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Original Reports

Spinal DN-9, a Peptidic Multifunctional Opioid/Neuropeptide FF Agonist Produced Potent Nontolerance Forming Analgesia With Limited Side Effects

Zilong Wang,* Biao Xu,* Changyu Jiang,† Ting Zhang,* Mengna Zhang,* Ning Li,* Qinqin Zhang,* Kangtai Xu,* Dan Chen,* Jian Xiao,* and Quan Fang*

*Key Laboratory of Preclinical Study for New Drugs of Gansu Province, School of Basic Medical Sciences, Lanzhou University, Lanzhou, China, †Department of Pain Medicine, Shenzhen Municipal Key Laboratory for Pain Medicine, Shenzhen Nanshan People's Hospital and the 6th Affiliated Hospital of Shenzhen University Health Science Center, Shenzhen, China

Abstract: **The development of multitarget opioid drugs has emerged as an attractive therapeutic strategy to eliminate opioid-related side effects. Our previous study developed a series of opioid and neuropeptide FF pharmacophore-containing chimeric peptides, including DN-9 (Tyr-*D*.Ala-Gly-NMe.Phe-Gly-Pro-Gln-Arg-Phe-NH_2), which produced potent nontolerance forming analgesia at the supraspinal level. In the present study, the antinociceptive effects of DN-9 in a series of preclinical pain models and the potential side-effects were investigated at the spinal level in mice. In the tail-flick test, intrathecal injection of DN-9 produced potent analgesia with an ED_{50} value at 1.33 pmol, and the spinal antinociception of DN-9 was mainly mediated by μ- and κ-opioid receptors. In addition, DN-9-induced spinal antinociception was augmented by the neuropeptide FF receptors antagonist. Furthermore, DN-9 could decrease both the frequency and amplitude of sEPSCs in lamina IIo neurons of the spinal cord, which were mediated by opioid receptors. In contrast to morphine, chronic intrathecal treatments with DN-9 did not induce analgesic tolerance, c-Fos expression or microglial activation. Intrathecal injection of DN-9 showed potent analgesia with antinociceptive ED_{50} values between .66 and 55.04 pmol in different pain models, including the formalin test, acetic acid-induced writhing test, carrageenan-induced inflammatory pain and neuropathic pain. Moreover, DN-9 did not show side effects in locomotor function and coordination, gastrointestinal transit inhibition, the cardiovascular system, and body temperature regulation at antinociceptive doses. Taken together, the present study showed DN-9 produced effective, nontolerance forming analgesia with reduced side effects at the spinal level. DN-9 might be a promising compound for developing multifunctional opioid analgesics with limited adverse effects.**

Perspective: ***This article presents the potent and nontolerance forming analgesia effects of DN-9 in a series of preclinical pain models with less opioid related adverse effects at the spinal level in mice. This study also demonstrates that DN-9 has translational potential into an intrathecal analgesic.***

Key Words: *Analgesic, neuropeptide FF (NPFF), opioid, pain, analgesic tolerance, opioid dependence.*

Abbreviations: *ANOVA, analysis of variance; AUC, area under the curve; β-FNA, beta-Funaltrexamine; BN-9, Tyr-D.Ala-Gly-Phe-Gln-Pro-Gln-Arg-Phe-NH_2; cAMP, cyclic adenosine monophosphate; CCI, chronic constriction injury; CFA, complete Freund's adjuvant; CPP, conditioned place preference;*

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Address reprint requests to Quan Fang, PhD, Key Laboratory of Preclinical Study for New Drugs of Gansu Province, School of Basic Medical Sciences, Lanzhou University, 199 Donggang West Road, Lanzhou, 730000, China. E-mail: fangq@lzu.edu.cn

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cox-2, cyclooxygenase-2; 1DMe, [D.Tyr^1,(N.Me)Phe^3] NPFF; DN-9, Tyr-D.Ala-Gly-NMe.Phe-Gly-Pro-Gln-Arg-Phe-NH_2; ED_{50}, effective dose 50% of maximum response; GIT, gastrointestinal inhibition; i.t., intrathecal; MAP, mean arterial pressure; %MPE, the percent maximum possible effect; NSAID, nonsteroidal anti-inflammatory drug; nor-BNI, nor-binaltorphimine; NPFF, Phe-Leu-Phe-Glu-Pro-Gln-Arg-Phe-NH_2; NTI, naltrindole; RF9, 1-adamantanecarbonyl-Arg-Phe-NH_2; SNL, spinal nerve ligation; sEPSCs, spontaneous excitatory postsynaptic currents; WHO, World Health Organization.

Pain is one of the leading characteristics of injuries and nearly all diseases. Chronic pain presents a major health burden, and millions of individuals are suffering from various pains worldwide.[1,7,13] According to the Medical Expenditure Panel Survey in 2008, in the United States, approximately 100 million adults were affected by chronic pain and the economic cost of pain was estimated to be between $560 and $635 billion annually.[12] A large number of analgesics, including nonopioid analgesics (eg, COX-2 inhibitors and other nonselective NSAIDs, acetaminophen, ketamine, and ketorolac) and opioid analgesics (eg, morphine, fentanyl, etorphine, sufentanil, and hydromorphone), have been approved.[34,44] However, pain management remains a major problem that physicians must deal with in the clinic.[23]

According to the WHO pain ladder, opioid analgesics should be used for the treatment of moderate to severe pain, including acute and cancer pain. Opioids have also been approved to be an effective and safe treatment for chronic noncancer pain.[4,40] Although opioids have been widely used as analgesics in the clinic, the unwanted side effects, such as tolerance, dependence, constipation, respiratory depression, nausea, sedation, dysphoria, and hallucinations, remain major problems that hamper their clinical usage, particularly in chronic pain.[21,47,49] Chronic opioid treatment may induce significant analgesic tolerance, which results in insufficient pain control and dose escalation to maintain the same analgesic effects. Another common adverse effect is physical dependence. Both tolerance and dependence may lead to opioid overdose and abuse. There are tens of thousands of deaths from opioid overdose in the United States.[36,38] Thus, effective and safe opioid analgesics are urgently needed.

Neuropeptide FF (NPFF) plays an important role in modulating opioid activities.[5,10,31,45] Our previous study developed a multifunctional agonist at opioid and NPFF receptors, BN-9, which produced potent nontolerance forming antinociception.[26,48] Moreover, based on the structure-based optimization of BN-9, we found DN-9 (Tyr-*D*.Ala-Gly-NMe.Phe-Gly-Pro-Gln-Arg-Phe-NH_2), which produced substantially more robust nontolerance forming analgesia in both acute and chronic pain models at the supraspinal level.[41] In addition, DN-9 has been demonstrated to produce robust antinociception in the mouse tail-flick test, formalin test and CFA-induced chronic inflammatory pain after i.c.v. administration.[41] However, supraspinal drug delivery remains an obstacle in the development of centrally acting drugs. Recently, intrathecal (i.t.) drug delivery has been considered an alternative therapy in chronic pain management.[9] Moreover, 2 medications, morphine and ziconotide, have been approved by the US Food and Drug Administration for i.t. analgesia.[6,37] Thus, it is extremely valuable to evaluate the analgesic effects of DN-9 at the spinal level.

In the present study, the spinal antinociceptive profiles of DN-9 were investigated in a series of preclinical pain models, including the tail-flick test, formalin test, acetic acid writhing test, carrageenan-induced inflammatory pain, and neuropathic pain models. Furthermore, its side effects were also evaluated in antinociceptive tolerance, rotarod, conditioned place preference (CPP), physiological dependence, gastrointestinal transit (GIT), body temperature, and cardiovascular tests.

Methods

Animals

The animals used in the blood pressure test were adult male Wistar rats, and the animals used in the other behavioral experiments were adult male KM mice (24−30 g), which were provided by the Experimental Animal Center of Lanzhou University and housed in a standard animal room that was maintained at 22 ± 2°C with a 12-hour light/12-hour dark cycle. The type of facility was specific pathogen free. The animals were provided with free access to food and water. The animal behavioral experiments were approved by the Ethics Committee of Lanzhou University (permit number: SYXK Gan 2009-0005) and were carried out in accordance with the European Community guidelines for the use of experimental animals (2010/63/EU). The animals used in the electrophysiology and immunostaining were C57BL/6 mice. All animals were used only once in the present study and were sacrificed via CO_2 inhalation after the experiments.

Animals were randomly assigned into the experimental groups. Each group included more than 5 animals, and the specific number of animals in each group was indicated in the figure legends. All data were collected and analyzed by 2 independent observers, who were blinded to the group assignment of the animals or the treatment group during analysis.

Chemicals

DN-9 (Tyr-*D*.Ala-Gly-NMe.Phe-Gly-Pro-Gln-Arg-Phe-NH_2) and RF9 used in this study were synthesized by manual solid-phase synthesis using standard N-fluorenylmethoxycarbonyl (Fmoc) chemistry, which was conducted on Rink amide MBHA resin as indicated in our previous report.[41] HBTU, HOBt, and DIEA were used as coupling agents. The established peptides were desalted by Gel

filtration, purified using semipreparative reversed-phase high-pressure liquid chromatography (RP-HPLC) and characterized by RP-HPLC and an electrospray ionization mass spectrometer (ESI-Q-TOFmaXis-4G, Bruker Daltonics). The peptides with purities that exceeded 95% as characterized by analytical RP-HPLC were used in the following studies. Beta-funaltrexamine hydrochloride dihydrate (β-FNA, Tocris, 0926), nor-binaltorphimine (nor-BNI, ab120078), naltrindole hydrochloride (NTI, Tocris, 0740) were purchased from Tocris or Abcam. Naloxone hydrochloride dihydrate (N7758), λ-carrageenan, acetic acid, formalin, sucrose, $NaHCO_3$, KCl, NaH_2PO_4, $CaCl_2$, $MgCl_2$, NaCl, KCl, K-gluconate, EGTA, HEPES, Mg-ATP, picrotoxin, strychnine, and glucose were purchased from Sigma Chemical Company. Morphine sulfate was purchased from Hospira. All drugs used in the behavioral tests were dissolved in sterilized saline and stored in 1.5 mL tubes at −20°C.

Administration of Drugs

The intrathecal injection procedure was conducted in conscious mice according to the report by Hylden and Wilcox.[20] Briefly, a 28-gauge needle connected to a 25-μl microsyringe was directly inserted between the L5 and L6 segment in the mice. Puncture of the dura was indicated by a reflexive lateral flick of the tail or formation of an 'S' shape by the tail. Drugs were injected into the subarachnoid space at a volume of 5 μl. The selective antagonists for μ-, κ- and δ- receptors, ie, β-FNA, nor-BNI or NTI, respectively, were i.t. injected 4 hours, 30 minutes or 20 minutes, respectively, prior to the injection of DN-9. Naloxone and RF9 were i.t. injected 10 minutes or 5 minutes, respectively, prior to the injection of DN-9.

Radiant Heat Tail-Flick Test

The radiant heat tail-flick test was performed as indicated in our previous reports.[26,41] The animals were gently restrained by hand, and the underside of the tail 3 cm from its distal end was placed on the radiant heat source. The time for the mouse to flick its tail off the heat source is defined as the tail-flick latency. The radiant heat intensity was adjusted to produce a baseline response in naÿve mice within 3 to 5 seconds. A cut-off time was set at 10 second to avoid tissue damage. Before the experiments, the mice were allowed to acclimatize to the environment in the behavior room for 30 minutes. The tail-flick latency was determined 3 times before injection as the baseline. The mice with a very high (>5 second) or lower (<3 second) baseline were excluded from the experiments. Tail-flick latency was tested at 10, 20, 30, 40, 50, and 60 minutes after drug injection.

Formalin-Induced Nociceptive Behavioral Test

The formalin-induced nociceptive behavioral test was conducted following our previous protocol.[26] The animals were acclimatized in a transparent acrylic observation chamber with a mirror positioned below the floor for a period of 15 minutes. Five minutes after drug administration, one hindpaw was administered an intraplantar injection of 20 μl of 5% formalin. After injection, the mice were immediately placed back in the observation chamber, and the time spent licking, shaking, and biting the injected paw at 0 to 5 minutes and 15 to 30 minutes was measured with a stopwatch.

Carrageenan-Induced Inflammatory Pain

The carrageenan model was used to evaluate the spinal antinociception of DN-9 in inflammatory pain. The carrageenan-induced inflammatory pain model was implemented following our previous report.[48] On the first day of the experiment, the mice were placed in a clear plastic chamber on the glass surface of a Hargreaves radiant heat apparatus (Taimeng Technology Corporation of Chengdu, China) and allowed to acclimatize for 30 minutes. The basal threshold to thermal stimulation was measured 5 times at 5-minute intervals, and a cutoff latency of 25 seconds was employed to prevent tissue damage. Then, 20 μl of 2% λ-carrageenan was intraplantar injected into the right hind paw. Twenty-four hours after the carrageenan injection, thermal hyperalgesia was tested 5 times at 5-minute intervals. The mice then received i.t. injection of drugs, and the paw withdrawal threshold was measured at 15, 30, 45, 60, and 75 minutes; each time point was measured 3 times at 2-minute intervals.

Acetic Acid-Induced Writhing Test

The acetic acid-induced writhing test has been widely used in investigating visceral pain.[16,43] At the beginning of the experiments, the mice were acclimatized in a transparent acrylic observation chamber for 30 minutes. The mice were i.t. injected with saline or DN-9. Each mouse was intraperitoneally injected with a .6% (v/v) acetic acid solution (10 mL/kg of body weight) after 5 minutes as an irritant stimulus and placed back in the plastic cage for observation. A writhe was defined as a contraction of the abdominal muscle together with a stretching of the hind limbs. The number of writhes within the 5 to 15 minutes after acetic acid injection was recorded.

Neuropathic Pain

The chronic constriction injury (CCI) model was used in the present study to evaluate the analgesic effects of DN-9 on neuropathic pain. The CCI model was carried out as indicated in previous reports.[39,43] The mice were anesthetized with pentobarbital sodium (80 mg/kg, intraperitoneal [i.p.]), and the sciatic nerve was exposed. The adhering tissue around the sciatic nerve was separated, and 3 of 4-0 chromic gut ligatures with a 1 mm interval between each were tied loosely around it proximal to the sciatic trifurcation.

The behavioral testing of neuropathic pain was detected for both thermal hyperalgesia and mechanical allodynia. The basal thermal and mechanical thresholds

were tested 5 times at 5-minutes intervals before the surgery using the Hargreaves radiant heat apparatus and electric von Frey (IITC Life Science), respectively. Thermal hyperalgesia was tested on day 10 after surgery. The mice were then i.t. administered drugs, and the paw withdrawal threshold was measured at 15, 30, 45, 60, 75, and 90 minutes; each time point measure 3 times at 2-minute intervals. Mechanical allodynia was measured on day 11 after surgery. The analgesic effects of DN-9 on mechanical allodynia were also measured at 15, 30, 45, 60, 75, and 90 minutes after i.t. administration, and each time point measure 3 times at 2-minute intervals.

Antinociceptive Tolerance

The antinociceptive tolerance test was carried out as indicated in our previous report.[41] Briefly, the mice were administered repeated i.t. injections of DN-9 for 6 days to evaluate the development of antinociceptive tolerance. The antinociceptive effects were tested on the first and sixth days using the tail-flick test.

Conditioned Place Preference Test

The conditioned place preference experiment was performed according to our previous study.[18] The CPP apparatus has 3 compartments. Two large compartments ($20 \times 20 \times 20$ cm) were connected by a narrower compartment ($5 \times 20 \times 20$ cm). The large compartments are visually and tactually distinct (black-and-white striped walls with a rough floor vs black-dotted white wall with a smooth floor). The day before the experiments, the mice were provided with free access to the entire apparatus for 15 minutes for acclimatizing to the apparatus. On the first day (day 1), the time that the mice spent in each compartment within 15 minutes was recorded. The following 3 days were conditioning days (days 2–4), and the mice were i.t. injected with saline and confined to one of the compartments for 15 minutes. After approximately 6 hours, the animals were i.t. injected with saline or DN-9 and confined to the opposite compartment. On the postconditioning day (day 5), the mice were also provided with free access to the entire apparatus for 15 minutes, and the time spent in each compartment was measured.

Naloxone Induced Withdrawal Response

The mice were administered repeated i.t. injections of saline, DN-9 or morphine for 6 days. Mice were injected subcutaneously with naloxone (subcutaneous, 10 mg/kg) 2 hours after the last challenge and placed in a separate cylinder at a height of 32 cm and diameter of 12 cm. The number of jumps was recorded for 30 minutes.

Rotarod Test

The rotarod test has been widely used to determine potential motor impairment.[41] The mice were trained for 2 days through 3 consecutive sessions to remain on the rod (16 revolutions per minute). The cut-off time was 300 seconds, and the animals that did not remain on the rod for the 180 seconds at the second training day were eliminated from the experiments. On the third day, the animals were i.t. injected with saline or DN-9 and tested at 15, 30, 60, and 90 minutes after administration. The latency to fall off the rod was recorded at each time point.

GIT Test

The GIT test was performed following our previous report.[28] Briefly, the mice were fasted for 16 hours with water available ad libitum before the experiments. DN-9 was i.t. injected 5 minutes before oral administration of a charcoal meal (.1 mL/10 g body weight), an aqueous suspension of 5% charcoal and 10% gum arable. Thirty minutes after the charcoal meal administration, the animal was sacrificed. The length of the whole small intestine and the farthest distance that the charcoal meal had traveled were measured. For each animal, the percent of GIT was calculated as the percentage of the small intestine tract that was traversed.

Body Temperature Measurement

The protocol of the body temperature measurement was performed according to our previous report.[17] The experiments were conducted between 10:00 and 14:00, and the ambient temperature was controlled within 20.5 to 21.5°C. Briefly, the mice were placed in a restraining device with their tails taped lightly to horizontal. The rectal temperature was measured with a thermistor probe linked to a recorder system (model BL-420F, Taimeng Technology Corporation of Chengdu, China). The thermistor probe was inserted 2.5 cm in depth into the rectum. The body temperature was recorded before and at 10, 20, 30, 40, 50, and 60 minutes after i.t. injection of DN-9. The change in body temperature was expressed as the difference between the body temperature before injection and after drug administration.

Blood Pressure Recording

The procedures were performed as previously described.[27] Male Wistar rats (220–250 g) were anesthetized with urethane (l.3 g/kg, i.p.). Additional doses of urethane were administered to maintain a uniform depth of anesthesia. The trachea was incised to avoid respiratory disorders. The rats spontaneously breathed room air. A PE-10 catheter was positioned at the level of the T12–L1 intervertebral space for the i.t. injection of drugs. Another PE-10 catheter, which was connected to a PT100 blood pressure transducer (Taimeng Technology Corporation of Chengdu, China), was placed in the left femoral artery. The blood pressure transducer was connected to a BL-420F recorder system (Taimeng Technology Corporation of Chengdu, China) to record the blood pressure. The mean arterial pressure (MAP) was continuously recorded from the BL-420F recorder system. The rats were then allowed to recover for 30 to 40

minutes. The position of the i.t. catheter was checked at necropsy after each experiment.

Immunofluorescence

The procedure of immunofluorescence followed a previous study.[2] The mice daily received single i.t. injection of saline, 10 pmol DN-9 or 2 nmol morphine. Two hours after the last injection, the mice were deeply anesthetized with isoflurane and perfused through the ascending aorta with phosphate-buffered saline (PBS), followed by 4% paraformaldehyde. After the perfusion, the L4 to L5 spinal cord was removed and postfixed in 4% paraformaldehyde overnight. The samples were then dehydrated with 30% sucrose solution, imbedded, and sliced into sections (30 μm, free floating) with a cryostat. The sections were blocked with 1% BSA for 1 hour at room temperature and then incubated overnight at 4°C with the following primary antibodies: anti-c-Fos antibody (goat, 1:200, Santa Cruz, catalog: sc-52-G) and anti-IBa1 antibody (rabbit, 1:1000, Wako, catalog: 019-19741). After being washed with PBS, the sections were incubated with the following secondary antibodies (1:400, Jackson Immunoresearch): Cy3-donkey anti-rabbit (catalog: 711-165-152) and FITC-donkey anti-goat (catalog: 705-165-003) for 2 hours at room temperature. The sections were washed with PBS, mounted in fluorescent mounting medium, and observed under a Nikon fluorescence microscope, and images were captured with a CCD Spot camera. For each animal under study, at least 5 sections were analyzed. Each group had at least 3 to 4 mice. Densitometry analysis of the anti-IBa1 immunoreactivity in the spinal cord dorsal horn (SDH) to assess microglial activation was performed using ImageJ. C-Fos positive neurons were counted from the whole dorsal horn.

Spinal Cord Slice Preparation

The adult (5−7 weeks old) male mice were anesthetized with urethane (1.5−2.0 g/kg, i.p.). The lumbosacral spinal cord was rapidly removed and placed in ice-cold sucrose-ACSF (Sucrose 240 mM, $NaHCO_3$ 25 mM, KCl 2.5 mM, NaH_2PO_4 1.25 mM, $CaCl_2$ 0.5 mM and $MgCl_2$ 3.5 mM) with 95% O_2 and 5% CO_2. After extraction and under anesthesia, the mice were sacrificed by decapitation. Transverse slices (300−400 μm) were cut using a vibrating microslicer (VT1200s Leica). The slices were incubated at 32°C for at least 30 minutes in regular ACSF (NaCl 126 mM, KCl 3 mM, $MgCl_2$ 1.3 mM, $CaCl_2$ 2.5 mM, $NaHCO_3$ 26 mM, NaH_2PO_4 1.25 mM, and glucose 11 mM) equilibrated with 95% O_2 and 5% CO_2.

Electrophysiological Recording

The slice was placed in the recording chamber and was completely submerged and superfused at a rate of 2 to 4 mL/min with ACSF, which was saturated with 95% O_2 and 5% CO_2 and maintained at room temperature. Lamina II neurons in lumbar segments were identified as a translucent band under a microscope (BX51WIF; Olympus), as previously described.[22] Whole-cell voltage-clamp recordings were made from lamina II neurons using patch-pipettes fabricated from thin-walled, fiber-filled capillaries, and the patch-pipettes had a resistance of 8 to 15 MΩ. The patch-pipette solution used to record spontaneous excitatory postsynaptic currents (sEPSCs) contained: K-gluconate 135 mM, KCl 5 mM, $CaCl_2$ 0.5 mM, $MgCl_2$ 2 mM, EGTA 5 mM, HEPES 5 mM, and Mg-ATP 5 mM (pH 7.3 adjusted with KOH, 300 mOsm). The sEPSC recordings were made at a holding potential (VH) of −70 mV in the presence of 10 μM picrotoxin and 2 μM strychnine to abolish the contamination from inhibitory postsynaptic currents. Signals were acquired using an Axopatch 700B amplifier. The data were analyzed using pCLAMP 10.3 software. sEPSC events were detected and analyzed using Mini Analysis Program ver. 6.0.3. The relative frequency and amplitude compared to the basal value are provided as the mean ± SEM.

Statistical Analysis

All data were provided as the means ± SEM. Data obtained from the antinociception test were analyzed with one-way analysis of variance (ANOVA) followed by Dunnett's or Bonferroni's post hoc test. The ED_{50} values and the corresponding 95% confidence intervals for the antinociception were calculated using GraphPad Prism 5. The antinociceptive tolerance was analyzed according to one-way ANOVA followed by the Tukey HSD post hoc test. A probability level of .05 or less was considered statistically significant.

Results

Spinal DN-9 Produced Antinociceptive Effects in the Mouse Tail-Flick Test

The acute antinociceptive effects of i.t. administration of DN-9 were investigated in the mouse radiant heat tail-flick test. Intrathecal administration of DN-9 produced dose-dependent increases in the tail-flick latencies (Fig 1A, $F_{18,162} = 17.03$, $P < .0001$), which peaked at 20 minutes. The antinociceptive ED_{50} value of DN-9 after i.t. injection in the tail-flick test was 1.33 (1.10, 1.61) pmol (Table 1).

Moreover, the opioid receptor antagonist naloxone and the NPFF receptors antagonist RF9 were used to determine the roles of opioid and NPFF receptors in DN-9 spinal antinociception. Pretreatment with naloxone (5 nmol, i.t.) 10 minutes prior to i.t. administration of DN-9 significantly blocked its antinociception, which suggests the spinal antinociception of DN-9 is mediated by the opioid receptor (Fig 1B, $F_{18,144} = 25.98$, $P < .0001$). Furthermore, 5 minutes pretreatment with the NPFF receptors antagonist RF9 (10 nmol, i.t.) significantly augmented the spinal antinociception of DN-9 (Fig 1C, $F_{18,180} = 26.68$, $P < .0001$).

To further identify the roles of individual opioid receptor subtypes in the acute antinociceptive effects of DN-9 after i.t. administration, the selective antagonists for μ-, κ- and δ- receptors, ie, β-FNA, nor-BNI or NTI,

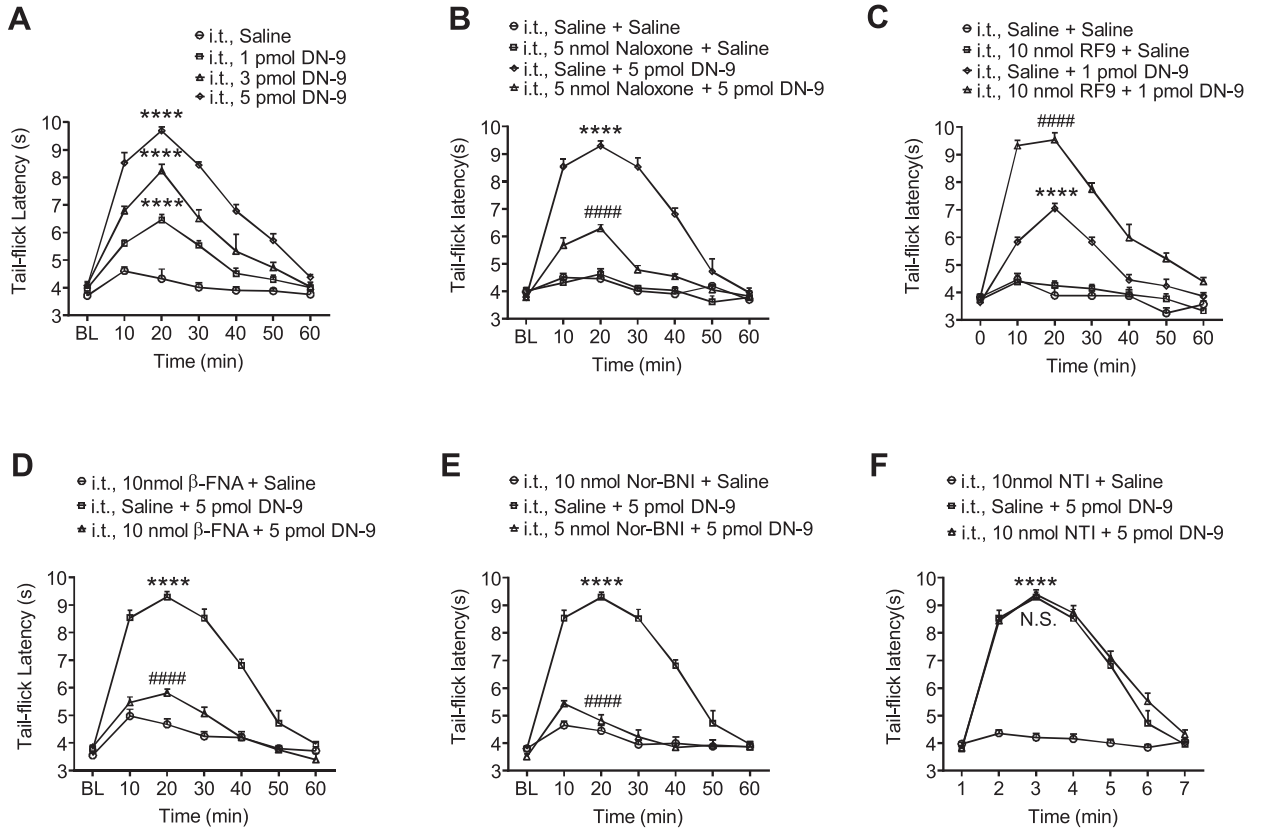

Figure 1. Antinociceptive effects of DN-9 in the mouse tail-flick test after intrathecal administration. **(A)** Antinociceptive dose- and time-response curve for DN-9 after i.t. administration. n = 8, 7, 8, 8 mice. **(B)** Effect of pretreatment with naloxone (5 nmol, i.t.) on the spinal antinociception of DN-9. n = 6, 8, 6, 8 mice. **(C)** Effect of pretreatment with RF9 (10 nmol, i.t.) on the spinal antinociception of DN-9. n = 8, 7, 9, 10 mice. **(D)** μ-opioid receptor selective antagonist β-FNA (10 nmol) could block the spinal antinociception of DN-9. n = 8, 8, 8 mice. **(E)** κ-opioid receptor selective antagonist nor-BNI (10 nmol) could block the spinal antinociception of DN-9. n = 8, 8, 8 mice. **(F)** δ-opioid receptor selective antagonist naltrindole (10 nmol) had no effect on the spinal antinociception of DN-9. n = 8, 8, 11 mice. Each value represents the mean ± S.E.M. $^{****}P < .0001$ indicate significant differences compared with the saline group, $^{\#\#\#\#}P < 0.0001$ indicates significant differences compared with the Saline + DN-9 group, n.s., no significance, according to Two-way ANOVA, followed by Bonferroni's post hoc test.

respectively, were i.t. injected 4 hours, 30 minutes or 20 minutes, respectively, prior to the injection of DN-9. The data are shown in Fig 1 D-F; the spinal antinociception of DN-9 was significantly blocked by pretreatment with the μ- and κ-opioid receptor antagonists β-FNA (10 nmol) ($F_{12,\ 126} = 27.07$, $P < .0001$) and nor-BNI (10 nmol) ($F_{12,\ 126} = 34.91$, $P < .0001$), but not the δ-opioid receptor antagonist NTI (10 nmol, $P > .05$).

Table 1. Analgesic Effects of DN-9 and Morphine After i.t. Administration in Mouse Pain Models

Pain models		*DN-9*			*Morphine*	*Morphine /DN-9*
		**ED_{50} (95% CI) pmol/mouse*	*Naloxone*	*RF9*	*[A]ED_{50} (95% CI) nmol/mouse*	
Tail-flick test		1.33 (1.10, 1.61)	↓	↑	0.35 (0.30, 0.40)†	263
Formalin test	Phase 1	37.69 (35.53, 39.99)	↓	-	19.55 (6.86, 55.69)	519
	Phase 2	55.04 (51.26, 59.10)	↓	↑	4.48 (3.94, 5.10)	81
Carrageenan induced inflammatory pain		2.98 (2.50, 3.54)	↓	↑	0.16 (0.09, 0.26)	54
CCI model	Thermal hyperalgesia	1.74 (1.38, 2.20)	↓	-	0.71 (0.47, 1.06)	408
	Mechanical allodynia	1.11 (0.63, 1.96)	↓	-	0.71 (0.52, 0.96)	639
Acetic acid induced writhing test		0.66 (0.60, 0.73)	↓	-	0.07 (0.05, 0.08)	106

↓Indicated the antagonist could decrease the effect of DN-9;
↑Indicated the antagonist could increase the effect of DN-9;
- Indicated the antagonist could not modulate the effect of DN-9.
*ED_{50} was determined at the time of peak effect.
†Data cited from Li. et.al.[26]

DN-9 Decreased Spontaneous Excitatory Transmission in Spinal Lamina II Neurons

We prepared spinal cord slices from WT mice for patch-clamp recordings in lamina IIo neurons. The sEPSC frequency gradually decreased over the time after DN-9 application, with a peak at approximately 4 minutes, accompanied by a reduction in the sEPSC amplitude (Fig 2A). Morphine (10 μM) also induced an inhibition on the sEPSC frequency and amplitude, which reached the maximal effects at approximately 4 minutes after morphine application (Fig 2B). At approximately 4 minutes after DN-9 addition, the sEPSC frequency decreased to 45.7 $\pm$ 6.3% (n = 7; $F_{2,18} = 52.79$, $P < .0001$) compared with the control (8.0 $\pm$ 1.5 Hz) in all recorded neurons. The sEPSC amplitude was reduced to 83.9 $\pm$ 4.6% (n = 7; $F_{2,18} = 7.751$, $P = .0038$) compared with the control (14.3 $\pm$ 1.4 pA) (Fig 2C). The frequency and amplitude after morphine application were decreased to 47.6 $\pm$ 6.4% (n = 7; $F_{2,18} = 52.79$, $P < .0001$) and 87.2 $\pm$ 3.8% (n = 7; $F_{2,18} = 7.751$, $P = .0263$) of the control (7.2 $\pm$ 1.3 Hz and 10.6 $\pm$ 1.3 pA), respectively (Fig 2C). Moreover, DN-9 (.1 μM) and morphine (10 μM) produced nearly equivalent effects on both the frequency and amplitude of sEPSCs (Fig 2C). In the presence of the opioid receptor antagonist naloxone (3 μM), it significantly blocked the ability of DN-9 to decrease the sEPSC frequency and amplitude (Fig 2D, E) in all neurons recorded, producing sEPSC frequency and amplitude of 80.7 $\pm$ 10.2% (n = 5; $F_{3,18} = 14.28$, $P = .0139$) and 98.5 $\pm$ 3.3% (n = 5; $F_{3,18} = 5.298$, $P = .0394$), respectively, of the control (8.8 $\pm$ 1.4 Hz, 10.5 $\pm$ 2.7 pA) at approximately 4 minutes after DN-9 treatment (Fig 2E). Naloxone alone did not affect the sEPSC frequency and amplitude (Fig 2E, $P = 1$).

Chronic Administration of DN-9 Did Not Produce Antinociceptive Tolerance at the Spinal Level

In the present study, the potential development of tolerance to the spinal antinociceptive effects of DN-9 was also evaluated. As shown in Fig 3A, i.t. administration of 5 pmol of DN-9 or 2 nmol of morphine occurred once daily for 6 days. Compared to day 1, 5 pmol DN-9 produced equivalent antinociceptive effects on day 6 ($P = 1$). However, the antinociceptive effects of morphine were significantly decreased after 6 days of repeated treatment. Therefore, these data indicated that i.t. administration of DN-9 produces a nontolerance forming antinociception.

Chronic treatment with opioids could induce microglial activation and increase c-Fos expression, which participate in hyperalgesia and tolerance development. In the present study, spinal chronic treatment with morphine could increase the c-Fos expression in the (SDH) (Fig 3B, C, $F_{2,9} = 118.6$, $P < .0001$). However, spinal chronic treatment with DN-9 could not induce a c-Fos expression increase ($F_{2,9} = 118.6$, $P = .24$). Similar to c-Fos, morphine could also induce significant microglial activation after 6 days of i.t. repeated treatment (Fig 3D-E, $F_{2,8} = 25.67$, $P = .0072$). Spinal chronic treatment with DN-9 could not induce microglial activation ($F_{2,8} = 25.67$, $P = .05$).

Spinal DN-9 Produced Antinociceptive Effects in Mouse Inflammatory Pain Models

In the formalin test, an intraplantar injection of formalin produces a biphasic pain response. Phase I (0 −5 minute) is thought to be induced by the direct activation of nociceptors, whereas the subsequent phase II (15−30 minutes) is proposed to reflect the combined effects of nociceptor input and spinal cord sensitization. As shown in Fig 4A, i.t. injection of DN-9 dose-dependently inhibited both the phase I and phase II flinching behaviors induced by formalin ($F_{3,32} = 43.20$, $P < .0001$). The reference ED_{50} values of DN-9 were 37.69 (35.53, 39.99) pmol and 55.04 (51.26, 59.10) pmol for phase I and phase II, respectively (Table 1). Intrathecal injection of morphine also showed dose-dependent analgesia effects in formalin test (Fig 4B). The reference ED_{50} values of morphine were 19.55 (6.86, 55.69) nmol and 4.48 (3.94, 5.10) nmol for phase I and phase II, respectively (Table 1). Furthermore, the opioid receptor antagonist naloxone and the NPFF receptors antagonist RF9 were used to determine the roles of opioid and NPFF receptors in the spinal antinociception of DN-9. Pretreatment with naloxone (5 nmol, i.t.) 10 minutes prior to i.t. administration of DN-9 significantly blocked the antinociceptive effects of DN-9 in both phase I and phase II of formalin pain (Fig 4C, $F_{3,32} = 42.09$, $P < .0001$). However, pretreatment with RF9 (10 nmol, i.t.) 5 minutes prior to DN-9 did not modify DN-9-induced antinociception in the first phase of formalin pain (Fig 4D, $F_{3,38} = 17.73$, $P = 1$), although it produced a significant enhancement in the spinal antinociception of DN-9 in the second phase of formalin pain (Fig 4D, $F_{3,38} = 17.73$, $P = .0442$). However, at the same doses, RF9 was not able to alter flinching behaviors in the formalin test alone ($P = 1$).

The acetic acid writhing test is a pain model that assesses irritant chemical stimulation induced visceral pain. The antinociceptive effects of i.t. administration of DN-9 in the acetic acid writhing test were also investigated in the present study. In Fig 5, intraperitoneal injection of 10 mL/kg 0.6% acetic acid solution induced a characteristic writhing response. The average writhing number in the 5 to 15 minutes after i.p. injection of acetic acid in the naive mice was 28.22 $\pm$ 2.83. Intrathecal injection of DN-9 could dose-dependently inhibit the writhing response with an ED_{50} value of 0.66 (.60, .73) pmol (Fig 5A, $F_{3,35} = 32.09$, $P < .0001$; Table 1). Intrathecal injection of morphine also dose-dependently inhibited the writhing response with an ED_{50} value of .07 (.05, .08) nmol (Fig 5B, $F_{3,22} = 115.4$, $P < .0001$; Table 1). Pretreatment with naloxone (5 nmol, i.t.) could significantly attenuate the analgesic effects of DN-9 at the spinal level in the acetic acid writhing test (Fig 5C, $F_{3,35} = 19.57$, $P = .0006$). However, i.t. pretreatment with RF9 produced a slight, but not statistically significant,

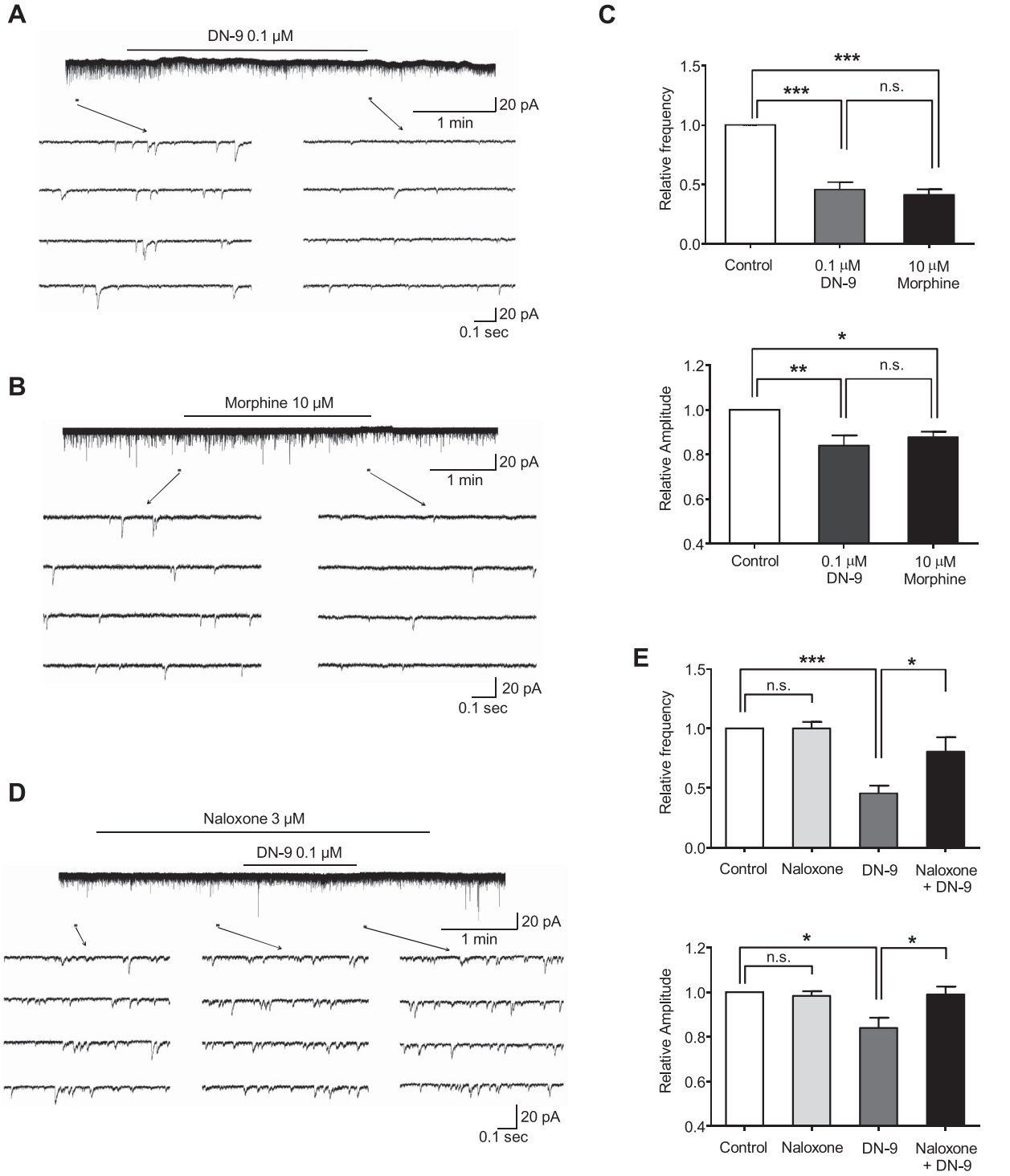

Figure 2. DN-9 (0.1 μM) decreases the frequency and amplitude of spontaneous excitatory postsynaptic currents (sEPSCs) at the spinal level by activating opioid-receptor. **(A)** Recordings of sEPSCs in the absence and presence of DN-9 (.1 μM). In this and subsequent figures, the duration of drug superfusion is shown by a horizontal bar above the chart recording, and 4 consecutive traces of sEPSCs for a period indicated by a short bar below the chart recording are shown in an expanded time scale. **(B)** Recordings of sEPSCs in the absence and presence of morphine (10 μM). **(C)** sEPSC frequency (upper) and amplitude (lower) after DN-9 and morphine treatment, relative to control, measured between 3 and 3.5 min after drug perfusion. **(D)** Effects of DN-9 on sEPSCs in the presence of an opioid-receptor antagonist, naloxone (3 μM). **(E)** Relative values of sEPSCs frequency (upper) and amplitude (lower) after DN-9 treatment in the absence or presence of naloxone (3 μM). Each value represents the mean $\pm$ S.E.M. n = 6-7 slices per group. n.s., no significance; $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$, according to one-way ANOVA for AUC data, followed by Bonferroni's post hoc test.

enhancement in the spinal analgesic effects of DN-9 (Fig 5D, $F_{3,38} = 12.60$, $P = .9814$).

Carrageenan is a polysaccharide, which is isolated from sea plants, and λ-carrageenan has been widely used to induce acute inflammatory pain. In the present study, intraplantar administration of 20 μl 2% λ-carrageenan into the hind paw of mice produced significant thermal hyperalgesia. Intrathecal injection of DN-9

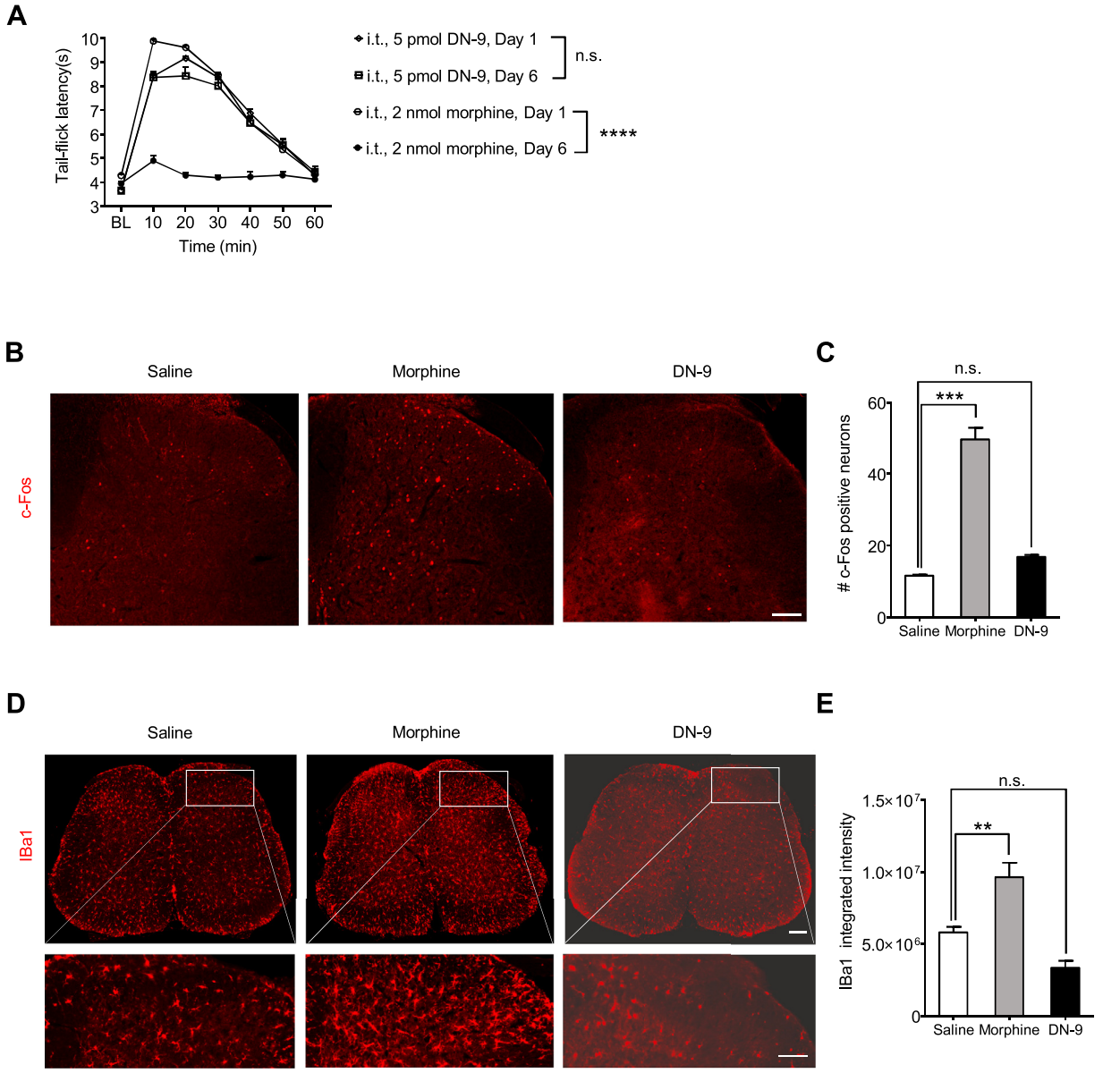

Figure 3. Antinociceptive tolerance evaluation of DN-9 after 6 days repeated i.t. administration in the mouse tail-flick test. **(A)** Dose- and time-response curves for 5 pmol DN-9 and 2 nmol morphine after i.t. administration at Day 1 and Day 6 after repeated administration. Each value represents mean ± S.E.M. n = 8, 10 mice. n.s., no significance; ****$P < .001$, according to two-way ANOVA for AUC data, followed by Bonferroni's post hoc test. **(B)** Representative images of immunochemistry staining of c-Fos after 6 days i.t. repeated injection of saline, 5 pmol DN-9 or 2 nmol morphine. **(C)** Quantification of c-Fos positive neurons. Each value represents mean ± S.E.M. from 4 mice per group. n.s., no significant; ***$P < .001$, one-way ANOVA, followed by Bonferroni's post hoc test. **(D)** Representative images of immunochemistry staining of Iba1 after 6 days i.t. repeated injection of saline, 5 pmol DN-9 or 2 nmol morphine. **(E)** Quantification of Iba1 integrated intensity. Each value represents mean ± S.E.M. from 3 to 4 mice per group. n.s., no significant; **$P < .01$, one-way ANOVA, followed by Bonferroni's post hoc test.

dose-dependently inhibited the thermal hyperalgesia induced by carrageenan (Fig 6A, $F_{2,240} = 6.852$, $P < .0001$), with a peak at 15 minutes after DN-9 injection. The antinociceptive ED_{50} value of DN-9 was 2.98 (2.50, 3.54) pmol in the inflammatory pain model (Table 1). Intrathecal injection of morphine dose-dependently inhibited the thermal hyperalgesia induced by carrageenan (Fig 6B, $F_{18,192} = 33.59$, $P < .0001$), with ED_{50} value of .16 (.09, .26) nmol in the inflammatory pain model (Table 1). In addition, pretreatment with naloxone (5 nmol, i.t.) completely blocked the analgesic effects of DN-9 (Fig 6C, $F_{18,186} = 9.779$, $P < .0001$). In contrast, pretreatment with RF9 (10 nmol, i.t.) significantly enhanced the analgesic effects of DN-9 (Fig 6D, $F_{18,246} = 7.787$, $P < .0001$).

Spinal DN-9 Produced Antinociceptive Effects in Neuropathic Pain

In the present study, the sciatic nerve chronic constriction injury model was used to evaluate the spinal analgesic effects of DN-9 in neuropathic pain. The mice showed robust decreases in the thermal threshold from 14 to 5 seconds on day 10 after the sciatic nerve chronic constriction surgery. Intrathecal injection of DN-9

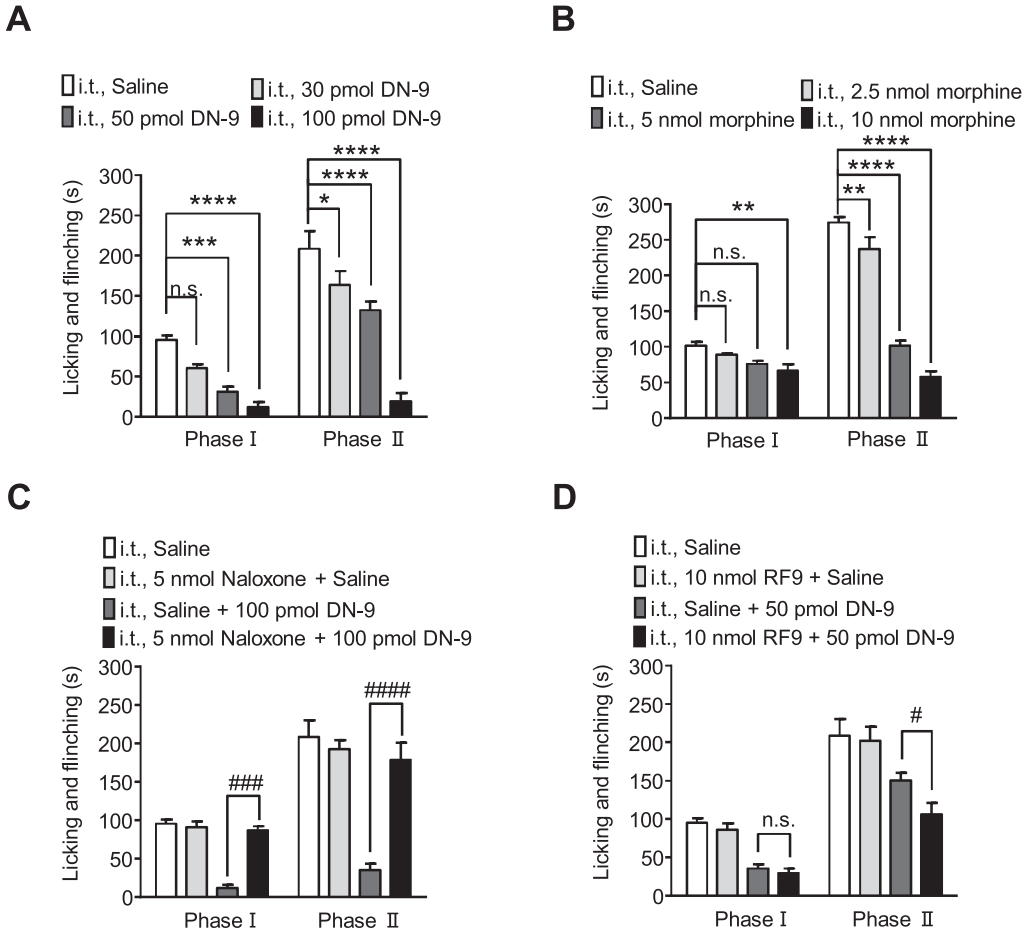

Figure 4. Antinociceptive effects of i.t. injection of DN-9 in the mouse formalin test. **(A)** Antinociceptive dose-response effects of DN-9 after i.t. administration. n = 9, 9, 9, 9 mice. **(B)** Antinociceptive dose-response effects of morphine after i.t. administration. n = 7, 6, 6, 6 mice. **(C)** Effect of pretreatment with naloxone (5 nmol, i.t.) on the antinociception of DN-9. n = 9, 9, 9, 9 mice. **(D)** Effect of pretreatment with RF9 (10 nmol, i.t.) on the antinociception of DN-9. n = 9, 12, 9, 12 mice. Each value represents the mean ± S.E. M. n.s., no significance, $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$, $^{****}P < .0001$ versus saline group according to one-way ANOVA, followed by Dunnett's post hoc test in dose-response effects. n.s., no significance, $^{\#\#\#}P < .001$, $^{\#\#\#\#}P < .0001$ indicates significant differences compared with the Saline + DN-9 group, according to one-way ANOVA, followed by Bonferroni's post hoc test in the antagonism results.

dose-dependently increased the thermal threshold, which indicates DN-9 has significant analgesia in CCI-induced thermal hyperalgesia (Fig 7A, $F_{21,245} = 5.975$, $P < .0001$). The antinociceptive ED_{50} value of DN-9 was 1.74 (1.38, 2.20) pmol. Intrathecal injection of morphine also showed dose-dependent analgesia effects in the thermal hyperalgesia in CCI model (Fig 7B, $F_{21,133} = 5.197$, $P < .0001$). The antinociceptive ED_{50} value of morphine was .71 (.47, 1.06) nmol (Table 1). Moreover, pretreatment with naloxone (5 nmol, i.t.) could completely block the analgesic effects of DN-9 at the spinal level (Fig 7C, $F_{21,203} = 10.08$, $P < .0001$). Interestingly, pretreatment with RF9 (10 nmol, i.t.) alone had significant analgesic effects on CCI-induced thermal hyperalgesia (Fig 7D, $F_{21,210} = 3.229$, $P < .0001$). Intrathecal pretreatment with 10 nmol RF9 prior to 3 pmol DN-9 resulted in a weak increase in the DN-9 analgesic effect, which was not significant ($P = .1655$). The sciatic nerve chronic constriction could also induce mechanical allodynia. The spinal analgesic effects of DN-9 on mechanical allodynia in the CCI model were also investigated in the present study. The mice showed a significant decrease in the mechanical threshold on day 11 after the sciatic nerve chronic constriction surgery. Intrathecal injection of both DN-9 and morphine could dose-dependently reduce the mechanical allodynia in the CCI model (Fig 7E, $F_{28,266} = 4.243$, $P < .0001$; Fig 7F, $F_{21,133} = 3.589$, $P < .0001$). The ED_{50} values for DN-9 and morphine are 1.11 (.63, 1.96) pmol and .71 (.52, .96) nmol, respectively (Table 1). Similar to thermal hyperalgesia, pretreatment with naloxone (5 nmol, i.t.) could completely block the antiallodynia effects of DN-9 at the spinal level (Fig 7G, $F_{21,213} = 5.827$, $P < .0001$). In addition, i.t. injection of RF9 had significant antiallodynia effects in the CCI model (Fig 7H, $F_{21,161} = 4.577$, $P < .0001$). Intrathecal pretreatment with 10 nmol RF9 prior to 3 pmol DN-9 had a slight, but not statistically significant, increase in the DN-9-induced analgesic effect ($P = .2921$).

The Side Effect Evaluation of Spinal DN-9

Firstly, we assessed the addiction potential of DN-9 in the conditioned place preference test and naloxone induced withdrawal response in 6 days repeated

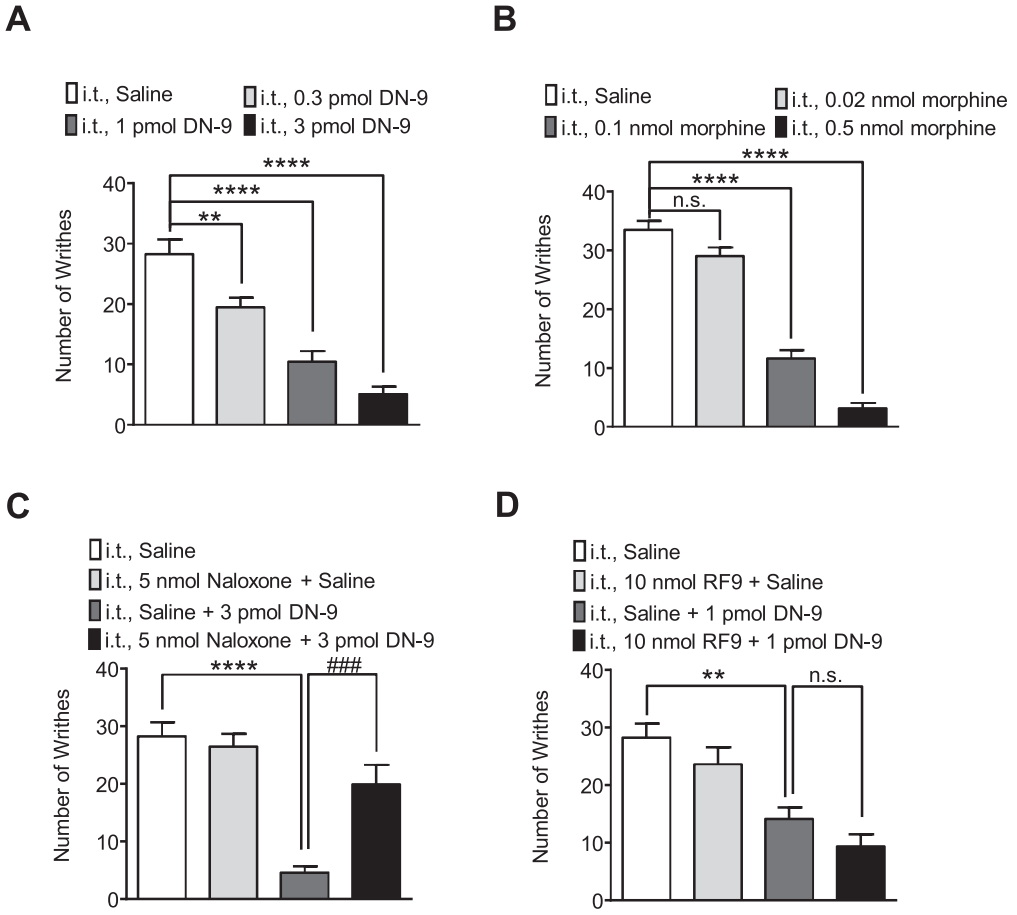

Figure 5. Antinociceptive effects of the i.t. injection of DN-9 in the mouse acetic acid writhing test. **(A)** Antinociceptive dose response of DN-9 after i.t. administration. n = 9, 9, 9, 9 mice. **(B)** Antinociceptive dose response of morphine after i.t. administration. n = 6, 6, 7, 7 mice. **(C)** Effect of pretreatment with naloxone (5 nmol, i.t.) on the antinociception of DN-9. n = 9, 9, 9, 9 mice. **(D)** Effect of pretreatment with RF9 (10 nmol, i.t.) on the antinociception of DN-9. n = 9, 10, 10, 10 mice. Each value represents the mean ± S.E. M. n.s., no significance, $^{**}P < .01$, $^{***}P < .001$, $^{****}P < .0001$ versus saline group according to one-way ANOVA, followed by Dunnett's post hoc test in dose-response effects. n.s., no significance, $^{###}P < .001$ indicates significant differences compared with the Saline + DN-9 group, according to one-way ANOVA, followed by Bonferroni's post hoc test in the antagonism results.

treatments. In the CPP test, 2 nmol (an analgesic dose) morphine is enough to induce a significant preference (Fig 8A, $P < .0001$). However, only 100 pmol DN-9 induced a significant preference in CPP test. Intrathecal injection of saline did not induce a place preference change. We also evaluated the naloxone induced withdrawal response in the mice which received 6 days i.t. injection of 10 pmol DN-9 or 2 nmol morphine. Six days repeated i.t. injected 2 nmol morphine produced significant withdrawal response, but 6 days repeated i.t. injected 10 pmol DN-9 did not show significant withdrawal response (Fig 8B).

The effects of DN-9 on the GIT, cardiovascular system and body temperature were also evaluated in rodents at the spinal level. As shown in Fig 8C, the GIT% of the i.t. injected saline group was 66.75 ± 3.32. Intrathecal injection of DN-9 dose-dependently inhibited the GIT ($F_{3,34} = 9.87$, $P < .0001$). At the high doses of 300 and 500 pmol, DN-9 exhibited significant inhibition of GIT in the mice (GIT%, 31.40 ± 7.69, $P = .0022$; GIT%, 21.67 ± 6.51, $P = .0002$, respectively). The ED_{50} values for DN-9 are 242.4 (135.4, 434.1) pmol in GIT assay. Intrathecal injection of 10 nmol morphine did not show significant decrease in GIT. The cardiovascular response to i.t. administration of DN-9 on the mean arterial pressure was investigated in rats. The results are shown in Fig 8D; i.t. administration of DN-9 produced a dose-dependent decrease in the MAP in urethane-anesthetized rats ($F_{4,20} = 41.38$, $P < .0001$). The MAP decreased 11.51 ± 1.66, 16.76 ± 2.08, 21.86 ± 2.87, and 32.46 ± 0.67 mm Hg from the baseline at 1, 3, 10, and 30 nmol of DN-9, respectively. Intrathecal injection of 15 nmol morphine showed significant decrease in the MAP. The body temperature modulation results are shown in Fig 8E; i.t. injection of DN-9 dose-dependently decreased the body temperature in mice, and at the high dose of 500 pmol DN-9 induced significant hypothermia ($F_{3,42} = 5.124$, $P = .0041$).

The potential influence of i.t. administration of DN-9 on the motor function was evaluated in the rotarod test. As shown in Fig 8F, compared with the saline group, i.t. injection of 10 pmol DN-9 did not change the rotarod performance ($P > .05$). Intrathecal injection of a high dose (100 pmol) of DN-9 had a slight, but not statistically significant, decrease in the rotarod performance ($P > .05$). Intrathecal injection of 2 nmol morphine did not change the rotarod performance.

Collectively, our results indicate that i.t. administration of DN-9 produces a nontolerance forming

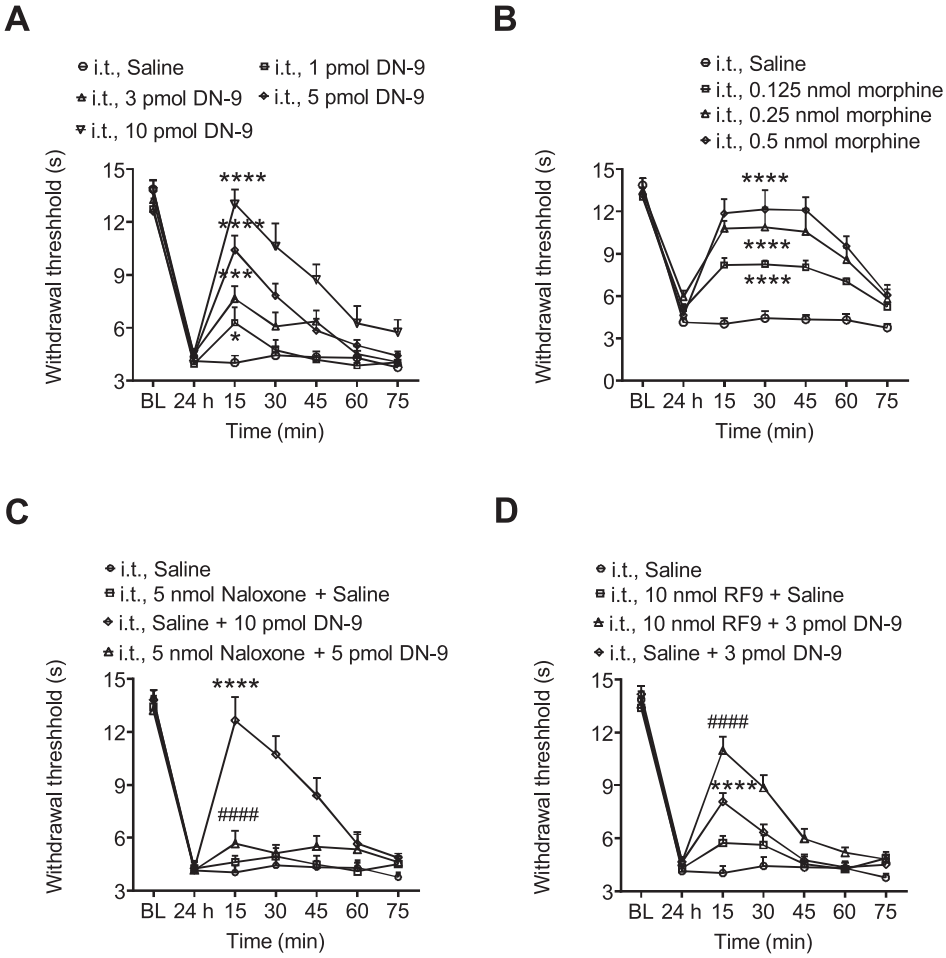

Figure 6. Antinociceptive effects of the i.t. injection of DN-9 in the carrageenan-induced inflammatory pain. **(A)** Antinociceptive dose- and time-response curve of DN-9 after i.t. administration. n = 9, 9, 9, 9, 9 mice. **(B)** Antinociceptive dose- and time-response curve of morphine after i.t. administration. n = 9, 6, 6, 6 mice. **(C)** Effect of pretreatment with naloxone (5 nmol, i.t.) on the antinociception of DN-9. n = 9, 8, 9, 9 mice. **(D)** Effect of pretreatment with RF9 (10 nmol, i.t.) on the antinociception of DN-9. n = 9, 12, 12, 12 mice. Each value represents the mean ± S.E.M. n.s., no significance, $^{*}P < .05$, $^{***}P < .001$, $^{****}P < .0001$ versus saline group according to one-way ANOVA, followed by Dunnett's post hoc test in dose-response effects. n.s., no significance, $^{****}P < .0001$ versus Saline group, $^{\#\#\#\#}P < .0001$ indicates significant differences compared with the Saline + DN-9 group, according to one-way ANOVA, followed by Bonferroni's post hoc test in the antagonism results.

antinociception. The antinociceptive ED_{50} values of DN-9 in various pain models were at the pmol level (Table 1), which suggests that at effective antinociceptive doses, i.t. injection of DN-9 cannot induce significant effects on GIT inhibition, withdrawal response, body temperature, and blood pressure regulation. However, DN-9 did not eliminate the opioid induced rewarding effects, high dose of DN-9 showed conditioned place preference.

Discussion

In this study, we identified that DN-9 might be a promising compound for developing multifunctional opioid analgesics with limited adverse effects. Intrathecal injection of DN-9 could produce potent analgesia with ED_{50} values between .66 and 55.04 pmol in mouse preclinical pain models. Furthermore, DN-9 could decrease both the frequency and amplitude of sEPSCs in lamina IIo neurons of the spinal cord with high potency. In contrast to morphine, chronic i.t. treatments with DN-9 could not develop analgesic tolerance, c-Fos expression or microglial activation. Moreover, DN-9 showed limited side effects in locomotor function and coordination, GIT inhibition, abuse potential, cardiovascular, and body temperature regulation.

First, i.t. administration of DN-9 produced potent analgesic effects with an ED_{50} value as low as 1.33 pmol in the mouse tail-flick test. In our previous study, the antinociceptive ED_{50} value of DN-9 after i.c.v. injection was 16.3 pmol, which indicates DN-9 could produce approximately 12-fold more potent analgesic effects at the spinal level than that at the supraspinal level.[41] Moreover, according to our previous study, the spinal analgesic ED_{50} values of BN-9 and morphine were 0.29 and 0.35 nmol, which indicates DN-9 significantly exhibited an improved antinociceptive potency that was approximately 218- and 263-fold more potent compared with its parent peptide BN-9 or morphine, respectively.[26] Similar to the supraspinal level, the spinal analgesic effects of DN-9 were blocked by the universal opioid receptor antagonist naloxone and the μ- and κ-opioid selective antagonists β-FNA and nor-BNI, respectively. Furthermore, the NPFF receptors antagonist RF9 significantly enhanced the spinal antinociception of DN-9 in the mouse tail-flick test. These results

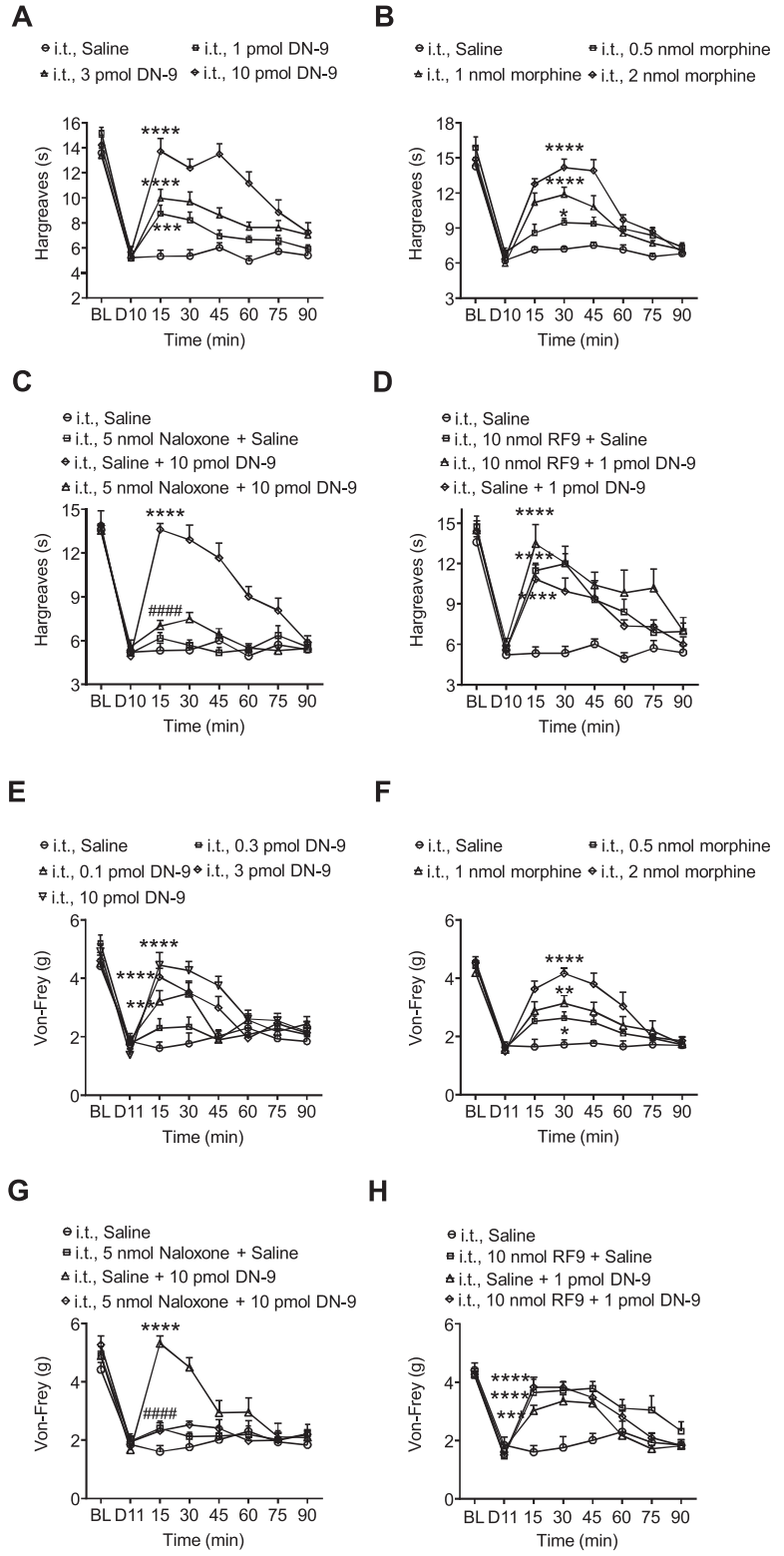

Figure 7. Antinociceptive effects of the i.t. injection of DN-9 in the mouse CCI model. (A) Antinociceptive dose- and time-response curve of the i.t. injection of DN-9 on thermal hyperalgesia in the mouse CCI model. n = 9, 12, 9, 9 mice. **(B)** Antinociceptive dose- and time-response curve of the i.t. injection of morphine on thermal hyperalgesia in the mouse CCI model. n = 5, 6, 6, 6 mice. **(C)** Effect of pretreatment with naloxone (5 nmol, i.t.) on the antinociception of DN-9 on thermal hyperalgesia. n = 9, 8, 7, 9 mice. **(D)** Effect of pretreatment with RF9 (10 nmol, i.t.) on the antinociception of DN-9 on thermal hyperalgesia. n = 9, 9, 8, 8 mice. **(E)** Antinociceptive dose- and time-response curve of the i.t. injection of DN-9 on mechanical allodynia in the mouse CCI model. n = 9, 8, 9, 8, 9 mice. **(F)** Antinociceptive dose- and time-response curve of the i.t. injection of morphine on mechanical allodynia in the mouse CCI model. n = 4, 6, 6, 7 mice. **(G)** Effect of pretreatment with naloxone (5 nmol, i.t.) on the antinociception of DN-9 on mechanical allodynia. n = 9, 8, 9, 9 mice. **(H)** Effect of pretreatment with RF9 (10 nmol, i.t.) on the antinociception of DN-9 on mechanical allodynia. n = 9, 6, 5, 7 mice. Each value represents the mean ± S.E.M. $^{**}P < .01$, $^{***}P < .001$, $^{****}P < .0001$ versus saline group according to one-way ANOVA, followed by Dunnett's post hoc test. $^{\#\#\#\#}P < .0001$ indicates significant differences compared with the Saline + DN-9 group, according to one-way ANOVA, followed by Bonferroni's post hoc test.

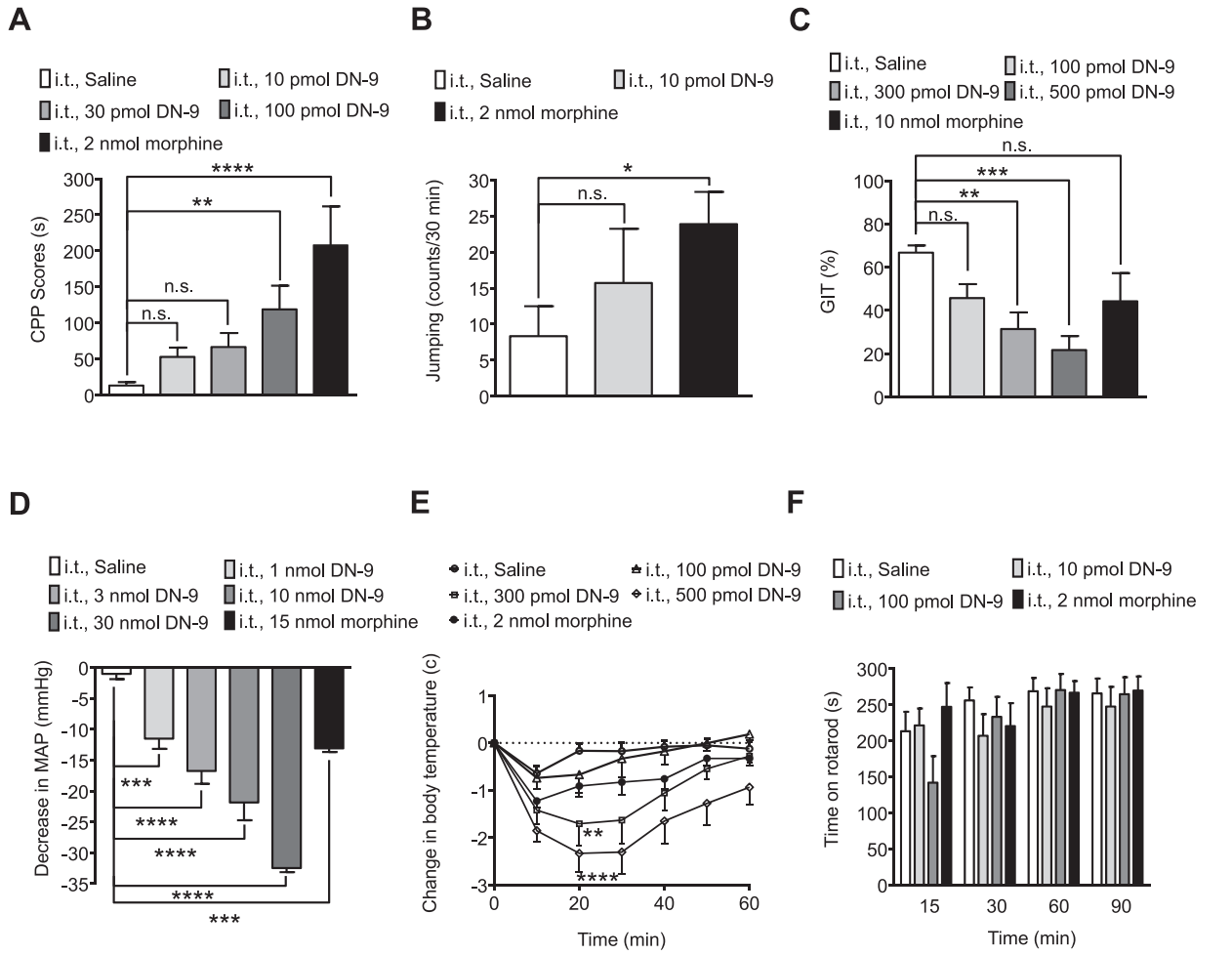

Figure 8. Evaluation of side effects of the i.t. administration of DN-9. **(A)** Effect of i.t. administration of DN-9 in mouse conditioned place preference. n = 15, 18, 5, 6, 5 mice. Data points represent means ± S.E.M. n.s. no significance, $^{**}P < .01$, $^{****}P < .0001$ indicate significant differences compared to saline group according to one-way ANOVA followed by the Dunnett's post hoc test. **(B)** Effect of i.t. administration of DN-9 in the naloxone induced withdrawal response in the 6 days DN-9 or morphine repeated mice. n = 6, 7, 8 mice. Data points represent means ± S.E.M. n.s. no significance, $^{*}P < .01$, unpaired t-test. **(C)** The effects of i.t. administration of DN-9 on gastrointestinal transit in mice. n = 9, 9, 9, 8, 5 mice. Data points represent means ± S.E.M. n.s. no significance, $^{**}P < .01$, $^{***}P < .001$ indicate significant differences compared to saline group according to one-way ANOVA followed by the Dunnett's post hoc test. **(D)** The effects of i.t. administration of DN-9 on blood pressure in rats. n = 5 rats per group. Data points represent means ± S.E.M. $^{***}P < .001$, $^{****}P < .0001$ indicate significant differences compared to saline group according to one-way ANOVA followed by the Dunnett's post hoc test. **(E)** The effects of i.t. administration of DN-9 on body temperature change in mice. n = 9, 10, 13, 14, 6 mice. Data points represent means ± S.E.M. $^{**}P < .01$, $^{****}P < .0001$ indicate significant differences compared to saline group according to two-way ANOVA followed by the Dunnett's post hoc test. **(F)** The effects of i.t. DN-9 on the motor performance of mice in the rotarod test. n = 11, 11, 10, 7 mice. Data points represent means ± S.E.M. n.s. no significance, according to one-way ANOVA followed by the Dunnett's post hoc test.

indicate that the spinal antinociception of DN-9 in the mouse tail-flick test is mainly mediated by the μ- and κ-opioid receptors and its NPFF moiety might exert antiopioid activities and partially reduce its opioid moiety-mediated antinociception. One mechanism for opioid analgesia is to inhibit neurotransmitter release and nociceptive synaptic transmission via presynaptic regulation in the SDH.[3,8] In the present study, DN-9 could markedly inhibit the sEPSC frequency and amplitude in out lamina II neurons (IIo), which are predominantly excitatory and form a nociceptive circuit with primary C-afferents and lamina I project neurons. Furthermore, 0.1 μM DN-9 produced equal effects with 10 μM morphine, which indicate the effect of DN-9 in inhibiting nociceptive synaptic transmission is 100-fold more potent than that of morphine. This finding is consistent with the present in vivo data, which demonstrated that DN-9 has more than 54- to 639-fold potent analgesia effects than morphine at the spinal level.

Tolerance is one of the major problems accompanied by chronic opioid treatment.[45,49] Intrathecal administration of morphine could easily develop antinociceptive tolerance. In our results, repeated injection of morphine nearly lost antinociceptive effects over 6 days, which is in line with previous report.[26] Noteworthy, repeated i.t. administration of DN-9 over 6 days did not develop antinociceptive tolerance. A lack of antinociceptive tolerance could significantly reduce the risk of opioid overdose, which is the leading cause of opioid induced death. These results also suggest that DN-9 might be used as a long-term opioid analgesic in chronic pain management.

In the present study, DN-9 showed dose-dependent analgesic effects in all animal preclinical pain models tested. It showed high potencies with different pain types, such as acute heat or chemical stimulation (tail-flick or formalin test phase I, respectively), inflammatory pain (formalin test phase II and carrageenan-induced inflammatory pain), neuropathic pain (CCI model), and visceral pain (acetic acid-induced writhing test). DN-9 also has potent analgesia in different pain readouts, such as evoked pain (thermal hyperalgesia and mechanical allodynia) and spontaneous pain behavior (formalin-induced flinching, licking and biting, and acetic acid-induced writhing). The detail analgesic ED_{50} values are summarized in Table 1. DN-9 produced potent analgesia with antinociceptive ED_{50} values between .66 and 55.04 pmol in these preclinical pain models. Compared to morphine, DN-9 showed 54- to 639-fold more potent analgesic effects. Moreover, DN-9 showed more potent analgesic effects in acetic acid induced writhing test and neuropathic pain, and relatively lower efficacy in formalin test. It has similar tendency compare to morphine (Table 1). It has been demonstrated that morphine or other opioids have different analgesic efficacy in different pain models.[19,33,35] Previous studies have shown that morphine or endomorphins need higher doses in formalin test to have enough analgesic effects.[19,33,35]

In the present study, antagonism studies with naloxone and RF9 were performed in all tested animal pain models. Pretreatment with naloxone could block the spinal analgesic effects of DN-9, which indicated the spinal analgesic effects of DN-9 were mainly mediated by opioid receptors. Moreover, opioids have been demonstrated to have abundant analgesic effects at the spinal level in different pain types.[21,49]

The roles of NPFF receptors in the spinal analgesic effects of DN-9 were complicated. In this study, pretreatment with the NPFF receptors antagonist RF9 significantly augmented the analgesic effects of DN-9 in both formalin and carrageenan-induced inflammatory pain. According to our previous cAMP assay, DN-9 could simultaneously activate both $NPFF_1$ and $NPFF_2$ receptors.[41] As the expression of the $NPFF_1$ receptor in the spinal cord was not detectable,[14,15] the additive effects of RF9 on the spinal DN-9 analgesic effects might be mediated by the $NPFF_2$ receptor in inflammatory pain. Moreover, recent studies have indicated that both the expression of the NPFF precursor and $NPFF_2$ receptor were upregulated at the spinal cord during carrageenan or CFA-induced inflammatory pain.[29,46] Our present results were consistent with the results obtained from the Chen J.C. group that the overexpression of $NPFF_2$ receptor could facilitate pain transmission and has pronociceptive effects in inflammatory pain.[29] In theory, DN-9 could activate the $NPFF_2$ receptor at the spinal level and produced pronociceptive effects, which would subsequently decrease the analgesic effects of its opioid moiety. Thus, pretreatment with RF9 antagonized the pronociceptive effects of the NPFF moiety of DN-9 and resulted in the enhancement of spinal analgesic effects in inflammatory pain. In addition, these results confirm the NPFF receptors agonistic characterization of DN-9 at the spinal level.

In the mouse CCI neuropathic model, the NPFF receptors antagonist RF9 could induce potent analgesic effects in both thermal hyperalgesia and mechanical allodynia. These results indicated that blocking the spinal NPFF receptors could have significant analgesic effects in neuropathic pain. These results were consistent with the previous report that selective $NPFF_1$ receptor antagonists (ie, AC-262620 and AC-262970) could produce a dose-dependent reversal of spinal nerve ligation-induced mechanical allodynia after i.p. administration.[25] However, in other literature, the stable analog of NPFF, 1DMe, has been demonstrated to have significant antiallodynia in the neuropathic pain models, independent of the opioid system.[42] These data indicated that both the NPFF receptors agonist and antagonist might have antinociceptive effects in neuropathic pain. Because of the complicated effects of the NPFF system in neuropathic pain modulation and the lack of very specific NPFF receptors antagonists to date, we cannot identify the role of the NPFF moiety of DN-9 in its antinociceptive response to neuropathic pain. However, previous studies have indicated that most opioid analgesics showed weak analgesic effects in neuropathic pain.[11,30] In our present study, i.t. injection of DN-9 showed potent analgesic effects in both thermal hyperalgesia and mechanical allodynia in a mouse model of neuropathic pain. The improved antinociceptive potency of DN-9 in a neuropathic pain model might result from its NPFF moiety.

Opioid-induced constipation is another side effect that limits the clinical usage of opioid analgesics.[24] In our present study, i.t. injection of analgesic doses of DN-9 could not produce GIT inhibition. The ED_{50} value for GIT is 182-fold higher than the ED_{50} value in tail-flick test. Furthermore, similar results were obtained in the body temperature modulation of i.t. administration of DN-9. 225-fold higher does compare to ED_{50} value in tail-flick test, induce significant hypothermia in mice. The rats were used for the cardiovascular modulation experiment. The effective dose to induce a significant decrease in the MAP was greater than 1 nmol (approximately 500 pmol for mouse based on normalization of dose to body surface area[32]), which is 376-flod higher does compare to ED_{50} value in tail-flick test. In CPP assay, analgesic doses of DN-9 did not shown significant conditioned preference. Only a very high does (100 pmol) of DN-9 developed significant conditioned preference, but this effect is still weaker than the effect of an analgesic (2 nmol) dose of morphine. The naloxone withdrawal assay showed that analgesic dose of morphine can induced significant withdrawal response, but not analgesic dose DN-9. Taken together, at potently analgesic doses, DN-9 did not have significant effects on conditioned place preference, withdrawal response, GIT, body temperature, and cardiovascular modulation.

In summary, the present study was carried out to characterize the spinal analgesic effects and opioid-like side effects of our newly developed opioid/NPFF agonist DN-9. First, in the mouse tail-flick test, i.t. injection of DN-9

produced potent nontolerance forming analgesia with an ED_{50} value at 1.33 pmol, which is mainly mediated by the μ- and κ-opioid receptors. Second, DN-9 could decrease both the frequency and amplitude of sEPSCs in lamina IIo neurons of the spinal cord via opioid receptors. Third, spinal DN-9 produced potent analgesia with antinociceptive ED_{50} values between .66 and 55.04 pmol in a series of preclinical pain models, including the formalin test, carrageenan-induced inflammatory pain, acetic acid writhing test, and neuropathic pain. Finally, DN-9 showed limited side effects in locomotor function and coordination, GIT inhibition, abuse potential, cardiovascular, and body temperature regulation. Taken together, the present study showed that DN-9 produced robust, nontolerance forming analgesia with reduced side effects at the spinal level. Thus, DN-9 might be a promising compound for developing novel multifunctional opioid analgesics with less opioid related adverse effects.

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