorsal horn neurons, a series of signal transduction cascades including ionotropic and metabotropic receptors, protein kinases-PKA, PKC or mitogen-activated protein kinase family and so on transmits noxious signals inside nerve cells[5,6].

Especially,α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR)s are responsible for the majority of excitatory synaptic transmission and play a crucial role in synaptic plasticity in the mammalian central nervous system[7]. Several genetic studies indicate that GluR2, as a key subunit in the AMPAR complex, determines activity-induced nociceptive hypersensitivity and suggest that removal or internalization of synaptic GluR2 in dorsal horn neurons might contribute to spinal central sensitization, a specific form of synaptic plasticity, in persistent inflammatory pain. Further, Park et al. reported that GluR2 phosphorylation was related to CFA-induced persistent inflammation[21].

Acupuncture has been used to treat a variety of illness including inflammatory pain in traditional Korean medicine[8] and electroacupuncture (EA) which is one of acupuncture techniques has widely been used to relieve acute and chronic inflammatory pain in patients. Studies on EA using experimental pain models also show that EA can effectively attenuate hyperalgesia associated with inflammatory pain[9,10]. Frequency-dependent EA analgesia is mediated by different opioid receptor subtypes. EA and disrupting glial function synergistically suppress inflammatory pain. EA analgesia is likely associated with its counter-regulation to spinal glial activation[11].

Therefore, in the present study, we investigated whether molecular mechanism of EA analgesia is related to attenuation of membrane GluR2 internalization in the CFA-induced inflammatory pain model. In addition, we studied the effect of EA on phosphorylation of GluR2 in neuron and glial cells in

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CFA-induced inflammatory pain model.

Materials and Methods

1. Animal and CFA injection

Male Sprague-Dawley rats (8 weeks) were purchased from Dooyeol biolab (Seoul, Korea) and adapted at 12:12h light-dark cycle for 3 day before the study. Food and tap water were provided ad libitum. The experimental procedures were conducted under the Animal Care Guideline of the Animal Experimental Committee of Pusan National University. To produce inflammation response, 0.1 ml of CFA (Sigma, St. Louis, MO, USA) emulsion was injected subcutaneously into the plantar side of one hind paw. Naive animals were used as normal group.

2. EA stimulation

In all experiment, two pairs of stainless-steel acupuncture needles (Dong-Bang, South Korea) of 0.25 mm diameter were bilaterally inserted into rat's hind legs on the same acupoint as Zusanli (ST36) and Sanyinjiao (SP6) in human. ST36 and SP6 are commonly used acupoints both in clinical practice and basic science research for the treatment of a variety of symptoms, including pain relief[12-14]. The needles at Zusanli and Sanyinjiao acupoint were connected to the cathode and anode of Electro-pulse generator (A300 Pulsemaster, World precision instruments, USA), respectively. Electrical pulses were given to rats in the following constant conditions: 2Hz frequency and 1ms width for 20 minute. All manipulations (EA stimulation, CFA injection, and normal group treatment) were carried out under 2% isoflurane (Choongwae pharm corporation, South Korea) anesthesia.

3. Subcellular Fractionation

The L_{4-5} segments of the spinal cords were homogenized in homogenization buffer [250 mM sucrose, 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 1 mM PMSF, 1 mM benzamidine]. The homogenate was centrifuged at 900 × g for 15 min at 4℃, and then the supernatant (T, total soluble fraction) was collected. After centrifugation at 20,500 rpm for 1 hour at 4℃, the supernatant (C, crude cytosolic fraction) and pellet (M, crude plasma membrane fraction) were collected. Pellet was dissolved in buffer [10 mM Tris-HCl (pH 7.4), 1.5% SDS, 0.1% Triton X-100].

4. Western blot analysis

For Western blot analysis, T, M, C samples were collected and the amount of total protein was measured by protein assay kit (BioRad, Hercules, CA, USA). Equal amounts of protein were separated by 7.5% SDS-PAGE and transferred to nitrocellulose membranes (Whatman protran™, Dassel, Germany). Membranes were blocked with 5% skim milk in PBST buffer [0.1% (v/v) Tween-20 in PBS buffer]. After washing with PBST buffer, the membranes were incubated overnight with the following primary antibody: rabbit polyclonal to GluR2 (ab20673, Abcam, Cambridge, UK), rabbit polyclonal to phospho-GluR2 (pGluR2 ser 880, ab52180, Abcam), rabbit polyclonal anti N-cadherin (sc-7939, SantaCruz Biotechnology, Santa Cruz, CA, USA) mouse anti α-adaptin (sc-17771, SantaCruz Biotechnology). The membranes were then washed with PBST buffer for 30 min and incubated for 1 h with the following secondary antibody: goat anti-rabbit IgG-HRP (sc-2004, SantaCruz Biotechnology), goat anti-mouse IgG-HRP (sc-2005, SantaCruz Biotechnology). After washing with PBST buffer, antibody-specific protein were detected by chemiluminescence method (Fluorchem Q, Alpha Innotech, San Leandro, CA, USA). Each immunoblot area of GluR2 and pGluR2 at each time point was captured and analyzed using Image J program (version 1.42q, NIH, USA).

5. Immunohistochemistry

The L_{4-5} segments of the spinal cords were fixed in 4% paraformaldehyde and immersed in 30% sucrose for 48 h at 4℃ for cryoprotection. Frozen 14 ㎛-thick section were then prepared, and pre-incubated in a blocking solution (CAS-block, Invitrogen-Molecular Probes, Camorillo, CA, USA) for 9 min at room temperature. The sections were incubated with the following primary antibodies overnight in PBS at 4℃: rabbit polyclonal to pGluR2 (ab52180, Abcam), anti-microglia (47704, BD Biosciences, San Jose, CA, USA) and anti-neuronal nuclei (NeuN, Millipore-Chemicon, Billerica, MA, USA). After being washed with PBST, the sections were incubated with secondary antibody: fluorescein anti-rabbit IgGc (Vector Laboratories, Inc. Burlingame, CA, USA), goat anti-rabbit IgG-TR (SantaCruz Biotechnology) for 2 h at room temperature and then washed with PBST. Slides were mounted in the mounting medium for fluorescence (Vector Laboratories, Inc.) and captured by a laser scanning confocal microscope (LSM 510, Zeiss, Oberkohen, Germany)

6. Data Analysis

Data was expressed as mean±SEM. Calculations of means, standard errors, one way ANOVA for the pairwise multiple comparison were performed using SigmaPlot version 11.2 software (SPSS, Chicago, IL, USA). $P<0.05$ was considered to indicate statistically significant differences.

Results

1. Effect of EA treatment on GluR2 internalization

This experiment was designed to test the effect of EA on membrane GluR2 internalization in CFA-induced pain model. EA was applied just after intraplantar injection of CFA. Rats were divided into the following 4 groups (n=3): normal, CFA-injected (CFA), EA-treated (EA) and CFA-injected group with EA stimulation (C+E)group. Each group (CFA, EA, C+E), except normal group, was then divided into two : day 1 and day 2. The L_{4-5} horn tissues were collected from day 1 group immediately after CFA injection and again the next day at the same time after EA stimulation. The L_{4-5} horn tissues were collected form day 2 group 3 times : immediately after CFA injection, the next day at the same time and two days after at the same time after adding EA stimulation.

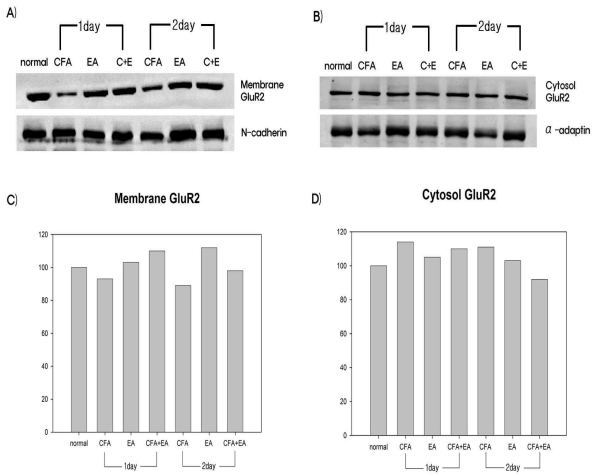

Fig. 1. Western blotting and densitometric analysis for membrane (A and C) and cytosolic GluR2 (B and D) in the ipsilateral dorsal portion of the L_{4-5} segment of the spinal cord. Densitometric data are shown normalized to levels detected in normal group which ran on the same gel.

As shown in Fig. 1, when one day had elapsed, in CFA group, the expression of membrane GluR2 was decreased (around 90%) while that of cytosolic GluR2 was increased (around 110%) in comparison with normal group, In EA group, there was little difference in both membrane and cytosolic GluR2 when compared with normal group (103% and 105%, respectively). In C+E group, the expression of GluR2 was increased (from 93% to 110%) in membrane, but there was little difference in cytosol in comparison with CFA group (114% and 110%, respectively). When two days had elapsed, in CFA group, the expression of membrane GluR2 was decreased (from 93% to 89%) a little more than day 1, but that of cytosolic GluR2 was decreased more than day 1 (from 114% to 111%). In EA group, the expression of membrane GluR2 was increased (around 110%), whereas that of cytosolic GluR2 was similar to that of normal group (103%). In C+E group, both membrane and cytosolic GluR2 were slightly decreased (98% and 92%, respectively).

2. Effect of EA treatment on phosphorylation of GluR2

This experiment was designed to determine the effect of CFA injection and EA treatment on phosphorylation of GluR2 in dorsal horn. In EA group and C+E group, tissues were collected after CFA injection and EA treatment under the same conditions as day 2. We got a graph by counting the number of the pGluR2-expressing cells from the picture obtained by immunohistochemistry(Fig. 2A). As illustrated in Fig. 2B, in the CFA group, the spinal pGluR2 increased compared to normal group after CFA injection ($p<0.001$). In EA group, the pGluR2 decreased compared to normal group ($p<0.001$) and compared to CFA group ($p<0.001$). The CFA+EA group showed a significant decrease of the pGluR2 compared to the CFA group ($p<0.05$).

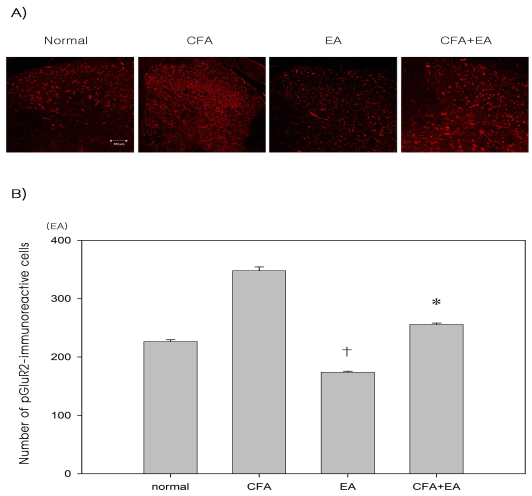

Fig. 2. Effect of EA on phosphorylation of GluR2 in CFA induced inflammation at 2 days after CFA-injection. Confocal microscopic images for pGluR2 in dorsal horn (A, Scale bar = 100 ㎛). Graph showing results on the number of cells expressing pGluR2. The results are presented as mean±SEM *P < 0.001 as compared to the CFA group, and † P < 0.001, as compared to the CFA+EA group.

3. Localization of pGluR2 in dorsal horn of the spinal cord

Confocal images demonstrated that pGluR2-immunoreaction (red) was colocalized with most of NeuN-positive cell (neuron, green) in the dorsal horn(Fig. 3A). The expression level of pGluR2 was also increased (red) compared with normal group. In EA group, the number of pGluR2-labelled neuron was decreased a little, compared with normal group. The number of pGluR2-labelled neuron was decreased when compared with CFA group in C+E group.

CFA group appeared dark yellow in merged image in comparison with normal group. In EA group and C+E, the intensity of yellow appeared to be much lower in comparison with CFA group. Such condition also can be seen in higher magnification(Fig. 3B).

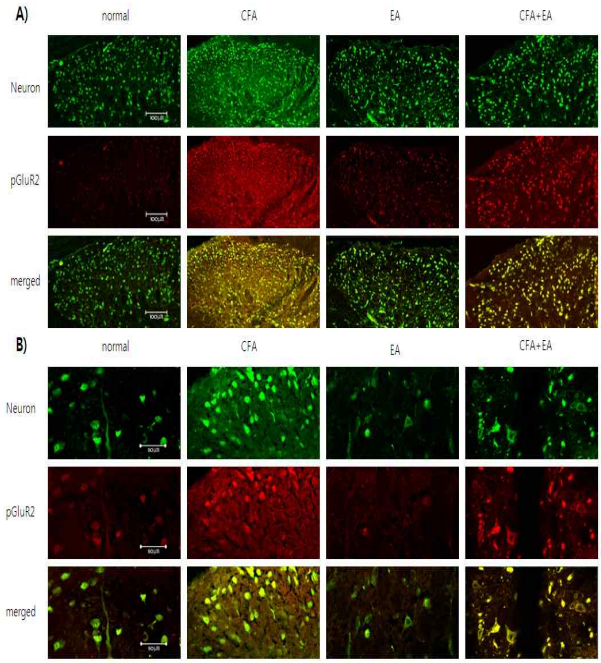

Fig. 3. Confocal microscopic images for pGluR2 and NeuN in dorsal horn. NeuN-positive cells (neuron) were usually colocalized with pGluR2 (red), and these cells were observed as yellow fluorescence in combination image (A, Scale bar = 100 ㎛). A higher magnification (B, scale bar = 50 ㎛).

4. Effect of EA treatment on activation of microglia

As shown in Fig. 4, the number of microglial cells in CFA group was remarkably increased in comparison with that of normal group (green). Further, the processes of microglial cells in CFA group were shortened and thickened in comparison with those of normal group. However, EA group and C+E group showed similar aspects with normal group.

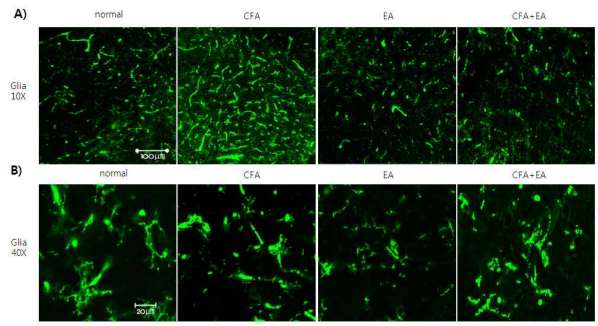

Fig. 4. Confocal microscopy of microglial cells in dorsal horn. Microglial cells were detected with microglial marker OX42 (A, scale bar = 100 ㎛). A higher magnification (B, scale bar=20 ㎛).

Discussion

EA has been shown to alleviate effectively the pain induced by inflammation in various types of rat model[15,16]. Meanwhile, among all four AMPAR subunits, GluR1 and GluR2 are the most abundant and are highly concentrated on the postsynaptic neuronal membranes of the superficial dorsal horn[17,18]. Targeted disruption of GluR2 gene produces a deficiency of dorsal horn GluR2-containing AMPARs, facilitates dorsal horn nociceptive plasticity, enhances long-term potentiation of primary afferent neurotransmission, and increases inflammatory hyperalgesia[19,20]. Nevertheless, little is known about the involvement between EA and GluR2 internalization with phosphorylation yet. In this study, we induced inflammation on rats' hindpaw by injection of CFA and then, examined whether there is close association between the inhibition of GluR2 internalization and EA analgesia by using immunoblot and Immunohistochemical analysis.

A main finding in our study is that EA treatment suppressed the spinal GluR2 internalization and GluR2 phosphorylation induced by CFA. When 1 day had elapsed after CFA injection, we could only ascertain that GluR2 were trafficked away from the synaptic membrane and moved into cytosol by observing that the expression of membrane GluR2 was decreased whereas that of cytosolic GluR2 was increased in day 1 CFA(Fig. 1). We could examine that the expression of membrane GluR2 was increased by day 1 EA treatment. But though day 1 CFA+EA group was administrated by CFA, the expression of GluR2 was increased more than that of normal group. Two days after CFA injection, it is shown that, as inflammation induced by CFA got aggravated, anti-inflammatory effects of EA also became reinforced. In day 2 CFA group, the number of cytosolic GluR2 was more increased whereas that of membrane GluR2 was more decreased. This suggests that CFA-induced inflammation might have become aggravated. Looking at day 2 CFA+EA group, we found that GluR2 internalization by CFA was remarkably attenuated in comparison with that of CFA group.

CFA injection produced a decrease in the expression of GluR2 in the postsynaptic membrane and a corresponding increase in cytoplasm. CFA injection did not lead to a significant change in level of total GluR2 in either side of dorsal horn. Normally, AMPAR subunits are constitutively recycled between synaptic membrane and the intercellular compartment. Inflammatory noxious input breaks the balance of GluR2 recycling and promotes GluR2 trafficking away from synaptic membrane[21]. In our result, we examined that when CFA was injected, the amount of membrane GluR2 was

decreased, whereas that of cytosolic GluR2 was increased, and EA treatment tends to suppress these changes. This suppression made by EA turned out to be more evident in day 2 than day 1. However, we should admit that these results are not statistically significant because the amount of tissue samples was not large enough that the margin of error might have been also great. We could have obtained more accurate result if we had got more absolute quantity of membrane or cytoplasm GluR2. But, to our regret, we could only see the tendency in this study.

Therefore, we investigated a confocal microscopic analysis whether EA could significantly decrease phosphorylation of GluR2 in the tissue samples that were not divided into membrane and cytoplasm. Park et al. reported that the number of pGluR2 in the ipsilateral dorsal horn was significantly increased after CFA injection[21]. It began at 2h and was maintained for at least 7d after CFA injection. That idea was consistent with our result. Looking at the graph obtained by immuohistochemistry, although we did not observe time-dependent changes, we could examine significant increases in pGluR2 on day 2 after CFA injection and found that, after CFA injection, EA treatment could suppress increases in pGluR2(Fig. 2). It suggests that EA treatment can significantly attenuate inflammation induced by CFA. In addition, EA treatment without CFA injection could still decrease the level of pGluR2 in comparison with normal group. This result supports the hypothesis that EA stimulation is also effective in preventing inflammation.

Lin et al. reported that microglial cells were increased in CFA injected animal[11]. Also Raghavendra et al. reported that in CFA-injected rats, the morphology of the microglial cells varied from that of typical resting microglia (with thin, highly ramified processes), through various stages of reaction in which the processes were shortened and greatly thickened, reaching morphology characteristic of activated microglia[22]. In our study, increasing of microglial cells in CFA group was observed in comparison with normal group, and with magnified observation, the processes of microglial cells in CFA group were shortened and thickened in comparison with those of normal group. However, EA group and CFA with EA group showed similar aspects with normal group.

Consequently, our results indicate that EA treatment can attenuate the CFA-induced inflammation by directly or indirectly suppressing GluR2 internalization in dorsal horn during maintenance of inflammatory pain. But to get more significant results, further studies are required. Moreover, we found that EA treatment could suppress increases in the number of pGluR2-labelled neuron. These findings show that EA might alleviate CFA-induced inflammation by coordinating mechanisms at the upstream step of neuron activation and GluR2 phosphorylation.

Acknowledgements

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References

1. Zhi-Qi Zhao. Neural mechanism underlying acupuncture analgesia. Progress in Neurobiology 85: 355-375, 2008.
2. Galan, A., Lopez-Garcia, J.A., Cervero, F., Laird, J.M. Activation of spinal extracellular signaling-regulated kinase-1 and -2 by intraplantar carrageenan in rodents. Neuroscience letters. 322: 37-40, 2002.
3. Ji, R.R., Baba, H., Brenner, G.J., Woolf, C.J. nociceptive-specific activation of ERK in spinal neurons contributes to pain hypersensitivity. Nature neuroscience. 2: 1114-1119, 1999.
4. Adwanikar, H., Karim, F., Gereau, R.W.4th. Inflammation persistently enhances nocifensive behaviors mediated by spinal group I mGluRs through sustained ERK activation. pain. 111: 125-135, 2004.
5. Kawasaki, Y., Kohno, T., Zhuang, Z.Y., Brenner, G.J., Wang, H., Van DEr Meer, C., et al. Ionotropic and metabotropic receptors, protein kinase A, protein kinase C, and Src contribute to C-fiber-induced ERK activation and cAMP response element-binding protein phosphorylation in dorsal horn neurons, leading to central sensitization. The Journal of neuroscience. 24: 8310-8321, 2004.
6. Ji, R.R., Gereau, R.W.4th., Malcangio, M., Strichartz, G.R. MAP kinase and pain. Brain Research Reviews. 60: 135-148, 2009.
7. Burnashev, N., Monyer, H., Seeburg, P.H., Sakmann, B. Divalent ion permeability of AMPA receptor channels is dominated by edited form of a single subunit. Neuron. 8: 189-198, 1992.
8. Hsu, D.T. Acupuncture: A review. Regional anesthesia. 21: 361-370, 1996.
9. Zhang, R.X., Lao, L., Wang, X., Fan, A., Wang, L., Ren, K., et al. Electroacupuncture attenuates inflammation in a rat model. Journal of alternative and complementary medicine. 11: 135-142, 2005.
10. Lin, J.G., Chen, W.L. Acupuncture analgesia: a review of its mechanism of actions. The American Journal of Chinese Medicine. 36: 635-645, 2008.

11. Ting Lin, Kai Li, Fei-Yu Zhang, Zhen-Kang Zhang, Alan R. Light., Kai-Yuan Fu. Dissociation of spinal microglia morphological activation and peripheral inflammation in inflammatory pain models. Journal of Neuroimmunology 192: 40-48, 2007.
12. Y. Wan, J.S. Han, J.E. Pintar. Electroacupuncture analgesia is enhanced in transgenic nociceptin/orphanin FQ knock-out mice. Beijing Xue Xue Bao. 41(3):376-379, 2009.
13. J.E. Jang, H.E. Kim, S.A. Koo, H.Y. Shin, E.S. Choe, B.T. Choi. Synergistic antinociceptive effects of N-methyl-d-aspartate receptor antagonist and electroacupuncture in the complete Freund's adjuvant-induced pain model. International Journal of Molecular Medicine. 28: 669-675, 2011.
14. J.R. Silva, M.L. Silva and W.A. Prado. Analgesia induced by 2- or 100-Hz electroacupuncture in the rat tail-flick test depends on the activation of different descending pain inhibitory mechanisms. Pain. 12(1):51-60, 2011.
15. Zhang, R.X., Wang, L., Liu, B., Qiao, J.T., Ren, K., Berman, B.M., et al. Mu opioid receptor-containing neurons mediate electroacupuncture-produced anti-hyperalgesia in rats with hind paw inflammation. Brain research. 1048: 235-240, 2005.
16. Koo, S.T., Park, Y.I., Lim, K.S., Chung, K., Chung, J.M. Acupuncture analgesia in a new rat model of ankle sprain pain. pain. 99: 423-431, 2002.
17. Kerr, R.C., Maxwell, D.J., Todd, A.J. GluR1 and GluR2/3 subunits of the AMPA-type glutamate receptor are associated with particular types of neurone in laminae Ⅰ-Ⅲ of the spinal dorsal horn of the rat. Journal of Neuroscience 10: 324-333, 1998.
18. Lu, C.R., Hwang, S.J., Phend, K.D., Rustioni, A., Valtschanoff, J.G. Primary afferent terminals in spinal cord express presynaptic AMPA receptors. Journal of Neuroscience. 22: 9522-9529, 2002.
19. Hartmann, B., Ahmadi, S., Heppenstall, P.A., Lewin, G.R., Scoff, C., Borchardt, T., Seeburg, P.H., Zeilhofer, H.U., Sprengel, R., Kuner, R. The AMPA receptor subunits GluR-A and GluR-B reciprocally modulate spinal synaptic plasticity and inflammatory pain. Neuron. 44: 637-650, 2004.
20. Youn, D.H., Royle, G., Kolaj, M., Vissel, B., Randic, M. Enhanced LTP of primary afferent neurotransmission in AMPA receptor GluR2-deficient mice. Pain. 2007.
21. Jang-Su Park, Nana Voitenko, Ronald S. Petralia, Xiaowei Guan, Ji-Tian Xu, Jordan P. Steinberg, Kogo Takamiya, et al. Persistent inflammation induces GluR2 internalization via NMDA receptor-triggered PKC activation in dorsal horn neurons. Journal of neuroscience. 29: 3206-3219, 2009.
22. Vasudeva Raghavendra, Flobert Y. Tanga1, Joyce A. DeLeo1. Complete Freunds adjuvant-induced peripheral inflammation evokes glial activation and proinflammatory cytokine expression in the CNS. European Journal of Neuroscience. 20: 467-473, 2004.