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Accepted Manuscript

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PII: S1526-5900(17)30514-X

DOI: 10.1016/j.jpain.2017.02.438

Reference: YJPAI 3394

To appear in: *Journal of Pain*

Received Date: 22 October 2016

Revised Date: 21 February 2017

Accepted Date: 26 February 2017

Please cite this article as: Zhou Y-Q, Chen S-P, Liu D-Q, Manyande A, Zhang W, Yang S-B, Xiong B-R, Fu Q-C, Song Z-p, Rittner H, Ye D-W, Tian Y-K, The Role of Spinal GABAB Receptors in Cancelr-Induced Bone Pain in Rats, *Journal of Pain* (2017), doi: 10.1016/j.jpain.2017.02.438.

The role of spinal GABAB receptors in cancer-induced bone pain in rats

Ya-Qun Zhou[1, 2], Shu-Ping Chen[1, 2], Dai-Qiang Liu[1, 2], Anne Manyande[3], Wen Zhang[1, 2], Shao-Bing Yang[1, 2], Bing-Rui Xiong[1, 2], Qiao-Chu Fu[1, 2], Zhen-peng Song[1, 2], Heike Rittner[4], Da-Wei Ye[5], Yu-Ke Tian[1, 2]

1. Anesthesiology Institute, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China.

2. Department of Anesthesiology and Pain Medicine, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China.

3. School of Human and Social Sciences, University of West London, London, UK.

4. Department of Anesthesiology, University Hospital of Würzburg, Würzburg, Germany.

5. Cancer Center, Tongji Hospital, Tongji Medical college, Huazhong University of Science and Technology, Wuhan, China.

Corresponding authors:

Da-Wei Ye, MD, PhD.

Cancer Center, Tongji Hospital, Tongji Medical college, Huazhong University of Science and Technology, Wuhan, China.

TEL: 00862783663409, FAX: 00862783662853, E-mail: dy0711@gmail.com.

Yu-Ke Tian, MD, PhD.

Anesthesiology Institute, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China.

TEL: 00862783663173, FAX: 00862783662853, E-mail: yktian@tjh.tjmu.edu.cn.

Running title: The role of GABAB receptors in bone cancer pain

Disclosures: This work was supported by grants from National Natural Science Foundation of P.R. China (Grant No. 81400917, 81371250 and 81571053), and

Specialized Research Fund for the Doctoral Program of Higher Education No.20130142120102, HUST No. 2014QT021. The authors have no conflict of interest.

Abstract

Cancer-induced bone pain (CIBP) remains a major challenge in advanced cancer patients due to our lack of understanding of its mechanisms. Previous studies have demonstrated the vital role of GABAB receptors (GABABRs) in regulating nociception and various neuropathic pain models have shown diminished activity of GABABRs. However, the role of spinal GABABRs in CIBP remains largely unknown. In this study, we investigated the specific cellular mechanisms of GABABRs in the development and maintenance of CIBP in rats. Our behavioral results show that both acute and chronic intrathecal treatment with baclofen, a GABABR agonist, significantly attenuated CIBP-induced mechanical allodynia and ambulatory pain. The expression levels of GABABRs were significantly decreased in a time-dependent manner and colocalized mostly with neuron and a minority with astrocyte and microglia. Chronic treatment with baclofen restored the expression of GABABRs and markedly inhibited the activation of cAMP-dependent protein kinase (PKA) and the cAMP-response element-binding protein (CREB) signaling pathway. **Perspective:** Our findings provide the first evidence that downregulation of GABABRs contribute to the development and maintenance of CIBP and restore diminished GABABRs attenuates CIBP-induced pain behaviors at least partially by inhibiting the PKA/CREB signaling pathway. Therefore, spinal GABABR may become a potential therapeutic target for the management of CIBP.

Keywords: Cancer-induced bone pain; GABAB receptor; PKA; CREB; Baclofen

1. Introduction

More than one-third patients with metastatic bone disease experience moderate to severe pain, which significantly debilitates their quality of life [39, 41, 56]. Cancer-induced bone pain (CIBP) is a complex pain state involving background, spontaneous and evoked pain [21, 35, 54]. Currently, the management of CIBP is limited to the use of opioids [28, 55]. However, high dose of opioids often lead to unwanted side effects including addiction, sedation, constipation, pruritus, nausea and vomiting [6, 9]. Although marked advances have been made in recent years, the mechanisms of CIBP are far from clear. Therefore, further elucidation of the mechanisms of CIBP is warranted to reveal novel therapeutic targets.

γ-aminobutyric acid (GABA), the main inhibitory neurotransmitter in the central nervous system (CNS), exerts its action via ionotropic GABAA receptors (GABAARs) and metabotropic GABAB receptors (GABABRs) [4]. GABABRs, composed of GABABR1 and GABABR2, are G-protein-coupled receptors (GPCRs) that elicit primarily inhibitory effects via the inhibition of presynaptic voltage-gated Ca^{2+} channels, activation of postsynaptic K^+ channels, and inhibition of adenylyl cyclase [5, 30]. In the spinal cord, GABABRs are mainly located on laminae I-III where nociceptive primary afferent fibers principally terminate [13]. Evidence is accumulating that GABABRs play a vital role in regulating nociception [20, 34, 40]. Moreover, studies suggeste that impairment of GABABRs contribute to neuropathic pain in various models including the spinal nerve ligation (SNL) [23], spinal cord injury [16] and diabetic neuropathy [3, 26, 44]. However, the role of spinal GABABRs in CIBP remains unknown. It has been shown that activation of GABABRs could inhibit adenylyl cyclase, and thus reduce the activity of cAMP-dependent protein kinase (PKA) signaling [11]. The

PKA complex consists of two catalytic and two regulatory subunits, which bind cAMP and phosphorylated cAMP-response element-binding protein (p-CREB), respectively [53]. Several lines of evidence have demonstrated that upregulation of PKA and p-CREB contribute to the development of chronic pain including CIBP [17, 19, 29, 57]. If activation of GABABRs could alleviate CIBP, maybe downregulation of PKA and p-CREB is one of its intracellular mechanisms, which needs to be investigated. In the present study, using a CIBP model induced by intramedullary injection of Walker 256 cells into the tibia of rats, we tested the hypothesis that activation of GABABRs could alleviate CIBP via inhibiting PKA/CREB signaling pathway.

2. Material and Methods

2.1. Animals and ethical statement

Since female rats are more susceptible to Walker 256 mammary gland carcinoma cells, we chose virgin female Sprague-Dawley rats (180-200 g, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, PR China) in this study. Rats were kept under controlled conditions (24 ± 0.5℃, 12 h alternating light-dark cycle, with *ad libitum* access to water and food). All experimental protocols were approved by the Animal Care and Use Committee of Huazhong University of Science & Technology and were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the guidelines of the International Association for the Study of Pain [58]. All efforts were made to minimize the number of animals used and their suffering.

2.2. Intrathecal catheters and drug administration

As described previously, the intrathecal catheters were administered 5 days prior to the establishment of CIBP models [22, 37, 49]. In brief, the rats were anesthetized by intraperitoneal injection (i.p.) of pentobarbital sodium (50 mg/kg). A PE-10

polyethylene catheter was then inserted into the subarachnoid space of the spinal cord between L5 and L6 spinous processes. The correct position of the catheter was verified by a tail flick response immediately after inserting the catheter and further confirmed by an intrathecal injection (i.t.) of 2% lidocaine (10 μL). Animals exhibiting motor dysfunction were excluded from the experiments.

R(+)-Baclofen hydrochloride, a GABAB receptor agonist, was purchased from Sigma-Aldrich (USA) and dissolved in saline. 10 μL baclofen (0.1 μg, 0.5 μg or 1 μg) or vehicle (saline) was intrathecally injected via the implanted catheter, followed by 10 μL saline for flushing. The dose of baclofen was based on our preliminary results and previous reports [3, 26]. The drug administration protocol was as follows: for acute treatment, baclofen (i.t., 0.1 μg, 0.5 μg or 1 μg /10 μL) or vehicle (10 μL) was administered on day 21 following CIBP model establishment; for chronic treatment, baclofen (i.t., 0.5 μg/10 μL, once a day) or vehicle (10 μL) was administered from day 3 to day 14.

2.3. Preparation of carcinoma cells

Walker 256 rat mammary gland carcinoma cells (4×10^7 cells/mL, 1 mL) were inoculated into the abdominal cavity of a female Sprague-Dawley rat. After 7 days, cells were harvested from the ascitic fluid of the above rat. Cells were diluted to achieve a final concentration (4×10^7 cells/mL) for injection using a hemocytometer. The single-cell suspensions were maintained on ice prior to surgery.

2.4. Bone cancer pain model

The model of CIBP was established as described previously [10, 15, 27]. Briefly, rats were anesthetized with pentobarbital sodium (50 mg/kg, i.p.). After shaving and disinfecting the left leg, a minimal incision was made to expose the proximal tibia. Then, the prepared Walker 256 cells (4×10^7 cells/mL, 10 μL) were slowly injected

into the bone cavity using a 10 μL Hamilton syringe. For the sham group, 10 μL D-Hank's solution was injected instead. The injection site was closed using bone wax as soon as the syringe was removed. Finally, the wound was sutured with 3-0 silk thread.

2.5. Behavioral tests

Behavioral tests were performed on day 0, 3, 7, 14 and 21 after surgery between 8:00 and 12:00 to avoid diurnal variation. Mechanical allodynia was assessed by ipsilateral hind paw withdrawal threshold (PWT) to von Frey filament stimulation as described previously [38]. Briefly, animals were placed in individual plastic boxes on a metal mesh floor and allowed to acclimatize for 30 min. Von Frey filaments, with ascending order of forces (1, 2, 4, 6, 8, 10, and 15 g), were applied for up to 6 s per filament to the mid-plantar of the right hind paw. Abrupt paw withdrawal, lickings, and shaking were considered as positive responses. Once a positive response was established, the paw was re-tested after a 5 min-rest, starting with the next descending von Frey filament until no response occurred. The lowest amount of force required to elicit a positive response was recorded as the PWT (in grams). Ambulatory pain was assessed by ipsilateral limb use during normal ambulation in a plastic observation box as described previously [14]. Limb use during spontaneous ambulation was scored on a scale of 0 to 4: 0 = complete lack of limb use, 1 = marked limping, 2 = extent between 1 and 3, 3 = slight limping, 4 = normal use. All the behavioral tests were performed by an investigator who was blinded to the experimental design.

2.6. Immunohistochemistry

Under deep anesthesia with pentobarbital sodium (60 mg/kg, i.p.), the rats were perfused intracardially with saline followed by 4% ice-cold paraformaldehyde in 0.1 M phosphate buffer saline (PBS). The L4-L5 spinal segments were removed and post-fixed in 4% paraformaldehyde for 4 h, and subsequently dehydrated in 30 % sucrose

solution overnight at 4℃. The samples were sectioned 30 μm thick in a cryostat (CM1900, Leica, Germany) and stored in PBS.

2.6.1. Single immunostainings

After washing three times in PBS, the sections were blocked with 5% donkey serum and 0.3% Triton X-100 for 1 h at 37°C, and then incubated overnight at 4°C with rabbit anti-GABABR1 antibody (1:50; DF4934; Affinity) or rabbit anti-GABABR2 antibody (1:50; A6594; ABclonal) or rabbit anti-PKAca antibody (1:50; ab26322; Abcam) or rabbit anti-phospho-CREB (pSer133) antibody (1:50; MA5-11192; Thermo). After washing three times in PBS, the sections were incubated with Alexa Fluor 488-labeled donkey anti-rabbit secondary antibody (1:500; A-21206; Invitrogen) for 2 h at 37°C and were washed in PBS. Nonspecific staining was determined by replacing primary or secondary antibodies with an antibody dilution buffer. Sections were rinsed, mounted and cover-slipped with 50% glycerol. Images were captured using a fluorescence microscope (DM2500, Leica, Germany). The GABABR1/GABABR2/PKA/p-CREB-immunolabeled surface areas were measured in laminae I-IV of the spinal cord dorsal horn using Image Pro Plus software. Quantification of the immunoreactivity was accomplished by calculating the percentages of immunostaining ([positive immunofluorescent surface area]/[total measured picture area] × 100). Six rats of each group were used for statistical analysis. All the image analyses were performed by an investigator who was blinded to the experimental design.

2.6.2. Double-labeling procedures

For double immunofluorescence, the sections were incubated with a mixture of two primary antibodies followed by a mixture of Alexa 488-conjugated and Alexa 594-conjugated secondary antibodies (1:500; A-21207; Invitrogen). Specifically, to

identify the cell types that expressed GABABR1, GABABR2, PKA, and p-CREB, each of the antibodies for these molecules was mixed with mouse anti-neuronal nuclei antibody (NeuN; neuronal marker; 1:200; MAB377; Millipore), mouse anti-glial fibrillary acidic protein antibody (GFAP, astrocytic marker; 1:300; 3670; Cell Signaling Technology), or goat anti-Iba1 antibody (microglial marker; 1:300; ab5076; Abcam).

2.7. Western blotting

Under deep anesthesia with pentobarbital sodium (60 mg/kg, i.p.), the L4-L5 spinal segments were removed and homogenized in RIPA lysis buffer containing 50mM Tris (pH 7.4), 150mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1mM NaF and 2mM EDTA. Then the homogenates were centrifuged at 12000 g for 30 minutes at 4°C. The supernatants were collected and the protein concentration was measured using the Bradford method. The samples were then heated at 95°C for 10 min in a loading buffer (pH 6.8): 250 mM Tris–HCl, 200 mM Sucrose, 300 mM DTT, 0.01% Coomassie brilliant blue-G, 8% sodium dodecyl sulfate (SDS). Equivalent amounts of proteins (50 μg) were separated using 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride membranes (PVDF; IPVH00010; Millipore). After blocking with 5% bovine serum albumin in Tris-buffered saline and Tween 20 (TBST, 0.1%) for 2 h at 37°C, the membranes were incubated overnight at 4 °C with rabbit anti-GABABR1 antibody (1:1000; DF4934; Affinity), rabbit anti-GABABR2 antibody (1:500; A6594; ABclonal), rabbit anti-PKAca antibody (1:1000; ab26322; Abcam), rabbit anti-phospho-CREB (pSer133) antibody (1:500; MA5-11192; Thermo) and mouse anti-glyceraldehyde-3-phosphate dehydrogenase antibody (GAPDH; 1:5000; AS1039; Aspen), respectively. The membranes were then washed in TBST and

incubated with horseradish peroxidase-conjugated goat-anti-rabbit (1:5000; A21020; Abbkine) or goat-anti-mouse secondary antibody (1:5000; A21010; Abbkine) for 2 h at 37°C. The bands were finally visualized with SuperLumia ECL Plus HRP Substrate Kit (K22030; Abbkine) and then detected by a computerized image analysis system (BIO-RAD, ChemiDoc XRS+, USA). The intensity of protein blots was quantified using System with image Lab software (Bio-rad Laboratories), normalized to loading control GAPDH and expressed as the fold of control. The blot density of control groups was set as 1.

2.8. Statistical analysis

All data are presented as mean ± SEM and analyzed using the GraphPad Prism version 5.01 for Windows (Graph Pad Software, San Diego, CA, USA). One-way analysis of variance (ANOVA) followed by Bonferroni *post hoc* test was used for Western blot and immunochemistry data. Two-way ANOVA with repeated measures, followed by Bonferroni *post hoc* test was used for PWT and limb use scores. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Role of spinal GABABRs in CIBP-related behaviors

No significant differences in pain-related behaviors were observed among all groups at baseline. The ipsilateral PWT of CIBP rats were significantly decreased from day 7 after tumor cell implantation (TCI) to the last observation on day 21 (Figure 1A). Similarly, the ipsilateral limb use scores of CIBP rats were clearly decreased over the same time frame (Figure 1B). These results indicate that CIBP rats developed mechanical allodynia and ambulatory pain.

To investigate the role of spinal GABABRs in CIBP rats, we intrathecally injected baclofen, a GABABR agonist, into sham and CIBP rats. We first tested the analgesic

effect of a single dose of baclofen on day 21 following TCI (i.t., 0.1 μg, 0.5 μg or 1 μg /10 μL). The behavioral tests were conducted at 0, 0.25, 0.5, 1, 1.5, 2, 2.5, 3 h after baclofen injection. Compared with that of the vehicle group, intrathecal injection of 0.1 μg baclofen had no significant influence on PWT and limb use scores. However, baclofen (i.t., 0.5 μg or 1 μg/10 μL) treatment notably reversed the CIBP-related behaviors, beginning at 0.25 h, peaking at 0.5 h and lasting for at least 2 h (Figure 1C and D). Moreover, in terms of PWT and limb use scores, significant differences were found between these two groups (0.5 μg vs. 1 μg). These results indicate that the antinociceptive effects of baclofen are dose-dependent. For chronic treatment, baclofen (i.t., 0.5 μg/10 μL, once a day) or vehicle (10 μL) was administered for 12 days (from day 3 following TCI to day 14), and the behavioral tests were conducted before baclofen injection at day 0, 3, 7, 14 and 21. The results showed that both mechanical allodynia and ambulatory pain were alleviated considerably in CIBP rats treated with baclofen rather than vehicle (Figure 1E and F). To examine whether baclofen affect the pain threshold of naïve rats, we intrathecal injection of baclofen (0.1 μg, 0.5 μg or 1 μg /10 μL) into naïve rats. The behavioral tests were conducted at 0, 0.5, 1, 2 h after baclofen injection. The results showed that intrathecal injection of baclofen had no significant influence on PWT and limb use scores compared with vehicle group (Figure 1G and H).

Taken together, these results demonstrate that activation of GABABRs attenuates the development and maintenance of mechanical allodynia and ambulatory pain following TCI, which indicates an important role of GABABRs in CIBP.

3.2. Expression and cellular localization of spinal GABABRs in CIBP rats

To investigate the expression and cellular localization of GABABs in the spinal cord of CIBP rats, we performed western blot analysis and immunohistochemistry. As

shown in Figures 2A-B and 3A-B, the expression of GABABR1 and GABABR2 protein were significantly and time dependently decreased in CIBP rats, beginning at day 7 after TCI and remained at low levels until day 21, the last test day. In contrast, GABABR1 and GABABR2 proteins were at a higher level in the spinal cord of sham rats. Moreover, our immunohistochemistry data also showed that the expression of GABABR1 and GABABR2 were evidently reduced in the ipsilateral spinal cord of CIBP rats compared with sham rats (Figures 2C-E and 3C-E). We further conducted double immunofluorescence staining of GABABR1 or GABABR2 with NeuN (a neuronal marker), GFAP (an astrocytic marker), or Iba-1 (a microglial marker). As illustrated in Figures 2F-N and 3F-N, GABABR1 and GABABR2 were predominantly colocalized with NeuN, and a minority with GFAP and Iba-1 in the superficial spinal cord of CIBP rats. These results demonstrate that spinal GABABRs are downregulated after TCI, and expressed mostly in neurons and a minority in astrocyte and microglia.

3.3. Chronic treatment with baclofen restored the expression of GABABRs.

To investigate whether baclofen exerts its analgesic effect by restoring the diminished expression of GABABRs, we chronically treated CIBP rats with baclofen (i.t., 0.5 μg/10 μL) once a day from day 3 to day 14 following TCI. Three hours after the final administration, spinal samples were collected to detect the expressions of GABABR1 and GABABR2 using western blot and immunohistochemistry. As shown in Figure 4A-B, the protein expression of GABABRs were significantly upregulated after baclofen treatment. Consistent with the western blot data, our immunohistochemistry also showed increased immunoreactivity of GABABRs in the superficial dorsal horn of spinal cord (Figure 4C-L). These results suggested that chronic treatment with baclofen could restore the expression of GABABRs.

3.4. Involvement of spinal PKA/CREB signaling pathway in the analgesic effect of baclofen

The PKA/CREB signaling pathway has been shown to participate in the development of chronic pain including CIBP. We consistently found that the expression of spinal PKA and p-CREB were significantly increased in a time-dependent manner, beginning at day 3 after TCI and remained at high levels until day 21, whereas PKA and p-CREB were at lower levels in the spinal cord of sham rats (Figures 5A-B and 6A-B). Moreover, our immunohistochemistry data also showed that the expression of PKA and p-CREB was significantly increased in the ipsilateral spinal cord of CIBP rats compared with sham rats (Figures 5C-E and 6C-E). However, the cellular localization of PKA and p-CREB in the spinal cord under CIBP condition remains unknown. Our double immunofluorescence staining showed that PKA was colocalized with NeuN, GFAP and Iba-1 and (Figures 5F-N), whereas p-CREB was strongly colocalized with NeuN (Figures 6F-N). These results confirm the activation of PKA/CREB signaling pathway in the CIBP condition.

To further examine the involvement of spinal PKA/CREB signaling pathway in the analgesic effect of baclofen, we intrathecally injected baclofen (0.5 μg/10 μL) or vehicle (10 μL) into CIBP rats or sham rats daily for 12 consecutive days (from day 3 following TCI to day 14). Three hours after the final administration, spinal samples were collected to detect the expressions of PKA and p-CREB using western blot and immunohistochemistry. Our results showed that the upregulated protein levels of PKA and p-CREB were considerably attenuated by repeated intrathecal injection of baclofen (Figures 7A-B). Additionally, our immunofluorescence results showed that CIBP-induced activation of PKA and p-CREB was markedly suppressed by baclofen

treatment (Figures 7C-L). These results indicate that restored expression of GABABRs could suppress the activation of PKA/CREB signaling pathway. Collectively, these data suggest that activation of GABABRs alleviates CIBP at least partly by inhibiting spinal PKA/CREB signaling pathway.

4. Discussion

The current study demonstrated that (1) activation of GABABRs via intrathecal administration of baclofen, a GABABR agonist, significantly attenuated CIBP-induced mechanical allodynia and ambulatory pain in a dose-dependent manner, (2) inoculation of Walker 256 mammary gland carcinoma cells into a rat tibia induced downregulation of GABABRs in the spinal cord in a time-dependent manner, (3) spinal GABABRs were mostly expressed in neurons and a minority in astrocytes and microglia, (4) chronic treatment with baclofen restored the expression of GABABRs and significantly inhibited the activation of PKA/CREB signaling pathway in CIBP rats. Taken together, these results provide the first evidence that activation of spinal GABABRs mitigates CIBP at least partially by inhibiting the PKA/CREB signaling pathway.

Despite advanced progressed has been made in the last decades for the management of chronic pain[1, 24, 43, 47], its mechanisms remains far from clear. It has long been suggested that diminished inhibitory neurotransmission plays a pivotal role in chronic pain states [46, 50]. As the main inhibitory neurotransmitter in the CNS, GABA exerts its effect via ionotropic GABAARs and metabotropic GABABRs [7]. Spinal GABABRs are abundantly expressed in primary afferent fibers and interneurons on laminae I-III [48]. Genetic deletion of GABABR1 or GABABR2 produces prominent hyperalgesia [12, 36]. Previously, impairment of GABABRs has been demonstrated to participate in the development of neuropathic pain induced by SNL [23], spinal cord injury [16] and diabetic

neuropathy [3, 26, 44]. Moreover, activation of GABABRs by intrathecal injection of baclofen produces analgesic effects [2, 42, 51]. In our study, we provided consistent results showing that GABABRs were significantly decreased in the ipsilateral spinal cord dorsal horn after TCI (Figures 2A-E and 3A-E). Furthermore, the decreased expression of GABABRs matches the time frame that coincides with the development of mechanical allodynia and ambulatory pain. Notably, both acute and chronic treatment with baclofen markedly alleviated the CIBP-induced mechanical allodynia and ambulatory pain (Figures 1C-F). These findings indicate that downregulation of GABABRs may be important contributors to diminished GABAergic inhibition under CIBP condition. More importantly, repeated intrathecal administration of baclofen upregulated the protein expression (Figure 3A-B) and immunoreactivity of GABABRs (Figure 3C-L), indicating restored expression of GABABRs after baclofen treatment. However, it is worth mentioning that several studies reported that loss of GABAergic neurons is not necessary for the development of neuropathic pain [31-33]. These inconsistent results might be due to the difference in animal model, time of behavioral tests and detection methods.

PKA has been demonstrated to play a vital role in the processing of nociception at both peripheral and central levels [8, 53]. Moreover, electrophysiological results also illustrate that PKA is involved in modulating neuronal excitability in response to noxious stimuli [19, 52]. When PKA is activated, its catalytic subunit can phosphorylate CREB in the spinal cord, which contributes to the prolonged synaptic plasticity strengthened during central sensitization [25, 45]. Previous studies have established that activation of PKA/CREB signaling pathway is involved in various types of chronic pain including CIBP [17, 29], and intrathecal treatment with H-89, a PKA inhibitor, significantly attenuates CIBP-related behaviors [18]. It is also shown that GABABR-

mediated inhibition of adenylyl cyclase reduces PKA activity, thereby influencing the regulation of gene expression [7]. Therefore, we determined whether inhibiting PKA/CREB signaling pathway is one of the mechanisms underlying the analgesic effect of the GABABR agonist. Consistent with previous researches, we found upregulated expression of PKA and p-CREB in the ipsilateral spinal cord of CIBP rats, whereas their expression in sham rats was at lower levels (Figures 5A-E and 6A-E). Furthermore, we performed double immunofluorescence to examine their cellular localization. We provided the first evidence that PKA was colocalized with neurons, astrocytes and microglia, whereas p-CREB was strongly colocalized with neurons under the CIBP condition (Figures 4F-N and 5F-N). In addition, CIBP rats that received chronic treatment with baclofen showed decreased expression of PKA and p-CREB in the ipsilateral spinal cord dorsal horn (Figure 7A-L). These results indicate that inhibition of PKA/CREB signaling pathway may contribute to the analgesic effect of the GABABR agonist.

In summary, the present study suggests that downregulation of GABABRs in the spinal cord contributes to the initiation and development of CIBP, and restores diminished GABABRs attenuates CIBP-induced mechanical allodynia and ambulatory pain at least partially by inhibiting the PKA/CREB signaling pathway. Therefore, spinal GABABR may become a potential therapeutic target for the management of CIBP.

Conflict of interest

The authors declare no conflict of interests

Acknowledgements

This work was supported by grants from National Natural Science Foundation of P.R. China (Grant No. 81400917, 81371250 and 81571053) and Specialized Research

Fund for the Doctoral Program of Higher Education No.20130142120102, HUST No. 2014QT021.

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Figure legends

Fig. 1. Activation of GABAB receptors (GABABRs) alleviated cancer-induced bone pain (CIBP)-related behaviors. (A, B) The ipsilateral paw withdrawal thresholds (PWT) and limb use scores were significantly decreased from day 7 after tumor cell implantation (TCI) to the last observation on day 21 (***P < 0.001 compared with the naive group, n = 6 in each group). (C, D) A single dose of baclofen (i.t., 0.5 μg and 1 μg, but not 0.1 μg, on day 21) significantly reversed the CIBP-induced mechanical allodynia and ambulatory pain (*P < 0.05, **P < 0.01, ***P < 0.001 compared with the vehicle group, #P < 0.05, ##P < 0.01 compared with the group treated with 0.5 μg baclofen, n = 6 in each group). The behavioral tests were conducted at 0, 0.25, 0.5, 1, 1.5, 2, 2.5, 3 h after baclofen injection. (E, F) For chronic treatment, baclofen (i.t., 0.5 μg/10 μL, once a day) or vehicle (10 μL) was administered for 12 days (from day 3 following TCI to day 14). The behavioral tests were conducted before baclofen injection at day 0, 3, 7, 14 and 21 after surgery. The development of ipsilateral mechanical allodynia and ambulatory pain of CIBP rats were significantly delayed and attenuated by baclofen (**P < 0.01, ***P < 0.001 compared with the sham+vehicle group. #P < 0.05, ##P < 0.01, ###P < 0.001 compared with the CIBP+vehicle group. n = 6 in each group). (G, H) Intrathecal injection of baclofen (0.1 μg, 0.5 μg or 1 μg /10 μL) into naïve rats had no significant influence on PWT and limb use scores compared with vehicle group (n = 3 in each group). The behavioral tests were conducted at 0, 0.5, 1, 2 h after baclofen injection.

Fig. 2. Expression and cellular localization of GABAB receptor 1 (GABABR1) in spinal cord dorsal horn of cancer-induced bone pain (CIBP) rats. (A, B) Western blot analysis showed the time course of GABABR1 expression in sham and CIBP rats (***P < 0.001 compared with the sham group. n = 6 in each group). The fold change for the density of GABABR1 was normalized to GAPDH for each sample

respectively. The fold change of GABABR1 in the sham group was set at 1 for quantification. (C-E) Immunohistochemistry data showed that GABABR1 was downregulated in the ipsilateral spinal cord dorsal horn at day 21 after tumor cell implantation (TCI) (n = 4 in each group). (F-N) Representative photomicrographs of GABABR1 (green) double fluorescence labeling with NeuN (red) for neurons, GFAP (red) for astrocytes and Iba1 (red) for microglia in the ipsilateral spinal cord at day 21 after TCI. Photomicrographs were taken from ipsilateral spinal cord dorsal horns (laminae I-II; as indicated in C) of CIBP rats (n = 4 in each group). The results showed that GABABR1 was co-expressed mostly with neurons (yellow) and a minority with astrocytes (yellow) and microglia (yellow).

Fig. 3. Expression and cellular localization of GABAB receptor 2 (GABABR2) in spinal cord dorsal horn of cancer-induced bone pain (CIBP) rats. (A, B) Western blot analysis showed the time course of GABABR2 expression in sham and CIBP rats (***P* < 0.05, ****P* < 0.001 compared with the sham group, n = 6 in each group). The fold change for the density of GABABR2 was normalized to GAPDH for each sample respectively. The fold change of GABABR2 in the sham group was set at 1 for quantification. (C-E) Immunohistochemistry data showed that GABABR2 was downregulated in the ipsilateral spinal cord dorsal horn at day 21 after tumor cell implantation (TCI) (n = 4 in each group). (F-N) Representative photomicrographs of GABABR2 (green) double fluorescence labeling with NeuN (red) for neurons, GFAP (red) for astrocytes and Iba1 (red) for microglia in the ipsilateral spinal cord at day 21 after TCI. Photomicrographs were taken from ipsilateral spinal cord dorsal horns (laminae I-II; as indicated in C) of CIBP rats (n = 4 in each group). The results showed that GABABR2 was co-expressed mostly with neurons (yellow) and a minority with astrocytes (yellow) and microglia (yellow).

Fig. 4. Repeated intrathecal administration of baclofen reversed tumor cell implantation (TCI)-induced downregulation of GABABRs in the spinal cord. (A, B) Western blot and data summary showed that baclofen restored the protein expression of GABABR1 and GABABR2 (**P < 0.01, ***P < 0.001 compared with the sham+vehicle group. #P < 0.05 compared with the CIBP+vehicle group. n = 6 in each group). Baclofen (i.t., 0.5 μg/10 μL) or vehicle (10 μL) was administered once a day from day 3 following tumor cell implantation (TCI) to day 14. Tissues were collected three hours after the last spinal injection. (C-J) Representative photomicrographs showed that baclofen reversed TCI-induced downregulation of GABABRs in the spinal cord. Baclofen (i.t., 0.5 μg/10 μL) or vehicle (10 μL) was administered once a day from day 3 following tumor cell implantation (TCI) to day 14. Tissues were collected three hours after the last spinal injection. (K, L) GABABRs-immunolabeled surface area were quantified from the spinal dorsal horn (laminae I-IV; as indicated in C) using Image Pro Plus software. Quantification of GABABRs immunoreactivity was accomplished by calculating the percentages of immunostaining ([positive immunofluorescent surface area]/[total measured picture area] × 100). Six rats of each group were used for statistical analysis (**P < 0.01, ***P < 0.001 compared with the sham+vehicle group. #P < 0.05 compared with the CIBP+vehicle group. n = 6 in each group).

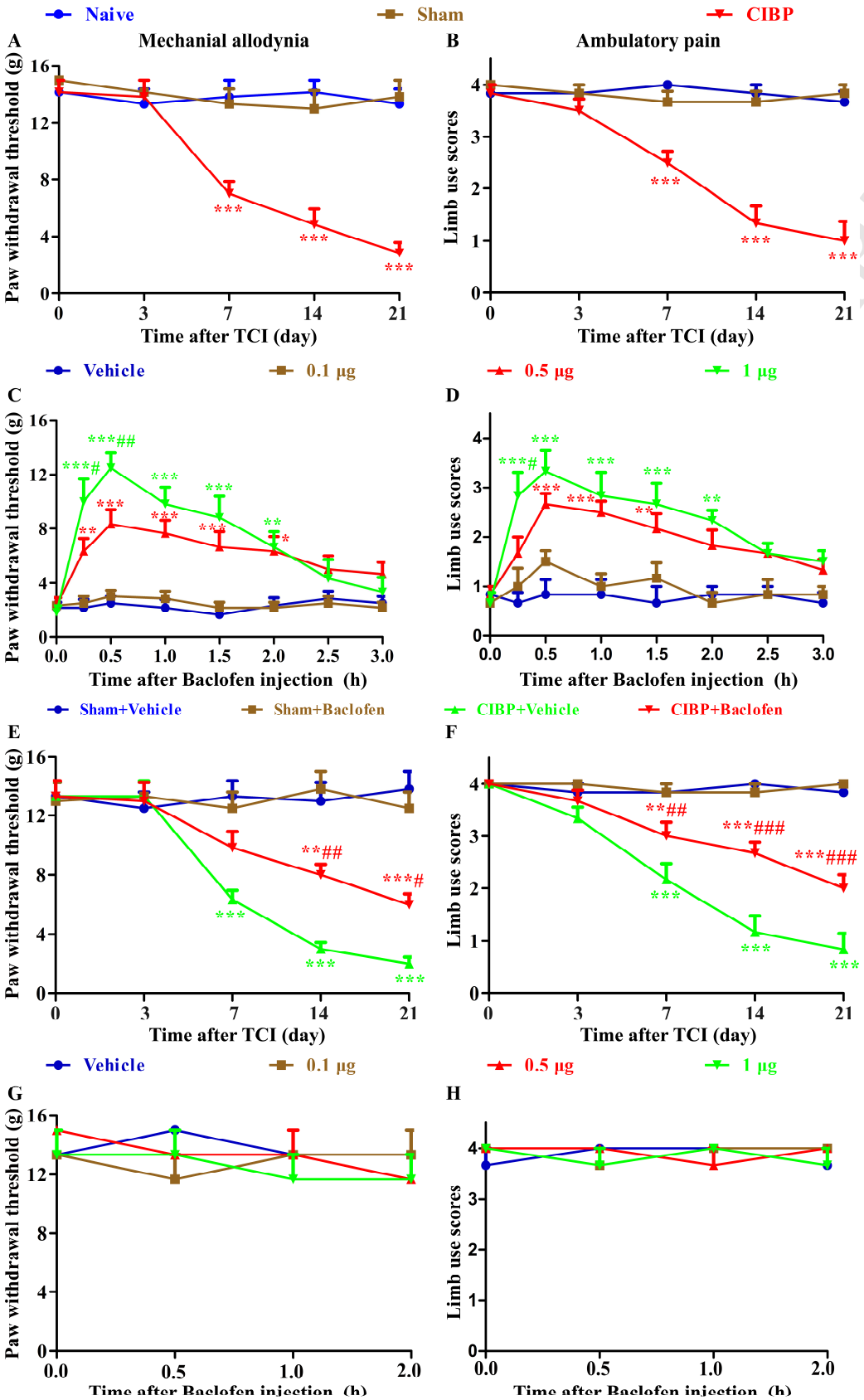
Fig. 5. Expression and cellular localization of cAMP-dependent protein kinase (PKA) in spinal cord dorsal horn of cancer-induced bone pain (CIBP) rats. (A, B) Western blot analysis showed the time course of PKA expression in CIBP rats (****P* < 0.001 compared with the sham group, n = 6 in each group). The fold change for the density of PKA was normalized to GAPDH for each sample respectively. The fold change of PKA in the sham group was set at 1 for quantification. (C-E)

Immunohistochemistry data showed that PKA was upregulated in the ipsilateral spinal cord dorsal horn at day 21 after tumor cell implantation (TCI) (n = 4 in each group). (F-N) Representative photomicrographs of PKA (green) double fluorescence labeling with NeuN (red) for neurons, GFAP (red) for astrocytes and Iba1 (red) for microglia in the ipsilateral spinal cord at day 21 after TCI. Photomicrographs were taken from ipsilateral spinal cord dorsal horns (laminae I-II; as indicated in E) of CIBP rats (n = 4 in each group). The results showed that PKA was colocalized mostly with astrocytes (yellow) and microglia (yellow) and a minority with neurons (yellow).

Fig. 6. Expression and cellular localization of phosphorylated cAMP-response element-binding protein (p-CREB) in spinal cord dorsal horn of cancer-induced bone pain (CIBP) rats. (A, B) Western blot analysis showed the time course of p-CREB expression in CIBP rats (***P < 0.001 compared with the sham group, n = 6 in each group). The fold change for the density of p-CREB was normalized to GAPDH for each sample respectively. The fold change of p-CREB in the sham group was set at 1 for quantification. (C-E) Immunohistochemistry data showed that p-CREB as upregulated in the ipsilateral spinal cord dorsal horn at day 21 after tumor cell implantation (TCI) (n = 4 in each group). (F-N) Representative photomicrographs of p-CREB (green) double fluorescence labeling with NeuN (red) for neurons, GFAP (red) for astrocytes and Iba1 (red) for microglia in the ipsilateral spinal cord at day 21 after TCI. Photomicrographs were taken from ipsilateral spinal cord dorsal horns (laminae I-II; as indicated in E) of CIBP rats (n = 4 in each group). The results showed that p-CREB was colocalized mostly with neurons (yellow) and a minority with astrocytes (yellow) and microglia (yellow).

Fig. 7. Repeated intrathecal administration of baclofen reversed tumor cell implantation (TCI)-induced upregulation of PKA and p-CREB in the spinal cord.

(A, B) Western blot and data summary showed inhibitory effect of baclofen on CIBP-induced increased expression of PKA and p-CREB protein (*P < 0.05, ***P < 0.001 compared with the sham+vehicle group. ###P < 0.001 compared with the CIBP+vehicle group. n = 6 in each group). Baclofen (i.t., 0.5 μg/10 μL) or vehicle (10 μL) was administered once a day from day 3 following tumor cell implantation (TCI) to day 14. Tissues were collected three hours after the last spinal injection. (C-J) Representative photomicrographs showed that baclofen inhibited TCI-induced upregulation of PKA and p-CREB in the spinal cord. Baclofen (i.t., 0.5 μg/10 μL) or vehicle (10 μL) was administered once a day from day 3 following tumor cell implantation (TCI) to day 14. Tissues were collected three hours after the last spinal injection. (K, L) PKA/p-CREB-immunolabeled surface area were quantified from the spinal dorsal horn (laminae I-IV; as indicated in C) using Image Pro Plus software. Quantification of PKA/p-CREB immunoreactivity was accomplished by calculating the percentages of immunostaining ([positive immunofluorescent surface area]/[total measured picture area] × 100). Six rats of each group were used for statistical analysis (***P < 0.001 compared with the sham+vehicle group. ##P < 0.01 compared with the CIBP+vehicle group. n = 6 in each group).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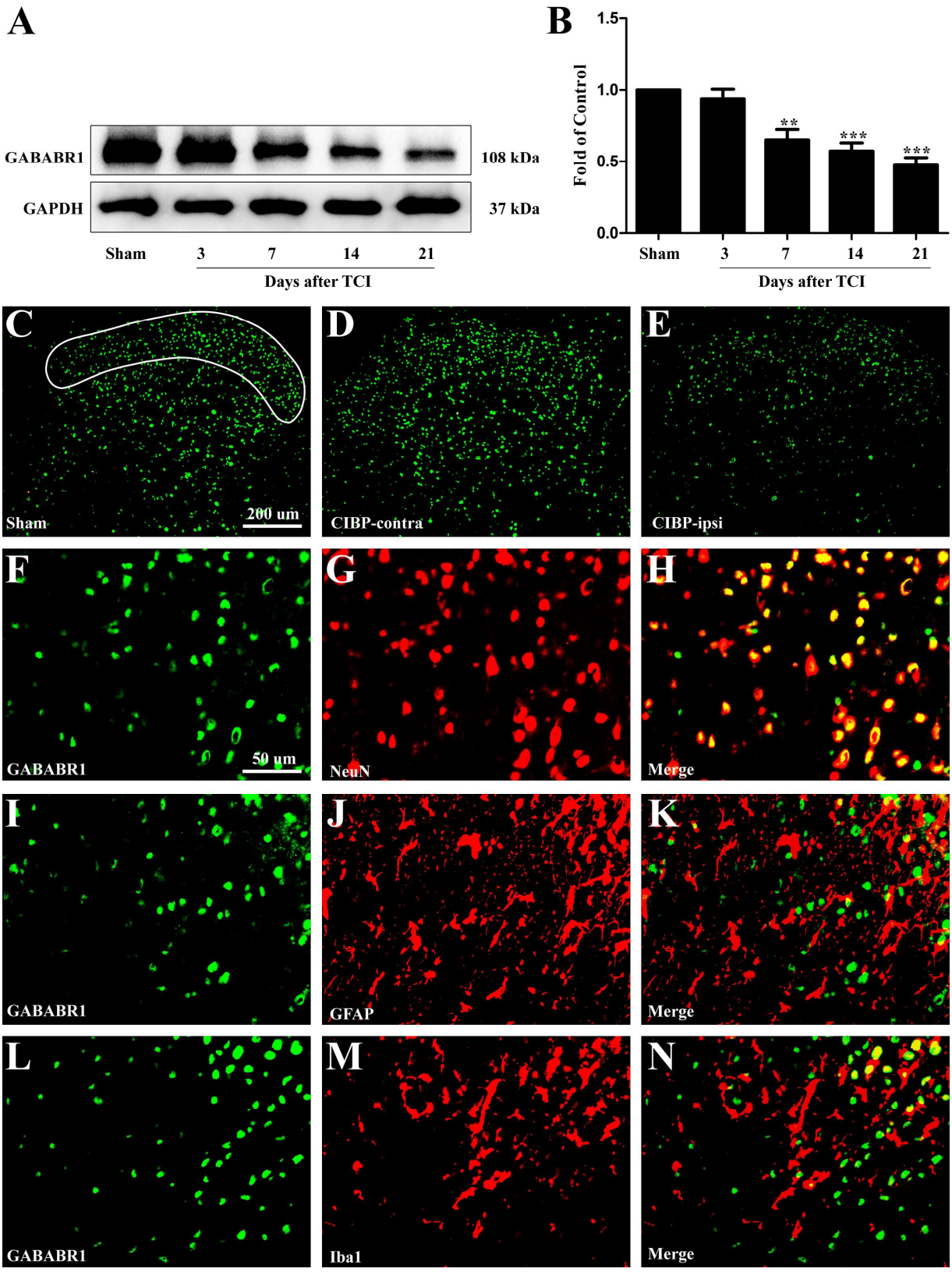
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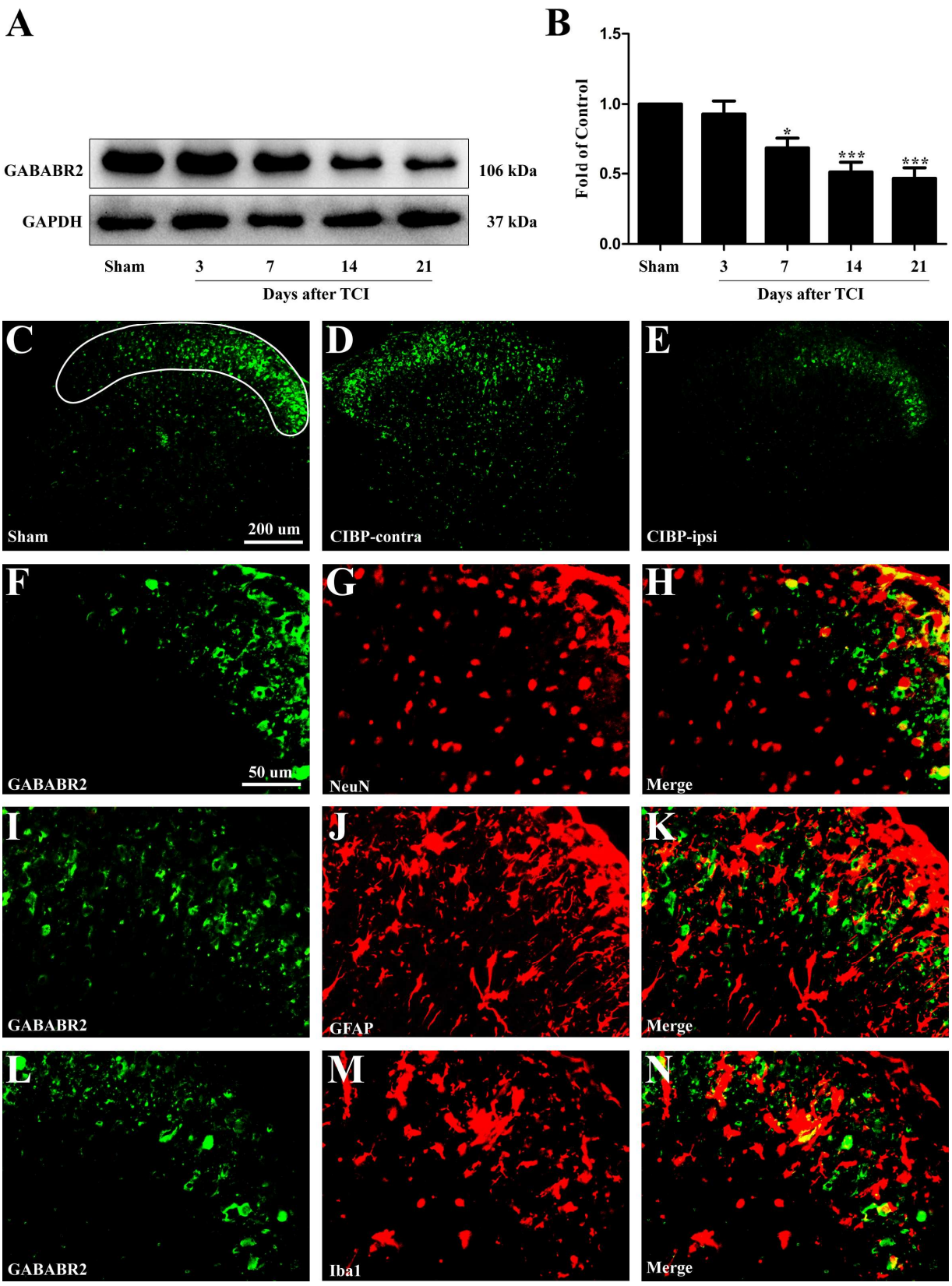
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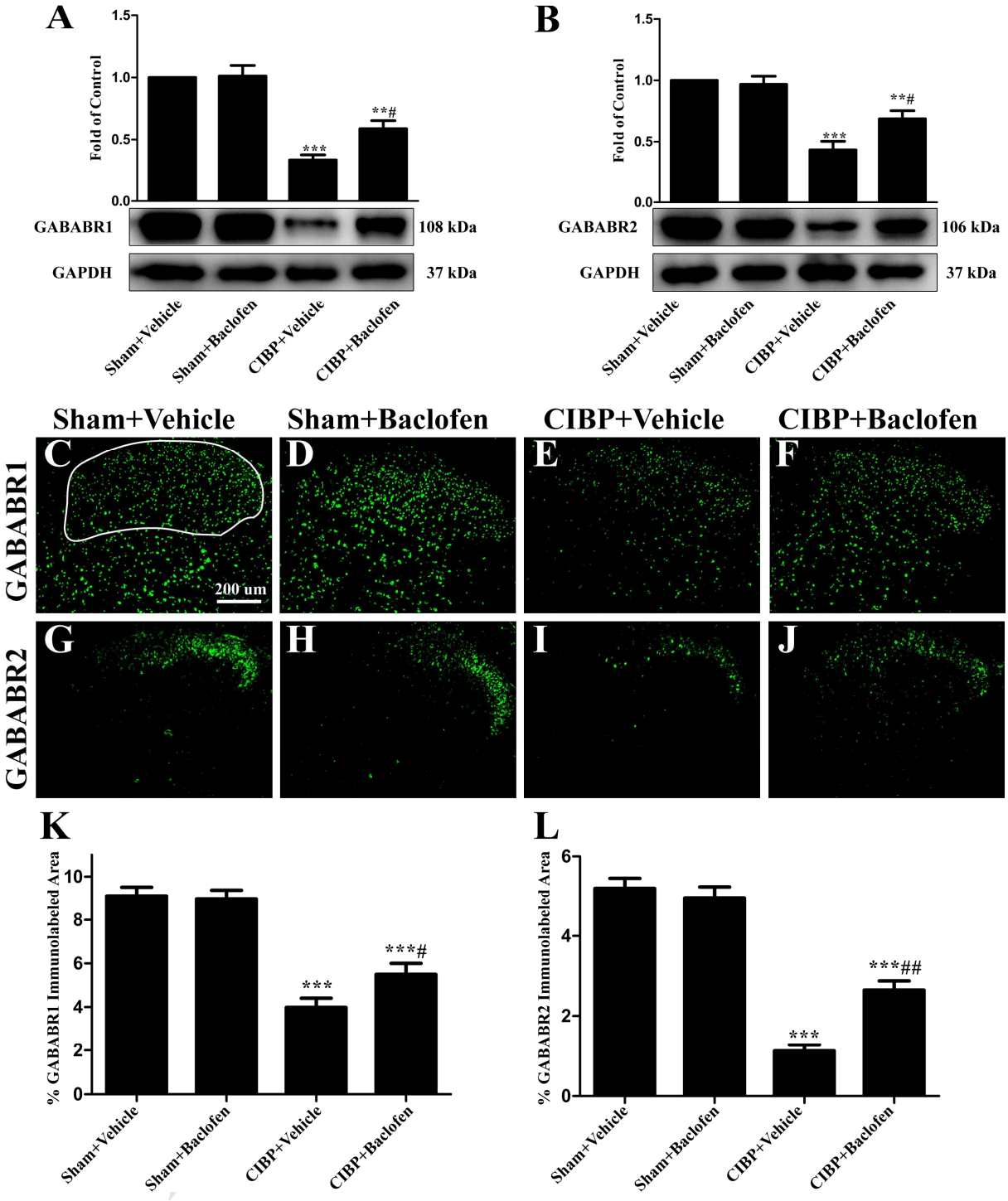
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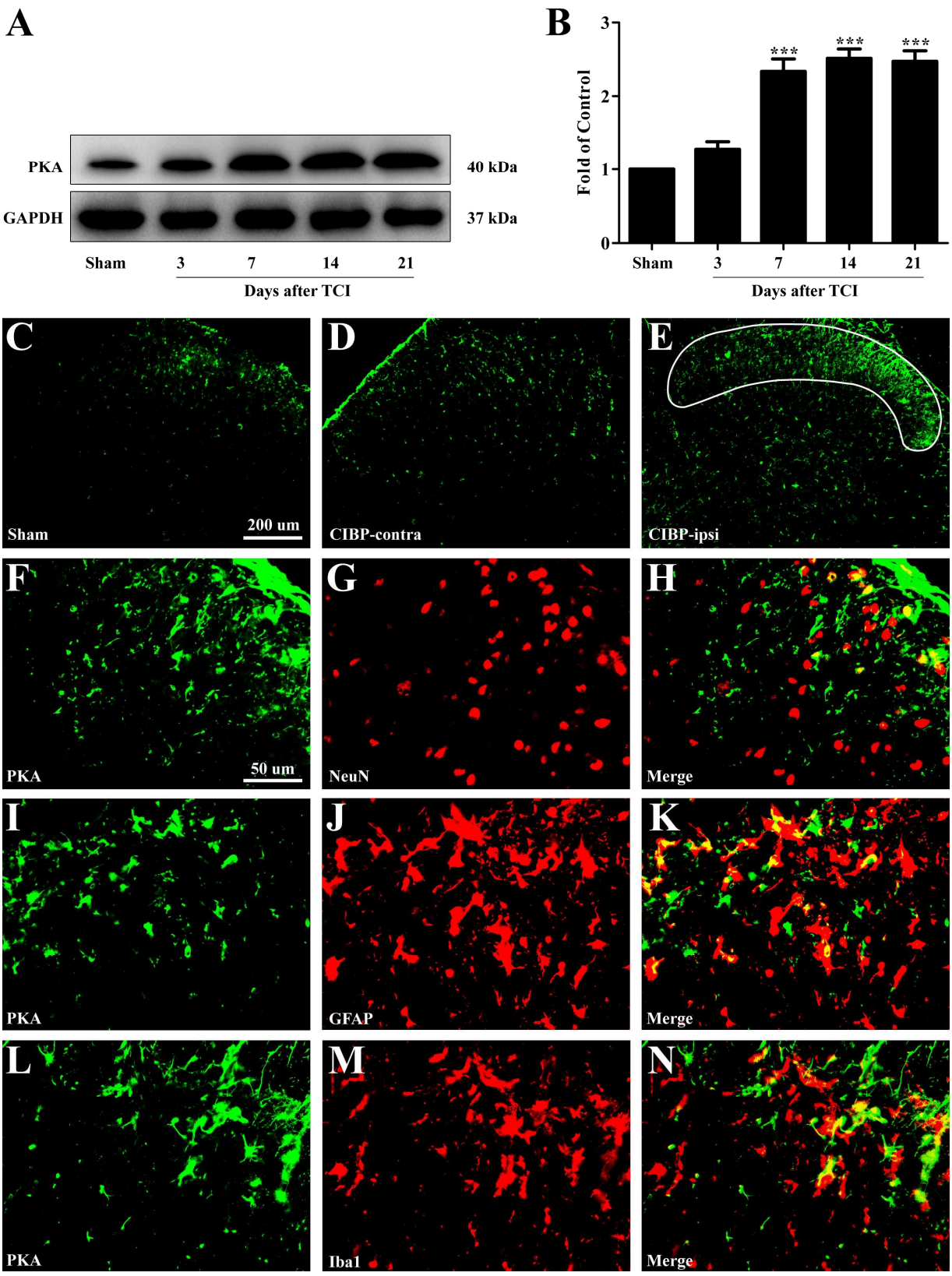
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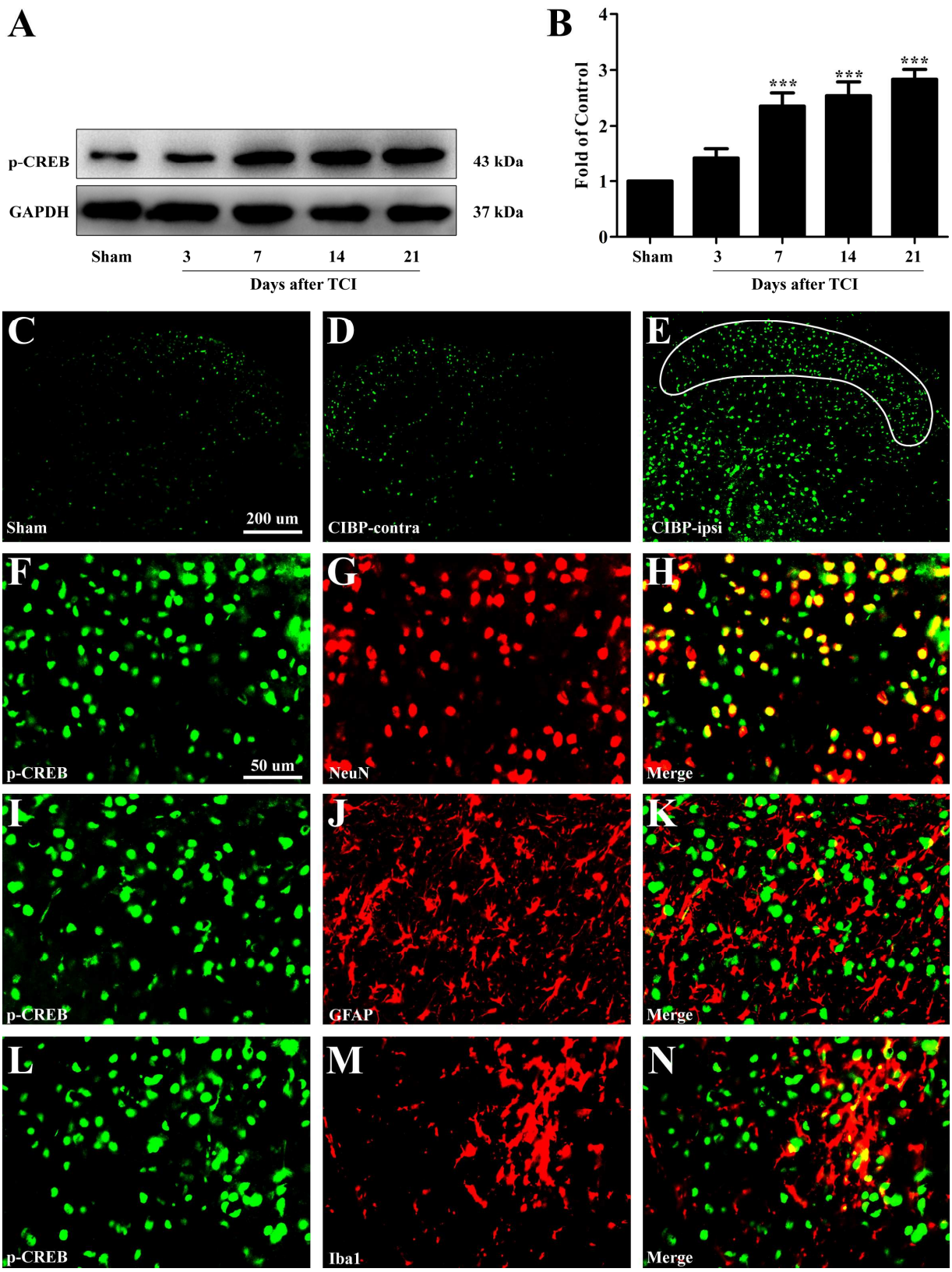
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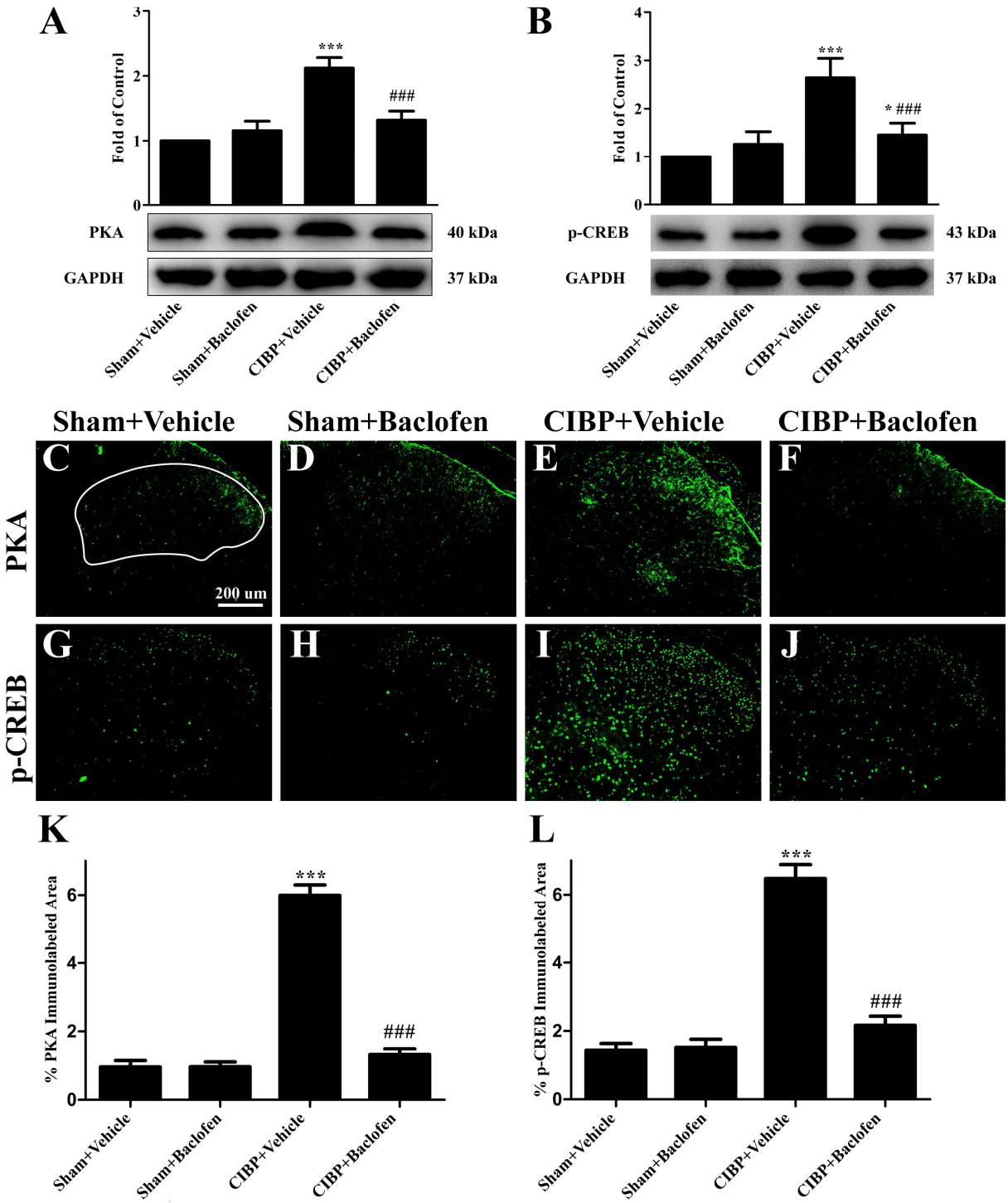
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CIBP+Baclofen

Highlights

1. The expression levels of GABABRs are significantly decreased in CIBP rats.
2. Restore diminished GABABRs attenuates CIBP-induced pain behaviors.
3. Inhibition the PKA/CREB signaling contributes to the analgesic effect of baclofen.