

Spinal Endogenous Acetylcholine Contributes to the Analgesic Effect of Systemic Morphine in Rats

Shao-Rui Chen, M.D.,* Hui-Lin Pan, M.D., Ph.D.†

Background: Systemic morphine is known to cause increased release of acetylcholine in the spinal cord. Intrathecal injection of the cholinergic receptor agonists or acetylcholinesterase inhibitors produces antinociception in both animals and humans. In the present study, we explored the functional importance of spinal endogenous acetylcholine in the analgesic action produced by intravenous morphine.

Methods: Rats were implanted with intravenous and intrathecal catheters. The antinociceptive effect of morphine was determined by the paw-withdrawal latency in response to a radiant heat stimulus after intrathecal treatment with atropine (a muscarinic receptor antagonist), mecamylamine (a nicotinic receptor antagonist), or cholinergic neurotoxins (ethylcholine mustard aziridinium ion [AF64A] and hemicholinium-3).

Results: Intravenous injection of 2.5 mg/kg morphine increased significantly the paw-withdrawal latency. Intrathecal pretreatment with 30 μ g atropine (n = 7) or 50 μ g mecamylamine (n = 6) both attenuated significantly the antinociceptive effect of morphine. The inhibitory effect of atropine on the effect of morphine was greater than that of mecamylamine. Furthermore, the antinociceptive effect of morphine was significantly reduced in rats pretreated with intrathecal AF64A (n = 7) or hemicholinium-3 (n = 6) to inhibit the high-affinity choline transporter and acetylcholine synthesis. We found that intrathecal AF64A reduced significantly the [³H]hemicholinium-3 binding sites but did not affect its affinity in the dorsal spinal cord.

Conclusions: The data in the current study indicate that spinal endogenous acetylcholine plays an important role in mediating the analgesic effect of systemic morphine through both muscarinic and nicotinic receptors.

SYSTEMIC morphine is extensively used for the treatment of acute and chronic pain. Previous studies have shown that the therapeutic effect of morphine is mediated selectively through μ -opioid receptors, and the intrinsic descending inhibitory system is closely involved in its analgesic action.^{1,2} Intravenous morphine may act at multiple sites in the central nervous system, including the spinal cord, to produce analgesia.²⁻⁴ Cholinergic neurons in the spinal cord are an important component of the descending inhibitory pathways for modulation of nociception. We have shown that intravenous injection of morphine increases the release of acetylcholine in the

spinal dorsal horn.⁵ Intravenous morphine also increases acetylcholine concentrations in the spinal dorsal horn and cerebrospinal fluid.^{5,6} However, the functional importance of spinal endogenous acetylcholine in the analgesia produced by systemic morphine has not been fully investigated.

Activation of the spinal cholinergic system is involved in antinociception. For example, intrathecal injection of muscarinic or nicotinic receptor agonists is effective to relieve acute and chronic pain in animals.⁷⁻⁹ Intrathecal injection of acetylcholinesterase inhibitors also produces antinociception in both animals and humans.^{7,10} Furthermore, intrathecal coadministration of acetylcholinesterase inhibitors and morphine produces a synergistic analgesic effect.^{7,10,11} The spinal cholinergic receptors also play an important role in the analgesic action of intrathecal α_2 receptor agonists. We have shown that spinal muscarinic and nicotinic receptors are important for the analgesic effect produced by intrathecal clonidine.¹² In this regard, blockade of spinal muscarinic or nicotinic receptors attenuates spinal nitric oxide release and the antiallodynic effect of intrathecal clonidine in a rat model of neuropathic pain.^{12,13}

The role of spinal muscarinic receptors in morphine analgesia is unclear. Although one study found that intrathecal atropine blocks the analgesic effect of intraperitoneal morphine,¹⁴ a recent study has failed to demonstrate the role of spinal muscarinic receptors in analgesia produced by intravenous morphine in rats.¹⁵ Like many other tissues, the spinal cord contains both muscarinic and nicotinic cholinergic receptors.^{16,17} However, the role of spinal nicotinic receptors in the analgesic effect of morphine has not been studied previously. Although data from previous neurochemical studies suggest that spinally released acetylcholine may be involved in the analgesic action of morphine,⁵ there is no substantial evidence to support the functional importance of spinal endogenous acetylcholine in the analgesic action of systemic morphine. It remains uncertain whether and to what extent spinal muscarinic and nicotinic receptors contribute to the analgesic action produced by morphine. Therefore, in the current study, we tested a hypothesis that endogenous spinal acetylcholine mediates the analgesic effect of systemic morphine through both spinal muscarinic and nicotinic receptors.

Materials and Methods

Male rats (Harlan Sprague-Dawley, Indianapolis, IN) weighing 250-275 g were used in this study. The surgi-

* Research Associate, Department of Anesthesiology, † Associate Professor and Director of Basic Research, Departments of Anesthesiology and Neuroscience and Anatomy.

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Address reprint requests to Dr. Pan: Department of Anesthesiology, H187, Pennsylvania State University College of Medicine, 500 University Drive, Hershey, Pennsylvania 17033-0850. Address electronic mail to: hpan@psu.edu. Individual article reprints may be purchased through the journal Web site, www.anesthesiology.org.

cal preparations and experimental protocols were approved by the Animal Care and Use Committee of the Pennsylvania State University College of Medicine (Hershey, PA). The rats were anesthetized with 2% halothane, and the right jugular vein was cannulated with PE-50 tubing. Intrathecal catheters (PE-10 tubing) were inserted through an incision in the cisternal membrane and advanced 8 cm caudal so that the tip of each catheter was positioned at the lumbar spinal level. Both intravenous and intrathecal catheters were externalized to the back of the neck and sutured to the musculature and skin at the incision site. The rats were used for analgesic studies after recovery for 4 or 5 days after cannulation.

Behavioral Assessment of Nociception

To quantitatively assess the nociceptive threshold of the hind paw, rats were placed on the glass surface of a thermal plantar testing apparatus (Model 336; IITC Inc./Life Science Instruments, Woodland Hills, CA). The rats were allowed to acclimate for 30 min before testing. The temperature of the glass surface was maintained constant at 30°C. A mobile radiant heat source located under the glass was focused onto the hind paw of the rats. The paw-withdrawal latency was recorded by a digital timer. The withdrawal latencies for the left and right paws were averaged, and the mean value was used to indicate the sensitivity to noxious heat stimulation.¹⁵ The apparatus was adjusted at the beginning of the study in six separate rats so that the baseline paw-withdrawal latency was approximately 10 s. This setting (*i.e.*, the light beam intensity) was kept unchanged for the remainder of the study. The cut-off of 30 s was used to prevent potential tissue damage.¹⁵

Motor function was evaluated by the placing or stepping reflex and the righting reflex.¹⁸ The former was evoked by drawing the dorsum of either hind paw across the edge of the table. The latter was assessed by placing the rat horizontally with its back on the table, which normally gives rise to an immediate, coordinated twisting of the body to an upright position. Changes in motor function was scored as follows: 0, normal; 1, slight deficit; 2, moderate deficit; and 3, severe deficit.

Role of Spinal Muscarinic or Nicotinic Receptors in Morphine Analgesia

After the baseline was measured, three groups of rats were treated intrathecally with 30 μ g atropine (a specific muscarinic receptor antagonist, $n = 7$), 50 μ g mecamylamine (a specific nicotinic receptor antagonist, $n = 6$), or the vehicle (saline, $n = 6$). The maximal effective doses of these antagonists upon intrathecal injection in rats have been systematically determined in our previous studies.¹² Fifteen minutes later, rats received intravenous injection of 2.5 mg/kg morphine, and the paw-withdrawal latency was determined every 10

min for 60 min after morphine injection. This dose of morphine was selected based on our previous study showing that it produces 60% maximal possible effect in rats.¹⁵ To assess the possibility that blockade of both muscarinic and nicotinic receptors may attenuate the antinociceptive action of morphine to a greater extent, we also tested the effect of intravenous morphine in six other rats 15 min after intrathecal injection of both 30 μ g atropine and 50 μ g mecamylamine. Drugs for intrathecal injections were dissolved in normal saline and administered in a volume of 5 μ l followed by a 10- μ l flush with normal saline. Atropine and mecamylamine were obtained from Sigma Chemical Company, St. Louis, MO. Morphine was supplied by Astra Pharmaceutical, Westborough, MA.

Role of Spinal Endogenous Acetylcholine in Morphine-produced Analgesia

To further demonstrate the role of spinal endogenous acetylcholine in the effect of morphine on antinociception, separate rats were injected intrathecally with either 5 nM ethylcholine mustard aziridinium ion (AF64A; $n = 6$) or its vehicle ($n = 6$). This dose of AF64A has been shown to effectively reduce the acetylcholine content in the dorsal spinal cord in rats.¹⁹ AF64A is a specific cholinergic neurotoxin that disrupts the high-affinity choline transporter (HACHT).²⁰ Because the HACHT is a rate-limiting step for the synthesis of acetylcholine,²¹ AF64A is capable of inducing irreversible inhibition of acetylcholine synthesis. AF64A was prepared according to the method described previously.²² Briefly, acetyethylcholine mustard HCl (RBI, Natick, MA) was diluted in distilled water and brought to pH 11.5 for 30 min with 1 N NaOH. The AF64A was formed by decreasing the pH to 7.0 with 0.1 N HCl and then adjusting to pH 7.4 with NaHCO₃. The cyclized solution was kept at 4°C and injected within 2 h. The vehicle control solution was distilled water to which an equivalent amount of 1 N NaOH was added, followed by pH adjustment with HCl and NaHCO₃, as with the AF64A solution. One week after intrathecal AF64A treatment, the analgesic effect produced by intravenous injection of 2.5 mg/kg morphine was tested. In addition, 1 μ g hemicholinium-3 (HC-3; Sigma) dissolved in saline, a reversible inhibitor of choline transporter,^{23,24} was injected intrathecally in six other rats. Three hours later, the antinociceptive effect of 2.5 mg/kg intravenous morphine in these rats was tested, as described earlier.

Effect of AF64A on Spinal High-affinity Choline Transporter Bindings

To estimate the effect of intrathecal AF64A on HACHT, the spinal cords of AF64A- and vehicle-treated rats ($n = 6$ in each group) were rapidly removed under 2-3% halothane anesthesia 1 week after intrathecal treatment. The dorsal halves of the spinal cord were dissected and used

for the [^3H]HC-3 binding experiments to confirm the neurotoxic effect of AF64A on the dorsal spinal HAcHT. The HC-3 binds specifically to the cholinergic transporter, and the [^3H]HC-3 binding has been used as a marker of cholinergic neuronal activity and of the integrity of cholinergic terminals.²⁵ The [^3H]HC-3 binding experiments were performed using membrane preparations.²⁶ Briefly, an aliquot of membrane was added to the tubes containing 50 mM glycylglycine buffer, 200 mM NaCl, and 10 nM [^3H]HC-3 (NEN, Boston, MA). After 30 min of incubation at 25°C, the reaction was terminated by adding ice-cold Tris-HCl buffer (pH 7.4) followed by rapid filtration using a Brandel Harvester (Model M48, Gaithersburg, MD). Radioactivity on Whatman GF/B filters was determined in a liquid scintillation counter (Model LS 6500; Beckman Coulter, Inc., Fullerton, CA). The nonspecific binding was determined by adding 10 μM HC-3 to the reaction mixture.

Data are presented as mean \pm SEM. The effects of atropine and mecamylamine on morphine-produced antinociceptive action and the difference between treatment with AF64A/HC-3 and the vehicle on the effect of morphine were determined by repeated-measures analysis of variance followed by Tukey *post hoc* test. The saturation binding data were processed using nonlinear regression analysis (Prism; GraphPad Software, San Diego, CA) to calculate maximal specific binding (B_{max}) and dissociation constant (K_{D}). Differences in B_{max} and K_{D} between the control and AF64A-treated groups were compared by the Student paired *t* test. $P < 0.05$ was considered to be statistically significant.

Results

Effect of Intrathecal Atropine and Mecamylamine on Morphine Analgesia

The withdrawal latency of the hind paw in response to the radiant heat stimulation before morphine injection was 10.3 ± 0.6 s ($n = 6$, saline group). The paw-withdrawal latency increased significantly (25.6 ± 1.4 s, $P < 0.05$) 10 min after intravenous injection of 2.5 mg/kg morphine in rats being given intrathecal saline (fig. 1). The effect of intravenous morphine on the paw-withdrawal latency lasted for 40–50 min. Intrathecal pretreatment with 30 μg atropine or 50 μg mecamylamine attenuated significantly the effect of morphine (fig. 1). Furthermore, the inhibitory effect of atropine on the effect of morphine was significantly greater than that of mecamylamine (fig. 1). In rats subjected to intrathecal injection of a combination of atropine and mecamylamine, the antinociceptive effect produced by subsequent intravenous injection of 2.5 mg/kg morphine was similar to that in rats pretreated with intrathecal atropine. Intrathecal administration of atropine or mecamylamine was not associated with any overt behavioral or motor function changes, which were assessed by

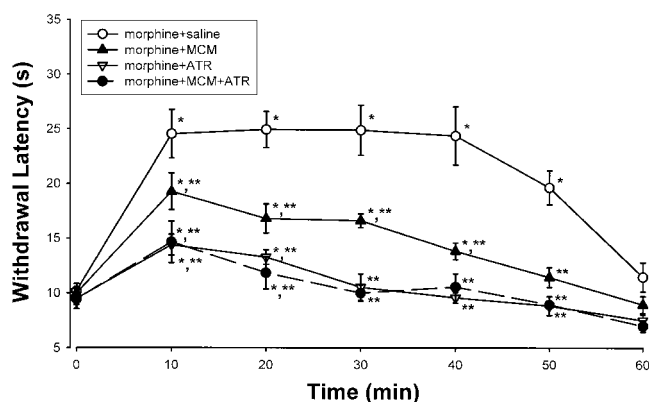


Fig. 1. Inhibitory effects of intrathecal injection of saline ($n = 6$), 30 μg atropine (ATR, $n = 7$), 50 μg mecamylamine (MCM, $n = 6$), or a combination of atropine and mecamylamine ($n = 6$) on the analgesic action of intravenous injection of 2.5 mg/kg morphine in rats. The nociceptive response was determined by the withdrawal latency of the hind paw to a heat stimulus. Data presented as mean \pm SEM. * $P < 0.05$ versus respective baseline control. ** $P < 0.05$ versus saline-plus-morphine group.

testing the animals' ability to stand and ambulate in a normal posture and to place and step with the hind paw.¹⁸

Effect of Intrathecal Pretreatment with AF64A and Hemicholinium-3 on Morphine-produced Analgesia

The baseline withdrawal threshold in response to the radiant heat stimulus was not significantly altered by intrathecal injection of AF64A or HC-3, compared to the vehicle group (fig. 2). The motor function, based on the placing/stepping reflex and the righting reflex, appeared normal in AF64A- and HC-3-treated rats. In rats subjected to intrathecal injection of AF64A, the effect of morphine on the paw-withdrawal latency was attenuated significantly, compared to that in rats pretreated with intrathecal vehicle (fig. 2). Intrathecal pretreatment with HC-3 also significantly reduced the effect of intravenous mor-

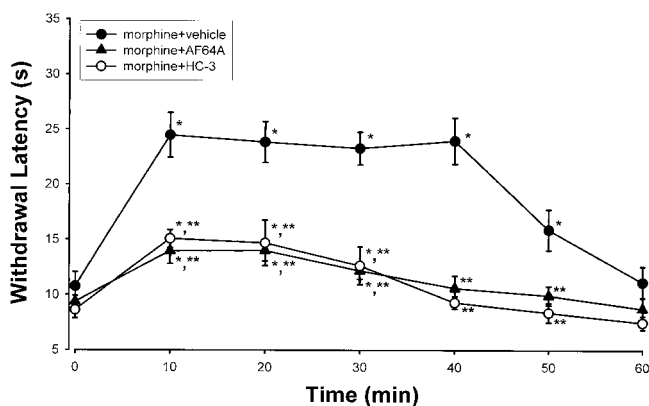


Fig. 2. Effects of intrathecal administration of hemicholinium-3 (HC-3; 1 μg , $n = 6$), ethylcholine mustard aziridinium ion (AF64A; 5 nm, $n = 7$), or its vehicle ($n = 6$) on the analgesic effect of intravenous injection of 2.5 mg/kg morphine in rats. Data presented as mean \pm SEM. * $P < 0.05$ versus respective baseline control. ** $P < 0.05$ versus vehicle-treated group.

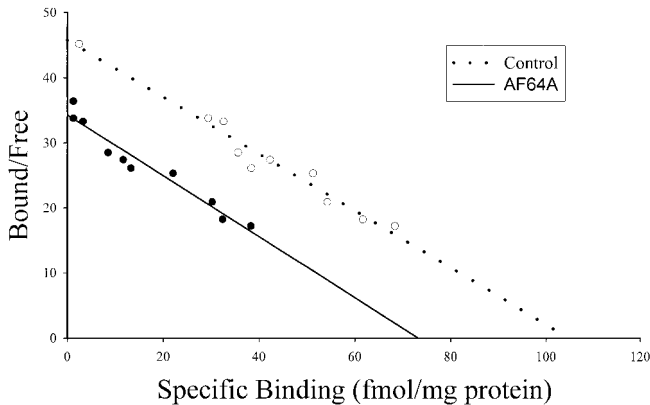


Fig. 3. Scatchard plot transformed from the saturation binding data showing the effect of intrathecal ethylcholine mustard aziridinium ion (AF64A; $n = 6$) or its vehicle ($n = 6$) on [^3H]hemicholinium-3 binding sites in the dorsal spinal cord.

phine to the level similar to that observed in the AF64A treated group (fig. 2).

Effect of Intrathecal AF64A on Spinal [^3H]hemicholinium-3 Binding Sites

Scatchard plot transformed from the saturation binding data demonstrates the effect of intrathecal AF64A on the B_{max} and K_D of [^3H]HC-3 bindings in the dorsal spinal cord (fig. 3). Compared with the vehicle-treated group ($B_{\text{max}} = 102.5 \pm 5.58$ fmol/mg protein; $K_D = 8.86 \pm 1.03$ nM), intrathecal AF64A significantly reduced the B_{max} (76.2 ± 3.86 fmol/mg protein) of the [^3H]HC-3 binding without an apparent effect on its K_D (7.91 ± 0.73 nM) in the dorsal spinal cord.

Discussion

In the present study, we examined the role of spinal endogenous acetylcholine and cholinergic receptors in the analgesic effect produced by intravenous morphine in rats. A previous study has reported that intrathecal treatment with atropine reduces the analgesic effect of intraperitoneal morphine.¹⁴ In the present study, we have provided new evidence that pretreatment with muscarinic or nicotinic receptor antagonists significantly attenuates the analgesic action of intravenous morphine. Another important finding of the current study is that reduction of endogenous spinal acetylcholine through inhibition of HAcHT with AF64A or HC-3 significantly reduces the analgesic effect of morphine. Therefore, this study provides important information that endogenous acetylcholine in the spinal cord mediates the analgesic action of intravenous morphine through both spinal muscarinic and nicotinic receptors.

Systemic morphine causes spinal acetylcholine release in animals and humans.^{5,6} Also, intrathecal administration of cholinergic receptor agonists or cholinesterase

inhibitors produces analgesia.^{8,10,27} Thus, we reasoned that endogenously released acetylcholine in the spinal cord is involved in morphine analgesia. A unique tool to study the role of endogenous acetylcholine in the spinal cord is the neurotoxin AF64A. The neurotoxin AF64A is an analog of choline and contains a highly reactive aziridinium ion capable of nucleophilic attack. It binds to catalytic sites of enzymes using choline as a substrate and reacts covalently causing irreversible inhibition.^{20,26} The HAcHT system is a specific marker for cholinergic neurons and is important for the acetylcholine synthesis.²⁸⁻³⁰ Administration of AF64A in animals results in a long-lasting reduction in the number of functional Na^+ -dependent HAcHT sites, leading eventually to a long-term reduction in the steady-state levels of tissue acetylcholine.²⁹ The effect of intrathecal AF64A on the HAcHT in the dorsal spinal cord has not been shown directly. In the present study, we found a significant reduction in the spinal [^3H]HC-3 binding site after AF64A treatment, suggesting the spinal high-affinity choline transporters and cholinergic presynaptic terminals are probably damaged by AF64A. This is consistent with a recent report that spinal acetylcholine content is reduced by the similar treatment with AF64A.¹⁹ The decrease in the spinal endogenous acetylcholine concentration after AF64A treatment could be a result of an irreversible alkylation of nucleophilic active sites on the choline carrier. Thus, it could render the cholinergic nerve terminal deficient in newly synthesized acetylcholine because of its prevention of choline from gaining access to the site of acetylcholine synthesis. As an alternative, the decrease in acetylcholine may result from cholinergic nerve terminal degeneration. In this regard, AF64A could be accumulated in the cholinergic neurons through HAcHT, which could disrupt the metabolic processes required for neuronal viability.²⁹

We found that intrathecal treatment with either of the two structurally dissimilar choline transporter inhibitors, AF64A or HC-3, both reduced significantly the morphine analgesia to the same extent. These data strongly suggest that endogenous acetylcholine in the spinal cord is important for the full manifestation of morphine analgesia. Acetylcholine is formed in cholinergic neurons from the cosubstrates, choline and acetyl coenzyme A, through a reaction catalyzed by the enzyme choline acetyltransferase. Because cholinergic neurons cannot synthesize choline *de novo*, their function depends on choline uptake.^{25,28} The HAcHT is considered to be present specifically in cholinergic neurons because a substantial portion of choline is converted to acetylcholine only when taken up through the high-affinity system.²⁸ In the spinal cord, the high-affinity choline transporters are found at high density in the dorsal horn of the spinal cord,²⁵ which could be the reason why intrathecal AF64A and HC-3 had no apparent effect on the motor function.

The role of spinal muscarinic receptors in morphine analgesia is supported by the finding in the present study that intrathecal atropine largely attenuated the effect of intravenous morphine. It has been reported that intrathecal atropine blocks the analgesic effect of intraperitoneal morphine in rats.¹⁴ We observed previously that the analgesic effect of intravenous morphine is not attenuated by intrathecal atropine if given 15–30 min after intravenous morphine.¹⁵ Thus, pretreatment of cholinergic antagonists appears to be important to demonstrate the role of spinal cholinergic receptors in morphine-produced analgesia. The discrepancy between this and our previous studies is most likely attributable to the fact that pretreatment of atropine can effectively block nitric oxide production caused by intrathecal morphine. We have shown that there is a rapid generation of nitric oxide in the spinal cord dorsal horn after intravenous morphine, which is completely blocked by pretreatment with intrathecal atropine.⁵ Intrathecal injection of nitric oxide synthase inhibitors eliminates the analgesic effect of intravenous morphine, indicating that spinal nitric oxide is essential for the analgesic action of systemic morphine.¹⁵ The important observation that pretreatment, but not posttreatment, with atropine can attenuate the effect of morphine supports an intermediate role of spinal cholinergic receptors in morphine-produced analgesia (*i.e.*, morphine → cholinergic receptors → nitric oxide → analgesia). There are at least three different subtypes of muscarinic receptors in the spinal cord.¹⁶ Because of a lack of highly specific antagonists for muscarinic receptor subtypes, we were unable to further study precisely the subtypes of muscarinic receptors in intravenous morphine analgesia using pharmacologic approaches at present. Importantly, Duttaroy *et al.*³¹ have recently reported that the analgesic potency of morphine is reduced in M₄ and M₂/M₄ receptor knockout mice. Therefore, the M₂ and M₄ receptor subtypes probably play an important role in the analgesic action of intravenous morphine.

Our study provides the first evidence that spinal nicotinic receptors mediate the analgesic action of intravenous morphine. Both spinal muscarinic and nicotinic receptors are present in the spinal cord.^{16,17} Stimulation of spinal nicotinic receptors can produce antinociception in animals.²⁷ The α_4 and β_2 subunits of neuronal nicotinic receptors are directly related to the analgesia produced by nicotinic agonists and are also probably involved in morphine analgesia.^{9,32} We found that intrathecal pretreatment with mecamylamine significantly attenuated the effect of intravenous morphine. This observation is consistent with our recent finding that nicotinic receptors are involved in spinal nitric oxide release caused by clonidine and the antiallodynic effect of intrathecal clonidine in a rat model of neuropathic pain.^{12,13} Similar to our recent finding that spinal muscarinic receptors play a greater role in the clonidine-produced

antiallodynia in neuropathic pain,¹² we found that intrathecal atropine attenuated the morphine analgesia to a greater extent than intrathecal mecamylamine. A combination of muscarinic and nicotinic antagonists did not further reduce the morphine analgesia, suggesting that a common final pathway is responsible for both muscarinic and nicotinic receptor-mediated analgesia. In this regard, spinal nitric oxide has been shown to be a prerequisite mediator for morphine analgesia in rats.^{5,15}

We have shown that spinal acetylcholine release after intravenous morphine depends on supraspinal sites.⁵ It has been shown that the noradrenergic neurons innervating the spinal cord dorsal horn are mainly located in the supraspinal site, especially the A7 cell group in the dorsolateral pontine tegmentum.^{4,33} On the other hand, the spinal cholinergic system appears to be intrinsic because spinal cholinergic neurons and nerve terminals are mainly located in the deep dorsal horn. Recent studies have suggested that systemically administered morphine probably acts on μ -opioid receptors in the ventromedial medulla and periaqueductal gray to cause disinhibition of noradrenergic neurons located in the A7 cell group,^{34–36} which leads to norepinephrine spillover in the spinal cord dorsal horn.^{4,37} Activation of spinal α_2 receptors could stimulate spinal cholinergic interneurons to increase acetylcholine, which then produces analgesia through spinal nitric oxide release.^{12,13} Consistent with this notion, it has been shown that cholinergic neurons are colocalized with neurons expressing nitric oxide synthase in the spinal cord dorsal horn.³⁸ We have demonstrated that clonidine-induced spinal nitric oxide release is mediated by spinal muscarinic and nicotinic receptors.¹³ Therefore, it is likely that spinally released acetylcholine functions as an important mediator of spinal nitric oxide generation and the analgesic effect of systemic morphine.

In summary, the current study indicates that the spinal cholinergic system plays an important role in the analgesic action of intravenous morphine. Our data, together with previous studies,^{5,14} provide complementary functional evidence that intravenous morphine activates the descending inhibitory system leading to increased release of endogenous acetylcholine in the spinal cord, which produces analgesia through activation of spinal muscarinic and nicotinic receptors. Thus, the current study has an important clinical implication that intrathecal cholinergic receptor agonists or acetylcholinesterase inhibitors could potentiate the analgesic effect of systemically administered opioids.

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