Spinal Muscarinic and Nicotinic Subtypes Activated by Clonidine in Postincisional Pain

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Background: A recent model of acute incisional pain has been characterized that strongly parallels the postoperative period in patients experiencing evoked pain. In that setting, abundant literature has revealed antihypersensitive effects produced by intrathecally administered α₂-adrenergic receptor agonists, such as clonidine, in both animals and humans. Recent reports have suggested an obligatory role of spinal acetylcholine receptors in the analgesic action of intrathecal clonidine. The authors sought to determine the involvement of spinal muscarinic and nicotinic receptor subpopulations in the antihypersensitivity effect of intrathecal clonidine in a rodent model for human postoperative pain.

Methods: After intrathecal catheterization, rats underwent superficial plantar incision. Clonidine or a combination of clonidine and muscarinic receptor subtype antagonists (M1, M2, M3, and M4) or nicotinic receptor subtype antagonists (α₂β₂ and α₆) were intrathecally administered, and withdrawal thresholds to mechanical stimuli were examined.

Results: Spinal clonidine maximally reduced hypersensitivity adjacent to the wound 30 min after its injection. When animals were intrathecally pretreated with the M1 muscarinic antagonist toxin MT-7, the M3 muscarinic antagonist 4-diphenylacetoxy-N-methylpiperidine, and the M4 muscarinic antagonist toxin MT-3, clonidine lost its antihypersensitive action. When animals were intrathecally pretreated with the α₂β₂ nicotinic receptor antagonist dihydro-β-erythroidine, but not with the α₆ nicotinic receptor antagonist methyllycaconitine, the antihypersensitivity action of clonidine was abolished.

Conclusions: These data indicate for the first time that the clonidine-induced increase in punctuate mechanical threshold is mediated via the activation of all but M2 muscarinic receptor subtypes, and via the activation of α₂β₂ but not α₆ nicotinic receptor subtypes in a rodent model for human postoperative pain.

**POSTOPERATIVE** pain has commonly been considered a unique form of acute pain because surgical incision induces mechanical hyperalgesia surrounding the wound in patients. Recently, a rat model of incisional pain has been developed that demonstrates reproducible, quantifiable mechanical allodynia lasting for several days after the incision, displaying similarities to the human postoperative pain state.¹ Cholinergic agents exert antinociceptive properties both in animals and humans, and intrathecal administration of muscarinic ligands or the injection of the cholinesterase inhibitor neostigmine produces postoperative antinociception. Five muscarinic receptors have been characterized by molecular cloning in both rats and humans (M1, M2, M3, M4, and M5), and among them, four selective antagonists are available (M1, M2, M3, and M4). In addition, activation of cholinergic receptors by nicotinic ligands produces antinociception in both animals and humans. Nicotinic acetylcholine receptors are pentameric ligand-gated ion channels composed of varying combinations of α (α₂–α₁₀) and β (β₁–β₄) subunits that are expressed throughout the central and peripheral nervous systems, and among nicotinic acetylcholine receptors, two subclasses (α₂β₂ and α₆) are pharmacologically characterized and selective antagonists are available. The α₂-adrenerceptor agonists yield antinociception in acute pain states, including pain after incision, in both animals and humans.²,³ However, α₂-adrenergceptor agonists, such as dexmedetomidine or clonidine, also produce sedation and cardiovascular depression after systematic or intrathecal injection, limiting their use as an adjuvant for postoperative analgesia. The mechanisms underlying the analgesic action of intrathecally administered clonidine are not fully known in that situation. Several lines of evidence suggest that intrathecally administered clonidine increases concentrations of acetylcholine in cerebrospinal fluid and microdialysates from spinal cord dorsal horn,⁴–⁶ and cholinergic and α₂-adrenergceptor receptors are localized within the same superficial laminae of the spinal cord dorsal horn.⁷,⁸ In addition, we have recently shown in animals after superficial surgery that the antiallodynic effect of clonidine was totally antagonized by atropine (a muscarinic receptor antagonist) and partially by mecamylamine (a nicotinic receptor antagonist), suggesting the reliance of intrathecal clonidine on spinal cholinergic systems for postoperative pain relief.² However, it remains unknown whether and to what extent spinal muscarinic/nicotinic subtypes contribute to analgesia produced by intrathecally administered clonidine in postoperative pain state. Therefore, we sought to determine among the muscarinic subtypes (M1, M2, M3, and M4) and among the nicotinic subclasses (α₂β₂ and α₆) which ones are activated by intrathecal clonidine for postoperative pain relief. In addition, to investigate the subtypes of muscarinic receptors involved in reduction in tactile hypersensitivity by intrathecal clonidine after plantar incision, we used muscarinic antagonists isolated from the venom of African mamba snakes, including two...
that are the most selective ligands for M1 and M4 receptors known.\(^9\)

### Materials and Methods

Male Sprague-Dawley rats (230–280 g) were housed in a temperature-controlled room and maintained on a 12-h day–night cycle. Water and food were available ad libitum. Drugs were obtained from Sigma-Aldrich (Lyon, France) or Peptides International (Louisville, KY) and administered intrathecally. All experimental procedures were approved by the Institutional Animal Care and Use Committee at the University of Claude Bernard Lyon 1 (Lyon, France) and conform to guidelines of the International Association for the Study of Pain.

#### Surgical Preparation

For intrathecal drug administration, polyethylene-10 catheters (Intermedic; Becton Dickinson France, Le Pont-De-Claix, France) was inserted during pentobarbital anesthesia (50 mg/kg intraperitoneal), as previously described.\(^10\) The catheter was passed caudally from the cisterna magna to the level of lumbar enlargement (7.5–8.0 cm). Only animals without evidence of neurologic dysfunction after catheter insertion were studied. Correct position of the lumbar catheter was verified by injection of 10 µl lidocaine, 2%, 1 day after the operation and observation of transient motor blockade. All studies were performed at least 5 days after insertion of the intrathecal catheter, and paw incision was performed as previously described.\(^1\) Animals were anesthetized with pentobarbital anesthesia, the plantar surface of the left hind paw was prepared with 70% ethanol, and a 1-cm longitudinal incision was made through the skin and fascia, starting 0.5 cm from the edge of the heel and extending toward the toes. The plantaris muscle was elevated and incised longitudinally. The wound was closed with two silk 5.0 sutures.

#### Behavioral Testing

For determining withdrawal threshold, rats were placed individually in plastic cages with a mesh floor. Animals were tested after acclimation to the environment, typically 30 min after being placed in the cage. Withdrawal threshold to punctuate mechanical testing was determined using calibrated von Frey filaments (Stoelting, Wood Dale, IL). The von Frey filaments used were 3.84, 4.08, 4.31, 4.56, 4.74, 4.93, 5.18, 5.46, and 5.88, corresponding to 0.5, 0.9, 1.7, 3.7, 5.5, 8.0, 12.4, 21.5, and 53.0 g, respectively. Filaments were applied vertically to an area adjacent to the wound at the heel for 4 s while the hair was bent. Brisk withdrawal or paw flinching was considered a positive response. In the absence of a response, the filament of next greater force was applied. In the presence of a response, the filament of next lower force was applied. The tactile stimulus producing a 50% likelihood of withdrawal was determined using the up–down method, as previously described.\(^11\) Tests were performed in duplicate, with an approximate 3-min test-free period between withdrawal responses, and their average was used. Studies were performed on the first day after paw incision surgery. Only rats with a withdrawal threshold less than 5 g were included in the study.

### Experimental Treatments and Drugs and Their Administration

All experimental drug treatments were conducted 24 h after plantar incision. The α\(_2\)-adrenoceptor agonist was used in this study was clonidine hydrochloride (20 µg; Sigma-Aldrich). The muscarinic subtype antagonists used in the study were muscarinic toxin (MT)-7 isolated from the venom of mamba snakes (1 and 10 µg; Peptides International), selective for the M1 muscarinic subtype; methoctramine (10 and 100 µg; Sigma-Aldrich), selective for the M2 muscarinic subtype; 4-diphenylacetoxy-N-methylpiperidine methiodide (4-DAMP, 1 and 10 µg; Sigma-Aldrich), selective for the M3 muscarinic subtype; and MT-3 (0.1 and 1 µg; Sigma-Aldrich), selective for the M4 muscarinic subtype. The nicotinic subtype antagonists used in the study were dihydro-β-erythroidine hydrobromide (DH\(_{BE}\), 5 and 50 µg; Sigma-Aldrich), selective for the α\(_2\)β\(_2\) nicotinic subtype, and methyllycaconitine (MLA, 5 and 50 µg; Sigma-Aldrich), selective for the α\(_4\)-nicotinic subtype. Antagonists or vehicles were injected intrathecally in a volume of 10 µl, 15 min before clonidine administration. Drugs were dissolved in normal saline or, when necessary, in sterile distilled water according to the manufacturer’s instructions. Intrathecal doses of selected antagonists have been previously shown to inhibit corresponding cholinergic ligands in rats subjected to mechanical stimuli.\(^12\)--\(^14\) Drugs were administered intrathecally in volumes of 10 µl, and thresholds for withdrawal were determined 30 min after clonidine administration. The probe dose of clonidine (20 µg) and the timing (30 min) were determined based on pilot experiments.

#### Statistical Analysis

Data are represented as median with lower and upper quartiles. Paw withdrawal thresholds in response to mechanical stimulation before and after paw incision were compared using a Wilcoxon signed rank test. Effects of time on withdrawal thresholds (not including preincision time points) were determined using a Friedman one-way repeated-measures analysis of variance on ranks followed by the Tukey test for multiple comparisons. Effects of individual drugs on withdrawal thresholds (not including preincision time points) were determined using a Kruskal-Wallis analysis of variance on ranks followed by the Dunn test for multiple comparisons. (Sig-
Results

One hundred eleven rats were included in the study. The median withdrawal threshold to punctuate mechanical stimulus before paw incision surgery was 27.7 g (22.3–37.3 g) and decreased to 2.6 g (1.6–3.1 g) within 24 h after plantar incision. 

Muscarinic Antagonism of Clonidine in Rats after Plantar Incision

Intrathecal injection of 20 μg clonidine significantly increased the withdrawal threshold 30 min after injection (figs. 1 and 2). Intrathecal injection of MT-7, 4-DAMP, and MT-3 significantly inhibited the antihypersensitivity effect of intrathecal clonidine (figs. 1A and 2). In contrast, intrathecal injection of methoctramine did not influence the antihypersensitivity effect of intrathecal clonidine (fig. 1B). Intrathecal injection of vehicle or either antagonist alone did not alter the withdrawal threshold (control time points: figs. 1 and 2).

Nicotinic Antagonism of Clonidine in Rats after Plantar Incision

Intrathecal injection of 20 μg clonidine significantly increased the withdrawal threshold 30 min after injection (figs. 3A and B). Intrathecal injection of DHβE significantly inhibited the antihypersensitivity effect of intrathecal clonidine (fig. 3A). In contrast, spinal injection of MLA did not influence the antihypersensitivity effect of intrathecal clonidine (fig. 3B). Intrathecal injection of vehicle or either antagonist alone did not alter the withdrawal threshold (control time points: figs. 3A and B).

Discussion

Hypersensitivity to punctuate mechanical stimuli after superficial incision exhibits unique pharmacology of in-
M3, and M4 muscarinic subtypes and their interaction to relieve pain, and shows that spinal M1, recently suggested that intrathecal pretreatment with vehicle, dihydro-β-erythroidine (DHβE, αβ), nicotinic subtype antagonist; A) and methyllycaconitine (MLA, α, nicotinic subtype antagonist; B) on punctuate mechanical withdrawal thresholds before and after paw incision and after intrathecal injection of 20 μg clonidine. Each symbol represents the median with first and third quartiles of four to seven animals. *P < 0.05 versus postincision and control times. $P < 0.05 versus vehicle.

Intrathecal administration of α2-adrenergic agonists such as clonidine clearly reduces hypersensitivity surrounding the wound after surgery. In addition, we have recently suggested that intrathecal α2-adrenergic agonist efficacy after surgery requires close interactions with spinal muscarinic and nicotinic receptors to produce antinociception, providing the rationale for the clinical use of central clonidine–neostigmine combination. The current study uses the acute pain model developed by Brennan et al.1 that closely parallels postoperative pain in humans to better understand underlying mechanisms of α2-adrenergic agonist–cholinergic system interaction to relief pain, and shows that spinal M1, M3, and M4 muscarinic subtypes and αβ nicotinic subtypes, but not M2 muscarinic subtypes or α2 nicotinic subtypes, are activated by intrathecal clonidine for postoperative pain relief.

**Spinal Muscarinic Receptors Subtypes Activated by Intrathecal Clonidine to Reduce Hypersensitivity after Plantar Incision**

Anatomical studies of ligand binding have revealed the presence of muscarinic receptors in the spinal cord of animals and humans. Interestingly, both muscarinic and α2-adrenergic receptors are localized within the same superficial laminae of the spinal cord dorsal horn, and substantial studies have demonstrated functional interaction between descending brainstem–spinal monoaminergic systems and medullar cholinergic neurons in various pain states, including postoperative pain. Recently, most of the autoradiographic studies, pharmacologic works, or reverse transcriptase–polymerase chain reaction analysis of messenger RNA has demonstrated the distribution of all muscarinic subtypes in the central nervous system, including the spinal cord dorsal horn. Under normal condition, spinal M1 and M3 receptor subtypes, but not M2 receptor subtypes, have been suggested to mediate spinal antinociception in the rat subjected to a radiant heat noxious stimulus. In addition, molecular studies conducted in genetically altered mice subjected to a thermal noxious stimulus revealed that M1 and M2 receptors subtypes, but not M4 receptor subtypes, are likely to be involved in antinociception from cholinergic agonists. Finally, Honda et al. suggested the involvement of spinal M3 receptor subtypes, but not M1 receptor subtypes, in an inflammatory pain model, because intrathecally administered 4-DAMP, but not the M1 receptor subtype antagonist pirenzepine, causes depletion of endogenous acetylcholine in the spinal cord from peripherally formalin-injected mice, although 4-DAMP displays close affinities for the M1 and M4 receptor subtypes as well. However, none of these studies have evaluated the interaction between the spinal monoaminergic system and the muscarinic receptor subtypes. In an attempt to pharmacologically characterize the spinal muscarinic receptor subtypes activated by intrathecal clonidine to produce antinociception, previous reports have shown discrepant results to our findings, possibly because spinal clonidine antinociceptive effects may vary across pain states or may vary pending the noxious stimulus or the animal species used. For example, in normal mice, Honda et al.26 have suggested that spinally injected α2-adrenoceptor agonists reduce withdrawal thresholds to punctuate mechanical stimulus via activation of M1 and M3 receptor subtypes but not M2 receptor subtypes. In addition, in streptozotocin-induced diabetic mice, Koga et al.28 have demonstrated that intrathecal clonidine did not alleviate allodynia to punctuate mechanical stimulus when animals were pretreated with intrathecal M1 receptor subtypes antagonists but not M2 or M3 antagonists. In contrast, in spinal nerve–ligated rats subjected to a punctuate mechanical stimulus and spinally pretreated with MT-7 and MT-3, the highly selective antagonists from the venom of African mamba snakes for M1 and M4 subtypes, respectively, Kang and Eisenach suggested a key role for spinal M4 receptor subtypes, but not M1, in the antihypersensitivity effect of spinally administered α2-adrenoceptor ago-
Spinal Nicotinic Receptors Subtypes Activated by Intrathecal Clonidine to Reduce Hypersensitivity after Plantar Incision

It is well documented that nicotinic acetylcholine receptors are expressed throughout the central nervous system, including descending noradrenergic fibers of the spinal cord of the rat. We and others have recently shown in vivo and in vitro evidence documenting a direct interaction between spinal \( \alpha_2 \)-adrenergic and nicotinic cholinergic receptors for antinociception in animals after plantar incision or spinal nerve ligation, but no previous reports have examined the role of spinal nicotinic receptors subtypes in the analgesic effect of clonidine in the setting of postoperative pain. Although mecamylamine partially reversed the antihypersensitivity effect of clonidine in a previous work, the current study supports that pretreatment with intrathecally administered \( \alpha_4 \beta_2 \) nicotinic receptor subtypes antagonists, but not \( \alpha_\text{ch} \), significantly attenuated the antihypersensitivity effect of clonidine in rats after superficial surgery. We believe that attenuation of the effect of clonidine by nicotinic receptor antagonists is unlikely attributable to a nonspecific effect because DHBE attenuated the analgesic effect of clonidine in a dose-dependent manner. Further, there is growing evidence suggesting a substantial role played by the heteropentameric nicotinic receptor \( \alpha_4\beta_2 \) in the setting of acute and chronic pain, possibly by stimulating the spinal cholinergic- \( \gamma \)-aminobutyric acidergic neurons. The role of non-\( \alpha_4\beta_2 \), including \( \alpha_\text{ch} \), nicotinic receptor subtypes in nicotinic analgesia has received little attention. In fact, no reports have examined the effect of intrathecally or systematically administered \( \alpha_\text{ch} \), or \( \alpha_4\beta_2 \), nicotinic receptor subtypes antagonists in the setting of postoperative pain.

First, \( \alpha_4 \) and \( \beta_2 \) subunits are the predominant nicotinic acetylcholine receptors subunits expressed in the spinal cord. Second, recent studies have led to discrepant results regarding the role played by the \( \alpha_\text{ch} \) nicotinic receptor subtypes in nociceptive transmission. For example, in mice subjected to a thermal noxious stimulus, a high dose of MLA, but not \( \alpha \)-bungarotoxin, both \( \alpha_\text{ch} \) nicotinic receptor subtype antagonists, significantly blocked the antinociceptive effects of nicotinic ligands after intrathecal injection. In addition, centrally administered MLA did not alter the time course of tail-flick responses elicited by nicotine in rats. Finally, in contrast, recent studies strongly suggest an important role of \( \alpha_\text{ch} \) nicotinic receptor subtypes for pain relief because antinociception elicited by spinal injection of nicotinic agonists in rats was reversed by MLA pretreatment.

The detailed mechanisms of the interaction between cholinergic neurons and noradrenergic neurons in the spinal cord remain unclear. The current study suggests that released acetylcholine evoked by intrathecally administered clonidine may act on \( M_1 \), \( M_3 \), and \( M_4 \) receptors and on \( \alpha_2 \beta_2 \) receptors to increase mechanical thresholds to von Frey filaments after plantar incision. The source of spinal acetylcholine release from \( \alpha_2 \)-adrenergic agonists is presumably from spinal cholinergic interneurons possibly through excitatory \( \alpha_2 \)-adrenergic receptors on cholinergic interneurons under neuropathic condition, but no such evidence is available in face of postoperative pain state.

Although the current study uses highly selective antagonists, i.e., peptides isolated from snake venoms, we acknowledge that the results obtained with these pharmacologic probes should be interpreted cautiously. In fact, the distribution of these peptides in the intrathecal space and spinal cord and their nonspecific effects are unknown, but previous work with intrathecal injection and the current study suggest that specific antagonism is possible with these drugs.

In summary, recent studies have provided substantial evidence indicating that cholinergic circuitry mediate the analgesic effect of intrathecally administered clonidine, the \( \alpha_2 \)-adrenergic receptor agonist, in animal models of postoperative and neuropathic pain. Our data clearly indicate an obligatory role of muscarinic and nicotinic receptors subtypes in the antihypersensitivity action of intrathecally administered clonidine for postoperative pain relief. The current study suggests that the spinal \( \alpha_2 \)-adrenoceptor-induced increase in punctuate mechanical thresholds is mediated through neuronal pathways involving all muscarinic receptor subpopulations but \( M_2 \), and \( \alpha_2\beta_2 \), but not \( \alpha_\text{ch} \), nicotinic receptor subtypes.

References

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