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Original Article

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Functional up-regulation of Nav1.8 sodium channel on dorsal root ganglia neurons contributes to the induction of scorpion sting pain

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Abstract

BmK I, purified from the venom of scorpion *Buthus martensi* Karsch (BmK), is a receptor site-3-specific modulator of voltage-gated sodium channels (VGSCs) and can induce pain-related behaviors in rats. The tetrodotoxin-resistant (TTX-R) sodium channel Nav1.8 contributes to most of the sodium current underlying the action potential upstroke in dorsal root ganglia (DRG) neurons and may serve as a critical ion channel targeted by BmK I. Herein, using electrophysiological, molecular, and behavioral approaches, we investigated whether the aberrant expression of Nav1.8 in DRG contributes to generation of pain induced by BmK I. The expression of Nav1.8 was found to be significantly increased at both mRNA and protein levels following intraplantar injection of BmK I in rats. In addition, the current density of TTX-R Nav1.8 sodium channel is significantly increased and the gating kinetics of Nav1.8 is also altered in DRG neurons from BmK I-treated rats. Furthermore, spontaneous pain and mechanical allodynia, but not thermal hyperalgesia induced by BmK I, are significantly alleviated through either blockade of the Nav1.8 sodium channel by its selective blocker A-803467 or knockdown of the Nav1.8 expression in DRG by antisense oligodeoxynucleotide (AS-ODN) targeting Nav1.8 in rats. Finally, BmK I was shown to induce enhanced pain behaviors in complete freund's adjuvant (CFA)-inflamed rats, which was partly due to the over-expression of Nav1.8 in DRG. Our results suggest that functional up-regulation of Nav1.8 channel on DRG neurons contributes to the development of BmK I-induced pain in rats.

Key words: pain, voltage-gated sodium channel, Nav1.8, BmK I

Introduction

Pain sensation originates from the activation of primary nociceptive sensory neurons (nociceptors) in periphery and transmits pain signaling to the spinal cord dorsal horn. The processing of sensory information from peripheral nociceptors to the spinal cord dorsal horn may change significantly following tissue and nerve injury [1,2]. The cell bodies of nociceptors are located in dorsal root ganglia (DRG) and the trigeminal ganglia (TG) are responsible for production

of pain-related receptors, ion channels or signaling molecules. Voltage-gated sodium channels (VGSCs) have been thought to play a pivotal role in the initiation and propagation of action potentials (APs) whose dysfunction may result in severe neurological disorders, including epilepsy and chronic pain [3]. A number of distinct VGSCs, primarily Nav1.7, Nav1.8, and Nav1.9, play key roles in the generation of APs in nociceptors [4]. Due to their selective distributions in nociceptors, these subtypes of VGSCs are considered as ideal drug targets for peripheral analgesia [5,6]. However, the dynamic plasticity for the expression and function of Nav1.8 under chronic pain conditions, such as inflammatory pain and neuropathic pain, is still elusive [7–10].

Scorpion envenomation can cause intense pain, skin edema, and burning sensation at the site of the sting in human [11–14]. We previously reported that Asian scorpion *Buthus martensi* Karsch (BmK) venom induces rat pain-related responses and clarified several peripheral and spinal cord mechanisms, including mast cell degranulation and histamine release at injury site, dynamic release of amino acid transmitters, activation of extracellular signal-regulated kinase (ERK) signaling pathway and spinal glial activation, increase of c-Fos expression, and up-regulation of nNOS expression [15–19]. BmK I, a polypeptide consisting of 64 amino acid residues purified from the BmK venom, serves as a main contributor of scorpion sting-induced pain, as application of BmK I can mimic BmK venom-induced spontaneous nociceptive response [14,20] and c-Fos expression in rat spinal cord dorsal horn [21]. Our previous work showed that BmK I can be classified into the α-like subgroup in the family of scorpion neurotoxins and it putatively binds to and acts on receptor site-3 of VGSCs [22,23]. BmK I can increase peak tetrodotoxin-sensitive (TTX-S) and tetrodotoxin-resistant (TTX-R) sodium currents and inhibit the inactivation of both TTX-S and TTX-R channels on small DRG neurons in rats [24]. Recently, we demonstrated that BmK I preferably inhibits the inactivation of Nav1.8 expressed in small-sized DRG neurons and produces increased peak currents and altered gating kinetics, resulting in increased neuronal hypersensitivity in rats [25]. Thus, BmK I may directly activate Nav1.8 in peripheral nerve endings to induce pain, and thus BmK I model may be a valuable model to investigate the molecular mechanism, especially the role of VGSCs, in pathological pain.

In the present study, we report that peripheral administration of BmK I can induce up-regulation of Nav1.8 expression in DRG neurons at both mRNA and protein levels following intraplantar injection of BmK I in rats. Concurrently, the current density of TTX-R Nav1.8 sodium channel is also increased significantly and the gating kinetics of Nav1.8 is altered in small-sized DRG neurons from BmK I-treated rats. Behavioral testing showed that both selective Nav1.8 sodium channel blocker A-803467 and knockdown of the Nav1.8 expression by antisense oligodeoxynucleotide (AS-ODN) can significantly attenuate the spontaneous flinching behavior and mechanical allodynia, but not thermal hyperalgesia induced by BmK I in rats. These results strongly suggest that functional up-regulation of Nav1.8 channel on DRG neurons contributes to the induction of BmK I-induced pain in rats.

Materials and Methods

Experimental animals

Adult male Sprague–Dawley rats weighing 220–250 g were used in all animal experiments. The rats were provided by Shanghai Experimental Animal Center, Chinese Academy of Sciences (Shanghai, China). The experiment protocols were reviewed and approved by the Shanghai University Animal Care and Use Committee. The guidelines of International Association for the Study of Pain (IASP) for experimental pain research in conscious animals were also followed [26]. The rats were housed in plastic boxes in groups of five at 22–26°C with food and water available *ad libitum* in the colony room. A 12 : 12 h light–dark cycle was maintained and behavioral testing was done between 9:00 and 18:30.

Drug preparation and administration

The crude BmK venom was purchased from a private scorpion culture farm in Zhengzhou, Henan province of China. BmK I was purified according to the previously described procedures [23]. The working solution was freshly prepared by dissolving 20 μg BmK I in 50 μl saline, which was then injected into the left hind paw of rat. Complete freund's adjuvant (CFA) (Sigma, St Louis, USA) was injected into the left hind paw at 100 μl per rat. A-803467 (Sigma), a selective Nav1.8 blocker, was dissolved in polyethylene glycol (PEG400) at 4 μg/μl as the stock solution [27,28]. The stock solution of A-803467 and vehicle were diluted in saline to the appropriate final concentrations and then injected intrathecally (1 ng and 10 ng in 10 μl) by direct lumbar puncture between the L5 and L6 in rats, or injected into the left hind paw (1 μg and 10 μg in 10 μl) as previously described [29].

RNA isolation and quantitative real-time RT-PCR

Total RNA was isolated from ipsilateral L4-L5 DRG of adult male rats ($n = 3$ for each time course) by using Trizol Total RNA Extractor (Sangon Biotech, Shanghai, China), and then reverse transcribed with PrimeScript®RT Master Mix (TaKaRa, Dalian, China), according to the manufacturer's protocol. Primer sequences targeting Nav1.8, Nav1.9, and β-actin were designed by Primer Premier 6.0 software with the following respective sequences: Nav1.8-S: 5′-TCCTCTC ACTGTTCCGCCTCAT-3′; Nav1.8-A: 5′-TTGCCTGGCTCTGCTC TTCATAC-3′; Nav1.9-S: 5′-TTCCTGGTGGTGTTCCGCATCC-3′; Nav1.9-A: 5′-TGAGCAGCAAGGCAATGAAGAGG-3′; β-actin-S: 5′-ACTATCGGCAATGAGCGGTTCC-3′; β-actin-A: 5′-AGCACTG TGTTGGCATAGAGGTC-3′. All primers were synthesized by Invitrogen (Shanghai, China). Quantitative PCR was performed in SYBR® Premix Ex TaqTM (TaKaRa), using the CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, Hercules, USA). The Nav1.8 mRNA was normalized to the β-actin mRNA level. Data were analyzed using the ΔΔCt method.

Western blot analysis

Western blot analysis was performed according to the previous reports [30,31]. Briefly, after the BmK I injection at different time points (2 h, 8 h, and 1 day), the rats were anesthetized with intraperitoneal (i.p.) injection of sodium pentobarbital (60 mg/kg), while naïve rats were taken as the control group. L4-L5 DRGs of all rats were labeled and snap frozen in liquid nitrogen. The DRG samples were homogenized in ice-cold RIPA Lysis Buffer (Beyotime, Shanghai, China) and centrifuged at 18,514 *g* for 20 min. Protein concentrations were measured by using a Bradford Protein Assay Kit (Beyotime). Protein samples (20 μg protein) were resolved by SDS–PAGE (8%) and transferred onto PVDF membrane. The membranes were blocked with 5% non fat milk at 4°C for whole night and then incubated with respective antibodies for 4 h at room temperature. The antibodies used were as follows: rabbit polyclonal to Nav1.8 (1:2000; Abcam, Cambridge, UK), rabbit polyclonal to β-actin (1:1000; Santa Cruz Biotech, Santa Cruz, USA), peroxidase-conjugated goat anti-rabbit IgG(H + L) (1:10,000; Kangchen, Shanghai, China). The blots were developed in ECL reagent (PerkinElmer Life Sciences, Waltham, USA), and

GelDoc-2000 Image System (Bio-Rad) was used for the densitometric quantification. The standardization ratio of Nav1.8 to β-actin band densitometric data was used to calculate the change in Nav1.8 expression.

Whole-cell patch clamp recording in isolated DRG neurons of rats

Neurons were isolated from the DRG of adult rats at 2 h after BmK I injection as described previously [24]. Whole-cell voltage-clamp recordings were performed on rat small diameter DRG neurons (<25 μm) according to the previous report [25]. The pipette solution contained 140 mM CsCl, 1 mM $MgCl_2$, 10 mM EGTA, 5 mM Na_2ATP, 0.4 mM Na_2GTP, and 10 mM HEPES (pH 7.2) (osmolarity, danwei, 280). The external solution contained 0.0005 mM TTX, 140 mM NaCl, 3 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 0.1 mM $CdCl_2$, 10 mM D-glucose, and 10 mM HEPES (pH 7.3) (osmolarity, danwei, 320). The external solution was saturated with O_2 before use.

Whole-cell voltage-clamp experiments were performed by using an EPC-10 amplifier (HEKA Elektronik, Lambrecht, Germany) at room temperature. Patch pipettes were fabricated from glass capillary tubes by a PP-830 Puller (Narishige, Tokyo, Japan) with the resistance of 3–5 MΩ. Data acquisition and stimulation protocols were controlled by Pulse/PusleFit 10.0 software (HEKA Elektronik). Capacitance transients and series resistance errors were compensated by 80%. Cells were discarded when the series resistance values were over 20 MΩ. Linear leakage was subtracted using P/4 protocol. Data were sampled at 50 kHz and low-pass filtered at 10 kHz.

Electrophysiological protocols and data analysis

Mean conductance (G) was calculated from peak current–voltage relationship using the equation $G = I/(V - V_r)$, where I is the peak current elicited upon depolarization, V is the membrane potential, and V_r is the reversal potential.

The voltage dependence for the activation was fitted with the Boltzmann relationship, $G/G_{max} = 1/[1 + \exp(V - V_m)/k_m]$, where V is the membrane potential, V_m is the voltage for half-maximum activation, and k_m is the slope factor.

The voltage-dependence inactivation data were described with the two-state Boltzmann equation, $I/I_{max} = 1/[1 + \exp(V - V_{1/2})/k]$, where V is the membrane potential of the conditioning step, $V_{1/2}$ is the membrane potential at which half-maximal inactivation is achieved, and k is the slope factor.

Data were analyzed using Origin 8.5 (OriginLab, Northampton, USA) and were presented as the mean ± SEM. The number of cells examined was presented by n value.

Measurement of spontaneous pain responses in rats

According to our previous reports [12,32], the rat was placed in a transparent plexiglas test box (20 × 20 × 30 cm) with a transparent glass floor placing on a supporting frame of 75 cm high above the experimental table. Rats were habituated for at least 1 h before experiments. After the intraplantar injection of BmK I, the rat was put back to the test box, and observation of pain behaviors was recorded for 2 h. The number of spontaneous flinching behavior was counted during 5-min intervals by an experimenter in a blinded manner.

Measurement of paw withdrawal mechanical threshold of rats

According to our previous reports [12,32], rats were placed in plexiglas test box (20 × 20 × 30 cm) on a mesh floor with 1 cm^2 openings for 30 min habituation before examination. Mechanical allodynia was determined by using a series of 10 calibrated von Frey filaments with forces from 0.6 to 26 g (#58011; Stoelting Co., Wood Dale, USA). Filaments were applied from underneath the metal mesh floor to the bilateral hindpaws. Each filament was probed for the same duration of 2–3 s with an interval of 10 s. The positive response was indicated by brisk withdrawal or flinching of the tested paw. The rat paw withdrawal mechanical threshold was defined as the lowest force that caused at least 5 withdrawals out of the 10 consecutive applications

Measurement of paw withdrawal thermal latency of rats

The previous reported procedure for radiant heat stimuli was used [12,32]. Briefly, rats were placed on the surface of a 2-mm-thick glass plate covered with the transparent plexiglas test box (20 × 20 × 30 cm) to measure the sensitivity to heat stimuli with radiant heat stimulator (RTY-3; Xi'an Fenglan Instrumental Factory, Xi'an, China). The radiant heat source was a high intensity projector halogen lamp bulb (150 W, 24 V) positioned under the glass door directly beneath the targeting area on the hind paw. The heat stimuli were applied to the bilateral hind paws. The cutoff time was set at 25 s to avoid potential tissue injury. Three stimuli were repeated with interval of 10 min and the paw withdrawal thermal latency was averaged.

Antisense oligodeoxynucleotide delivery

The specific antisense AS-ODN sequence targeting Nav1.8 was 5′-TCCTCTGTGCTTGGTTCTGGCCT-3′ and the mis-match oligodeoxynucleotide (MM-ODN) sequence was 5′-TCCTTCGTGCTGT GTTCGTGCCT-3′ according to the previous report [32]. The ODNs were synthesized by Genscript (Nanjing, China). Intrathecal delivery of ODNs (45 μg/5 μl, dissolved in nuclease-free ultrapure water) was made twice daily for three consecutive days. After treatment with A-803467 for 30 min, BmK I was subcutaneously injected into the rat hind paw. Behavioral testing was performed according to methods described previously [33].

Statistical analysis

All values were presented as the mean ± SEM. Student's t-test was used for two-group comparison. One-way ANOVA followed by post hoc Bonferroni test was used for multiple comparisons. Two-way repeated-measured ANOVA was also used to analyze the data with multiple time points. Differences with $P < 0.05$ were considered as statistically significant.

Results

BmK I increases the expression of Nav1.8 channel in primary sensory neurons in ipsilateral DRG

Previous studies suggested that increased expression of VGSCs contributes to the generation of peripheral sensitization of nociceptors [3,34]. Thus, we used quantitative real-time RT-PCR and western blotting to investigate the expression changes of Nav1.8 in DRG induced by intraplantar injection of BmK I in rats. The results showed that the mRNA expression of *Nav 1.8* was increased in ipsilateral L4-L5 DRG neurons following BmK I administration at 2 h and 8 h compared with the control group and the expression of Nav1.8 returned to basal level after 1 day (**Fig. 1A**). In contrast, the mRNA expression of *Nav 1.9* in ipsilateral L4-L5 DRG neurons was not affected by BmK I treatment (**Fig. 1A**). Consistently, the protein expression level of Nav1.8 was also significantly increased at 2 h (from 100.00% ± 13.23% to 236.42% ± 20.30%), and at 8 h (from 100.00% ± 13.23% to 186.87% ± 2.00%) following BmK I

treatment (**Fig. 1B,C**). Thus, both mRNA and protein levels of Nav1.8 expression are increased in ipsilateral DRG following intraplantar injection of BmK I.

By using immunohistochemistry, we further evaluated whether the cellular distribution of Nav1.8 in DRG neurons was dynamically altered by intraplantar injection of BmK I. It was found that the percentage of co-localization of Nav1.8 and calcitonin gene-related peptide (CGRP), a molecular maker of peptidergic DRG neurons, was increased from 33.30% ± 0.78% to 41.90% ± 0.92% at 2 h after BmK I injection, while the percentage change in control group was slight (from 33.30% ± 0.86% to 34.30% ± 2.20%) (**Fig. 2**), suggesting that the up-regulation of expression of Nav1.8 mainly occurs in small-sized CGRP-positive DRG neurons.

Current density and gating kinetics of Nav1.8 are significantly altered by BmK I treatment

Based on the methods described previously [33], we isolated the small diameter neurons from ipsilateral DRG on rats 2 h after BmK I injection. In order to isolate the contribution of the TTX-R Nav1.8 current from TTX-sodium channel, we used 500 nM TTX in the extracellular solution [24]. Furthermore, in this set of experiments, the cell membrane potential was held at −65 mV to inhibit the potential contribution of Nav1.9 channel, thus leaving only the Nav1.8 current to be measured [32,35]. The consequent Nav1.8 activation currents were elicited by applying a series of 150 ms test pulses between −55 mV and +40 mV in 5 mV increments, and the inactivation currents were elicited by the test pulse of 0 mV with a range of 100 ms pre-pulse from −60 mV to 0 mV in 5 mV increments. Representative recordings of Nav1.8 currents on DRG neurons from both control and BmK I-treated groups were shown in **Fig. 3A**. It was found that after treatment with BmK I, Nav1.8 current density was significantly increased compared with the control in small DRG neurons. The current density under a range of voltages revealed that the BmK I injection induced a significant increase of the maximum current density after 2 h (−314.87 ± 34.21 pA/pF, $n = 13$) compared with the control DRG neurons (−189.24 ± 24.75 pA/pF, $n = 10$) (**Fig. 3B**).

Meanwhile, I-V curve implies that a slight shift exists in the activation of Nav1.8 current from the neurons of BmK I-treated group compared with that of the control, indicating that the modulation of Nav1.8 kinetics may occur in small DRG neurons after BmK I injection. Therefore, we tested whether the voltage-dependence activation and steady-state inactivation of Nav1.8 currents are altered. Our results showed that the midpoint of activation ($V_{1/2}$) left was shifted by −8.61 mV, and the activation of Nav1.8 current was remarkably hyperpolarized compared with the control (**Fig. 3C** and **Table 1**). The inactivation of Nav1.8 was also considerably shifted in the hyperpolarizing direction by −6.4 mV compared with the control (**Fig. 3D** and **Table 1**). However, the slope factors of activation and inactivation only changed slightly, with no significant statistical difference between the control group and the BmK I-treated group (**Table 1**). Despite the shift in steady-state inactivation induced by BmK I, the curve of inactivation (**Fig. 3D**) implies that the channel will be partially inactive under a holding membrane potential around −65 mV. However, the drastic augmentation of more than 60% in Nav1.8 current density (**Fig. 3B**) indicates that, more likely, the inactivation of channels was compensated largely by the enhanced expression of Nav1.8 channel.

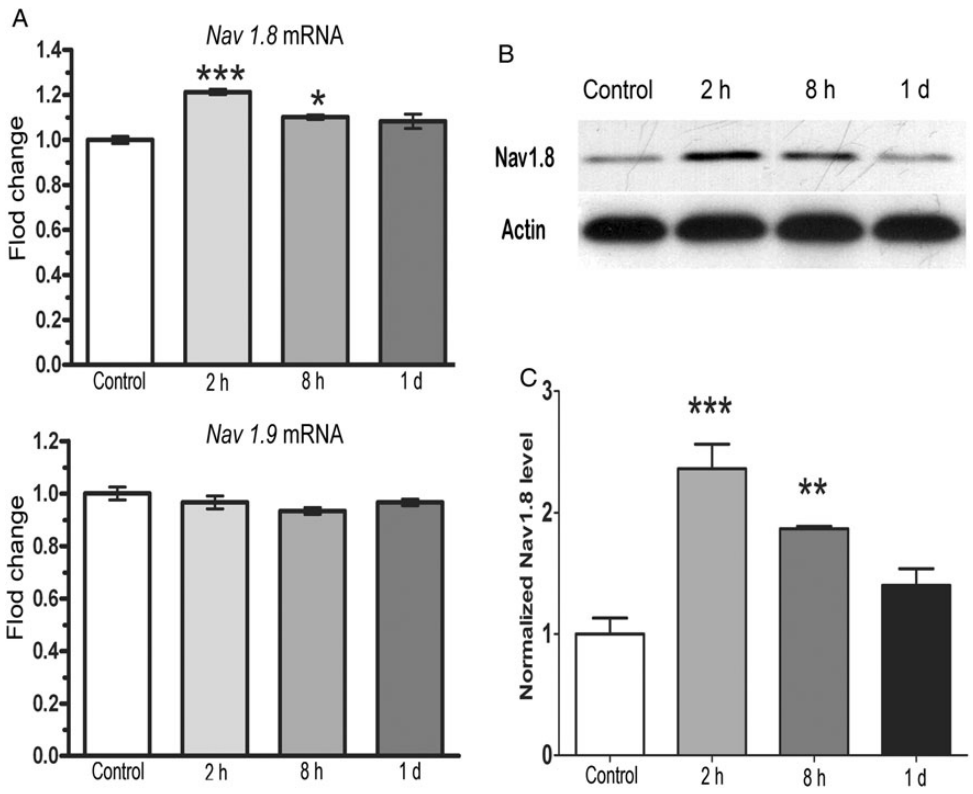

Figure 1. Expression of Nav1.8 channel is up-regulated by administration of BmK I in rats (A) Expressions of *Nav 1.8* and *Nav 1.9* channels at mRNA level in L4-L5 DRG. Change of mRNA expression was normalized to that of *β-actin*. (B) Western blot analysis of Nav1.8 protein in DRG. (C) Densitometry analysis revealed that Nav1.8 protein level was increased significantly after BmK I injection. ***$P < 0.001$ and **$P < 0.01$ are compared with the control group ($n = 3$). Data were presented as the mean ± SEM.

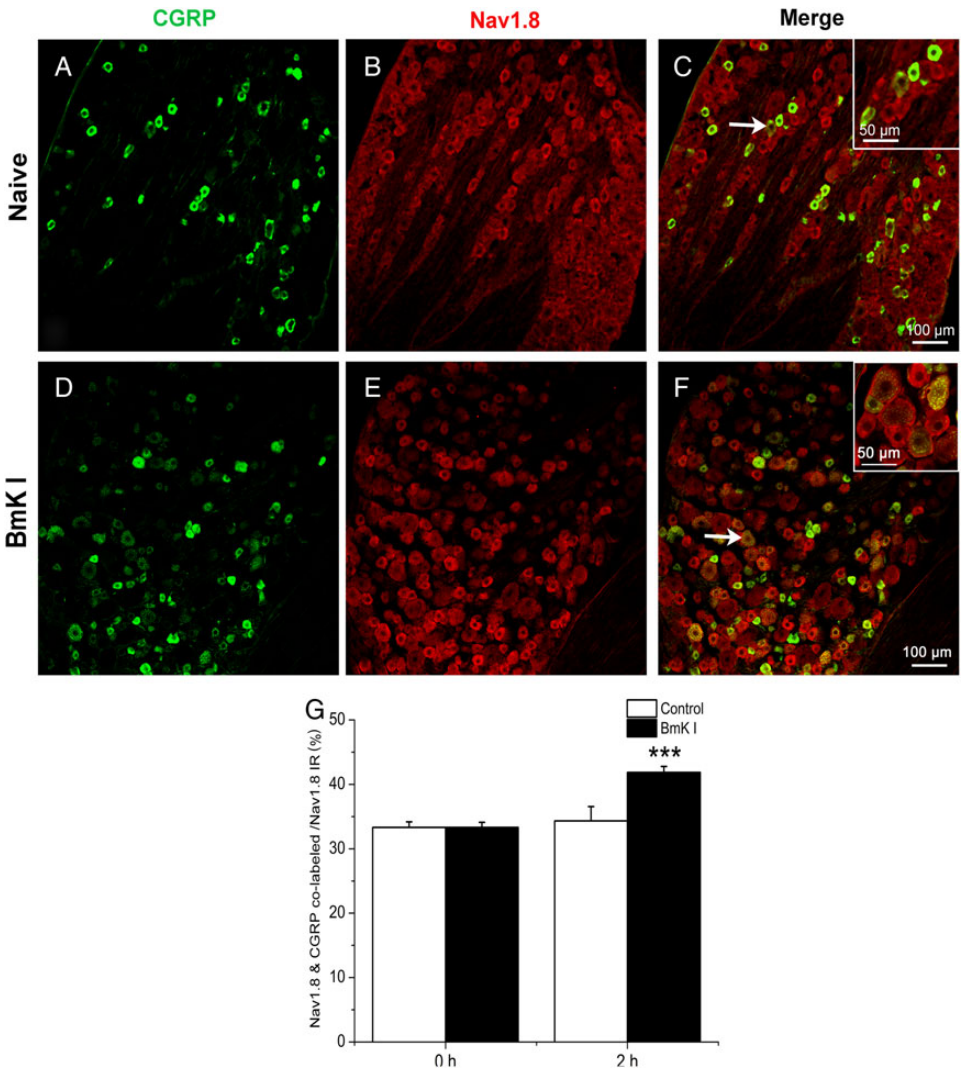

Figure 2. Co-localization of Nav1.8 is increased in small diameter DRG neurons (A–F) Nav1.8 was shown in red and co-markers in green (CGRP), while co-labeling in yellow (as arrows). (G) Quantitative analysis of the number of co-localization neurons in total Nav1.8 positive cells. Comparison of the control group with the BmK I-injected group, ***$P < 0.001$, $n = 3$. Data were presented as the mean ± SEM.

It also demonstrates that the increase of Nav1.8 current density is not due to the changes in the availability of the channels.

BmK I-induced pain responses are attenuated by intrathecal injection of a selective Nav1.8 blocker in rats

The up-regulation of Nav1.8 expression in small diameter DRG neurons by BmK I injection in rats may cause an increase of Nav1.8 current with the alteration of kinetics, which leads to lowered threshold for triggering action potentials, and modulate the transmission of the neuronal electrical impulse, which consequently influences nociception [35,36]. Thus, we determined whether Nav1.8 participates in the BmK I-induced pain behaviors in rats.

Our previous studies showed that diverse pain-related behaviors, including spontaneous nociceptive behaviors, mechanical and thermal hypersensitivity could be induced by intraplantar injection of BmK I [33]. A-803467, a selective Nav1.8 blocker, was administrated to investigate the involvement of Nav1.8 in BmK I-induced pain-related behaviors in rats [27,28]. Prior to administration of BmK I in rats, A-803467 was intrathecally injected into the spinal cord via direct lumbar puncture and diffused for 30 min. Results showed that the spontaneous flinching behavior was suppressed by injection of 1 ng and 10 ng A-803467 (**Fig. 4A**). The suppression of the nociceptive behavior by A-803467 lasted for 2 h (**Fig. 4B**). Meanwhile, BmK I-induced mechanical hypersensitivity of the ipsilateral hind paw was significantly inhibited by administration of A-803467 (**Fig. 4C**), but the thermal hyperalgesia was not affected by administration of A-803467 (**Fig. 4E**). However, the contralateral mechanical and thermal sensitivity was not affected significantly by BmK I or A-803467 (**Fig. 4D,F**).

BmK I-induced pain responses are attenuated by intraplantar injection of a selective Nav1.8 blocker in rats

To provide direct evidence for the notion that Nav1.8 plays a key role in BmK I-induced pain in peripheral nociceptor, we locally administrated A-803467 (1 μg and 10 μg in 10 μl) at the same hind paw of rats 30 min before the injection of BmK I. Local administration of A-803467 still significantly suppressed the spontaneous flinching behavior and the effect lasted for 2 h (**Fig. 5A**). The total number of

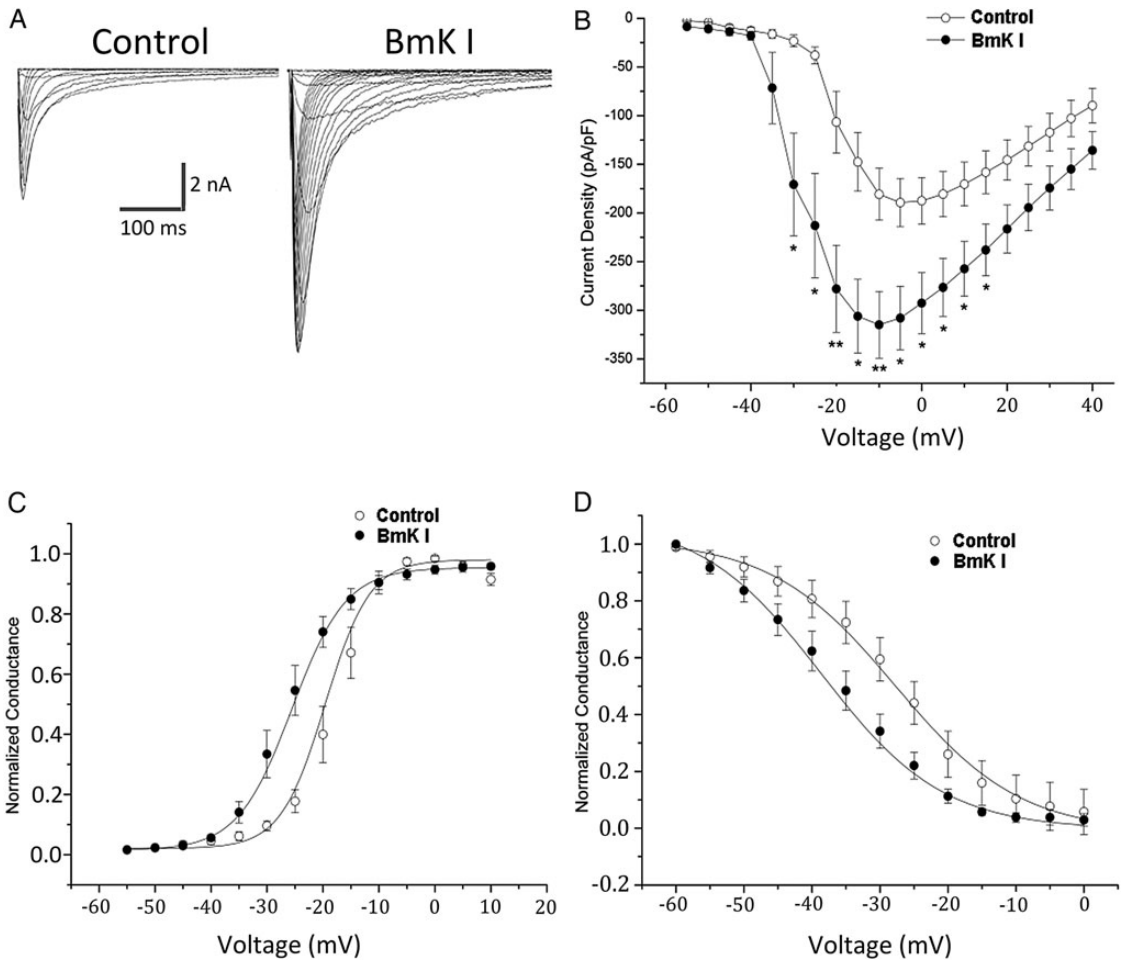

Figure 3. Nav1.8 currents are enhanced in DRG neurons from rats treated with BmK I (A) Representative currents are elicited by the pulse protocol described in the Materials and Methods. Isolation of TTX-resistant Nav1.8 currents in small diameter DRG neurons from the control and BmK I-treated rats, which are significantly increased at 2 h after BmK I injection. (B) I-V curves of Nav1.8 current obtained from DRG neurons and all currents was shown as the amplitude divide the membrane capacitance. The peak maximum current is observed at −5 mV (control) and −10 mV (BmK I-treated group), while peak Nav1.8 current densities were significantly increased at 2 h after BmK I treatment (*P< 0.05, **P< 0.01 compared with the control by Student's t-test). (C,D) Kinetic properties of Nav1.8 current in small DRG neurons. BmK I treatment induced a left shift of the activation (C) and inactivation (D) curves of Nav.18 current. The parameters of kinetic are summarized in Table 1.

Table 1. Parameters of kinetics of Nav1.8 currents

n	Control		n	BmK I	
	$V_{1/2}$ (mV)	k_m (mV)		$V_{1/2}$ (mV)	k_m (mV)
Activation					
7	−17.20 ± 1.39	3.32 ± 0.36	14	−25.81 ± 1.43***	3.88 ± 0.57
Steady-state inactivation					
10	−26.57 ± 1.51	5.22 ± 0.26	17	−32.97 ± 1.93*	4.83 ± 0.19

***P < 0.001, *P < 0.5 compared with the control.

flinches was deceased by ~38% and 41% (from 1267 ± 79 to 779 ± 46 and 753 ± 65), respectively (**Fig. 5B**). Furthermore, after administration of A-803467, the mechanical paw withdrawal threshold was increased from 5.20 ± 0.85 to 6.90 ± 1.12 and 11.33 ± 1.27 g, respectively (**Fig. 5C**). In contrast, BmK I-induced thermal hyperalgesia was not affected by A-803467 (**Fig. 5E**). However, the contralateral mechanical and thermal sensitivity was not affected significantly by BmK I or A-803467 (**Fig. 5D,F**).

BmK I-induced pain responses are attenuated by down-regulation of Nav1.8 in DRG neurons in rats

To confirm the functional significance of the up-regulation of Nav1.8 for BmK I-induced pain responses, we used AS-ODN specifically targeting Nav1.8 to knockdown the expression of Nav1.8 in DRG neurons [32]. After three days of intrathecal administration of Nav1.8 AS-ODN, the mRNA level of Nav1.8 in DRG neurons declined significantly (**Fig. 6A**). Treatment of Nav1.8 AS-ODN also remarkably

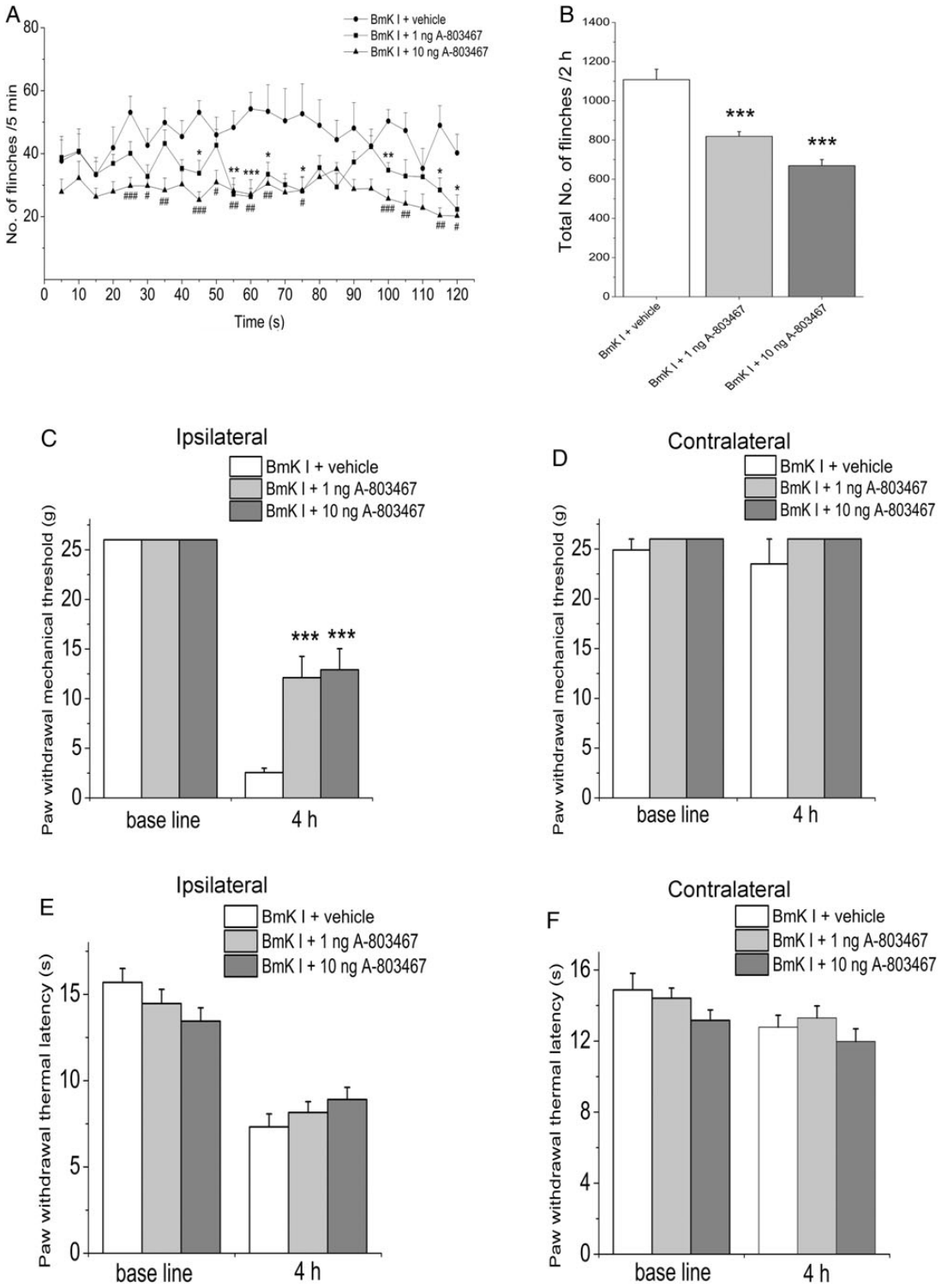

Figure 4. BmK I-induced spontaneous flinching behavior and mechanical hypersensitivity are suppressed by blocking Nav1.8 in spinal cord (A) Rat flinch behavior was attenuated when rats were intraspinally pretreated with A-803467 (1 and 10 ng) 30 min before BmK I administration. The number of rat hind paw flinches was recorded at every 5 min starting from the injection of BmK I to 2 h ($^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, $n=9$, 1 ng A-803467 group compared with the vehicle group, $n=9$; $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$, $n=9$, 10 ng A-803467 group compared with the vehicle group). (B) The total number of rat flinches in 2 h was suppressed by A-803467 significantly ($^{***}P<0.001$, compared with the vehicle group, respectively). (C,D) Ipsilateral mechanical hypersensitivity (C) was suppressed by blocking Nav1.8 ($^{***}P<0.001$, 1 ng A-803467 group, $n=9$; $^{***}P<0.001$, 10 ng A-803467 group, $n=11$; compared with the vehicle group, $n=8$), while slight change was also observed in contralateralmechanical (D). (E,F) Ipsilateral and contralateral thermal hypersensitivity were not significantly changed ($n=9$–11). Data were analyzed by One-way ANOVA and Two-way ANOVA followed by a Bonferroni's *post hoc* test. Data were presented as the mean ± SEM.

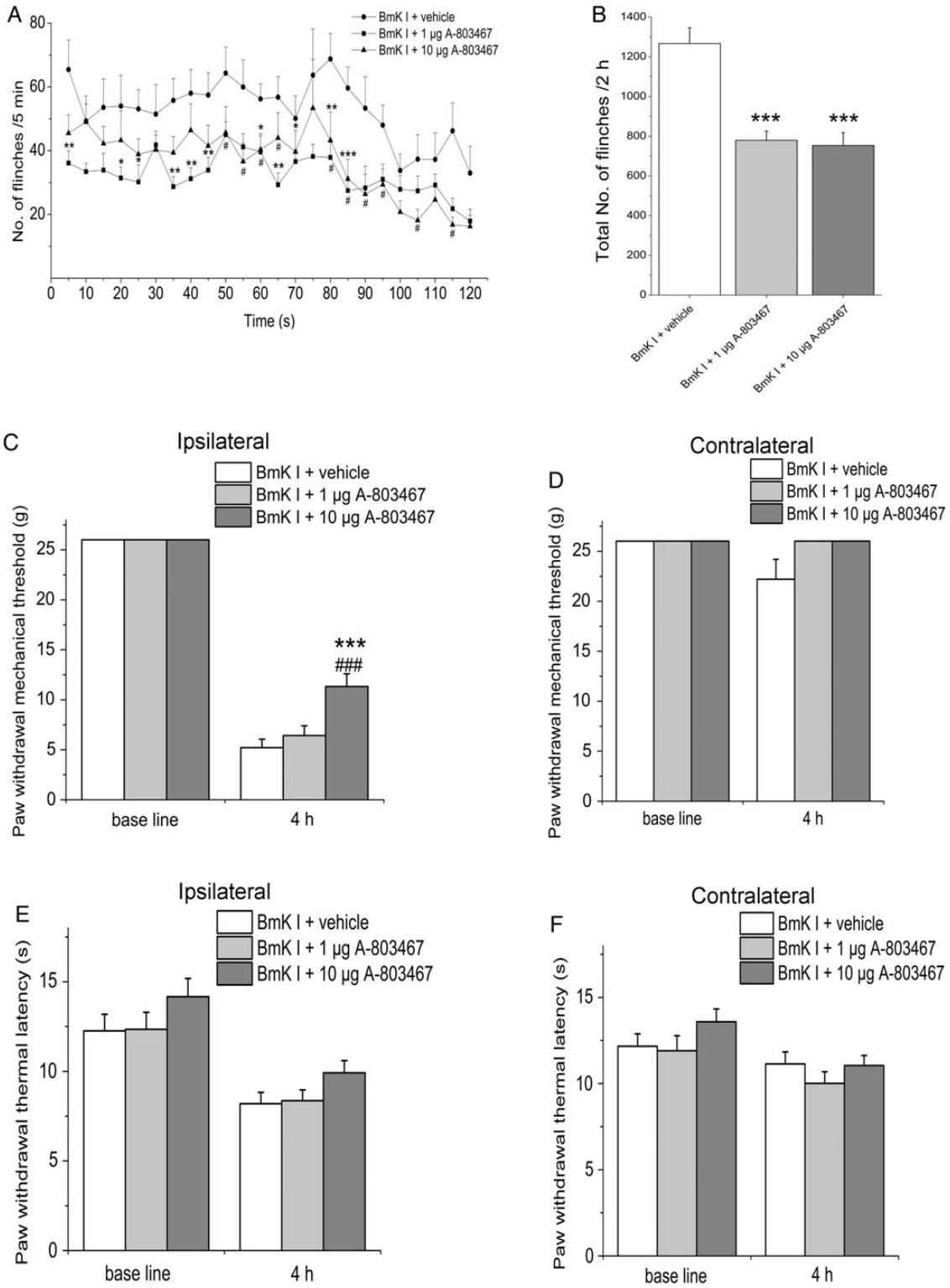

Figure 5. BmK I-induced spontaneous flinching behavior and mechanical hypersensitivity are suppressed by blocking Nav1.8 in peripheral nociceptors (A) Rat flinching behavior was suppressed after intraplantarly pretreated with A-803467 (1 and 10 μg) 30 min before BmK I administration. The number of rat hind paw flinches was recorded at every 5 min starting from BmK I injection to 2 h ($^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, $n=10$, 1 μg A-803467 group compared with the vehicle group, $n=9$; $^{\#}P<0.05$, $n=9$, 10 μg A-803467 group compared with the vehicle group). (B) The total number of rat flinches was suppressed by A-803467 prominently ($^{***}P<0.001$ compared with the vehicle group, respectively). (C) Ipsilateral mechanical hypersensitivity was suppressed by intraplantarly blocking Nav1.8 ($^{***}P<0.001$, 10 μg A-803467 group, $n=9$, compared with the vehicle group, $n=10$; $^{\#\#\#}P<0.001$, 10 μg A-803467 group compared with 1 μg A-803467 group, $n=12$). (D) Contralateral mechanical hypersensitivity was also slightly suppressed by pretreatment with A-803467. (E,F) Ipsilateral and contralateral thermal hypersensitivity were still not significantly changed ($n=9$–11). Data were analyzed by One-way ANOVA and Two-way ANOVA followed by a Bonferroni's *post hoc* test. Date were presented as the mean ± SEM.

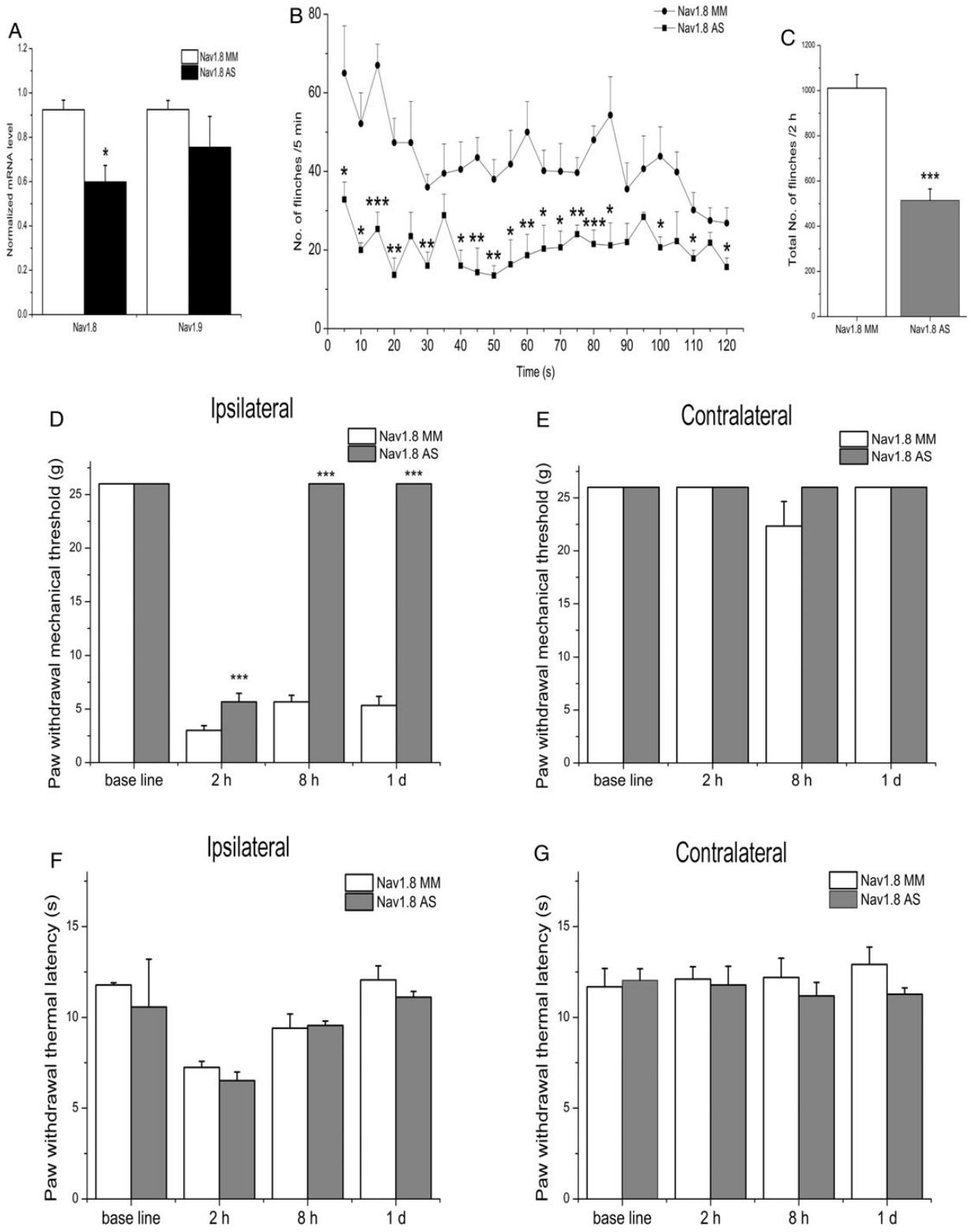

Figure 6. BmK I-induced spontaneous flinching behavior and mechanical hypersensitivity are suppressed by down-regulation of Nav1.8 in DRG neurons (A) The mRNA level of Nav1.8 was down-regulated in DRG neurons by specific AS-ODN. DRG neurons were isolated from Nav1.8 AS/MM-ODN-treated rats 2 h after BmK I injection. In contrast, the mRNA level of Nav1.9 was not affected significantly. (B) Rat spontaneous flinching behavior was attenuated by the treatment of Nav1.8 AS-ODN (*P< 0.05, **P< 0.01, ***P< 0.001, n = 6, compared with Nav1.8 MM-ODN group). (C) The total number of rat flinches was reduced compared with Nav1.8 MM-ODN group (***P< 0.001). (D) Ipsilateral mechanical hypersensitivity was suppressed in Nav1.8 AS-ODN group (*P< 0.05,***P< 0.001, n = 6, compared with Nav1.8 MM-ODN group). (E) Contralateral mechanical hypersensitivity was also suppressed slightly. (F,G) Ipsilateral and contralateral thermal hypersensitivity were not significantly changed (n = 6). The data of B and C were analyzed by the Student's t-test, D and E were analyzed by Two-way ANOVA followed by a Bonferroni's *post hoc* test. Data were presented as the mean ± SEM.

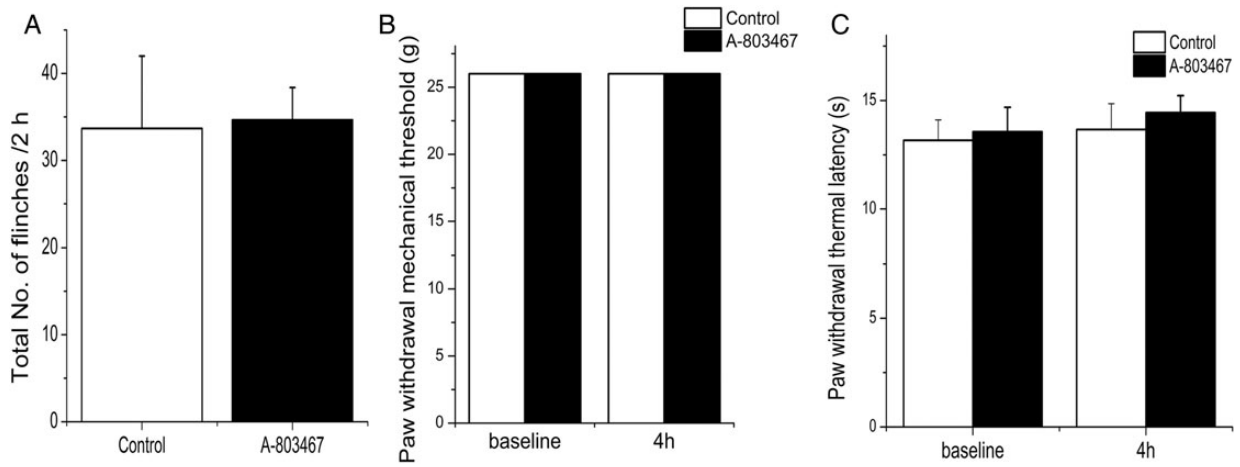

Figure 7. Basal pain threshold is not changed by intraplantar injection of A-803467 (10 μg) alone (A) Spontaneous flinching behavior did not exist in 2 h ($n = 6$). The flinch was just exist for 3 min after injection. (B) Mechanical sense threshold was not affected ($n = 6$). (C) Thermal-pain sense was not changed ($n = 6$).

reduced the BmK I-induced spontaneous flinching behavior (**Fig. 6B**). The total number of flinching in 2 h was decreased from 1010 ± 60 (MM-ODN-treated rats) to 514 ± 50 (Nav1.8 AS-ODN-treated rats) (**Fig. 6C**). In Nav1.8 AS-ODN-treated rats, the mechanical hypersensitivity caused by BmK I injection was attenuated at 2 h, 8 h, and 1 day (**Fig. 6D**). But the BmK I-induced thermal hypersensitivity was not affected in Nav1.8 AS-ODN-treated rats (**Fig. 6F**). However, the contralateral mechanical and thermal sensitivity was not affected significantly by BmK I or Nav1.8 AS-ODN (**Fig. 6E,G**). These results suggest that the up-regulation of Nav1.8 contributes to the spontaneous flinching behavior and mechanical hypersensitivity, but not thermal hyperalgesia after the application of BmK I. Compared with the vehicle control, intraplantar injection of Nav1.8 blocker A-803467 did not induce spontaneous flinching behavior and did not affect basal mechanical or thermal-pain threshold (**Fig. 7**).

BmK I-induced pain responses are enhanced in rats by pretreatment with CFA

The above 'loss-of-function' study revealed that the activation and up-regulation of Nav1.8 expression are essential for BmK I-induced pain. Subsequently, we performed 'gain-of-function' study to test the effects of over-expression of Nav1.8 in DRG on BmK I-induced pain in rats. CFA was used to induce inflammation in rat hind paw, which could induce over-expression of Nav1.8 in DRG neurons for 2 weeks in rats [35]. Thus, CFA was injected 8 h before BmK I administration, and then the behavioral test was performed. It was found that BmK I-induced spontaneous flinching behavior was increased in CFA-inflamed rats (**Fig. 8A**). The total number of flinches was increased from 1011 ± 60 (saline-treated rats) to 1356 ± 127 (CFA-inflamed rats) (**Fig. 8B**). Meanwhile, in CFA-inflamed rats, the threshold of mechanical hypersensitivity was further reduced from 3.00 ± 0.40 g to 0.01 ± 0.002 g, and from 5.70 ± 0.60 g to 3.20 ± 0.50 g, respectively, at 2 and 8 h after BmK I injection (**Fig. 8C**). But, BmK I-induced thermal hypersensitivity was not further increased in CFA-inflamed rats (**Fig. 8D**). These results indicate that over-expression of Nav1.8 may prominently enhance BmK I-induced spontaneous pain and mechanical hypersensitivity.

Discussion

VGSCs play an essential role in pain sensation under both physiological and pathological conditions [3,37]. Among them, Nav1.8 channel has been shown to regulate sensory neuron excitability and thus to participate in the peripheral sensitization associated with the development of chronic pain [3,38]. Nav1.8 is highly expressed and contributes to the majority (80%–90%) of action potential electrogenesis in small DRG neurons [39,40]. Our recent study demonstrated that acute perfusion of BmK I on isolated DRG neurons could induce the hyperexcitability of DRG neurons, due to increased peak currents and altered gating kinetics of Nav1.8 [25]. However, whether BmK I can cause changes of Nav1.8 expression in DRG neurons is not clear. In the present study, we show that the up-regulation in Nav1.8 expression and activity might substantially result in neuronal hyperexcitability in primary sensory neurons, thus contributing to the persistent pain hypersensitivity induced by BmK I. Our data suggest that BmK I can not only directly modulate Nav1.8 activity but also further induce the alteration of Nav1.8 expression in primary sensory neurons, which contributes to the BmK I-induced pain.

The roles of Nav1.8 on pain behaviors appear to be very complex and may depend on distinct pain animal models used in the studies. Nav1.8-null mice display decreases in behavioral responses to noxious thermal and mechanical stimulus. AS-ODNs that can knock down Nav1.8 mRNA expression are also effective in reducing pain behaviors associated with peripheral inflammation [41,42]. The thermal hyperalgesia induced by NGF or carrageenan is also reduced obviously [43]. However, Wood *et al.* [44] found that Nav1.8-null mice exhibited an increased acute pain threshold to noxious mechanical stimuli, but not thermal stimuli, applied to the neuronal peripheral receptive field. Nav1.8 AS-ODNs or ambroxol (a Nav1.8 blocker) administrated to the spinal cord in rats reduced the mechanical hypersensitivity induced by CFA [35,42], but was not effective in the mechanical and thermal hypersensitivity induced by melittin [30]. A-803467, a selective Nav1.8 blocker, dose-dependently reduces mechanical allodynia in a variety of rat inflammation pain models, including capsaicin-induced secondary mechanical allodynia and thermal hyperalgesia after intraplantar CFA injection, but is inactive against formalin-induced nociception and acute thermal and postoperative pain [27]. In this study, we demonstrated that Nav1.8 participates in spontaneous neociception and mechanical allodynia, but not thermal hyperalgesia in BmK I-induced pain. Our data suggest that the participation of Nav1.8 in the pain behaviors induced by BmK I is different from other models. Additionally, BmK I-induced pain model is also different from other inflammatory pain models.

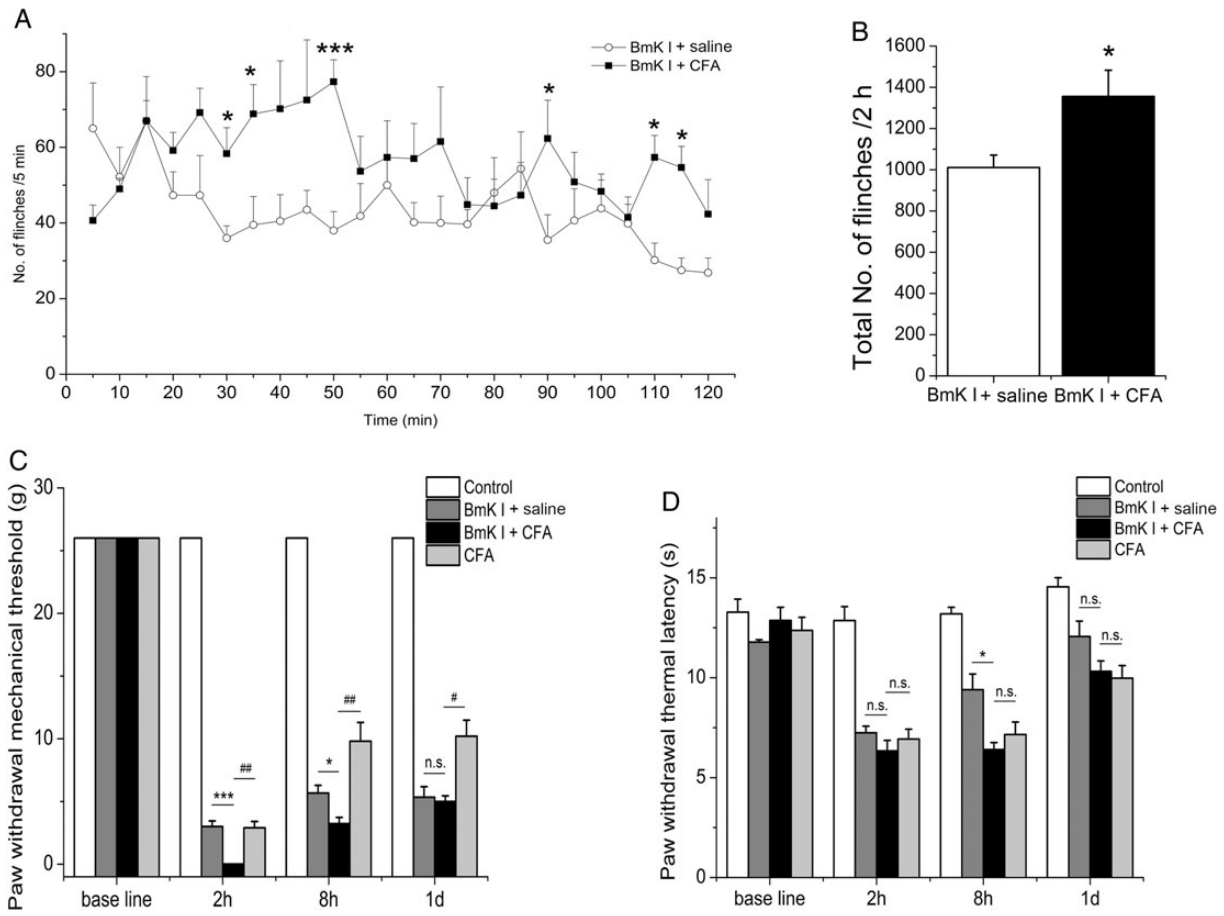

Figure 8. BmK I-induced pain is enhanced after CFA injection (A) Spontaneous flinching behavior induced by BmK I was enhanced in CFA-injected rats (*$P<0.05$, ***$P<0.001$, $n=6$, compared with the group only injected with BmK I). (B) The total number of rat flinches was increased (*$P<0.05$, $n=6$). (C) Mechanical hypersensitivity was enhanced significantly. The threshold was reduced from 3.0 ± 0.4 g to 0.01 ± 0.002 g, 5.7 ± 0.6 g to 3.2 ± 0.5 g, 5.3 ± 0.8 g to 5.0 ± 0.4 g at 2 h, 8 h, and 1 day, respectively, after BmK I injection (***$P<0.001$, *$P<0.05$, $n=6$, compared with the group only injected with BmK I; $^{\#\#}P<0.01$, $^{\#}P<0.05$, $n=6$, compared with the group only injected with CFA). (D) Thermal hypersensitivity was not affected obviously ($n=6$). In all experiments the contralateral pain sense was not changed compared with the control group. The data of B were analyzed by the Student's *t*-test, C and D were analyzed by Two-way ANOVA followed by a Bonferroni's *post hoc* test. Data were presented as means ± SEM.

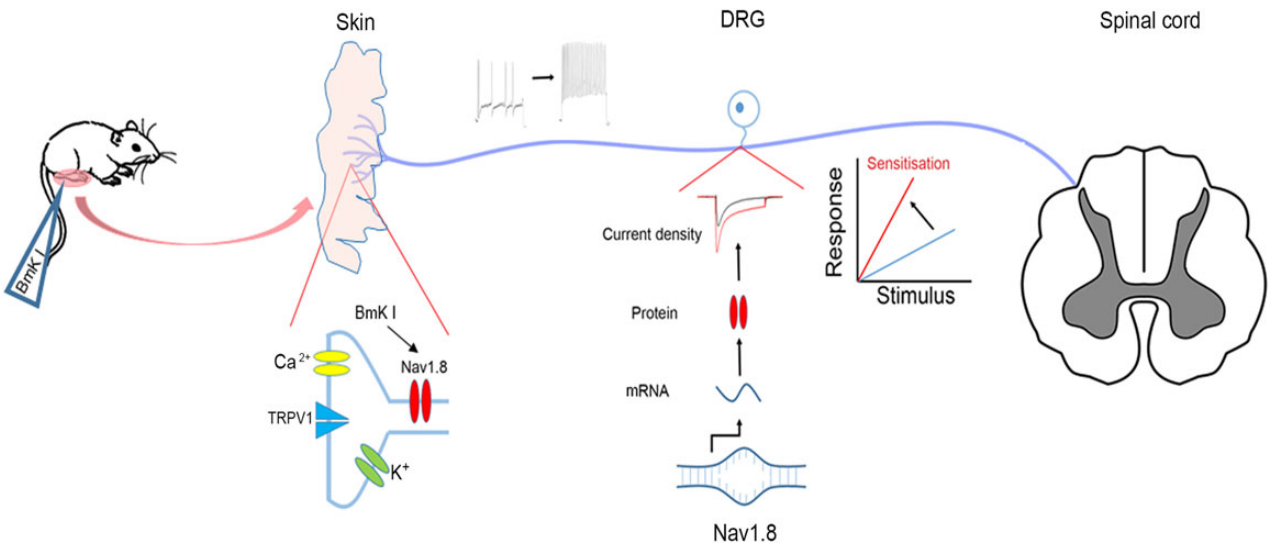

Figure 9. Working hypothesis of the mechanism by which Nav1.8 participates in the BmK I-induced pain

It should be noted that the up-regulation of Nav1.8 expression and alteration of kinetics in DRG have been highlighted by others following one to four days post-insult exposure to carrageenan or CFA [35,45–47]. In contrast, in this study, all these changes in Nav1.8 could be observed in 2 h after BmK I injection. We speculate that the inflammatory responses at injury site, such as mast cell degranulation and histamine release may contribute to the up-regulation of Nav1.8 expression. In our previous studies, we found that after BmK I injection, the activation of ERK signaling pathway and the up-regulation of c-Fos expression and nNOS expression occur in spinal cord [15,33,48]. Interestingly, the up-regulation of Nav1.8 is consistent with the time course change of c-Fos and nNOS. We postulate that the up-regulation of Nav1.8 in peripheral nervous system may drive these signalings in spinal cord. In DRG neurons, we also found that several intracellular signal pathways are activated, including p38 and mTOR, which are in parallel with the up-regulation of Nav1.8 [30,31]. It is possible that the mTOR and p38 signaling pathways may be involved in the alteration of Nav1.8 expression and function in DRG neurons in 2 h. However, the exact roles of these signaling pathways in the regulation of Nav1.8 expression are unclear and require further investigation.

Meanwhile, some reports showed that injection of A-803467 into the L4 DRG or into the hind paw receptive field could reduce evoked but not spontaneous wide-dynamic range (WDR) firing in spinal nerve ligation (SNL) rats [28]. In contrast, intraspinal injection of A-803467 decreases both evoked and spontaneous discharges of WDR neurons. Thus, Nav1.8 on the L4 DRG and Nav1.8 on the peripheral and central terminals of primary afferent neurons were suggested to regulate the inflow of low-intensity mechanical signals to the spinal WDR neurons, while Nav1.8 on central terminals seemed to be critical to the modulation of spontaneous firing in SNL rats. However, our results show that injection of A-803467 into the hind paw receptive field and intraspinal injection both decrease the spontaneous flinching behavior, and reduce the mechanical hypersensitivity in BmK I-injected rats. It was demonstrated that Nav1.8 on the central terminals of DRG neurons in spinal cord and Nav1.8 on peripheral terminals of DRG neurons in the hind paw receptive fields regulate these pain signals of spontaneous firing and mechanical sensitivity.

In our recent study, we found that BmK I directly activates Nav1.8 to induce the hyperexcitability of DRG neurons, which may occur in nerve terminals [25]. In this study, we identified that functional up-regulation of Nav1.8 also contributes to the induction of BmK I-induced pain. When pain signals initiated in primary nociceptors are transmitted into the cell bodies of DRG neurons, several intracellular signaling pathways, including p38 and mTOR pathways, are activated. The transcription and translation of Nav1.8 are enhanced, although the roles of these signaling pathways in Nav1.8 up-regulation are unclear. The working hypothesis on the mechanism by which Nav1.8 participates in BmK I-induced pain is shown in **Fig. 9**.

In summary, we demonstrate that functional up-regulation of Nav1.8 expression contributes to BmK I-induced pain-related behaviors in rats. Our work provides the potential mechanisms of scorpion sting pain, and suggests that targeting Nav1.8 may be a promising strategy for chronic pain. BmK I-induced pain model might be valuable to understand pain mechanims.

Funding

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