Activation of neuronal voltage-gated potassium Kv7/KCNQ/M-current by a novel channel opener SCR2682 for alleviation of chronic pain

Authors: Jing Wang¹#, Yani Liu¹, ², ³#, Fang Hu¹, Jiuyong Yang¹, Xiaoyu Guo¹, Xingming Hou¹, Chuanxia Ju¹* and KeWei Wang¹, ², ³*

# These authors contributed equally
* Correspondence

Affiliations:
¹Department of Pharmacology, School of Pharmacy at Qingdao University Medical College, #1 Ningde Road, Qingdao 266073, China; ²Center for Brain Science and Brain-Inspired Intelligence, Guangdong-Hong Kong-Macao Greater Bay Area, China; ³Institute of Innovative Drugs, Qingdao University, #38 Dengzhou Road, Qingdao 266003, China
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Running title: Kv7 opener SCR2682 alleviates pain

* Correspondence: Associate Prof. Chuanxia Ju¹ (jucx@qdu.edu.cn) and Prof. KeWei Wang¹,²,³ (wangkw@qdu.edu.cn)

¹Department of Pharmacology, School of Pharmacy at Qingdao University Medical College, #1 Ningde Road, Qingdao 266073, China; ²Center for Brain Science and Brain-Inspired Intelligence, Guangdong-Hong Kong-Macao Greater Bay Area, China; ³Institute of Innovative Drugs, Qingdao University, #38 Dengzhou Road, Qingdao 266003, China

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Abbreviations: NSAIDs, nonsteroidal anti-inflammatory drugs; DRG, dorsal root ganglia; RTG, retigabine; PGB, pregabalin; CFA, complete Freund's adjuvant; PWMT, paw withdrawal mechanical threshold; PWTL, paw withdrawal thermal latency; SNI, spared nerve injury; RMP, resting membrane potential
Abstract

Treatment of chronic pain remains an unmet medical need. The neuronal voltage-gated potassium Kv7/KCNQ/M channel has been implicated as a therapeutic target for chronic pain. However, whether pharmacological activation of Kv7 channel can alleviate pain remains elusive. In this study, we show that selective activation of native M currents by a novel channel opener SCR2682 reduces repetitive firings of dorsal root ganglia (DRG) sensory neurons. Intraperitoneal administration of SCR2682 relieves mechanical allodynia and thermal hyperalgesia in rat models of pain induced by complete Freund's adjuvant (CFA) or spared nerve injury (SNI) in a dose-dependent manner without affecting locomotor activity. The anti-nociceptive efficacy of SCR2682 can be reversed by the channel specific blocker XE991. Furthermore, SCR2682 increases Kv7.2/KCNQ2 mRNA and protein expression in DRG neurons from rats in the SNI model of neuropathic pain. Taken together, pharmacological activation of neuronal Kv7 channels by opener SCR2682 can alleviate pain in rats, thus possessing therapeutic potential for chronic pain or hyperexcitability-related neurological disorders.

Key words: SCR2682, Kv7/KCNQ/M channel, chronic pain, XE991, retigabine
Significance Statement: A novel Kv7 opener SCR2682 inhibits action potential firings in DRG sensory neurons and exhibits an efficacy in antinociception, thus possessing a developmental potential for treatment of chronic pain or epilepsy.
Introduction

Neuronal hyperexcitability defines the fundamental mechanism of neurological diseases such as chronic pain and epilepsy (Snowball and Schorge, 2015). Therefore, pharmacological inhibition of repetitive neuronal firing represents an attractive strategy for therapy of chronic pain (Payne et al., 2015). The most commonly prescribed medications for chronic pain are nonsteroidal anti-inflammatory drugs (NSAIDs), skeletal muscle relaxants and opioid analgesics (van Hecke et al., 2013; Tsantoulas and McMahon, 2014; Hsu, 2017). However, clinical use of these drugs for treatment of pain renders either limited efficacy or serious side effects (Gordon, 2003; Hu et al., 2016; Volkow et al., 2018). Therefore, the treatment of chronic pain still remains an unmet medical need.

Potassium channels are considered to be key targets for control of membrane excitability and treatment of chronic pain (Liu and Wang, 2019). The voltage-gated Kv7 potassium channels encoded by KCNQ gene subfamily, include five members of Kv7.1-Kv7.5 (Cooper and Jan, 2003; Brown and Passmore, 2009). Neuronal Kv7 channels formed by Kv7.2, Kv7.3 and Kv7.5 subunits are mainly expressed in the central nervous system or peripheral sensory system, such as dorsal root ganglia (DRG) sensory neurons and dorsal horn neurons (Passmore et al., 2003; Wickenden and McNaughton-Smith, 2009; Wang and Li, 2016). Kv7 channels are slow activating, non-inactivating and voltage-dependent K^+ currents that exert an inhibitory control on neuronal excitability (Wang et al., 1998; Cooper and Jan, 2003; Wulff et al., 2009). Loss-of-function or inhibition of Kv7 channels increases cell excitability through depolarizing resting membrane potential and increasing firings of nociceptive neurons (Barkai et al., 2017), and is involved in the pathogenesis of epilepsy, migraine and neuropathic pain (Biervert et al., 1998; Charlier et al., 1998; Schroeder et al., 1998; Blackburn-Munro and Jensen, 2003a; Gribkoff, 2003). Therefore, enhancing Kv7 function in nociceptors may provide a promising treatment for chronic pain (Passmore et al., 2003; Wulff et al., 2009).

Kv7 opener retigabine, or ezogabine, was the first approved by U.S. Food and Drug Administration for the adjuvant treatment of partial seizures (GlaxoSmithKline, 2016). In preclinical studies, retigabine exhibits anti-nociceptive effects on inflammatory, neuropathic and bone cancer pain in rodent models (Hayashi et al., 2014; Cai et al., 2015; Pottabathini et al., 2015). Flupirtine, a structural analog of retigabine, was used for decades as a centrally acting, nonopioid analgesic for treatment of a variety of acute and chronic pain (Puls et al., 2011; Szelenyi, 2013). However, both retigabine and flupirtine have recently been discontinued because of their safety issues associated with retina and skin discoloration and liver toxicity (Puls et al., 2011; Garin Shkolnik et al., 2014). Therefore, it is necessary to identify more selective and
potent Kv7 channel openers with fewer side effects for therapy of chronic pain.

In this study, we evaluated the anti-nociceptive effect of a novel Kv7/KCNQ/M channel opener SCR2682 on neuronal firing and nociception in rat models of chronic pain. SCR2682 potently activates Kv7.2-Kv7.5 channels in dose-dependent manner with an EC 50 of 9.8 ± 0.4 nM on Kv7.2/7.3 (Zhang et al., 2019), which is 100-fold more potent than retigabine in activating Kv7.2/Kv7.3 channel.
Materials and Methods

Animals

The 8-week-old male Sprague-Dawley (SD) rats and 7-day-old SD rats were purchased from Beijing Vital River Laboratory Animal Technology Co. Ltd. Animals were housed in 3 to 5 per cage and received food and water ad libitum. Before behavioral experiments, rats were acclimated to 1 week in a quiet animal breeding room with a 12-hour light/dark cycle (7 a.m. to 7 p.m.) under controlled temperature (23 ± 2 °C) and humidity (50 ± 5%). All in vivo experiments were conducted from 9:00 a.m. to 5:00 p.m. in a double-blind manner. All experimental procedures were approved by the Animal Ethics Committee of Qingdao University College of Medicine, and complied with the ethical guidelines of the International Association for the Study of Pain.

Regents

Compounds SCR2682 (4-(2-bromo-6,7-dihydrothieno[3,2-c]pyridin-5(4H)-yl)-2,6-dimethylphenyl)-3,3 dimethylbutanamide), XE991 and retigabine (RTG) were synthesized with above 95% purity by Shanghai Zhimeng Biopharma Co. Ltd (Shanghai, China). Pregabalin (PGB) was purchased from Beijing Solarbio Science & Technology Co, Ltd (Beijing, China). GAPDH antibody and secondary antibody were purchased from Abcam (Shanghai, China). KCNQ2 antibody was purchased from Santa Cruz Biotechnology (USA). The primers for KCNQ2 and β-actin were synthesized by Invitrogen (Shanghai, China). All drugs were dissolved in DMSO stock solutions and stored at -20 °C before use. The stock solutions were diluted to working concentrations with saline or electrophysiological bath solution on the day of experiment.

Culture of rat dorsal root ganglion (DRG) neurons

DRG neurons were isolated from the 7-day-old SD rats as previously described (Zhang et al., 2013). Briefly, DRG neurons acutely dissected from the intervertebral foramina were digested at 37 °C with 1 mg/ml collagenase for 30 min and followed by another 30 min digestion with 2.5 mg/ml trypsin before subsequently suspended at least twice in DMEM plus 10% fetal calf serum to stop the digestion. Thereafter, the acutely dissociated neurons from DRGs were plated on poly-D-lysine-coated glass coverslips and cultured for 4 days before electrophysiological recordings within 48 h.

Electrophysiology

For current recordings of DRG neurons, the perforated patch-clamp recording technique was used with amphotericin B (250 μg/ml, Sigma, USA) in the pipette (Zhang et al., 2013) and an HEKA EPC10 patch-clamp amplifier. The acquisition rate of data was at 10 kHz before filtered at 2.5 kHz. Patch electrodes
were pulled using a micropipette puller (Sutter Instruments, Novato, CA, USA) and fire-polished to a final resistance of 1-2 MΩ. Series resistance was compensated by 60-80%. The internal solution for recordings of DRG neurons was as follows (in mM): KCl 150, MgCl₂ 5, HEPES 10, pH 7.4 adjusted with KOH; and external solution (in mM) contained: NaCl 160, KCl 2.5, MgCl₂ 1, CaCl₂ 2, Glucose 10, HEPES 20, and pH 7.4 adjusted with NaOH.

**Locomotor activity**

The open field test was used to measure the general locomotor activity. SD rats were randomly divided into groups consisting of vehicle control (10% Tween-80-saline), RTG (7 mg/kg) and SCR2682 group (0.5, 1, 2, 4 and 8 mg/kg). After 1 h of intraperitoneal (i.p.) administration, each rat was placed in a 40 cm × 40 cm open field box for 5 min, and its total distance and the mean travel speed in the apparatus were recorded and analyzed using the SMART 3.0 (Panlab, Barcelona, Spain). The apparatus was wipe-cleaned with 75% alcohol before the next testing.

**Chronic pain models**

**Complete Freund’s adjuvant (CFA)-induced inflammatory pain model**

Adult male SD rats were anaesthetized with 10% chloral hydrate (i.p.) before injected with 50% CFA (in a 100 μl injection volume, Sigma, USA) into the planter surface of the right hindpaw (Peng et al., 2012). Rats in the sham control group underwent the same procedures, except saline injected into the right hindpaw (Teng et al., 2016). SCR2682 (0.5, 1 and 2 mg/kg, i.p.) or RTG (7 mg/kg, i.p.) was administered after CFA injection for 24 h, and rats in the sham control and CFA model group were injected with 10% Tween-80-saline. The paw withdrawal mechanical threshold (PWMT) and paw withdrawal thermal latency (PWTL) were performed at time points 0, 0.5, 1, 2 and 3 h after compound administration.

**The spared nerve injury (SNI)-induced model of persistent peripheral neuropathic pain**

Adult male SD rats were anesthetized by intraperitoneal injection with 10% chloral hydrate. The rat SNI model was generated based on previous descriptions (Decosterd and Woolf, 2000). Briefly, the skin of left posterior leg was incised and the biceps femoris muscles were separated for the exposure of the three branches of sciatic nerve (tibial, common peroneal and sural nerves). The tibial and common peroneal nerves the surrounding tissues were isolated and ligated with removal about 2-3 mm of distal nerve stump to avoid regeneration while ensuring the sural nerve intact. The same procedure was performed for sham-operated rats without nerve ligation and cut. After the operation, muscles and skin were closed with absorbable sutures. After the establishment of SNI model, rats were divided into 8 groups: Sham, SNI + Vehicle, SNI + SCR2682 (0.5 mg/kg), SNI + SCR2682 (1 mg/kg), SNI + SCR2682 (2 mg/kg), SNI +
SCR2682 (2 mg/kg) + XE991 (3 mg/kg), SNI + RTG (7 mg/kg) and SNI + PGB (10 mg/kg). The rats of sham and SNI + Vehicle groups were injected with 10% Tween-80-saline. Drugs were injected (i.p.) for consecutive 9 days (Chen et al., 2015), and the PWMT was determined after 1 h of administration on days 1, 3, 5, 7, and 9. After the behavioral experiments, the animals were decapitated for isolation of DRGs from the L4 and L5 spinal segment. The isolated DRGs were stored at -80 °C until further use for detection of Kv7.2 mRNA and protein levels in quantitative RT-PCR and western blot assays, respectively.

Measurement of pain threshold

Paw withdrawal mechanical threshold (PWMT)

The PWMT induced by von Frey hair was measured by an up-and-down method for evaluation of mechanical allodynia. Rats were individually placed in a transparent plexiglass cover on a metal mesh for 20-30 min until the disappearance of basic combing and exploring activities. A series of von Frey hairs with different forces were applied to stimulate the plantar of rat hindpaws until a bend in a slight S-shape was observed for 5-6 s. If rats showed paw withdrawal reaction, the result was positive and otherwise, it was negative. The PWMT was calculated by the following formula: PWMT (g) =10^[Xf+Kδ]. Where Xf is the log value of the last von Frey hair force, K is the value looked up from the standardized table based on the up-and-down pattern, and δ is the average of the difference between the log values of the adjacent von Frey hair force.

Paw withdrawal thermal latency (PWTL)

The PWTL of thermal hyperalgesia was measured using a fully automatic plantar analgesia meter (BME-410C). An individual rat was placed on a glass plate with a transparent plexiglass cover. After 15 min of adaptation, the plantar of rat hindpaws was heated and the time from contact with thermal radiation source to paw withdrawal was recorded as the PWTL with cut-off latency of 35 s to prevent potential tissue damage. Each rat was repeatedly measured 5 times at an interval of 5 min, and an average PWTL was calculated.

Quantitative RT-PCR and Western blot

The total RNA was extracted from DRGs neurons of the L4 and L5 spinal segment using Trizol reagent. The FastKing RT Kit (With gDNase) was used to remove genomic DNA contamination and synthetize cDNA. The real-time PCR was performed using Taq PCR Mastermix in a Bio-Rad iCycler Thermal Cycler and using SYBR Green as a reporter. The primer sequences used were as follows: KCNQ2, sense 5'-CAGTGCGGATCAGAGTCTCG-3', antisense 5'-CTTGCTTCTTTCTGAGTTCTGCC-3'; β-actin sense
5′-CATTGCTGACAGGATGCAGAAGG-3′, antisense 5′-TGCTGGAAGGTGGACAGTGAGG-3′. Expression levels were normalized to the β-actin. Data were analyzed using the 2\(^{-\Delta\Delta CT}\) method.

The total protein was extracted using RIPA lysis buffer containing 1% protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific, USA). The BCA protein assay kit (Thermo Fisher Scientific, USA) was used to determine the total protein concentration. The equal amount of proteins was denatured and separated with 10% SDS-PAGE before transferred to the PVDF membrane. The PVDF membranes were blocked with 5% no-fat milk in 0.5% Tween-20-TBS (TBST) for 2 h at room temperature before incubation with primary antibodies of mouse anti-KCNQ2 (1:500) and rabbit anti-GAPDH (1:10,000) overnight at 4 °C. The HRP-conjugated secondary antibody (1:10,000) was incubated at room temperature for 1 h after wash of primary antibody with TBST. The membrane was placed in chemiluminescence reagent for 2-3 min and imaged under dark condition. The bands were quantified using Image Lab software.

**Statistical analysis**

Statistical analysis was performed using GraphPad Prism 5 software. All data were expressed as the mean ± SEM (standard error of means). Statistical analysis for difference between groups in electrophysiological experiments was carried out using paired Student’s t tests. Statistical differences among groups for *in vivo* behavioral tests were compared by one-way or two-way analysis of variance (ANOVA), followed by Bonferroni post-hoc test. The Bonferroni post hoc tests were conducted only if F in ANOVA achieved *P* < 0.05 and there was no significant variance inhomogeneity. When *P* < 0.05, the difference was considered to be statistically significant.
Results

Compound SCR2682 enhances native M current in rat DRG neurons

To examine the effect of SCR2682 on activation of native M current, we carried out recordings of acutely isolated DRG neurons from rats. The M current was activated by a depolarizing voltage at -20 mV, and the tail current was observed at -60 mV (Figure 1A). Application of SCR2682 at 0.1 μM or retigabine (RTG) at 10 μM as a positive control increased M current by approximately 30% measured at -20 mV, which was blocked by the channel specific inhibitor XE991 (3 μM) (Figure 1A). SCR2682 also induced a significant hyperpolarization of the resting membrane potential (RMP) with a leftward shift about 10 mV to -66.4 ± 3.7 mV from -56.7 ± 2.8 mV (Figure 1B). Similarly, perfusing SCR2682 at 0.1 μM also abolished the firing spikes of DRG neurons, whereas M channel specific blocker XE991 significantly increased the number of action potentials elicited by injection of 270 pA inward current (Figure 1C). These results indicate that activation of native M-current by SCR2682 significantly suppresses the firing of DRG neurons, suggesting that SCR2682 may have anti-nociceptive activity.

Anti-nociceptive effect of SCR2682 on CFA-induced inflammatory pain in rats

We further examined the effect of SCR2682 on mechanical allodynia and thermal hyperalgesia in CFA-induced inflammatory pain in rats. As shown in Figure 2A and B, subcutaneous (s.c.) injection of CFA resulted in development of hypersensitivity to both mechanical and thermal stimuli at 24 h after the injection, and the hyperalgesic effect was observed and maintained for 5 days before a slow recovery over time to baseline, consistent with our previous observations (Teng et al., 2016). In contrast, administrations of SCR2682 at different concentrations (0.5, 1 and 2 mg/kg, i.p.) caused an increase of both PWMT and PWTL in a dose-dependent manner with maximal effect after 2 h injection (Figure 2C and D). As a positive control, RTG (7 mg/kg, i.p.) also increased the PWMT and PWTL with maximum effect at 0.5 h (Figure 2C and D). The effects of the highest doses of SCR2862 and RTG were comparable in the PWMT procedure, with RTG at 0.5 h producing greater effect in PWTL compared to PWMT. These results demonstrate that SCR2682 can alleviate the mechanical allodynia and thermal hyperalgesia induced by CFA.

SCR2682 alleviates SNI-induced neuropathic pain

To further evaluate the effect of SCR2682 on chronic pain, we generated the neuropathic pain model of SNI in rats. Compared with the sham group, rats in SNI vehicle groups showed hypersensitivity response to von Frey hair stimuli on day 9 after surgery, and the mechanical alldynia was observed and maintained
during the 10-day period of experiments. As shown in Figure 3 and Table 1, there was no difference in the baseline threshold for mechanical pain response determined in von Frey hair assay among the different groups before surgical operation. In contrast, administration of SCR2682 (i.p.) at different doses (0.5, 1 and 2 mg/kg) resulted in a dose-dependent increase of ipsilateral PWMT, as compared with the vehicle group (Figure 3 and Table 1). Co-treatment of Kv7 channel blocker XE991 (3 mg/kg, i.p.) and SCR2682 (2 mg/kg, i.p.) resulted in a decrease of PWMT, indicating the antagonism of XE991 on anti-nociception of SCR2682 through the inhibition of Kv7 (Figure 3 and Table 1). As positive controls, RTG at 7 mg/kg (i.p.) or PGB (10 mg/kg, i.p.) also exhibited a significant increase of PWMT in SNI rats (Figure 3 and Table 1). These results demonstrate that selective activation of Kv7 by SCR2682 can alleviate neuropathic pain induced by SNI.

Effect of SCR2682 on locomotor activity

Locomotor activity is commonly used for evaluation of psychostimulative effect or sedative activity. We next tested the effect of SCR2682 on the locomotor activity by assessing total travel distance and average speed in the open field test. As depicted in Figure 4, rats treated with different doses of SCR2682 (0.5, 1 and 2 mg/kg, i.p.) or RTG (7 mg/kg, i.p.) had no significant differences in the total travel distance and the average speed, as compared with the control group. However, dosing SCR2682 at higher concentrations of 4 mg/kg and 8 mg/kg caused the reduction of total travel distance and the mean travel speed (Figure 4), suggesting the sedative effect of SCR2682 at high doses.

Increased expressions of Kv7.2/KCNQ2 by SCR2682 in isolated DRG neurons from rats with spared nerve injury

To investigate the effect of SCR2682 on Kv7.2/KCNQ2 channel subunit expressions in DRG neurons from rats with SNI, we examined the mRNA and protein expression levels of KCNQ2 using the quantitative RT-PCR and western blot analysis. As illustrated in Figure 5, the expression levels of KCNQ2 mRNAs and proteins in DRG neurons from the SNI group were significantly lower than that of sham control group. In contrast, treatment with SCR2682 (2 mg/kg, i.p.) or RTG (7 mg/kg, i.p.) significantly increased KCNQ2 mRNA and protein expression levels as compared with the SNI + vehicle group (Figure 5). To further confirm the increased expression of KCNQ2 in DRG neurons, we also co-injected SCR2682 with XE991 (3 mg/kg, i.p.) that caused the reduction of KCNQ2 mRNA and protein expression levels (Figure 5). These results show that SCR2682 upregulates KCNQ2 expression in DRG neurons which can be reversed by blocker XE991.
Discussion

Neuronal Kv7/KCNQ/M channels serve as promising therapeutic target for chronic pain and epilepsy (Wulff et al., 2009). Our recent identification of small molecule SCR2682 shows potent and selective activation of Kv7/KCNQ/M channel with nM range of IC50 (9.8 nM) (Zhang et al., 2019). In the present study, we aimed at investigating whether this novel SCR2682 could alleviate chronic pain. We find that in vitro SCR2682 activates the native M current and suppresses action potential firing of DRG neurons. In vivo behavioral test, SCR2682 also alleviates inflammatory and neuropathic pain in rats without affecting locomotor activity at dose of 2 mg/kg or lower dose, thus demonstrating the therapeutic potential of SCR2682 for chronic pain without sedative activity.

Over the past years, many small molecules including retigabine, flupirtiline, ICA-27243 and QO-58 have been reported to exhibit anti-nociceptive activities in various pain models through activation of Kv7/KCNQ/M channels (Munro and Dalby-Brown, 2007; Zhang et al., 2013; Hayashi et al., 2014). Our SCR2682 is effective in relieving mechanical allodynia and/or thermal hyperalgesia in inflammatory or neuropathic pain in a dose dependent manner. The anti-nociceptive effect of SCR2682 can be reversed by M channel inhibitor XE991, indicating that SCR2682-mediated anti-nociception is directly involved in activation of Kv7 channel (Blackburn-Munro and Jensen, 2003b; Hayashi et al., 2014; Cai et al., 2015; Zhang et al., 2015; Di Cesare Mannelli et al., 2017). The efficacy of SCR2682 on chronic pain is consistent with our previous report that SCR2682 is more potent in activating neuronal homomeric Kv7.2, Kv7.3, Kv7.4 and heteromeric Kv7.2/7.3 and Kv7.3/7.5 channels than RTG (Zhang et al., 2019). The anti-nociceptive effect of SCR2682 can be reversed by M channel inhibitor XE991, indicating that SCR2682-mediated anti-nociception is directly involved in activation of Kv7 channel (Blackburn-Munro and Jensen, 2003b; Hayashi et al., 2014; Cai et al., 2015; Zhang et al., 2015; Di Cesare Mannelli et al., 2017).

Our spontaneous locomotion tests show that SCR2682 at low doses of 0.5, 1, 2 mg/kg has no effect on altering the total travel distance. However, dosing at higher concentrations such as 4 and 8 mg/kg starts to reduce total travel distance and mean speed, suggesting the sedative effect induced by SCR2682 at the high doses. It has been reported that retigabine at high doses can increase inhibitory neurotransmission through potentiation of GABA_\text{A} receptor response (Otto et al., 2002), which may help explain the reduction of total travel distance caused by SCR2682 at higher doses likely through increasing GABA activity. For cardiac safety evaluation, SCR2682 exhibits a weak inhibition on cardiac hERG channels in comparison with retigabine (Zhang et al., 2019), suggesting that SCR2682 is less likely to cause liability concerns as seen in
retigabine for corrected QT interval prolongation and urinary retention (Daniluk et al., 2016).

Previous studies indicate that Kv7.2 expressions or Kv7.2-positive neurons are significantly decreased in DRG from SNI model (Cisneros et al., 2015) or bone cancer model in rats (Rose et al., 2011; Zheng et al., 2013). The relative expression of KCNQ2 mRNA is approximately 4-fold greater than KCNQ3 or 180-fold greater than KCNQ5 (Rose et al., 2011). In line with these observations, our findings show that expression of KCNQ2 mRNA and protein in DRGs of SNI model group is significantly lower than that of sham group, and KCNQ2 mRNA and protein expressions are significantly increased after administration of SCR2682 or retigabine, which can be reversed by XE991. These observations suggest that SCR2682 attenuates neuropathic pain by up-regulating Kv7.2 channels expression and increasing channel activity in the DRG neurons.

In conclusion, our findings show that the novel opener SCR2682 potently activates neuronal Kv7 currents and reduces excitability of DRG neurons in vitro. SCR2682 also alleviates mechanical allodynia and/or thermal hyperalgesia in models of inflammatory and persistent peripheral neuropathic pain. Therefore, Kv7 channel activator SCR2682 may have therapeutic potential for chronic pain.

Authorship Contributions
Participated in research design: Jing Wang, Yani Liu, Chuanxia Ju and KeWe Wang
Conducted experiments: Jing Wang, Yani Liu, Fang Hu, Jiuyong Yang, Xiaoyu Guo and Xingming Hou
Performed data analysis: Jing Wang, Yani Liu
Wrote or contributed to the writing of the manuscript: Jing Wang, Yani Liu, Chuanxia Ju and KeWei Wang

Conflict of interest statement: No author has an actual or perceived conflict of interest with the contents of this article
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Footnotes

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### Table 1. Effect of SCR2682 on alleviation of SNI-induced neuropathic pain

<table>
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<tr>
<th>Days after SNI</th>
<th>Sham</th>
<th>Vehicle</th>
<th>SCR2682 (0.5 mg/kg)</th>
<th>SCR2682 (1 mg/kg)</th>
<th>SCR2682 (2 mg/kg)</th>
<th>SCR2682 + XE991a</th>
<th>RTG (7 mg/kg)</th>
<th>PGB (10 mg/kg)</th>
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<td>12.50 ± 1.63</td>
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<td>15</td>
<td>12.41 ± 0.70</td>
<td>0.88 ± 0.20</td>
<td>1.94 ± 0.40</td>
<td>4.12 ± 1.02*</td>
<td>6.30 ± 0.53***</td>
<td>1.19 ± 0.26***</td>
<td>6.93 ± 1.84###</td>
<td>5.73 ± 1.79###</td>
</tr>
<tr>
<td>17</td>
<td>12.92 ± 0.62</td>
<td>0.76 ± 0.14</td>
<td>2.73 ± 0.17</td>
<td>3.84 ± 0.79†</td>
<td>5.22 ± 0.64***</td>
<td>1.35 ± 0.36***</td>
<td>5.85 ± 0.85###</td>
<td>5.89 ± 1.66###</td>
</tr>
<tr>
<td>19</td>
<td>11.28 ± 0.89</td>
<td>0.63 ± 0.13</td>
<td>1.67 ± 0.45</td>
<td>4.67 ± 1.002###</td>
<td>5.50 ± 0.73***</td>
<td>1.66 ± 0.37***</td>
<td>4.94 ± 0.91###</td>
<td>5.29 ± 0.72###</td>
</tr>
</tbody>
</table>

Note: SCR2682, RTG and PGB were administrated (i.p., once a day) from day 11 to 19 in rats after SNI surgery. The PWMT (g) was determined 1 hour post compound administration. a indicates the administration of SCR2682 (2 mg/kg) plus XE991 (3 mg/kg). ***P < 0.001 vs Sham; #P < 0.05, ##P < 0.01, ###P < 0.001 vs SNI + Vehicle; $P < 0.01, $$$P < 0.001 vs SNI + SCR2682 (2 mg/kg). Data were expressed as the mean ± SEM, n=6-8. Comparison was made by two-way ANOVA, followed by posthoc Bonferroni’s test.
Legends for Figures

Figure 1. Enhancement of native M-currents in DRG neurons and attenuation of neuronal firings by SCR2682.

A) Left panel, representative M-current traces were recorded in DRG neurons with holding potential at -20 mV for activation of M-current and -60 mV for deactivation of M-current. SCR2682 (0.1 μM) or RTG (10 μM) as a positive control enhanced the amplitude of M-current. A specific blocker XE991 (3.0 μM) was used to inhibit the M-current. A compound effect was washed out before addition of another compound. Right panel, a summary for effect of SCR2682 (0.1 μM), RTG (10 μM) or XE991 (3.0 μM) on M-current measured at -20 mV; n=9.  

B) Left panel, representative resting membrane potential (RMP) before and after application of SCR2682 at 0.1 μM or XE991 at 3.0 μM. Right panel, a summary for RMP with -56.7 ± 2.8 mV for control, -66.4 ± 3.7 mV for SCR2682 and -51.7 ± 4.6 mV for XE991.  

C) Left panel, representative traces for action potentials in the absence or presence of SCR2682 (0.1 μM), RTG (10 μM), or XE991 (3.0 μM). The action potential was evoked by injection of 270 pA depolarizing current as indicated at the top of the panel. Right panel, a summary for the numbers of the evoked action potential recorded in cultured DRG neurons in the presence and absence of M channel modulators. *P < 0.05, ***P < 0.001, indicates statistical significance in comparison with the control.
Figure 2. Anti-nociceptive effects of SCR2682 on inflammatory pain induced by CFA.

A) The change of PWMT between 0 and 5 days after CFA injection in rats. n=3. B) The change of PWTL between 0 and 5 days after CFA injection. n=3. *P < 0.05, **P < 0.01, ***P < 0.001 vs sham control C) Effect of SCR2682 (0.5, 1 and 2 mg/kg, i.p.) on mechanical allodynia in rat model of CFA-induced inflammatory pain, n=6-8. D) Effect of SCR2682 (0.5, 1 and 2 mg/kg, i.p.) on thermal hyperalgesia in rat model of CFA-induced inflammatory pain, n=6-8. **P < 0.01 vs Sham; #P < 0.5, ##P < 0.01, ###P < 0.001 vs CFA + Vehicle; $P < 0.5, $$$P < 0.001 vs CFA + SCR2682 (2 mg/kg). Data were expressed as the mean ± SEM. Comparison was made by two-way ANOVA, followed by posthoc Bonferroni’s test.

Figure 3. Effect of SCR2682 on alleviation of SNI-induced neuropathic pain.

A) A schematic drawing of timeline and treatment in rat spared nerve injury (SNI) model of rats. B) Intraperitoneal (i.p.) injections of SCR2682 at different concentrations of 0.5, 1 and 2 mg/kg were made for 9 days and the PWMT was determined after 1 hour of compounds administration on days 1, 3, 5, 7, and 9. Retigabine (RTG) at 7 mg/kg and pregabalin (PGB) at 10 mg/kg were used as positive controls. ***P < 0.001 vs Sham; ###P < 0.001 vs SNI + Vehicle; $$$P < 0.001 vs SNI + SCR2682 (2 mg/kg). Data were expressed as the mean ± SEM, n=6-8. Comparison was made by one-way ANOVA, followed by posthoc Bonferroni’s test.
**Figure 4. Effect of SCR2682 on locomotor activity.**

A) Representative traces of mice in the open field test after injections of SCR2682 (0.5, 1, 2, 4 and 8 mg/kg, i.p.) and RTG (7 mg/kg, i.p.). B) The total distance traveled in the open field for 5 min after injections of RTG (7 mg/kg) or SCR2682 (0.5, 1, 2, 4 and 8 mg/kg, i.p.). C) The mean travel speed in open field for 5 min after injection of RTG (7 mg/kg, i.p.) or SCR2682 (0.5, 1, 2, 4 and 8 mg/kg, i.p.). *$P < 0.05$, **$P < 0.01$ vs Vehicle. Data were expressed as the mean ± SEM, n=6-7. Comparison was made by one-way ANOVA, followed by posthoc Bonferroni’s test.

**Figure 5. Effect of SCR2682 on the expression levels of KCNQ2 in DRG of rats with neuropathic pain induced by SNI.**

A) Quantification of the relative changes in KCNQ2 mRNA expression. B) **Top panel:** Representative western blots of KCNQ2 and GAPDH in ipsilateral L4 and L5 DRG from 9 days after saline- and compounds-injected rats. **Bottom panel:** Quantification of the relative changes in KCNQ2 protein expression. *$P < 0.05$ vs SNI + Vehicle, #$P < 0.05$ vs SNI + SCR2682 (2 mg/kg). Data were expressed as the mean ± SEM, n=6. Comparison was made by one-way ANOVA, followed by posthoc Bonferroni’s test.
Figure 1

A

-20 mV -60 mV
500 pA 200 ms
SCR2682 RTG Control XE991

B

XE991
SCR2682
RMP (mV)

Time(s)
0 100 200 300 400 500

C

270 pA

20 mV -50 mV
-64 mV
-38 mV

Control SCR2682 XE991

No. of elicited action potentials

Control SCR2682 XE991
Figure 2

A

![Graph A](image)

Days after CFA injection

B

![Graph B](image)

Days after CFA injection

C

![Graph C](image)

Time after administration (h)

D

![Graph D](image)

Time after administration (h)
Figure 3

A

Determination of baseline threshold
↓ 1 day ↓ Surgery
↓ 1 day ↓ Determination of PWMT (every other day for 9 days)
↓ 1 day ↓ Administration of drugs (i.p., once a day for 9 days)

B

Administration of drugs

Days after surgery

PWMT (g)

- Sham
- SNI+Vehicle
- SNI+SCR2682 (0.5)
- SNI+SCR2682 (1)
- SNI+SCR2682 (2)
- SNI+SCR2682 (2)+XE991 (3)
- SNI+RTG (7)
- SNI+PGB (10)