

Investigation of the role of Z944 related to muscarinic receptors on hyperalgesia in rats

Z944'ün hiperaljezide muskarinik reseptörler ile ilişkili rolünün sıçanlarda incelenmesi

Meryem Dilek Acar¹, Ömer Bozduman², Arzu Erdal³

¹Department of Physiology, Samsun University Faculty of Medicine, Samsun, Türkiye

²Orthopedic and Traumatologist, Private Practice, Samsun, Türkiye

³Department of Pharmacology, Ondokuz Mayıs University Faculty of Medicine, Samsun, Türkiye

ABSTRACT

Background: This study aims to investigate the muscarinic receptor-associated Z944 responses on hyperalgesia resulting from chronic constriction injury (CCI) of the rat sciatic nerve.

Materials and Methods: A total of 72 male Wistar Albino rats (200-250 g) were randomly divided into eight groups. The right sciatic nerve CCI of Wistar Albino rats was performed except the Saline intraperitoneal (i.p.) group. Two weeks after the operations, pressure test of both paws and hot plate test were performed in the groups of Saline, CCI + Saline, CCI + DMSO (i.p.), CCI + 1 mg/kg Atropine (i.p.), CCI + Z944 1, 3, 10 mg/kg (i.p.) dose and CCI + 1 mg/kg Atropine + 10 mg/kg Z944. The locomotor activity test was performed before the tests.

Results: The results of right paw pressure test ($p < 0.001$) and hot plate test ($p = 0.032$) of CCI + Saline group, compared to the Saline group test results, confirmed the development of mechanical hyperalgesia and thermal hyperalgesia of the CCI + Saline group. The locomotor activities of Saline and other CCI groups were higher than the activity of CCI + 1 mg/kg Atropine group ($p = 0.017$). The dose-dependent increasing effect of Z944 in dose groups compared to the CCI + Saline group was determined, respectively, in the right paw pressure test ($p = 0.472$, $p = 0.006$, $p < 0.001$) and the hot plate test ($p = 0.001$, $p < 0.001$, $p < 0.001$). The left paw pressure test results of CCI + Saline group and Z944 dose groups were not statistically different ($p = 0.995$, $p = 0.159$, $p = 0.110$, respectively). Also, there were no statistically differences between the CCI + Saline and the CCI + 1 mg/kg Atropine + 10 mg/kg Z944 groups according to right paw pressure ($p = 0.945$), left paw pressure ($p = 0.763$), and hot plate ($p = 0.817$) tests results.

Conclusion: The attenuation of the analgesic effect of Z944 by the non-selective muscarinic receptor antagonist atropine suggests that the analgesic activity of Z944 may be mediated, at least in part, through muscarinic receptor-dependent mechanisms.

Keywords: Neuropathic pain, sciatic nerve ligation, T-type calcium channels.

ÖZ

Amaç: Bu çalışmada, sıçan siyatik sinirinde oluşturulan kronik konstriksiyon hasarı (CCI) sonucunda gelişen hiperaljezide muskarinik reseptörler ile ilişkili Z944 yanıtları araştırıldı.

Gereç ve Yöntemler: Toplam 72 erkek Wistar Albino sıçan (200-250 g) rastgele sekiz gruba ayrıldı. Salin intraperitoneal (i.p.) grubu dışındaki Wistar Albino sıçanlarda sağ siyatik sinire CCI uygulandı. Operasyondan iki hafta sonra, Salin, CCI + Salin, CCI + DMSO (i.p.), CCI + 1 mg/kg Atropin (i.p.), CCI + Z944 1, 3 ve 10 mg/kg (i.p.) doz ve CCI + 1 mg/kg Atropin + 10 mg/kg Z944 gruplarında her iki pençeye basınç testi ve sıcak plaka testi uygulandı. Lokomotor aktivite testi, diğer testlerden önce gerçekleştirildi.

Bulgular: CCI + Salin grubunun sağ pençe basınç testi ($p < 0.001$) ve sıcak plaka testi ($p = 0.032$) sonuçlarının Salin grubu ile karşılaştırılması, CCI + Salin grubunda mekanik hiperaljezi ve termal hiperaljezi geliştiğini doğruladı. Salin ve diğer CCI gruplarının lokomotor aktiviteleri, CCI + 1 mg/kg Atropin grubuna kıyasla daha yüksekti ($p = 0.017$). CCI + Salin grubuyla karşılaştırıldığında, Z944 doz gruplarında Z944'ün doza bağlı artan etkisi; sırasıyla sağ pençe basınç testinde ($p = 0.472$, $p = 0.006$, $p < 0.001$) ve sıcak plaka testinde ($p = 0.001$, $p < 0.001$, $p < 0.001$) olarak belirlendi. Ayrıca CCI + Salin grubu ile Z944 doz gruplarının sol pençe basınç testi sonuçları arasında istatistiksel olarak anlamlı bir fark bulunmadı (sırasıyla $p = 0.995$, $p = 0.159$, $p = 0.110$). Sağ pençe basıncı ($p = 0.945$), sol pençe basıncı ($p = 0.763$) ve sıcak plaka ($p = 0.817$) testi sonuçları açısından CCI + Salin grubu ile CCI + 1 mg/kg Atropin + 10 mg/kg Z944 grupları arasında istatistiksel olarak anlamlı bir fark bulunmadı.

Sonuç: Z944'ün analjezik etkisinin nonselektif muskarinik reseptör antagonist atropin tarafından azaltılması, Z944'ün analjezik aktivitesinin en azından kısmen muskarinik reseptör aracılı mekanizmalar üzerinden gerçekleşebileceğini düşündürmektedir.

Anahtar sözcükler: Nöropatik ağrı, siyatik sinir ligasyonu, T-tipi kalsiyum kanalları.

Corresponding author: Meryem Dilek Acar, MD.

E-mail: drmeryemdilekacar@gmail.com

Doi: 10.5606/agri.2026.95

Received: March 02, 2026 | Accepted: July 10, 2026 | Published online: September 26, 2026

Cite this article as: Acar MD, Bozduman Ö, Erdal A. Investigation of the role of Z944 related to muscarinic receptors on hyperalgesia in rats. Agri 2026;38(4):272-281. doi: 10.5606/agri.2026.95.

© 2026 The Author(s). This is an open access article published under the terms of the Creative Commons Attribution 4.0 International License (CC BY 4.0), which permits unrestricted use, distribution, reproduction, and adaptation in any medium, provided the original work is properly cited. <https://creativecommons.org/licenses/by/4.0/>

T-type voltage-gated calcium channels in the spinal cord are defined as one of the important targets for pain cure, as their abnormal activity is associated with pain.^[1] The T-type or low voltage-activated subfamily has Cav3.1-Cav3.3 subtypes.^[2] The decrease due to knockdown in Cav3.2 expression increases pain thresholds of mechanical and thermal in the neuropathic pain model and in pure rodents.^[3] Neuropathic pain is a somatosensory nervous system dysfunction and is defined as chronic severe pain.^[4,5] There are also studies reflecting the roles of Cav3.1 and Cav3.3 subtypes during the pathophysiology of chronic pain.^[6,7] Depending on the location of the injury, there are two types of neuropathic pain: central neuropathic pain and peripheral neuropathic pain.^[1,5,8] Peripheral neuropathic pain is often seen in patients with persistent diabetes, sternotomy, cancer, lumbar disc syndrome, mastectomy, thoracotomy, herpes, and acquired immunodeficiency syndrome (AIDS).^[9] The symptoms of neuropathic pain are allodynia (i.e., increased pain reaction to stimuli which do not usually trigger pain), dysesthesia (burning pain sensation), hyperalgesia (exaggerated response to noxious stimuli), and neuralgia (shooting/stabbing/electric shock-like pain).^[10] T-type low-voltage-activated calcium channels are crucial for the subthreshold neuronal excitability control in pain processing areas in the dorsal horn of the spinal cord.^[11] The superficial area of the dorsal horn in the spinal cord (lamina I and lamina II) plays a role in receiving painful stimuli from the periphery and transmitting them to the brain.^[1] An experimental electrophysiological study performed by isolating the spinal cord demonstrated that Z944 inhibited T-type voltage-gated channels and reduced the excitability in lamina I and lamina II dorsal horn neurons.^[12] In addition, Cav3.1 and Cav3.2 have been identified as molecular targets of Z944 and are inhibited by this agent.^[13,14]

A patch-clamp study demonstrated that the currents through T-type calcium channels are reduced by muscarinic receptor stimulation in granulos cells.^[15] The cholinergic system has a role in the modulation of pain in the dorsal horn of the spinal cord.^[16] The stimulation of muscarinic receptors is also effective in relieving neuropathic pain.^[17] The muscarinic acetylcholine receptors of the spinal cord are more densely located in the superficial dorsal horn in both rats and humans. Although muscarinic acetylcholine receptors have the analgesic effect through inhibition of glutamate release and activation of gamma-aminobutyric acid (GABA) transmission in

the dorsal horn of the spinal cord,^[18] there are also some studies that researched the complex mechanism between muscarinic receptors and T-type calcium channels.^[19-22] In the present study, we, for the first time, aimed to investigate the possible effect related to muscarinic receptors in the activity of Z944 acting through Cav3.1 and Cav3.2 Ca⁺² channels in sciatic nerve-ligated rats.

MATERIALS AND METHODS

Animals

A total of 72 male Wistar Albino rats (200-250 g) were randomly divided into eight groups. The study was approved by the Ondokuz Mayıs University Animal Experiments Local Ethics Committee (Date: 31.05.2023, No: 2023/36). Animals were obtained from Ondokuz Mayıs University Experimental Animal Research Center. The rats were housed in 12 h of dark-light cycle at a 22 ± 2°C temperature and 60 ± 5% humidity environment with access to water and food.

Medicines and chemicals

Ulixacaltamide (Lixfrevo; Praxis Precision Medicines, MA, USA), with the development code Z944, is a novel experimental drug for treating essential tremor.^[23] The drug, as a selective T-type calcium channel blocker, reduces abnormal cerebello thalamo cortical activity, which manifests clinically as tremors.^[24] The drug critic Phase 3 was resulted in October 2025.^[25] In April 2026 United States Food and Drug Administration (FDA) accepted a New Drug Application (NDA), and after reviewing the expected action date is in January 2027.^[26]

Z944 (Sigma, SML2635) was injected intraperitoneally (i.p.) at doses of 1, 3 and 10 mg/kg, 30 min before the tests.^[27] 10% DMSO (C.ERBA 445103) and 90% carboxymethylcellulose (TCI C0603) (0.5% in saline) were used to dissolve Z944.^[28] Also, DMSO was evaluated in a group.^[12] A total of 1 mg/kg dose of atropine sulphate (TCI A0754) was injected i.p. 30 min before the agent.^[29]

Chronic constriction injury

The rats were kept away from food for 12 h before the operation.^[30] After induction of anesthesia with 80 mg/kg (i.p.) ketamine hydrochloride (Keta-Control®, Doğa İlaç, İstanbul, Türkiye), the hair on the mid-thigh and surrounding areas was shaved.^[31] The sciatic nerve was exposed at the mid-level of the right hind leg, and blunt dissection was made via the biceps femoris. Proximal to the

sciatic nerve branch, approximately 7 mm of the nerve was released from the attached tissue. Four ligatures (4.0 silk) were tied limply around the nerve at approximately 1-mm intervals, which ultimately affects 4 to 5 mm of the sciatic nerve's length. The target constriction level was to achieve reduction, but not complete obstruction of the superficial epineurial vascular circulation.^[30] Two rats died after the operations; two rats from the Saline group were transferred to the chronic constriction injury (CCI) groups.

Behavioral tests

As described in the literature, hyperalgesia caused by mechanical/thermal effects resulting from sciatic nerve constriction is more pronounced on Day 14 compared to Day 7 after surgery.^[32] The behavioral tests were performed 14 days after CCI.

Locomotor activity test

The locomotor activity cage apparatus (41 cm²-33 cm wall) (Ugo Basile, Biological Research Apparatus, VA, Italy) was used for investigating the effects of drugs on locomotor activity. Rats were observed individually in the activity cage.^[33] The numerical data in the horizontal plane were automatically detected by the device for 5 min, and the data of each rat was recorded. The inner surfaces of the device were cleaned with 20% alcohol, and the alcohol was allowed to dry after each test.

Paw pressure test

A Randall-Selitto model analgesimeter (Model: 37215, Ugo Basile, VA, Italy) was used for the paw pressure test to evaluate mechanical hyperalgesia. The increasing pressure was applied via the equipment to the middle parts of both hind paws of the rats. The pressure level in grams (g) that caused withdrawal was recorded for each rat.^[34]

Hot plate test

The hot plate test was performed via a metal platform with transparent walls (Model: 7280, Ugo Basile, VA, Italy) for determining thermal hyperalgesia. Rats were placed on a hot plate which was set at $54 \pm 0.4^{\circ}\text{C}$. The reactions such as paw licking/jumping were observed. The reaction time in seconds (sec) was recorded for each rat.^[33]

Statistical analysis

The PASS version 15.0.5 software (NCSs, LLC. Kaysville, Utah, USA) was used for sample size calculation. Accordingly, the minimum number of rats for the investigation was 72, when the power of the test 0.8588 and the alpha error is 0.05.^[33]

Statistical analysis was performed using the IBM SPSS version 23.0 software (IBM Corp., Armonk, NY, USA). The descriptive data were presented in mean $\pm$ standard deviation (SD), median (25th-75th quartiles), or number and frequency, where applicable. The compliance of the measurements with normal distribution without distinguishing the groups was examined with the Shapiro-Wilks test. The Kruskal-Wallis test was used in the comparison of the eight groups and the groups causing significant differences were determined with the post-hoc Dunn test. The right and left paw pressure test results of each group were compared with the Wilcoxon signed-rank test. The median and quartiles of the groups were presented graphically with box plot. A p value of < 0.05 was considered statistically significant.

RESULTS

Effect of CCI on the development of hyperalgesia

The CCI + Saline group withdrawal response was at a lower pressure (g) than that of the Saline group in the right paw pressure test ($p < 0.001$). The reaction of CCI + Saline group developed in a shorter time (sec) compared to the Saline group in the hot plate test ($p = 0.032$), (Figure 1, 2).

Locomotor activity test results

The mean locomotor activities of Saline, CCI + Saline, CCI + DMSO, CCI + 1 mg/kg Z944, CCI + 3 mg/kg Z944, CCI + 10 mg/kg Z944, CCI + 1 mg/kg Atropine + 10 mg/kg Z944 groups were significantly higher than the mean activity of the CCI + 1 mg/kg Atropine group ($p = 0.017$) (Figure 3).

Right paw pressure test results

The pressure (g) that caused withdrawal response in the groups of Saline and CCI + 10 mg/kg Z944 were significantly higher than all other groups in the right paw pressure test. These two groups were followed by the CCI + 3 mg/kg Z944 group, and the mean pressure (g) causing withdrawal reaction of this group was also significantly higher than the other groups ($p < 0.001$) (Figure 1).

Left paw pressure test results

The withdrawal responses of Saline, CCI + 3 mg/kg Z944 and CCI + 10 mg/kg Z944 groups were for higher pressure than that of the CCI + 1 mg/kg Atropine group according to left paw pressure test results ($p = 0.030$) (Figure 1).

Comparison of right and left paw pressure test results

There was no statistically significant difference between right and left paw pressure test results of the Saline group ($p = 0.932$). The right side mean pressure (g) that elicited withdrawal response was lower than left side mean pressure (g) causing withdrawal in the groups of CCI, including CCI +

Saline ($p = 0.008$), CCI + DMSO ($p = 0.008$), CCI + 1 mg/kg Atropine ($p = 0.011$), CCI + 1 mg/kg Z944 ($p = 0.008$), CCI + 3 mg/kg Z944 ($p = 0.008$), CCI + 10 mg/kg Z944 ($p = 0.039$) and CCI + 1 mg/kg Atropine + 10 mg/kg Z944 ($p = 0.008$) (Figure 1).

Effect of DMSO on hyperalgesia

There were no statistically significant differences according to the right paw pressure test ($p = 0.889$)

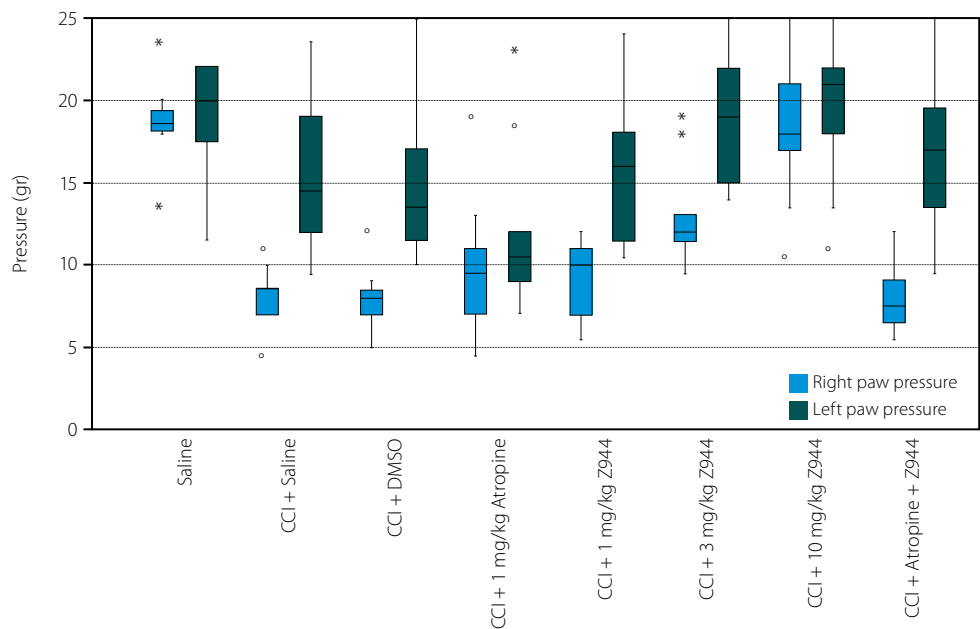


Figure 1. The medians and the quartiles of pressure (gram) causing withdrawal in right and left paw pressure tests, according to groups.

CCI, chronic constriction injury; °, Q1 (25th) – 1.5 (IQR) or Q1 (75th) + 1.5 (IQR), *, Q1 (25th) – 3.0 (IQR) or Q1 (75th) + 305 (IQR).

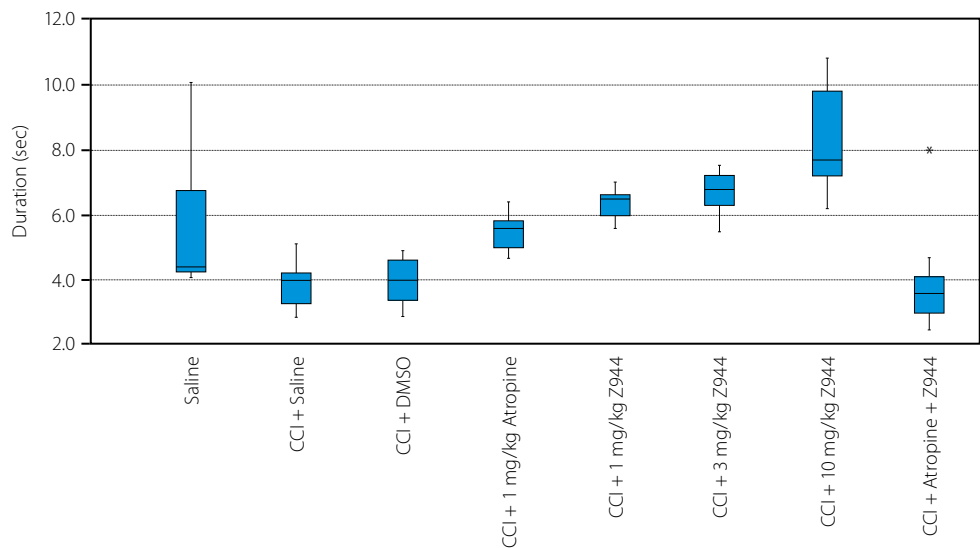


Figure 2. The medians and the quartiles of duration (sec) for reaction in hot plate test, according to groups.

CCI, chronic constriction injury; *, Q1 (25th) – 3.0 (IQR) or Q1 (75th) + 305 (IQR).

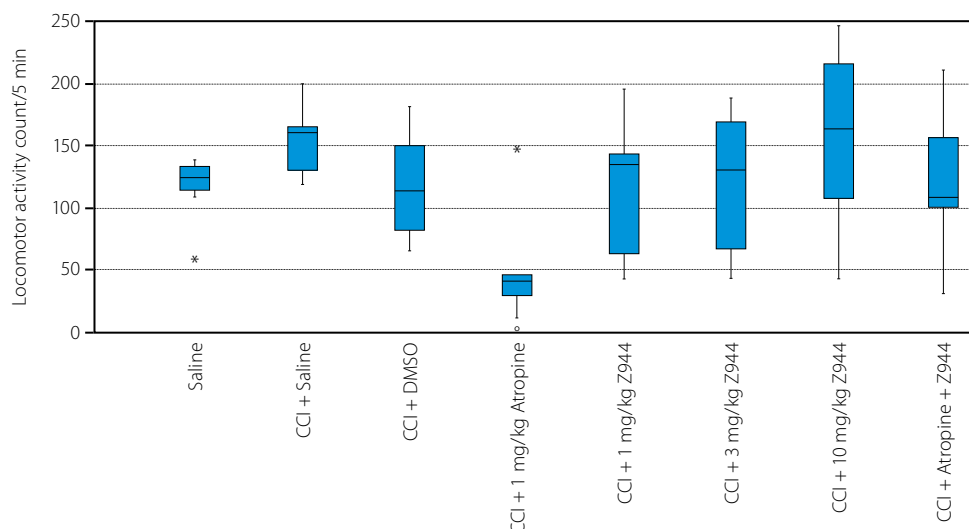


Figure 3. The medians and quartiles of locomotor activities, according to groups.

CCI, chronic constriction injury; *, Q1 (25th) – 3.0 (IQR) or Q1 (75th) + 305 (IQR).

and the hot plate test ($p = 0.817$) between the groups of CCI + Saline and CCI + DMSO (Figures 1 and 2).

Effect of Atropine on hyperalgesia

The right paw pressure test results of CCI + Saline and CCI + 1 mg/kg Atropine groups were not statistically different ($p = 0.350$). The CCI + 1 mg/kg Atropine group had a longer duration (sec) for reaction than the CCI + Saline group in the hot plate test ($p = 0.026$) (Figure 1 and 2).

Effect of Z944 on hyperalgesia

The results of CCI + Saline and CCI + 1 mg/kg Z944 groups were not statistically different according to the right paw pressure test ($p = 0.472$). The withdrawal response of CCI + 3 mg/kg Z944 and CCI + 10 mg/kg Z944 groups were for higher pressure (g) than that of the CCI + Saline group in right paw pressure test ($p = 0.006$, $p < 0.001$, respectively) (Figure 1).

The CCI + 1 mg/kg Z944, CCI + 3 mg/kg Z944 and CCI + 10 mg/kg Z944 groups had longer duration (sec) for reaction than the CCI + Saline group in the hot plate test ($p = 0.001$, $p < 0.001$, $p < 0.001$, respectively) (Figure 2).

Effect of 1 mg/kg Atropine + 10 mg/kg Z944 on hyperalgesia

The results of CCI + Saline and CCI + 1 mg/kg Atropine + 10 mg/kg Z944 groups were not statistically different according to the right paw pressure test ($p = 0.945$) and the hot plate test ($p = 0.817$) (Figures 1 and 2).

DISCUSSION

In the present study, we investigated the possible effect related to muscarinic receptors in the activity of Z944 acting through Cav3.1 and Cav3.2 Ca^{+2} channels in sciatic nerve-ligated rats. Our study results showed that the analgesic effect of Z944 was suppressed by atropine in sciatic nerve-ligated rats. This finding suggests that the analgesic effect achieved through the inhibition of T-type voltage-gated calcium channels in neuropathic pain may involve a mechanism associated with muscarinic receptors.

Chronic constriction injury is a mononeuropathy model which was created by the operations aiming loosely constriction of the common sciatic nerve in rats. Mechanical and thermal hyperalgesia induced by this model in rats provide advantages for investigating the pathophysiological mechanisms of neuropathic pain in humans.^[30] The pain-related changes of this study on Day 14 after surgery were compatible with the previously reported studies with sciatic nerve-ligated rats.^[8,32] The withdrawal response of the CCI + Saline group was at a lower pressure (g) than that of the Saline group indicating mechanical hyperalgesia in the CCI + Saline group. The lower reaction time (sec) of the CCI + Saline group compared to the Saline group in the hot plate test demonstrated thermal hyperalgesia of the CCI + Saline group. The presence of four ligaments around the sciatic nerve on the right side probably led to an exaggerated response to painful stimuli.

Locomotor activity of the CCI + 1 mg/kg Atropine group decreased compared to the other groups, including the Z944 dose groups. A study of Weldon et al.^[35] showed a statistically significant reduction in the running response of Sprague-Dawley rats with atropine (1 mg/kg, i.p). Although, Z944 has a high affinity for T-type voltage-gated calcium channels, Z944 has no effect on high-voltage-activated calcium channels/sodium channels at the dose that blocks T-type voltage-gated calcium channels, and Z944 does not cause motor deficit/sedation in behavioral tests in the dose range of 1 to 10 mg/kg (i.p.).^[12,36]

The doses of 3 mg/kg and 10 mg/kg of Z944 had a dose-dependent increasing analgesic effect in the right paw pressure test of the sciatic nerve-ligated rats. The study of Antunes et al.^[37] demonstrated that Z944 inhibited mechanical hypersensitivity when given systemically to mice with a neuropathic pain model produced by partial sciatic nerve injury. The Cav3.1 subtype has an important role for analgesic effect in persistent neuropathic pain, despite the current medical treatments,^[38] and the Cav3.2 subtype has also been shown to be selectively upregulated in dorsal root ganglion peripheral sensory neurons and in the spinal dorsal horn in chronic pain rodent models.^[39-41] T-type current density increases in small sensory neurons originating from L4-5 dorsal root ganglia ipsilateral to sciatic nerve injury in rats.^[42] The upregulation of T-type channels following sciatic nerve injury has been found to cause hyperexcitability of small dorsal root ganglion cells and thus hyperalgesia in animal models of neuropathic pain resulting from mechanical nerve injury.^[11] During the development of neuropathic pain, probably the upregulation of T-type channels in spinal dorsal horn and dorsal root ganglion cells ipsilateral to the CCI, may have caused the dose-dependent effect of Z944 in the right paw pressure test.

The fact that lack of analgesic effect of the CCI + 1 mg/kg Atropine + 10 mg/kg Z944 group compared to the CCI + Saline group in the right paw pressure test suggests the role of muscarinic receptors in the mechanism of action of Z944. In previous different experimental studies, atropine was used to evaluate the role of muscarinic receptors in the analgesic activity of the tested agent.^[29,43] Atropine has also been reported to reverse the effect of gabapentin on mechanical hypersensitivity after CCI.^[44] The neurons of the superficial dorsal horn (lamina II, substantia gelatinosa) receive sensory

information from nociceptive primary afferent nerves. Glutamate is released from the terminals of primary afferent nerves.^[45] The muscarinic receptors are G protein-coupled metabotropic receptors and they are divided into two main subtypes: excitatory (M1, M3, and M5) and inhibitory (M2 and M4).^[46] The synapse between primary afferents and second-order sensory neurons in the dorsal horn is the first transmission for the regulation of sensory input. The inhibition of glutamate release from primary afferents is associated with the analgesic effects of Gi/o-coupled M2, M4 muscarinic receptors.^[47,48] The muscarinic receptor activation, which provides presynaptic inhibition of the synaptic input to lamina II neurons, is critical. The muscarinic receptor activation can also excite lamina II GABAergic interneurons and increase local GABA release,^[49] and not only the stimulation of M2, M4 muscarinic receptors but also M3 muscarinic receptors play a role in presynaptic GABA release and in GABA-B receptor-mediated analgesia in the spinal cord.^[49,50]

In the present study, according to the paw pressure test of left side without CCI, 1 mg/kg Atropine caused a withdrawal response for lower pressure (g) than that of the 3 mg/kg, 10 mg/kg dose groups of Z944 and the Saline group. Atropine is a competitive antagonist of muscarinic receptors.^[51] The inhibition of muscarinic receptors causes nociceptive hypersensitivity.^[52] Meanwhile, low-voltage-activated (near resting membrane potential) T-type voltage-gated calcium channels are important in the generation of neuronal excitability^[19] due to their subthreshold excitability and action potential firing properties in physiological (nociceptive) and pathological (neuropathic) pain.^[11] However, the left paw pressure test results of CCI + Saline and the dose groups of Z944 were not different. The withdrawal responses of all CCI groups were for lower pressure (g) in the right paw pressure test than that of the left paw pressure test. This right-left paw pressure test result difference was not present in the non-operated Saline group. These results also support the probable development of T-type voltage-gated channel upregulation in spinal dorsal horn and dorsal root ganglion cells ipsilateral to CCI.

In the current study, there was no significant difference between the results of CCI + Saline and CCI + 1 mg/kg Atropine groups according to the right paw pressure test. However, the CCI + 1 mg/kg Atropine group had a longer duration (sec) for reaction compared to the CCI + Saline group

in the hot plate test. In a previous study, atropine (1 mg/kg, i.p.) was also found to reverse the effects of some antinociceptive agents in mice in the hot plate test, but had no effect on the latent period alone.^[53] The locomotor activity decrease in the CCI + 1 mg/kg Atropine group may have caused a delay in more complex reactions such as paw licking/jumping in the hot plate test, compared to the withdrawal response in the paw pressure test, mimicking the analgesic effect.

The dose-dependent increasing analgesic effect of Z944 was detected for 1 mg/kg, 3 mg/kg and 10 mg/kg via the hot plate test of sciatic nerve-ligated rats, according to the results of the current study. Systemically administered Z944 is known to inhibit thermal hyperalgesia in rats with sciatic CCI.^[54] The knockout and knockdown studies^[55,56] showed the role of T-type voltage-gated channels (particularly Cav3.2) in the dorsal root ganglion in amplifying nociceptive signals originating from the periphery and in the development of central sensitization in the dorsal horn.^[21] Although it has not yet been elucidated which Cav3.2 subtype is involved in this mechanism, the activity of this channel is critical to the neuronal circuits underlying chronic pain.^[57] The doses of 1 mg/kg, 3 mg/kg, 10 mg/kg of Z944 has been reported to have a dose-dependent manner increasing effect in preventing mechanical allodynia, which was three days after intraplantar injection of Freund's adjuvant for creating inflammatory pain in rats.^[12] Also, Z944 was an effective analgesic in a dose-dependent manner at doses of 1 mg/kg, 3 mg/kg, and 10 mg/kg in acetic acid writhing test of mice.^[27]

The muscarinic signaling has a critical importance in neuropathic pain.^[46] The endogenous acetylcholine, originating from cholinergic interneurons in the dorsal horn, is an important modulator of sensory transmission, particularly at the spinal level where nociceptive stimuli enter the central nervous system.^[58] Xanomeline, a highly selective agonist of M1 muscarinic receptors, did not produce an analgesic effect^[59] may be due to the fact that M1 muscarinic receptor activation had no/moderate stimulatory effect on Cav3.1 and Cav3.2 T-type calcium channels in rats and humans.^[19] Based on the literature,^[19] there was a possibility that blockade of M1 muscarinic receptors with atropine in sciatic nerve-ligated rats would not alter the analgesic effect of Z944 mediated by Cav3.1 and Cav3.2 T-type calcium channels, or might even enhance it. However,

no analgesic effect was detected in the CCI + 1 mg/kg Atropine + 10 mg/kg Z944 group during behavioral tests.

Neuropathic pain is associated with epigenetic mechanisms that cause permanent changes in gene expression of the primary sensory neurons.^[60] The peripheral nerve injury causes upregulation of M2 muscarinic receptors in primary sensory afferent neurons, and that M2 muscarinic receptors are upregulated in small and medium-sized dorsal root ganglion neurons and their undamaged ipsilateral neighbors after nerve injury by axotomy, but not in contralateral dorsal root ganglion neurons compared to normals.^[61] However, another study showed that the nerve damage was found to reduce M2 muscarinic receptor (*Chrm2*) gene expression in the dorsal root ganglion and the M2 muscarinic receptor encoded by this gene, and consequently, the analgesic efficacy.^[60] It has also been reported that excessive down-regulation of systems, including M2 muscarinic receptors or defective upregulation associated with compensatory mechanisms may contribute to the continuation of neuropathic pain.^[62] Although T-type calcium currents did not change after acetylcholine application to cells which were transfected with M2/M4 receptors,^[20] according to the study of Zhang et al.,^[22] alpha-cobratoxin-activated M4 muscarinic receptors had the inhibiting effect of T-type calcium channel currents in mice dorsal root ganglion neurons. The statistically significant difference between right paw pressure and hot plate test results of the CCI + 10 mg/kg Z944 and the CCI + 1 mg/kg Atropine + 10 mg/kg Z944 groups revealed muscarinic activity in the effect of Z944, which may be related to M4 muscarinic receptor activity. However, further studies are needed to confirm the results.

T-type calcium channel activity was elevated by cells transfected with M3 receptors, according to a patch clamp study.^[20] It has also been shown that M3 muscarinic receptors do not mediate antinociception at the spinal level in Sprague-Dawley rats.^[59] However, another study revealed that the activation of M3 muscarinic receptors in mice dorsal root ganglion neurons strongly inhibited T-type calcium channel currents and also mechanical, thermal and inflammatory nociception via behavioral tests. The aforementioned experimental study showed that the analgesic effect of cobratoxin was blocked by atropine or the M3 selective antagonist 4-DAMP.^[21] In the current study,

the test results obtained by blocking muscarinic receptor activity in the CCI + 1 mg/kg Atropine + 10 mg/kg Z944 group suggest that M3 muscarinic receptors may play a role in the mechanism of Z944 and this suggestion also requires further investigation.

The activation of M5 muscarinic receptors expressed in primary afferent neuron terminals potentiates primary afferent input, while increased glutamate release from spinal interneurons leads to indirect inhibition of primary afferent input via glutamate receptors. However, this excitatory potentiation effect of the M5 receptor was detected in M2 and M4 knockout mice.^[47] Furthermore, no hybridization signal was detected for mRNAs expression of M1 and M5 muscarinic receptor subtypes in rat dorsal root ganglion via an *in situ* hybridization study.^[63]

Nonetheless, there are some limitations to this study that should be acknowledged. First, the relationship between T-type voltage-gated calcium channels and muscarinic receptors in neuropathic pain involves complex cellular mechanisms and remains an area of active research, despite accumulating evidence in the literature. Second, the present study, using Z944—a novel experimental drug that inhibits the Cav3.1 and Cav3.2 subtypes of T-type voltage-gated calcium channels—investigates the behavioral consequences of this interaction.

In conclusion, our study results showed that Z944 had a dose-dependent increasing effect on mechanical hyperalgesia and thermal hyperalgesia of sciatic nerve-ligated rats. However, Z944 was not an effective analgesic on the non-sciatic nerve-ligated sides of rats. The right withdrawal responses of all CCI groups were also at lower pressure (g) than the left withdrawal responses of non-operated paws. This difference was not present in the non-operated Saline group. The analgesic effect of Z944 on sciatic nerve-ligated rats was inhibited by the non-specific muscarinic receptor competitive antagonist, atropine. Taken together, these results indicate that muscarinic activity is an contributing factor of Z944 analgesic effect on hyperalgesia of sciatic nerve-ligated rats. Building on the existing literature, the novel findings of the present study may provide a basis for further investigation of the underlying mechanisms involved. Future studies employing selective muscarinic antagonists, together with epigenetic approaches, may help elucidate the mechanisms underlying the observed effects.

Acknowledgement

We would like to thank Prof. Dr. Handan Ankaralı for her guidance in the statistical analyses.

Author Contributions

M.D.A.: Conception and design, supervision, literature review, writing and critical review; M.D.A., Ö.B., A.E.: Materials, data collection and processing; M.D.A., A.E.: Analysis and interpretation.

Conflict of Interest

The authors declared no conflicts of interest with respect to the authorship and/or publication of this article.

Funding

The project was supported by TUBITAK 1002-B with the approval number 124S367 on 22.03.2024. The project support was also provided by Samsun University Project Office with the approval number BAP.TIP.5501.2023.001 on 14.09.2023.

Data Sharing Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

AI Disclosure

The authors declare that artificial intelligence (AI) tools were not used, or were used solely for language editing, and had no role in data analysis, interpretation, or the formulation of conclusions. All scientific content, data interpretation, and conclusions are the sole responsibility of the authors. The authors further confirm that AI tools were not used to generate, fabricate, or 'hallucinate' references, and that all references have been carefully verified for accuracy.

REFERENCES

1. Bourinet E, Altier C, Hildebrand ME, Trang T, Salter MW, Zamponi GW. Calcium-permeable ion channels in pain signaling. *Physiol Rev* 2014;94:81-140. doi: 10.1152/physrev.00023.2013.
2. Zamponi GW, Striessnig J, Koschak A, Dolphin AC. The physiology, pathology, and pharmacology of voltage-gated calcium channels and their future therapeutic potential. *Pharmacol Rev* 2015;67:821-70. doi: 10.1124/pr.114.009654.
3. Bourinet E, Alloui A, Monteil A, Barrère C, Couette B, Poirot O, et al. Silencing of the Cav3.2 T-type calcium channel gene in sensory neurons demonstrates its major role in nociception. *EMBO J* 2005;24:315-24. doi: 10.1038/sj.emboj.7600515.
4. Finnerup NB, Attal N, Haroutounian S, McNicol E, Baron R, Dworkin RH, et al. Pharmacotherapy for neuropathic pain in adults: A systematic review and meta-analysis. *Lancet Neurol* 2015;14:162-73. doi: 10.1016/S1474-4422(14)70251-0.
5. Treede RD, Jensen TS, Campbell JN, Cruccu G, Dostrovsky JO, Griffin JW, et al. Neuropathic pain: redefinition and a grading system for clinical and research purposes. *Neurology* 2008;70:1630-5. doi: 10.1212/01.wnl.0000282763.29778.59.
6. Na HS, Choi S, Kim J, Park J, Shin HS. Attenuated neuropathic pain in Cav3.1 null mice. *Mol Cells* 2008;25:242-6.
7. Yue J, Liu L, Liu Z, Shu B, Zhang Y. Upregulation of T-type Ca²⁺ channels in primary sensory neurons in spinal nerve injury. *Spine (Phila Pa 1976)* 2013;38:463-70. doi: 10.1097/BRS.0b013e318272fbf8.
8. Khangura RK, Bali A, Kaur G, Singh N, Jaggi AS. Neuropathic pain attenuating effects of perampamil in an experimental model of chronic constriction injury in rats. *Biomed Pharmacother* 2017;94:557-63. doi: 10.1016/j.biopha.2017.07.137.
9. Verma S, Estanislao L, Simpson D. HIV-associated neuropathic pain: epidemiology, pathophysiology and management. *CNS Drugs* 2005;19:325-34. doi: 10.2165/00023210-200519040-00005.

10. Jaggi AS, Jain V, Singh N. Animal models of neuropathic pain. *Fundam Clin Pharmacol* 2011;25:1-28. doi: 10.1111/j.1472-8206.2009.00801.x.
11. Todorovic SM, Jevtovic-Todorovic V. Neuropathic pain: role for presynaptic T-type channels in nociceptive signaling. *Pflugers Arch* 2013;465:921-7. doi: 10.1007/s00424-012-1211-y.
12. Harding EK, Dedek A, Bonin RP, Salter MW, Snutch TP, Hildebrand ME. The T-type calcium channel antagonist, Z944, reduces spinal excitability and pain hypersensitivity. *Br J Pharmacol* 2021;178:3517-32. doi: 10.1111/bph.15498.
13. Ciotu CI, Fischer MJM. Novel analgesics with peripheral targets. *Neurotherapeutics* 2020;17:784-825. doi: 10.1007/s13311-020-00937-z.
14. Gambeta E, Gandini MA, Souza IA, Zamponi GW. Ca V 3.2 calcium channels contribute to trigeminal neuralgia. *Pain* 2022;163:2315-25. doi: 10.1097/j.pain.0000000000002651.
15. Platano D, Magli MC, Ferraretti AP, Gianaroli L, Aicardi G. L- and T-type voltage-gated Ca²⁺ channels in human granulosa cells: Functional characterization and cholinergic regulation. *J Clin Endocrinol Metab* 2005;90:2192-7. doi: 10.1210/jc.2004-1819.
16. Avery MC, Krichmar JL. Neuromodulatory systems and their interactions: A review of models, theories, and experiments. *Front Neural Circuits* 2017;11:108. doi: 10.3389/fncir.2017.00108.
17. Kimura M, Saito S, Obata H. Dexmedetomidine decreases hyperalgesia in neuropathic pain by increasing acetylcholine in the spinal cord. *Neurosci Lett* 2012;529:70-4. doi: 10.1016/j.neulet.2012.08.008.
18. Zhang HM, Chen SR, Pan HL. Regulation of glutamate release from primary afferents and interneurons in the spinal cord by muscarinic receptor subtypes. *J Neurophysiol* 2007;97:102-9. doi: 10.1152/jn.00586.2006.
19. Hildebrand ME, David LS, Hamid J, Mulatz K, Garcia E, Zamponi GW, et al. Selective inhibition of Cav3.3 T-type calcium channels by Galphaq/11-coupled muscarinic acetylcholine receptors. *J Biol Chem* 2007;282:21043-55. doi: 10.1074/jbc.M611809200.
20. Pemberton KE, Hill-Eubanks LJ, Jones SV. Modulation of low-threshold T-type calcium channels by the five muscarinic receptor subtypes in NIH 3T3 cells. *Pflugers Arch* 2000;440:452-61. doi: 10.1007/s004240000303.
21. Zhang Y, Zhang L, Wang F, Zhang Y, Wang J, Qin Z, et al. Activation of M3 muscarinic receptors inhibits T-type Ca(2+) channel currents via pertussis toxin-sensitive novel protein kinase C pathway in small dorsal root ganglion neurons. *Cell Signal* 2011;23:1057-67. doi: 10.1016/j.cellsig.2011.02.001.
22. Zhang L, Zhang Y, Jiang D, Reid PF, Jiang X, Qin Z, et al. Alphacobratoxin inhibits T-type calcium currents through muscarinic M4 receptor and Go-protein $\beta\gamma$ subunits-dependent protein kinase A pathway in dorsal root ganglion neurons. *Neuropharmacology* 2012;62:1062-72. doi: 10.1016/j.neuropharm.2011.10.017.
23. Ulixacaltamide - Praxis Precision Medicine. AdisInsight. Springer Nature Switzerland AG. Available from: <https://adisinsight.springer.com/drugs/800035366> [Accessed: April 14, 2026]
24. Scott L, Puryear CB, Belfort GM, Raines S, Hughes ZA, Matthews LG, et al. Translational pharmacology of PRAX-944, a novel T-type calcium channel blocker in development for the treatment of essential tremor. *Mov Disord* 2022;37:1193-201. doi: 10.1002/mds.28969.
25. Praxis Precision Medicines. Praxis Precision Medicines announces positive topline results from two pivotal Phase 3 studies of ulixacaltamide HCl in the Essential3 program for essential tremor [press release]. 2025. Available from: https://investors.praxismedicines.com/news-releases/news-release-details/praxis-precision-medicines-announces-positive-topline-results-1?utm_source=chatgpt.com. [Accessed: January 13, 2026]
26. Praxis Precision Medicines. Praxis Precision Medicines announces FDA acceptance of new drug application for ulixacaltamide HCl in patients with essential tremor [press release]. 2026 Apr 14. Available from: <https://investors.praxismedicines.com/news-releases/news-release-details/praxis-precision-medicines-announces-fda-acceptance-new-drug> [Accessed: April 21, 2026]
27. Xia F, Du SZ, Wu MK, Liu R, Ye YS, Yang J, et al. Icetexane diterpenoids as Cav3.2 T-type calcium channel inhibitors from *Salvia pratii* and analgesic effect of their Semi-synthesized derivatives. *Bioorg Chem* 2022;128:106059. doi: 10.1016/j.bioorg.2022.106059.
28. Henbid MT, Marks WN, Collins MJ, Cain SM, Snutch TP, Howland JG. Sociability impairments in genetic absence epilepsy rats from Strasbourg: Reversal by the T-type calcium channel antagonist Z944. *Exp Neurol* 2017;296:16-22. doi: 10.1016/j.expneurol.2017.06.022.
29. Pinardi G, Sierralta F, Miranda HF. Atropine reverses the antinociception of nonsteroidal anti-inflammatory drugs in the tail-flick test of mice. *Pharmacol Biochem Behav* 2003;74:603-8. doi: 10.1016/s0091-3057(02)01046-8.
30. Naik AK, Tandan SK, Dudhgaonkar SP, Jadhav SH, Kataria M, Prakash VR, et al. Role of oxidative stress in pathophysiology of peripheral neuropathy and modulation by N-acetyl-L-cysteine in rats. *Eur J Pain* 2006;10:573-9. doi: 10.1016/j.ejpain.2005.08.006.
31. Owoyele BV, Bakare AO, Ayinla MT, Adeshina KA, Onietan D, Azeez SO. Antinociceptive effects of lead acetate in sciatic nerve chronic constriction injury model of peripheral neuropathy in male Wistar rats. *Naunyn Schmiedeberg's Arch Pharmacol* 2021;394:117-25. doi: 10.1007/s00210-020-01951-w.
32. Kukkar A, Singh N, Jaggi AS. Attenuation of neuropathic pain by sodium butyrate in an experimental model of chronic constriction injury in rats. *J Formos Med Assoc* 2014;113:921-8. doi: 10.1016/j.jfma.2013.05.013.
33. Hallak M, Balci H, Günaydin C, Bilge SS. The role of muscarinic and serotonergic-2A receptors in the antinociceptive effect of pregabalin. *Physiol Pharmacol* 2019;23:302-8.
34. Rayiti RK, Munnangi SR, Bandarupalli R, Chakka V, Nimmagadda SL, Sk LS, et al. Effect of chrysin on mechanical hyperalgesia in chronic constriction injury-induced neuropathic pain in rat model. *Int J Appl Basic Med Res* 2020;10:189-93. doi: 10.4103/ijabmr.IJABMR_58_19.
35. Weldon DT, O'Hare E, Kuskowski MA, Cleary J, Mach JR Jr. Alternating lever cyclic-ratio schedule analysis of the effects of atropine sulfate. *Pharmacol Biochem Behav* 1996;54:753-7. doi: 10.1016/0091-3057(96)00014-7.
36. Tringham E, Powell KL, Cain SM, Kuplast K, Mezeyova J, Weerapura M, et al. T-type calcium channel blockers that attenuate thalamic burst firing and suppress absence seizures. *Sci Transl Med* 2012;4:121ra19. doi: 10.1126/scitranslmed.3003120.
37. Antunes FTT, Huang S, Chen L, Zamponi GW. Effect of ABT-639 on Cav3.2 channel activity and its analgesic actions in mouse models of inflammatory and neuropathic pain. *Eur J Pharmacol* 2024;967:176416. doi: 10.1016/j.ejphar.2024.176416.
38. Choi S, Yu E, Hwang E, Llinás RR. Pathophysiological implication of Cav3.1 T-type Ca²⁺ channels in trigeminal neuropathic pain. *Proc Natl Acad Sci U S A* 2016;113:2270-5. doi: 10.1073/pnas.1600418113.
39. García-Caballero A, Gadotti VM, Stenkowski P, Weiss N, Souza IA, Hodgkinson V, et al. The deubiquitinating enzyme USP5 modulates neuropathic and inflammatory pain by enhancing Cav3.2 channel activity. *Neuron* 2014;83:1144-58. doi: 10.1016/j.neuron.2014.07.036.
40. Gomez K, Calderón-Rivera A, Sandoval A, González-Ramírez R, Vargas-Parada A, Ojeda-Alonso J, et al. Cdk5-dependent phosphorylation of Cav3.2 T-Type channels: Possible role in nerve ligation-induced neuropathic allodynia and the compound action potential in primary afferent C fibers. *J Neurosci* 2020;40:283-96. doi: 10.1523/JNEUROSCI.0181-19.2019.
41. Li Y, Tatsui CE, Rhines LD, North RY, Harrison DS, Cassidy RM, et al. Dorsal root ganglion neurons become hyperexcitable and increase expression of voltage-gated T-type calcium channels (Cav3.2) in paclitaxel-induced peripheral neuropathy. *Pain* 2017;158:417-29. doi: 10.1097/j.pain.0000000000000774.
42. Jagodic MM, Pathirathna S, Joksovic PM, Lee W, Nelson MT, Naik AK, et al. Upregulation of the T-type calcium current in small rat sensory neurons after chronic constrictive injury of the sciatic nerve. *J Neurophysiol* 2008;99:3151-6. doi: 10.1152/jn.01031.2007.

43. Amorim JL, Lima FA, Brand ALM, Cunha S, Rezende CM, Fernandes PD. Two new β N-alkanoyl-5-hydroxytryptamides with relevant antinociceptive activity. *Biomedicines* 2021;9:455. doi: 10.3390/biomedicines9050455.
44. Takasu K, Honda M, Ono H, Tanabe M. Spinal alpha(2)-adrenergic and muscarinic receptors and the NO release cascade mediate supraspinally produced effectiveness of gabapentin at decreasing mechanical hypersensitivity in mice after partial nerve injury. *Br J Pharmacol* 2006;148:233-44. doi: 10.1038/sj.bjp.0706731.
45. Pan YZ, Pan HL. Primary afferent stimulation differentially potentiates excitatory and inhibitory inputs to spinal lamina II outer and inner neurons. *J Neurophysiol* 2004;91:2413-21. doi: 10.1152/jn.01242.2003.
46. Colangelo C, Shichkova P, Keller D, Markram H, Ramaswamy S. Cellular, synaptic and network effects of acetylcholine in the neocortex. *Front Neural Circuits* 2019;13:24. doi: 10.3389/fncir.2019.00024.
47. Chen SR, Chen H, Yuan WX, Wess J, Pan HL. Differential regulation of primary afferent input to spinal cord by muscarinic receptor subtypes delineated using knockout mice. *J Biol Chem* 2014;289:14321-30. doi: 10.1074/jbc.M114.550384.
48. Pan HL, Wu ZZ, Zhou HY, Chen SR, Zhang HM, Li DP. Modulation of pain transmission by G-protein-coupled receptors. *Pharmacol Ther* 2008;117:141-61. doi: 10.1016/j.pharmthera.2007.09.003.
49. Li DP, Chen SR, Pan YZ, Levey AI, Pan HL. Role of presynaptic muscarinic and GABA(B) receptors in spinal glutamate release and cholinergic analgesia in rats. *J Physiol* 2002;543:807-18. doi: 10.1113/jphysiol.2002.020644.
50. Zhang HM, Li DP, Chen SR, Pan HL. M2, M3, and M4 receptor subtypes contribute to muscarinic potentiation of GABAergic inputs to spinal dorsal horn neurons. *J Pharmacol Exp Ther* 2005;313:697-704. doi: 10.1124/jpet.104.079939.
51. Rang HP, Dale MM, Ritter JM, Moore P. *Pharmacology*. 5th ed. Edinburgh: Elsevier; 2003.
52. Fiorino DE, Garcia-Guzman M. Muscarinic pain pharmacology: Realizing the promise of novel analgesics by overcoming old challenges. *Handb Exp Pharmacol* 2012;(208):191-221. doi: 10.1007/978-3-642-23274-9_9.
53. Yue CQ, Ye J, Li CL, Li RT, Sun Q. Antinociceptive effects of the novel spirocyclopiperazinium salt compound LXM-10 in mice. *Pharmacol Biochem Behav* 2007;86:643-50. doi: 10.1016/j.pbb.2007.02.009.
54. LeBlanc BW, Lii TR, Huang JJ, Chao YC, Bowary PM, Cross BS, et al. T-type calcium channel blocker Z944 restores cortical synchrony and thalamocortical connectivity in a rat model of neuropathic pain. *Pain* 2016;157:255-63. doi: 10.1097/j.pain.0000000000000362.
55. Bourinet E, Zamponi GW. Voltage gated calcium channels as targets for analgesics. *Curr Top Med Chem* 2005;5:539-46. doi: 10.2174/1568026054367610.
56. Choi S, Na HS, Kim J, Lee J, Lee S, Kim D, et al. Attenuated pain responses in mice lacking Ca(V)3.2 T-type channels. *Genes Brain Behav* 2007;6:425-31. doi: 10.1111/j.1601-183X.2006.00268.x.
57. Bourinet E, Francois A, Laffray S. T-type calcium channels in neuropathic pain. *Pain* 2016;157:S15-22. doi: 10.1097/j.pain.0000000000000469.
58. Mesnage B, Gaillard S, Godin AG, Rodeau JL, Hammer M, Von Engelhardt J, et al. Morphological and functional characterization of cholinergic interneurons in the dorsal horn of the mouse spinal cord. *J Comp Neurol* 2011;519:3139-58. doi: 10.1002/cne.22668.
59. Cai YQ, Chen SR, Han HD, Sood AK, Lopez-Berestein G, Pan HL. Role of M2, M3, and M4 muscarinic receptor subtypes in the spinal cholinergic control of nociception revealed using siRNA in rats. *J Neurochem* 2009;111:1000-10. doi: 10.1111/j.1471-4159.2009.06396.x.
60. Zhang J, Chen SR, Chen H, Pan HL. RE1-silencing transcription factor controls the acute-to-chronic neuropathic pain transition and Chrm2 receptor gene expression in primary sensory neurons. *J Biol Chem* 2018;293:19078-91. doi: 10.1074/jbc.RA118.005846.
61. Hayashida KI, Bynum T, Vincler M, Eisenach JC. Inhibitory M2 muscarinic receptors are upregulated in both axotomized and intact small diameter dorsal root ganglion cells after peripheral nerve injury. *Neuroscience* 2006;140:259-68. doi: 10.1016/j.neuroscience.2006.02.013.
62. Taylor BK. Spinal inhibitory neurotransmission in neuropathic pain. *Curr Pain Headache Rep* 2009;13:208-14. doi: 10.1007/s11916-009-0035-8.
63. Tata AM, Vilaró MT, Mengod G. Muscarinic receptor subtypes expression in rat and chick dorsal root ganglia. *Brain Res Mol Brain Res* 2000;82:1-10. doi: 10.1016/s0169-328x(00)00165-0.