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Accepted Manuscript

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PII: S0014-2999(19)30427-3

DOI: https://doi.org/10.1016/j.ejphar.2019.172475

Article Number: 172475

Reference: EJP 172475

To appear in: *European Journal of Pharmacology*

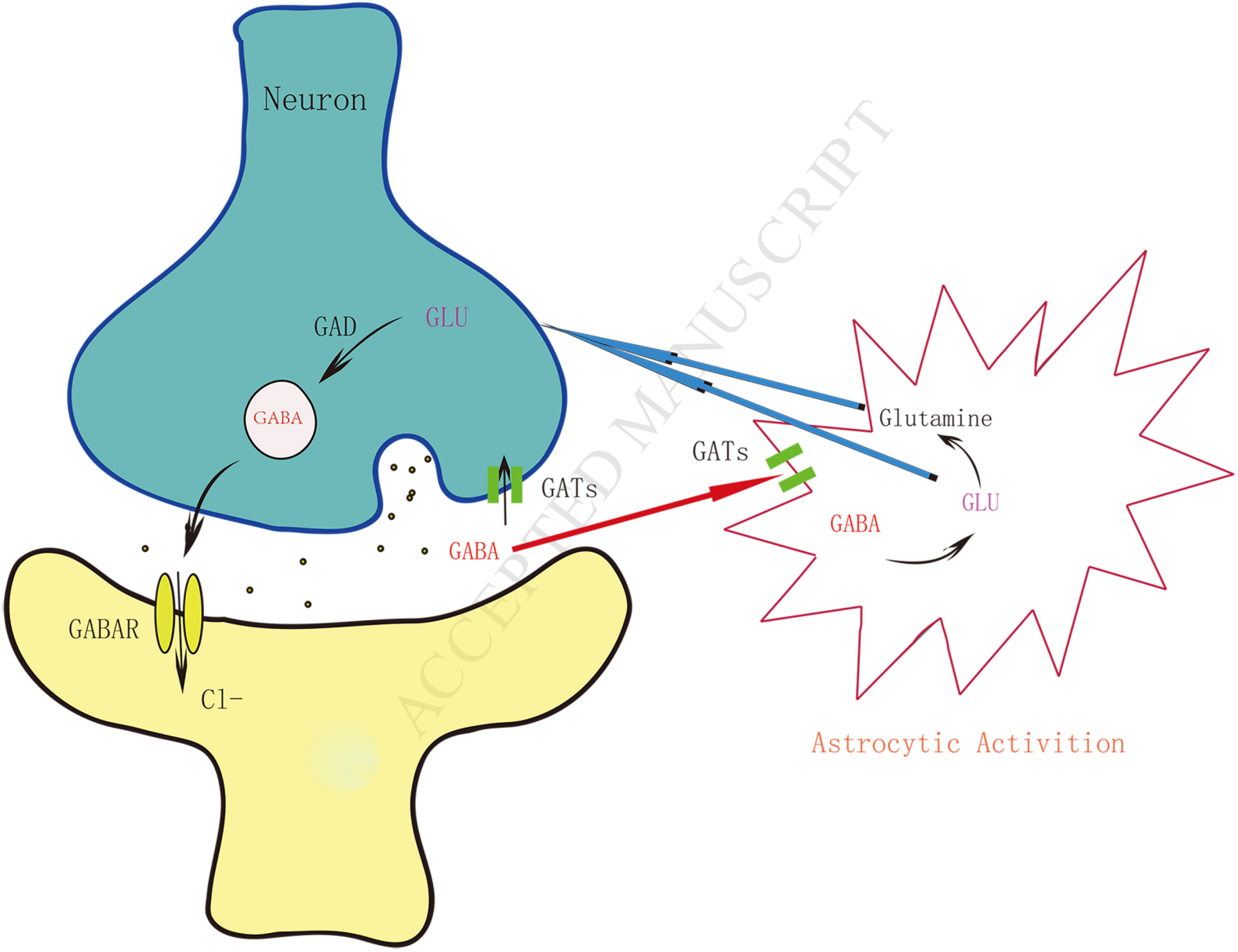
Received Date: 18 February 2019

Revised Date: 17 June 2019

Accepted Date: 18 June 2019

Please cite this article as: Ge, M.-M., Chen, S.-P., Zhou, Y.-Q., Li, Z., Tian, X.-B., Gao, F., Manyande, A., Tian, Y.-K., Yang, H., The therapeutic potential of GABA in neuron-glia interactions of cancer-induced bone pain, *European Journal of Pharmacology* (2019), doi: https://doi.org/10.1016/j.ejphar.2019.172475.

Cancer-Induced Bone Pain



The therapeutic potential of GABA in neuron-glia interactions of cancer-induced bone pain

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Abstract

The development of effective therapeutics for cancer-induced bone pain (CIBP) remains a tremendous challenge owing to its unclear mechanisms. Gamma-aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the central nervous system (CNS). Emerging studies have shown that disinhibition in the spinal cord dorsal horn may account for the development of chronic pain. However, the role of GABA in the development of CIBP remains elusive. In addition, accumulating evidence has shown that neuroglial cells in the peripheral nervous system, especially astrocytes and microglial cells, played an important role in the maintenance of CIBP. In this study, we investigated the expression of GABA and Gamma-aminobutyric acid transporter-1 (GAT-1), a transporter of GABA. Our results demonstrate that GABA was decreased in CIBP rats as expected. However, the expression of glutamic acid decarboxylase (GAD) 65 was up-regulated on day 21 after surgery, while the expression of GAD 67 remained unchanged after surgery. We also found that the expression of GAT-1 was up-regulated mainly in the astrocytes of the spinal cord. Moreover, we evaluated the analgesic effect of exogenous GABA and the GAT-1 inhibitor. Intrathecal administration of exogenous GABA and NO-711 (a GAT-1 selective inhibitor) significantly reversed CIBP-induced mechanical allodynia in a dose-dependent manner. These results firstly show that neuron-glia interactions, especially on the GABAergic pathway, contribute to the development of CIBP. In conclusion, exogenous GABA and GAT-1 inhibitor might be alternative therapeutic strategies for the treatment of CIBP.

Keywords: Cancer-induced bone pain; Gamma-Aminobutyric acid; Glutamic acid decarboxylases; GABA transporters; NO-711; Astrocyte.

1. Introduction

Cancer-induced bone pain (CIBP) is chronic pain caused by primary or distant metastases of non-bone tumors, notably those in the breast, prostate, and lung (Liu et al., 2011; Liu et al., 2018; Zhou et al., 2016). It not only brings tremendous physical injury, but also causes severe negative emotions which dramatically affect the quality of a patient's life (Zhou et al., 2017). Currently, drug therapeutics of CIBP are ineffective due to the escalating doses and unwanted side effects (Edwards et al., 2018; Lu et al., 2015). The development of effective therapeutics for CIBP remains a target of ongoing studies.

Gamma-aminobutyric acid (GABA), the major inhibitory neurotransmitter in the central nervous system (CNS), plays an important part in controlling the transmission of pain information (Kontinen et al., 2001; Polgár et al., 2003). GABA is synthesized from glutamate by the action of two glutamic acid decarboxylases GAD65 and GAD67 (Kami et al., 2016). GAD65, predominantly localized in the nerve terminal in the laminae I–II of the spinal cord, is preferentially synthesize GABA for neurotransmission. While GAD67, evenly spread throughout the neurons in the deep laminae of the spinal cord, is preferentially synthesize cytoplasmic GABA (Pinal and Tobin, 1998; Soghomonian and Martin, 1998; Walls et al., 2010).

Gamma-aminobutyric acid transporters (GATs), which transport GABA in the

synaptic cleft, play a critical role in regulating the homeostasis of extracellular GABA (Yadav et al., 2015). GATs are divided into four subtypes: gamma-aminobutyric acid transporter-1 (GAT-1), gamma-aminobutyric acid transporter-2 (GAT-2), gamma-aminobutyric acid transporter-3 (GAT-3), and Betaine/GABA transporter 1 (BGT-1). GAT-1 is the most predominant and makes up about 80% of all GATs (Li et al., 2011; Smith et al., 2007). Up-regulation of GABA transporters may result in faster removal of GABA from the synaptic cleft of GABAergic axon terminals, which may trigger certain CNS disorders (Ng and Ong, 2001; Sharopov et al., 2014). Moreover, GABA transporter inhibitors such as Tiagabine, and NO-711 (a GAT-1 selective inhibitor) provide analgesic effect in neuropathic pain (Kataoka et al., 2013; Masocha and Parvathy, 2016). These results suggest that GAT-1 may be involved in the transmission of pain information.

Several studies have shown that there is apoptosis of GABAergic neuron in the spinal cord which could result in the reduction of GABA and its synthesizing enzyme in neuropathic pain model (Fu et al., 2016; Lorenzo et al., 2014; Moore et al., 2002). However, other studies suggest that GABAergic neurons in the spinal cord do not induce apoptosis (Polgar et al., 2004). The apoptosis cells were likely to be microglia, rather than neurons (Polgár et al., 2005). Moreover, other studies demonstrated that disinhibition in the spinal cord could induced by up-regulation of GAT-1, which resulted in the reduction of GABA in the synaptic cleft of the spinal cord (Polgár and Todd, 2008). In this study, we firstly demonstrated that the neuron-glia interactions, especially on GABAergic pathway, contributed to the development of CIBP.

Moreover, intrathecal administration of exogenous GABA or GAT-1 inhibitor may be alternative therapeutic strategies for the management of CIBP.

2. Material and methods

2.1. Animals and ethical statement

Due to female adult rats being more susceptible to Walker 256 mammary gland carcinoma cells, all experiments were performed on female adult Sprague-Dawley rats (180-200 g), provided by Tongji Medical College, Huazhong University of Science and Technology (HUST), Wuhan, People's Republic of China and kept in a temperature-controlled (21 ± 1□) environment under a standard 12-h light/12-h dark cycle with abundant food and drinking water. All experimental protocols received approval from the Animal Care and Used Committee of Huazhong University of Science & Technology and were in accordance with the National Institutes of Health Guide for the Care (Zimmermann, 1983). All efforts were made to minimize the number of animals used and their suffering. Animals were divided randomly into different groups. The number in each group is shown in all the figure legends.

2.2. Intrathecal catheters and drug administration

For intrathecal administration of drugs, intrathecal catheter implantation was completed as previously described (Gwak et al., 2008). Briefly, under pentobarbital sodium (50 mg/kg) anesthesia, a PE-10 polyethylene tubing (I.D. 0.25 mm and O.D. 0.5 mm) was inserted into the intrathecal space between L5 - L6 of the spinal cord.

Different doses of GABA (0.5 μg, 1.0 μg, Sigma; Cat No. PARUI-QE) and NO-711(10 μg, 50 μg, 100 μg, Sigma) were dissolved in saline, and then injected into the intrathecal space in a volume of 10 μl, followed by 10 μl saline for flushing. The control group received the same volume of saline. The doses of GABA and NO-711 were determined based on previous reports (Eaton et al., 1999; Li et al., 2011). The drug administration protocols are shown in the corresponding drug administration schedules (Fig. 2A, Fig. 7A).

2.3. Preparation of tumor cells

Tumor cells were prepared as described previously (Hu et al., 2017a). Briefly, Walker 256 mammary gland carcinoma cell suspension (1 ml, 4×10^7 cells/mL) was injected into the abdominal cavity of female rats. After 7-10 days, cells were extracted from the ascitic fluid of the rat and suspended in phosphate buffered saline (PBS). Cell suspension was adjusted to 4×10^7 cells/mL for injection using a hemocytometer.

2.4. Cancer-induced bone pain model

The CIBP model was carried out by inoculating Walker 256 mammary gland carcinoma cells into the right tibia based on previous studies (Chen et al., 2018; Zhou et al., 2018). After anesthesia with pentobarbital sodium (50 mg/kg), a minimal incision in the right leg was made to expose the tibia. Then, prepared carcinoma cells (4×10^7 cells/mL,10 μL) were injected into the intramedullary of the right tibia using a 10-μL Hamilton syringe. Rats in the sham group were injected 10 μL PBS solution

instead. The injection site was quickly sealed with bone wax while the syringe was removed. Then, the wound was sutured using 3-0 silk thread.

2.5. Evaluation of behavioral pain

All animals were habituated, and basal pain sensitivity was performed before surgery. Animals that showed obvious abnormal data were discarded. In order to observe the development of mechanical allodynia after tumor cell inoculation (TCI), the pain behavioral test was performed on day 5, 7,14, 21 after surgery. After administration of GABA, NO-711 or vehicle, the pain behavior test protocols were as follows: for acute treatment, the pain behavior test was performed on day 14 after TCI; for chronic treatment, the pain behavior test was continuously performed from day 14 to day 18 once daily after TCI. The mechanical allodynia was evaluated using the hind paw withdrawal threshold (PWT) according to Electronic Von-Frey methods as previously described (Liu et al., 2017). Rats were first placed in Plexiglas boxes with a wire mesh floor for about 30 min. Continuous and enhancive force was applied on the hind paw until the paw was withdrawn. The minimum force to evoke a positive response was recorded as the PWT (grams). The maximum force applied was 50 g in order to prevent any tissue damage. The test was performed three times with 5 min intervals and the average of three measurements was considered as the PWT (grams). All of the behavioral tests were performed by an investigator who was blind to the experimental design.

2.6. Immunofluorescence

Rats were deeply anesthetized with pentobarbital sodium (50 mg/kg) and intracardially perfused with 0.1M PBS followed by 4% paraformaldehyde in 0.1M PBS (Hu et al., 2017b). L3-L5 spinal segments of rats were dissected and post-fixed in 4% paraformaldehyde overnight, and subsequently transferred to 30% sucrose solution until the tissues sank to the bottom at 4 □. The spinal cord was sectioned 20 μm thick and processed in accordance with standard immunofluorescence protocols. After washing in 0.1M PBS, the sections were blocked with 5% goat serum for 1 h at 37□, and then incubated overnight at 4 □ with the following primary antibodies: mouse anti-GAD65 (1:100; Abcam; ab26113; Cat No. GR3184556-1), mouse anti-GAD67 (1:100; Abcam; ab26116; Cat No. GR3188743-1), rabbit anti-GABA (1:100; Sigma; A2052; Cat No. 047M4852V), rabbit anti-NEUN (1:500; Abcam; ab177487; Cat No. GR249899-54), rabbit anti-GAT-1 (1:100; Abcam; ab426; Cat No. GR3202352-3), mouse anti-glial fibrillary acidic protein (GFAP) (1:500; Cell Signaling Technology, CST; #34001S; Lot 6), goat anti-ionized calcium bindingadaptor molecule-1 (Iba-1) (1:200; Abcam; ab5076; Cat No.GR256200-1). After washing 30 min in PBS, the sections were incubated for 2 h with the corresponding Alexa Fluor 488-conjugated secondary antibodies (1:100; Protomer) or Cy3-conjugated secondary antibodies (1:100; Protomer) then washed in PBS three times, followed by 4', 6-diamidino-2-phenylindole (DAPI) (Beyotime Biotechnology) and incubated for 15 min. Sections were cover slipped with 50% glycerol. Fluorescent images were captured using a fluorescence microscope (DM2500; Leica) and then

measured by image-pro plus software.

2.7. Western blot

Under deep anaesthesia with pentobarbital sodium (50 mg/kg), the L3-L5 spinal cord was immediately removed and homogenized in ice-cold radio-immunoprecipitation assay (RIPA) lysis buffer containing protease inhibitor cocktail (Guo et al., 2017). The protein concentration of supernatants was measured using the Bradford method. 40 μg proteins from each group were separated by 10% sodium dodecyl sulfate polyacrylamide gel and then transferred to polyvinylidene fluoride membranes (IPVH00010; EMD Millipore). After incubation in a blocking solution of 5% skimmed milk in Tris-buffered saline and 0.1% Tween 20 for 2 h at room temperature, the membrane was incubated overnight at 4 □ with anti–glutamic acid decarboxylase-GAD65 (1:1000; Abcam; ab26113; Cat No. GR3184556-1) or -GAD67 antibody (1:1000; Abcam; ab26116; Cat No. GR3188743-1), anti-GAT-1 (1:1000; Abcam; ab426; Cat No. GR3202352-3), anti-GFAP (1:5000; Cell Signaling Technology, CST; #34001S; Lot 6), polyclone anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (1:2000; Promoter; Cat No. 60004-1-Ig), followed by corresponding incubation with horseradish peroxidase linked to secondary antibodies for 90 min at 37 □. For visualization, Super-Lumia ECL Plus HRP Substrate Kit (K22030; Abbkine) was used and then detected using a computerized image analysis system (ChemiDoc XRS1, Bio-Rad, Hercules,CA). Intensity of each band was normalized to the internal control (GAPDH) and measured using the image

lab software (Bio-Rad).

2.8. Statistical analysis

All statistical analyses are presented with mean ± S.E.M. and performed using Prism 6 (GraphPad Software). The behavioral responses to mechanical stimuli were tested using two-way analysis of variance with repeated measures followed by the Bonferroni test. Western blot data was analyzed using one-way analysis of variance with repeated measures followed by the Bonferroni test. $P < 0.05$ was considered as significant.

3. Results

3.1. Mechanical allodynia induced by tumor cell inoculation in female rats.

PWTs were measured at baseline prior to surgery and on day 5, 7, 14, 21 after surgery in order to observe the development of mechanical allodynia in each group. Prior to TCI, the average mechanical threshold was 23.34 ± 0.74 g (ipsilateral) in the CIBP group, which showed no significant differences compared with other groups at baseline. The ipsilateral PWTs were significantly decreased from day 5 (ipsilateral 16.44 ± 1.33 g) to the last observation day 21 (ipsilateral 10.25 ± 0.35 g) after TCI compared with the naïve or the sham group (Fig. 1A).

3.2. Changes of spinal GABA in CIBP rats.

In order to clarify the changes of GABA in the spinal cord, sections of the spinal cord

in each group were analyzed using immunofluorescence staining. GABA+ immunoreactivities were mainly presented in the superficial dorsal horn of the spinal cord. GABA+ immunoreactivities in the spinal cord of CIBP rats were obviously weaker than the naïve and sham groups (Fig. 2A-E). In the images that emerged, a number of rounded GABA+ cells were co-expressed with NeuN (a neuron marker) and some were expressed in the neuropils surrounding NeuN which are consistent with previous reports (Fig. 2K-T).

3.3. Expression of spinal GABA synthesizing enzymes GAD65 and GAD67 in CIBP rats.

To further evaluate the signaling of GABA in the spinal cord and to the occurrence of CIBP, we performed western blot and immunofluorescence staining to evaluate GABA synthesis, including GABA synthesizing enzymes GAD65 and GAD67. For western blot, the protein levels of GAD65 on day 21 of the CIBP group were significantly increased compared to the naïve group (Fig. 3A-B). While, no significant difference was observed in the protein expression of GAD67 between the CIBP group and the naïve group (Fig. 4A-B). The immunofluorescence data also showed that the expression level of GAD65 was increased in the lamina II of the ipsilateral spinal dorsal horn of the CIBP group on day 21 compared to the naïve group (Fig. 3C-G). And in the enlarged merged images, GAD65 immunoreactivities were presented as punctate structures in neuropils mostly in the superficial dorsal horn of the spinal cord (Fig. 3R-V). GAD67 immunoreactivities were detected in neuronal cell bodies and

co-expressed with NeuN (Fig. 4M-V). In conclusion, GAD65 was elevated in the ipsilateral spinal cord of the CIBP group, while, the expression level of GAD67 showed no significant difference in each group.

3.4. Attenuation of mechanical allodynia by intrathecal administration of GABA.

To determine whether GABA in the spinal cord was responsible for the development of CIBP, rats were intrathecally injected exogenous GABA. The results showed that intrathecal administration of a single dose of GABA (intrathecal injection (i.t.) 0.5 μg/10 μl or 1.0 μg/10 μl) significantly reversed CIBP-induced mechanical allodynia on day 14 after TCI both on the ipsilateral and contralateral hind limbs compared with before the intrathecal drug administration and the vehicle group. The behavioral tests were conducted at 0, 0.25, 0.5, 1.0, 2.0, 3.0 h after GABA injection. The analgesic effect of GABA began at 0.25 h, peaked at 1.0 h, and lasted for 2.0 h (Fig. 5B-C). GABA (1.0 μg/10 μl, once a day) or vehicle (10 μl, once a day) was conducted from day 14 to day 18 in order to observe chronic analgesic effect and behavioral tests were conducted 1.0 h after GABA injection from day 14 to day 18. The results showed that continuous administration of 5 days GABA (1.0 μg /10 μl) could significantly alleviate CIBP-induced mechanical allodynia rather than the vehicle (Fig. 5D).

3.5. Expression and cellular localization of spinal GABA transporter GAT-1 in CIBP rats.

We also evaluated the expression of spinal GABA transporter GAT-1 in order to show

the clearance of GABA in the synaptic cleft in each group. For western blot, the expression levels of GAT-1 protein on day 14 and day 21 of CIBP group were significantly increased when compared with the naïve group (Fig. 6A-B). Meanwhile, as we expected the protein expression of GFAP (an astrocyte marker) was consistent with GAT-1 in each group (Fig. 6C-D). We also tested the cellular localization of GAT-1 (green) in the lumbar dorsal horn by double immunofluorescence with NeuN (red) for neurons, GFAP (red) for astrocytes, and Iba-1 (red) for microglia to further confirm the cellular localization of GABA transporter GAT-1. As shown in Fig. 6E-P, GAT-1 was mostly co-expressed with GFAP, and a minority with NeuN in the superficial spinal cord. These results showed that GAT-1 was up-regulated on day 14 and day 21 after TCI and expressed mostly in astrocytes, and a minority in neurons.

3.6. Effect of intrathecal injection of GAT-1 inhibitor NO-711 on the development of CIBP-induced mechanical allodynia.

To explore the effect of GAT-1 inhibitor NO-711 on CIBP-induced mechanical allodynia, we intrathecally injected a single dose of NO-711 into CIBP rats on day 14 after TCI. Results show that NO-711 administration dramatically attenuated CIBP-induced mechanical allodynia on ipsilateral hindlimb in a dose-dependent manner compared with the vehicle group, and the efficiency of NO-711 (100 μg/10 μl, and 50 μg/10 μl, on day 14) began at 0.25 h, reached a peak at 0.5 h, and lasted for 3.0 h, except for the 10 μg/10 μl NO-711 (Fig. 7B). However, there was no significant analgesic effect of NO-711 on the contralateral hindlimb compared with the vehicle

group (Fig. 7C).

For chronic treatment, we intrathecally administered NO-711 (100 μg/10 μl, once a day) or vehicle (10 μl, once a day) from day 14 to day 18 and PWTs were measured 0.5 h after injection. As shown in Fig. 7D, continuous administration of NO-711 (100 μg/10 μl, once a day) obviously attenuated CIBP-induced mechanical allodynia compared with the vehicle group.

3.7. Chronic administration of GAT-1 inhibitor NO-711 suppressed the up-regulation of GABA transporters GAT-1 and astrocytes in CIBP rats.

To confirm whether NO-711 exerts its analgesic effect by suppressing the expression of GABA transporters GAT-1 and GFAP, we intrathecally injected NO-711 (100 μg/10 μl, once a day) into CIBP rats from day 14 to day 18. Spinal cords were collected to examine the expression of GABA transporters GAT-1 and astrocytes using western blot and immunofluorescence 0.5 h after injection. The expression of GAT-1 and GFAP were significantly suppressed after NO-711 treatment (Fig. 8A-B). As illustrated in Fig. 8C-H, immunofluorescence staining also showed that the immunoreactivities of GAT-1 and GFAP in the superficial dorsal horn of the spinal cord in the CIBP + NO-711 group were weaker than the CIBP + vehicle group. These results confirm that chronic treatment with GAT-1 inhibitor NO-711 could suppress the expression of GABA transporters GAT-1 and astrocytes in CIBP rats.

4. Discussion

Our present study showed that: 1) The expression of GABA was decreased in the spinal cord in CIBP rats which is consistent with previous reports and intrathecal administration of exogenous GABA significantly alleviated CIBP-induced mechanical allodynia. 2) The increase of GAD65 might serve as a compensation mechanism to supplement the absence of GABA in the synaptic cleft of the spinal cord, while the expression of GAD67 did not change. 3) The expression of GABA transporters GAT-1 was up-regulated on day 14 and day 21 in CIBP rats. 4) Chronic treatment of GAT-1 inhibitor NO-711 significantly suppressed the up-regulation of both GAT-1 and GFAP in the ipsilateral spinal cord. In conclusion, these results firstly indicate that the neuron-glia interactions, especially on the GABAergic pathway, contributed to CIBP-induced mechanical allodynia.

Disinhibition in the spinal cord is an important factor leading to mechanical allodynia in rats with neuropathic pain. One mechanism proposed accounting for the disinhibition was loss of GABA or its synthesizing enzyme (Moore et al., 2002). All these changes have been considered to result from the apoptosis of GABAergic neurons in the spinal cord, as indicated by a reduction of GABA-immunoreactive and the presence of the apoptotic marker (Lorenzo et al., 2014; Scholz et al., 2005). However, other studies using stereological count opposed these findings by suggesting that there was no significant loss of GABAergic neurons in the spinal cord of spared nerve injury (SNI) or chronic constriction nerve injury (CCI) model rats (Lee et al., 2010; Polgár et al., 2005; Polgár et al., 2003). In contrast to these studies, others provided evidence for an increase in GABA-immunoreactive in the spinal cord

(Castro-Lopes et al., 1992; Ko et al., 2018). These studies demonstrate that the increased GABAergic inhibitory tone measured by electrophysiology in the spinal cord may serve as a compensation mechanism for increased excitability in neuropathic pain (Kontinen et al., 2001). Thus, there are explicit discrepancies in the findings of these studies with regard to the complex anatomic changes related to the functional role of GABAergic neurons in neuropathic pain (Polgár et al., 2003). Therefore, it is important to investigate whether the changes of spinal GABAergic elements are related to the genesis and development of CIBP by comparing the quantities of GABA, its synthesizing enzymes GAD65 and GAD67 in the spinal cord. In contrast to previous studies, our results showed that the expression of GAD65 was elevated on day 21 after TCI. We used both western blot and immunofluorescence staining to analyze its expression, which could testify to the reliability of our results. It is difficult to illustrate the discrepancy between our results and those of previous studies. One possible explanation might be that the mechanism of CIBP is distinct from neuropathic pain. Some studies have reported that CIBP rats showed remarkable activation of glia cells but there was no change in the number of neurons (Schwei et al., 1999). Another possible reason for the change of GAD65 might be that it serves as a compensation mechanism to control the balance of GABA and glutamate which is consistent with the electrophysiologic results of Kontinen study (Kontinen et al., 2001).

Another possible mechanism of disinhibition was depletion of GABA in the synaptic cleft. GABA transporters play a vital role in the homeostasis of GABA in the synaptic

cleft of the spinal cord. In normal conditions, the expression of GABA is controlled by GABAergic neurons and astrocytes GABA transporters through the mechanism of glutamate-GABA cycle (Gwak and Hulsebosch, 2011). GABA transporters control the homeostasis between excitatory and inhibitory information by clearing GABA released from presynaptic terminals in the spinal cord (Yadav et al., 2015). In recent years, accumulating evidence has shown that neuroglial cells in the peripheral nervous system, especially astrocytes and microglial cells, played an important role in the genesis and maintenance of CIBP (Guo et al., 2016; Zhang et al., 2005). It has also been confirmed that the expression of GAT-1 was elevated in the paclitaxel or CCI model (Daemen et al., 2008; Gosselin et al., 2010). However, the neuron-glia interactions on the GABAergic pathway in CIBP rats were not exactly shown. In our study, we found that the up-regulation of astrocytes and GABA transporters GAT-1 in the ipsilateral spinal cord contributed to the genesis and development of CIBP. In addition, intrathecal administration of GAT-1 inhibitor NO-711 significantly attenuated CIBP-induced mechanical allodynia.

5. Conclusion

In conclusion, the present study suggests that loss of inhibition in the spinal dorsal horn contribute to the development of CIBP. Our data showed that intrathecal delivery of GABA or pharmacologically inhibition of GAT-1 could attenuate CIBP-induced mechanical allodynia. Therefore, spinal GABA and GAT-1 might be a potential therapeutic target for cancer pain.

Acknowledgements and conflicts of interest

The study was supported by a grant from National Natural Science Foundation of People's Republic of China (grant 81371250). The authors have no conflicts of interest to declare.

Author contributions

Hui Yang, Yu-Ke Tian, Xue-Bi Tian, and Feng Gao designed the research study; Meng-Meng Ge, Shu-Ping Chen, Ya-Qun Zhou and Zheng Li performed the study; Meng-Meng Ge prepared the manuscript; Hui Yang, Ya-Qun Zhou, Anne Manyande and Xue-Bi Tian revised the content of the manuscript. All authors approved the final version of manuscript.

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Figure legends

Fig. 1.: Tumor cell inoculation induced mechanical allodynia in female rats. (A) CIBP ipsilateral PWTs were significantly decreased from day 5 after TCI to the last observation on day 21 (*** $P < 0.001$ compared with the naïve group, ### $P < 0.001$ compared with the sham group, N = 10 in each group). While, the naïve and sham groups showed no obvious change during 21 days.

Fig. 2.: Changes of GABA in the ipsilateral spinal cord after TCI. (A-J) Immunoreactivities of GABA (green) and NeuN (red) in the ipsilateral spinal cord in each group. The fluorescence intensity of GABA (green) in the spinal cord of CIBP rats was weaker than the naïve and sham groups (N = 4 in each group). (K-T) As we detected in the merged images, some GABA (green) immunoreactivities were co-expressed with NeuN (red) and some were expressed in the neuropils surrounding NeuN (red) (N = 4 in each group).

Fig. 3.: Expression of spinal GABA synthesizing enzymes GAD65 in CIBP rats. (A-B) Western blot analysis showed that GAD65 protein expression in the dorsal horn

was significantly up-regulated on day 21 post TCI of CIBP group (*** $P < 0.001$ compared with the naïve group, N = 6 in each group). (C-G) Immunofluorescence staining showed that the increased GAD65 (green) was mainly expressed in the lamina II of lumbar dorsal horn of ipsilateral spinal cord (N = 4 in each group). (R-V) The enlarged merge images showed that GAD65 (green) immunoreactivities were expressed as punctate structures in neuropils (N = 4 in each group).

Fig. 4.: Expression of spinal GABA synthesizing enzymes GAD67 in CIBP rats. (A-B) Western blot analysis showed that GAD67 protein expression in the spinal dorsal horn had no difference in each group ($P > 0.05$ compared with the naïve group, N = 6 in each group). (C-G) Immunofluorescence staining showed that GAD67 (green) was spread evenly throughout GABAergic neurons in the deep laminae of the spinal cord (N = 4 in each group). (R-V) The enlarged merge images showed that GAD67 (green) immunoreactivities were expressed in neuronal cell bodies and co-expressed with NeuN (a neuron marker) (N = 4 in each group).

Fig. 5.: Intrathecal administration of GABA alleviated CIBP-induced mechanical allodynia. (A) Scheme of GABA administration and behavioral assessment in CIBP rats. (B-C) A single dose of GABA (i.t. 0.5 μg/10 μl and 1.0 μg/10 μl on day 14) significantly alleviated CIBP-induced mechanical allodynia both on the ipsilateral and contralateral hindlimbs (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with the CIBP + vehicle group. # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ compared with before

intrathecal drug administration, N = 6 in each group). (D) The development of CIBP-induced mechanical allodynia was attenuated by the continuous administration of 5 days GABA (i.t. 1.0 μg/10 μl, once a day) (*** $P < 0.001$ compared with the CIBP + vehicle group. # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ compared with the naïve group, N = 6 in each group).

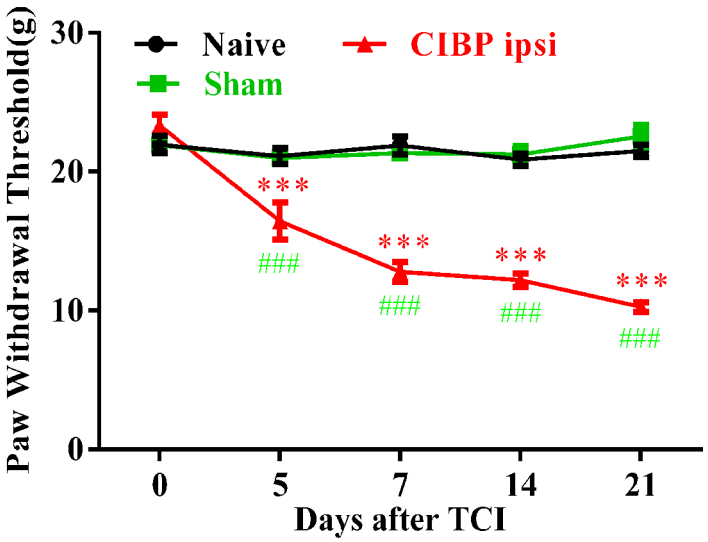
Fig. 6.: Expression and cellular localization of GAT-1 in the dorsal horn of spinal cord in CIBP rats. (A-B) Western blot analysis showed that the time course of GAT-1 protein expression in the dorsal horn on day 7, day 14 and day 21 post TCI (* $P < 0.05$, ** $P < 0.01$ compared with the naïve group, N = 6 in each group). (C-D) As we respect that the expression of GFAP (an astrocyte marker) is consistent with GAT-1 (** $P < 0.01$, *** $P < 0.001$ compared with the naïve group, N = 6 in each group). (E-P) Representative photomicrographs of GAT-1 (green) double fluorescence labeling with NeuN (red) for neurons, GFAP (red) for astrocytes, and Iba-1 (red) for microglia in the ipsilateral spinal cord on day 14 after TCI. The results showed that GAT-1 was co-expressed mostly with GFAP (yellow) and minority with NeuN (yellow) (N = 4 in each group).

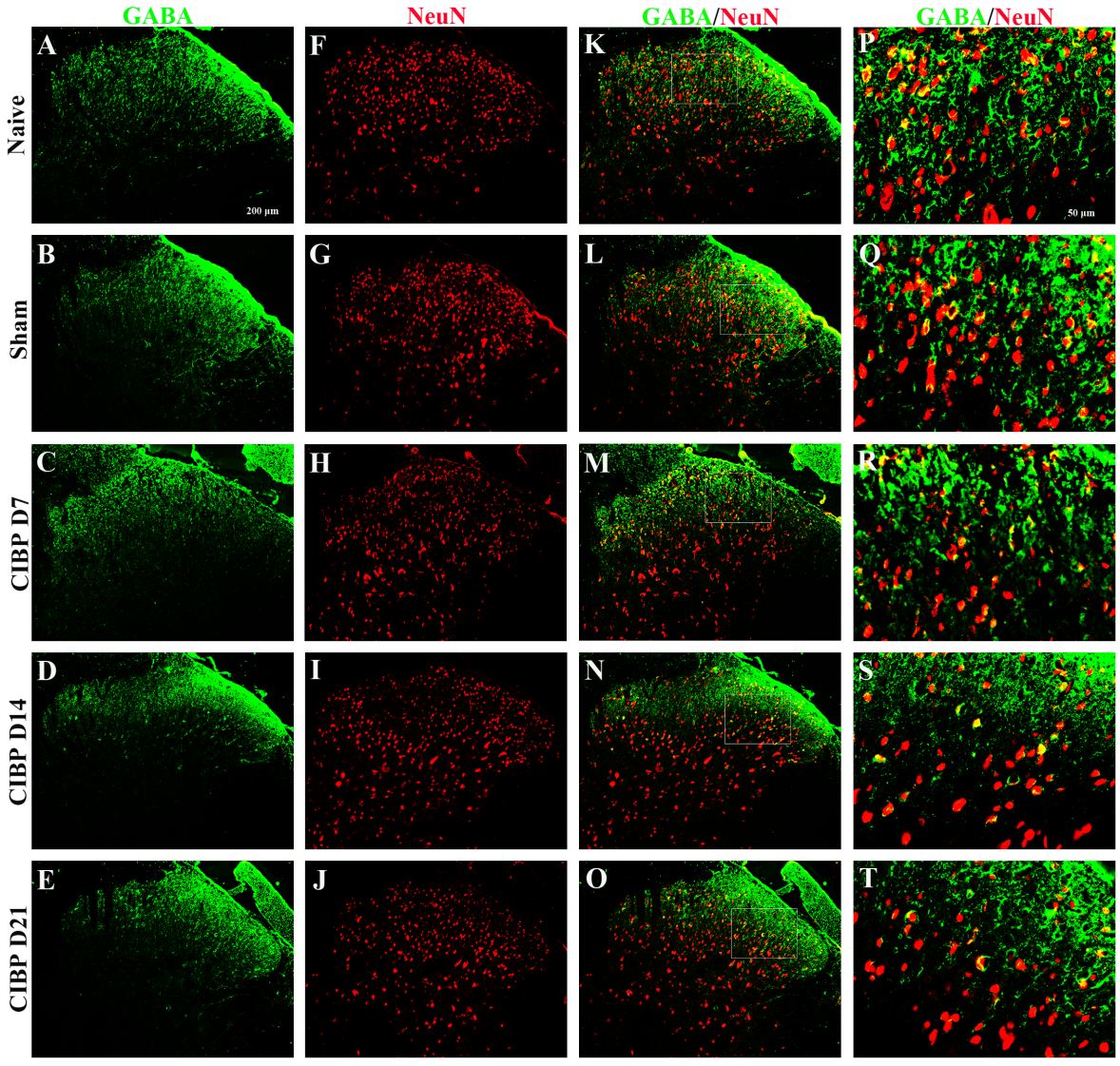
Fig. 7.: Intrathecal administration of GAT-1 inhibitor NO-711alleviatedCIBP-induced mechanical allodynia. (A) Scheme of NO-711 administration and behavioral assessment in CIBP rats. (B-C) A single dose of NO-711 (i.t. 100 μg/10 μl and 50 μg/10 μl, but not 10 μg/10 μl, on day 14)

significantly reversed the CIBP-induced mechanical allodynia on ipsilateral hindlimb. However, there was no significant analgesic effect of NO-711 on the contralateral hindlimb. The result showed that only a single dose of 100 μg NO-711 reversed the CIBP-induced mechanical allodynia on contralateral hindlimb at 0.5 h after NO-711 administration (* $P < 0.05$, *** $P < 0.001$ compared with the CIBP + vehicle group, N = 4 in each group). (D) For chronic treatment, NO-711 (i.t. 100 μg/10 μl, once a day) or vehicle was injected for 5 days (from day 14 to day 18 after TCI) and PWTs were measured 0.5 h after injection. The ipsilateral mechanical allodynia was obviously reversed by NO-711 (100 μg/10 μl) (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with the CIBP + vehicle group. ## $P < 0.01$, ### $P < 0.001$ compared with the naïve group, N = 4 in each group).

Fig. 8.: Repeated intrathecal administration of NO-711 (100 μg/10 μl) reversed CIBP-induced up-regulation of GAT-1 and astrocytes in the spinal cord.

(A) Western blot data showed that the protein expression of GAT-1 was down-regulated in the CIBP + NO-711 group (** $P < 0.01$ compared with the CIBP + vehicle group, N = 4 in each group). (B) The expression of GFAP was also suppressed in the CIBP + NO-711 group (* $P < 0.05$ compared with the CIBP + vehicle group, N = 4 in each group). (C-H) Immunofluorescence staining also demonstrated that NO-711 (100 μg/10 μl) reversed the increase of GABA transporters GAT-1and astrocytes in the spinal cord in CIBP rats.




GABA
NeuN
GABA/NeuN
GABA/NeuN
Naive
Sham
CIBP D7
CIBP D14
CIBP D21
A
B
C
D
E
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I
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K
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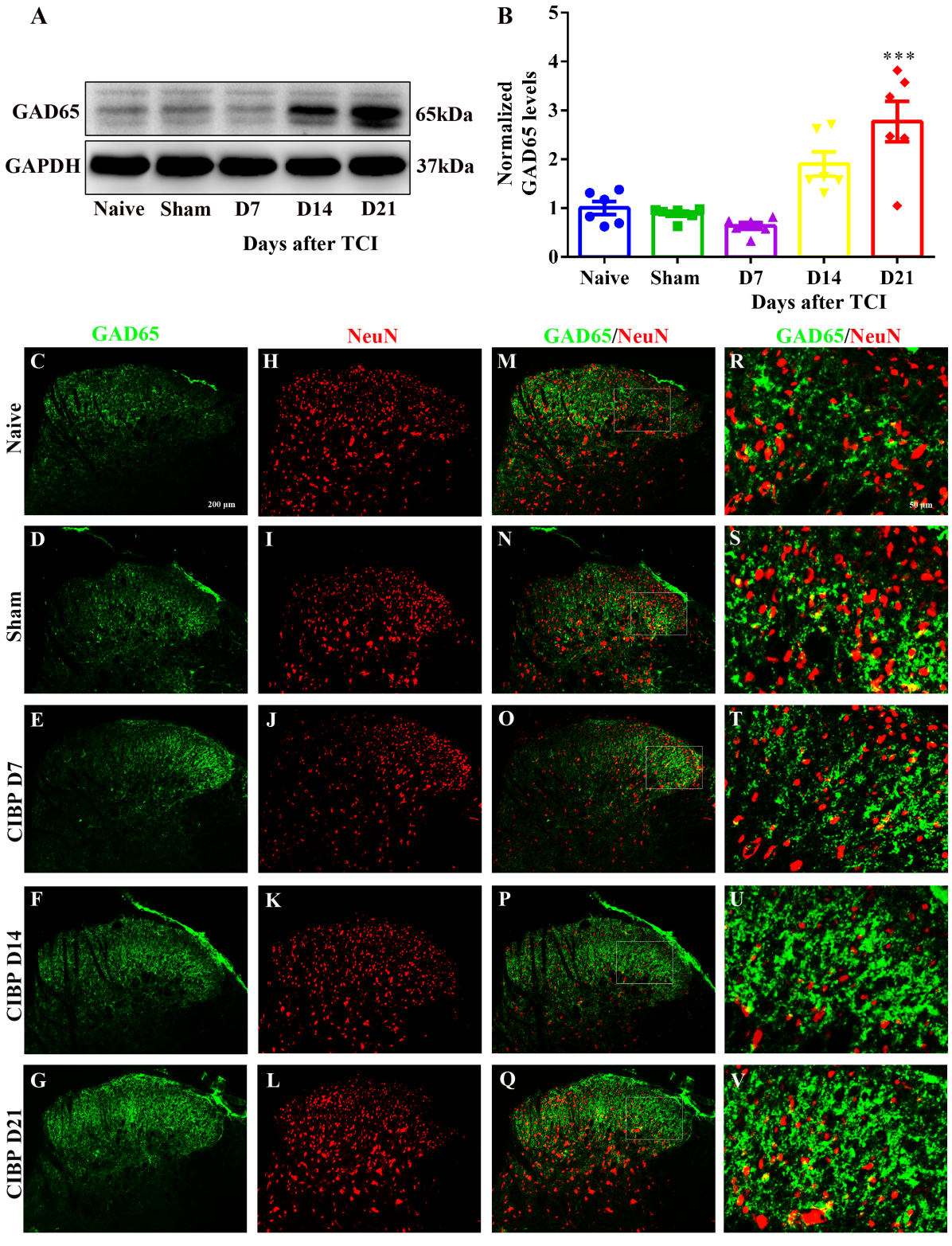
M
N
O
P
Q
R
S
T
200 μm
50 μm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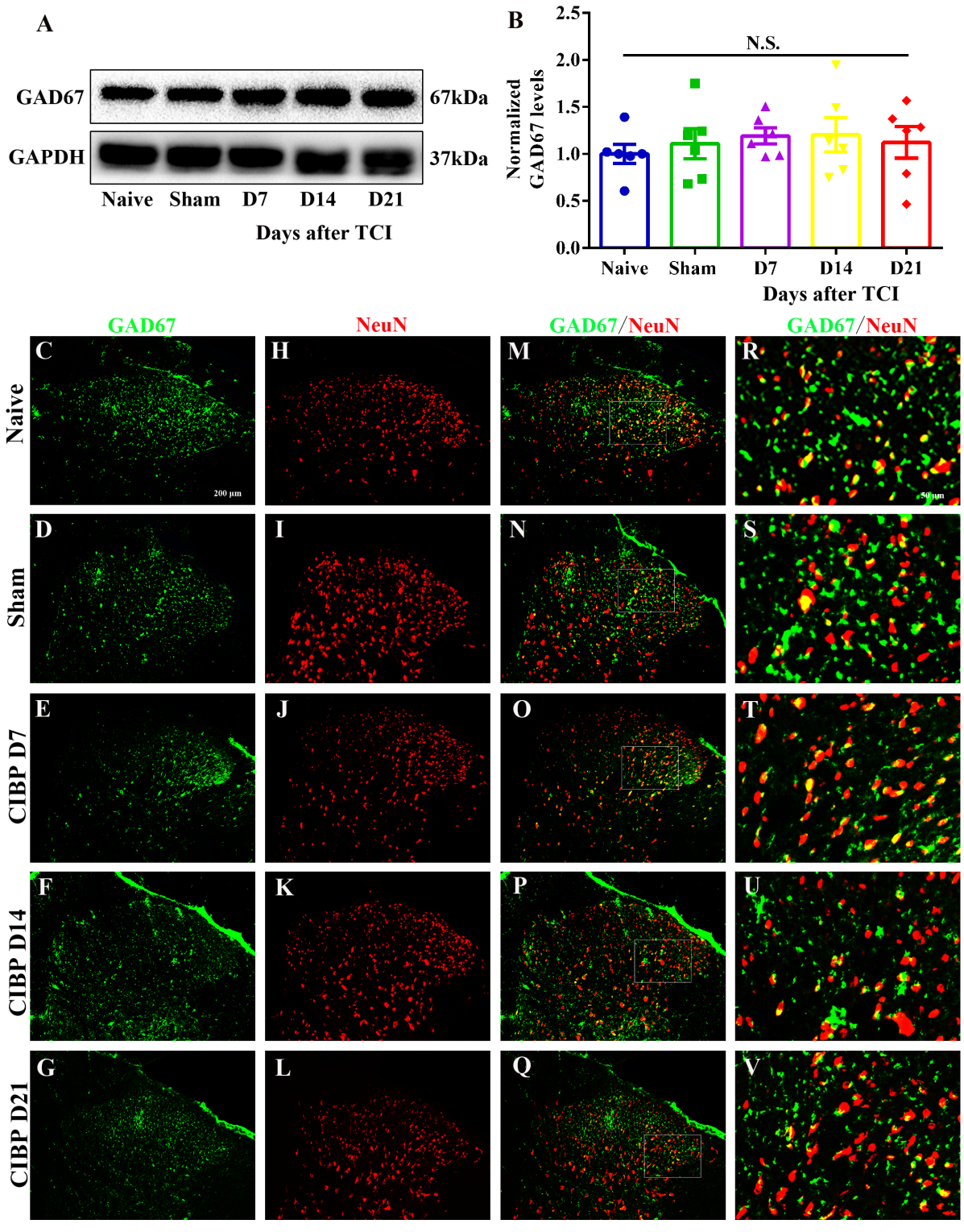
GAD65
GAPDH
65kDa
37kDa
Naive
Sham
D7
D14
D21
Days after TCI
B
Normalized GAD65 levels
***
Naive
Sham
D7
D14
D21
Days after TCI
GAD65
NeuN
GAD65/NeuN
GAD65/NeuN
Naive
Sham
CIBP D7
CIBP D14
CIBP D21
200 μm
50 μm


A
GAD67
67kDa
GAPDH
37kDa
Naive
Sham
D7
D14
D21
Days after TCI
B
N.S.
Normalized GAD67 levels
2.5
2.0
1.5
1.0
0.5
0.0
Naive
Sham
D7
D14
D21
Days after TCI
GAD67
NeuN
GAD67/NeuN
GAD67/NeuN
Naive
Sham
CIBP D7
CIBP D14
CIBP D21
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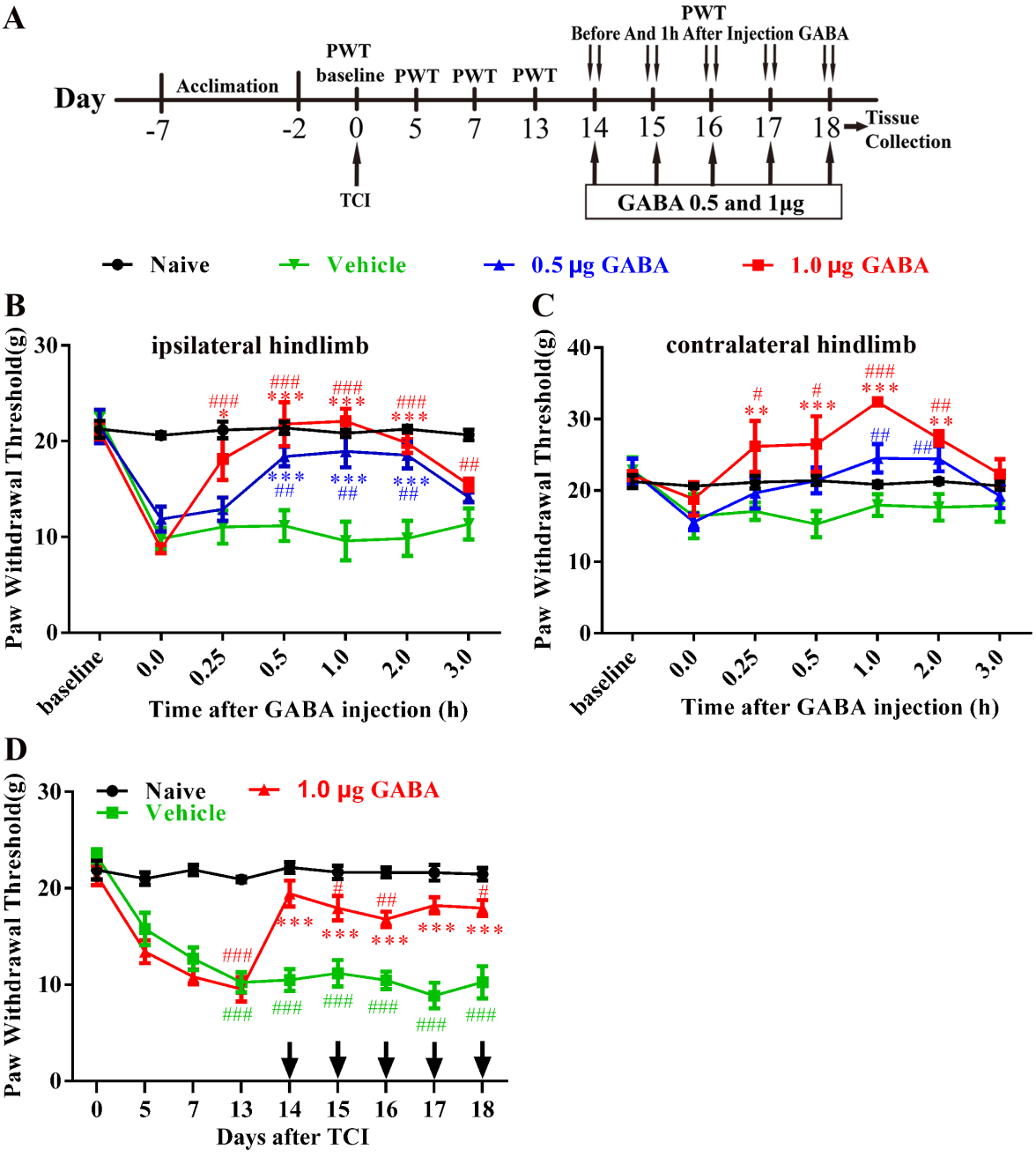
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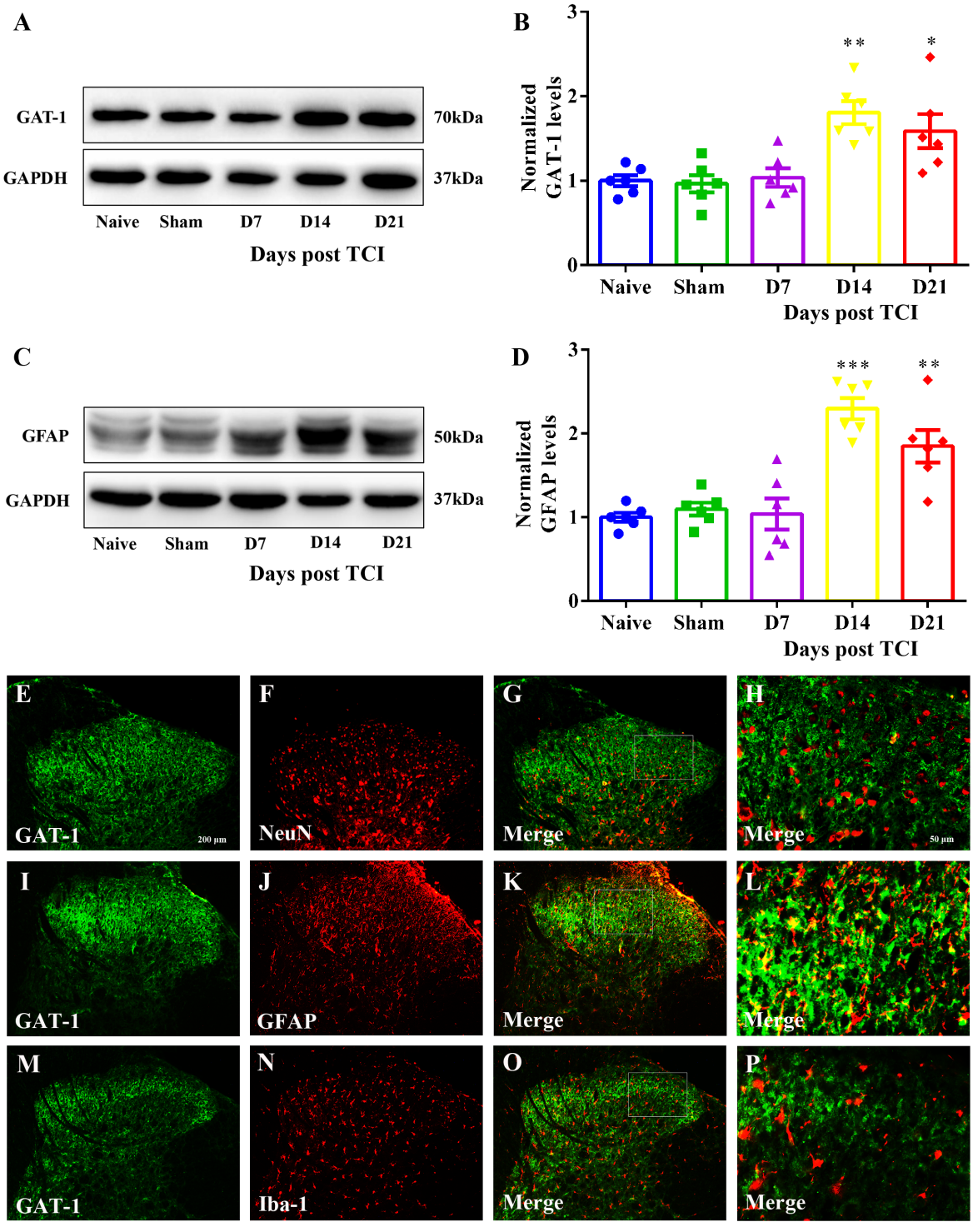
PWT
Before And 1h After Injection GABA
PWT
baseline
PWT
PWT
PWT
Day
Acclimation
-7
-2
0
5
7
13
14
15
16
17
18
Tissue
Collection
TCI
GABA 0.5 and 1μg
Naive
Vehicle
0.5 μg GABA
1.0 μg GABA
B
ipsilateral hindlimb
Paw Withdrawal Threshold(g)
Time after GABA injection (h)
baseline
0.0
0.25
0.5
1.0
2.0
3.0
C
contralateral hindlimb
Paw Withdrawal Threshold(g)
Time after GABA injection (h)
D
Naive
Vehicle
1.0 μg GABA
Paw Withdrawal Threshold(g)
Days after TCI


A
GAT-1
70kDa
GAPDH
37kDa
Naive
Sham
D7
D14
D21
Days post TCI
B
Normalized GAT-1 levels
**
*
Naive
Sham
D7
D14
D21
Days post TCI
C
GFAP
50kDa
GAPDH
37kDa
Naive
Sham
D7
D14
D21
Days post TCI
D
Normalized GFAP levels
***
**
Naive
Sham
D7
D14
D21
Days post TCI
E
GAT-1
200 μm
F
NeuN
G
Merge
H
Merge
50 μm
I
GAT-1
J
GFAP
K
Merge
L
Merge
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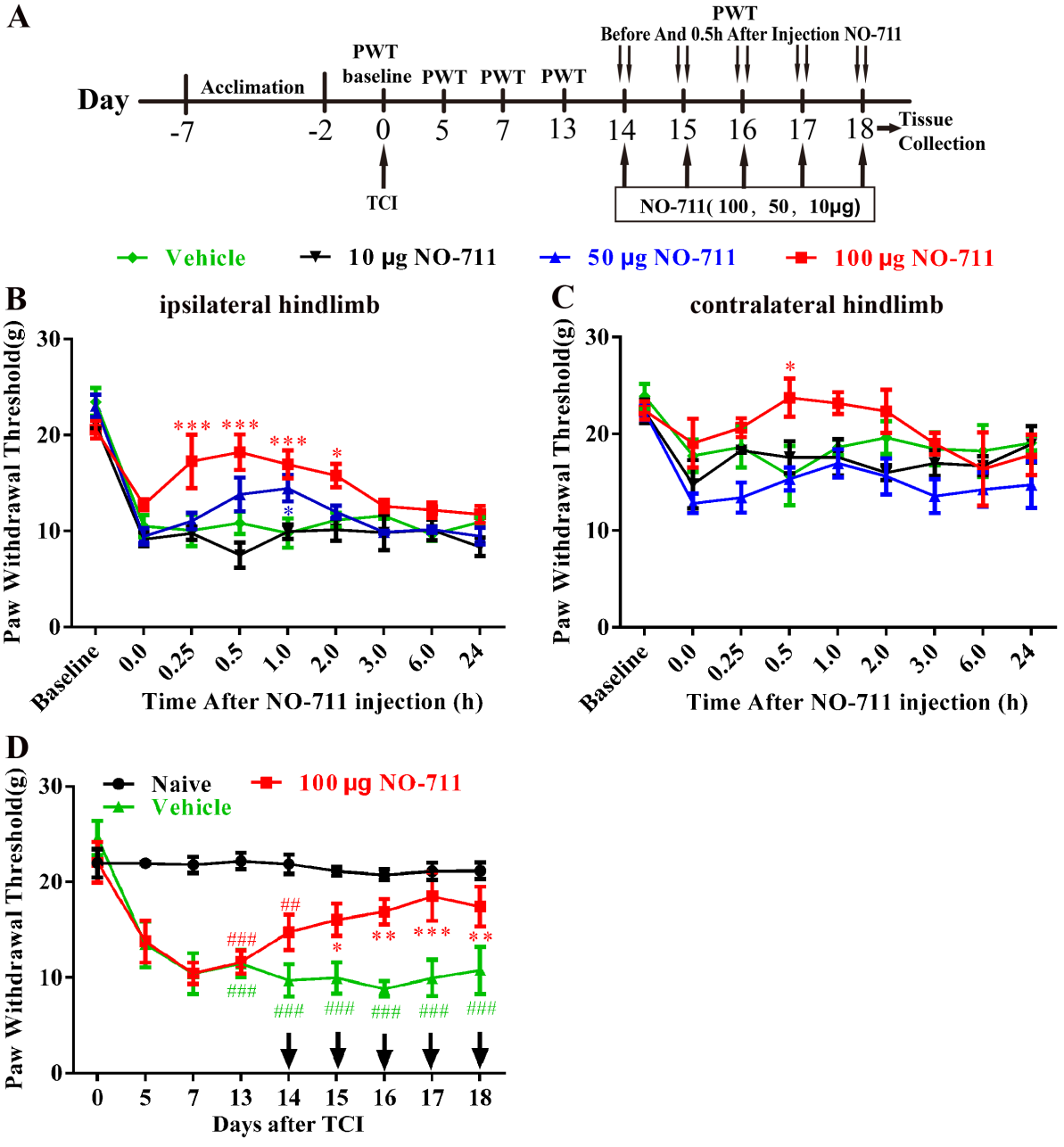
GAT-1
N
Iba-1
O
Merge
P
Merge


A
PWT
Before And 0.5h After Injection NO-711
PWT
baseline
PWT
PWT
PWT
Day
Acclimation
-7
-2
0
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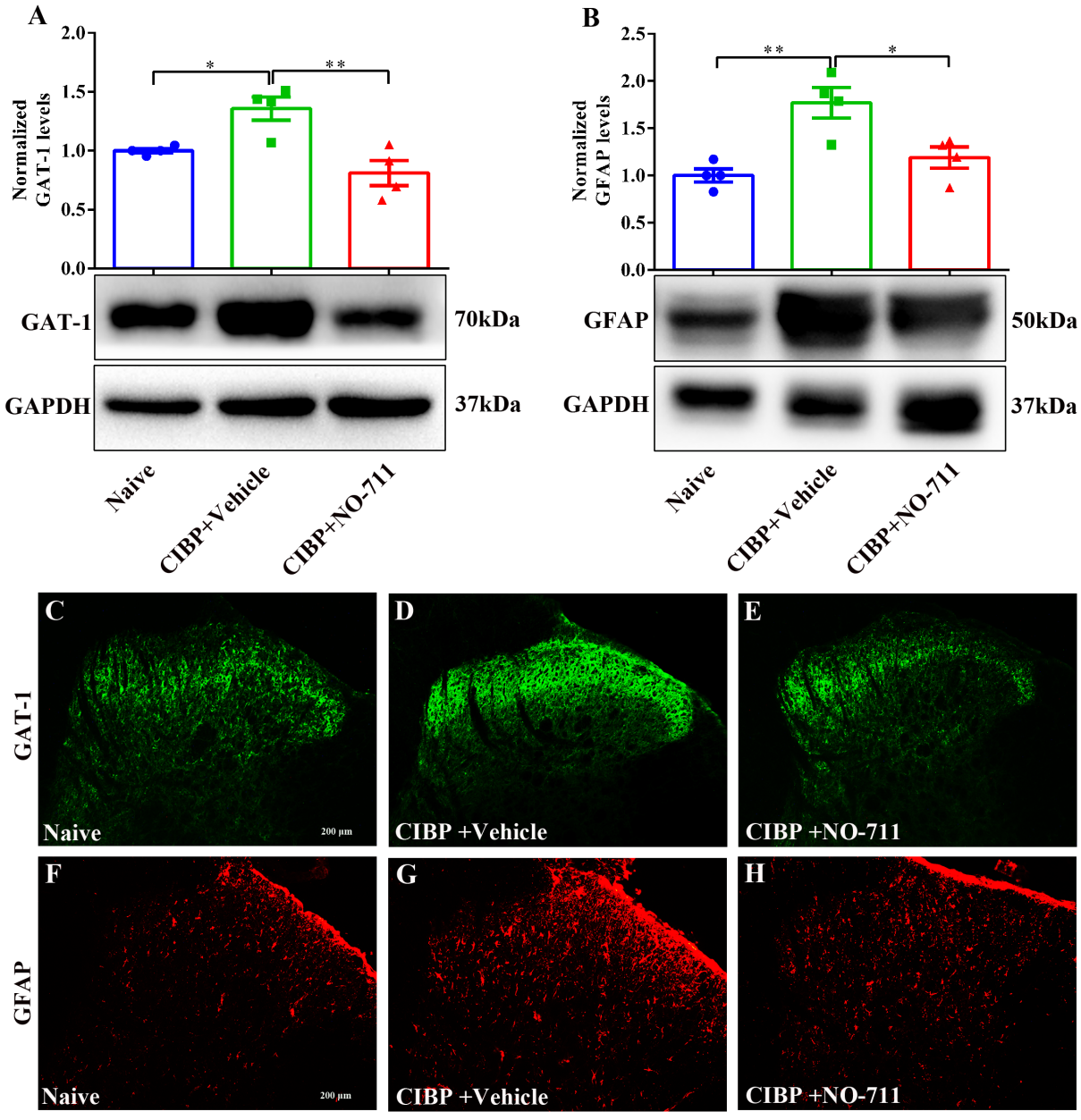
7
13
14
15
16
17
18
Tissue
Collection
TCI
NO-711(100， 50， 10μg)
Vehicle
10 μg NO-711
50 μg NO-711
100 μg NO-711
B
ipsilateral hindlimb
Paw Withdrawal Threshold(g)
Baseline
0.0
0.25
0.5
1.0
2.0
3.0
6.0
24
Time After NO-711 injection (h)
C
contralateral hindlimb
Paw Withdrawal Threshold(g)
Time After NO-711 injection (h)
D
Naive
100 μg NO-711
Vehicle
Paw Withdrawal Threshold(g)
Days after TCI


A
Normalized GAT-1 levels
2.0
1.5
1.0
0.5
0.0
*
**
GAT-1
70kDa
GAPDH
37kDa
Naive
CIBP+Vehicle
CIBP+NO-711
B
Normalized GFAP levels
2.5
2.0
1.5
1.0
0.5
0.0
**
*
GFAP
50kDa
GAPDH
37kDa
Naive
CIBP+Vehicle
CIBP+NO-711
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D
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GAT-1
Naive
200 μm
CIBP +Vehicle
CIBP +NO-711
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GFAP
Naive
200 μm
CIBP +Vehicle
CIBP +NO-711