Expression of gamma-aminobutyric acid type A receptor α2 subunit in the dorsal root ganglion of rats with sciatic nerve injury*

Yu Lian1,2, Yang Wang1,2,3, Ketao Ma1,2, Lei Zhao1,2, Zhongshuang Zhang1,2, Yuanyuan Shang1,2, Junqiang Si1,2,3, Li Li1,2

1 Department of Physiology, Shihezi University School of Medicine, Shihezi 832002, Xinjiang Uygur Autonomous Region, China
2 Key Laboratory of Xinjiang Endemic and Ethnic Disease, Shihezi University School of Medicine, Shihezi 832002, Xinjiang Uygur Autonomous Region, China
3 Fundamental Medical School of Wuhan University, Wuhan 430071, Hubei Province, China

Abstract
The γ-aminobutyric acid neurotransmitter in the spinal cord dorsal horn plays an important role in pain modulation through primary afferent-mediated presynaptic inhibition. The weakening of γ-aminobutyric acid-mediated presynaptic inhibition may be an important cause of neuropathic pain. γ-aminobutyric acid-mediated presynaptic inhibition is related to the current strength of γ-aminobutyric acid A receptor activation. In view of this, the whole-cell patch-clamp technique was used here to record the change in muscimol activated current of dorsal root ganglion neurons in a chronic constriction injury model. Results found that damage in rat dorsal root ganglion neurons following application of muscimol caused concentration-dependent activation of current, and compared with the sham group, its current strength and γ-aminobutyric acid A receptor protein expression decreased. Immunofluorescence revealed that γ-aminobutyric acid type A receptor α2 subunit protein expression decreased and was most obvious at 12 and 15 days after modeling. Our experimental findings confirmed that the γ-aminobutyric acid type A receptor α2 subunit in the chronic constriction injury model rat dorsal root ganglion was downregulated, which may be one of the reasons for the reduction of injury in dorsal root ganglion neurons following muscimol-activated currents.

Key Words
γ-aminobutyric acid; γ-aminobutyric acid type A Receptor α2 subunit; neuropathic pain; dorsal root ganglion; whole-cell patch clamp; immunofluorescence; primary afferent depolarization; paw withdrawal latency; muscimol

Research Highlights
(1) Gamma-aminobutyric acid type A receptor α2 subunit participates in the pathogenesis of neurological pain.
(2) Gamma-aminobutyric acid type A receptor α2 subunit expression was downregulated in the dorsal root ganglion in rats with chronic constriction injury, which may be one of the reasons for the decreased current of dorsal root ganglion activation at the ipsilateral side after chronic constriction injury in the sciatic nerve.
(3) The activation current of the gamma-aminobutyric acid type A receptor α2 subunit was decreased on the ipsilateral side, but increased on the contralateral side. This change resulted from the compensation mechanism of nerves.
INTRODUCTION

Neuropathic pain is defined as ‘pain initiated or caused by a primary lesion or dysfunction in the nervous system’. Neuropathic pain is often reported as having a lancinating or continuous burning character and is often associated with the appearance of abnormal sensory signs, such as spontaneous pain-related behavior, allodynia (pain as a result of a stimulus that does not normally provoke pain) or hyperalgesia (an increased response to a stimulus which is normally painful)\[1-4\]. In normal primary afferent neurons, it is rare for a firing threshold to be reached without the input of a stimulus. However, following nerve injury, it has been demonstrated that there is a large increase in the level of spontaneous firing in afferent neurons linked to the injury site. This has been termed ectopic discharge and has also been demonstrated in humans suffering from neuropathic pain.

\(\gamma\)-aminobutyric acid (GABA) is a classical inhibitory neurotransmitter. GABA receptors can be divided into GABA\(_\alpha\)R, GABA\(_\beta\)R and GABA\(_\gamma\)R. GABA\(_\alpha\)R are ligand-gated ion channel receptors. Spinal dorsal horn GABA\(_\alpha\) receptors are found both postsynaptically on central neurons and presynaptically on axons and/or terminals of primary sensory neurons, where they mediate primary afferent depolarization and presynaptic inhibition. GABA can act at GABA\(_\alpha\)R localized on primary afferent neurons to inhibit presynaptic neurotransmitter release and produce analgesia via a process of primary afferent depolarization. GABA mediates fast synaptic inhibition by activating ionotropic GABA\(_\alpha\)R, which are assembled from a large family of constituent subunits\[5-6\]. GABA\(_\alpha\)R is made up from 19 known subunits (\(\alpha_1\)-6, \(\beta_1\)-3, \(\gamma_1\)-3, \(\delta\), \(\epsilon\), \(\theta\), \(\pi\) and \(\rho_1\)-3)\[7-8\] with an integral channel that is permeable to Cl\(^-\) ions. The majority of GABA\(_\alpha\)R consist of \(\alpha_2\beta_2\gamma_2\)\[9\] that can interact with a range of compounds via specific binding sites that include agonists (e.g., GABA and muscimol). After injury, GABA\(_\alpha\)R positive allosteric modulators inhibit robust analgesia in animals via restoration of post-synaptic GABA\(_\alpha\)R \(\alpha_2\) and GABA\(_\alpha\)R \(\alpha_3\) subunit function within the spinal cord\[10\]. Subunit \(\alpha_2\) is expressed at pre- and post-synaptic elements within the spinal dorsal horn and is predominantly found within superficial layers that receive nociceptive input from primary afferents\[11\]. Recent molecular, genetic, and pharmacological data point to \(\alpha_1\)-containing GABA\(_\alpha\)R as the "sedative" and \(\alpha_2\) and/or \(\alpha_3\)-containing receptors as the "anxiolytic" subtypes\[12\].

The contribution of primary afferent depolarization to the processing of nociceptive signals and to the antihyperalgesic effect of GABA\(_\alpha\)R modulators is unknown, mainly because of the lack of suitable tools for the specific targeting of presynaptic GABA\(_\alpha\)R. In this study, through the establishment of a chronic constriction injury (CCI) model of sciatic nerve, we monitored GABA\(_\alpha\)R \(\alpha_2\) subunit function and expression changes in L4,5 dorsal root ganglion neurons using whole-cell patch-clamp and immunofluorescence methods to further understand the contribution of the GABA\(_\alpha\)R \(\alpha_2\) subunit in pathological pain.

RESULTS

Quantitative analysis of experimental animals

Ninety experimental Sprague-Dawley rats were randomly divided into three groups: sham-surgery group (\(n = 30\)), CCI ipsilateral side model group (\(n = 30\)) and CCI contralateral side model group (\(n = 30\)). CCI models were established on the ipsilateral side and contralateral side of the latter groups, and the number of modeling success was both 26. The sham-surgery group was treated the same as that in the CCI modeling method, with the exception of mp ligation with chromium suture, and 26 rats were randomly selected from the sham group. A total of 78 rats in three groups were involved in the final experiment, 20 in each group for electrophysiology experiments and six in each group for immunofluorescence experiments.

Thermal hyperalgesia of the rat CCI model

We explored the behavioral performance of hyperalgesia after CCI. Thermal hyperalgesia was measured before operation (baseline) and on 1, 3, 7, 12 and 15 days after operation. Thermal hyperalgesia was assessed using the hot plate test. Heat-evoked thermal withdrawal latency of CCI rats began to significantly decrease from 1 to 15 days post-surgery. The sham-operated and CCI contralateral side showed no changes in baseline and after surgery (\(P > 0.05\)); the thermal withdrawal latency of the CCI ipsilateral side were significantly different before and
after surgery ($P < 0.01$), and compared with sham-operated rats, thermal withdrawal latency of CCI rats significantly decreased ($P < 0.05$; Figure 1).

Changes in GABA$_A$R electrophysiological features on dorsal root ganglion neurons in CCI rats

Freshly isolated neurons from the L$_{4-6}$ dorsal root ganglion in the diameter range of 15–45 μm were used in the present study. In the majority of neurons examined (82.4%, 89/108), muscimol ($10^{-8}$–$10^{-3}$ M) induced a concentration-dependent inward current. We recorded three typical types of muscimol-activated currents: fast desensitization type (3/108), mixed (93/108) and slow desensitization type (12/108) (Figure 2A). This muscimol-activated current could be reversibly blocked by bicuculline ($10^{-4}$ M), a selective antagonist of GABA$_A$R, indicating this current was mediated by GABA$_A$R (Figure 2B).

Muscimol induced a concentration-dependent ($10^{-8}$–$10^{-3}$ M) inward current on L$_{4-6}$ dorsal root ganglion neurons from the sham-operated, CCI ipsilateral side model, and CCI contralateral side model. Figure 3A shows the sham-operated, CCI ipsilateral side, and CCI contralateral side curve traces for muscimol. The muscimol-activated current amplitude ($I_{	ext{muscimol}}$) was significantly reduced in the CCI ipsilateral side model, while $I_{	ext{muscimol}}$ significantly increased on the CCI contralateral side. $I_{	ext{muscimol}}$ ($10^{-4}$ M) in the sham-operated, CCI ipsilateral side model, and CCI contralateral side model rats was $1.487 \pm 92.9$ pA ($n = 6$), $541 \pm 63.3$ pA ($n = 6$) and $1.945 \pm 28.4$ pA ($n = 6$), respectively, among the three groups, which were significantly different ($P < 0.01$). From concentration-response curves of muscimol-activated neurons (Figure 3B), it can be seen that, (a) the dose-response curve for the CCI ipsilateral side is shifted downwards compared with sham-operated rats; the CCI contralateral side is shifted upwards; (b) the EC$_{50}$ value in the sham-operated, CCI ipsilateral side model, and CCI contralateral side model groups are very close (14.7 ± 1.8 μM; 14.3 ± 0.9 μM; 14.4 ± 1.6 μM; $n = 6$), and are not significantly different between groups ($P > 0.05$).

Distribution of GABA$_A$R α$_2$ subunit expressing neurons in the dorsal root ganglion of CCI rats

Immunofluorescence staining displayed that GABA$_A$R α$_2$ subunit expression was widely distributed in dorsal root ganglion neurons of all three groups, and were mainly located in the cell membranes of various diameters at 12 days after modeling (Figure 4). Quantitative analysis demonstrated that GABA$_A$R α$_2$ subunit expression was decreased in the CCI ipsilateral side model group when compared with the sham-surgery group at 12 days after modeling ($P < 0.01$), and expression levels were highest in the CCI contralateral side model group.

Based on the above experiments, GABA$_A$R α$_2$ subunits levels in sensory neurons may underlie the development of pain. We examined alterations to GABA$_A$R α$_2$ subunits...
in the ipsilateral L4-6 dorsal root ganglion on days 3, 7, 12 and 15 after operation using immunofluorescence. GABA$_{n}$R $\alpha_2$ subunit-positive signals in the membrane of dorsal root ganglion neurons decreased (Figure 5).

**Figure 3**  Comparison of muscimol-activated currents of dorsal root ganglion neurons.

(A) Sequential current-traces illustrating the concentration-dependent muscimol-activation current amplitude. 

(B) The concentration-response curves for muscimol-activation. Data at each point are represented as mean ± SEM of 6–17 neurons. All muscimol-induced currents were normalized. $^{a}P<0.05$, vs. sham-surgery group; $^{b}P<0.01$, vs. chronic constriction injury (CCI) contralateral side model group. 

The EC$_{50}$ (half number of effective concentrations) value in the sham-surgery, CCI ipsilateral side model, and CCI contralateral side model groups are very close (14.7 ± 1.8 $\mu$M, 14.3 ± 0.9 $\mu$M, 14.4 ± 1.6 $\mu$M; $n=6$), and no significant difference among the three groups was observed ($P > 0.05$). A homogeneity test for variance was performed followed by one-way analysis of variance, and two-group comparison was conducted using the least significant difference $t$-test.

Changes in absorbance values on the CCI contralateral side were not seen on day 3 (34.9 ± 5.21; $n=6$) or day 7 (31.1 ± 3.50; $n=6$) after surgery. However, the mean absorbance began to significantly attenuate by day 12 (20.2 ± 2.24; $n=6$) after operation when compared with the sham-surgery group (37.2 ± 5.31; $n=6$; $P < 0.05$). This significant decrease continued from 12 to 15 days (20.2±2.24; $n=6$).

**Figure 4** Confocal images of $\gamma$-aminobutyric acid type A receptor $\alpha_2$ (GABA$_{n}$R $\alpha_2$) subunit expression in the chronic constriction injury (CCI)-induced L4-6 dorsal root ganglion (immunofluorescence staining, fluorescence microscope, ×20) at day 12 after injury.

GABA$_{n}$R $\alpha_2$ subunit immunoreactivity was seen in dorsal root ganglion neuron cell membranes. Neurites of dorsal root ganglion neurons formed networks. Positive cells presented green fluorescent labeling: Mild fluorescence intensity in the sham group (A), weak fluorescence intensity in the CCI surgery group (B), high fluorescence intensity in the CCI contralateral group(C). Scale bars (A–C): 50 $\mu$m.

(D) The histogram shows the expression of GABA$_{n}$R $\alpha_2$ in the dorsal root ganglion. $^{a}P < 0.01$, vs. sham-surgery group; $^{b}P < 0.01$, vs. CCI contralateral side model group. Data are expressed as mean ± SEM of six rats in each group.

**DISCUSSION**

In the present study, we found that the CCI ipsilateral side muscimol-activated current amplitudes were significantly lower than that in the sham-surgery group, while the CCI contralateral side model group was greater. Although there was no nerve damage to the contralateral side, it still produced a series of changes in the electrophysiological properties of GABA$_{n}$R and the expression of the GABA$_{n}$R $\alpha_2$ subunit. Such compensatory changes may be due to injury information from the unilateral nerve into the bilateral spinal cord. Several possible explanations could account for this compensatory enhancement. Synaptic plasticity of neurons is believed to be fundamental to pathological pain and the importance of spinal sensitization is well recognized. This synaptic arrangement can be the substrate of local disinhibition, which may play a key role in sensory processing$^{13}$-$^{14}$. In the spinal cord, the GABA$_{n}$R $\alpha_2$ subunit is densely expressed in the superficial layers of the dorsal horn, the main termination area of
Indeed, GABAergic interneurons intercalated within the circuitry linking primary afferents and spinal projection neurons may play a dual role in controlling network excitability\[13,19\]. This study found that the GABA\(_{\alpha_2}\) subunit was involved in neuropathic pain. The GABA\(_{\alpha_2}\) containing \(\alpha_2\) subunit and \(\alpha_2\) subunit are current targets in the battle to develop new pain medications, as they are expressed in the spinal cord where increasing inhibitory drive should result in analgesia\[25\]. We have found by immunofluorescence staining that the GABA\(_{\alpha_2}\) subunit is widely distributed in dorsal root ganglion neuron cell membranes of various diameters. Being consistent with electrophysiological experiments, at 12 days after CCI, the positive signal of the CCI ipsilateral side is reduced, however the contralateral side shows a compensatory increase. Fukuoka et al\[21\] examined the effects of unilateral L\(_3\) spinal nerve ligation using in situ hybridization, and found that GABA\(_{\alpha_2}\) subunit mRNA decreased in ipsilateral L\(_3\) dorsal root ganglion neurons but did not reach statistical significance. This result may explain why different damage models cause varying degrees of neuropathic pain. The CCI model is reported to be more sensitive to mechanical stimuli than the spinal nerve ligation model\[22\]. According to time-dependent changes of GABA\(_{\alpha_2}\) subunit expression, a significant decrease was observed at day 12 and continued until day 15. However, in the hot plate test, values for heat-evoked thermal withdrawal latency of CCI rats began to significantly decrease from 1 to 15 days post-surgery. This result may be due to the fact that there was no damage to dorsal root ganglion GABA\(_{\alpha_2}\) involved in neuropathic pain occurrence and development of hyperalgesia, and that thermal hyperalgesia could not afford a leading role.

GABA, the most abundant and important inhibitive neurotransmitter in the central nervous system, plays a regulatory role in the regeneration of various nerve cells\[23-24\]. GABA receptor activation has been shown to lead to the depolarization of primary afferent terminals in the spinal dorsal horn, and significantly reduce the transmission of excitatory impulses in primary afferent fibers\[25\]. If GABA receptor-mediated presynaptic inhibition reduced, this may cause damage to transfer to the message center and the spinal cord, causing sensitization of neurons, leading to neuropathic pain. We have found a loss of presynaptic GABA\(_{\alpha_2}\)-mediated inhibition in the dorsal root ganglion of CCI rats. This can be regarded as a key feature that contributes to the signs and symptoms of pain associated with neuropathic injury. After CCI, there is no specific loss to GABAergic neurons in the rat spinal dorsal horn after CCI in the sciatic

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At this site, the GABA\(_{\alpha_2}\) subunit is found not only postsynaptically on central neurons, where they cause classical hyperpolarization, but most likely presynaptically on the terminals of primary sensory neurons\[15-16\]. These terminals are depolarized by GABA\(_{\alpha_2}\) receptors\[17\], because their intracellular chloride concentration is kept above the electrochemical equilibrium by the chloride importer NKCC1. This primary afferent depolarization causes presynaptic inhibition, i.e., a reduction in synaptic glutamate release, possibly through inactivation of presynaptic sodium channels and/or through activation of a shunting conductance, both of which can result in inhibition of action potential propagation into presynaptic terminals\[18\]. Both processes will result in the reduction of nociceptive input to the spinal cord.
Other studies have shown this in the spared nerve injury model (e.g., partial sciatic nerve injury) suggesting that GABA synthesis is down-regulated. However, in wild-type (α2−) mice and mice lacking α2-GABAR, specifically in primary nociceptors (sns-α2−), GABAR currents and dorsal root potentials are of normal amplitude in vitro, and mice show normal response thresholds to thermal and mechanical stimulation in vivo, and develop normal inflammatory and neuropathic pain sensitization. However, the effect of benzodiazepines such as diazepam and midazolam was significantly reduced.

Studies have demonstrated that the GABAR α2 subunit participates in pain perception conduction in CCI rats. In patch clamp experiments, we found that there is a change in GABAR function in CCI rats. At the same time, molecular biology studies confirmed the change in GABAR α2 subunit expression. In addition, weakening of presynaptic inhibition of primary afferents can be due to the reduction in presynaptic GABAR expression. On the other hand, weakening of presynaptic inhibition of primary afferents can also be interpreted as presynaptic GABAR phosphorylation or dephosphorylation to reduce the activity of GABAR. In the progression of neuropathic pain, a large number of intracellular signaling pathways are involved in GABAR subunit phosphorylation, such as cAMP, PKA, cGMP/PKC, NO, CaMKII and tyrosine kinase. GABA\(\alpha\)R phosphorylation or dephosphorylation will be our future research direction, and we hope to shed further light on neuropathic pain development.

### MATERIALS AND METHODS

#### Design

A randomized, controlled, animal experiment.

#### Time and setting

Animal experiments were performed at the Department of Physiology, Shihezi University Medical College, China from August 2010 to November 2011.

#### Materials

Female, healthy, Sprague-Dawley rats, aged 8–10 weeks, weighing 250–280 g, were provided by the Experimental Animal Center of Xinjiang Medical University, China (license No. SCXK 2003-0001). Rats were housed in separate cages with a specific pathogen-free level barrier environment at 24 ± 3°C, relative humidity of 40–70%, 100–120 lx/12-hour light illumination, and allowed free access to food and water. The protocol was conducted in accordance with the Guidance Suggestions for the Care and Use of Laboratory Animals, formulated by the Ministry of Science and Technology of China.

#### Methods

**Establishment of the rat CCI model**

Surgical procedures were performed as previously described by Bennett et al. Rats were anaesthetized with an intraperitoneal injection of 1% (w/v) sodium pentobarbital, and the left sciatic nerve trunk was exposed under sterile conditions. Proximal to the sciatic trifurcation, approximately 7 mm of nerve was freed, and 4 tight ligatures of chrome catgut were placed around the sciatic nerve with about 1 mm spacing. The surgical procedure was followed until the sciatic nerve was exposed, but no ligatures were applied. All surgical procedures were performed by the same person under aseptic conditions. There were significant differences between the CCI contralateral side model and sham-surgery groups, and paw withdrawal latency values in the CCI ipsilateral side model group were declined by at least 30%, which indicated modeling success. Six rats were randomly selected from each group.

**Behavioral testing of thermal sensitivity of the rat CCI model**

Thermal hyperalgesia was measured using a 58°C hot plate, and expressed as paw withdrawal latency of the left hind paw. The constant temperature water bath apparatus was produced in Jintan, China (type SHZ-82). Three measurements of thermal nociceptive threshold were taken for each rat, at 4-minute intervals, and the mean of three measurements was regarded as the paw withdrawal latency. Maximum latency was defined as 30 seconds, after which time the animals were removed from the hot plate to prevent tissue damage. Behavioral testing at the following different time points was performed before operation on 1, 3, 5, 7, 12 and 15 days after operation. After behavioral testing at each time point, all six rats from each group were killed, and L4,5 dorsal root ganglia were dissected out for experimentation.

**Immunofluorescence staining for GABA\(\alpha\)R α2 expression in the dorsal root ganglion**

Rats were anaesthetized with 1% (w/v) sodium pentobarbital, and then perfused through the aorta with 0.9% (w/v) normal saline, followed by fresh 4% (w/v) paraformaldehyde in PBS for 10 minutes for tissue fixation. The lumbar dorsal root ganglion at the level of L4,5 to nerve injury was removed rapidly and placed in 4% (w/v)
paraformaldehyde in PBS for 24 hours. The L4/L5 dorsal root ganglia were cut into 5 μm slices with a freezing microtome. The sections were treated with 0.3% (v/v) H2O2 for 10 minutes and blocked with 3% (w/v) bovine serum albumin in 0.3% (v/v) H2O2. Immunofluorescence staining was performed with goat anti-GABA_A R_α2 polyclonal antibody (1:200; Santa Cruz Biotechnology, CA, USA), overnight at 4°C. The sections were incubated for 1 hour in solution containing FITC-labeled rabbit anti-goat secondary antibody (1:100; Zhongshan Golden Bridge, Beijing, China) at room temperature. Slides were then examined by confocal microscopy. Quantitative analysis of GABA_A R_α2 expression in the dorsal root ganglion was performed by measuring the mean absorbance following laser confocal microscopy (LSM510; Carl Zeiss, Jena, Germany) and using analysis software (hp9001; Carl Zeiss, Jena, Germany).

Electrophysiological recordings of dorsal root ganglion neurons
Whole-cell patch clamp recordings were carried out at room temperature (22–24°C) using a whole-cell patch clamp amplifier.[36] Currents were recorded from single dorsal root ganglion neurons in vitro using an Axon 700B amplifier (Axon, San Jose, USA) and the pCLAMP 10.2 hardware and software (Axon, Silicon Valley, USA). The micropipettes were filled with internal solution containing (mM): KCl 140, MgCl_2 2.5, HEPES 10, EGTA 11 and ATP 5. The pH was adjusted to 7.2 with KOH, and osmolarity was adjusted to 310 mOsm/L with glucose. Cells were bathed in an external solution containing (mM): NaCl 150, KCl 5, CaCl_2 2.5, MgCl_2 2, HEPES 10, D-glucose 10. Osmolarity was adjusted to 340 mOsm/L with glucose, and pH was adjusted to 7.4 with NaOH. The resistance of the recording pipette was in the range of 2–5 MΩ. A small patch of membrane underneath the tip of the pipette was aspirated to form a gigaseal, and then a negative pressure was applied to rupture it, thus establishing a whole-cell configuration. The adjustment of capacitance compensation and series resistance compensation was carried out before the membrane currents were recorded. The holding potential was set at −60 mV, except when indicated otherwise. Membrane currents were filtered at 10 kHz.

Statistical analysis
Data were analyzed with SPSS 13.0 software (SPSS, Chicago, IL, USA) and presented as mean ± SEM. A homogeneity test for variance was performed followed by one-way analysis of variance, and two-group comparison was conducted using the least significant difference t-test. A P < 0.05 was considered statistically significant.

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Conflicts of interest: None declared.

Ethical approval: The experiment is approved by the Policy for Rodent Survival Surgery provided by the Animal Care Committee of Shihezi University School of Medicine, China.

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