THE ROLE OF MUSCARINIC RECEPTORS IN PAIN MODULATION

Nurcan Bektas*, Dilara Nemutlu, Gamze Ulugbay, Rana Arslan

Anadolu University Faculty of Pharmacy, Department of Pharmacology, 26470 Eskisehir, TURKEY.

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ABSTRACT
Pain is an unpleasant experience comes along with any kind of damage and effects daily routine negatively. Although there are various drugs, many of them could not completely succeed in relieving pain due to pain modulation is a complex process involving numerous mediators and receptors. Therefore, it is a rational approach identifying the components involved in this complex process and develop new agents act on these components. In this respect, the involvement of muscarinic receptors in pain modulation has drawn attention in recent years. The aim of the review is to exhibit the involvement of the muscarinic receptor subtypes that contribute to pain modulation. The search strategy was performed with MeSH terms and free text words, using the bibliographic databases Science Direct and PubMed. The articles have been collected from the experimental animal studies. It is obvious that muscarinic receptors that are located in both peripheral and central areas are extensively involved in the pain process, besides the regional effectiveness of these receptors and their subtypes may vary. Since the muscarinic receptors are various and involve in many physiologic processes, the possibility of adverse effects is a problem in their clinical use. Thus, determining the receptor specificity is an important issue to understand what types of muscarinic receptors involve in pain modulation and to develop new drugs. The agonists of muscarinic receptors are promising for relieving pain although there are lots of unanswered questions.

Keywords: pain; peripheral muscarinic receptors; spinal muscarinic receptors; supraspinal muscarinic receptors.

INTRODUCTION
Pain is experienced by all the people, and various pharmacological groups are used to relieve pain. Various problems such as drug interactions, adverse effects, and tolerability problems could be observed with current agents. Studies are devoted to gain a better understand of pain and discover new agents, however, a lot of questions remain unanswered. International Association for the Study of Pain (IASP) describes pain as an unpleasant sensory and emotional experience arising from any part of the body and associated with actual or potential tissue damage, or described in terms of such damage. The pain is an ‘experience’ and in this respect it differs from ‘nociception’. Nociception is called a neural process provides transduction and transmission of a noxious stimulus to the brain by using pain pathways. Noxious stimuli are detected by nociceptors that are found in skin, muscle, connective tissues, blood vessels, and viscera. They are sensory neurons giving rise to a nerve fiber. They have two main fiber types: Aδ and C fibers. The nociceptors travel through the spinal cord and make synaptic connections with second order neurons in the gray matter column of the dorsal horn (DH). A part of second-order neurons have ascending axons and project to the brainstem or the thalamocortical system. The impulses originated from brain stem nuclei, “descend” to the spinal level and affect the transmission of pain signals at the DH. The relative balance between descending inhibition and facilitation can be changed by the type and intensity of the stimulus and also by the time following the injury. Somatosensory system that detects destructive and potentially tissue injurious stimuli plays a critical role as an essential protective mechanism including numerous interacting peripheral and central mechanisms. These mechanisms are the highly complex process involving various mediators and receptors as seen in Table 1. Pain control is provided by the interaction of these chemicals and receptors over an extensive network from the periphery to the CNS. The rate of participation of the chemicals and receptor types in the modulation depend on the pain types and noxious stimulus.

It is clear that the muscarinic acetylcholine receptors (mAChRs) that discussed in this review, apparently play a role both directly and indirectly in pain modulation. The activation of mAChRs provides pain control by contributing to releasing various modulators and changing the permeability of various ion channels. Moreover, they also mediate the analgesic effect of other analgesic agents. In this review, the experimental
MUSCARINIC RECEPTORS IN PAIN

Acetylcholine and muscarinic receptor subtypes in pain modulation

Acetylcholine (ACh) is a neurotransmitter found in both the peripheral and central nervous system in many organisms as well as humans. According to several reports, ACh plays a role in the inhibition and regulation of the pain transmission. The physiological effects of ACh are mediated by mAChRs or nicotinic acetylcholine receptors (nAChRs). It is known that mAChRs are also involved in pain modulation as well as mAChRs; however, it will be touched on mAChRs in this review.

Molecular cloning studies have identified five different mAChRs termed as M₁–M₅. There is consensus on mAChRs in peripheral tissues and the nervous system. The M₁, M₂ and M₅ subtypes are selectively bind to G₉/₁₁ proteins to activate phospholipase C, whereas the M₂ and M₅ subtypes are selectively coupled to the pertussis toxin-sensitive G₁₅ proteins that mediate the adenyl cyclase inhibition. mAChRs activating drugs have been in use for a long time to treat both acute and chronic pain. It is well known that treatment of post-operative pain, labor pain and cancer pain with cholinesterase inhibitors lead to strong and stable analgesia via cholinergic stimulation and following spinal mAChRs activation. Some research reports that cholinergic agonists also have analgesic effects on animal experiments, as well as cholinesterase inhibitors. Additionally, mAChRs agonists are better alternative than opioid analgesics in that they do not show physical dependence. However, some cholinergic side effects such as bradycardia, hypotension, diarrhea, frequent urination and lacrimation prevent their clinical benefits. That’s why using drugs that are selective for the subtypes taking roles in the modulation of pain, are gaining importance and lots of research triggering to investigate the mAChRs subtypes that participate in the antinociception. Using the genetic knockout (KO) animals, contributes to examine the importance of receptor subtypes and their ligands and receptor subtypes that have a high affinity.

The perception and control of pain are provided through an extensive network from the periphery to the CNS. mAChRs involve in pain modulation at all levels. In peripheral antinociception, the functional roles of peripheral mAChR have been showed with a series of electrophysiological and neurochemical studies. It is suggested that the transmission of pain impulses may be suppressed via activation of mAChRs that are located on peripheral nociceptors of the skin. Neuronal and non-neuronal ACh released from peripheral sources such as sensory neurons or separate cell types of the skin such as keratinocytes and fibroblasts, respectively, following cutaneous injury can activate sensory afferents through muscarinic receptors as well as nicotinic receptors. The activation of mAChR provides desensitization in sensory neuron.

The investigations are more focused on the spinal and supraspinal muscarinic pain modulation since the mAChRs are also expressed in central pain processing regions such as the spinal cord, thalamus, periaqueductal gray (PAG), and rostral ventrolateral medulla (RVM). Although M₁, M₂, M₅, and M₆ subtypes existed in the central area, the intensity and the localization of these muscarinic subtypes are different. A primary site of action for cholinomimetics in nociceptive processing is the spinal cord. M₂ is the major mAChR subtype expressed in the spinal cord, whereas M₁ and M₅ subtypes represent only a fraction of the total mAChRs in the spinal cord. These subtypes, especially M₂ and M₅, are important ones for pain, located in the spinal cord DH and nociceptive pathways as shown in behavioral, pharmacological, neurochemical and electrophysiological studies. The contribution of these subtypes in spinal pain modulation is emphasized in the further parts of the review. Additionally, investigations emphasize the M₁, M₂, and M₅ subtypes are involved in supraspinal pain modulation. There are proofs about the regulation of pain perception by mAChRs via supraspinal mechanisms, as well as spinal mechanisms. The supraspinal administration of muscarinic ligands shows that they have analgesic effects at the levels of the hypothalamus, PAG, RVM and amygdala. It has been reported that mAChRs activation may play an analgesic role by affecting the electric activities of pain excited neurons and pain inhibited neurons in the caudate putamen, a region is known that contribute to nociceptive modulation. Moreover, stimulation of mAChRs in the thalamus can influence the emotional part of analgesia. The studies about which subtype is involved in pain control and how they contribute this process have been accelerated since the role of muscarinic pain modulation was elucidated.

M₁ subtypes

Previously Ghezzi and Zidovick, [23] reported that M₁ receptors participate in the central antinociception, and Zhuo and Gebhart [24] supported that the role of M₁ receptor subtype in the spinal cholinergic modulation. Afterward, Sheardown et al. [25] studied rat models of acute pain and indicated that M₁ receptor subtype is not necessary for antinociception. More recently, it has been shown that i.t. application of putative M₁ agonist McN-A-343 (β-[4-[[N-(cholinophenyl)carbamoyl]oxy]-2-butyln] trimethylammonium chloride) caused dose-dependent antinociceptive effect in tail-flick test. [25] Although there is a little evidence about M₁ receptors involve in spinal cholinergic modulation, its role is predominantly distinct in supraspinal cholinergic antinociception. In one of the studies suggesting its role, knockdown of the alpha subunit of G₉/₁₁ proteins, provided by intracerebroventricular (i.c.v.) administration of antisense oligodeoxyribonucleotide,
and in another one knockdown of central M2 prevented the antinociception induced by systemically injection of oxotremorine (OXO), non-selective mAChR agonist, and physostigmine, acetylcholine esterase inhibitor.[26, 27] Moreover, the contribution of supraspinal M1 mAChRs in morphine analgesia was investigated as discussed below.[28] In another study, the antinoceptive mechanism of xanomeline, an M1/M2-prefering agonist, was determined by using nonselective (scopolamine and pirenzepine), and selective mAChR antagonists MT-7 (muscarinic toxin-7), for M1 receptor, and MT-3 (muscarinic toxin-3) for M3 receptor in several models of inflammatory and neuropathic pain. Scopolamine and pirenzepine entirely antagonized the analgesic effect of xanomeline, supporting that the analgesic effect is associated with the muscarinic system. In addition, MT-7, the highly selective M1 receptor toxin, nearly suppress the whole analgesic effect of xanomeline when injected supraspinal although, MT-3, the highly selective M3 receptor toxin, reversed the analgesia relatively MT-3 also had no effect when given spinally. These results have been indicated the supraspinal M3 receptors’ weak role, as well as the predominant role of supraspinal M1 receptors in analgesia.[29]

**M3 subtypes**

M3 receptor subtypes seem to handle both peripheral and central muscarinic antinociception. Reduction of the sensitivity of peripheral nociceptors to different painful stimuli by muscarinic agonist via the activation of cutaneous M2 mAChRs has been shown in M2 KO mice.[17] Thus, M2 mAChR agonists have potential as a peripheral analgesic, particularly when administered topically owing to the possessing various side-effects when administered systemically.

The M2 subtype is the most crucial mAChR that mediate analgesia produced by muscarinic agonists in the spinal cord. Gomez et al.[30] used the M2 KO mice to investigate the pharmacological role of M2 mAChRs. Antinociceptive effect of the non-selective mAChR agonist OXO on thermal thresholds evaluated by using tail-flick and hot-plate tests. Even though the tail-flick method estimates pain sensitivity mainly at the spinal level; the hot-plate test assesses pain responses and analgesia at the supraspinal level. The antinociception evoked by OXO disappeared in M2 KO mice. This study obviously proves the role of M2 subtypes in central muscarinic pain modulation. In a study, the effects of systemic arecaidine, M2 mAChR agonist, administration on nociceptive responses evaluated in a murine model of nerve growth factor-induced pain. Antinociception of arecaidine by activation of M2 mAChRs exerted analgesic action on DRG sensory neurons by negatively modulating vanilloid receptor subtype 1 (VR1) activity.

These evidence also informative to show that there is a cross interaction between M2 mAChRs and VR1 activity.[31] It has been predicted that M2 subtypes are mostly involved in acute pain modulation. In a study that performed with WAY-132983 ((3R,4R)-3-(3-hexylsulfanyl-pyrazin-2-yl oxy)-1-aza-bicyclo[2.2.1] heptane), M1/M2-prefering agonist, this agent could not found effective in acute pain model and it was claimed that this ineffectiveness occurred due to its low affinity and potency for M2 receptors.[32] In general, M1 mAChR subtypes are also expressed on presynaptic terminals, as well as postsynaptic neurons, to modulate the releasing of some neurotransmitters, such as GABA, glutamate and ACh itself, as touched on below. Jeong et al. [33] searched if the stimulation of mAChRs may regulate glutamate releasing from primary afferents onto medullary DH neurons which receive Aδ- and C-fibers from orofacial tissues and contribute orofacial pain process including migraine and trigeminal neuralgia and showed that the stimulation of presynaptic M2 mAChRs reduce action potential-dependent glutamate releasing onto medullary DH neurons. Therefore, M2 mAChRs may also be promising targets for the management of pain arising from orofacial tissues.

Additionally, M2 mAChRs seem to be involved in the affective dimension of pain. Raisings in vocalization thresholds (Pain behaviors evoked by noxious tail shock) produced by intra-nucleus parafascicularis (nPf) carbachol were reversed dose-dependently by local administration of the non-specific mAChR antagonist atropine. Localization studies show moderate to high expression of M2 receptors in nPf, a thalamic site that takes part in the creation of affective responses to painful stimulus. Thereby, the antinoceptive effects induced by intra-nPf carbachol are most presumably mediated by M2 receptors.[32]

**M3 subtypes**

A study performed to understand the role of M3 mAChRs of the spinal cord in pain modulation showed that the release of ACh modulated by presynaptic M3 mAChRs which are involved in the second phase of nociception evoked by formalin due to significant increase of the ACh level in the second phase was inhibited by it injection of M3 antagonist 4-DAMP.[34] This study supports the results obtained from Dawson et al.[35] in which M1/M3 receptor agonists L-689,660 and AF102B were found effective in the tail-flick test. In contrast, in the study performed by Cai et al. [36], it was shown that M3 subtypes did not contribute to antinociception at the spinal level. As mentioned above, some contrary results also obtained for M1 mAChRs. The reasons for this controversy are not apparent but are attributed to differences in animal strains, agents and assessment methods. Further investigations are required to identify this controversy. In Matera et al. study [37], new bis(ammonio)alkane-types mAChR agonists that

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incorporate the orthosteric muscarinic agonist iperoxo into a molecular fragment of the M₁-selective allosteric modulators W84 or naphemothium, was synthesized and their analgesic action was assayed in vivo in the acetic acid writhing test. Among these synthesized compounds, the naphemothium-related compound, named as 8b, which showed the most potent antinociception without muscarinic side effects such as cardiovascular unwanted effects and the lowest intrinsic activity at M₁ mAChRs when compared with those measured at M₁ and M₂ subtypes. This fact may be explained that M₁ mAChRs prevent the muscarinic side effects and also they are involved in pain modulation less than other receptor subtypes.

M₄ subtypes
The antinociceptive effects of various centrally active muscarinic agonists has been evaluated by using M₄ KO, M₃ KO, and M₃/M₄ double-KO mice in order to understand the involvement of the M₂ and M₄ mAChRs in muscarinic agonist-induced analgesia in the tail-flick and hot-plate tests. The analgesic activity induced by subcutaneous (s.c.) administration of the non-selective mAChR agonist OXO entirely disappeared in M₂/M₄ double-KO mice in both tests. Previously, Gomez et al. showed that the analgesic action of OXO was significantly decreased in M₄ KO mice. However, it was indicated that non-M₂ mAChRs can also mediate profound antinociception because maximum analgesia could still be elicited in M₂ KO mice by increasing doses of OXO. The wholly disappearing of antinociception in M₂/M₄ double-KO mice suggests that both M₂ and M₄ mAChRs participate in mediating muscarinic antinociception at both spinal and supraspinal levels, and non-M₂/M₄ mAChRs do not involve in this effect.[38] Similarly, CMI-936 (2-exo-[5-(3-methyl-1,2,4-oxadiazolyl)]-221]-7-azabicycloheptane) and CMI-1145 (2-exo-[5-(3-amino-1,2,4-oxadiazolyl)]-221]-7-azabicycloheptane) (s.c.), M₄ preferring agonists, showed potential antinociceptive efficacy in the tail-flick test and this efficacy was reduced by M₂/M₄ preferring antagonists like hymbacin (s.c.), pertussis toxin (i.t.) and M₄ selective peptide antagonist, MT-3 (i.t.).[39] In another study, the changes of muscarinic M₄ receptor levels have been investigated by using M₄ mAChR subtype selective ligands with receptor autoradiography, on rats with acute and chronic arthritis, the model of pain. The heat-killed Mycobacterium butyricum was applied intradermally to rats and then observed 12 days for acute, 30 days for the chronic group. An important reduction of M₄ mAChR level, the down-regulation of M₄ mAChRs spinal cord of rats that have acute and chronic arthritis, occurred as a result of prolonged ACh, released highly against to the pain stimulus, stimulation.[40] In addition, M₃/M₄ preferring agonist WAY-132983, the agent which was found ineffective in acute pain model as aforementioned, generated strong and efficient antihyperalgesic and antiallodynic effects in rodent models of chemical irritant-induced visceral pain, chronic inflammatory, neuropathic, and incisional pain.[52] These findings show that M₄ mAChRs are participating muscarinic mechanisms of analgesia at the level of the spinal cord and M₄ mAChR selective agonists promise hope for using as analgesics.

M₅ subtypes
There are limited studies on M₅ receptors induced antinociception, and the involvement of M₅ mAChRs in pain modulation has not been exactly proved yet. However, because the existence of mRNA’s of this subtype in the DRG area was shown, development of novel drugs act on M₅ mAChRs are on the agenda.[13] In the one recent of these studies, a complex role of M₅ mAChRs was also revealed. It has been demonstrated that the activation of the M₅ subtype expressed at primary afferent terminals potentiates primary afferent input, whereas stimulation of the M₅ subtype can also indirectly inhibit nociceptive primary afferent input through increased glutamate release from spinal interneurons and subsequent activation of group II/III metabotropic glutamate receptors (mGlurRs).[36]

mAChRs involved in the antinociception induced by other analgesic treatments
mAChRs mediate the antinociceptive effect of not only own agonist but also the other analgesic treatments such as morphine, clonidine, and spinal cord stimulation (SCS), so they are also called as mAChR ligands. It is proved that SCS can be used for neuropathic pain treatment.[41] An increased release of spinal ACh acting on mAChRs has been reported to be one of the mechanisms involved in SCS.[42] It has been shown that sub-effective dose of OXO may have a synergistic effect with SCS against painful hypersensitivity in SCS non-responding rats.[43] In another study analgesic effect of SCS were completely blocked by atropine but it was not susceptible to the nicotinic antagonist mecamylamine, and there was only a partial attenuation produced by M₁ and M₃ antagonists. Interestingly, M₄ selective antagonist MT-3, blocked the SCS induced analgesia selectively.[42] Therefore, M₄ subtype could be defined as a key subtype for SCS induced analgesia. Da Silva et al.[44] has recently shown that low-frequency electro acupuncture-induced analgesia utilizes muscarinic mechanism in the dorsal anterior pretectal nucleus, which is located in descending pathways from the dorsolateral funiculus to the spinal dorsal horn for mediating nociception.

It has been investigated that morphine and clonidine, analgesic agents, are capable of increasing ACh release at spinal level and this endogenous ACh has a significant role in mediating the analgesic effect of these drugs.[10,12] Spinal ACh assists to the analgesic effect of systemic morphine through cholinergic receptors.[10] It has been suggested by a study that demonstrates that the interaction between ACh and endogenous opiate peptides (EOP) as morphine. ACh and EOP increase the release of each other which antagonized by their receptor antagonists, opiate antagonist; naloxone, and mAChRs.
A modulation via non-cholinergic system

The antinociception of NSAIDs has been developed by NSAIDs in acute thermal pain model uptake inhibitor, antagonized the antinociception transmission and terminals that facilitate cholinergic neurotransmitters such as inhibitory GABA and glycine, excitatory glutamate, and ACh itself onto spinal DH neurons. GABA is the primary inhibitory neurotransmitter in the CNS. The interaction between the muscarinic system and GABAergic transmission in CNS has been studied for a long time. Moreover, GABA neurons and receptors are also distributed in supraspinal sites that organize the perception and response to painful impulse, and this neurotransmitter system regulates sensory information proceeding in the spinal cord. It has two receptors called ionotropic GABA_A, primarily postsynaptic, and metabotropic GABA_B, mostly presynaptic. The interaction between cholinergic system and GABAergic transmission in CNS has been studied for a long time and the various reports indicate that spinal mAChR activation produce antinociception via activation of mAChRs on the GABAergic interneurons and terminals to excite GABA release and then the DH neurons are inhibited by GABA_A receptor-mediated Cl- channels provoked by this released GABA. It is also clearly seen that globus pallidus and substantia gelatinosa are involved in pain modulation, considering the muscarinic modulation of GABA release. The stimulation of somatodendritic M_2, M_3, and M_4 on GABA interneurons assists the GABAergic transmission and causes inhibition of postsynaptic DH neurons. Contrary to rats, presynaptic M_2, M_3, and M_4 mAChR subtypes regulate GABAergic transmission in mice DH. The inhibitory GABAergic input to DH neurons is mostly weakened through the stimulation of M_2 or M_3 mAChRs, whereas M_3 activation assists the releasing of GABA. Endogenously released GABA in the spinal cord can preferentially activate presynaptic GABA_A receptors. For instance in Chen and Pan study, antinociception induced by it mAChR activation in streptozocin-treated rats blocked by it GABA_A receptor antagonist, CGP55845 ((1S)-1-(3,4-Dichlorophenyl)ethyl[amino]-2-hydroxypropyl) (phenylmethyl)phosphinic acid hydrochloride. The activated presynaptic GABA_A receptors may attenuate the spinal release of glutamate indirectly and contribute to spinal analgesia.

Muscarinic pain modulation via non-cholinergic systems

Muscarinic pain modulation also provided by non-cholinergic pain modulatory systems and it will be discussed briefly in this part of the review. mAChRs are broadly found in postsynaptic neurons and also in presynaptic terminals in the nervous system. Presynaptic mAChRs modulate the release of several neurotransmitters such as inhibitory GABA and glycine, excitatory glutamate, and ACh itself onto spinal DH neurons. GABA is the primary inhibitory neurotransmitter in the CNS. The interaction between the muscarinic system and GABAergic transmission in CNS has been studied for a long time. Moreover, GABA neurons and receptors are also distributed in supraspinal sites that organize the perception and response to painful impulse, and this neurotransmitter system regulates sensory information proceeding in the spinal cord. It has two receptors called ionotropic GABA_A, primarily postsynaptic, and metabotropic GABA_B, mostly presynaptic. The interaction between cholinergic system and GABAergic transmission in CNS has been studied for a long time and the various reports indicate that spinal mAChR activation produce antinociception via activation of mAChRs on the GABAergic interneurons and terminals to excite GABA release and then the DH neurons are inhibited by GABA_A receptor-mediated Cl- channels provoked by this released GABA. It is also clearly seen that globus pallidus and substantia gelatinosa are involved in pain modulation, considering the muscarinic modulation of GABA release. The stimulation of somatodendritic M_2, M_3, and M_4 on GABA interneurons assists the GABAergic transmission and causes inhibition of postsynaptic DH neurons. Contrary to rats, presynaptic M_2, M_3, and M_4 mAChR subtypes regulate GABAergic transmission in mice DH. The inhibitory GABAergic input to DH neurons is mostly weakened through the stimulation of M_2 or M_3 mAChRs, whereas M_3 activation assists the releasing of GABA. Endogenously released GABA in the spinal cord can preferentially activate presynaptic GABA_A receptors. For instance in Chen and Pan study, antinociception induced by it mAChR activation in streptozocin-treated rats blocked by it GABA_A receptor antagonist, CGP55845 ((1S)-1-(3,4-Dichlorophenyl)ethyl[amino]-2-hydroxypropyl) (phenylmethyl)phosphinic acid hydrochloride. The activated presynaptic GABA_A receptors may attenuate the spinal release of glutamate indirectly and contribute to spinal analgesia.

Glutamate is a major excitatory neurotransmitter in the spinal cord. It is crucial in the handling of sensual information in the spinal cord DH and is known to provide improved excitability of DH neurons in chronic pain conditions. The heteroreceptor function of GABA_A receptors also controls synaptic glycine release as well as glutamate release to spinal DH neurons. Glycine is the other inhibitory neurotransmitter, and blocking of its receptors in the spinal cord is known to

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cause oversensitiveness of DH neurons and allodynia. The findings suggest that any impairment of glycinergic inhibitory synaptic transmission in the spinal DH is associated with the progress of neuropathic pain.\textsuperscript{[13]}

mAChRs also directly contribute to the regulation of glycinergic, glutamatergic inputs on DH neurons in mice and rats as well as GABAergic modulation. Presynaptic M\textsubscript{2} on primary peripheral sensory neurons inhibit excitatory glutamatergic input to DH neurons through diminishing the Ca\textsuperscript{2+} influx into primary afferent terminals in rats\textsuperscript{[53]} and M\textsubscript{2}/M\textsubscript{4} and M\textsubscript{3} mAChRs subtypes on a subset of interneurons also inhibit. The inhibition of postsynaptic DH neurons by increasing glycinergic transmission is also provided by stimulation of somatodendritic M\textsubscript{2} and M\textsubscript{4} mAChRs on glycine interneurons. The M\textsubscript{1} subtype is mainly responsible for the muscarinic potentiation of synaptic glycine release in the rat spinal cord.\textsuperscript{[13, 62]}

As understood, glutamate transmission takes an important place in muscarinic pain modulation. Interactions between the muscarinic and glutamatergic neurotransmitter systems may change the neuronal excitability and synaptic transmission by synergistic activation of M\textsubscript{i} mAChRs and metabotropic glutamate receptors (mGlus), group I (mGlu1 and mGlu5).\textsuperscript{[54]}

M\textsubscript{i}, M\textsubscript{3}, M\textsubscript{4}, and M\textsubscript{4} subtypes may also alter the activation of some ion channels when modify the neurotransmitter release. Therefore, the interaction between the ion channels and the muscarinic system is also a valuable focus point. In mice and rats, the stimulation of presynaptic M\textsubscript{i} can help the release of neurotransmitter from sympathetic neurons by M\textsubscript{2} type K\textsuperscript{+} current suppression or weaken release through closing the voltage-activated N- and L-type Ca\textsuperscript{2+} channels. Stimulation of presynaptic M\textsubscript{i} and M\textsubscript{4} inhibit the release of neurotransmitter through fast inhibition of N- and P/Q-type Ca\textsuperscript{2+} channels.\textsuperscript{[13]} The N- and P/Q-type voltage-gated calcium channel subtypes are associated with the release of glutamate from trigeminal primary afferents\textsuperscript{[53]} and may be implicated in the mAChR-mediated presynaptic inhibition of glutamate release onto medullary DH neurons.

There are some evidence that the activation of mAChRs may contribute the pain modulation by acting on the pain modulatory ion currents. It was informed that increasing intracellular calcium content is important for cholinergic antinociception. T-type Ca\textsuperscript{2+} channels are important in modulating intracellular Ca\textsuperscript{2+} ion concentration nearby the resting membrane potential and the regulation of T-type currents in reaction to stimulation of an array of G protein-coupled receptors, as M\textsubscript{i} mAChRs.\textsuperscript{[85]} In Zhang et al.\textsuperscript{[86]} study, alpha-cobratoxin, a neurotoxic protein that has the analgesic effect, reversibly inhibited T-currents dose-dependently. Selective M\textsubscript{i} mAChR antagonist tropicamide blocked this inhibitory effect. As M\textsubscript{i} mAChRs, M\textsubscript{i} mAChRs activation by G\textsubscript{q,11} also inhibits T-type flow by way of an unclear mechanism pathway and stimulation of M3 mAChR blocks T-type currents via a new PKC isoform pathway in ice DRG neurons.\textsuperscript{[57]} It is known that mAChRs activate K\textsuperscript{+} channels and the agents that open K\textsuperscript{+} channels such as neuronal Kv7 and K\textsuperscript{+}ATP channels have been demonstrated to generate an antinociceptive effect in models of acute and chronic pain.\textsuperscript{[58, 59]} In one of the studies that show various types of K\textsuperscript{+} channels such as K\textsuperscript{+}ATP appeared to be related with the antinociception of mAChR agonists, i.c.v. glibenclamide (K\textsuperscript{+}ATP channel blocker) antagonized the antinociceptive effect of i.c.v. pilocarpine.\textsuperscript{[60]} In the further study, it was reported that the antinociception provoked by i.t. bethanecol was potentiated by nicorandil (K\textsuperscript{+} channel opener) and partially antagonized by glibenclamide and charybdotoxin (both K\textsuperscript{+}ATP channel and Ca\textsuperscript{2+}-activated K\textsuperscript{+} channel blockers).\textsuperscript{[59]} These outcomes indicated that the antinociception evoked by mAChRs agonists, especially through the activation of M\textsubscript{2} mAChR subtypes, both at the supraspinal and the spinal levels is reliant on opening of K\textsuperscript{+}ATP channel.\textsuperscript{[61]}

Also, group-I mGlus inhibit the mAChR-dependent K\textsuperscript{+} flow that is important for the after hyperpolarization that happens following an action potential and a leakage of K\textsuperscript{+} flow in neurons. Pharmacological and electrophysiological studies show that group-I mGlus on peripheral sensory neurons are crucial in chronic pain models.\textsuperscript{[62]} It is possible that prevention of weakening the muscarinic K\textsuperscript{+} flow may underlying the antinociception evoked by mGlur antagonists. Additionally, it should be noted that muscarinic stimulation decrease glutamate release as well, which provides mGlus activation.\textsuperscript{[62, 63]}

The activity of these neuropeptides may be reinforced via VR1 activity. One of the interactions of muscarinic pain modulation is with transient receptor potential VR1 known as TRPV1, a nonselective cation channel. It is triggered via injurious heat, protons and vanilloid agonists.\textsuperscript{[31]} De Angelis et al.\textsuperscript{[31]} show that the activation of M\textsubscript{2} mAChR leads to desensitization to mechanical and heat stimulus via a down-regulation of VR1 expression.

In the last step of muscarinic antinociception, it is possible to discuss muscarinic and opioidergic interaction on emotional modulation and defensive responses to pain. In Leite-Panissi study\textsuperscript{[64]} it is demonstrated that antinociception evoked by carbachol or morphone sulfate administered into the central nucleus of the amygdala (involved in diverse emotional and cognitive functions related to responses to fear and orientation, defensive behavior, pain) is prevented by pretreatment with nalozone in the same region. It is indicated that the action of the cholinergic system is opioid-dependent in descending inhibitory pathways as well. Moreover, it is known that mAChRs activation also raises EOP concentration in the spinal cord and assist to antinociception by improvement of EOP stimulation of μ receptors. EOP system includes enkephalin, endorphin, and dynorphin, all of them play an important role in analgesia by acting on μ-, δ- and κ- opioid receptors.\textsuperscript{[11]}

Even though, the antinociception gained in DH can be
referred to stimulation of cholinergic-opioidergic systems, it cannot be rejected the attendance of other hippocampal neurotransmitters in antinociceptive response mediating.

CONCLUSION
It is obvious that the muscarinic system is involved in pain process mediating by mAChRs as discussed in this review. Electrophysiological and neurochemical studies indicate that these receptors are located in both peripheral and central areas; however, the density of the receptors differs. Although muscarinic receptors are more involved in central pain control, at spinal and supraspinal levels, peripheral control cannot be negligible. In the periphery, among the mAChRs, M₂ subtypes seems to be responsible for cholinergic antinociception. It is suggested that the transmission of pain impulses may be suppressed via activation of mAChRs that are located on peripheral nociceptors of the skin. Neuronal and non-neuronal ACh released from peripheral sources such as sensory neurons or different cell types of the skin such as keratinocytes and fibroblasts, respectively, following cutaneous injury can activate sensory afferents through mAChRs. Because main task of M₂ mAChRs is on the heart and soft muscle physiology, they may cause several systemic side-effects, for this reason, M₂ agonists can be administrated as topical analgesics in the acute and chronic pain conditions which is managed peripherally with minimal adverse systemic effects.

It has been discussed in this review that there are some areas that intensely take part in pain modulation in CNS. The investigations are more focused on the spinal and supraspinal cholinergic pain modulation since the cholinergic receptor density is more excessive in spinal and supraspinal pain pathways. The obtained results from these investigations are considerable. In the respect of muscarinic antinociception; M₁, M₂, and M₄ mAChRs are important subtypes, especially M₂ and M₄, located in the spinal cord DH and nociceptive pathways. According to the preclinical pharmacological information, activations of M₂ and even M₁ receptors can be necessary for ACh releasing in the spinal level. This secretion leads to antinociception by the activation of M₂ receptors. Thereby, M₂ receptors are privileged for the mAChR-mediated spinal antinociception as well as peripheral antinociception. Also, investigations emphasize the M₂, M₄, and especially M₁ subtypes are involved in supraspinal pain modulation. It has been reported that mAChRs activation may possess an analgesic action via affecting the electric activities of pain-excited neurons and pain-inhibited neurons in the caudate putamen, a region is known that contribute to nociceptive modulation. When all the data is considered, it is remarkable that M₁ subtype predominantly involve in supraspinal muscarinic antinociception whereas M₂ and M₄ subtypes involve in spinal antinociception. mAChs utilize various mechanisms such as modulation of neurotransmitter release and ion channels permeability concurrently with muscarinic antinociception.

In conclusion, it is possible to say that mAChRs regulate analgesia peripherally and centrally at spinal and supraspinal levels, and muscarinic antinociception extensively takes part in pain control. Moreover, mAChRs are related to analgesia induced by different pain treatments. Because ACh-activated cholinergic receptors involve in many physiological processes, the drugs that act on this receptor system may cause undesirable side-effects. Thereby, it is so important to identify the best subtype to reduce or remove the cholinergic side effects that are seen with the nonselective agonists and to design new therapeutic strategies. The muscarinic receptor-mediated agents are promising. Muscarinic agonists seem to be efficient against numerous stimulus approaches and possess a wide efficacy against a series of clinically important acute and chronic pain conditions. The treatments targeting central pain pathways provide more effective results than peripheral-targeting treatments for both chronic pain types such as neuropathic and inflammatory pain and acute pain. Nevertheless, it is rational to use these agents for both peripheral and central management of these pain conditions. Thereby, the selective agonists targeting M₁, M₂ or M₄ mAChRs are valuable agents because of providing an acceptable analgesia in the management of acute and chronic pain conditions that are induced by several disorders. It is also possible to utilize these agonists in controlling post-operative pain, labor pain, and orofacial pain such as migraine.

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