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Antinociceptive effect of spirocyclopiperazinium salt compound LXM-15 via activating peripheral α7 nAChR and M4 mAChR in mice

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ABSTRACT

The aim of this study was to evaluate the antinociceptive effects and potential mechanisms of the spirocyclopiperazinium compound LXM-15. We found that LXM-15 produced significant antinociceptive effects in a dose- and time-dependent manner in mice. The maximum inhibition ratio was 70% in the acetic acid writhing test; the effect started at 1.0 h, peaked at 2.0 h with the MPEs of 61%, and persisted 3.5 h in the hot-plate test; LXM-15 reduced the time spent licking or biting the injected paw remarkably with inhibitions of 53% in formalin test. LXM-15 did not affect motor coordination, spontaneous activity, body temperature, heart rate, or liver enzyme activity, the LD_{50} values was 616.26 μmol/kg. The antinociceptive effect of LXM-15 was blocked by mecamylamine, hexamethonium, atropine or atropine methylnitrate, and was also blocked by MLA, tropicamide. In contrast, the effect was not blocked by naloxone. Meanwhile, competition receptor binding assays showed LXM-15 can bind to α7 nAChR or M4 mAChR. Our studies show that LXM-15 may be via activating peripheral α7 nicotnic and M4 muscarinic receptors, resulted in antinociceptive effects.

1. Introduction

There are many therapeutic approaches for pain relief, and chief among them are agonists of opioid receptors and inhibitors of cyclooxygenase, which are inadequately managed in many patients, and that there are inevitable side effects associated. It is urgently needed to discover efficient analgesics without side effects in pain management. In recent years, there has been more studies focusing on the pathway underling nAChR- or mAChR-mediated antinociceptive effects (Dussor et al., 2004; Millan, 2002). Agonist of nAChRs excites analgesic activity (Bernardini et al., 2001a,b; Rueter et al., 2003; Sudo et al., 2002). For example, epibatidine, ABT594 has a clear analgesic effect, which is mainly related with the central α4β2 nicotinic receptor (Flores, 2000), but administration produces strong and complex side effects such as ataxia, hypothermia, convulsions and so on (Boyce et al., 2000; Rowbotham et al., 2009). In the mean time, several studies have shown that the muscarinic acetylcholine receptors (mAChRs) modulate pain perception (Galeotti et al., 2008), but the use of mAChR agonists is limited by their undesirable shortcomings. Based on above, our group synthesized a series of novel spirocyclopiperazinium compounds with analgesic activity, which does not closely relate with α4β2 nAChR (Sun et al., 2007). Compound LXM-15 (8-(m-nitrophenyl)-5,8-diazaspiro[4.5]decane bromide Fig. 1) is one of them that cannot pass the blood–brain barrier easily and thereby avoid causing any central nervous system side effects. In this study, a thorough examination was done regarding the antinociceptive activities of LXM-15 by acetic acid writhing, hot-plate and formalin tests. An assessment was constructed upon acute toxicity and typical side effects, including motor coordination, locomotor activity, temperature, heart rate and hepatotoxicty. We also determined the ability of LXM-15 binding to α7 nAChR and M4 mAChR, and explored the potential signaling pathway of antinociception.

2. Materials and methods

2.1. Animals

C57/BL6 mice, weighing 20–22 g, and Wistar rats, weighing 170–230 g, were obtained from the Department of Laboratory Animal Science of Peking University. All animals were used in compliance with the guidelines set by the International Association for the Study of Pain (IASP) and the Institutional Animal Care and Use Committee of Peking University. All behavioral measurements were performed in

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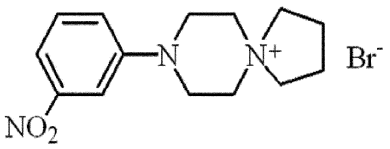

Fig. 1. The chemical structure of the spirocyclopiperazinium compound LXM-15.

awake, age-matched adult mice of both sexes and were conducted in a blinded manner.

2.2. Drugs

Drug use: morphine hydrochloride (Neuroscience Research Institute of Peking University, Beijing, PR. China), Aspirin, naloxone hydrochloride, atropine sulphate, atropine methylnitrate, mecamylamine hydrochloride, hexamethonium chloride, methyllycaconitine citrate, tropicamide, bovine serum albumin (BSA), polyethyleneimine and nicotine (Sigma Chemical Co., St. Louis, MO, USA), [^{3}H]Methyllycaconitine ([^{3}H]MLA, 100 Ci/mmol, American Radiolabeled Chemicals, Inc.), [N-methyl-^{3}H]scopolamine methyl chloride ([^{3}H]NMS, 3.03 TBq/mmol) and Liquid Scintillation Cocktails (PerkinElmer Life Sciences, Boston, MA), Modified Lowry Protein Assay Kit (Beijing Applygen Technologies Inc. PR China). LXM-15 was synthesized by Runtao Li and Qi Sun. The doses of drugs were selected on the basis of previous reports and our preliminary studies (Beirith et al., 1998; Boccia et al., 2001; Decker et al., 1998). All drugs were dissolved in distilled water immediately before use and administered in a volume of 10 ml/kg by subcutaneous injection (s.c.), intraperitoneal injection (i.p.) or intragastric injection (i.g.).

2.3. Antinociceptive tests

2.3.1. Acetic acid writhing test

Acetic acid writhing test was performed as previously described (Galeotti et al., 2008). Mice were administered LXM-15 (20, 10 or 5 μmol/kg, s.c.), aspirin (1665 μmol/kg, i.g.) or vehicle (distilled water) 30 min prior to injection of 0.6% acetic acid (10 ml/kg, i.p.). The number of stretching movements was recorded for 10 min, starting 5 min after acetic acid injection. Analgesia percentage was expressed by following formula:

$$\text{Inhibition (\%)} = 100 \times (\text{number of control} - \text{number of experiment})/\text{number of control}$$

2.3.2. Hot-plate test

The hot-plate test was performed according to the method previously described (Sulaiman et al., 2008). The mice were placed on a plate maintained at 55 °C, and the latency of licking hind paws, shaking or jumping off the surface was evaluated as an index of nociception. Prior to drug administration, baseline response latency of each mouse was measured three times. The hot-plate response latency was measured at 0.5, 1, 1.5, 2, 2.5, 3 and 3.5 h after administering LXM-15 (20, 10 or 5 μmol/kg, s.c.), morphine (18 μmol/kg, s.c.) or vehicle. Mice with baseline latency shorter than 5 s or longer than 20 s were eliminated to exclude hyposensitive or hypersensitive mice. Cut-off time was 20 s. Analgesia was quantified with the Maximum Possible Effect (MPE), which was calculated as:

$$\text{MPE (\%)} = [(\text{response latency} - \text{baseline latency})/(\text{cut-off time} - \text{baseline latency})] \times 100$$

2.3.3. Formalin test

The formalin test was conducted according to methods published previously (Seo et al., 2008; Tjolsen et al., 1992). We administered LXM-15 (20, 10 or 5 μmol/kg, s.c.), morphine (18 μmol/kg, s.c.) or vehicle to each mouse 1 h before injecting 20 μl of 1% formalin (v/v in distilled water) subcutaneously into the right forepaw. The time mice spent licking or biting their injected paws was recorded at 5 min intervals for 1 h. The first 10 min was considered as early phase (Phase I) and the period of 10–60 min as the late phase (Phase II) of the nociceptive response. The percentage of inhibition was calculated using the time mice spent licking or biting their injected paws:

$$\text{Inhibition (\%)} = (\text{control} - \text{experiment})/\text{control} \times 100$$

2.3.4. Tail-flick test

The responsiveness to radiant heat was assessed in the tail-flick test (D'Amour and Smith, 1941). The latency to remove the tail from the heat source was recorded for each animal. Test latency was recorded at 1, 2, 3 h after administering LXM-15 (20 μmol/kg, s.c.). A control response (2–4 s) was determined for each mouse before treatment, and cut-off time used was 10 s.

2.4. The antinociceptive mechanisms of LXM-15

To illustrate the antinociceptive mechanisms of LXM-15, mice were pretreated with different receptor antagonists in the hot-plate test and formalin test.

2.4.1. Effect of receptor antagonists on the antinociception of LXM-15 in the hot-plate test

In the hot-plate test, naloxone (a non-selective opioid receptor antagonist 5 μmol/kg, i.p.), mecamylamine (a central and peripheral neuronal nicotinic acetylcholine receptor antagonist, 5 μmol/kg, i.p.), hexamethonium (a peripheral neuronal nicotinic acetylcholine receptor antagonist, 18 μmol/kg, i.p.), atropine (a central and peripheral muscarinic acetylcholine receptor antagonist, 7 μmol/kg, i.p.), atropine methylnitrate (a peripheral muscarinic acetylcholine receptor antagonist, 14 μmol/kg, i.p.), Methyllycaconitine (an α7 nAChR antagonist, 3, 0.3 or 0.03 μmol/kg, i.p.), tropicamide (an M4 muscarinic receptor antagonist, 3, 0.3 or 0.03 μmol/kg, i.p.) or vehicle were pretreated 15 min before LXM-15 (20 μmol/kg, s.c.), morphine (18 μmol/kg, s.c.) or vehicle injected. The hot-plate response latencies were measured at 1, 2 and 3 h after the second drug administration.

2.4.2. Effect of receptor antagonists on the antinociception of LXM-15 in the formalin test

In the formalin test, Methyllycaconitine (3, 0.3 or 0.03 μmol/kg, i.p.), tropicamide (3, 0.3 or 0.03 μmol/kg, i.p.) or vehicle were pretreated 15 min before LXM-15 (20 μmol/kg, s.c.) or vehicle injected. 1 h later, 20 μl of 1% formalin was injected subcutaneously into the right forepaw.

2.5. Receptor binding assays

2.5.1. Brain membrane preparations

12 male Wistar rats were sacrificed by cervical dislocation. Hippocampus and corpus striatum fractions of brain were dissected out and homogenized in 10 volumes of ice-cold 0.32 M sucrose respectively. The homogenates were centrifuged at 1000 × g for 10 min. The supernatant was then centrifuged in 50 mM Tris–HCl (pH 7.4) at 20,000 × g for 30 min. This procedure was repeated, and the final pellets were suspended in a buffer and stored at −20 °C for the binding assay. Protein concentrations were measured by the modified Lowry protein assay according to manufacturer's instruction.

2.5.2. [^{3}H]MLA binding assay

For competition assay, binding affinity of [^{3}H]-MLA to hippocampus membranes was measured as described by Davies et al. (Davies et al., 1999). In brief, hippocampus membranes (containing 0.4 mg of protein) were incubated in borosilicate glass tubes with 2 nM [^{3}H]MLA (100 Ci/mmol) in a final volume of 0.5 ml for 2 h at 4 °C, in the absence or presence of increasing concentrations of LXM-15 from 1.52×10^{-9} to 1.00×10^{-5} M. Non-specific binding was determined from tubes containing 1 mM nicotine. Incubation buffer consisted of phosphate buffer (20 mM Na_2HPO_4, 5 mM KH_2PO_4, 150 mM NaCl, pH 7.4) supplemented with 0.1% (w/v) BSA. Incubation was completed by rapid filtration under vacuum through Whatman GF/B glass fibre filters (Whatman Intl. Ltd., Maidstone, UK) pre-soaked in 0.3% polyethyleneimine overnight. Tubes and filters were washed rapidly three times with 3 ml ice-cold PBS. Filters were then dried and counted on liquid scintillation spectrometry (Tri-Carb 2100 TR, PerkinElmer, USA). The IC_{50} value for LXM-15 from competition binding assay was determined by nonlinear regression analysis with SPSS16.0 software.

2.5.3. [^{3}H]NMS binding assay

[^{3}H]NMS binding assay was performed according to previously described (Ehlert and Tran, 1990; Oki et al., 2005). Corpus striatum membranes (containing 0.2 mg of protein) were incubated in tubes with 0.5 nM [^{3}H]NMS (60 Ci/mmol) in a final volume of 1 ml for 1 h at 25 °C. Competitive binding was determined in the presence of increasing concentrations of LXM-15 from 1.00×10^{-6} to 3.20×10^{-5} M. Non-specific binding was defined in the presence of 10 μM atropine. Incubation buffer consisted of Tris–HCl buffer (50 mM, pH 7.4). The remainder of the procedure was done as in the MLA binding assay.

2.6. Side effect tests

Following tests were performed at 0.5, 2 and 3.5 h after administering LXM-15 (80, 40 or 20 μmol/kg, s.c.), Diazepam (7 μmol/kg, i.p.) or vehicle.

2.6.1. Rota-rod test

Motor coordination was assessed by the rota-rod test. The apparatus (Model DXP-2, Institute of Materia Medica, Chinese Academy of Medical Sciences, PR China) consisted of a base platform and a rotating rod with a diameter of 2.5 cm, subdivided into four equal sections. Each mouse was trained to run on the rota-rod at a constant

speed (40 rev/min) for 60 s without falling. The time mice were able to maintain their balance on the rod was measured. The cut-off time was 120 s.

2.6.2. Spontaneous activity

Locomotor activity was determined using the Opto-Varimex infrared photocell-based activity monitor (Model DXP-2, Institute of Materia Medica, Chinese Academy of Medical Sciences, PR China). Horizontal (ambulation) and vertical (rearing) activities were recorded within 5 min.

2.6.3. Body temperature

Body temperature was measured by digital thermometer. The probe was inserted 3.0 cm into the rectum.

2.6.4. Heart rate

Animals were anesthetized with pentobarbital sodium, and heart rate was recorded with a Cardiofax ECG-6511 (Nihon Konden Corp, Tokyo).

2.6.5. Serum hepatic enzymes

The activities of serum lanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) were measured in mice treated with LXM-15 (40, 20 μmol/kg, s.c.), CCl_4 (15%, i.p.) or vehicle 24 or 48 h later. The blood of mice were collected and stored on ice before centrifugation at 1000 × g at 4 °C for 10 min, then the serum were collected. The activities of ALT, AST and ALP were determined with assay kits (Nanjing Jiancheng Bioengineering Institute, PR China). Absorbance of the reaction mixture was measured by a spectrophotometer at 505 nm for ALT and AST and 520 nm for ALP.

2.7. Acute toxicity test

We used 50% lethal dose (LD_{50}) to determine the acute toxicity of LXM-15 in mice. A series of concentrations of LXM-15 was administered by subcutaneous injection. The percentages of mortality were recorded after 24 h of treatment. LD_{50} values were calculated by the method of Bliss (probit regression analysis).

2.8. Statistical analysis

Each group contained 10 mice. All data were analyzed by using repeated-measures analysis of variance. Differences were considered significant at $P < 0.05$.

3. Results

3.1. Antinociceptive effect of LXM-15

As shown in Fig. 2A, LXM-15 significantly reduced the number of abdominal constrictions as compared with the control group at the doses of 20, 10 and 5 μmol/kg, inhibition ratios were 70%, 61% and 45%, respectively. In the hot-plate test, LXM-15 prolonged response latency times, showing a significant increase in MPE compared with control ($P < 0.01$) (Fig. 2B), and the effect was both dose- and time-dependent. The antinociceptive effect of LXM-15 began at 1.0 h, peaked at 2.0 h, and persisted until 3.5 h after s.c. administration. Peak MPEs were 61%, 38% and 25%, respectively, at the doses of 20, 10 or 5 μmol/kg, s.c. Repeated-measures ANOVA showed significant differences for treatment groups [$F(4,45) = 139.314$, $P < 0.001$], times [$F(6,270) = 20.998$, $P < 0.001$], and interaction between treatment groups and times [$F(24,270) = 20.407$, $P < 0.001$] in the hot-plate test. Consistent with its effects on the previous test, LXM-15 markedly reduced the time mice spent licking or biting the injected paw in the

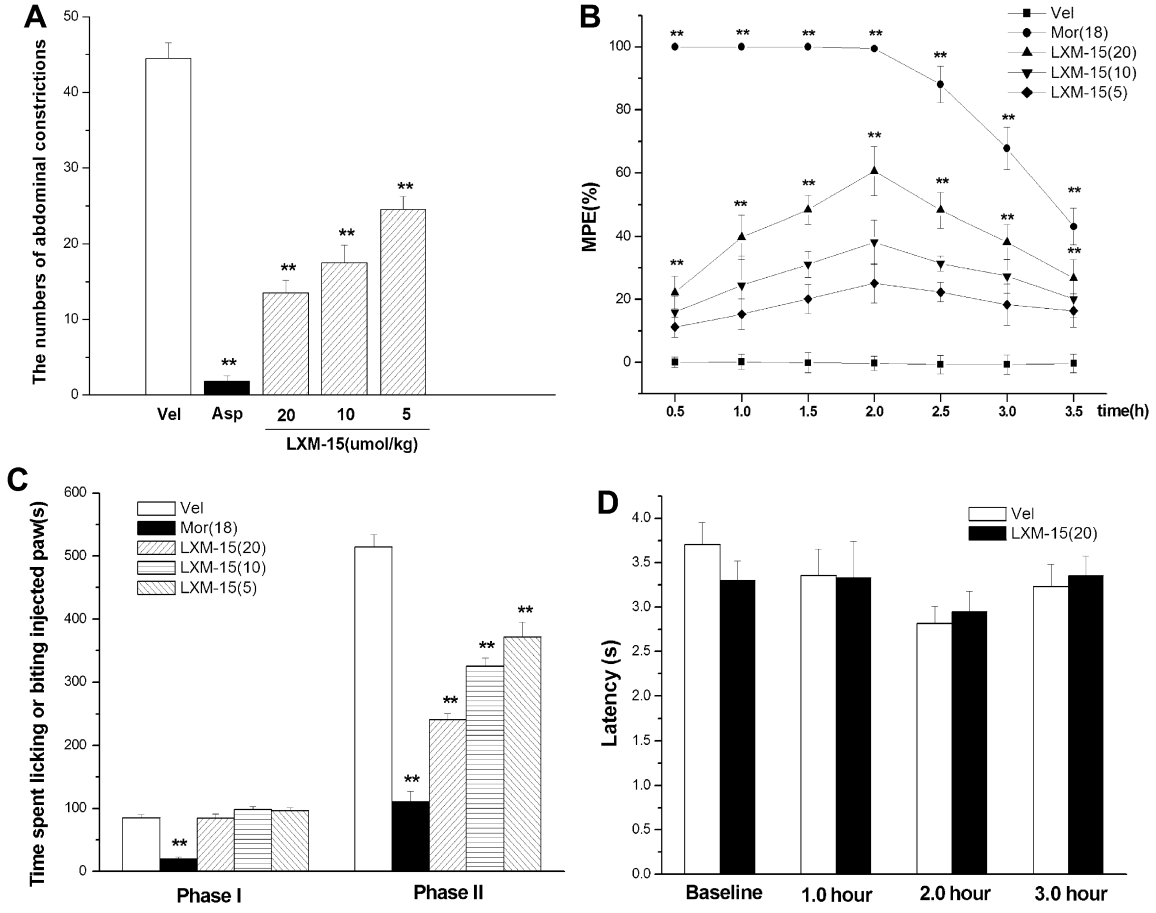

Fig. 2. Antinociceptive effect of LXM-15. The mice were administered LXM-15 (20, 10 or 5 μmol/kg, s.c.), aspirin (1665 μmol/kg, i.g.), morphine (18 μmol/kg, s.c.) or vehicle (distilled water). (A) Effect of LXM-15 in the acetic acid writhing test. LXM-15 significantly reduced the number of abdominal constrictions. (B) Effect of LXM-15 in the hot-plate test. LXM-15 increased the MPEs in a dose- and time-dependent fashion. (C) Effect of LXM-15 in the formalin test. LXM-15 reduced the time spent licking or biting the injected paw in a dose- and time-dependent fashion. (D) Effect of LXM-15 in the tail-flick test. LXM-15 did not affect MPEs. All data are mean ± S.E.M. of 10 mice per group. $^*P < 0.05$, $^{**}P < 0.01$ compared with vehicle group.

formalin test, compared with the control group in phase II ($P < 0.01$; Fig. 2C), and produced inhibitions of 54%, 37% and 28%, respectively, at the doses of 20, 10 or 5 μmol/kg, i.p. However, LXM-15 had no effect during phase I (Fig. 2C). No antinociception effect was observed in the tail-flick test (Fig. 2D).

3.2. Potential antinociceptive mechanisms of LXM-15

3.2.1. Involvement of opioid system

Fig. 3 shows that the non-selective opioid antagonist naloxone (5 μmol/kg, i.p.) did not block the effect produced by LXM-15 at 1.0, 2.0 and 3.0 h after administration, whereas it blocked the antinociception caused by morphine (18 μmol/kg, s.c.) in the hot-plate test. Repeated-measures ANOVA showed significant differences for treatment groups [$F(4,45) = 224.494$, $P < 0.001$], times [$F(2,90) = 38.465$, $P < 0.001$], and interaction between treatment groups and times [$F(8,90) = 35.074$, $P < 0.001$] in the hot-plate test.

3.2.2. Involvement of peripheral α7 nicotinic acetylcholine system

As shown in Fig. 4A, when mice were predosed with mecamylamine (5 μmol/kg, i.p.), the efficacy of LXM-15 was inhibited to 0.6% MPE at peak time in the hot-plate test. At the same time, the peripheral neuronal nicotinic acetylcholine receptor antagonist hexamethonium (18 μmol/kg, i.p.) also blocked the effect to −1.01% MPE. Repeated-measures ANOVA showed significant differences for treatment groups [$F(3,36) = 53.617$, $P < 0.001$], times[$F(6,216) = 3.148$, $P < 0.01$], and interaction between treatment groups and times [$F(18,216) = 3.342$, $P < 0.001$].

After pretreatment with methyllycaconitine (3 and 0.3 μmol/kg, i.p.), the antinociceptive effect of LXM-15 was attenuated in both tests. MPEs were reduced to 10% and 27% at peak time in the hot-plate test (Fig. 4B) and the time spent licking or biting injected paws were reduced to 12% and 25% during phase II of the formalin test (Fig. 4C). The lowest dose of methyllycaconitine citrate had no effect in either model. Repeated-measures ANOVA showed significant differences for treatment groups [$F(4,45) = 48.857$, $P < 0.001$], times [$F(6,270) = 33.212$, $P < 0.001$], and interaction between treatment groups and times [$F(24,270) = 5.927$, $P < 0.001$] in the hot-plate test.

3.2.3. Involvement of peripheral M4 muscarinic acetylcholine system

As shown in Fig. 5A, when mice were predosed with atropine (7 μmol/kg, i.p.), the efficacy of LXM-15 was inhibited to 2.8% MPE at peak time. Meanwhile, the peripheral muscarinic acetylcholine receptor antagonist atropine methylnitrate (14 μmol/kg, i.p.) also blocked the effect to −0.3% MPE in the hot-plate test. Repeated-measures ANOVA showed significant differences for treatment groups [$F(3,36) = 63.745$, $P < 0.001$], times [$F(6,216) = 5.033$, $P < 0.001$], and interaction between treatment groups and times [$F(18,216) = 3.888$, $P < 0.001$].

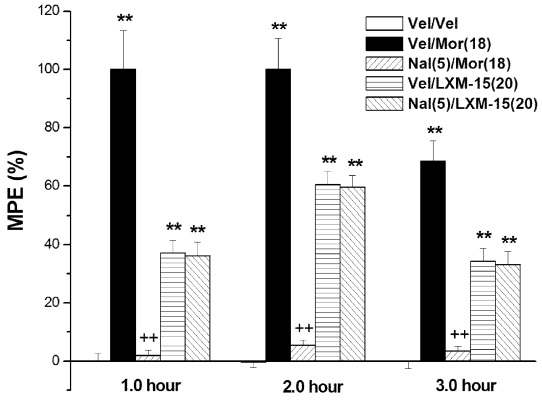

Fig. 3. Effect of naloxone on the LXM-15-induced antinociception in the hot-plate test. Mice were pretreated with naloxone (Nal. 5 μmol/kg, i.p.) or vehicle before administration of LXM-15 (20 μmol/kg, s.c.), morphine (Mor. 18 μmol/kg, s.c.) or vehicle. Naloxone blocked the morphine-induced MPE increase, but did not block the effect of LXM-15. All data are mean ± S.E.M. of 10 mice per group. $^{**}P < 0.01$ vs Vel/Vel group; $^{++}P < 0.01$ vs Vel/Mor group at the same time.

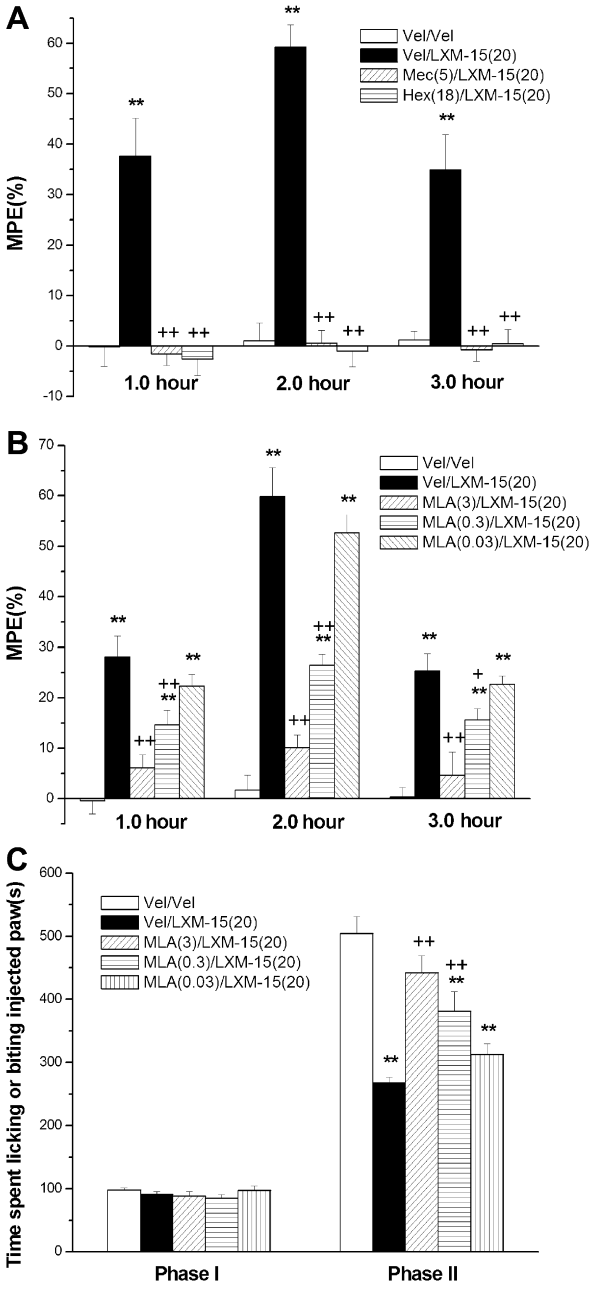

Fig. 4. Effect of mecamylamine (Mec), hexamethonium (Hex), methyllycaconitine (MLA) on LXM-15-induced antinociception. The mice were pretreated with Mec (5 μmol/kg, i.p.), Hex (18 μmol/kg, i.p.), MLA (3, 0.3 or 0.03 μmol/kg, i.p.) or vehicle before administration of LXM-15 (20 μmol/kg, s.c.) or vehicle. (A) Effect of Mec or Hex on LXM-15-induced antinociception in the hot-plate test. Mec or Hex blocked LXM-15-induced MPE increase. (B) Effect of MLA on LXM-15-induced antinociception in the hot-plate test. MLA blocked LXM-15-induced MPE increase at doses of 3 and 0.3 μmol/kg, i.p. (C) Effect of MLA on LXM-15-induced antinociception in the formalin test. MLA blocked LXM-15-induced decrease in the time spent licking or biting at doses of 3 and 0.3 μmol/kg, i.p. All data are mean ± S.E.M. of 10 mice per group. $^{**}P < 0.01$ vs Vel/Vel group; $^{+}P < 0.05$, $^{++}P < 0.01$ vs Vel/LXM-15 group at the same time.

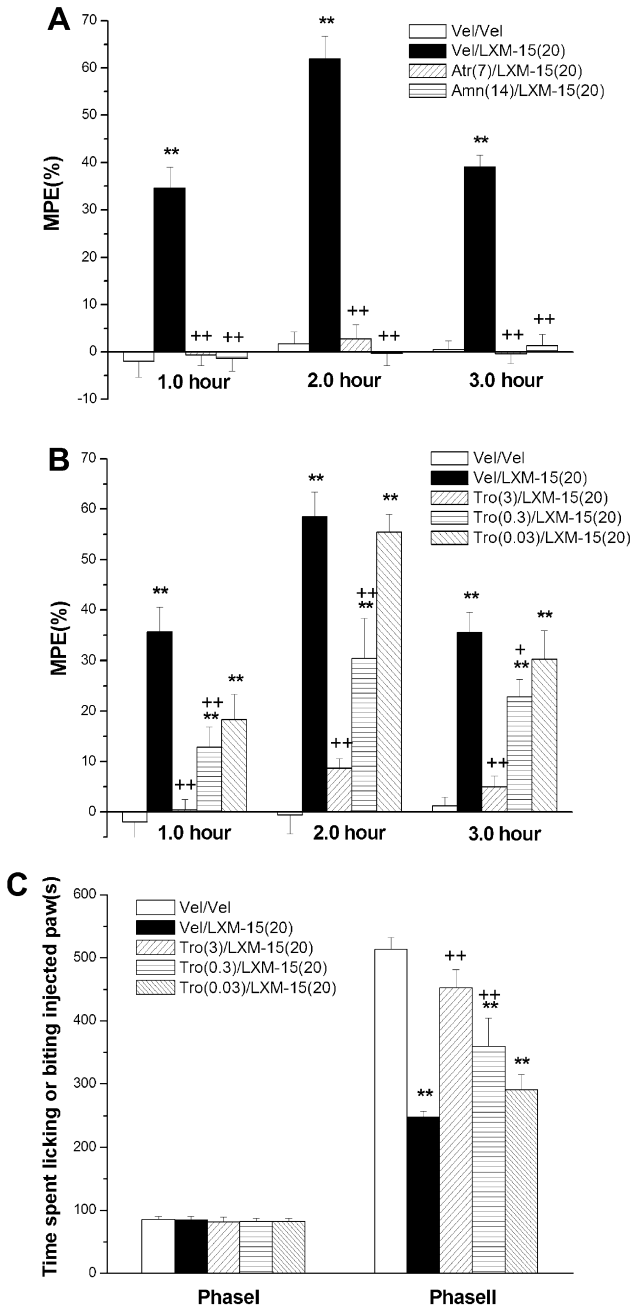

Fig. 5. Effect of atropine (Atr), atropine methylnitrate (Amn) or tropicamide (Tro) on LXM-15-induced antinociception. The mice were pretreated with Atr (7 μmol/kg, i.p.), Amn (14 μmol/kg, i.p.), Tro (3, 0.3 or 0.03 μmol/kg, i.p.) or vehicle before administration of LXM-15 (20 μmol/kg, s.c.) or vehicle. (A) Effect of Atr or Amn on LXM-15-induced antinociception in the hot-plate test. Atr or Amn blocked the LXM-15-induced MPE increase. (B) Effect of Tro on LXM-15-induced antinociception in the hot-plate test. Tro blocked the LXM-15-induced MPE increase at doses of 3 and 0.3 μmol/kg, i.p. (C) Effect of Tro on LXM-15-induced antinociception in the formalin test. Tro blocked the LXM-15-induced decrease in time spent licking or biting at doses of 3 and 0.3 μmol/kg, i.p. All data are mean ± S.E.M. of 10 mice per group. $^{**}P < 0.01$ vs Vel/Vel group; $^{+}P < 0.05$, $^{++}P < 0.01$ vs Vel/LXM-15 group at the same time.

Furthermore, for the mice which were pretreated with Tropicamide (3 and 0.3 μmol/kg, i.p.), the antinociceptive effect of LXM-15 was attenuated in both tests. Peak MPEs were reduced to 9% and 30% (Fig. 5B) in the hot-plate test, and the inhibitions were reduced to 12% and 30% (Fig. 5C) in the formalin test, respectively. Tropicamide had no effect on LXM-15 activity at the lowest dose (0.03 μmol/kg, i.p.) in either model. Repeated-measures ANOVA showed significant differences for treatment groups [$F(4,45) = 40.444$, $P < 0.001$], times [$F(6,270) = 16.937$, $P < 0.001$], and interaction between treatment groups and times [$F(24,270) = 2.684$, $P < 0.001$] in the hot-plate test.

3.2.4. Receptor binding assays

The competition α7 nicotinic acetylcholine receptor binding assay in vitro shows that LXM-15 displaced [^{3}H]MLA binding to hippocampus membranes of rat brains concentration-dependently, with an IC_{50} of 8.72×10^{-7} M. Similarly, LXM-15 also displaced [^{3}H] NMS binding in rat corpus striatum fractions, the IC_{50} was 1.35×10^{-5} M.

3.3. Assessment of side effects

The compound LXM-15 (80, 40 or 20 μmol/kg, s.c.) had no significant effect on motor performance (Table 1) or spontaneous activity (Table 2); nor did it decrease body temperature (Table 3) or heart rate (Table 4) at 0.5, 2 or 3.5 h after administration in mice. However, diazepam (7 μmol/kg, i.p.) reduced performance and activity significantly, as well as mice's body temperature. Moreover, LXM-15 (40, 20 μmol/kg) had no significant effect on serum ALT, AST, and ALP activities, whereas mice treated with CCl_4 developed significant hepatotoxicty at 24 and 48 h, the enzyme activity were increased markedly compared with the normal group (Table 5).

3.4. Assessment of acute toxicity

When mice were administered LXM-15 at doses of 914.3, 800, 700, 612.5, 410.3, 314.2 μmol/kg, s.c., the death rates were 90%, 80%, 60%, 50%, 20%, and 10% respectively. The median lethal dose (LD_{50}) was 616.26 μmol/kg, 95% confidence interval 516.03–709.03 μmol/kg.

4. Discussion

The present results demonstrate that monospirocyclopiperazinium salt compound LXM-15 exhibits antinociceptive effects in mice subjected to either acute thermal or persistent chemical pain stimuli, in a dose-dependent and time-dependent manner. There were significant reductions induced by the highest does of LXM-15 in number of abdominal constrictions (by 70%) and time spent licking or biting the injected paw (by 54%), while MPE had increased by 61%. LXM-15 did not reduce nociception in the tail-flick test, which means LXM-15 did not produce central antinociceptive effect. Further characterization of the antinociceptive effect of LXM-15 using the hot-plate test revealed that the antinociceptive effect peaked at 2 h and was still present at 3.5 h after s.c. administration, the LD_{50} values was 616.26 μmol/kg (95% confidence interval 516.03–709.03 μmol/kg),

Table 1
Effect of LXM-15 on motor performance in mice (rota-rod test).

Group	Dose (μmol/kg)	Before treatment (s)	After treatment (s)		
			0.5 h	2.0 h	3.5 h
Vel	–	99.7 ± 4.18	102.9 ± 4.19	95.6 ± 4.25	102.1 ± 3.68
Diazepam	7	98.5 ± 3.62	46.0 ± 2.40**	71.7 ± 2.29**	100.9 ± 3.93
LXM-15	20	95.1 ± 4.68	100.9 ± 4.09	96.8 ± 3.74	101.2 ± 3.75
	40	94.2 ± 3.94	96.8 ± 4.46	100.1 ± 3.58	100.3 ± 3.78
	80	94.4 ± 4.17	97.9 ± 3.67	95.2 ± 3.48	102.2 ± 3.79

Note: The time for mice staying on the rotating rod was measured before and 0.5 h, 2.0 h, 3.5 h after administered of LXM-15 (80, 40 or 20 μmol/kg, s.c.), Diazepam (7 μmol/kg, i.p.) or vehicle. All data are mean ± S.E.M. of 10 mice per group. $^{**}P < 0.01$ vs Vel group at the same time.

Table 2
Effect of LXM-15 on the spontaneous activities in mice.

Group	Dose (μmol/kg)	Before treatment (n)	Numbers of crossing after treatment (n)		
			0.5 h	2.0 h	3.5 h
Vel	–	350.20 ± 13.07	352.00 ± 14.53	345.60 ± 10.83	335.70 ± 17.39
Diazepam	7	354.50 ± 18.71	172.50 ± 12.05**	209.10 ± 15.30**	270.50 ± 28.30*
LXM-15	20	354.20 ± 12.20	362.70 ± 11.48	341.80 ± 21.58	334.60 ± 19.04
	40	351.30 ± 11.85	368.00 ± 12.63	344.40 ± 23.20	344.20 ± 12.72
	80	357.30 ± 12.79	358.70 ± 16.02	356.40 ± 11.43	351.90 ± 8.97

Note: The spontaneous activities were tested before and 0.5 h, 2.0 h, 3.5 h after administered of LXM-15 (80, 40 or 20 μmol/kg, s.c.), Diazepam(7 μmol/kg, i.p.), or vehicle. All data are mean ± S.E.M. of 10 mice per group. *$P < 0.05$, **$P < 0.01$ vs Vel group at the same time.

the results illustrates that LMX-15 had efficacious antinociceptive effect with lower toxicity.

To explore the antinociceptive mechanism of LXM-15, we used the naloxone antagonism test. We discovered that naloxone blocked the antinociceptive effect of morphine entirely, but did not block the effect of LXM-15. The result indicates that the opioid system does not interfere with the antinociception of LXM-15, which means that LXM-15 may lack the problems associated with opioid analgesia.

Reports show that peripheral neuronal nicotinic acetylcholine receptors (nAChRs) participate in the modulation of pain perception (Wang et al., 2005). The stimulation of nAChRs excites peripheral sensory nerve fibers and exerts antinociceptive effects (Bernardini et al., 2001a,b; Rueter et al., 2003). LXM-15 produces significant antinociceptive effects that are fully blocked by mecamylamine or hexamethonium, showing that the antinociceptive effect of LXM-15 may be related to peripheral nAChR. Muscarinic acetylcholine receptors (mAChRs) have been widely reported as pharmacologic targets for pain treatment. Most of them focused on central nervous system mAChR (Schechtmann et al., 2008). Recent evidence suggests that activation of mAChRs present on peripheral nociceptors can also suppress the transmission of pain impulses (Bernardini et al., 2001a,b). Our results show that the antinociception of LXM-15 was fully blocked by atropine or atropine methylnitrate, making possible connection between antinociceptive effect of LXM-15 and peripheral mAChR.

As we know, nicotinic and muscarinic receptors agonists are associated with typical acetylcholine-like effects, such as reduced motor activity, hypothermia, tremor, incoordination, polysialia, and bradycardia (Barocelli et al., 2001; Decker et al., 1994). Consequently, we assessed the effect of LXM-15 on motor coordination, spontaneous activity, body temperature, heart rate, and hepatotoxicty in animals. The results show that LXM-15 did not affect their behavior, body temperature or heart rate, nor did it change animals' gross behavior or polysialia at the minimal lethal dose (314.20 μmol/kg, s.c.) in acute toxicity tests. LXM-15 also had no significant effects on the liver function. Thus, it suggests that LXM-15 does not produce any typical side effects of muscarinic or nicotinic agonists. However, the reason for LXM-15 to produce antinociceptive effects without any side effects still remained unknown. So it was essential for us to find out which subtypes are involved in the peripheral antinociception of LXM-15.

Reports show that α7 nAChR participates in antinociceptive pathways (Damaj et al., 2000; Jones and Dunlop, 2007). Our findings indicate that methyllycaconitine reduced the antinociceptive effects of LXM-15 dose-dependently, indicating α7 nAChR activation was involved in the antinociceptive effect of LXM-15. Activation of M4 muscarinic receptor leads to strong analgesic effects (Ellis et al., 1999), which does not seem to regulate critical peripheral physiological functions (Caulfield, 1993; Duttaroy et al., 2002), for example, a reduced heart rate (mediated by M2R) (Gomeza et al., 1999), an increased smooth-muscle contractility and a glandular secretion (mediated by M3R) (Caulfield, 1993). LXM-15 produced antinociceptive effect by activating peripheral mAChR without affecting heart rate, glandular secretion in mice. Therefore, we assumed that the analgesic effect of LXM-15 is likely to involve M4 receptor subtype. Our results show that M4 muscarinic receptor antagonist tropicamide reduced the antinociceptive effect of LXM-15 dose-dependently, revealing the involvement of M4 mAChR activation in the antinociceptive effect of LXM-15.

In order to determine whether the antinociception of LXM-15 was acting via α7 nAChR and M4 mAChR, we introduced a further investigation using [^{3}H]MLA and [^{3}H]NMS binding assays to explore the specificity of LXM-15 to bind at α7 nAChR or M4 mAChR. Results show that LXM-15 can bind to α7 nAChR (IC_{50} of 8.72×10^{-7} M) or M4 mAChR (IC_{50} of 1.35×10^{-5} M), which confirms the results in vivo. In [^{3}H]NMS binding assay, we chose corpus striatum which enriched M1 and M4 receptors (Gerber et al., 2001). [^{3}H]NMS combined with both M1 receptors and M4 receptors, so the IC_{50} value (1.35×10^{-5} M) showed a higher affinity of LXM-15 to M4 receptor. Our studies indicate LXM-15 binding with α7 nAChR or M4 mAChR, and thus, result in antinociception.

In summary, we found that spirocyclopiperazinium salt compound LXM-15 is a powerful agent for suppressing pain without obvious side effects. The antinociception signaling pathway probably involves binding and activating peripheral α7 nAChR and M4 mAChR, and results in antinociceptive effects. The study suggests that the activation of both peripheral α7 nAChR and

Table 3
Effect of LXM-15 on the temperature in mice.

Group	Dose (μmol/kg)	Before treatment (°C)	Rectal temperature after treatment (°C)		
			0.5 h	2.0 h	3.5 h
Vel	–	37.77 ± 0.10	37.83 ± 0.07	37.39 ± 0.08	37.80 ± 0.13
Diazepam	7	37.88 ± 0.14	36.00 ± 0.29**	36.38 ± 0.25**	37.34 ± 0.15
LXM-15	20	37.87 ± 0.10	37.59 ± 0.21	37.33 ± 0.15	37.50 ± 0.19
	40	37.77 ± 0.13	37.49 ± 0.23	37.53 ± 0.22	37.42 ± 0.23
	80	37.70 ± 0.17	37.48 ± 0.29	37.78 ± 0.24	37.75 ± 0.24

Note: The Rectal temperature were tested before and 0.5 h, 2.0 h, 3.5 h after administered of LXM-15 (80, 40 or 20 μmol/kg, s.c.), Diazepam(7 μmol/kg, i.p.) or vehicle. All data are mean ± S.E.M. of 10 mice per group. **$P < 0.01$ vs Vel group at the same time.

Table 4
Effect of LXM-15 on the heart rate in mice.

Group	Dose (μmol/kg)	Heart rate after treatment		
		0.5 h	2.0 h	3.5 h
Vel	–	483.17 ± 23.25	463.43 ± 30.92	430.34 ± 40.82
LXM-15	20	456.22 ± 9.04	399.18 ± 19.97	364.64 ± 23.47
	40	456.38 ± 12.41	407.04 ± 22.48	392.03 ± 24.68
	80	496.54 ± 18.53	457.80 ± 15.62	399.01 ± 23.90

Note: The heart rate was tested before and 0.5 h, 2.0 h, 3.5 h after administered of LXM-15 (80, 40 or 20 μmol/kg, s.c.) or vehicle. All data are mean ± S.E.M. of 10 mice per group.

Table 5
Effect of LXM-15 on ALT, AST, and ALP activities in mice serum.

Groups	Dose (μmol/kg)	ALT (U/L)		AST (U/L)		AKP (U/L)	
		24 h	48 h	24 h	48 h	24 h	48 h
Vel	–	2.44 ± 0.21	2.12 ± 0.20	2.41 ± 0.15	2.19 ± 0.16	9.57 ± 0.20	9.68 ± 0.11
CCl_4	–	14.68 ± 0.32**	14.23 ± 0.38**	5.99 ± 0.38**	4.48 ± 0.38**	11.98 ± 0.45**	17.82 ± 0.22**
LXM-15	20	2.82 ± 0.23	2.10 ± 0.12	2.46 ± 0.13	2.13 ± 0.11	9.45 ± 0.38	10.36 ± 0.11
	40	3.13 ± 0.33	2.16 ± 0.19	2.64 ± 0.10	2.39 ± 0.22	9.64 ± 0.27	10.01 ± 0.30

Note: The serum activities of ALT, AST and ALP were determined at 24 or 48 h after administered of LXM-15 (40, 20 μmol/kg, s.c.), CCl_4 (15%, i.p.) or vehicle. The values represent mean ± S.E.M. of 10 mice per group. $^{**}P < 0.01$ vs Vel group at the same time.

M4 mAChR was required to produce the antinociception. Our findings may shed some light on the development of novel analgesic drugs with fewer side effects.

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References

Barocelli, E., Ballabeni, V., Bertoni, S., de Amici, M., Impicciatore, M., 2001. Evidence for specific analgesic activity of a muscarinic agonist selected among a new series of acetylenic derivatives. Life Sciences 68, 1775–1785.

Beirith, A., Santos, A.R., Rodrigues, A.L., Creczynski-Pasa, T.B., Calixto, J.B., 1998. Spinal and supraspinal antinociceptive action of dipyrone in formalin, capsaicin and glutamate tests. Study of the mechanism of action. European Journal of Pharmacology 345, 233–245.

Bernardini, N., Reeh, P., Sauer, S., 2001a. Muscarinic M2 receptors inhibit heat induced CGRP release from isolated rat skin. Neuroreport 12, 2457–2460.

Bernardini, N., Sauer, S.K., Haberberger, R., Fischer, M.J., Reeh, P.W., 2001b. Excitatory nicotinic and desensitizing muscarinic (M2) effects on C-nociceptors in isolated rat skin. Journal of Neuroscience 21, 3295–3302.

Boccia, M.M., Acosta, G.B., Baratti, C.M., 2001. Memory improving actions of gabapentin in mice: possible involvement of central muscarinic cholinergic mechanism. Neuroscience Letters 311, 153–156.

Boyce, S., Webb, J.K., Shepheard, S.L., Russell, M.G., Hill, R.G., Rupniak, N.M., 2000. Analgesic and toxic effects of ABT-594 resemble epibatidine and nicotine in rats. Pain 85, 443–450.

Caulfield, M.P., 1993. Muscarinic receptors—characterization, coupling and function. Pharmacology & Therapeutics 58, 319–379.

Damaj, M.I., Meyer, E.M., Martin, B.R., 2000. The antinociceptive effects of alpha7 nicotinic agonists in an acute pain model. Neuropharmacology 39, 2785–2791.

D'Amour, F., Smith, D., 1941. A method for determining loss of pain sensation. Journal of Pharmacology and Experimental Therapeutics 72, 74–79.

Davies, A.R., Hardick, D.J., Blagbrough, I.S., Potter, B.V., Wolstenholme, A.J., Wonnacott, S., 1999. Characterisation of the binding of [^{3}H]methyllycaconitine: a new radioligand for labelling alpha 7-type neuronal nicotinic acetylcholine receptors. Neuropharmacology 38, 679–690.

Decker, M.W., Bannon, A.W., Buckley, M.J., Kim, D.J., Holladay, M.W., Ryther, K.B., Lin, N.H., Wasicak, J.T., Williams, M., Arneric, S.P., 1998. Antinociceptive effects of the novel neuronal nicotinic acetylcholine receptor agonist, ABT-594, in mice. European Journal of Pharmacology 346, 23–33.

Decker, M., Buckley, M., Brioni, J., 1994. Differential effects of pretreatment with nicotine and lobeline on nicotine-induced changes in body temperature and locomotor activity in mice. Drug Development Research 31, 52–58.

Dussor, G.O., Helesic, G., Hargreaves, K.M., Flores, C.M., 2004. Cholinergic modulation of nociceptive responses in vivo and neuropeptide release in vitro at the level of the primary sensory neuron. Pain 107, 22–32.

Duttaroy, A., Gomeza, J., Gan, J.W., Siddiqui, N., Basile, A.S., Harman, W.D., Smith, P.L., Felder, C.C., Levey, A.I., Wess, J., 2002. Evaluation of muscarinic agonist-induced analgesia in muscarinic acetylcholine receptor knockout mice. Molecular Pharmacology 62, 1084–1093.

Ehlert, F.J., Tran, L.P., 1990. Regional distribution of M_1, M_2 and non-M_1, non-M_2 subtypes of muscarinic binding sites in rat brain. Journal of Pharmacology and Experimental Therapeutics 255, 1148–1157.

Ellis, J.L., Harman, D., Gonzalez, J., Spera, M.L., Liu, R., Shen, T.Y., Wypij, D.M., Zuo, F., 1999. Development of muscarinic analgesics derived from epibatidine: role of the M4 receptor subtype. Journal of Pharmacology and Experimental Therapeutics 288, 1143–1150.

Flores, C.M., 2000. The promise and pitfalls of a nicotinic cholinergic approach to pain management. Pain 88, 1–6.

Galeotti, N., Quattrone, A., Vivoli, E., Bartolini, A., Ghelardini, C., 2008. Type 1 and type 3 ryanodine receptors are selectively involved in muscarinic antinociception in mice: an antisense study. Neuroscience 153, 814–822.

Gerber, D.J., Sotnikova, T.D., Gainetdinov, R.R., Huang, S.Y., Caron, M.G., Tonegawa, S., 2001. Hyperactivity, elevated dopaminergic transmission, and response to amphetamine in M_1 muscarinic acetylcholine receptor-deficient mice. Proceedings of the National Academy of Sciences of the United States of America 98, 15312–15317.

Gomeza, J., Shannon, H., Kostenis, E., Felder, C., Zhang, L., Brodkin, J., Grinberg, A., Sheng, H., Wess, J., 1999. Pronounced pharmacologic deficits in M_2 muscarinic acetylcholine receptor knockout mice. Proceedings of the National Academy of Sciences of the United States of America 96, 1692–1697.

Jones, P.G., Dunlop, J., 2007. Targeting the cholinergic system as a therapeutic strategy for the treatment of pain. Neuropharmacology 53, 197–206.

Millan, M.J., 2002. Descending control of pain. Progress in Neurobiology 66, 355–474.

Oki, T., Takagi, Y., Inagaki, S., Taketo, M.M., Manabe, T., Matsui, M., Yamada, S., 2005. Quantitative analysis of binding parameters of [^{3}H]N-methylscopolamine in central nervous system of muscarinic acetylcholine receptor knockout mice. Brain Research Molecular Brain Research 133, 6–11.

Rowbotham, M.C., Rachel, D.W., Thomas, J., Nothaft, W., Backonja, M.M., 2009. A randomized, double-blind, placebo-controlled trial evaluating the efficacy and safety of ABT-594 in patients with diabetic peripheral neuropathic pain. Pain.

Rueter, L.E., Kohlhaas, K.L., Curzon, P., Surowy, C.S., Meyer, M.D., 2003. Peripheral and central sites of action for A-85380 in the spinal nerve ligation model of neuropathic pain. Pain 103, 269–276.

Schechtmann, G., Song, Z., Ultenius, C., Meyerson, B.A., Linderoth, B., 2008. Cholinergic mechanisms involved in the pain relieving effect of spinal cord stimulation in a model of neuropathy. Pain 139, 136–145.

Seo, Y., Kwon, M., Choi, H., Choi, S., Nam, J., Lee, J., Jung, S., Park, S., Suh, H., 2008. The differential effects of acetaminophen on lipopolysaccharide induced hyperalgesia in various mouse pain models. Pharmacology Biochemistry and Behavior 91, 121–127.

Sudo, S., Sudo, M., Simons, C.T., Dessirier, J.M., Carstens, E., 2002. Sensitization of trigeminal caudalis neuronal responses to intraoral acid and salt stimuli and desensitization by nicotine. Pain 98, 277–286.

Sulaiman, M.R., Hussain, M.K., Zakaria, Z.A., Somchit, M.N., Moin, S., Mohamad, A.S., Israf, D.A., 2008. Evaluation of the antinociceptive activity of *Ficus deltoidea* aqueous extract. Fitoterapia 79, 557–561.

Sun, Q., Yue, C., Ye, J., Li, C., Cheng, T., Li, R., 2007. Unique spirocyclopiperazinium salt III: further investigation of monospirocyclopiperazinium (MSPZ) salts as potential analgesics. Bioorganic & Medicinal Chemistry Letters 17, 6245–6249.

Tjolsen, A., Berge, O.G., Hunskaar, S., Rosland, J.H., Hole, K., 1992. The formalin test: an evaluation of the method. Pain 51, 5–17.

Wang, Y., Su, D.M., Wang, R.H., Liu, Y., Wang, H., 2005. Antinociceptive effects of choline against acute and inflammatory pain. Neuroscience 132, 49–56.