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Blockade of BK channels attenuates chronic visceral hypersensitivity in an IBS-like rat model

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Abstract

Background: Visceral hypersensitivity in irritable bowel syndrome (IBS) is still poorly understood, despite that chronic abdominal pain is the most common symptoms in IBS patients. To study effects of BK channels on visceral hypersensitivity in IBS rats and the underlying mechanisms, IBS rats were established by colorectal distention (CRD) in postnatal rats. The expression of large-conductance calcium and voltage-dependent potassium ion channels (BK channels) of the thoracolumbar spinal cord was examined in IBS and control rats. The effects of BK channel blockade on visceral hypersensitivity were evaluated. The interaction of BK channels and N-methyl-D-aspartate acid (NMDA) receptors was explored, and synaptic transmission at superficial dorsal horn (SDH) neurons of the thoracolumbar spinal cord was recorded by whole-cell patch clamp in IBS rats.

Results: The expression of the BK channels of the thoracolumbar spinal cord in IBS rats was significantly reduced. The blockade of BK channels could reduce the visceral hypersensitivity in IBS rats. There was an interaction between BK channels and NMDA receptors in the spinal cord. The frequency of spontaneous inhibitory postsynaptic currents (sIPSCs) in SDH neurons is significantly reduced in IBS rats. The blockade of BK channels depolarizes the inhibitory interneuron membrane and increases their excitability in IBS rats.

Conclusions: BK channels could interact with NMDA receptors in the thoracolumbar spinal cord of rats and regulate visceral hypersensitivity in IBS rats.

Keywords

IBS, BK channel, visceral hypersensitivity, NMDA, spinal cord

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Background

Irritable bowel syndrome (IBS) is a heterogeneous disorder associated with chronic abdominal pain, bloating and changes in bowel habits; IBS imposes a significant socioeconomic burden.[1–3] However, the underlying mechanisms of visceral hypersensitivity remain largely unknown and there is still no satisfactory treatment. At present, some studies have concluded that visceral hypersensitivity in IBS patients is related to the upregulation of Cav ion channels and may also be closely related to the dysfunction of Nav1.5 ion channels. Previous studies have also found that blocking the hyperpolarized activation of cyclic nucleotide-gated cation channels could alleviate visceral hypersensitivity in IBS rats,

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indicating that ion channels play an important role in the development of IBS visceral pain.[4–6] Studies have found that intrathecal injection of large-conductance calcium and voltage-dependent potassium ion channel (Big/Large-conductance Ca^{2+}-activated K^+ channels, BK channels) blockers can relieve neuropathic pain.[7] The BK channel regulates membrane excitability and intracellular calcium ion signaling through selective connection of large BK α subunits and accessory β or γ subunits.[8–10] Recently, the full-length structure of the BK channel in the open state was analyzed by cryo-electron microscopy, which provides a more detailed structural basis for the study of the BK channel.[11] BK channel blockers can alleviate morphine-induced hyperalgesia by blocking BK channels on microglia.[12] Earlier studies found that the activity of N-methyl-D-aspartate acid (NMDA) receptors was related to the visceral pain sensitization associated with IBS.[13,14] Protein profiling and coimmunoprecipitation confirmed that the BKα S0–S1 loop and NMDA receptor subunit GluN1 physiologically combine to form a BK-NMDA receptor complex, which is widely present in different brain regions of rats.[15] Early research found that calcium influx mediated by NMDA receptors in rat olfactory bulb granule cells can activate BK channels.[16] This shows that BK channels and NMDA receptors may work synergistically. However, whether spinal cord BK channels interact with NMDA receptors to jointly regulate visceral pain transmission and visceral hypersensitivity in IBS and their mechanism of action in the spinal cord need further experimental confirmation.

In the present study, an IBS rat model was established and assessed by colorectal distention (CRD). The expression of BK channels in the thoracolumbar spinal cord was measured by Western blotting. The effects of BK channel blockade on visceral hypersensitivity were evaluated. The interaction of BK channels and NMDA receptors was confirmed by coimmunoprecipitation and immunofluorescence staining. Inhibitory synaptic transmission at superficial dorsal horn (SDH) neurons in lamina II of the thoracolumbar spinal cord was recorded by whole-cell patch clamp.

Results

Evaluation of IBS

Rats that had undergone CRD during the neonatal period were evaluated at 7 weeks to determine whether the model was successful, and then corresponding follow-up experiments were performed (Figure 1(a)). Visceral hypersensitivity was assessed by measuring the Electromyogram (EMG) response to CRD pressure and indicated that neonatal CRD caused visceral hypersensitivity in adult rats (Figure 1(b) and (c)). The percentage

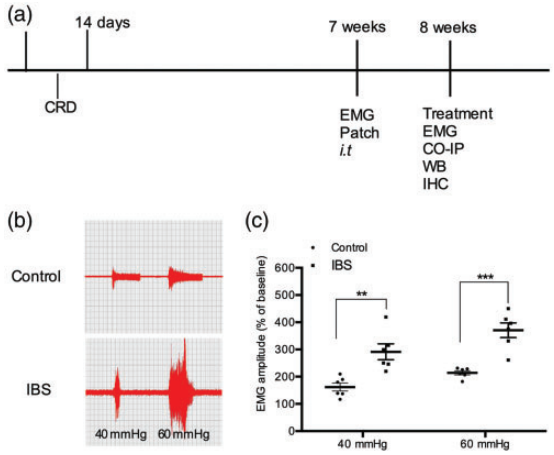

Figure 1. Timeline of the experiment and assessment of visceral hypersensitivity in rats. (a) Timeline of the experimental design. (b) Representative traces of EMG recordings from control and IBS rats to assess visceral hypersensitivity. (c) Bar graph of the EMG amplitude of control and IBS rats at 40 and 60 mmHg CRD. n = 6 per group; **: $P < 0.01$, ***: $P < 0.001$, *t*-test, compared to control rats.

increases in the average amplitude of EMG in control rats and IBS rats were $162 \pm 14.80\%$ and $291.50 \pm 29.32\%$, respectively under 40 mmHg pressure ($P = 0.0028$); the average amplitude increases in EMG in control rats and IBS rats under 60 mmHg pressure were $214.20 \pm 7.56\%$ and $370.80 \pm 27.12\%$, respectively ($P = 0.0002$). The EMG amplitude of IBS rats was increased by more than 20% compared with that of control rats, suggesting that the IBS model was successfully established.

The expression of the BK channels in the thoracolumbar spinal cord in IBS rats was significantly reduced

To determine whether the BK channel is involved in the development of visceral pain in IBS rats, Western blotting was used to detect the expression of the BKα protein in the spinal thoracolumbar segment of rats. Comparing the total protein of BKα with a corresponding internal reference, the obtained optical density ratios were: control group, 1.05 ± 0.23; and IBS group, 0.38 ± 0.08 ($P = 0.0314$, n = 6, Figure 2(a) and (c)). Comparing the BKα membrane protein with the corresponding internal reference, the obtained optical density ratios were: control group, 0.83 ± 0.12; and IBS group, 0.37 ± 0.08 ($P = 0.0141$, n = 6, Figure 2(b) and (d)). The results showed that the expression of total IBS rat BKα protein and membrane protein were significantly reduced and that BK channels participated in the development of visceral hypersensitivity in IBS rats.

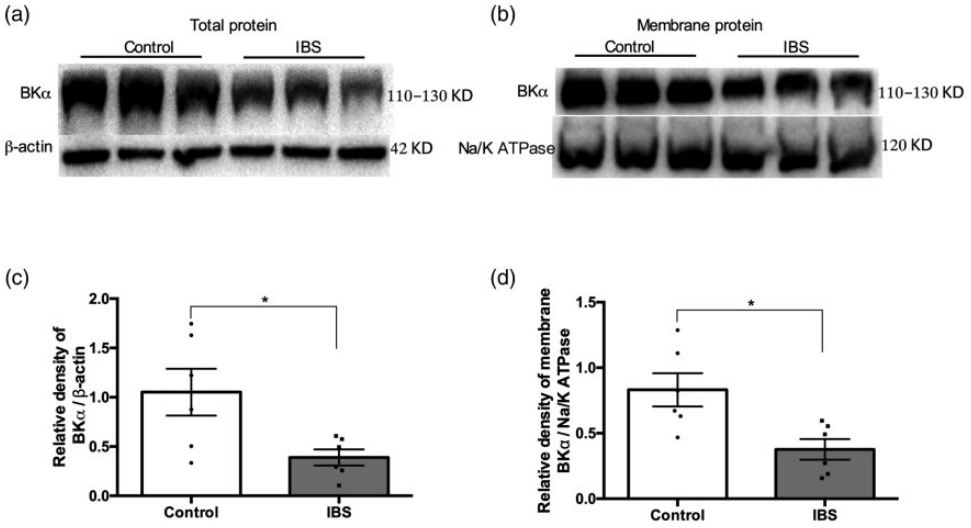

Figure 2. The protein expression of BKα in rats. (a, b) Western blotting for the total protein and membrane protein expression of BKα. (c, d) Bar graph of the protein expression of BKα normalized to that of β-actin or Na/K ATPase. n = 6 per group. *: $P < 0.05$, compared to control rats.

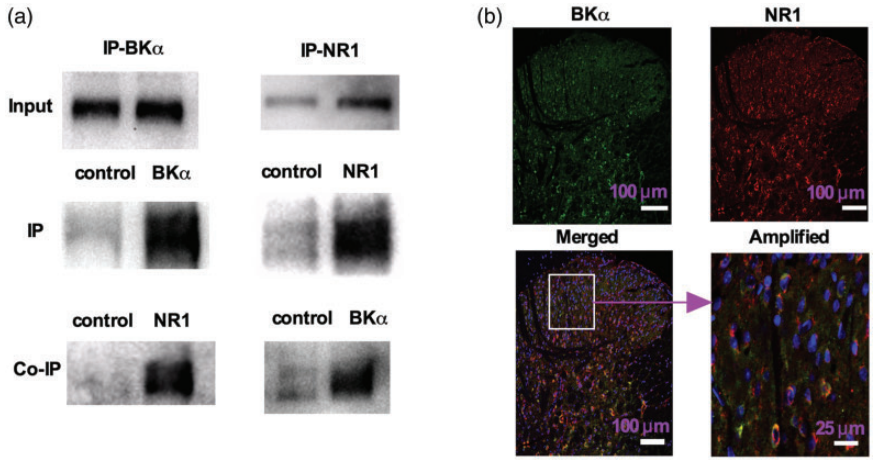

Figure 3. Co-IP of membrane proteins from IBS rats. (a) Western blotting for the protein expression of BKα and NR1 after IP. Control: mIgG or rIgG. (b) Neurons showing colocalization of antibodies against NR1 (red) and BKα (green) in IBS rats. n = 6 per group. *: $P < 0.05$, compared to control rats.

The spinal BK channels could interact with NMDA receptors

To determine whether the NMDA receptors and BK channels in the spinal cord can interact to regulate visceral pain and visceral hypersensitivity in IBS rats, the membrane proteins of the thoracolumbar segment of the spinal cord of IBS rats were extracted, and an anti-mouse BKα antibody and protein A agarose with a rabbit anti-NR1 antibody were incubated with the isolated membrane proteins to evaluate binding. The negative control (control) was coimmunoprecipitated (coimmunoprecipitation, Co-IP) with mouse IgG and rabbit IgG, and the immunoprecipitated complex was detected by Western blotting. Colocalization of BK channel and NMDAR in spinal cord SDH neurons was detected by immunofluorescence staining. These results showed that NMDARs and BK channels could interact with each other in the spinal cord of IBS rats (Figure 3).

Effect of the BK channel blocker paxilline on EMG discharge in rats

The Western blotting results showed that the expression of BK channels in the thoracolumbar segment of the spinal cord of IBS rats was reduced and BK channels were involved in the development of visceral

hypersensitivity in IBS rats. Rats were given 1% DMSO intrathecally and different concentrations of paxilline (0.02 μM, 0.2 μM, 2 μM, or 10 μM) to observe the EMG amplitude induced by colorectal dilation at different pressures (40 or 60 mmHg). For control rats, the results showed that the EMG amplitude measured under a pressure of 40 mmHg did not change significantly under the action of DMSO but exhibited a significant decrease under the effects of different concentrations of paxilline (Figure 4(a)). However, the EMG amplitude measured under 60 mmHg pressure did not change significantly under the action of DMSO and paxilline (0.02 μM) but a significant decrease was found under the effects of higher concentrations of paxilline (0.2 μM, 2 μM, and 10 μM) (Figure 4(b)). For IBS rats, the results showed that the EMG amplitude measured under 40 or 60 mmHg pressure did not change significantly under the action of DMSO and paxilline (2 μM) but showed a significant decrease under the action of high-dose paxilline (10 μM) (Figure 4(e) and (f)).

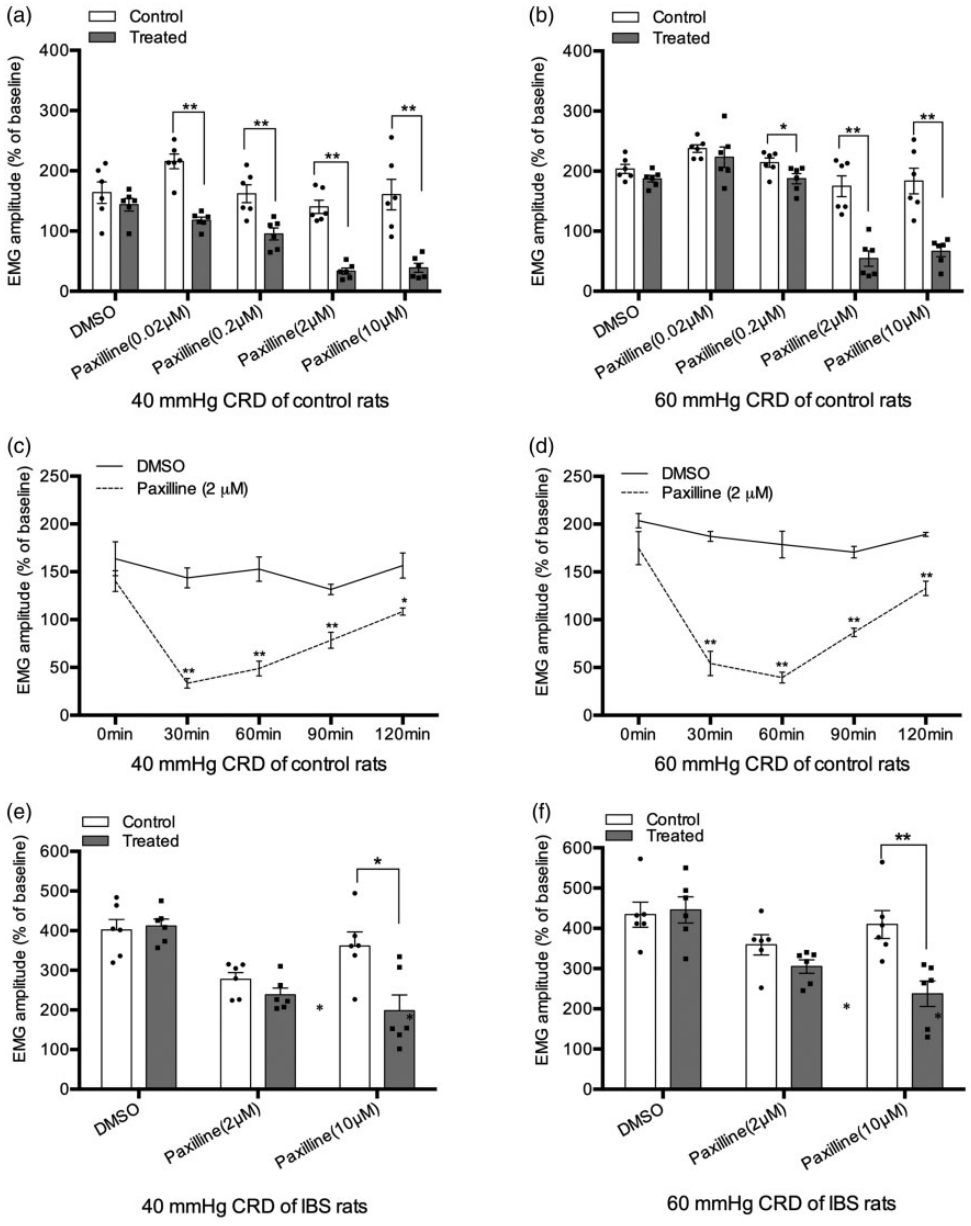

Figure 4. Effect of paxilline on rats. (a, b) Bar graphs of the EMG amplitude 30 min after spinal intrathecal injection of DMSO or paxilline (0.02, 0.2, 2, or 10 μM) with CRD at 40 mmHg or 60 mmHg in control rats, respectively; n = 6 per group. (c, d) Time curves of the effects of DMSO or paxilline (2 μM) at 40 and 60 mmHg CRD in control rats, respectively; n = 6 per group; *: $P < 0.05$, **: $P < 0.01$, compared to DMSO. (e, f) Bar graphs of the EMG amplitude 30 min after spinal intrathecal injection of DMSO or paxilline (2 or 10 μM) with CRD of 40 mmHg and 60 mmHg in IBS rats; n = 6 per group; *: $P < 0.05$, **: $P < 0.01$, compared to DMSO.

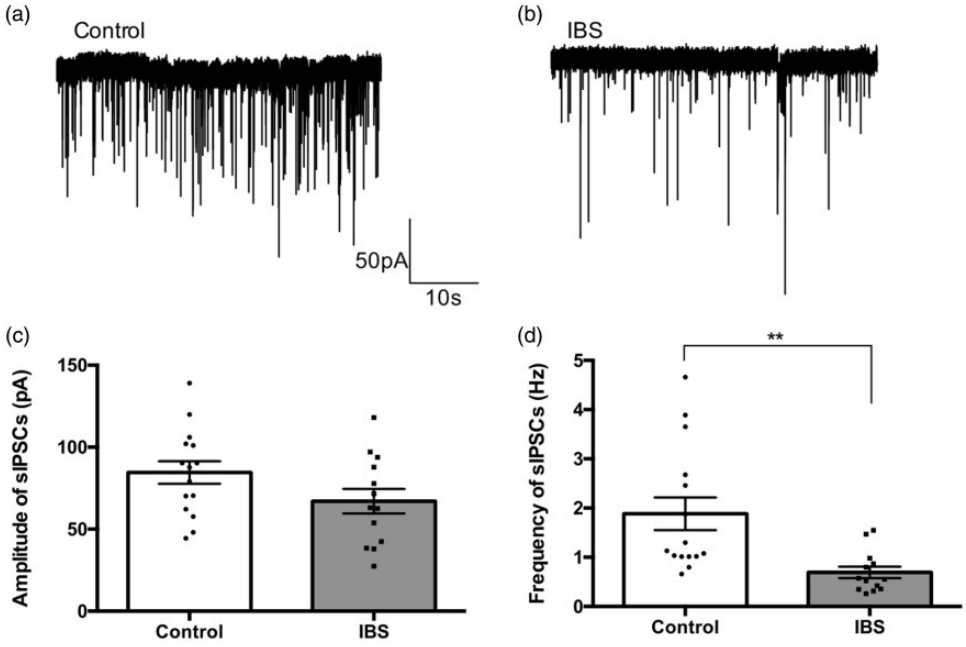

Figure 5. sIPSCs of spinal cord lamina II interneurons in control and IBS rats. (a, b) Representative traces of sIPSC recordings from lamina II neurons in thoracolumbar spinal cord slices taken from control and IBS rats. (c, d) Bar graphs of the amplitude and frequency of sIPSCs in control and IBS rats. Neurons: n = 15 or 13 per group, animals: n = 5 or 6 per group; **: $P < 0.01$, *t*-test, compared to control rats.

To further assess the time course of the effect of paxilline, the EMG amplitude was measured at different CRD pressures (40 and 60 mmHg) every 30 min after intrathecal injection of paxilline (2 μM). The apparent amplitude decreased and gradually returned to normal over 120 min (Figure 4(c) and (d)).

The frequency of sIPSCs in lamina II interneurons of the spinal dorsal horn is significantly reduced in IBS rats

To clarify the changes in the inhibitory synaptic transmission of neurons in the dorsal horn of IBS rats, intermediate neuron sIPSCs were recorded. Compared with that of control rats, the frequency of sIPSCs recorded in the intermediate neurons of IBS rats was significantly reduced but without significant changes in the amplitude of sIPSCs. Figure 5(a) and (b) shows a representative current diagram of recorded neurons; the average amplitude of normal rats was 84.55 ± 6.87 pA, and the average amplitude of IBS rats was 67.05 ± 7.53 pA ($P = 0.0985$). The average frequency of sIPSCs in normal rats was 1.89 ± 0.33 Hz, and the average frequency of sIPSCs in IBS rats was 0.69 ± 0.12 Hz ($P = 0.0034$, Figure 2(c) and (d)).

The BK channel blocker paxilline depolarizes the inhibitory interneuron membrane of spinal lamina II neurons and increases their excitability in IBS rats

In the voltage clamp mode, the voltage was clamped at −70 mV and then switched to the current clamp mode, giving a current of 0 to 300 pA, and the current was increased at 50 pA intervals to evaluate inhibitory interneurons (Figure 6(a)). The changes in spontaneous excitatory postsynaptic potential (sEPSP) and resting membrane potential (RMP) before and after paxilline were recorded under the current clamp model. The inhibitory interneurons showed obvious spontaneous discharge approximately 10 min after paxilline administration. After elution, the drug effect could last for at least 20 min. A total of 11 inhibitory interneurons were recorded, all of which showed spontaneous discharge after administration. The average membrane potential increased from -63.84 ± 2.92 mV to -53.14 ± 2.39 mV ($P < 0.0001$, Figure 6(b)). The cells showed a depolarized state and were excited to exhibit AP firing (Figure 6(c)). This indicates that blocking the BK channels can cause depolarization of the inhibitory interneuron cell membrane and that the increase in excitability leads to the enhancement of spontaneous discharge.

To further clarify whether paxilline can also cause stimulation-induced changes in the evoked excitatory postsynaptic potential (eEPSP), the stimulation electrode was placed in the dorsal root entry area of the spinal cord, and the eEPSP was recorded in lamina II of the spinal cord. Figure 6(d) shows the eEPSP of representative neurons before and after administration of paxilline. The eEPSP of inhibitory interneurons increased significantly after paxilline administration. The amplitudes before and after administration were 26.17 ± 5.24 mV and 37.82 ± 4.98 mV respectively ($P = 0.0050$,

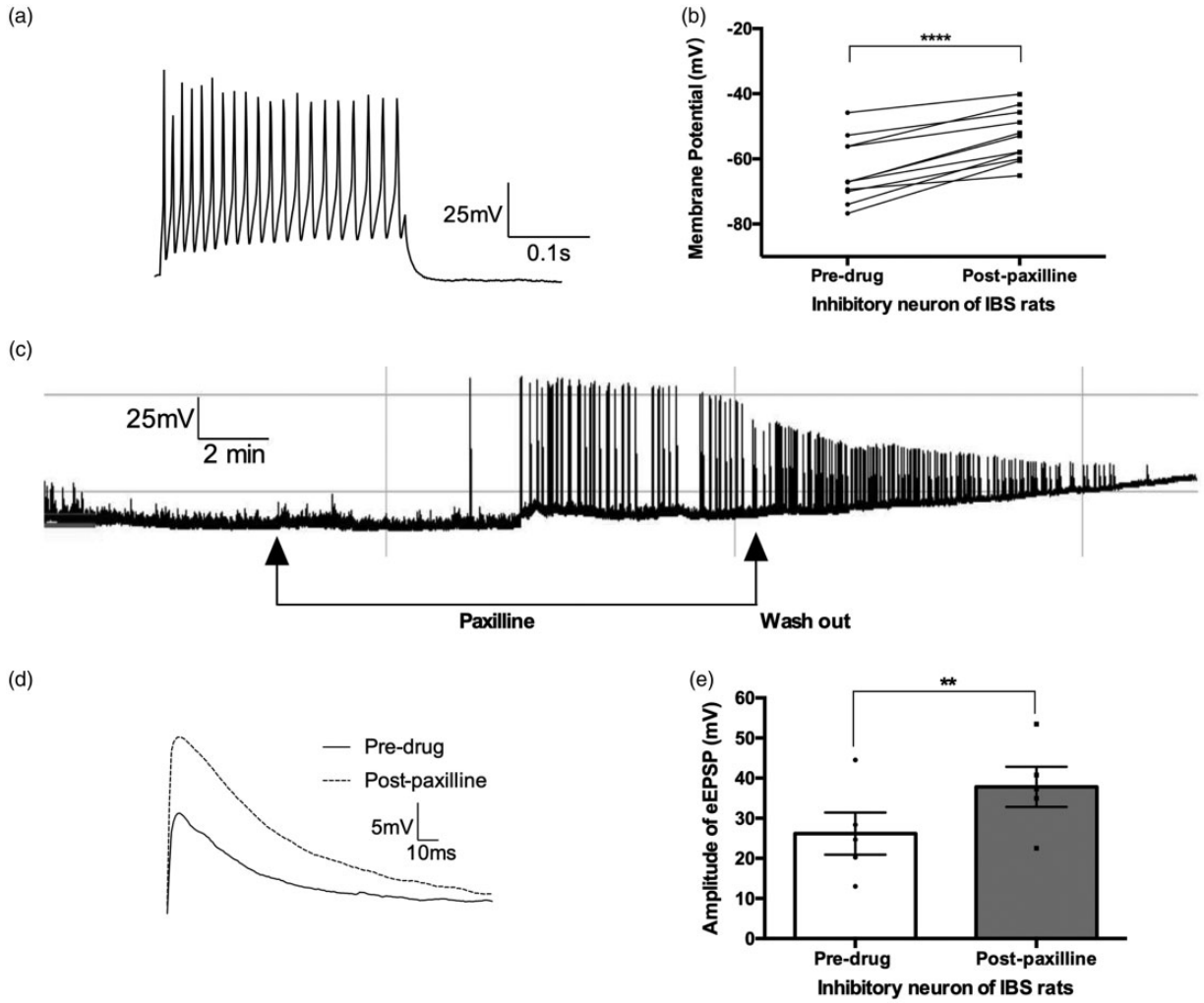

Figure 6. The RMP and EPSP of spinal cord lamina II neurons in IBS rats before and after the application of paxilline. (a) Representative action potential trace of an inhibitory interneuron. (b) Bar graph of the membrane potential of inhibitory neurons. Neurons, n = 11; animals, n = 9; ****: $P < 0.0001$, paired *t*-test, compared to predrug. (c) Representative traces of the predrug and post-paxilline treatment sEPSPs of inhibitory interneurons; neurons = 11, animals = 9. (d) Representative traces of the eEPSP of inhibitory interneurons before and after paxilline applications. (e) Bar graph of the amplitude of the eEPSP of inhibitory interneurons before and after paxilline applications. Neurons, n = 5; animals, n = 5; **: $P < 0.01$, paired *t*-test, compared to predrug.

Figure 6(e)). This shows that after stimulation of afferent nerves, blocking the BK channels causes increased excitability of inhibitory interneurons in lamina II of the dorsal horn of the spinal cord in IBS rats.

The amplitude and frequency of sIPSCs recorded from interneurons of the spinal lamina II increased after paxilline treatment in IBS rats

To clarify the effect of paxilline on the inhibitory synaptic transmission of lamina II neurons in the dorsal horn of the spinal cord in IBS rats, the spontaneous inhibitory postsynaptic currents (sIPSCs) of interneurons before and after administration were recorded. When recording, QX-314 was added to the electrode fluid to block the corresponding sodium channel. Figure 7(a) and (b) show the representative sIPSC current map of the recorded neurons. The average amplitudes before and after administration were 62.44 ± 8.14 pA and 96.37 ± 14.87 pA, respectively ($P = 0.0028$, Figure 7(c)). The mean frequencies before and after administration were 0.64 ± 0.12 Hz and 0.93 ± 0.15 Hz, respectively ($P = 0.0424$, Figure 7(d)). The results showed that after paxilline administration, the amplitude and frequency of sIPSCs increased significantly, indicating that blockade of BK channels could cause the release of inhibitory synaptic transmitters from the interneurons of the spinal cord.

To further confirm that paxilline mainly caused the release of inhibitory transmitters, the evoked excitatory

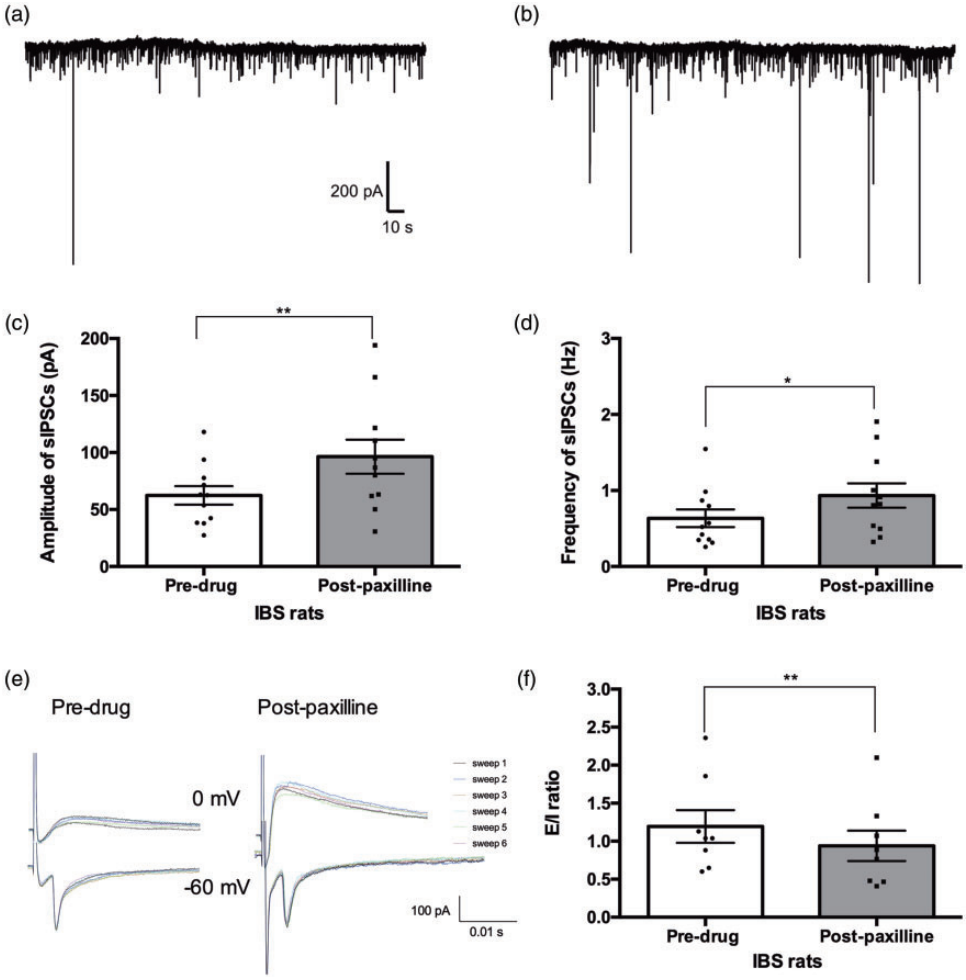

Figure 7. The sIPSCs and E/I of spinal cord lamina II neurons in IBS rats before and after application of paxilline. (a, b) Representative traces of sIPSC recorded from lamina II neurons in thoracolumbar spinal cord slices taken from IBS rats. (c, d) Bar graphs of the amplitude and frequency of sIPSCs in IBS rats. Neurons, n = 11; animals, n = 5 per group. (e) Representative traces of eEPSC (voltage clamped at −60 mv) and eIPSC (voltage clamped at 0 mv) recorded in the neuron of thoracolumbar spinal cord slice from IBS rats. The amplitude of eIPSCs increased after application of paxilline compared to the baseline. (f) Bar graph of the E/I ratio. Neurons, n = 8; animals, n = 5 per group; $P = 0.0044$. *: $P < 0.05$, **: $P < 0.01$, paired *t*-test, compared to predrug.

postsynaptic currents (eEPSCs) and evoked inhibitory postsynaptic currents (eIPSCs) of the same neuron were recorded, and the ratio of the eEPSCs/eIPSCs after drug administration was observed. A single neuron was voltage-clamped at −60 mV to record the eEPSCs caused by stimulation before administration, and then the clamping voltage was changed to 0 mV to record the eIPSCs caused by stimulation. After 10 min of administration of paxilline, the voltage was clamped at −60 mV and 0 mV again, the stimulation intensity and frequency remained unchanged, and the eEPSCs and eIPSCs were recorded. Figure 7(e) shows the representative current graphs recorded. The ratio of eEPSCs/eIPSCs (E/I ratio) of the single neurons before and after administration were 1.19 ± 0.21 and 0.93 ± 0.20, respectively ($P = 0.0044$, Figure 7(f)). The results showed that the E/I ratio of individual neurons was significantly reduced after administration, indicating that after BK channel inhibition, the release of inhibitory transmitters increased.

Discussion

In this study, we hypothesized that inhibition of the BK channels could relieve the visceral hypersensitivity of IBS rats, probably due to the synaptic activity of interneurons in the lamina II of the spinal dorsal horn. First, the expression level of the BK channels in thoracolumbar spinal segments was decreased in IBS rats compared to control rats. Additionally, blockade of BK channels significantly suppressed visceral hypersensitivity, and BK channels could interact with NMDA receptors in the spinal cord. Finally, blockade of BK channels

could enhance inhibitory synaptic transmission in the lamina II of the spinal dorsal horn in IBS rats.

Inhibition of BK channels could reduce the rat response of external oblique muscle to CRD

The role of BK channels in pain is still controversial. Activation of BK channels can reduce neuropathic pain and inflammatory pain[17,18]; there are also studies showing that inhibition of BK channels can reduce neuropathic pain and morphine-induced hyperalgesia.[7,12] In this study, we found that BK channels contributed to the formation of IBS (Figure 2) and that blockade of the BK channels could inhibit visceral hyperalgesia induced by postnatal CRD (Figure 4).

Paxilline is an indole diterpenoid compound produced by Penicillium mycoides that can specifically block the opening of BK channels.[19–21] In this study, after intrathecal injection of different concentrations of paxilline, it was found that EMG responses was inhibited in a dose-dependent manner in control rats. According to drug time curve analysis, the onset time of the drug was 30 min at 2 μM, and the obvious suppression lasted for at least 120 minutes. However, the discharge response could only be significantly inhibited in IBS rats by increasing the dose of paxilline to 10 μM. The underlying mechanism is that IBS rats are characteristic of visceral hypersensitivity, therefore higher concentration of paxilline is needed to inhibit the pain response in IBS rats compared to the control rats. Furthermore, the BK channel of spinal cord in IBS rats is mostly open. However, paxilline is a type of channel blocker more sensitive to closed channel. There are two types of ion channel blockers: open channel blockers and closed channel blockers.[22] The binding capacity of paxilline for the closed BK channels is 500 times that for the open state and shows a linear increase at 2 μM.[23–25] Paxilline specifically binds to the S6-PH fissure structure of BKα in the closed state, preventing the opening of the channel, which indicates that paxilline is a closed channel blocker.[22,26] According to our study results, most of the BK channels in the spinal cord of IBS rats could be open. BK currents will be recorded in the future study.

BK channels could interact with NMDARs to affect visceral pain

In the previous study we found that NMDA receptors were excessively activated in the IBS rats.[14] In this study, the Co-IP and immunofluorescence staining results confirmed that there was an interaction between the spinal NMDA receptors and BK channels in IBS rats (Figure 3). It is very likely that direct interactions occur to form an additional negative regulatory system that regulates the excessive influx of calcium ions caused by excessive excitation of NMDA receptors, jointly regulating the excitability of spinal neurons and then affecting the development of visceral pain sensitization in IBS rats. The interaction of NMDARs and the BK channels further indicated that the distance between them could be very close, leading to the BK channel generally being open in IBS rats. In the record of rat olfactory bulb neurons, it was found that the NMDAR and BK channel were located at the beginning of the neuron axon, forming a physiological coupling to jointly regulate the excitability of the neuron.[16] BK channels were found that modulated excitatory synaptic transmission via functional coupling with NMDARs at postsynaptic sites of medial perforant path-dentate gyrus granule cell synapses.[15] According to these studies, BK channel and NMDAR could form a complex to regulate the excitability of neurons. To further clarify the location of the interaction between the two proteins in IBS rats, the protein profile and gene mutations could be further investigated in the future.

Blockade of the BK channels enhances inhibitory synaptic transmission

In a previous study, we found that the frequency and amplitude of sEPSCs increased significantly,[27] and here, we further confirmed that the frequency and amplitude of sIPSCs decreased significantly, which together indicated that the excitability of lamina II neurons in the dorsal horn of the spinal cord in IBS rats was significantly increased.

To investigate whether paxilline affects the synaptic transmission of lamina II in the dorsal horn of the spinal cord in IBS rats, we recorded the sEPSP, eEPSP, and E/I ratio of inhibitory interneurons. The results of this study showed that the sEPSPs and eEPSPs were recorded for isolated spinal cord slices from normal rats and IBS model rats after electrical stimulation. After paxilline was given to close the BK channels, the sEPSPs and eEPSPs of inhibitory interneurons were both significantly enhanced, and the membrane potential was decreased; these results indicated that paxilline enhanced the excitability of inhibitory interneurons, causing the depolarization of inhibitory interneuron membranes, generating action potentials and exciting neurons, which in turn caused the release of inhibitory neurotransmitters.

Conclusion

BK channels could interact with NMDA receptors in the thoracolumbar spinal cord of rats and regulate visceral pain in control rats and visceral hypersensitivity in IBS rats. Blockade of the BK channels could relieve visceral hypersensitivity in IBS rats. It could potentially provide

a new therapeutic method for treating visceral hypersensitivity in IBS patients.

Methods

Animals and assessment of visceral hypersensitivity

Department of the Experimental Animal Center of Fujian Medical University provided Male Sprague Dawley rats. IBS was established on the 8th to 14th day after birth. A colorectal dilation of 60 mmHg pressure was implemented with a 20 mm × 2.5 mm human vascular reconstruction balloon at the same time every day for 1 min.[28,29] The colorectal distention was applied once a day. Animals procedures were approved by the Committee for Care and Use of Laboratory Animals at Fujian Medical University. The study had a single-blind cross-sectional experimental design. EMG measurements were carried out as previously described to assess visceral hypersensitivity.[30]

Intrathecal catheter implantation and agent administration

The rats were anesthetized with barbanylum (8%, 0.1 mL/100 g). A sterile polyethylene catheter (BB31695-PE/1, Scientific Commodities Inc, USA) was inserted between the L6 and S1 vertebrae. After catheter implantation, rats showing neurological deficits were euthanized. The rats were intrathecal injected with lidocaine to confirm catheter in the lumbar enlargement.[31] Agents were administered 1 week after cannulation. Various dosages of paxilline (0.02 μM, 0.2 μM, 2 μM, 10 μM) (first dissolved in 100% DMSO to 1 mM, which was then diluted to final dosage with normal saline) were administered in a volume of 10 μL.

Immunofluorescence histochemical staining

Rats were intracardially perfused with ice-cold 4% paraformaldehyde (in 1× PBS) under deep anesthesia. Spinal cord were postfixed for 4 hours at 4 °C, then transfer to 0.1 M PB solution containing 30% (w/v) sucrose for dehydro before sectioning (20 μm; Leica CM1900). Spinal cord slices were permeabilized with 0.2% Triton X-100 in PBS at room temperature and then blocked in 3% goat serum and 5% bovine serum albumin (BSA) with 0.05% Triton X-100 in PBS for 1 hour. Then incubated in rabbit anti-BKα (AP107, Alomone lab, 1:500) and Guinea pig anti-NMDAR1 (GluN1) (1:500) in 5% BSA at 4 °C for 48 h. Slices were washed with PBS and then incubated in Alexa 488 labeled donkey anti-rabbit serum (1:1000) and Alexa 594 labeled goat anti-guinea pig (1:1000) in PBS for 2 hours and then washed again. All sections were stained with DAPI, mounted with antifade solution, and imaged with an Olympus SP8 confocal microscope.

Co-immunoprecipitation

Immunopurification of channel protein from rat spinal cord was done as described previously.[10,15] The cell membranes were prepared from freshly isolated adult thoracolumbar spinal cord via tissue homogenization and differential centrifugation at 2,000 × g for 10 min to collect supernatants and then at 100,000 × g for 30 min to collect cell membranes. Cell membranes were solubilized at 2% dodecyl maltoside(DDM) in a TBS buffer (150 mM NaCl, 20 mM Tris·HCl pH 8.0), then centrifugation at 16,605 × g for 15 min to collect supernatants and incubated with primary antibodies (1:1000): mouse anti-BKα monoclonal antibody (clone L6/60, Neuromab), rabbit anti-NMDAR1 (G8913, Sigma), mouse IgG, rabbit IgG. The mixer was slowly rotated and mixed for 3 hours in 4 °C, then add protein A-agarose beads to continue to rotate and mix for 1 hour. After repetitive washing with 2% DDM, the captured proteins were eluted by using 2× Laemmli SDS-PAGE loading buffer (4% SDS, 20% glycerol, 100 mM DTT, 0.001% bromophenol blue, and 125 mM Tris·HCl, pH 6.8). The eluted proteins were separated on an SDS/PAGE gel with a complete run for immunoblot and western blotting analysis.

Western blotting

The expression of BK channels in the thoracolumbar spinal cord in rats was measured by western blotting. A total of 30 μg of protein per sample was separated by electrophoresis and transferred to polyvinylidene fluoride (PVDF) membranes (Invitrogen, USA). The following antibodies were used: mouse anti-BKα monoclonal antibody (clone L6/60, Neuromab); mouse anti-β-Actin monoclonal antibody (Cat. No. EM31011-01, Beijing Emarbio Science & Technology Co., Ltd, China); mouse anti-Na/K ATPase antibody (Provided by Ye Lab). Then, the membranes were washed and probed with peroxidase-conjugated goat anti-mouse IgG (Cat. No. E030110-01. EarthOx Life Science). The bands were detected using an electrochemiluminescence system.

Electrophysiology

Acute thoracolumbar spinal cord slices (450 μm thickness) were prepared from 7-to 8-week-old rats using a vibratome (Leica VT1000S, Germany) as described previously[32,33] and kept at 31 °C for at least 30 min in artificial cerebral spinal fluid (ACSF) containing 95 mM NaCl, 1.8 mM KCl, 1.2 mM KH_2PO4, 7 mM $MgSO_4$, 0.5 mM $CaCl_2$, 26 mM $NaHCO_3$, and 15 mM D-glucose,

and 50 mM sucrose and aerated with 95% O_2 and 5% CO_2 [33]. For recording, each spinal cord slice was visualized under an infrared differential interference contrast optics microscope (Olympus). Whole-cell patch clamp recordings were made at room temperature from SDH neurons in lamina II of the spinal cord. The neurons that produced delayed firing of action potential under current-clamp conditions were selected for further experiments[34,35]; the data were collected with a MultiClamp 700B amplifier (Axon Instruments) and pCLAMP software (v.10.3, Axon Instruments) and digitized at 10 kHz (Digidata1322A, Axon Instruments). The spinal cord slice was continuously perfused with ACSF containing 127 mM NaCl, 1.8 mM KCl, 1.2 mM KH_2PO4, 1.3 mM $MgSO_4$, 2.4 mM $CaCl_2$, 26 mM $NaHCO_3$, and 15 mM D-glucose and aerated with 95% O_2 and 5% CO_2. Pipette electrodes (4–8 MΩ) were filled with different solution as following: sEPSP, eEPSP electrode solution: 133 mM K-Glucomate, 8 mM NaCl, 2 mM Mg·ATP, 0.3 mM Na·GTP, 0.6 mM EGTA, 10 mM Hepes, pH value adjusted to 7.2–7.3 with KOH. sIPSCs electrode solution: 135 mM CsCl, 1.2 mM MgCl, 2 mM Mg·ATP, 0.3 mM Na_2·GTP, 10 mM EGTA, 10 mM Hepes, 10 mM Na_2-phosphocreatine, 1 mM QX-314, pH is adjusted with CsOH To 7.2–7.3. E/I electrode solution: 130 mM Cs-methylsulphate, 10 mM CsCl, 10 mM HEPEs, 1.1 mM EGTA, 2 mM $MgCl_2$, 2.5 mM MgATP, 0.3 mM Na_2GTP, and the pH value was adjusted to 7.2–7.3 using CsOH.[36] Only cells with a resting membrane potential of at least −55 mV and a stable series resistance or capacitance were included in the analyses. Neurons were clamped at −70 mV, and spontaneous inhibitory postsynaptic currents (sIPSCs) or spontaneous excitatory postsynaptic potential (sEPSP) were recorded for at least 5 min to establish stable baseline values. The perfusion medium was changed to 10 μM paxilline for at least 10 min without altering the perfusion rate, then, sIPSCs or sEPSP were recorded for more than 10 min. When record the Evoked Excitatory Postsynaptic Potential (eEPSP) and E/I, the stimulation electrode is placed in the dorsal root entry area of the spinal cord. A single pulse is applied through the stimulation electrode in a constant current mode through the isolator, with a stimulation of 0.1 ms, 0.5 mA, and 0.5 Hz. If necessary, adjust the stimulation intensity to induce the subthreshold potential as much as possible, and record the eEPSP or E/I before and after administration. Paxilline was purchased from Alomone Lab (Israel), the other drugs were purchased from Sigma Aldrich (USA). Paxilline were dissolved in dimethyl sulfoxide to create stock solutions. Before each experiment, the stock solutions were diluted with ACSF to obtain the specific concentrations.

Statistical analysis

Data are presented as the mean ± SEM. The data for EMG were analyzed with one-way ANOVA and paired t-test. The data of western blotting were analyzed with two-tailed independent t-tests. Data for electrophysiology were analyzed with Clampfit 10.3 (Axon Instruments) and Mini-analysis 6.0 (Synaptosoft Inc). The cumulative fraction of the amplitudes and the inter-event intervals of the sIPSCs were compared using Kolmogorov-Smirnov tests; the amplitudes or frequencies of the 3 min period just before and after paxilline application (10 min) was compared. Group means were compared using a paired t-test. Statistical analysis was performed using GraphPad Prism 8.0. $p < 0.05$ was considered statistically significant.

Author Contributions

F.F., and C.L. designed research; F.F., Y.C., L.G., and P.S. performed research; F.F., Y.C., L.G., and P.S., contributed to acquisition of data; F.F., Y.C., and A.C. analyzed data; F.F., and C.L. wrote the paper.

Declaration of Conflicting Interests

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