Neurochemical Research (2019) 44:2786–2795
https://doi.org/10.1007/s11064-019-02899-x

ORIGINAL PAPER

Antinociceptive Effect of Spirocyclopiperazinium Salt Compound DXL-A-24 and the Underlying Mechanism

Ding Wang[1] · Hua Yang[1,4] · Yingying Liang[1] · Xin Wang[2] · Xiaolei Du[2] · Runtao Li[2] · Yimin Jiang[3] · Jia Ye[1]

Received: 26 May 2019 / Revised: 20 October 2019 / Accepted: 24 October 2019 / Published online: 6 November 2019

Abstract

The antinociceptive effects of spirocyclopiperazinium salt compound DXL-A-24 on neuropathic pain and chemical-stimulated pain were investigated in this study. After the administration of DXL-A-24, the paw withdrawal latency (PWL) and mechanical withdrawal threshold (MWT) were increased in rats suffering from neuropathic pain (chronic constriction injury, CCI) on days 1, 3, 5, 7 and 14 after surgery, and pain responses were inhibited in mice stimulated with chemicals (formalin or acetic acid). In the analysis of antinociceptive targets, the effect of DXL-A-24 was blocked by a peripheral nicotinic acetylcholine receptor (nAChR) antagonist (hexamethonium, Hex) or α7 nAChR antagonist (methyllycaconitine, MLA) in the formalin test. Meanwhile, the effect of DXL-A-24 was also blocked by a peripheral muscarinic acetylcholine receptor (mAChR) antagonist (atropine methylnitrate, Amn) or M4 mAChR antagonist (tropicamide, TRO). The antinociceptive signalling pathway was explored using molecular biology methods in ipsilateral dorsal root ganglions (DRGs) of CCI rats after the administration of DXL-A-24 for 7 days. Western blot analyses showed that the increased levels of phosphorylation of calcium/calmodulin-dependent protein kinase II alpha (CaMKIIα) and cAMP response element-binding protein (CREB) were eliminated, and the qRT-PCR assay showed that the increase in the expression of Tumor necrosis factor alpha (TNF-α) mRNA was reduced. Meanwhile, immunofluorescence staining revealed that the increase in calcitonin gene related peptide (CGRP) expression was inhibited by the administration of DXL-A-24, and the effect was blocked by MLA or TRO. In conclusion, DXL-A-24 exerts significant antinociceptive effects on neuropathic pain and chemical-stimulated pain. The antinociceptive effect of DXL-A-24 is probably attributed to the activation of peripheral α7 nAChR and M4 mAChR, the subsequent inhibition of the CaMKIIα/CREB signalling pathway, and finally the inhibition of TNF-α and CGRP expression.

Keywords Spirocyclopiperazinium salt compound DXL-A-24 · Neuropathic pain · α7 nAChR · M4 mAChR · Signalling pathway

Introduction

Neuropathic pain, which is defined as 'persistent pain resulting from peripheral nerve injury' by the IASP, is characterized by hyperalgesia, allodynia and spontaneous pain [1, 2]. Many diseases cause neuropathic pain, such as autoimmune diseases, metabolic disorders, infection, vascular diseases, injuries, tumours, etc. [3]. According to an epidemiological study, 6.9–10% people experience neuropathic pain [4]. Currently, antidepressants (amitriptyline) and antiepileptic drugs (pregabalin and gabapentin) are widely used in the clinical as analgesics for neuropathic pain [5, 6]. However, the prognosis is poor. Less than 40–60% of patients obtain partial relief of neuropathic pain, and these patients suffer from many side effects, including tolerance, addiction, cardiotoxicity, dizziness, etc. [7]. Therefore, investigations of

Ding Wang and Hua Yang contributed equally to this work.

✉ Jia Ye
yejia@bjmu.edu.cn

1 Department of Molecular and Cellular Pharmacology, School of Pharmaceutical Sciences, Peking University, Beijing, China

2 Department of Chemical Biology, School of Pharmaceutical Sciences, Peking University, Beijing, China

3 Medical and Healthy Analysis Center, Peking University, Beijing, China

4 Department of Medical Laboratory, Hunan University of Medicine, Huaihua, China

analgesics with high effects and low toxicity for neuropathic pain are urgently needed.

The spirocyclopiperazinium salt compound DXL-A-24 (Fig. 1) was designed and synthesized based on its parent compound LXM-15. As shown in a previous study, LXM-15 exerts significant antinociceptive effects on several acute pain models, without obvious toxicity. Receptor binding assays showed that LXM-15 achieved its antinociceptive effect by binding α7 nAChR and M4 mAChR [8]. Compared with LXM-15, the process used to synthesize DXL-A-24 is less time-consuming and has a lower cost. In this study, we investigated the antinociceptive effects of DXL-A-24 on neuropathic pain and chemical-stimulated pain, and further explored the antinociceptive mechanisms.

Materials and Methods

Animals

SD rats (200–250 g) and ICR mice (20–22 g) of both sexes (half males and half females) were purchased from the Department of Laboratory Animal Science of Peking University. Animals were housed under standard conditions (22 ± 0.5 °C, relative humidity of 55 ± 5% and an alternating 12 h light–dark cycle), and were provided food and water ad libitum. All experiments were approved by the Institutional Animal Care and Use Committee of Peking University, and complied with the recommendations of the Committee for Research and Ethical Issues of the International Association for the Study of Pain.

Drugs and Reagents

Drugs: Aspirin (Beijing Shuguang Pharmaceutical Co. Ltd, Beijing, China), Gabapentin (Jiangsu Hengrui Medicine, Lianyungang, China), methyllycaconitine citrate (MLA) (Abcam, Cambridge, MA, USA), hexamethonium (Hex), atropine methylnitrate (Amn), tropicamide (TRO) (Sigma Chemical Co., St. Louis, MO, USA), formalin and acetic acid (Beijing Chem. Works, Beijing, China) were used in the present study. DXL-A-24 was synthesized by Runtao Li and Xin Wang. All of the drugs were dissolved in double distilled water (DDW) or normal saline (NS) immediately before use and were administered via the intragastric (i.g.), subcutaneous (s.c.) or intraperitoneal (i.p.) route.

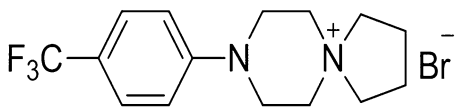

Fig. 1 The chemical structure of the Spirocyclopiperazinium salt compound DXL-A-24

Reagents: RIPA lysis buffer (Beijing Beyotime Institute of Biotechnology, Beijing, China), protease inhibitor cocktails, phosphatase inhibitors cocktails, the bicinchoninic acid (BCA) protein assay kit (Beijing Applygen Technologies Inc. Beijing, China), anti-phospho-calcium/calmodulin-dependent protein kinase IIα (Thr286) (pCaMKIIα) antibody, anti-CaMKIIα antibody, anti-calcitonin gene related peptide (CGRP) antibody, anti-phospho-cAMP response element-binding protein (Ser133) (pCREB) antibody, anti-CREB antibody (Abcam, Cambridge, MA, USA), β-actin (Beijing Zhongshan Golden Bridge Biotechnology, Beijing, China), and specific horseradish peroxidase (HRP)-conjugated secondary antibodies (M&G Technology Beijing Ltd, Beijing, China), high-capacity cDNA reverse transcription kits (Invitrogen, Carlsbad, CA, USA), Alexa Fluor-488-conjugated donkey secondary antibodies(Jackson ImmunoResearch Inc. PA, USA) were also used in this study.

Chronic Constriction Injury Model

A rat model of chronic constriction injury (CCI) was established according to the procedure described by Bennett and Xie in 1988 [9]. The baseline mechanical withdrawal threshold and paw withdrawal latency of each rat were measured prior to the operation. After rats were anesthetized with sodium pentobarbital (60 mg/kg, i.p.), the skin of right midthigh was incised and muscles were separated to expose the sciatic nerve. The sciatic nerve was ligated loosely for four times with 4–0 silk thread at 1 mm intervals. After ligation, incisions in the muscle and skin were sequentially sutured. In sham-operated animals, the sciatic nerve was exposed but not ligated. After the operation, the mechanical withdrawal threshold and paw withdrawal latency of rats were measured. The CCI rats that failed to exhibit thermal hyperalgesia and mechanical allodynia were excluded from the study.

Antiepileptics are the most frequently studied drugs in neuropathic pain, and gabapentin (a GABA inhibitor) has been strongly recommended as a first-line treatment for peripheral and central neuropathic pain [10]. In the present study, gabapentin was chosen as the positive drug. Thirty-six rats of both sexes were divided into six groups and treated with DXL-A-24 (1, 0.5 or 0.25 mg/kg, i.g.), gabapentin (100 mg/kg, i.g.) or vehicle (DDW, i.g.) daily after the operation. Behavioural tests were conducted 2 h after drug administration on days 1, 3, 5, 7 and 14 after ligation.

Mechanical Withdrawal Threshold Measurement

Rats were placed in transparent plastic boxes with a metal wire mesh floor 30 min to allow them to adapt to the

environment. Then, electronic von Frey filaments (IITC 2390, USA) were applied to the middle plantar surface of the right hind paws. The minimum force triggering paw withdrawal was recorded automatically as the mechanical withdrawal threshold (MWT) of rats. The MWT was measured for three times at 5 min intervals. The percentage of Pain Threshold Elevation (PTE %) was calculated using the following formula:

$$\text{PTE\%} = \frac{\text{MWT of experiment} - \text{MWT of vehicle}}{\text{MWT of vehicle}} \times 100$$

Paw Withdrawal Latency Measurement

Rats were placed on a heated surface (50.5 °C), and the intervals from placement to the first response (licking or biting their hind paws or jumping) were regarded as the paw withdrawal latency (PWL). Fourteen seconds was set as the cut-off time. The percentage of Maximal Possible Effect (MPE %) was calculated using the following formula:

$$\text{MPE\%} = \frac{\text{PWL of experiment} - \text{PWL of vehicle}}{\text{cut off time} - \text{PWL of vehicle}} \times 100$$

Chemical Stimulation-Induced Pain Model

Formalin Test

Five groups of 6 mice of both sexes were administered DXL-A-24 (0.5, 0.25 or 0.125 mg/kg, i.g.), aspirin (300 mg/kg, i.g.) or vehicle (DDW, i.g.) 2 h before the injection of 2.5% formalin (20 µl in NS, s.c.) into the right hind paw. Aspirin, a non-steroidal anti-inflammatory drug (NSAID), is now considered one of the most effective and versatile medications for the treatment of mild-to-moderate pain [11]. Aspirin was used as the positive drug in this study. The time spent licking or biting injured paws after the formalin injection was recorded. The first 5 min was regarded as phase I, and the interval from the 10th min to the 60th min was regarded as phase II. The percent inhibition was calculated using the following formula:

$$\text{Inhibition ratio (\%)} = \frac{\text{time of vehicle} - \text{time of experiment}}{\text{time of vehicle}} \times 100$$

Acetic Acid Test

Five groups of 6 mice of both sexes were administered DXL-A-24 (0.5, 0.25 or 0.125 mg/kg, i.g.), aspirin (300 mg/kg, i.g.) or vehicle (DDW, i.g.) 2 h before the injection of 0.6% acetic acid (0.2 ml in NS, i.p.). The number of abdominal constrictions was counted and recorded from the 5th min to the 20th min after the acetic acid injection. The percent inhibition was calculated using the following formula:

$$\text{Inhibition ratio (\%)} = \frac{\text{number of vehicle} - \text{number of experiment}}{\text{number of vehicle}} \times 100$$

Receptor Blocking Tests

Eight groups of 6 mice of both sexes were pretreated with hexamethonium (Hex, a peripheral neuronal nAChR antagonist, 18 µmol/kg, i.p.), atropine methylnitrate (Amn, a peripheral mAChR antagonist, 14 µmol/kg, i.p.), methyllycaconitine (MLA, an α7 nAChR antagonist, 3 µmol/kg, i.p.), tropicamide (TRO, a M4 mAChR antagonist, 3 µmol/kg, i.p.) or NS (i.p.), respectively. Fifteen min later, mice were administered DXL-A-24 (0.5 mg/kg, i.g.) or DDW (i.g.). The formalin tests were conducted as described in section "Formalin test".

Western Blot

Eighteen rats of both sexes were divided into three groups: Sham group (DDW, i.g.), Vehicle group (DDW, i.g.), DXL-A-24 group (1 mg/kg, i.g.). Sham-operated rats and CCI rats were sacrificed 2 h after the administration of DXL-A-24 or vehicle on day 7 after surgery. Then, the ipsilateral DRGs (L4 and L5) were harvested and homogenized in ice-cold RIPA lysis buffer supplemented with 2% protease inhibitors and 1% phosphatase inhibitors. Collected tissue samples were centrifuged at 12,000×*g* for 5 min and the supernatant was collected. The protein concentration of each supernatant was measured using the bicinchoninic acid (BCA) protein assay kit according to the manufacturer's instructions. Each sample was adjusted to an equal concentration, mixed with sodium dodecyl sulfated (SDS) sample buffer and boiled for 5 min. Samples containing equal amounts of protein were separated using 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently electrotransferred onto a polyvinylidene fluoride (PVDF) membrane (0.45 µm). After blocking with 5% skim milk in Tris-buffered saline containing Tween-20 (TBST) for 1.5 h at room temperature, the membrane was incubated with primary antibodies, including a rabbit anti-calcium/calmodulin-dependent protein kinase IIα (CaMKIIα) antibody, rabbit anti-phospho-CaMKIIα (Thr286) antibody, rabbit anti-cAMP response element-binding protein (CREB) antibody, rabbit anti-phospho-CREB (Ser133) antibody and mouse anti-β-actin monoclonal antibody (diluted in

5% skim milk in TBST, 1:1000–1:5000) overnight at 4 °C. Then, the membrane was washed three times with TBST and incubated with a horseradish peroxidase-conjugated goat anti-rabbit IgG or anti-mouse IgG (1:2000, diluted in 5% skim milk in TBST) for 1 h at room temperature. The membrane was washed three times with TBST again, and the blots were visualized using a ChemiDoc imaging system after an incubation with a chemiluminescence (ECL) reagent. The protein levels were normalized to the intensity of β-actin, a control protein.

qRT-PCR

As described in section "Western Blot", eighteen rats of both sexes were divided into three groups and DRGs were collected. Total RNA was extracted using Trizol reagent, and 2 μg of total RNA from each sample were reverse transcribed into first-strand complementary DNAs (cDNAs) using the high-capacity cDNA reverse transcription kits. Two microliters of cDNA products were used as templates for PCR and amplified with specific primers (for TNF-α, sense: 5′- GCA TGATCCGAGATGTGGAA-3′ and antisense: 5′- AGACAC CGCCTGGAGTTCTG-3′; for β-actin, sense: 5′- TGTGAT GGTGGGAATGGGTCAG-3′ and antisense: 5′- TTTGAT GTCACGCACGATTTCC-3′). PCR was conducted with the following parameters: 50 °C for 2 min, 95 °C for 2 min, 40 cycles of 95 °C for 15 s, 58 °C for 15 s, 72 °C for 1 min.

Immunofluorescence

Twenty rats of both sexes were divided into five groups: Sham group (NS/DDW), Vehicle group (NS/DDW), DXL-A-24 group (NS/DXL-A-24), MLA group (MLA/DXL-A-24), TRO group (TRO/DXL-A-24). After operation, rats were pretreated with MLA (3 mg/kg, i.p.), TRO (3 mg/kg, i.p.) or NS (i.p.), respectively, 20 min prior to the administration of DXL-A-24 (1 mg/kg, i.g.) or DDW (i.g.) for 7 days. The rats were anaesthetized with pentobarbital (60 mg/kg, i.p.) and perfused with physiological saline on day 7. The ipsilateral DRGs were harvested, post-fixed with 4% paraformaldehyde and dehydrated through successive incubations with 10%, 20% and 30% sucrose solutions. DRGs were cut to 16 μm frozen sections and stored in − 80 °C freezers until use. Frozen sections were processed for immunofluorescence staining for the CGRP protein as described below. Sections were hydrated in PBS for 10 min and then blocked with a blocking solution (PBS + 10% donkey serum + 1% Triton X-100) at 37 °C for 2 h. The sections were exposed to the goat primary antibody for CGRP (1:400, diluted in PBS + 2% donkey serum + 0.5% Triton X-100) at 4 °C overnight. After rinses with PBS, sections were incubated with Alexa Fluor-488-conjugated donkey secondary antibodies (1:400, diluted in PBS + 2% donkey serum + 0.5% Triton X-100) at 37 °C for 2 h. Then, sections were cover slipped. Images were captured with an Olympus fluorescence microscope.

Acute Toxicity Test

The limit test for the acute toxicity of DXL-A-24 was conducted according to Organisation for Economic Cooperation and Development (OECD) guideline 423. After six mice were administered DXL-A-24 (1000 mg/kg, i.g.), the behavioural, neurological, and autonomic states and any lethality, moribund state, or death were observed for 72 h with special attention and thereafter up to14 days [12].

Statistical Analysis

Analyses were performed with SPSS 23.0 software. Data are presented as mean ± SD. The mechanical withdrawal threshold and paw withdrawal latency in the chronic constriction injury (CCI) model were analysed with repeated-measures & mixed design ANOVAs. Other comparisons were analysed with one-way ANOVA followed by the Dunnett post hoc analysis. A value of $P < 0.05$ was considered statistically significant.

Results

Antinociceptive Effect of DXL-A-24 on Neuropathic Pain

CCI surgery induced significant thermal hyperalgesia and mechanical allodynia. As shown in Fig. 2, the paw withdrawal latency (PWL) and mechanical withdrawal threshold (MWT) of CCI rats were significantly shorter ($P < 0.01$). The PWL decreased to the minimum level on day 3 after surgery and remained stable, and the MWT decreased to lowest value on day 7 and remained stable. The administration of DXL-A-24 (1, 0.5 or 0.25 mg/kg, i.g.) prolonged the PWL on days 1, 3, 5, 7 and 14, and the MPE % was 74%, 38% and 26%, respectively, on day 7. The DXL-A-24 groups showed a significant difference compared with the vehicle group at all time points ($P < 0.01$) (Fig. 2a). The repeated-measures & mixed design ANOVA revealed a significant effect of treatment ($F(5,25) = 42.75$, $P < 0.01$), but not time ($F(5125) = 1.45$, $P > 0.05$) or the treatment × time interaction [$F(25,125) = 1.04$, $P > 0.05$], on CCI. Meanwhile, the administration of DXL-A-24 (1, 0.5 or 0.25 mg/kg, i.g.) increased the MWT, and the PTE % was 121%, 90% and 81%, respectively, on day 7. The

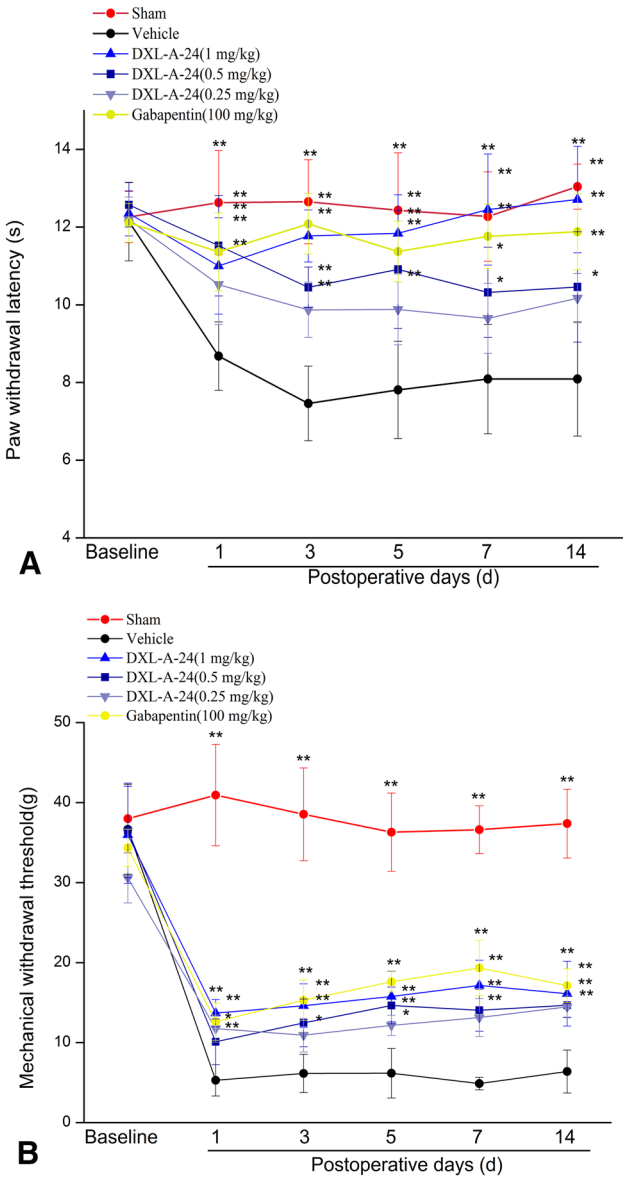

Fig. 2 Antinociceptive effect of DXL-A-24 on neuropathic pain in CCI rats. Sham or CCI rats were administered DXL-A-24 (1, 0.5 or 0.25 mg/kg, i.g.), gabapentin (100 mg/kg, i.g.) or vehicle (DDW, i.g.) daily after surgery. The paw withdrawal latency (PWL) (**a**) and mechanical withdrawal threshold (MWT) (**b**) were measured 2 h after drug administration on days 1, 3, 5, 7 and 14 after ligation. Data are presented as mean ± SD of 6 rats per group. *P<0.05 and **P<0.01 compared with the Vehicle group

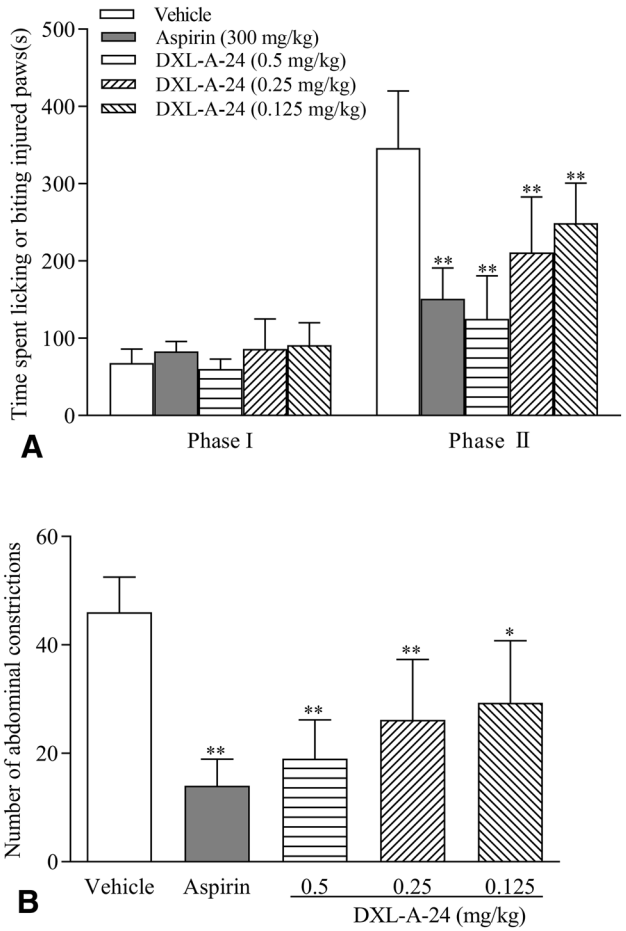

Fig. 3 Antinociceptive effect of DXL-A-24 on chemical-stimulated pain in mice. Mice were administered DXL-A-24 (0.5, 0.25 or 0.125 mg/kg, i.g.), aspirin (300 mg/kg, i.g.) or vehicle (DDW, i.g.). **a** Antinociceptive effect of DXL-A-24 on animals in the formalin test. At 2 h after the administration of DXL-A-24, the right hind paws of mice were injected with 2.5% formalin (20 µl in NS, s.c.), and time spent licking or biting the injected paw was recorded. The first 5 min was regarded as phase I, and the interval of time from the 10th min to the 60th min was regarded as phase II. **b** Antinociceptive effect of DXL-A-24 on mice in the acetic acid test. At 2 h after the administration of DXL-A-24, mice were injected with 0.6% acetic acid and the number of abdominal constrictions was recorded from the 5th min to the 20th min. Data are presented as mean ± SD of 6 mice per group. *P<0.05 and **P<0.01 compared with the Vehicle group

DXL-A-24 groups showed a significant difference compared with the vehicle group at all time points (P<0.01) (Fig. 2b). The repeated-measures & mixed design ANOVA revealed significant effects of treatment ($F(5,25)=207.67$, P<0.01), treatment × time interaction [$F(25,125)=5.84$, P<0.01], but not time ($F(5,125)=0.24$, P>0.05), on CCI. The gabapentin group exhibited an MPE % and PTE % of 62 and 142%, respectively, on day 7(P<0.01).

Antinociceptive Effect of DXL-A-24 on Chemical Stimulation-Induced Pain

Mice injected with 2.5% formalin displayed an obvious response of licking and biting the injured paws. As shown in Fig. 3a, the administration of DXL-A-24 (0.5, 0.25 or 0.125 mg/kg, i.g.) significantly attenuated the nociceptive behaviour ($F(4,25)=18.11$, P<0.01), and the percent inhibition was 64%, 39% and 28%, respectively. The positive control drug aspirin (300 mg/kg, i.g.) inhibited the nociceptive behaviour with a percent inhibition of 57%.

In the acetic acid test, 0.6% acetic acid induced severe abdominal constrictions in mice. The administration of DXL-A-24 (0.5, 0.25 or 0.125 mg/kg, i.g.) significantly reduced the number of abdominal constrictions ($F(4,25) = 12.05$, $P < 0.05$, $P < 0.01$), with a percent inhibition of 60%, 43% and 36%, respectively. Aspirin (300 mg/kg, i.g.) inhibited 70% of abdominal constrictions (Fig. 3b).

Potential Antinociceptive Targets of DXL-A-24

In the formalin test, DXL-A-24 exerted a significant analgesic effect. Pretreatment with Hex or Amn blocked the antinociceptive effect of DXL-A-24, and the percent inhibition was reduced from 54% to 11% and 8%, respectively (Fig. 4a) ($F(3,20) = 22.42$, $P < 0.01$). Furthermore, pretreatment with MLA and TRO also blocked the antinociceptive effect of DXL-A-24, and the percent inhibition was reduced from 61% to 17% and 16% respectively (Fig. 4b) ($F(3,20) = 32.61$, $P < 0.01$). Significant differences were not observed between the vehicle group and antagonists-pretreated groups ($P > 0.05$).

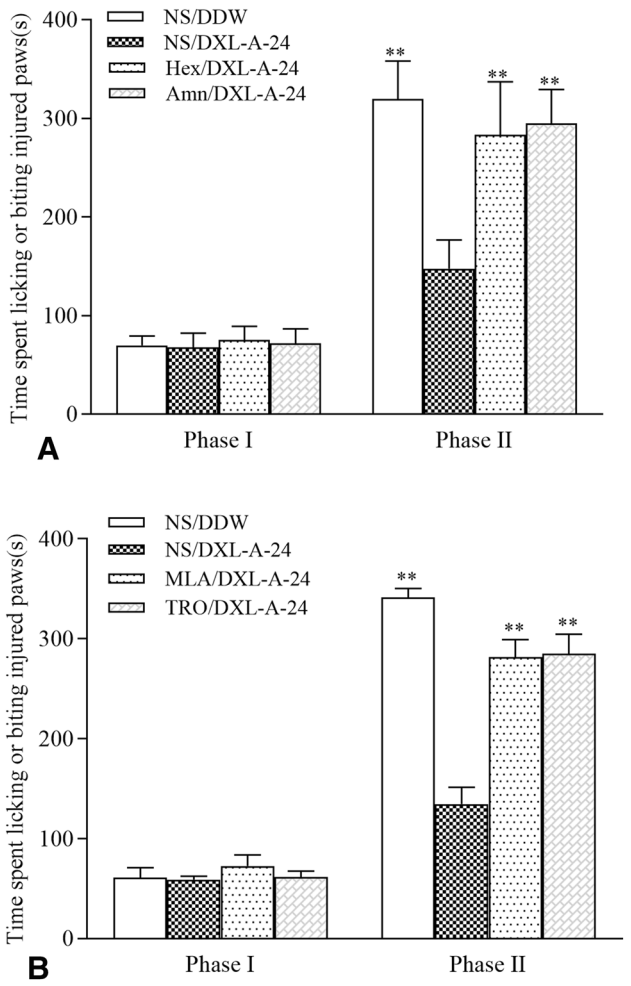

Fig. 4 Effects of Hex, Amn, MLA or TRO on the antinociceptive effect of DXL-A-24. **a** Mice were pretreated with Hex (18 μmol/kg, i.p.), Amn (14 μmol/kg, i.p.) or NS (i.p.) before the administration of DXL-A-24 (0.5 mg/kg, i.g.) or DDW (i.g.). **b** Mice were pretreated with MLA (3 μmol/kg, i.p.), TRO (3 μmol/kg, i.p.) or NS (i.p.) before the administration of DXL-A-24 (0.5 mg/kg, i.g.) or DDW (i.g.). Two hours later, formalin tests were performed. Data are presented as mean ± SD of 6 mice per group. $^{**}P<0.01$ compared with the Vehicle group

Potential Signalling Pathway

Level of the pCaMKIIα Protein in DRGs

In western blot tests, we evaluated the effect of DXL-A-24 on the level of CaMKIIα protein. In CCI rats, the phosphorylation of CaMKIIα in the DRGs was significantly increased compared with sham-operated rats ($P < 0.01$). The administration of DXL-A-24 decreased the phosphorylation of CaMKIIα by 38% ($F(2,15) = 23.73$, $P < 0.01$) (Fig. 5a). Meanwhile, the expression of CaMKIIα remained stable in each group ($F(2,15) = 1.80$, $P > 0.05$) (Fig. 5b).

Level of the pCREB Protein in DRGs

Subsequently, the levels of pCREB in DRGs were explored. In CCI rats, the phosphorylation of CREB in the DRGs was significantly increased compared with sham-operated rats ($P < 0.01$). The administration of DXL-A-24 inhibited the increase in CREB phosphorylation by 54% ($F(2,15) = 7.65$, $P < 0.01$) (Fig. 5c). A significant difference in CREB levels was not observed ($F(2,15) = 0.52$, $P > 0.05$) (Fig. 5d).

Expression of the TNF-α mRNA in DRGs

We next examined the effect of DXL-A-24 on TNF-α expression after CCI operation. Our quantitative real-time PCR analysis revealed increased expression of the TNF-α mRNA in the DRGs of CCI rats compared with the sham group ($P < 0.01$). DXL-A-24 (1 mg/kg) effectively inhibited this change by 60% ($F(2,15) = 14.60$, $P < 0.01$) (Fig. 6).

Expression of the CGRP in DRGs

Immunofluorescence staining was conducted to examine CGRP expression in the DRGs of CCI rats. A few CGRP-positive cells were observed in the DRGs of sham-operated rats (Fig. 7a). After CCI operation, CGRP expression was obviously increased in the DRGs (Fig. 7b). The administration of DXL-A-24 substantially inhibited the increase in CGRP expression (Fig. 7c). Pretreatment with MLA and TRO blocked the effect of DXL-A-24 (Fig. 7d and e). CGRP expression in the DRGs was calculated using the percentage of CGRP positive cells in the total DRG cells (Fig. 7f.)

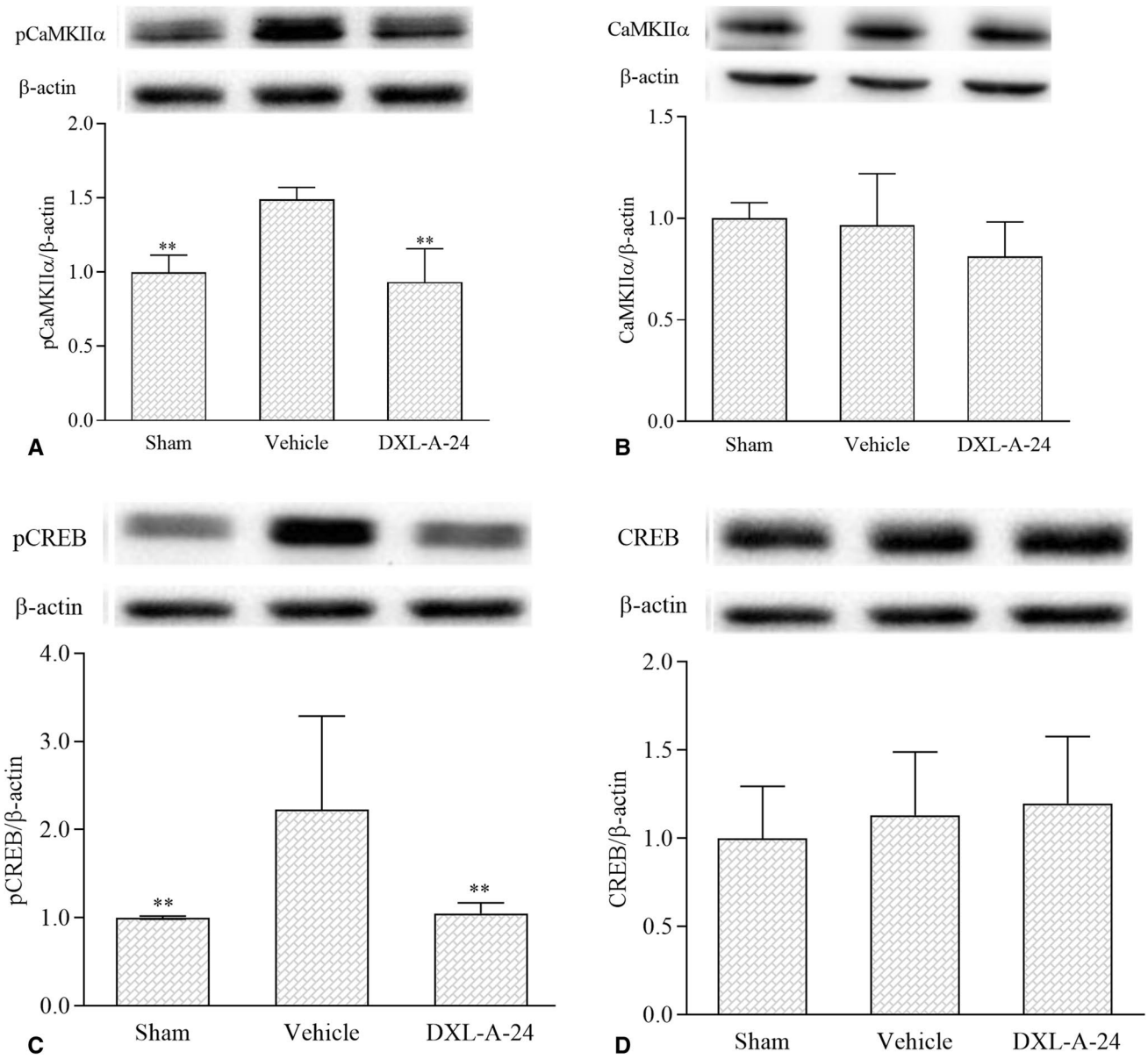

Fig. 5 Effects of DXL-A-24 on pCaMKIIα and pCREB levels in CCI rats. After nerve injury, rats were administered with DXL-A-24 daily and ipsilateral DRGs (L4 and L5) were harvested from the sham or CCI rats on day 7. The levels of pCaMKIIα (**a**), CaMKIIα (**b**), pCREB (**c**) and CREB (**d**) in DRGs were detected using western blotting test. Data are presented as mean ± SD of 6 rats per group. $^{**}P<0.01$ compared with the Vehicle group

Acute Toxicity

Mice administered DXL-A-24 (1000 mg/kg, i.g.) exhibited normal behavioural, neurological and autonomic states. No mortality or toxicity was observed during the observation period. Thus, the minimum lethal dose of DXL-A-24 is greater than 1000 mg/kg, which is equivalent to 2500 times the high-dose (0.4 mg/kg). This compound is very safe and non-toxic in mice.

Discussion

In the present study, DXL-A-24 significantly inhibited nerve injury-induced hyperalgesia and the chemical-stimulated pain response in a dose-dependent manner. In CCI rats, the MWT was increased and the PTE % was 121% after the administration of DXL-A-24 for 7 days; at the same time, the PWL was prolonged and the MPE % was

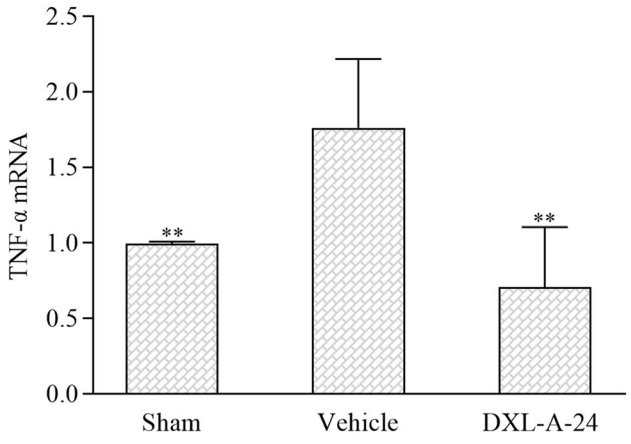

Fig. 6 Effect of DXL-A-24 on TNF-α mRNA expression. After nerve injury, rats were administered with DXL-A-24 daily, and ipsilateral DRGs (L4 and L5) were harvested from the sham or CCI rats on the 7th day. The expression of the TNF-α mRNA in DRGs was detected using RT-PCR test. Data are presented as mean ± SD of 6 rats per group. $^{**}P<0.01$ compared with the Vehicle group

74%. The administration of the positive drug gabapentin resulted in a PTE % of 142% and MPE % of 62%. In mice with chemical-stimulated pain, the administration of DXL-A-24 decreased the time spent licking or biting the injured paws by 64% and reduced the number of abdominal constrictions by 60%. Aspirin inhibited these phenomena by 57 and 70% respectively. In the acute toxicity test, DXL-A-24 showed no obvious toxicity. Taken together, DXL-A-24 produced similar antinociceptive effects to gabapentin and aspirin that are clinical analgesics, without obvious toxicity, implying that DXL-A-24 is probably a promising analgesic.

α7 nAChR is widely distributed in the central and peripheral nervous systems, and is associated with various physical functions, such as anxiety, learning, memory, movement and pain [13–15]. The activation of α7 nAChR has been shown to alleviate pain induced by chemical stimulation, inflammation or nerve injury [8, 16]. In the present study, the antinociceptive effect of DXL-A-24 was blocked by the peripheral nAChR antagonist Hex, and further blocked by the α7 nAChR antagonist MLA, suggesting that DXL-A-24 likely exerts its antinociceptive effect

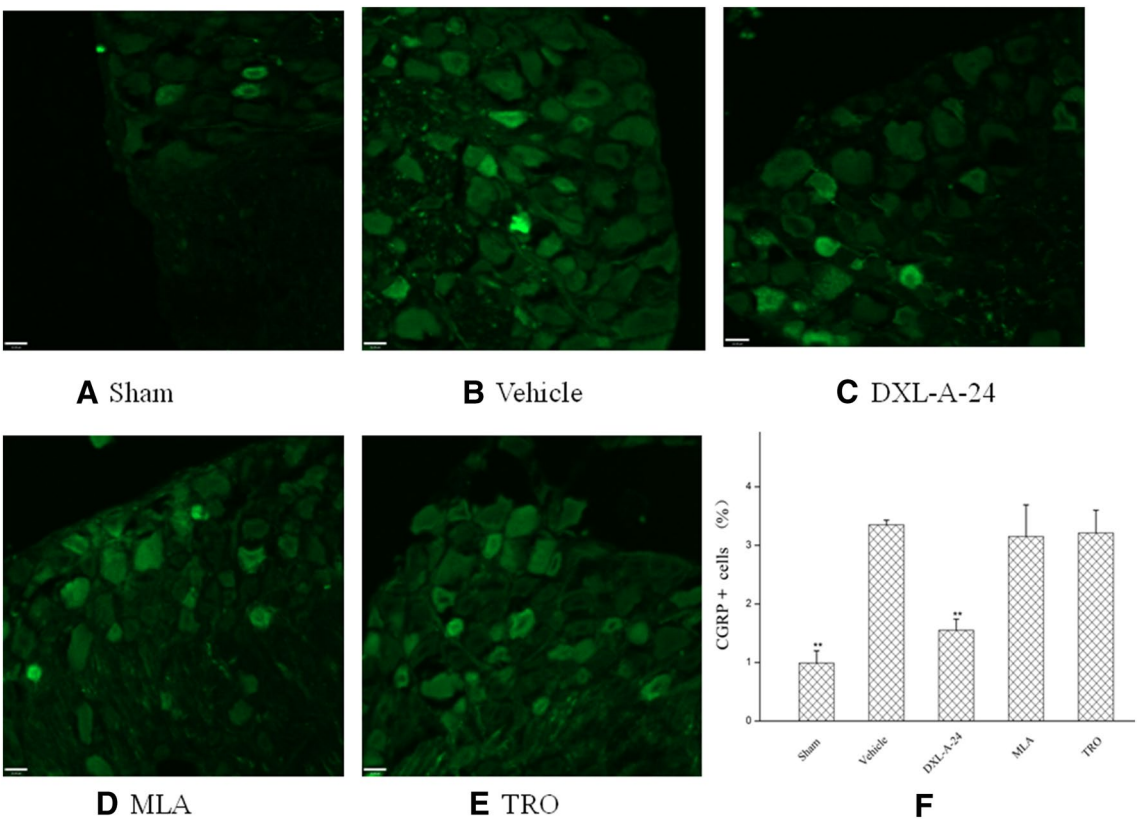

Fig. 7 Effect of DXL-A-24 on the expression of CGRP in the DRGs of CCI rats. CGRP expression in the DRGs was detected using immunofluorescence staining (scale bar 22 μm). **a** Sham group (NS/DDW). **b** Vehicle group (NS/DDW). **c** DXL-A-24 group (NS/DXL-A-24). **d** MLA group (MLA/DXL-A-24). **e** TRO group (TRO/DXL-A-24). **f** Percentage of CGRP positive cells in the total DRG cells. Data are presented as mean ± SD of 4 rats per group. $^{**}P<0.01$ compared with the Vehicle group

by activating peripheral α7 nAChR, and potentially avoids the adverse reaction triggered by α7 nAChR activation in the central nervous system.

Calcium/calmodulin-dependent protein kinase II (CaMKII), which includes α, β, γ and δ subtypes, is expressed in the nervous systems [17]. CaMKIIα is activated by an increase in the intracellular Ca^{2+} concentration and calmodulin, and then auto-phosphorylated at Thr-286 [18]. As an important factor in the development of chronic pain, pCaMKIIα levels are increased in the spinal cord of rats with neuropathic pain [19]. In our study, we initially confirmed that the ligation of the sciatic nerve increased pCaMKIIα levels, but did not alter CaMKIIα expression in rat DRGs. The increase in phosphorylation was inhibited by DXL-A-24. Base on these results, the increase of pCaMKIIα levels played an important role in neuropathic pain, and DXL-A-24 probably alleviated neuropathic pain by inhibiting CaMKIIα phosphorylation.

As a type of GPCR receptor, M4 mAChR is activated through an interaction with phosphorylated CaMKIIα, and then inhibits downstream signalling pathways [20]. Meanwhile, M4 mAChR is postulated to be a more attractive antinociceptive target because it does not influence physiological function [21, 22]. However, the role of M4 mAChR in neuropathic pain has not yet been investigated. In the present study, the antinociceptive effect of DXL-A-24 was blocked by Amn or TRO, indicating that the antinociceptive effect of DXL-A-24 is probably mediated by the activation of peripheral M4 mAChR.

The inhibition of the activity of cAMP-response element binding protein (CREB) alleviates the allodynia and hyperalgesia caused by nerve injury [23, 24]. At the same time, phosphorylated CREB (pCREB) contributes to maintaining hyperalgesia by triggering the production of related factors such as TNF-α [25]. The increase in pCREB expression caused by CCI surgery was inhibited by DXL-A-24 in the present study, suggesting that the antinociceptive effect of DXL-A-24 was associated with the inhibition of CREB phosphorylation.

TNF-α is a proinflammatory mediator that induces inflammation and facilitates the development of neuropathic pain [26]. Meanwhile, TNF-α plays a more important role in regulating pain [27]. The expression of the TNF-α mRNA was increased in the DRGs of CCI rats, and the administration of DXL-A-24 inhibited the expression of TNF-α mRNA in the present study, suggesting that the antinociceptive effect of DXL-A-24 was related to the inhibition of TNF-α expression.

Calcitonin gene-related peptide (CGRP), an excitatory neurotransmitter that is released in primary afferent nerves, promotes the occurrence of allodynia and hyperalgesia [28]. Currently, CGRP antagonists are being investigated as antinociceptive targets and have been proven to exert antinociceptive effects on different pain models [29, 30]. In the present study, the increase in CGRP expression was inhibited by DXL-A-24, and the effect was blocked by MLA and TRO, implying that DXL-A-24 alleviated neuropathic pain by inhibiting CGRP expression, which is probably mediated by the activation of α7 nAChR and M4 mAChR.

Conclusions

In conclusion, the spirocyclopiperazinium salt compound DXL-A-24 exerted significant antinociceptive effects on neuropathic pain and chemical-stimulated pain. The antinociceptive effect may be mediated by the activation of peripheral α7 nAChR and M4 mAChR, the subsequent inhibition of the CaMKIIα/CREB signalling pathway, and eventually the reduction of CGRP and TNF-α expression.

Acknowledgements This research was supported by grants from the National Natural Science Foundation of China, China (Grant nos. 81870876 and 81470050) and the Beijing Municipal Natural Science Foundation, China (Grant no. 7122097).

Compliance with ethical standards

Conflict of interest The authors have no conflicts of interest to disclose.

References

1. Jensen TS, Baron R, Haanpaa M, Kalso E, Loeser JD, Rice AS, Treede RD (2011) A new definition of neuropathic pain. Pain 152:2204–2205
2. Kaur P, Muthuraman A, Kaur J (2015) Ameliorative potential of angiotensin-converting enzyme inhibitor (ramipril) on chronic constriction injury of sciatic nerve induced neuropathic pain in mice. J Renin Angiotensin Aldosterone Syst. 16:103–112
3. Campbell JN, Meyer RA (2006) Mechanisms of neuropathic pain. Neuron 52:77–92
4. van Hecke O, Austin SK, Khan RA, Smith BH, Torrance N (2014) Neuropathic pain in the general population: a systematic review of epidemiological studies. Pain 155:654–662
5. Dworkin RH, O'Connor AB, Audette J, Baron R, Gourlay GK, Haanpaa ML, Kent JL, Krane EJ, Lebel AA, Levy RM, Mackey SC, Mayer J, Miaskowski C, Raja SN, Rice AS, Schmader KE, Stacey B, Stanos S, Treede RD, Turk DC, Walco GA, Wells CD (2010) Recommendations for the pharmacological management of neuropathic pain: an overview and literature update. Mayo Clin Proc 85:S3–S14
6. Pereira A, Gitlin MJ, Gross RA, Posner K, Dworkin RH (2013) Suicidality associated with antiepileptic drugs: implications for the treatment of neuropathic pain and fibromyalgia. Pain 154:345–349
7. Dworkin RH, O'Connor AB, Backonja M, Farrar JT, Finnerup NB, Jensen TS, Kalso EA, Loeser JD, Miaskowski C, Nurmikko TJ, Portenoy RK, Rice AS, Stacey BR, Treede RD, Turk DC,

Wallace MS (2007) Pharmacologic management of neuropathic pain: evidence-based recommendations. Pain 132:237–251
8. Zhao X, Ye J, Sun Q, Xiong Y, Li R, Jiang Y (2011) Antinociceptive effect of spirocyclopiperazinium salt compound LXM-15 via activating peripheral alpha7 nAChR and M4 mAChR in mice. Neuropharmacology 60:446–452
9. Bennett GJ, Xie YK (1988) A peripheral mononeuropathy in rat that produces disorders of pain sensation like those seen in man. Pain 33:87–107
10. Colloca L, Ludman T, Bouhassira D, Baron R, Dickenson AH, Yarnitsky D, Freeman R, Truini A, Attal N, Finnerup NB, Eccleston C, Kalso E, Bennett DL, Dworkin RH, Raja SN (2017) Neuropathic pain. Nat Rev Dis Primers. 3:17002
11. Hersh EV, Moore PA, Ross GL (2000) Over-the-counter analgesics and antipyretics: a critical assessment. Clin Ther 22:500–548
12. Paviaya US, Kumar P, Wanjari MM, Thenmozhi S, Balakrishnan BR (2013) Analgesic and anti-inflammatory activity of root bark of Grewia asiatica Linn. in rodents. Anc Sci Life. 32:150–155
13. Quik M, Zhang D, McGregor M, Bordia T (2015) Alpha7 nicotinic receptors as therapeutic targets for Parkinson's disease. Biochem Pharmacol 97:399–407
14. Shelukhina I, Paddenberg R, Kummer W, Tsetlin V (2015) Functional expression and axonal transport of alpha7 nAChRs by peptidergic nociceptors of rat dorsal root ganglion. Brain Struct Funct. 220:1885–1899
15. Wan D, Wang D, Sun Q, Song Y, Jiang Y, Li R, Ye J (2016) Antinociception of spirocyclopiperazinium salt compound LXM-10-M targeting alpha7 nicotinic receptor and M4 muscarinic receptor and inhibiting CaMKIIalpha/CREB/CGRP signaling pathway in mice. Eur J Pharmacol 770:92–98
16. Medhurst SJ, Hatcher JP, Hille CJ, Bingham S, Clayton NM, Billinton A, Chessell IP (2008) Activation of the alpha7-nicotinic acetylcholine receptor reverses complete freund adjuvant-induced mechanical hyperalgesia in the rat via a central site of action. J Pain. 9:580–587
17. Soderling TR, Chang B, Brickey D (2001) Cellular signaling through multifunctional Ca^{2+}/calmodulin-dependent protein kinase II. J Biol Chem 276:3719–3722
18. Ogawa A, Dai Y, Yamanaka H, Iwata K, Niwa H, Noguchi K (2005) Ca(2+)/calmodulin-protein kinase IIalpha in the trigeminal subnucleus caudalis contributes to neuropathic pain following inferior alveolar nerve transection. Exp Neurol 192:310–319
19. Boric M, Jelicic KA, Puljak L (2014) The expression of calcium/calmodulin-dependent protein kinase II in the dorsal horns of rats with type 1 and type 2 diabetes. Neurosci Lett 579:151–156
20. Guo ML, Mao LM, Wang JQ (2010) Modulation of M4 muscarinic acetylcholine receptors by interacting proteins. Neurosci Bull. 26:469–473
21. Duttaroy A, Gomeza J, Gan JW, Siddiqui N, Basile AS, Harman WD, Smith PL, Felder CC, Levey AI, Wess J (2002) Evaluation of muscarinic agonist-induced analgesia in muscarinic acetylcholine receptor knockout mice. Mol Pharmacol 62:1084–1093
22. Swedberg MD, Sheardown MJ, Sauerberg P, Olesen PH, Suzdak PD, Hansen KT, Bymaster FP, Ward JS, Mitch CH, Calligaro DO, Delapp NW, Shannon HE (1997) Butylthio[2.2.2] (NNC 11-1053/LY297802): an orally active muscarinic agonist analgesic. J Pharmacol Exp Ther 281:876–883
23. Liou JT, Liu FC, Hsin ST, Yang CY, Lui PW (2007) Inhibition of the cyclic adenosine monophosphate pathway attenuates neuropathic pain and reduces phosphorylation of cyclic adenosine monophosphate response element-binding in the spinal cord after partial sciatic nerve ligation in rats. Anesth Analg 105:1830–1837
24. Song XJ, Wang ZB, Gan Q, Walters ET (2006) cAMP and cGMP contribute to sensory neuron hyperexcitability and hyperalgesia in rats with dorsal root ganglia compression. J Neurophysiol 95:479–492
25. Yao CY, Weng ZL, Zhang JC, Feng T, Lin Y, Yao S (2016) Interleukin-17A acts to maintain neuropathic pain through activation of CaMKII/CREB signaling in spinal neurons. Mol Neurobiol 53:3914–3926
26. Ogawa N, Kawai H, Terashima T, Kojima H, Oka K, Chan L, Maegawa H (2014) Gene therapy for neuropathic pain by silencing of TNF-alpha expression with lentiviral vectors targeting the dorsal root ganglion in mice. PLoS ONE 9:e92073
27. Andrade P, Hoogland G, Del RJ, Steinbusch HW, Visser-Vandewalle V, Daemen MA (2014) Tumor necrosis factor-alpha inhibitors alleviation of experimentally induced neuropathic pain is associated with modulation of TNF receptor expression. J Neurosci Res 92:1490–1498
28. Ma W, Eisenach JC (2003) Intraplantar injection of a cyclooxygenase inhibitor ketorolac reduces immunoreactivities of substance P, calcitonin gene-related peptide, and dynorphin in the dorsal horn of rats with nerve injury or inflammation. Neuroscience 121:681–690
29. Hirsch S, Corradini L, Just S, Arndt K, Doods H (2013) The CGRP receptor antagonist BIBN4096BS peripherally alleviates inflammatory pain in rats. Pain 154:700–707
30. Romero-Reyes M, Pardi V, Akerman S (2015) A potent and selective calcitonin gene-related peptide (CGRP) receptor antagonist, MK-8825, inhibits responses to nociceptive trigeminal activation: role of CGRP in orofacial pain. Exp Neurol 271:95–103