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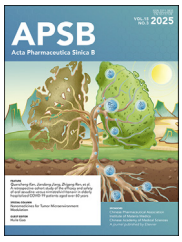

ORIGINAL ARTICLE

A synthetic peptide, derived from neurotoxin GsMTx4, acts as a non-opioid analgesic to alleviate mechanical and neuropathic pain through the TRPV4 channel

ShaoXi Ke[a,b,†], Ping Dong[a,c,†], Yi Mei[a,†], JiaQi Wang[a,†], Mingxi Tang[d,e,†], Wanxin Su[a,†], JingJing Wang[a,†], Chen Chen[a], Xiaohui Wang[a], JunWei Ji[a], XinRan Zhuang[a], ShuangShuang Yang[a], Yun Zhang[a], Linda M. Boland[f], Meng Cui[g], Masahiro Sokabe[h,i], Zhe Zhang[a,j,*], QiongYao Tang[a,j,*]

[a]*Jiangsu Province Key Laboratory of Anesthesiology, Xuzhou Medical University, Xuzhou 221004, China*
[b]*The Second Affiliated Hospital of Zhejiang University School of Medicine, Hangzhou 310007, China*
[c]*Ruijin Hospital, Shanghai Jiaotong University School of Medicine, Shanghai 201801, China*
[d]*Department of Pathology, Yaan People's Hospital (Yaan Hospital of West China Hospital of Sichuan University), Ya'an 625000, China*
[e]*Department of Pathology, Affiliated Hospital of Southwest Medical University, Luzhou 646000, China*
[f]*Department of Biology, University of Richmond, Richmond, VA 23173, USA*
[g]*Department of Pharmaceutical Sciences, Northeastern University School of Pharmacy, Boston, MA 02115, USA*
[h]*Mechanobiology Laboratory, Nagoya University, Graduate School of Medicine, Nagoya 464-8601, Japan*
[i]*Human Information Systems Lab, Kanazawa Institute of Technology, Kanazawa 921-8501, Japan*
[j]*NMPA Key Laboratory for Research and Evaluation of Narcotic and Psychotropic Drugs, Xuzhou Medical University, Xuzhou 221004, China*

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KEY WORDS

Peptide;

Abstract Mechanical pain is one of the most common causes of clinical pain, but there remains a lack of effective treatment for debilitating mechanical and chronic forms of neuropathic pain. Recently, neurotoxin GsMTx4, a selective mechanosensitive (MS) channel inhibitor, has been found to be effective,

*Corresponding authors.
E-mail addresses: Zhangzhe70@xzhmu.edu.cn (Zhe Zhang), qiongyaotang@xzhmu.edu.cn (QiongYao Tang).
[†]These authors made equal contributions to this work.

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Pain;
Non-opioid analgesic;
Mechanical pain;
TRPV4;
Tolerance addiction;
Mechanosensitive channel

while the underlying mechanism remains elusive. Here, with multiple rodent pain models, we demonstrated that a GsMTx4-based 17-residue peptide, which we call P10581, was able to reduce mechanical hyperalgesia and neuropathic pain. The analgesic effects of P10581 can be as strong as morphine but is not toxic in animal models. The anti-hyperalgesic effect of the peptide was resistant to naloxone (an μ-opioid receptor antagonist) and showed no side effects of morphine, including tolerance, motor impairment, and conditioned place preference. Pharmacological inhibition of TRPV4 by P10581 in a heterogeneous expression system, combined with the use of *Trpv4* knockout mice indicates that TRPV4 channels may act as the potential target for the analgesic effect of P10581. Our study identified a potential drug for curing mechanical pain and exposed its mechanism.

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1. Introduction

Mechanical pain is often caused by maternal labor, surgery, burns, inflammation, and neuropathy, among many other pathological conditions. Despite the relevance of treating mechanical nociception, there is no effective treatment available for the debilitating and chronic forms of mechanical pain[1]. In humans, inflammation and nerve injury can cause abnormal sensory processes that result in exaggerated responses to mechanical stimuli[2,3]. Tissue damage can lead to hypersensitivities, such as allodynia and hyperalgesia, which are usually resistant to analgesics[4-6]. Developing effective treatments to alleviate mechanical pain is a clinically important goal.

Many ion channels are known to mediate certain types of pain. For example, activation of the TRPV1 channel mediates inflammatory thermal hyperalgesia[7]. TRPM8 and TRPA1 mediate pain associated with cold[8-10] and several acid-sensing ion channels (ASIC) subtypes are suggested to be therapeutic targets in peripheral pain conditions[11-13]. Recently, the transient receptor potential vanilloid type 4 (TRPV4) channel was suggested to play an essential role in the mechanical hyperalgesia associated with pronociceptive inflammatory mediators and peripheral neuropathic pain[14-17], and the mechanosensitive (MS) ion channel Piezo1/Piezo2 are also shown to mediate sensitivity to mechanical pain in mice[3]. TRPV4 channel is particularly interesting due to its involvement in the development of hyperalgesia in inflamed tissue[18] and neuropathic pain symptoms[15]. This channel is expressed in sensory neurons and represents an innovative target to tackle pain signaling in models induced by trauma, surgery, chemotherapy, cancer, diabetes, and alcohol intake[19]. A promising approach to reducing mechanical hyperalgesia and nociceptor sensitization is to identify specific blockers for MS channels, the discovery of the targeting molecule is urgently needed for the treatment of mechanical pain.

Polypeptide toxins play a central role in understanding the physiological and pathophysiological functions in ion channel studies, they have the advantages of higher bioactivity, permeability, target potency, selectivity, and safety compared with small-molecule and protein drugs[20]. In the field of pain, they led to important advances in basic research and even in clinical applications[12,21,22]. The neuropeptide GsMTx4, from *Tramutola spatulata*, was reported to inhibit stretch-activated cationic currents in mammalian astrocytes, cardiomyocytes, and the central nervous system[23-26]. This peptide was also shown to reduce mechanical hyperalgesia and neuropathic pain in rats, presumably through its inhibitory effects on certain mechanically sensitive ion channels[1,27]. While this venom has potential clinical applications for treating hyperalgesia[1,27], the complex structure and folding make it a major barrier to drug development.

Structural analysis reveals that the six cysteines in the backbone of GsMTx4 form three cystine knots[28]. These inhibitor cystine-knot (ICK) motifs are common features of venom toxins[28-30]. Sequence comparison between the two mechanosensitive peptides (GsMTx4 and GsMTx2) and with other homologous ICK peptides (*e.g.*, VSTX1 and hanatoxin) shows more similarity between GsMTx4 with other ICK toxins[29,30]. However, none of the ICK peptides have been identified as MS channel blockers, suggesting that the channel-blocking actions of GsMTx4 may be explained by a specific feature of this toxin that is not shared with other peptides[28,30]. Based on the similarity shared by the two MS channel toxins in the backbone folds in loop2 and loop3, we have identified a 17-residue short peptide Pept 01 (named P10581 in this study), which acts to mimic the functional roles of GsMTx4 in inhibiting a mechanosensitive BK (SAKca) channel through the modification of the mechanogating[30].

The primary aim of the present study was to examine the novel effect of P10581 on mechanical hyperalgesia in rodent models. We have demonstrated that this natural toxin-based peptide P10581 selectively produces effective effects in reducing mechanical hyperalgesia and neuropathic pain in a way that is different from morphine. Pharmacological inhibition of TRPV4 by P10581 in expressed HEK293T cells, combined with *Trpv4*-gene deficient mice, we also identified the potential target for P10581 in alleviating mechanical pain. Our findings may offer a new opportunity for the development of a novel non-opioid analgesic in the treatment of mechanical forms of pain.

2. Materials and methods

2.1. Ethics and experimental animals

All animal studies complied with the ARRIVE guidelines[31,32]. Animal care and procedures were approved by the Ethics Committee of Xuzhou Medical University (Xuzhou, China). Experiments were performed on 180−220 g adult Sprague−Dawley (SD) rats (the Experimental Animal Center, Xuzhou Medical University, Xuzhou, China) or C57BL/6J (mixed population of male and female) or $Bk^{-/-}$, and $Trpv4^{-/-}$ knockout mice, backcrossed with C57BL/6J mice for more than 10 generations (GemPharmatech Co., Ltd., China) were used to examine the role of BK/TRPV4 involvements in anti-hyperalgesic effect of peptide.

Corresponding WT littermates (male and female) were used as genetic background controls. The genotype of the mice was confirmed by PCR using genomic DNA extracted from the mouse's tail as a template. The animals were housed in groups of 2−3 for rats or 2−5 for mice at a controlled temperature of 23 ± 1 °C. All animals had free access to food and water under a constant 12 h light−dark cycle (lights on at 7:00 a.m.). On the day of behavioral testing, animals were acclimatized to the environment for at least 30 min before testing. All animal behavior experiments were performed at room temperature (23−25 °C).

All animals were used only once to prevent drug or testing experience from confounding the study. We did not test the possible off-target effect. There were no significantly different effects in the anti-hyperalgesic effects of P10581 between males and females (Supporting Information Fig. S1).

2.2. *Inflammatory pain model*

Carrageenan (Carr.)-induced model of acute inflammatory pain: this was evoked by intraplate injection as described previously[1,12]. In brief, Carr. was injected intradermally (i.d.) into the right hindpaw of rats to induce inflammation immediately after the baseline testing of the paw withdrawal threshold (PWT). Peptides were administered intradermally (i.d. 1200 ng/kg in 5 μL) or intraperitoneally (i.p. 270 μg/kg in 5 μL) 1.5 h after Carr. injection in the same location of the inflamed area to test their effects. The doses of Carr. used were 5 μL for i.d. (1%) for i.d. or 50 μL (2%) for i.p. peptide testing[1,27]. Carr. was injected 30 min later after BL was measured.

CFA-induced model of chronic inflammatory pain: CFA (Complete Freund's Adjuvant) was injected (10 μL, intraplantar) into the plantar surface of the right hind paw[33,34]. Peptide/drugs were administrated by subcutaneous injection as described previously[35]. In brief, the unanesthetized mice in lightly restrained, a 30 G needle (attached to a microsyringe) was inserted through the skin, and the peptide/drug (100 μL volume) was injected into the subcutaneous space. The effects of short peptide P10581 (2 μg/kg) were compared in wild-type and *Kcnma1/Trpv4*-deficient mice 3−5 days after CFA injection. Behavioral tests were performed by an experimenter blinded to experimental conditions.

2.3. *Never injury model of neuropathic pain*

The neuropathic pain model of rats was established using the chronic constriction nerve injury (CCI) model[36]. Briefly, after baseline mechanical thresholds (BL) were measured, rats were anesthetized with isoflurane. Under anesthesia, the left common sciatic nerve of the hindpaw was exposed at the mid-thigh level through the biceps femoris; silk thread was tied loosely around the sciatic nerve until a brief twitch was observed. Great care was taken to secure the ligatures so that the nerve was constricted, but the circulation was not interrupted; the skin was then sutured. The sham control group was subjected to the same surgical operations except for the nerve ligature[36]. Gentamicin (10 mg/mL, 0.2 mL; Solarbio Science & Technology Co., Ltd., Shanghai, China) was injected at the rat's belly (i.p.) to prevent infection. From Day 6, a peptide (i.d. 1.2 μg/kg, in 5 μL) was administered each day, and paw withdrawal thresholds were measured 2 h after injection to determine the anti-hyperalgesic effects of the peptide. The short peptide P10581 reached the maximum/stable anti-hyperalgesic effects on rats from Day 7 after the surgery.

Rats that failed to respond to the mechanical stimuli after Carr. or CCI (before peptide/drug treatment) were excluded from the presentation and data analysis. The equivalent volumes of saline and/or morphine were used as the negative and positive controls, respectively. Morphine was injected 30 min prior to testing.

2.4. *Rotarod test*

The rotarod treadmills (ENV-577M, Med associates, St. Albans, VT, USA) were used to assess the motor coordination of rats[12]. In brief, on the day before testing, rats were trained on a fixed-speed (4 rpm) protocol until they could stay for 30 s. On the same day, rats were placed on the dowel rotating accelerated from 4 to 10 rpm at a constant rate of 5 rpm. The motor coordination of rats was evaluated by the time until the mouse fell from the rod which was recorded as the latency to fall. The effects of the peptide on rat motor impairment were assessed for five days and injected each day.

2.5. *Mechanical nociceptive threshold measurements*

2.5.1. *Randall−Selitto test*

Mechanical nociceptive thresholds were assessed using the hind paw-withdrawal test with a Randall−Sellito analgesia meter (Ugo-Basile Biological Research Apparatus, Comerio-Varese, Italy)[1,27]. Mechanical pressure was continuously increased to the dorsal surface of the affected hind paw by using a blunt conical probe in a Randall−Sillito test instrument until vocalization or a withdrawal reflex occurred while the rats were lightly restrained. The mechanical thresholds of the baseline (BL) were recorded as the mean of 3 measurements (with at least 5-min intervals). After pharmacological reagent injection, the mechanical threshold measurements for each paw were repeated five times at a 5-min interval and averaged to be considered an independent observation.

2.5.2. *Von Frey hair test*

Mechanical nociceptive thresholds were also assessed using Von Frey filaments (Stoelting)[37]. Rats were placed in transparent plastic domes with a metal mesh floor to allow access to the plantar surface of hindpaws. The filament was pressed perpendicular to the plantar surface of the hindpaw with sufficient force applied to cause a slight bucking for 6 s. Sharply withdrawing or flinching immediately after the removal of the filament was considered as a positive response. The force (in grams) producing a 50% likelihood of withdrawal was determined by the "up-down" method[37].

2.6. *Analgesic tolerance tests*

2.6.1. *Peptide/morphine tolerance*

Acute tolerance of peptide/morphine was induced in rats by repeated injections of peptide/morphine (5 mg/kg, i.d.) at 2 h intervals as described previously[38]. In the inflammatory pain model induced by Carr. (1%, 5 μL, i.d.), rats received six consecutive injections of morphine (i.d. 5 mg/kg) or peptide (i.d. 2 μg/kg, 5 μL) at 2 h intervals. In the CFA-induced inflammatory pain model, rats were subjected to Randall−Sellito tests 1 h after peptide/morphine injections. Normal saline (i.d. 5 μL) was used for the negative control.

2.6.2. *Chronic peptide/morphine tolerance*

Chronic tolerance was produced in rats by repeated injection of peptide/morphine (s.c., twice a day) for ~9 days. Briefly, seven days after CCI, rats received 8−9 consecutive administrations of morphine (i.d. 5 mg/kg)[12,39] or peptide (i.d. 8 μg/kg) twice a day (9:00 am and 5:00 pm). Rats were subjected to Randall−Sellito tests 1 h after morphine/peptide injections in the morning. The same amount of normal saline (i.d. 50 μL) was used for negative control.

2.7. *CPP test*

Conditioned place preference (CPP) test to peptide/drug was assessed using identical conditioning boxes that consisted of two chambers (20 cm × 20 cm × 20 cm), one with vertical black-white, and the other with horizontal stripes. The two chambers have different floor textures and were connected with a white central compartment (6 cm × 6 cm × 6 cm) that was not paired during peptide/drug treatment. Prior to testing, mice were habituated for 2−3 days (6 h per day) to the room where the behavioral tests were conducted as described previously[40,41] and then returned to the cage. Mice exhibiting signs of increased anxiety, (*i.e.*, shaking and/or vocalizations) received additional handling until these signs were reduced.

Peptide/drug CPP: before CPP training, the initial preference (pre-conditioning) of the mice for the two chambers was determined in a 15-min session. The mice were placed in the central chamber and were allowed access to the two compartments. Mice did not exhibit initial preference (bias) for the horizontal or vertical striped chamber and were randomly assigned to three groups (saline, morphine, and peptide). Subsequently, mice received eight conditioning sessions (four in the morning and four in the evening). During conditioning, mice in the peptide/drug groups received a saline injection (s.c.) in the morning and were immediately confined to one chamber (saline-paired conditioning chamber) for 30 min. Four hours later, mice in the peptide/morphine group received a peptide/morphine (s.c.) injection in the afternoon and were immediately confined to the other chamber (peptide/morphine-paired conditioning chamber) for 30 min. Mice for the saline group received saline in both conditioning sessions (in the morning and afternoon). The training was repeated for 4 days. 24 h after the final dose injection on Day 5, mice were placed in the central chamber and were allowed to access two chambers for 15 min. Place preference scores were calculated by subtracting the time spent on the drug-paired side during preconditioning from the time spent on the drug-paired side during postconditioning[41].

2.8. *Electrophysiology*

Electrophysiological Macropatch Recording in HEK293: whole-cell patch-clamp recordings were performed in HEK293 to test the effect of peptide P10581 on the mechanosensitive TRPV4 currents induced by GSK101, the selective TRPV4 activator. TRPV4 channels were transiently transfected in HEK293 cells using Lipofectamine™ 2000 (Invitrogen). *Trpv4*-eGFP cDNA was a gift from the laboratory of Dr. Fan Yang (Zhejiang Medical University, Zhejiang, China), where eGFP was used to monitor TRPV4 expression. HEK293 cell cultures were performed as previously described[42]. The pCLamp 10 (Molecular Devices) was used to drive stimulus protocols and data acquisition. Currents were filtered at 2 kHz and digitized at 10 kHz. Whole-cell voltage-clamp recordings were performed 24 h after transfection at room temperature (~25 °C), where minor TRPV4 currents were observed. These currents transfected in HEK293T cells could be activated by TRPPV4 selective activator GSK101, and inhibited by TRPV4 selective inhibitor GSK219. The recording solutions used were the same as used previously described. The pipette (intracellular) were (in mmol/L) are: 140 CsCl, 5 EGTA, 10 HEPES, 2 MgATP, 0.2 NaGTP titrated to pH 7.2 with CsOH, and the bath (extracellular) solution consisted of (in mmol/L): 140 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, titrated to pH 7.4 with NaOH[42]. Ramps (from −100 mV to +100 mV, 400 ms) were continuously repeated every 5 s.

Electrophysiological macropatch recording in oocytes: Two-electrode voltage-clamp (TEVC) recordings[43,44] were used to test the effects of peptide P10581 on the TRPV4 channel induced by exposure to extracellular hypotonic solution. *Xenopus laevis* oocytes were prepared and injected using standard protocols[45-47]. *Trpv4* cDNA was amplified with PCR and subcloned into the *Xenopus* oocyte expression vector pXoom, 3′ of the T7 promoter. The cRNA was prepared using the Ambion mESSAGE mACHINE T7 kit. The cRNA was injected at 10−25 ng/oocyte and TEVC recordings were carried out 2 days after injection. The base bath solution contained 66 mmol/L KCl, 100 mmol/L sorbitol, 1.8 mmol/L $BaCl_2$, and 5 mmol/L K-HEPES, pH 7.2. 4α-Phorbol 12,13-didecanoate (4α-PDD; 3 μmol/L) was added directly to the bath[48]. Sorbitol was omitted from the base solution to form the hypotonic solution[48]. Data were analyzed using both pClamp10 and Origin software.

2.9. *Statistical analyses*

To examine the dose−response relationship of peptides on mechanical hypersensitivity, data are presented as the increases in paw withdrawal threshold (ΔPWT) or latency (ΔPWL) post peptide injection, as shown in Eq. (1):

$$\Delta\mathrm{PWT} = \mathrm{PWT}_{(\text{Post-Drug})} - \mathrm{PWT}_{(\text{Pre-Drug})} \quad \left(\text{or}: \Delta\mathrm{PWL} = \mathrm{PWL}_{(\text{Post-Drug})} - \mathrm{PWL}_{(\text{Pre-Drug})}\right) \tag{1}$$

For comparison of the anti-hyperalgesia effect of P10581 to GsMTx4, the IC_{50} values (half-maximal inhibitory concentrations) for the anti-hyperalgesic effects of drugs were obtained by linear regression with the standard Hill equation[49,50]. Data from patch-clamp recordings were analyzed with Clampfit 10 (Molecular Devices) and plotted with GraphPad Prism 9.0.0 (Dotmatics) or Grapher (Golden).

The data and statistical analysis complied with pharmacology's recommendations and requirements on experimental design and analysis in pharmacology[51]. All peptide/drug treatment on animal experiments and data analysis were conducted blindly. Results are presented as means ± standard error of mean (SEM). Statistical differences were calculated by a Student's *t*-test or a one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test. *P*-value <0.05 was considered as statistically significant.

2.10. *Drugs and chemicals*

The peptide P10581 was commercially synthesized with a purity of >98% (Sangon Biotech, Shanghai, China). The full-length neuropeptide GsMTx4 was purchased from Alomone Labs (Jerusalem, Israel) or BioScience Inc. (TX, USA). All peptides were dissolved in distilled water to make a 5 or 10 mmol/L stock solution and stored at −80 °C. The stock solution was diluted with normal saline freshly at the time of injection or patch clamp

experiment. Other chemicals were purchased from Sigma−Aldrich unless otherwise noted.

3. Results

3.1. The effect of P10581 on inflammatory pain administrated intradermally as determined by Randall−Sellito test

Previously, we identified a short peptide based on the natural toxin GsMTx4, which we call P10581 in this study (Fig. 1A), mimics the action of GsMTx4 to inhibit SAKca channel[30]. In this study, we comprehensively tested whether peptide P10581 alleviates mechanical pain as GsMTx4 does[1,27].

P10581 contains both loop2, loop3, hydrophobic residue Trp-7 and the positively charged residue Lys-8 in loop1 in GsMTx4 (Fig. 1A). MD simulations with the free-product run method present a concave face commonly found in neurotoxins[12,52] and has a shape similar to GsMTx4 (Fig. 1B)[30]. To determine whether P10581 reduces pressure-evoked mechanical pain, rats were subjected to the Randall−Sellito test[1,27]. The paw withdrawal threshold (PWT) was significantly reduced from 201.5 ± 4.5 g

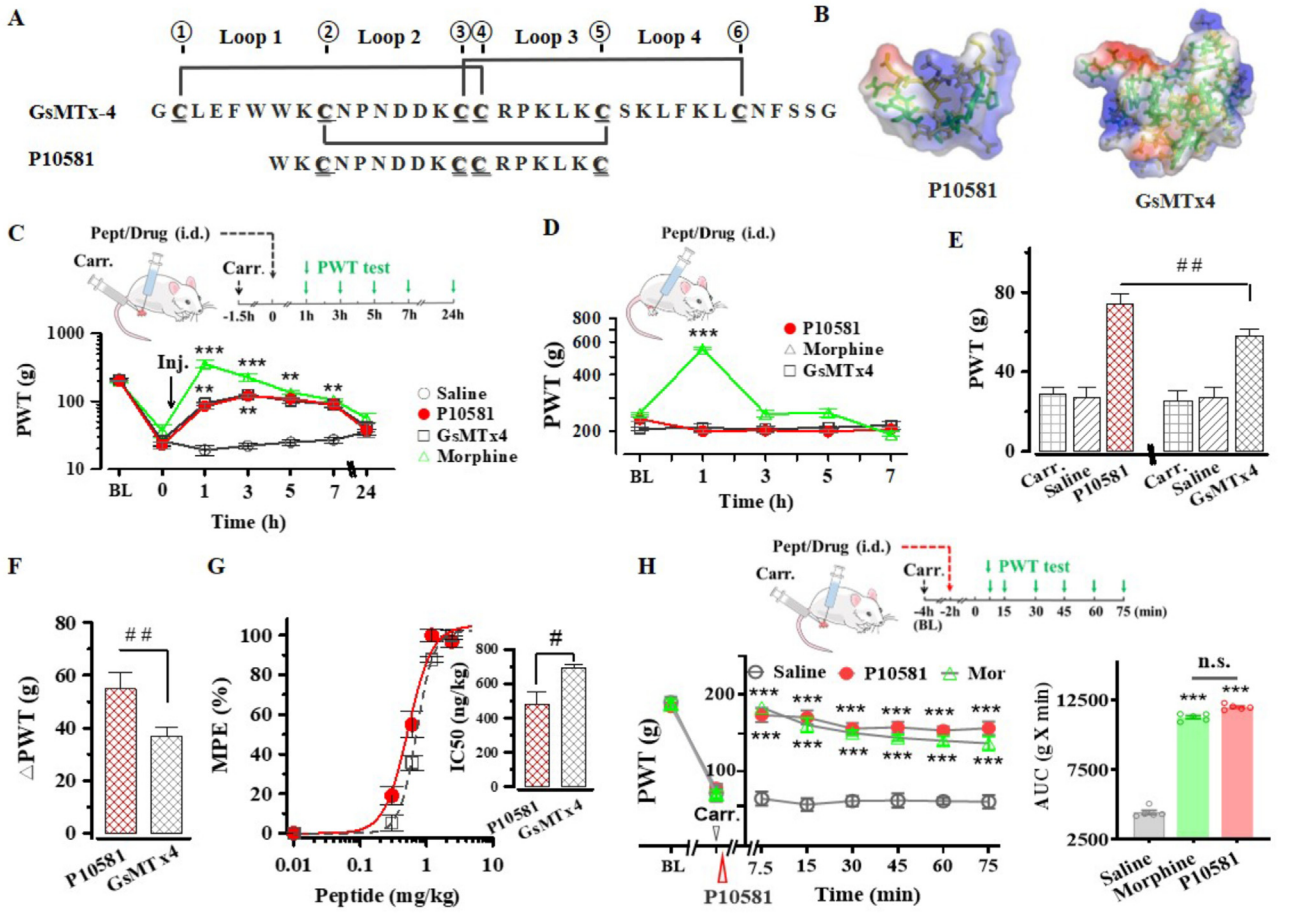

Figure 1 The synthetic peptide P10581 reduced mechanical hyperalgesia in a dose-dependent manner when given intradermally (i.d.). (A) Sequence for P10581 compared to neurotoxin GsMTx4. (B) The structure of P10581 obtained by MD simulations based on the solution structure of GsMTx4 (PDB code 1TYK). (C) The effect of P10581 (i.d. 1200 ng/kg, 5 μL) on mechanical pain induced by pressure by using Randall−Sellito test. Normal saline (i.d. 5 μL) was used for control. The inset above shows the animal protocol. The decreased paw withdrawal threshold (PWT) induced by carrageenan (Carr. 1%, 5 μL) was tested 1.5 h after Carr. injection. BL: Baseline. (D) Evaluation of P10581 (i.d. 1200 ng/kg, 5 μL) on the baseline of the nociceptive mechanical threshold without inflamed. Morphine (i.d. 5 mg/kg) was used for positive control. The effect of GsMTx4 (i.d. 1200 ng/kg, 5 μL) was shown for comparison. $n = 6$ per group (except $n = 10$ for morphine). (E) The effects of peptides P10581 (left) and GsMTx4 (right) on mechanical hyperalgesia when low amounts of peptides were administered intradermally (i.d. 600 ng/kg, 5 μL). $n = 6$ per group. (F) Comparison of analgesic effects (ΔPWT) between P10581 and GsMTx4. ΔPWT = $PWT_{(Post\text{-}Peptide)}$−$PWT_{(pre\text{-}Peptide)}$. ΔPWT were obtained in (E). $n = 6$ per group. (G) Dose-dependent effects (ΔPWT) for P10581 compared with GsMTx4. Rats were subjected to Randall−Sellito tests 2 h after peptide injections. Solid lines are fits to the standard Hill equation. The IC_{50} and Hill coefficient factors obtained were: 481.6 ± 71.2 ng/kg and 2.1 ± 0.6 for P10581, and 689.3 ± 21.5 ng/kg and 3.6 ± 0.2 for GsMTx4, respectively ($n = 5$ per group). The inset compared the IC_{50} for the anti-hyperalgesic effect of P10581 *vs*. GsMTx4. (H) Comparison of the acute anti-hyperalgesic effect of P10581 injected intradermally *vs*. morphine, 0.4 μg (2 μg/kg, i.d.) and 200 μg (1 mg/kg, i.d.) per rat, respectively. Peptide/morphine was administered 1.5 h after Carr. injection, the PWT was tested 2 h after administration. The inset above shows the animal protocol. $n = 5$ per group. Right: the area under the curves (AUC, g·min) calculated from each rat. Data are expressed as mean ± SEM, $^{***}P < 0.001$ and $^{**}P < 0.01$ *vs*. saline. $^{\#\#}P < 0.01$, $^{\#}P < 0.05$, P10581*vs*. GsMTx4.

(baseline, BL) to 23.5 ± 5.3 g after Carr., indicating the significant hyperalgesia induced by Carr. Intradermal (i.d.) injection of P10581 at 1200 ng/kg significantly increased PWT to 85.2 ± 6.6 g at 1 h post-peptide administration, and this effect reached the maximum at 3 h after injection (PWT was 120.6 ± 6.6 g) and lasted for 7 h as long as we tested. This anti-hyperalgesic effect of P10581 is comparable with that of the full-length toxin GsMTx4 under the same conditions (Fig. 1C) and was no longer detectable 24 h after peptide administration. Besides, although morphine increased the baseline of PWT greatly, neither P10581 nor GsMTx4 affected the baseline mechanical nociceptive threshold when the hindpaws were not inflamed (Fig. 1D).

A low dose at 0.6 μg/kg of P10581 (i.d.) modestly increased the threshold of PWT, whereas the same dose of GsMTx4 had only a mild impact on PWT (Fig. 1E), consistent with the peptide P10581 having a greater potency or efficacy than GsMTx4 on mechanical pain (Fig. 1F). The analgesic effects of P10581 and GsMTX4 were dose-dependent (Fig. 1G). The IC_{50}, required to reach half of the maximum effect in reducing the mechanical pain, was significantly lower for P10581 when compared with GsMTx4 (Fig. 1G, insert), indicating a more potent analgesic effect for P10581 when compared with the natural toxin.

We next compared the analgesic effect of P10581 with that of morphine when administered intradermally. Intradermal injection of peptide (0.4 μg per rat) produced a substantial increase in PWT (Fig. 1H), which is comparable with that of morphine (i.d., 200 μg per rat) (Fig. 1H, right), at which morphine reversed PWT to baseline before Carr. We suggested that the analgesic effect of peptide P10581 could be as strong as morphine against mechanical pain when given intradermally.

3.2. *Effect of P10581 on inflammatory pain administrated intraperitoneally as determined by Randall−Sellito test*

We next assessed the anti-hyperalgesic effect of P10581 when administered intraperitoneally (i.p.). As shown in Fig. 2A, administration of neither P10581 nor GsMTx4 (270 μg/kg) affected the baseline mechanical nociceptive threshold when hindpaws were not inflamed. However, following inflammation induced by Carr., the same dose of P10581 significantly increased the PWT in the Randall−Sellito test (from 29.8 ± 4.2 g to 159.9 ± 11.7 g), indicating the effective analgesic effect of peptide on rats (Fig. 2B). The anti-hyperalgesic effect of P10581 showed a dose-dependent effect (Fig. 2C). We did not observe significant differences in analgesic effects between P10581 and GsMTx4 at 270 μg/kg, consistent with the observation in Fig. 2B. Nevertheless, the maximum analgesic effect for P10581 was significantly greater than that induced by GsMTx4 (Fig. 2D), suggesting a greater potency for P10581 when compared with GsMTx4.

We also compared the acute anti-hyperalgesic effect of P10581 with morphine when administrated intraperitoneally. i.p. administration of P10581 (i.p. 0.08 mg/rat) produced a large increase in PWT, which is comparable with that of morphine at a dose of 0.4 mg/rat (Fig. 2E and F), suggesting that the analgesic effect of P10581 may be as strong as morphine on mechanical pain when administrated intraperitoneally.

3.3. *Analgesic effect of P10581 as determined by Von Frey hair test*

To assess the effect of P10581 on inflammation-induced mechanical allodynia, we used a Von Frey hair test[27]. As shown in Supporting Information Fig. S2, significant allodynia was observed after inducing inflammation with Carr., which resulted in a reduced PWT to the application of Von Frey hairs. When P10581 was administered intraperitoneally (i.p. 270 μg/kg, 50 μL), a significant increase in PWT was observed, which was not significantly different from that of GsMTx4 (Fig. S2A). Furthermore, local administration of P10581 (1200 ng/kg, i.d.) injected into the same side of the hindpaw inflamed by Carr., elicited a nearly complete block of Carr.-induced allodynia (Fig. S2B).

Taken together, these results suggest that P10581 reduces inflammation-evoked mechanical pain with an equivalent or greater potency than GsMTx4. The potent effect of P10581 can be as strong as morphine when given either intradermally or intraperitoneally.

3.4. *Effect of P10581 on neuropathic pain*

Neuropathic pain is induced by classic constriction injury of the sciatic nerve (CCI) model, which causes local neuropathy and can last for more than one month[36,53]. After sciatic nerve injury, rats showed signs of neuropathic pain, which was determined by the mechanical PWT subjected to the Randall−Sellito test (Fig. 3A). P10581 (1800 ng/kg) significantly raised PWT each day when compared with the pre-treatment values. It reversed ∼41.2% of PWT (71.5 ± 3.2 g before *vs.* 128.3 ± 5.5 g after), indicating a significant anti-hyperalgesic effect for P10581 on neuropathic pain (Fig. 3B and C), which is comparable with that of GsMTx4 at this tested dose (Fig. 3D).

The anti-hyperalgesic effects of P10581 became more prominent with the increases in the dose tested on Days 6, 7, 8, 10, and 14 after CCI (Fig. 3E), consistent with the dose-dependent effects of the peptide on neuropathic pain. The lower efficacy for P10581 obtained on Day 6 after CCI (Fig. 3E−G) may arise from the inflammation induced by chronic nerve injury. When compared with GsMTx4, the effect of P10581 on neuropathic pain reached a higher level of analgesia (Fig. 3H), suggesting a greater potency for P10581 than GsMTx4 (Fig. 3I).

3.5. *Peptide P10581 fails to reduce thermal or cold pain*

We also investigated whether P10581 modulates hot or cold pain. To measure analgesia to hot pain we measured paw withdrawal latency (PWL) in rats following the application of a thermal laser[1]. The time taken for rats to lick or lift hindpaws was recorded as an indication of hot pain. As shown in Supporting Information Fig. S3A, rats administered with either P10581 or GsMTx4 (i.d. 1200 ng/kg, 5 μL) did not change their PWL when compared to the saline group.

To determine whether P10581 modulates cold pain, the plantar surface of the rat hindpaw was subjected to the noxious cold using dry ice stimulation of the hind paw[54]. Although cold stimuli significantly decreased the rat PWL, intradermal injections of neither P10581 nor GsMTx4 at 1200 ng/kg (i.d. 5 μL) affected PWL following cold stimulus (Fig. S3B). Indeed, we did not observe significant differences in PWL response to cold stimuli even with a higher dose (∼4800 ng/kg) of the peptide (Supporting Information Fig. S4).

In conclusion, peptide P10581 fails to reduce either thermal or cold pain. These results may suggest a selective action of this nature-based peptide on the mechanisms that induce mechanical hyperalgesia.

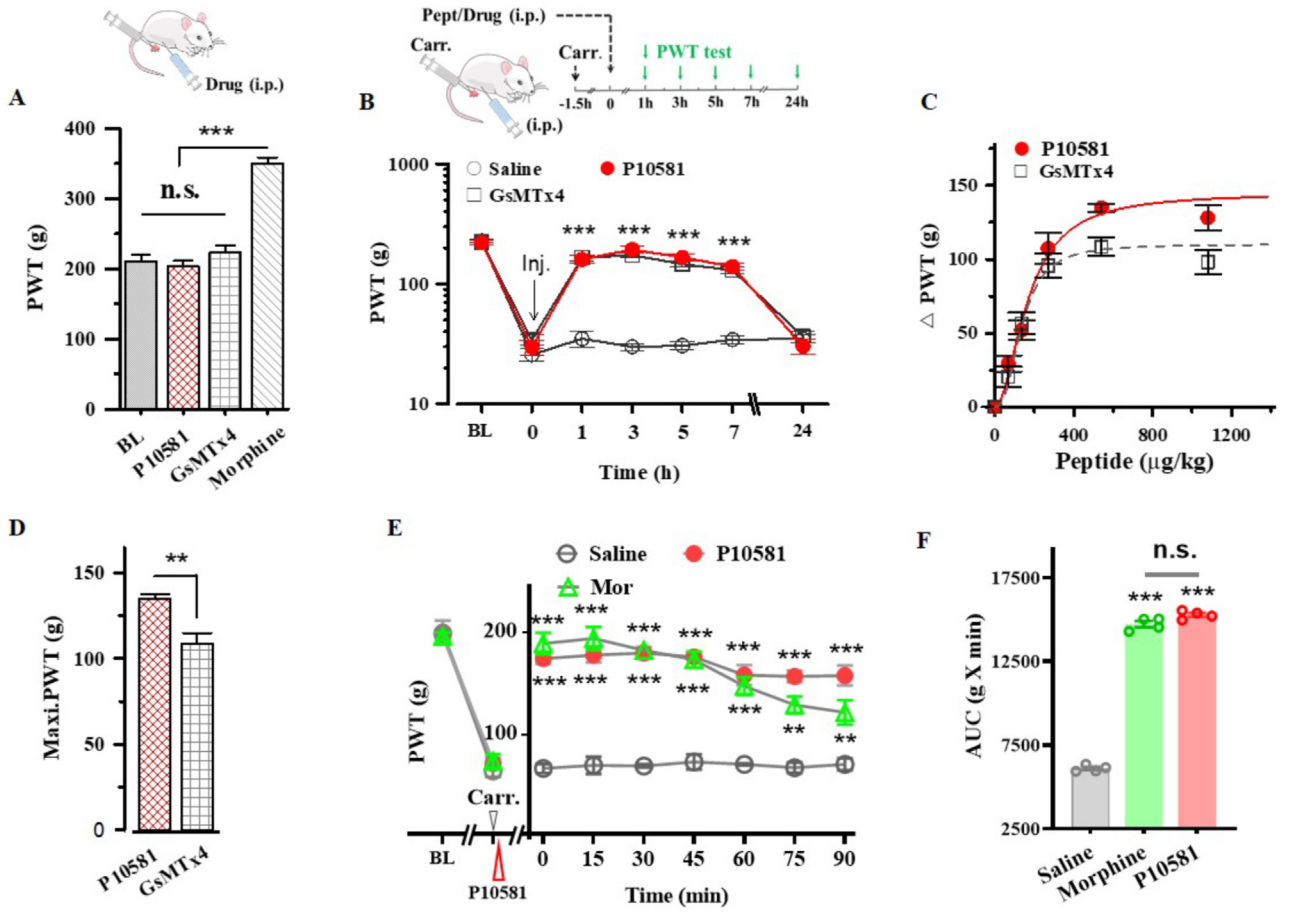

Figure 2 Synthetic short peptide P10581 reduces mechanical hyperalgesia in a dose-dependent manner when given intraperitoneally (i.p.). (A) Evaluation of i.p. injection of P10581 *vs*. GsMTx4 (270 μg/kg, 50 μL) on the baseline of the nociceptive mechanical threshold without inflamed. Morphine (i.p. 10 mg/kg) was used for positive control, $n = 6$ per group. (B) The effect of P10581 on mechanical pain induced by pressure on hindpaws. P10581 (i.p. 270 μg/kg, 50 μL) was administered intraperitoneally 1.5 h after the inflammatory model induced by Carr. (i.d. 1%, 5 μL). Normal saline (i.p. 50 μL) was used for control. $n = 6$ per group. (C) Dose-dependent effect for P10581 (i.p.) on mechanical hyperalgesia induced by Carr. (i.d. 2%, 50 μL) compared with GsMTx4. Rats were subjected to Randall−Sellito tests 3 h after peptide injection. $n = 4$−5 at per dose. (D) Comparison of the maximum anti-hyperalgesic effects (MaximΔPWT) between P10581 and GsMTx4. (E) Comparison of the acute anti-hyperalgesic effect of P10581 *vs*. morphine given intraperitoneally, 0.08 mg (400 μg/kg) and 0.5 mg (2.5 mg/kg) per rat, respectively. (F) The area under the curves (AUC, g·min) calculated from each rat. $n = 4$ per group. The peptide/drug was given intraperitoneally 1.5 h after Carr. (i.d. 2%, 5 μL). Rats were subjected to Randall−Sellito tests 1 h after peptide/drug treatment. Data are expressed as mean ± SEM, $^{***}P < 0.001$ and $^{**}P < 0.01$ *vs*. saline unless specified.

3.6. P10581 does not develop analgesic tolerance

Morphine is a powerful pain reliever, but also a potent inducer of tolerance[12,55]. We tested whether P10581 induces analgesic tolerance on mechanical pain as morphine does.

In the acute inflammatory pain model induced by Carr., rats received six consecutive repeated injections of 2 μg/kg P10581 (i.d.) at 2 h intervals (Fig. 4A), which produced the maximum analgesic effect (Fig. 1). As shown in Fig. 4B, repeated injections of morphine led to a time-dependent decrease in PWT, consistent with the tolerance developed in rats[12,38], whereas, under the same conditions, repeated injections of P10581 did not show a reduction in the anti-hyperalgesic effects, suggesting a lack of development of acute tolerance to peripherally administrated P10581.

In the chronic inflammatory pain model induced by Complete Freund's Adjuvant (CFA): injection of CFA into mouse hindpaw elicits tissue edema and hypersensitivity to mechanical stimulation, which results in a decreased mechanical PWT in the Randall−Sellito test (Fig. 4C). Intrathecal injections (i.t.) of P10581 induced a substantial central analgesic effect (Fig. 4C, inset) in a dose-dependent manner (Supporting Information Fig. S5). To investigate whether P10581 develops tolerance in the inflammatory CFA pain model, rats were subjected to peptide twice a day (at 9:00 am and 5:00 pm) from Day 4 after CFA, for 7 days of consecutive repeated injections (total 14 injections) (Fig. 4D) with 2 μg/kg, which produced the maximum central anti-hyperalgesic effect in mice (Fig. S5). We found central repeated injection of peptide did not reduce the analgesic effects (Fig. 4E), indicating that P10581 did not evoke central analgesic tolerance in rats. In contrast, repeated i.t. injection of morphine completely abolished the analgesic effect. We concluded that the central analgesic effect of P10581 did not develop tolerance.

In the chronic constriction nerve injury model induced by CCI: from Day 8 after CCI, rats were subjected to peptide twice a day

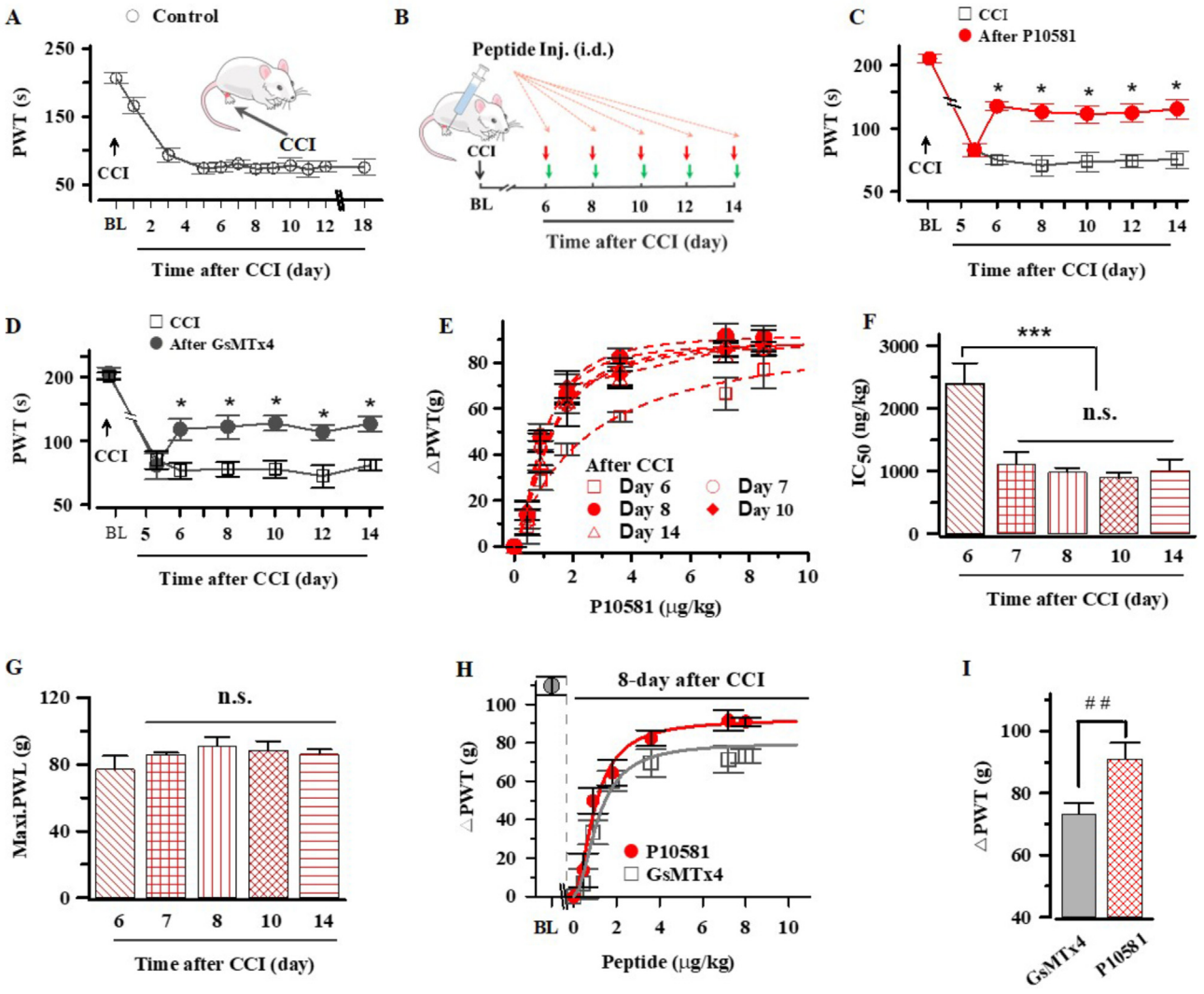

Figure 3 The synthetic peptide P10581 reduces neuropathic pain in a dose-dependent manner. Neuropathic pain was induced by chronic constriction nerve injury (CCI). (A) Rats were subjected to Randall−Sellito tests for neuropathic pain. Nerve injury lasted for 18 consecutive days as long as we tested ($n = 10$). (B) The animal protocol used for (C, D). (C) The effect of P10581 (i.d. 1.8 μg/kg, 5 μL) on neuropathic pain up to 14 days after CCI. $n = 7$ per group. (D) The same as in (C), but for the effect of GsMTx4 (i.d. 1.8 μg/kg, 5 μL), $n = 6$ per group. (E) Dose-dependent analgesic efficacies of P10581 on neuropathic pain obtained on Days 6, 7, 8, 10, and 14. $n = 5$−6 per dose. P10581 was administered intradermally 2 h before the rats were subjected to Randall−Sellito tests. (F) Summary of the IC_{50} for the anti-hyperalgesic effects of P10581 on neuropathic pain on the days as indicated. (G) Summary of the maximum anti-hyperalgesic effects (MaxiΔPWT) of P10581 on neuropathic pain tested on the days as indicated. (H) Comparison of the dose-dependent analgesic effect for P10581 compared with peptide GsMTx4. (I) Comparison of the maximum anti-hyperalgesic effects (MaxiΔPWL) between P10581 and GsMTx4 on neuropathic pain. Rats were subjected to Randall−Sellito tests 2 h after peptides injection (i.d.) on Day 8 after CCI. $n = 4$−5 per dose. Data are expressed as mean ± SEM, $^{**}P < 0.01$ and $^{*}P < 0.05$, post-treatment *vs*. pre-treatment unless specified.

(i.d., at 9:00 am and 5:00 pm) for 9 consecutive days (Fig. 4F). As a positive control, repeated injections of morphine (5 mg/kg, i.d.) led to a time-dependent decrease in PWT, and completely lost its anti-hyperalgesic effect from Day 4 (Fig. 4G). Interestingly, further injection of P10581 (7.2 μg/kg, i.d.) overcame the tolerance induced by morphine, suggesting again that peptide P10581 evokes an anti-hyperalgesic effect by a mechanism that may be distinct from that by morphine. On the other hand, repetitive injections of P10581 (18 total) did not produce a significant reduction in PWT (Fig. 4H and I). In another independent test, IC_{50} obtained for the analgesic effect of P10581 on Day 8 after CCI (before repeated injections) was not significantly different from that obtained on Day 16 (after 9 days of repeated injections, Fig. 4J and K), demonstrating again that peptide P10581 did not evoke tolerance on neuropathic pain.

3.7. P10581 does not produce CPP

Opioids are the mainstay of pain treatment in the current clinic. However, prolonged use of opioids is commonly accompanied by burdensome side effects[56]. And the addiction produced is a severe crisis for public health. To examine whether P10581 produces addition, the standard conditioned place preference (CPP) model[41] was conducted. First, we identified that P10581 does not affect

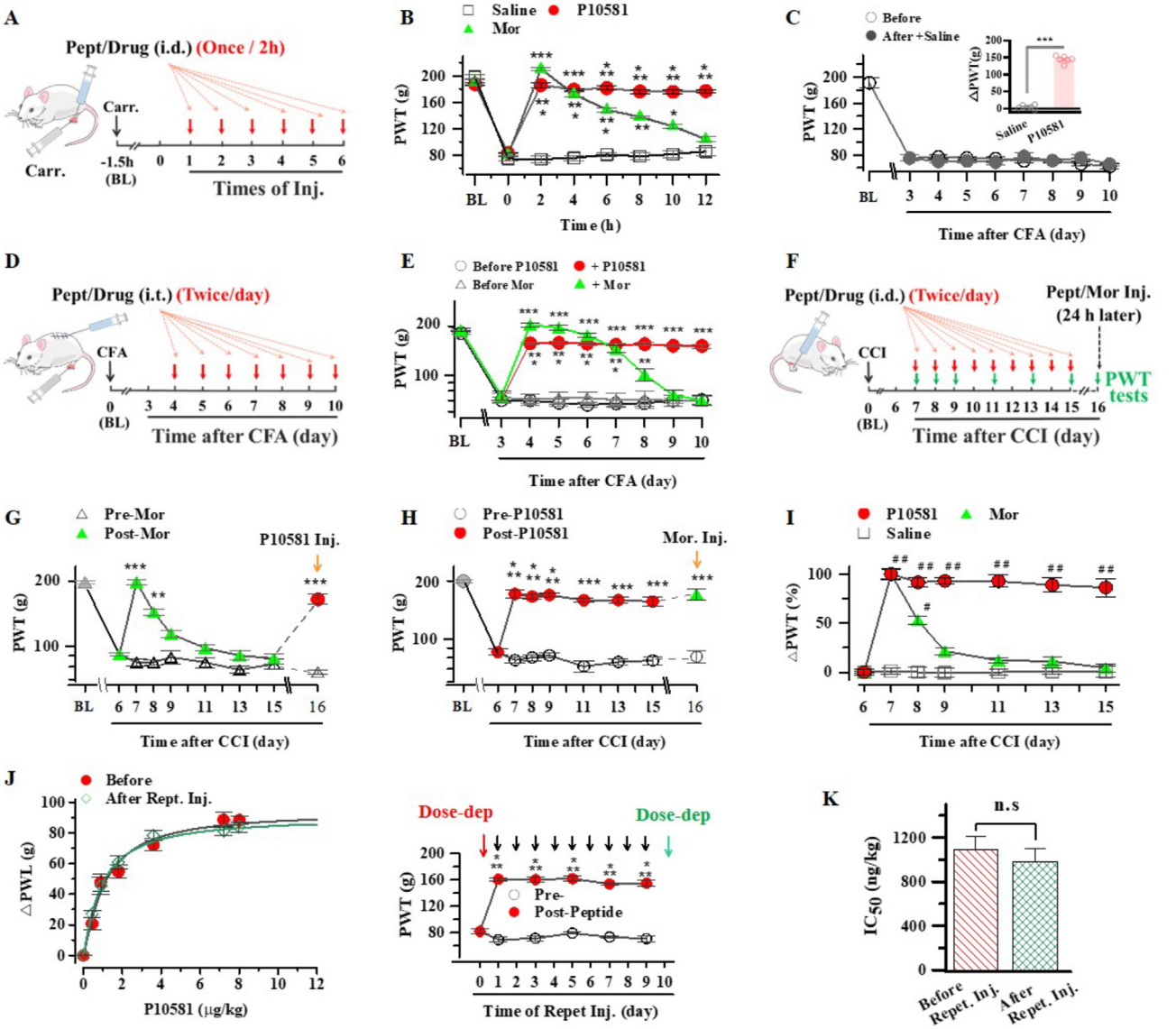

Figure 4 The analgesic effects of peptide P10581 do not develop analgesic tolerance in both inflammatory and neuropathic pain. (A) Animal protocol for repeated injections of peptide/drug after carrageenan for (B). (B) Repeated injections of P10581 (2 μg/kg, 5 μL, i.d.) did not reduce the analgesic effect in Carr. (i.d. 1%, 5 μL) inflammatory pain model, compared with morphine (i.d. 5 mg/kg). Peptide/morphine was given every 2 h for 12 h after. Rats were subjected to Randall−Sellito tests 1 h after peptide/drug injections. $n = 6$ per group. $^{***}P < 0.001$, $^{**}P < 0.01$ and $^{*}P < 0.05$, peptide/drug *vs*. Saline. (C) Injection of CFA into mouse hindpaw results in a decreased mechanical PWT in the Randall−Sellito test. Inset showing the analgesic effect of P10581 (i.t. 2 μg/kg, 5 μL) after CFA. (D) Animal protocols for repeated injections of peptide/drug after CFA for (E). (E) Repeated intrathecal (i.t.) injections of P10581 (2 μg/kg, 5 μL, twice a day) did not induce tolerance compared with morphine in CFA pain model. Peptide/drugs were given twice a day (at 9:00 am and 5:00 pm) for 7 consecutive days from Day 4 after CFA. Rats were subjected to Randall−Sellito tests 1 h after peptide/drug injections at 9 am. $n = 6$ per group. (F) Animal protocol for repeated injections of peptide/drug after CCI for (G−I). (G) 9 days of consecutive repeated injections of morphine (i.d. 5 mg/kg, twice a day) developed tolerance on neuropathic pain in CCI, whereas P10581 (red circle, i.d. 7.2 μg/kg, 5 μL) mostly reversed the tolerance induced by morphine. The effect of P10581 on Day 16 (after CCI) was obtained 24 h after the final injection of morphine. $n = 6$ per group. (H) the same as in G but for the effect of P10581 (i.d. 8 μg/kg, 5 μL, twice a day). The effect of morphine on Day 16 (after CCI) was obtained 24 h after the final injection of P10581. $n = 7$ per group. (I) Comparison of the normalized analgesic effects (ΔPWL (%)) for P10581 *vs*. morphine (obtained from G and H). ΔPWT (%) = $[(PWT_{(Post\text{-}Peptide/drug)} - PWT_{(Pre\text{-}Peptide/drug)})/(\text{Maximum } \Delta PWT)] \times 100$. (J) The dose-dependent analgesic efficacies for P10581 that were obtained before (Red arrow in inset) *vs*. after (Green arrow in inset) 9 days of consecutive repetitive injections (twice a day, at 9:00 am and 5:00 pm). The dose-dependent effect of P10581 after repetitive injections was obtained 24 h after the final dose administration. $n = 4-6$ per dose. Solid lines are fit to the standard Hill equation. $n = 4-6$ per dose. (K) Summary of IC_{50} obtained in (J). Data are expressed as mean ± SEM, $^{***}P < 0.001$, $^{**}P < 0.01$ and $^{*}P < 0.05$ for (C−I), $^{\#\#}P < 0.01$, $^{\#}P < 0.05$, Post-treatment *vs*. pre-treatment. n.s. not significantly different.

motor behavior evaluated by the Rotarod test (Supporting Information Fig. S6). To assess whether P10581 produces CPP, mice were randomly divided into three groups (saline, morphine, and P10581) and were habituated for 3 days (6 h/day) to the experimental room. The initial preferences (pre-conditioning) for two chambers (horizontal or vertical) were determined in 15 min on Day 0 (T1, Fig. 5A). Then each mouse was handled twice daily (from Days 1−4) and received eight conditioning sessions (s.c., morning saline and afternoon peptide/drug). In each session, mice were constrained in the chamber for 30 min (*e.g.* saline in the horizontal chamber, and peptide/morphine in the paired vertical one). 24 h after final dose injection on the test day (Day 5), mice were placed in the central chamber and the preferences for two chambers (horizontal or vertical) were determined for 15 min (T2, Fig. 5A). We found that morphine-paired group (10 mg/kg) significantly increased the time spent in morphine-paired (vertical) chamber, where mice were confined for 30 min in each session after morphine injection (Fig. 5B and C, middle). In contrast, 2 μmol/L/kg P10581, which produces the maximum analgesic effect in mice, did not show significant place reference. The preference scores for the P10581-paired group did not exhibit a significant increase compared to the saline group, whereas the morphine-paired group significantly increased CPP scores (Fig. 5D). We concluded that peptide P10581 does not exert conditioned place preference as morphine does.

3.8. *The effect of P10581 exerts potent resistance to naloxone*

To determine the role of the μ-opioid receptor in this process of the analgesic effect of peptide, naloxone, an μ-opioid receptor antagonist, was given subcutaneously (2 μg/kg) 2 h after peptide/morphine was administered (to ensure the maximum analgesic effect produced by peptide). Although naloxone completely prevented the increase in PWTs induced by morphine, P10581 (i.d. 2 μg/kg) showed a large increase in PWTs either in the absence or presence of naloxone (Fig. 5E), suggesting that naloxone did not significantly prevent the analgesic effect of P10581 (Fig. 5F). We concluded that the short peptide may not act as opioid-dependent analgesia in mice.

3.9. *TRPV4, rather than the BK channel, is required for the analgesic effect of P10581 in mice*

BK channels are expressed in sensory neurons and mediate both inflammatory and neuropathic pain in mice. We first tested whether BK channels contribute to the analgesic effects of P10581 by using a BK channel opener and a BK conditional knockout (*Kcnma1*-cKO) mice. As shown in Supporting Information Fig. S7, BK channel opener NS1619 injected intrathecally (i.t. 10 μg/kg, 10 μL) did not alter the analgesic effect of P10581 administered intraperitoneally. In addition, the deletion of *Kcnma1*-gene (Cre-*Bk*-cKO) also did not affect the anti-hyperalgesic effect of P10581 (2 μg/kg, 100 μL) administered subcutaneously (Supporting Information Fig. S8). Thus, it is most likely that BK (including both SAKca and regular BK) channels are not the molecular target for the analgesic effect of P10581. These results are consistent with our previous report. Although P10581 acts as a potent inhibitor of mechanosensitive BK (SAKc) channels in the heart, it does not inhibit regular/canonical BK (*e.g.* mSlo1) that are wildly expressed in peripheral and central neurons and have been shown to lack mechanosensitivity[30].

The mechanosensitive TRPV4 has been implicated in pathological pain conditions, including various inflammatory and neuropathic pain, but not in cold hypersensitivity[27,54]. Interestingly, GsMTx-4 inhibits the hyperalgesia for mechanical and hypotonic stimuli, to the same extent as treatment with oligodeoxynucleotides (ODN) antisense to *Trpv4*[27]. We used the *Trpv4*-gene deficient (*Trpv4*-KO) mice to assess the involvement of TRPV4 channels in the analgesic actions of P10581 (Fig. 6A). The genotypes for *Trpv4* KO mice were determined by PCR (Fig. 6A and B) using the primers (Table 1). Homozygous *Trpv4* ($Trpv4^{-/-}$) mice showed a significantly improved mechanical PWT (BL) when compared with wild-type (WT) littermates, a hallmark of mechanical hyperalgesia (Fig. 6C). The difference observed in our experiments for the increased baseline to other reports, may arise from the different methods used for the tests of the mechanical hyperalgesia. This is possible as TRPV4 channel expression is increased in wild-type channels, it is sensitive to shell-stretch and contributes to mechanical hyperalgesia[57].

Injection of CFA decreased the mechanical PWT and developed a similar degree from Days 3−10 (after CFA) (Fig. 6C). Subcutaneous (s.c.) injection of P10581 (2 μg/kg) in WT ($Trpv4^{+/+}$) mice significantly increased PWT of the hind paws (Fig. 6D), confirming the peripheral analgesic effects of P10581 evoked on wild-type mice. The anti-hyperalgesic effect of the peptide lasted 3 h as long as we tested. Interestingly, the same dose of P10581 showed a reduced analgesic potency in $Trpv4^{+/-}$ (Fig. 6D; at 1 h post-injection) and mostly abolished in *Trpv4*-deficient mice (Fig. 6E). In fact, P10581 was mostly ineffective on both $Trpv4^{+/-}$ and $Trpv4^{-/-}$ mice at 2 h (Fig. 6F) or 3 h (Fig. 6G) after injections, suggesting that the TRPV4 channel plays a necessary role in the anti-hyperalgesic effect developed by P10581.

Since morphine was equally effective in the anti-hyperalgesic effects among wild-type and *Trpv4*-deficient mice (Fig. 6H), it is most likely that P10581 produces its analgesic effect through a pain pathway or mechanism different from that accessed by morphine, consistent with the result that the analgesic effect of P10581 is resistance to naloxone (Fig. 5E and F).

3.10. *The peptide P10581 inhibits the TRPV4 channel when heterologously expressed in HEK 239T or Xenopus oocytes*

To further investigate the direct functional role of TRPV4 played in the analgesic actions of P10581, we examined the inhibitory effect of the peptide on TRPV4 by using whole-cell current recordings when they are heterologously expressed in HEK 239T cells. We observed some basal currents for TRPV4 channels under our recording conditions (∼25 °C), it is possible as the TRPV4 channel can be partially activated at 24−27 °C. Extracellular application of a potent and selective TRPV4 activator GSK101 elicited a robust increase in the currents, and this GSK101-evoked-TRPV4 current was completely inhibited by further application of peptide P10581 (5 μmol/L) in the intact cell from the extracellular side (bath solution) of the cell (Fig. 7A and B). The remaining (basal) currents were not further inhibited by the TRPV4 selective inhibitor GSK219, suggesting that non-mechanosensitive basal currents may arise from unknown endogenous currents in HEK293 cells.

It has been reported that the TRPV4 channel can be directly activated by mechanical force produced with hypotonicity[48], whereas GsMTx-4 markedly reduces nociceptive flinching in response to hypotonic stimulation. Thus, we asked whether P10581 could inhibit the TRPV4 current that is activated by hypotonicity. As shown in Fig. 7C and D, again, extracellular

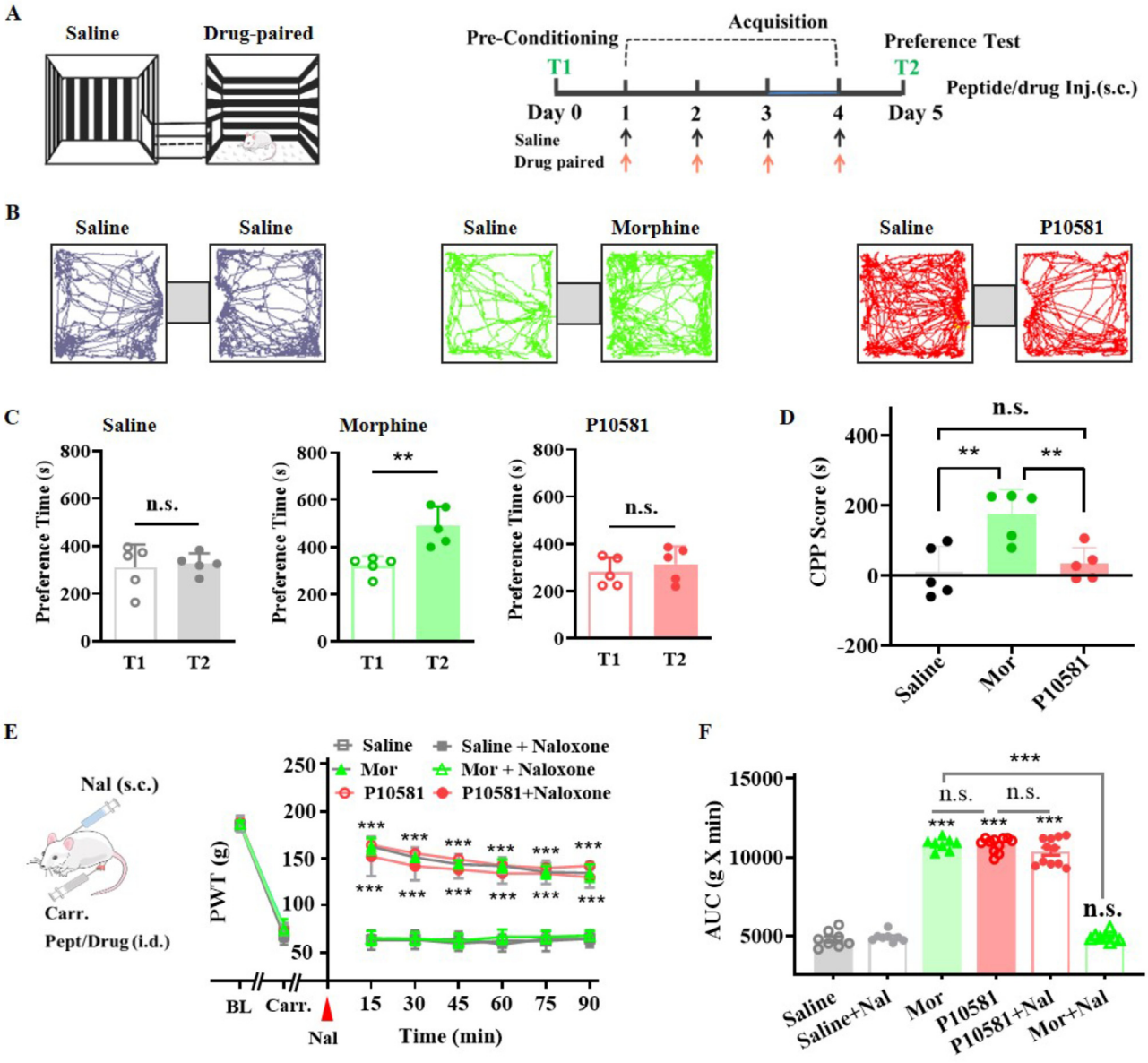

Figure 5 Peptide P10581 does not produce conditioned place preference (CPP) and exerts potent naloxone resistance in rats. (A−D) Peptide P10581 did not exhibit CPP. (A) Schematic of the experimental design for training and test of CPP. (B) Representative tracking for Saline (left)-, Morphine (middle)-, and P10581 (right)-paired groups after CCP training (See Method). (C) Mice treated with P10581 (2 μg/kg, s.c. 100 μL), at which produced a maximum anti-hyperalgesic effect, did not produce a significant increase in preference (T2). Saline (100 μL. left)- and morphine (10 mg/kg, s.c. middle)-paired groups were shown for negative and positive controls, respectively. (D) Mice treated with P10581 (2 μg/kg, 100 μL, s.c. left) did not produce a significant increase in preference scores relative to the saline-treated group. Preference scores were calculated by subtracting the time spent on the drug-paired side during pre-conditioning from the time spent on the drug-paired side during postconditioning[41]. $n = 5$ per group. (E, F) The analgesic effect of P10581 shows potent naloxone (Nal) resistance in rats. (E) P10581 (i.d. 2 μg/kg, 5 μL) on mechanical pain subjected to the Randall−Sellito tests, showed a large increase in response to PWT either in the absence or presence of naloxone. Note: morphine lost the anti-hyperalgesic effect on mechanical pain after co-application of naloxone. (F) The areas under the curves (AUC, g·min) calculated from each mouse. The peptide/morphine (1 mg/kg) was given 1 h after Carr. (i.d. 1%, 5 μL). Naloxone was injected subcutaneously (2 mg/kg, s.c.) 2 h after peptide/drug administration (to ensure the maximum anti-hyperalgesic effect of P10581). Note: peptide at 2 μg/kg (i.d.) can induce an analgesic effect against acute and inflammatory pain that can be as strong as morphine. Rats were subjected to the Randall−Sellito tests 15 min after naloxone was subcutaneously (s.c.) injected. $n = 8-11$ per group. Data are expressed as mean ± SEM, $^{***}P < 0.001$ and $^{**}P < 0.01$ *vs.* saline unless specified. n.s. not significant difference.

application of peptide P10581 (1 μmol/L) inhibited the hypotonically activated TRPV4 currents to the level of the basal current (before hypotonic solution application). Subsequent application of the TRPV4 selective inhibitor GSK219 did not further decrease the currents. The inhibitory effect of P10581 on TRPV4 was in a dose-dependent manner (Fig. 7E and F).

Taken together, these results demonstrate that peptide P10581 not only inhibits the TRPV4 currents activated by GSK101, but

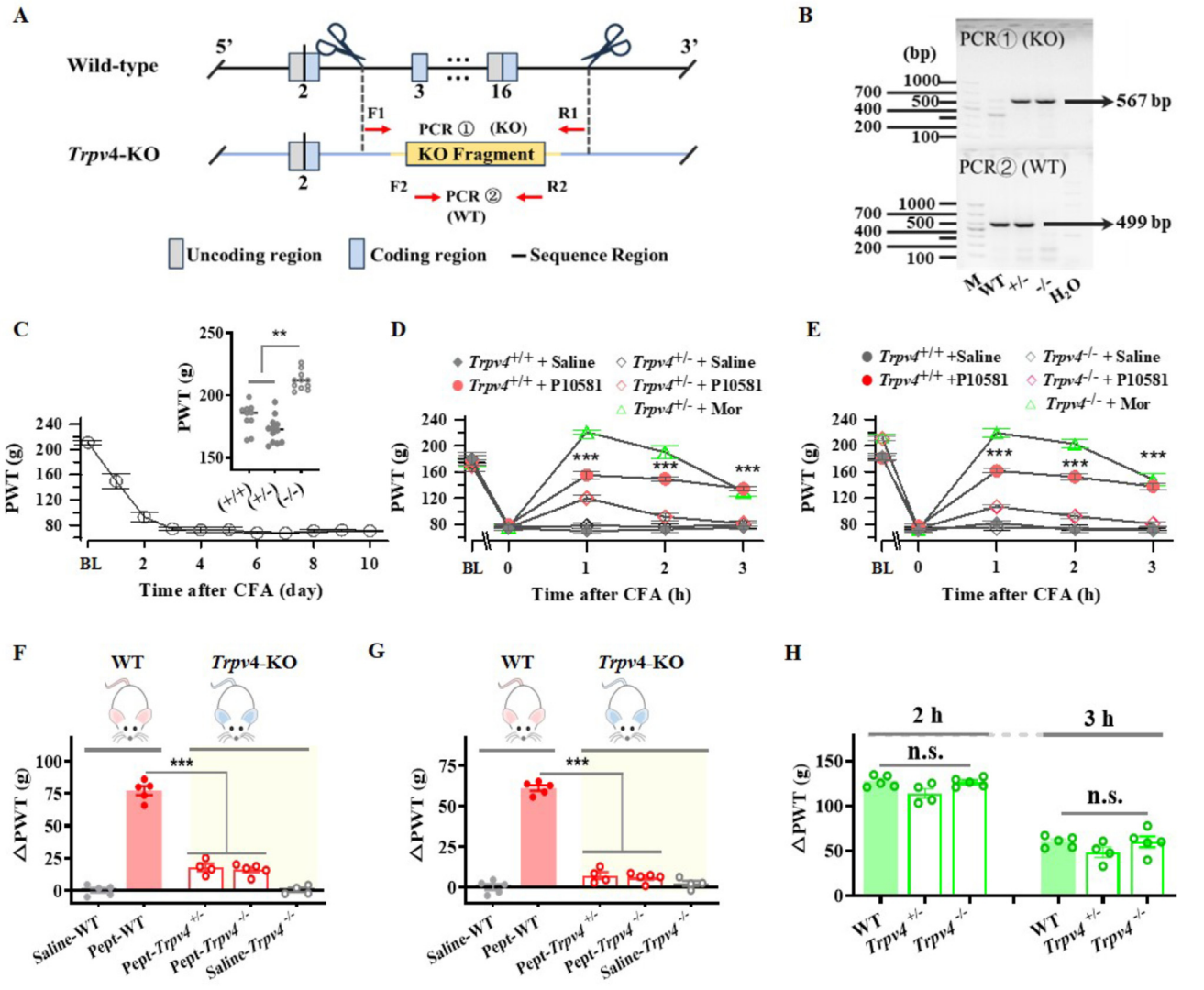

Figure 6 The analgesic effect of peptide P10581 was mostly abolished by *Trpv4*-gene deletion in mice. (A−H) Deletion of *Trpv4*-gene mostly abolished the analgesic effect of P10581: (A) Schematic diagram of *Trpv4* knock-out mice and PCR verification strategies. (B) PCR verifications of KO-fragment deletion in *Trpv4* KO mice. The 499 bp fragment band is generated from *Trpv4* wild-type mice, and the 567 bp fragment band is the expected PCR result of the *Trpv4* KO genotype. The primers used are listed in Table 1. (C) Injection of CFA in homozygous ($Trpv4^{-/-}$) mice decreased the mechanical PWT subjected to the Randall−Sellito test, which lasted for 10 days as long as we tested. $n = 11$. Inset compared the baseline (before CFA) response to mechanical PWT among wild-type ($Trpv4^{+/+}$) littermates, heterozygous ($Trpv4^{+/-}$), and homozygous ($Trpv4^{-/-}$) mice. $n = 10-12$. (D) The analgesic effects of P10581 (s.c. 2 μg/kg, 100 μL) on heterozygous ($Trpv4^{+/-}$) mice. The corresponding wild-type ($Trpv4^{+/+}$) littermates were used for controls. (E) The same as in (D), but for P10581 (s.c. 2 μg/kg, 100 μL) effects on homozygous ($Trpv4^{+/-}$) mice *vs*. WT littermates. (F) Comparison of analgesic effects (ΔPWT) induced by P10581 at 2 h post-injections among WT littermates and *Trpv4*-deficient mice. (G) The same as in (F), but obtained at 3 h post-injections. (H) Comparison of the analgesic effects (ΔPWT) of morphine (5 mg/kg) obtained at 2 h (left) or 3 h (right) post-injections. ΔPWT (g) = $PWT_{(Post\text{-}Drug)} - PWT_{(Pre\text{-}Drug)}$. $n = 4-5$ per group. Data are expressed as mean ± SEM, ***$P < 0.0001$ *vs*. saline. **$P < 0.01$ and *$P < 0.01$, *Trpv4*-deficient mice *vs*. WT littermates. n.s. not significant difference.

also abolishes TRPV4 currents that are activated by hypotonicity (as summarized in Fig. 7G). We propose that peptide P10581 may act as an inhibitor directly targeting the mechanosensitive TRPV4 channel to alleviate mechanical and neuropathic pain in the rodent pain model. Nevertheless, considering that P10581 showed a minor analgesic effect in TRPV4 knockout mice, we cannot rule out the possibility that other pain- and mechano-sensing ion channels (*e.g.*, Piezo) have a minor contribution to the anti-hyperalgesic effect of P10581 (see discussion).

4. Discussion

In the present study, we examined the anti-hyperalgesic effects of a natural toxin-based peptide (P10581) in the rodent models of inflammatory and neuropathic pain and explored the involvement of TRPV4 channel as the potential target for the peptide actions. We found that peptide P10581 effectively reduced the mechanical hyperalgesia and neuropathic pain subjected to the Randall−Sellito or Von Frey hair test, while it did not affect

Table 1 Real-time PCR information for TRPV4.

PCR No.	Primer	Sequence (5′−3′)	PCR products
PCR ①	F1	CGTGGTTCTCAGGAATGATGG	WT: 20460 bp
	R1	TTCCTGACATCTGGGATGATGG	Targeted: 567 bp
PCR ②	F2	TGGGAAGCTCAACTATGGCATC	WT: 499 bp
	R2	CAGGAGAATACTGACAACCGTCAC	Targeted: 0 bp

Note: Wild type: ① PCR reaction obtains a single WT band; ② PCR reaction obtains a single WT band. Heterozygote: ① PCR reaction obtains a WT band and a KO band; ② PCR reaction obtains a WT band. Homozygote: ① PCR reaction obtains a single KO band; ② PCR reaction without product. Note: If the WT band is too large, it may not be possible to obtain a WT band. In Supporting Information Fig. S7, PCR ① was used for genotype identification.

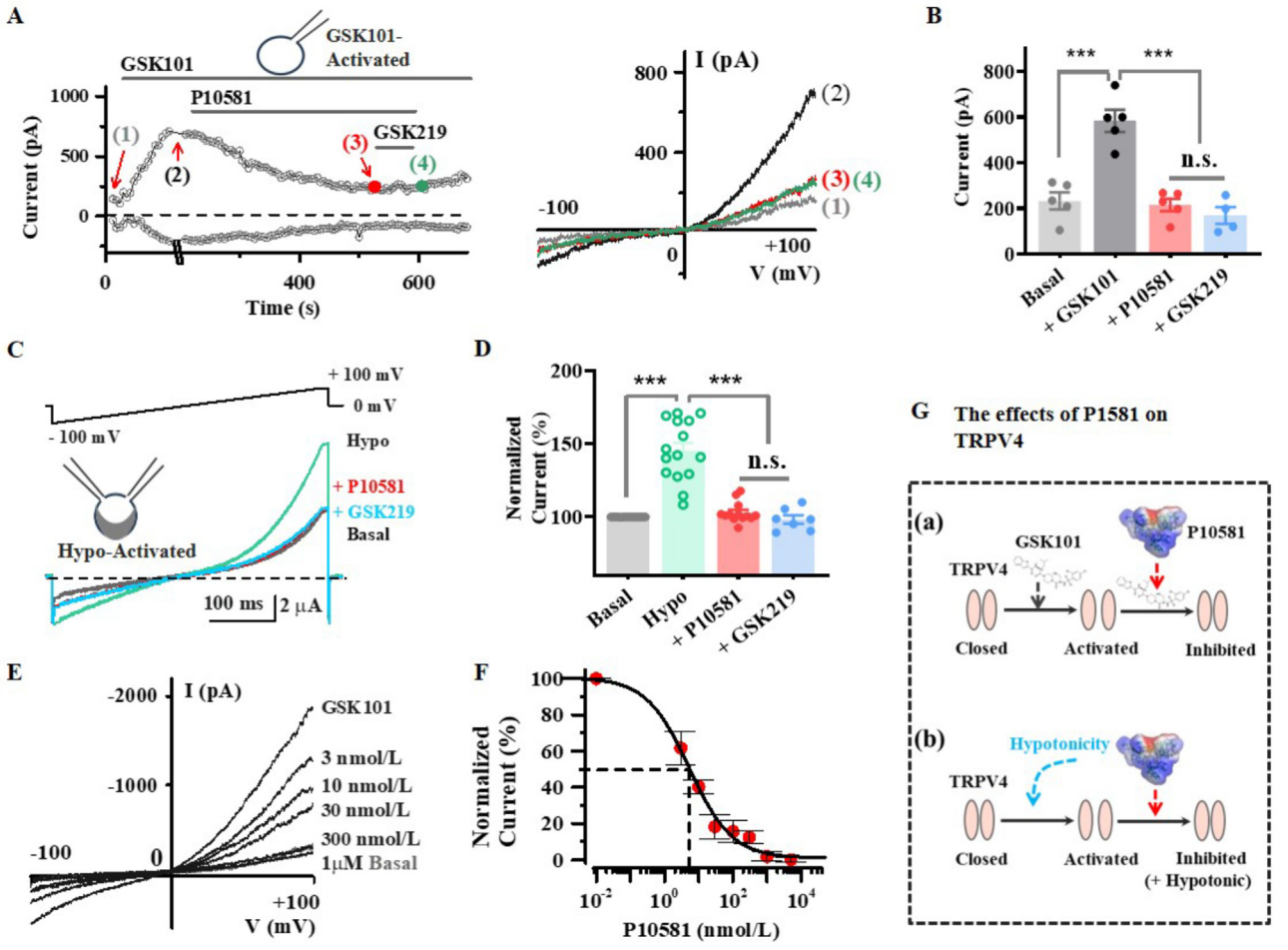

Figure 7 TRPV4 is inhibited by the peptide P10581. (A) Left: representative time course of whole-cell current recordings elicited in HEK293 that were transiently expressed with TRPV4. Right: representative ramp current traces taken at the time points at (1), (2), (3), and (4) from the time course recording. A ramp protocol elicited by a voltage ramp from −100 mV to +100 mV was used. Note, currents evoked by TRPV4 selective activator GSK101 (0.3 μmol/L) at +100 mV were inhibited by co-application of P10581 (5 μmol/L), further application of TRPV4 selective inhibitor GSK219 (1 μmol/L) did not produce further decreases in TRPV4 currents. Horizontal bars show the time durations of applications of GSK101, P10581, and GSK219 from both solutions. (B) Bar summarized the effect of P10581 on GSK101-activated TRPv4 whole-cell currents recorded at +100 mV. Further applied GSK219 did not produce further inhibition on the TRPV4 channel. $n = 4-5$ per group. (C, D) P10581 inhibits TRPV4 currents induced by hypotonicity. (C) Representative whole-cell current elicited in *Xenopus* oocytes that were transiently expressed with TRPV4. Note, TRPV4 current activated by hypotonicity was inhibited by P10581 (1 μmol/L). Currents were recorded by using standard two-electrode voltage clamp (TEVC) recording. (D) The normalized inhibitory effect of P10581 on TRPV4-induced currents by hypotonicity at +100 mV. Further applied TRPV4 selective inhibitor GSK219 did not produce further inhibition. (E, F) Dose-dependent inhibition effects of P10581 on TRPV4-channel. Solid line is fit to the standard Hill equation. The IC_{50} and Hill coefficient factors obtained were: 5.2 ± 1.2 nmol/L and 0.92 ± 0.12 ($n = 4-5$ per dose). (G) Schematic diagrams of the effects of P10581 on the TRPV4 channel, showing that P10581 not only inhibits GSK101-evoked TRPV4 activation (a) but also inhibits the currents activated by hypotonicity. Data are expressed as mean ± SEM, $^{***}P < 0.001$ *vs*. basal currents unless specified.

mechanical nociceptive thresholds to thermal or cold stimuli. The anti-hyperalgesic effect of P10581 is resistant to the opioid antagonist (naloxone) and can be as strong as morphine, but it did not produce the side effects of opioid-induced, *e.g.*, tolerance, addiction, or motor impairment. In addition, we found that the analgesic effect of P10581 was nearly absent in *Trpv4*-deficient mice, suggesting an essential role of TRPV4 in the therapeutic activity of the peptide. Further pharmacological inhibition of TRPV4 by P10581 when expressed in HEK 239T cells, suggested that this mechanosensitive channel may act as the direct target for the anti-hyperalgesic of peptide. These studies show that this short peptide may have considerable potential as a non-opioid analgesic in alleviating the form of mechanical pain, as well as in the treatment of *Trpv4*-driven diseases.

Natural products represent a reliable source of biologically active compounds that may have pharmacological potency and safety, and natural product-based drug design is regarded as one of the effective means for new drug development. The MS channel selective inhibitor GsMTx4 extracted from the venom of the tarantula *Grammostola spatulate*[26,30], showed substantial analgesic effects in reducing mechanical and neuropathic pain, presumably *via* some type of MS channel[1,27]. GsMTx4-based peptide Pept 01 (referred to as P10581 in this study) has been identified as a mechanosensitive BK (SAK_{Ca}) channel inhibitor[30]. In this study, we characterized the novel effects of this peptide in alleviating mechanical pain. In pain models, P10581 effectively attenuated mechanical hyperalgesia and neuropathic pain without significant efficacy on thermal and cold pain even with a considerably higher dose. It is most likely that this peptide acts as a novel analgesic selective to mechanical pain. The result that the P10581 exhibited a strong analgesic effect in reducing inflammation-, and constriction nerve injury-evoked mechanical pain, supports the idea that the regions between loop2 + loop3 in the natural toxin (Trp^{7}−Lys^{23} in GsMTx4) are responsible for its action on the pharmacological blockade on a mechanosensitive BK (SAKca) channel[30]. Nevertheless, the result that the analgesic effects of P10581 were retained in *Kcnma1*-gene-lacking mice (Fig. S6), makes it unlikely that the anti-hyperalgesic effect of peptide functions through BK (including SAKca and regular BK) channel as a target. Although P10581 is a potent inhibitor of mechanosensitive BK (SAKc) channels in the heart, it does not inhibit non-mechanosensitive regular/canonical BK (*e.g.*, mSlo1) that are wildly expressed in peripheral and central neurons[30], the result that the knockout of *Bk* has no impact on the analgesic effect of P10581 is consistent with the previous report.

Peptides possess low immunogenicity and high specificity, and having been increasingly developed as drugs for the treatment of various diseases[58,59]. In this study, we also identified a target channel for the anti-hyperalgesis actions of P10581. The results that the analgesic effect of the peptide was mostly abolished in *Trpv4*-deficient mice suggest that the mechanosensitive TRPV4 channel is a potential therapeutic target for peptide action. The result that IC_{50} for P10581 on TRPV4 is ∼5.2 nmol/L, is approximately consistent with the concentration (1200 ng/kg) we used in mice (that is 7.9 nmol/L, based on the molecular weight and blood volume in mice). Since this type of peptide acts as a gating modifier that targets mechano-gating of the MS channels by partitioning into the cell membrane[26,30,60], the local concentration of peptide accumulated on the cell membrane might be higher than the actual concentration used.

While P10581 analgesia was absent in *Trpv4*-deficient mice, morphine's analgesic effects were retained and not significantly altered by the deletion of *Trpv4*-gene, indicating a lack of a generalized defect in analgesic pathways for morphine in this mouse strain. It is most likely that peptide P10581 produces its anti-hyperalgesic effect *via* a different pathway or mechanism from that of morphine. As naloxone did not significantly prevent the analgesic effect of P10581, we suggest that the peptide may act as a non-opioid analgesic. This is pretty important as a non-opioid analgesic may hold promise to develop safer and non-addictive medications[61,62].

In addition to TRPV4, two GsMTx4-sensitive MS channels TRPC1 and TRPC6, expressed in dorsal root ganglion neurons (DRG), are known to cooperate with TRPV4 to mediate mechanical hyperalgesia and primary afferent nociceptor sensitization[27]. Although these two channels may have distinctive roles, one may assume that the outcome of the analgesic effect for P10581 in this study could also be explained by peptide acting on TRPV4 exerting analgesia of TRPC1/TRPC6-mediated noxious sensations. If TRPC1/TRPC6 were the direct analgesic target of P10581, P10581's effect would have been retained in *Trpv4*-gene-lacking mice. However, this was not the case, suggesting that TRPV4 is the key analgesic and pharmacological target for the anti-hyperalgesic effect of P10581. The minor anti-hyperalgesic effect (a spike, ∼15%) remained in *Trpv4*-deficient mice at 1 h after administration (Fig. 6E and F) suggests that other MS channels may contribute to the analgesic effect of peptide in mice. It has been shown that the mechanosensitive Piezo1/Piezo2 channel is sensitive to GsMTx4, and mediates inflammatory- and nerve injury-induced neuropathic pain in mice and humans[63,64]. Thus, one possibility is that the minor anti-hyperalgesic effect of P10581 remaining in *Trpv4*-KO mice may arise from the inhibition of the Piezo1/Piezo2 channel. Indeed, shear stress can produce a fast and transient, but minor Ca^{2+} influx *via* Piezo1 in *Trpv4* KO mice[65]. Whether P10581 inhibits Piezo1/Piezo2 and/or other pain-sensing ion channels, such as TRPV1 or ASIC, needs to be further identified in future studies. The lack of the anti-hyperalgesic effect of P10581 (tested 2 h post-injection) in both $Trpv4^{+/-}$/$Trpv4^{-/-}$ mice supports the conclusion that TRPV4 plays an essential role in the effect of peptide.

5. Conclusions

In conclusion, we discovered a short peptide derived from the natural toxin GsMTx4. This peptide, we call P10581, exhibited effective anti-hyperalgesic effects on mechanical forms of pain and these actions depend on the mechanosensitive TRPV4 ion channel. The anti-hyperalgesic effect of P10581 is comparable with morphine but does not show analgesic tolerance, addiction, or motor dysfunction as morphine. These findings offer new opportunities for the development of a non-opioid analgesic in the treatment of mechanical forms of pain.

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Author contributions

ShaoXi Ke: Project administration, Methodology, Investigation, Formal analysis, Data curation. Ping Dong: Project administration, Methodology, Investigation, Formal analysis, Data curation. Yi Mei: Project administration, Methodology, Investigation, Formal analysis, Data curation. JiaQi Wang: Project administration, Methodology, Investigation, Formal analysis, Data curation. Mingxi Tang: Writing − review & editing, Methodology, Investigation, Funding acquisition, Data curation. Wanxin Su: Methodology, Formal analysis, Data curation. JingJing Wang: Project administration, Methodology, Formal analysis, Data curation. Chen Chen: Project administration, Methodology, Formal analysis, Data curation. Xiaohui Wang: Project administration, Methodology, Data curation. JunWei Ji: Project administration, Methodology, Data curation. XinRan Zhuang: Project administration, Methodology, Formal analysis, Data curation. ShuangShuang Yang: Project administration, Methodology, Data curation. Yun Zhang: Project administration, Methodology, Data curation. Linda M. Boland: Writing − review & editing, Project administration, Methodology, Investigation. Meng Cui: Writing − review & editing, Methodology, Data curation. Masahiro Sokabe: Writing − review & editing, Supervision, Methodology, Investigation. Zhe Zhang: Writing − review & editing, Supervision, Project administration, Investigation, Funding acquisition, Conceptualization. QiongYao Tang: Writing − review & editing, Writing − original draft, Supervision, Resources, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization.

Conflicts of interest

The authors declare no conflicts of interest exist. Qiongyao Tang, Zhe Zhang, ShaoXi Ke, Ping Dong, and MingXi Tang have applied for a US patent related to this study.

Appendix A. Supporting information

Supporting information to this article can be found online at https://doi.org/10.1016/j.apsb.2024.12.028.

References

1. Park SP, Kim BM, Koo JY, Cho H, Lee CH, Kim M, et al. A tarantula spider toxin, GsMTx4, reduces mechanical and neuropathic pain. *Pain* 2008;**137**:208−17.
2. Costigan M, Scholz J, Woolf CJ. Neuropathic pain: a maladaptive response of the nervous system to damage. *Annu Rev Neurosci* 2009;**32**:1−32.
3. Murthy SE, Loud MC, Daou I, Marshall KL, Schwaller F, Kuhnemund J, et al. The mechanosensitive ion channel Piezo2 mediates sensitivity to mechanical pain in mice. *Sci Transl Med* 2018;**10**:eaat9897.
4. Yamada M, Fujita Y, Hayano Y, Hayakawa H, Baba K, Mochizuki H, et al. Increased expression of fibronectin leucine-rich transmembrane protein 3 in the dorsal root ganglion induces neuropathic pain in rats. *J Neurosci* 2019;**39**:7615−27.
5. Baron R, Binder A, Wasner G. Neuropathic pain: diagnosis, pathophysiological mechanisms, and treatment. *Lancet Neurol* 2010;**9**:807−19.
6. Woolf CJ, Salter MW. Neuronal plasticity: increasing the gain in pain. *Science* 2000;**288**:1765−9.
7. Caterina MJ, Leffler A, Malmberg AB, Martin WJ, Trafton J, Petersen Zeitz KR, et al. Impaired nociception and pain sensation in mice lacking the capsaicin receptor. *Science* 2000;**288**:306−13.
8. Jordt SE, Bautista DM, Chuang HH, McKemy DD, Zygmunt PM, Hogestatt ED, et al. Mustard oils and cannabinoids excite sensory nerve fibres through the TRP channel ANKTM1. *Nature* 2004;**427**:260−5.
9. McKemy DD, Neuhausser WM, Julius D. Identification of a cold receptor reveals a general role for TRP channels in thermosensation. *Nature* 2002;**416**:52−8.
10. Story GM, Peier AM, Reeve AJ, Eid SR, Mosbacher J, Hricik TR, et al. ANKTM1, a TRP-like channel expressed in nociceptive neurons, is activated by cold temperatures. *Cell* 2003;**112**:819−29.
11. Deval E, Noel J, Gasull X, Delaunay A, Alloui A, Friend V, et al. Acid-sensing ion channels in postoperative pain. *J Neurosci* 2011;**31**:6059−66.
12. Diochot S, Baron A, Salinas M, Douguet D, Scarzello S, Dabert Gay AS, et al. Black mamba venom peptides target acid-sensing ion channels to abolish pain. *Nature* 2012;**490**:552−5.
13. Lee JYP, Saez NJ, Cristofori Armstrong B, Anangi R, King GF, Smith MT, et al. Inhibition of acid-sensing ion channels by diminazene and APETx2 evoke partial and highly variable antihyperalgesia in a rat model of inflammatory pain. *Br J Pharmacol* 2018;**175**:2204−18.
14. Alessandri Haber N, Dina OA, Yeh JJ, Parada CA, Reichling DB, Levine JD. Transient receptor potential vanilloid 4 is essential in chemotherapy-induced neuropathic pain in the rat. *J Neurosci* 2004;**24**:4444−52.
15. Alessandri Haber N, Joseph E, Dina OA, Liedtke W, Levine JD. TRPV4 mediates pain-related behavior induced by mild hypertonic stimuli in the presence of inflammatory mediator. *Pain* 2005;**118**:70−9.
16. Alessandri Haber N, Yeh JJ, Boyd AE, Parada CA, Chen X, Reichling DB, et al. Hypotonicity induces TRPV4-mediated nociception in rat. *Neuron* 2003;**39**:497−511.
17. Choi G, Yang TJ, Yoo S, Choi SI, Lim JY, Cho PS, et al. TRPV4-mediated anti-nociceptive effect of suberanilohydroxamic acid on mechanical pain. *Mol Neurobiol* 2019;**56**:444−53.
18. Alessandri Haber N, Dina OA, Joseph EK, Reichling D, Levine JD. A transient receptor potential vanilloid 4-dependent mechanism of hyperalgesia is engaged by concerted action of inflammatory mediators. *J Neurosci* 2006;**26**:3864−74.
19. Rodrigues P, Ruviaro NA, Trevisan G. TRPV4 role in neuropathic pain mechanisms in rodents. *Antioxidants (Basel)* 2022;**12**:24.
20. Wang D, Deng B, Cheng L, Li J, Zhang J, Zhang X, et al. A novel and low-toxic peptide DR3penA alleviates pulmonary fibrosis by regulating the MAPK/miR-23b-5p/AQP5 signaling axis. *Acta Pharm Sin B* 2023;**13**:722−38.
21. Schmidtko A, Lotsch J, Freynhagen R, Geisslinger G. Ziconotide for treatment of severe chronic pain. *Lancet* 2010;**375**:1569−77.
22. Mazzuca M, Heurteaux C, Alloui A, Diochot S, Baron A, Voilley N, et al. A tarantula peptide against pain *via* ASIC1a channels and opioid mechanisms. *Nat Neurosci* 2007;**10**:943−5.
23. Li H, Xu J, Shen ZS, Wang GM, Tang M, Du XR, et al. The neuropeptide GsMTx4 inhibits a mechanosensitive BK channel through the voltage-dependent modification specific to mechano-gating. *J Biol Chem* 2019;**294**:11892−909.
24. Suchyna TM, Tape SE, Koeppe RE 2nd, Andersen OS, Sachs F, Gottlieb PA. Bilayer-dependent inhibition of mechanosensitive channels by neuroactive peptide enantiomers. *Nature* 2004;**430**:235−40.
25. Lee W, Leddy HA, Chen Y, Lee SH, Zelenski NA, McNulty AL, et al. Synergy between Piezo1 and Piezo2 channels confers high-strain mechanosensitivity to articular cartilage. *Proc Natl Acad Sci U S A* 2014;**111**:E5114−22.
26. Suchyna TM, Johnson JH, Hamer K, Leykam JF, Gage DA, Clemo HF, et al. Identification of a peptide toxin from Grammostola

spatulata spider venom that blocks cation-selective stretch-activated channels. *J Gen Physiol* 2000;**115**:583−98.
27. Alessandri Haber N, Dina OA, Chen X, Levine JD. TRPC1 and TRPC6 channels cooperate with TRPV4 to mediate mechanical hyperalgesia and nociceptor sensitization. *J Neurosci* 2009;**29**:6217−28.
28. Oswald RE, Suchyna TM, McFeeters R, Gottlieb P, Sachs F. Solution structure of peptide toxins that block mechanosensitive ion channels. *J Biol Chem* 2002;**277**:34443−50.
29. Nishizawa K, Nishizawa M, Gnanasambandam R, Sachs F, Sukharev SI, Suchyna TM. Effects of Lys to Glu mutations in GsMTx4 on membrane binding, peptide orientation, and self-association propensity, as analyzed by molecular dynamics simulations. *Biochim Biophys Acta* 2015;**1848**:2767−78.
30. Zhou N, Li H, Xu J, Shen ZS, Tang M, Wang XH, et al. Two types of peptides derived from the neurotoxin GsMTx4 inhibit a mechano-sensitive potassium channel by modifying the mechano-gate. *J Biol Chem* 2022;**298**:102326.
31. Percie du Sert N, Hurst V, Ahluwalia A, Alam S, Avey MT, Baker M, et al. The ARRIVE guidelines 2.0: updated guidelines for reporting animal research. *BMJ Open Sci* 2020;**4**:e100115.
32. Lilley E, Stanford SC, Kendall DE, Alexander SPH, Cirino G, Docherty JR, et al. ARRIVE 2.0 and the British Journal of Pharmacology: updated guidance for 2020. *Br J Pharmacol* 2020;**177**:3611−6.
33. Caceres AI, Liu B, Jabba SV, Achanta S, Morris JB, Jordt SE. Transient receptor potential cation channel subfamily M member 8 channels mediate the anti-inflammatory effects of eucalyptol. *Br J Pharmacol* 2017;**174**:867−79.
34. Honda K, Shinoda M, Kondo M, Shimizu K, Yonemoto H, Otsuki K, et al. Sensitization of TRPV1 and TRPA1 *via* peripheral mGluR5 signaling contributes to thermal and mechanical hypersensitivity. *Pain* 2017;**158**:1754−64.
35. Corder G, Tawfik VL, Wang D, Sypek EI, Low SA, Dickinson JR, et al. Loss of μ opioid receptor signaling in nociceptors, but not microglia, abrogates morphine tolerance without disrupting analgesia. *Nat Med* 2017;**23**:164−73.
36. Bennett GJ, Xie YK. A peripheral mononeuropathy in rat that produces disorders of pain sensation like those seen in man. *Pain* 1988;**33**:87−107.
37. Chaplan SR, Bach FW, Pogrel JW, Chung JM, Yaksh TL. Quantitative assessment of tactile allodynia in the rat paw. *J Neurosci Methods* 1994;**53**:55−63.
38. Tian JH, Zhang W, Fang Y, Xu W, Grandy DK, Han JS. Endogenous orphanin FQ: evidence for a role in the modulation of electroacupuncture analgesia and the development of tolerance to analgesia produced by morphine and electroacupuncture. *Br J Pharmacol* 1998;**124**:21−6.
39. Deng M, Chen SR, Chen H, Pan HL. $\alpha 2\delta$-1-Bound *N*-methyl-D-aspartate receptors mediate morphine-induced hyperalgesia and analgesic tolerance by potentiating glutamatergic input in rodents. *Anesthesiology* 2019;**130**:804−19.
40. Portugal GS, Gould TJ. Nicotine withdrawal disrupts new contextual learning. *Pharmacol Biochem Behav* 2009;**92**:117−23.
41. Portugal GS, Al Hasani R, Fakira AK, Gonzalez Romero JL, Melyan Z, McCall JG, et al. Hippocampal long-term potentiation is disrupted during expression and extinction but is restored after reinstatement of morphine place preference. *J Neurosci* 2014;**34**:527−38.
42. Bang S, Yoo S, Yang TJ, Cho H, Hwang SW. Nociceptive and pro-inflammatory effects of dimethylallyl pyrophosphate *via* TRPV4 activation. *Br J Pharmacol* 2012;**166**:1433−43.
43. Tang QY, Zhang Z, Xia J, Ren D, Logothetis DE. Phosphatidylinositol 4,5-bisphosphate activates Slo3 currents and its hydrolysis underlies the epidermal growth factor-induced current inhibition. *J Biol Chem* 2010;**285**:19259−66.
44. Xu XY, Zhang FF, Gan J, Zhang MY, Shen ZS, Guo Q, et al. Identification of the acid-sensitive site critical for chloral hydrate (CH) activation of the proton-activated chloride channel. *J Neurosci* 2023;**43**:526−39.
45. Tang QY, Zhang FF, Xu J, Wang R, Chen J, Logothetis DE, et al. Epilepsy-related slack channel mutants lead to channel over-activity by two different mechanisms. *Cell Rep* 2016;**14**:129−39.
46. Tang QY, Zeng XH, Lingle CJ. Closed-channel block of BK potassium channels by bbTBA requires partial activation. *J Gen Physiol* 2009;**134**:409−36.
47. Xu J, Lv YT, Zhao XY, Wang JJ, Shen ZS, Li J, et al. Identification of sodium- and chloride-sensitive sites in the slack channel. *J Neurosci* 2023;**43**:2665−81.
48. Loukin S, Zhou X, Su Z, Saimi Y, Kung C. Wild-type and brachyolmia-causing mutant TRPV4 channels respond directly to stretch force. *J Biol Chem* 2010;**285**:27176−81.
49. Tang QY, Zhang Z, Meng XY, Cui M, Logothetis DE. Structural determinants of phosphatidylinositol 4,5-bisphosphate (PIP2) regulation of BK channel activity through the RCK1 Ca^{2+} coordination site. *J Biol Chem* 2014;**289**:18860−72.
50. Wang GM, Zhong ZG, Du XR, Zhang FF, Guo Q, Liu Y, et al. Cloning and characterization of the rat Slo3 (K_{Ca} 5.1) channel: from biophysics to pharmacology. *Br J Pharmacol* 2020;**177**:3552−67.
51. Curtis MJ, Alexander S, Cirino G, Docherty JR, George CH, Giembycz MA, et al. Experimental design and analysis and their reporting II: updated and simplified guidance for authors and peer reviewers. *Br J Pharmacol* 2018;**175**:987−93.
52. Escoubas P, De Weille JR, Lecoq A, Diochot S, Waldmann R, Champigny G, et al. Isolation of a tarantula toxin specific for a class of proton-gated Na^+ channels. *J Biol Chem* 2000;**275**:25116−21.
53. Chen K, Wang T, Li Y, Wu J, Zhao CX, Liu S, et al. Rhodojaponin VI indirectly targets Cav2.2 channels *via* *N*-ethylmaleimide-sensitive fusion protein to alleviate neuropathic pain. *Acta Pharm Sin B* 2023;**13**:1326−36.
54. Ehlers VL, Sadler KE, Stucky CL. Peripheral transient receptor potential vanilloid type 4 hypersensitivity contributes to chronic sickle cell disease pain. *Pain* 2023;**164**:1874−86.
55. Bohn LM, Gainetdinov RR, Lin FT, Lefkowitz RJ, Caron MG. μ-opioid receptor desensitization by beta-arrestin-2 determines morphine tolerance but not dependence. *Nature* 2000;**408**:720−3.
56. Fraczek K, Ferraiolo M, Hermans E, Bujalska Zadrozny M, Kasarello K, Erdei A, et al. Novel opioid-neurotensin-based hybrid peptide with spinal long-lasting antinociceptive activity and a propensity to delay tolerance development. *Acta Pharm Sin B* 2020;**10**:1440−52.
57. Swain SM, Romac JM, Vigna SR, Liddle RA. Piezo1-mediated stellate cell activation causes pressure-induced pancreatic fibrosis in mice. *JCI Insight* 2022;**7**:e158288.
58. Zhu Q, Chen Z, Paul PK, Lu Y, Wu W, Qi J. Oral delivery of proteins and peptides: challenges, status quo and future perspectives. *Acta Pharm Sin B* 2021;**11**:2416−48.
59. Wang R, Shen Q, Li X, Xie C, Lu W, Wang S, et al. Efficacy of inverso isomer of CendR peptide on tumor tissue penetration. *Acta Pharm Sin B* 2018;**8**:825−32.
60. Bode F, Sachs F, Franz MR. Tarantula peptide inhibits atrial fibrillation. *Nature* 2001;**409**:35−6.
61. Alsaloum M, Higerd GP, Effraim PR, Waxman SG. Status of peripheral sodium channel blockers for non-addictive pain treatment. *Nat Rev Neurol* 2020;**16**:689−705.
62. Perez de Vega MJ, Ferrer Montiel A, Gonzalez Muniz R. Recent progress in non-opioid analgesic peptides. *Arch Biochem Biophys* 2018;**660**:36−52.
63. Szczot M, Liljencrantz J, Ghitani N, Barik A, Lam R, Thompson JH, et al. PIEZO2 mediates injury-induced tactile pain in mice and humans. *Sci Transl Med* 2018;**10**:eaat9892.
64. Lee W, Nims RJ, Savadipour A, Zhang Q, Leddy HA, Liu F, et al. Inflammatory signaling sensitizes Piezo1 mechanotransduction in articular chondrocytes as a pathogenic feed-forward mechanism in osteoarthritis. *Proc Natl Acad Sci U S A* 2021;**118**:e2001611118.
65. Swartz KJ, MacKinnon R. Mapping the receptor site for hanatoxin, a gating modifier of voltage-dependent K^+ channels. *Neuron* 1997;**18**:675−82.