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Sex Differences in Cholinergic Analgesia I

A Supplemental Nicotinic Mechanism in Normal Females

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Background: **Cholinergic agents produce analgesia after systemic and intrathecal administration. A retrospective review showed that intrathecal neostigmine was more potent in women than in men, suggesting a sex difference in this response. The purpose of this study was to determine whether such a sex difference exists in normal rats and to examine the pharmacologic mechanisms that underlie this difference.**

Methods: **Male and female rats with indwelling intrathecal catheters received injections of neostigmine, bethanechol (muscarinic agonist), or RJR-2403 (neuronal nicotinic agonist) alone or with atropine (muscarinic antagonist), mecamylamine (nicotinic antagonist), or phentolamine (α-adrenergic antagonist) with antinociception determined to a noxious heat stimulus to the hind paw. Time *versus* subcutaneous paw temperature relationships were defined for males and females.**

Results: **Neostigmine produced dose-dependent antinociception with five times greater potency in female than in male rats. Neostigmine-induced antinociception was reversed in male rats by atropine and unaffected by mecamylamine, whereas it was partially reduced by each antagonist alone in females and completely reversed after injection of both. RJR-2403 was more potent in females than in males, whereas there was no sex difference to bethanechol. Phentolamine partially reversed antinociception from RJR-2403 in females. Paw temperature increased more rapidly in females than in males for the same lamp intensity.**

Conclusions: **These data demonstrate a large sex difference in antinociception to intrathecal neostigmine that is primarily the result of a nicotinic component in females. Phentolamine reversal suggests that part of this nicotinic component may rely on spinal norepinephrine release. A better understanding of this sex difference could lead to development of novel pain therapy for women. (Key words: Muscarinic; neostigmine; noradrenergic; pain; women's health.)**

INTRATHECAL neostigmine produces dose-dependent analgesia and enhances analgesia from opioids and α_2-adrenergic agonists in volunteers[1-4] and patients with chronic pain[5] or acute postoperative pain.[6-8] In a retrospective review of the volunteer studies cited above, we noted that women demonstrated a twofold greater sensitivity to analgesia from neostigmine than did men. Thus, the percent reduction in pain score to a noxious thermal stimulus was reduced more in women (20 ± 7%, 24 ± 6%, and 46 ± 9% after 50, 100, and 200 μg neostigmine, respectively; mean ± SEM) than in men (11 ± 4%, 18 ± 5%, and 23 ± 6%, respectively; $P < 0.01$ by two-way analysis of variance; n = 5-12 per group). In contrast, neostigmine concentrations over time in cerebrospinal fluid did not differ between men and women volunteers. These data suggest a sex difference in humans from intrathecal neostigmine, which is pharmacodynamic rather than pharmacokinetic, and a 2-yr randomized trial of this hypothesis, as well as the hormonal dependence of this difference in volunteers, has begun.

Sex differences in cholinergic analgesia or spinal cholinergic activity have not previously been investigated. However, it has been noted that females show an estrogen-dependent increase in the brain in choline acetyltransferase mRNA[9] and enzymatic activity,[10] high affinity choline uptake,[11] and stimulated acetylcholine release.[12] These data suggest that estrogen, present in the premenopausal adult female, could also stimulate cholinergic activity or innervation in the spinal cord and that

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this could underlie the suggestive evidence of greater potency of intrathecal neostigmine in women.

Both muscarinic[13] and nicotinic[14] agonists produce antinociception in animals, and both types of receptors are present in the superficial laminae of the spinal cord. The primary purpose of this study was to determine whether there is a sex difference in the antinociceptive potency of intrathecal neostigmine in normal rats and to determine its reliance on muscarinic or nicotinic mechanisms. Because nicotinic agonists have been shown in the brain to stimulate norepinephrine release,[15] a secondary purpose was to determine whether part of the antinociception from nicotinic stimulation in the spinal cord could be caused by a noradrenergic mechanism. Finally, in the course of these studies, we noted that the intensity of the lamp that generated radiant heat to stimulate the paw needed to be reduced in females to obtain the same baseline latency to paw withdrawal as the males. We therefore studied the time/temperature relationship in the subcutaneous tissue of the hind paw in males and females to determine if this reduced lamp intensity in the females resulted in reduced noxious heat stimulus in the tissues surrounding the nociceptors.

Materials and Methods

After obtaining approval by the Animal Care and Use Committee of Wake Forest University School of Medicine, male (250–300 g) and female (200–250 g) Sprague Dawley rats (Harlan, Indianapolis, IN) aged 10 weeks were used for the study. We did not assess or influence the hormonal cycle of the female rats in this study. At least 5 days before experimentation, animals were anesthetized, and a polyethylene catheter was inserted through a nick in the cisternal membrane and advanced 8.5 cm such that its tip lay in the lumbar intrathecal space as previously described.[16] Rats that showed neurologic deficits after awakening were killed immediately. To confirm correct placement of the catheters, 10 μl of 2% lidocaine were injected, followed by a 10-μl saline flush the day after surgery. Only animals that developed transient bilateral motor and sensory blockade in the hind legs were included in the study. After implantation of the intrathecal catheters, rats were housed individually with free access to food and water and allowed to recover for 5 days before use. Each animal was studied twice in an experimental series, with a 4–6-day interval between studies. Animals were exposed to either one or two drugs.

Nociceptive Testing

To assess antinociception from an acute thermal stimulus, a commercially available paw thermal stimulator was used (Anesthesiology Research Laboratory, Department of Anesthesiology, University of California, San Diego, CA). This device has been previously described[17] and is based on the initial work of Hargreaves *et al.*[18] Briefly, rats were placed in Plexiglas cubicles (San Diego Plastics, Inc., San Diego, CA) on a raised floor of glass maintained at 30°C. After 30 min, when the animals became habituated to the environment, a radiant heat source was focused on the plantar surface of the hind paw. The stimulus intensity was controlled by a constant voltage source and was adjusted so that baseline latency to paw withdrawal from the heat source was 10–15 s. Both paws were tested in random order 1–2 min apart, and the average of their values was calculated. A 30-s cutoff was used to limit possible tissue damage after exposure to the stimulus. Six to 12 animals were tested in each experiment.

Experimental Paradigm

Before testing, intrathecal catheters were connected to polyethylene 10 tubing, and syringes were prefilled with all drugs to be administered during the study. Rats were then placed in the plastic box on the thermal testing device and habituated to the testing environment, and baseline hind paw withdrawal latencies were obtained. Cumulative doses of neostigmine (either 0.1, 0.3, 0.7, and 1.0 μg or 1, 3, 7, and 12 μg) and bethanechol (10, 30, 100, and 300 μg) were administered at 15-min intervals, and hind paw withdrawal latencies were obtained at 10 and 15 min after each dose. If episodic behavioral side effects (*e.g.*, grooming, urination) precluded assessment at both 10 and 15 min, then only one value was used; otherwise we used the average of the two determinations. Single doses of the nicotinic agonist RJR-2403 [(E)-*N*-methyl-4-(3-pyridinyl)-3-butene-1-amine fumarate] were injected, and hind paw withdrawal latencies were determined at 10 and 15 min after injection.

The effect of the antagonists (atropine 10 μg, mecamylamine 10 μg, phentolamine 30 μg, mecamylamine plus atropine, saline control) was determined for neostigmine in male and female rats. In these experiments, antagonists were injected intrathecally, followed 15 min later by neostigmine 1 μg. To examine the pharmacologic pathways involved in RJR-2403–induced antinociception, female rats were injected with an antagonist (atropine 10 μg, mecamylamine 10 μg, phentolamine 30 μg, saline control), followed by the just

maximally effective dose of RJR-2403 (20 μg) after 15 min. In all antagonist experiments, response latencies were determined before and every 5 min thereafter for a total of 60 min. Values obtained at 10 and 15 min after the agonist were averaged for the value of the antagonist effect on the agonist. Separate experiments examined the effects of antagonists alone. To assess analgesic effects of chronic intrathecal exposure of RJR-2403, antinociception was assessed in male and female rats before and after a 2-week exposure to RJR-2403 (20 μg three times per week).

To examine for differences in heating characteristics of male and female paws, six male and five female rats were tested to determine latency to withdrawal with lamp intensity set at 5.0 and 5.25 A. Animals were then anesthetized with intraperitoneal pentobarbital and positioned in the paw-heating device. A 32-gauge, calibrated thermister was inserted in the hind paw such that its sensing tip was located subcutaneously to the plantar paw surface at the midphalangeal level. Thermister temperature was recorded at 100-ms intervals before and for 30 s after initiating heating with lamp intensity at 5.0 and 5.25 A.

Intrathecal Drugs and Injection

The following drugs were used in the study: neostigmine methylsulfate (Gensia, Irvine, CA), bethanechol chloride (Research Biochemicals International, Natick, MA), RJR 2403 (RJ Reynolds Tobacco Co., Winston-Salem, NC), atropine sulfate, mecamylamine, and phentolamine methaneosulfonate salt (Sigma, St. Louis, MO). All drugs were dissolved in normal saline and were injected intrathecally in a volume of 5 μl over 30 s followed by a 10-μl saline flush. Each drug and dose was tested in six animals, with the exception of the 1-μg neostigmine dose, which represents 12 animals, because this dose overlapped the high- and low-dose-range sets.

Statistics

Data are presented as mean ± SEM. Because a cutoff value was used, analgesic data in rats were converted to percent maximum possible effect according to the formula:

$$\text{percent maximum possible effect} = (\text{observed} - \text{baseline})/(30\text{ s} - \text{baseline}) \times 100$$

where the maximum effect is 30 s. All data were normally distributed. ED_{50} was calculated by linear regression. Note that for neostigmine, two different cumulative dose ranges were used. An ED_{50} was calculated only in rats that had responses crossing the 50% maximum possible effect value. Data were analyzed by one- and two-way analysis of variance followed by Dunnett's test or Scheffé's test, as appropriate, with $P < 0.05$ considered significant.

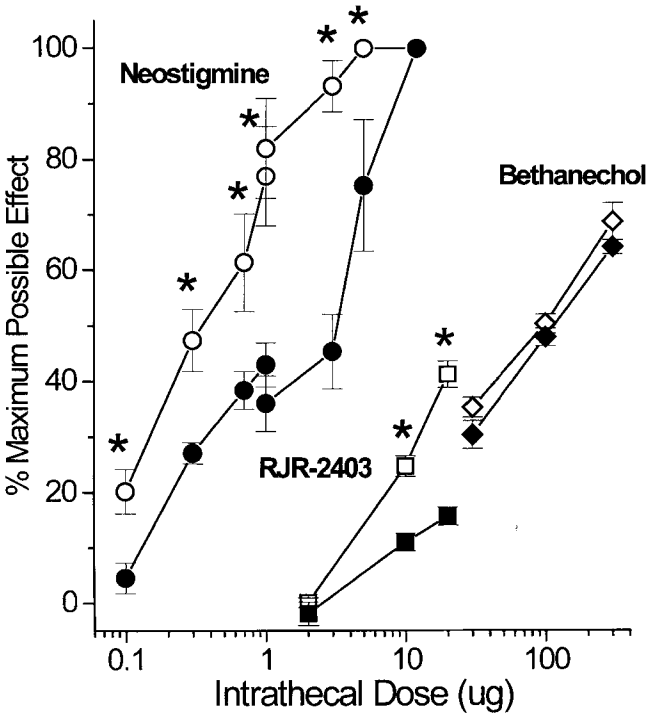

Fig. 1. Dose-dependent analgesic response of female (*open symbols*) and male (*closed symbols*) receiving intrathecal neostigmine (*circles*), the muscarinic agonist bethanechol (*diamonds*), or the neuronal nicotinic agonist RJR-2403 (*squares*). Values shown represent the mean ± SEM of observations at 10 and 15 min after injection and are expressed as percent maximum possible effect. *$P < 0.05$ compared with males by two-way analysis of variance.

Results

Intrathecal injection of neostigmine resulted in a dose-dependent increase in thermal response latency in male and female rats. In female rats, however, the antinociceptive potency of intrathecal neostigmine was five times greater than in the male rats (fig. 1; ED_{50}, 3.3 ± 0.4 μg in males *vs.* 0.57 ± 0.15 μg in females). The muscarinic agonist bethanechol also induced a dose-dependent increase in thermal paw withdrawal latency (fig. 1), but antinociception was not different between male and female rats at the doses used. In contrast, intrathecal injection of the nicotinic acetylcholine receptor

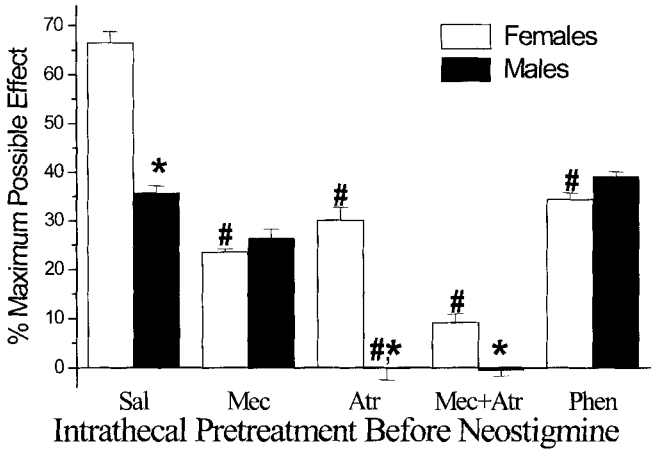

Fig. 2. Effect of pretreatment administered spinally 15 min before 1 μg spinal neostigmine on antinociception in female (*open bars*) and male (*solid bars*) rats. Pretreatment consisted of saline (Sal), mecamylamine (Mec; 10 μg), atropine (Atr; 10 μg), Mec plus Atr, or phentolamine (Phen; 30 μg). Values shown represent the mean ± SEM. Antinociception is expressed as percent maximum possible effect. *$P < 0.05$ compared with males; #$P < 0.05$ compared with saline pretreatment by analysis of variance.

(nAChR) agonist RJR-2403, which is highly selective for the $\alpha4\beta2$ nAChR subtype,[19] resulted in antinociception that was approximately twice as great in females as in males (fig. 1). Larger doses of RJR-2403 could not be examined because of severe agitation behavior observed in pilot experiments. Duration of antinociception was limited to 15–20 min with all doses.

Antagonist studies were performed to determine the pharmacologic mechanism for this greater analgesic effect in females. Antagonists alone had no effect on baseline withdrawal latency. Intrathecal pretreatment with the nAChR antagonist mecamylamine did not attenuate antinociception from intrathecal neostigmine in male rats, whereas it significantly reduced antinociception in female rats (fig. 2). As indicated, the degree of antinociception from 1 μg neostigmine, although significantly different between male and female rats (fig. 1), became equal after mecamylamine pretreatment (fig. 2) in male and female rats. Although intrathecal pretreatment with 10 μg of the muscarinic antagonist atropine completely blocked the antinociceptive effect of the 1-μg dose of neostigmine in male rats, the same dose significantly reduced but did not abolish antinociception in female rats. Pretreatment with mecamylamine and atropine reduced antinociception significantly in females. Intrathecal phentolamine pretreatment significantly reduced antinociception from intrathecal neostigmine in females but failed to attenuate the neostigmine-induced increase in response latencies in males (fig. 2).

Further characterization studies were performed in female rats that received RJR-2403. Pretreatment with saline or atropine did not affect antinociception elicited by RJR-2403, whereas mecamylamine significantly reduced RJR-2403's antinociception compared with the saline control (fig. 3, left panel). As with neostigmine, antinociception from RJR-2403 was significantly attenuated in female rats by intrathecal pretreatment with phentolamine (fig. 3, left panel).

Chronic exposure to nicotine or nAChR agonists can paradoxically increase the number of nAChR receptors and the overall response to exposure to agonist.[20] Chronic exposure to RJR-2403 by repeated intrathecal injection for 2 weeks resulted in an increased antinociception in males but not in females (fig. 3, right panel).

Side effects from intrathecal injections did not differ between male and female rats. Intrathecal neostigmine produced scratching, tail grooming, licking behavior, and sedation at doses ≥ 3 μg, and motor impairment at 12 μg. Similar effects, but not motor impairment, were observed with intrathecal bethanechol at doses ≥ 100

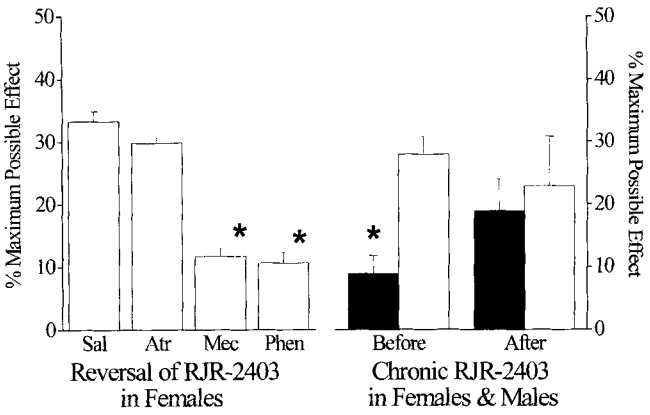

Fig. 3. (*Left*) Reversal of the effect of 20 μg spinal RJR-2403 by pretreatment (15 min before RJR-2403) with spinal injection of saline (Sal), the muscarinic antagonist atropine (Atr; 10 μg), the nicotinic antagonist mecamylamine (Mec; 10 μg), or the α-adrenergic antagonist phentolamine (Phen; 30 μg) in female rats. Values shown represent the mean ± SEM and are expressed as percent maximum possible effect at the time of peak effect (15 min after injection) in response to a noxious heat stimulus to the hind paw. *$P < 0.05$ compared with saline control by analysis of variance. (*Right*) Effect of chronic spinal exposure to RJR-2403 on antinociceptive effect to 20 μg spinal RJR-2403 in female (*open bars*) and male (*solid bars*) rats. Values shown represent the mean ± SEM. Antinociception was assessed before and after a 2-week exposure to RJR-2403 (20 μg three times per week) and is expressed as percent maximum possible effect. *$P < 0.05$ compared with females by two-way analysis of variance.

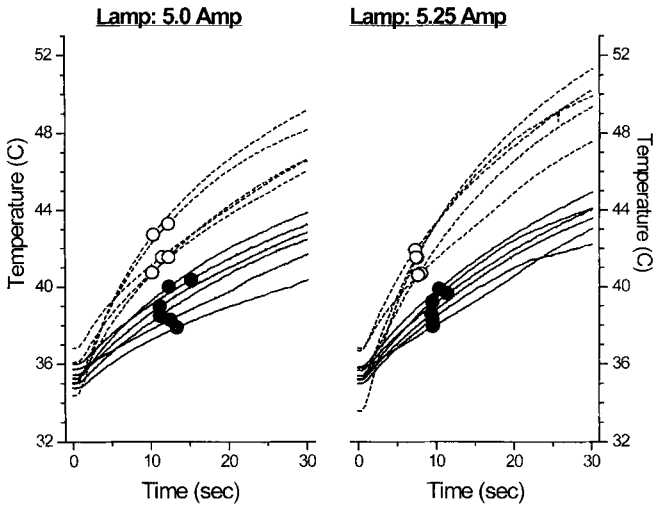

Fig. 4. Temperature in hind-paw subcutaneous tissue of female (*dotted line*) and male (*sold line*) anesthetized rats in the thermal testing device with lamp intensity set at 5.0 A (*left*) or 5.25 A (*right*). Each line represents the average of two determinations in one animal. For each lamp intensity, females differ from males by two-way analysis of variance ($P < 0.05$). Open symbols represent the time of withdrawal of the hind paw in that female animal when tested in the conscious state at that lamp intensity. Solid symbols represent the same for male animals.

μg. RJR-2403 at doses ≥ 10 μg caused intense grooming, rearing, turning around, and agitation. Atropine abolished the grooming but failed to alter any other side effects elicited by RJR-2403. As previously noted,[21] atropine pretreatment followed by neostigmine resulted in severe agitation, spontaneous vocalization, irritability to light touch, and abnormal posturing (back arched with weight bearing on front paws). This was not observed with mecamylamine or phentolamine pretreatment.

Withdrawal latency was shorter in female than male rats at both the 5.0-A lamp intensity (11.3 ± 0.51 s *vs.* 12.6 ± 0.67 s, respectively; $P < 0.05$) and at the 5.25-A lamp intensity (7.62 ± 0.14 s *vs* 9.97 ± 0.36 s, respectively; $P < 0.05$). Increasing the lamp intensity significantly reduced latency to withdrawal in both sexes. Subcutaneous temperature in the paw was similar in male and female rats when resting on the temperature-controlled surface of the testing device (fig. 4, time 0). At both lamp intensities, subcutaneous paw temperature increased more rapidly in females than in males (fig. 4). The symbols in figure 4 represent the withdrawal time for each animal when tested in the conscious state before the temperature experiment at each lamp intensity. Mean temperature at time of withdrawal was greater for females than males at both the 5.0-A intensity (42.0 ± 0.51°C *vs.* 39.0 ± 0.44°C; $P < 0.05$) and at the 5.25-A intensity (41.3 ± 0.29°C *vs.* 38.9 ± 0.35°C; $P < 0.05$). Although latency to withdrawal was affected by lamp intensity, subcutaneous temperature at the time of withdrawal was not for either sex.

Discussion

There is no precedent for this large increase in potency of an analgesic in females. In rats, opioids are more potent in males than in females, although this depends on the behavioral assay used,[22] whereas in humans there seem to be minor or no differences between the sexes in dose response or efficacy of μ-preferring opioids. In rats, pregnancy is associated with an increase in pain threshold caused by increased κ-opioid activity in the spinal cord,[23] although in humans the relevance of this observation has been questioned.[24] κ-Opioids have been recently demonstrated to produce greater analgesia in women than in men experiencing moderate pain (wisdom tooth extraction), although the difference in that study was in duration, not intensity of analgesia, suggesting that pharmacokinetic differences may play a role.[25] In contrast, retrospective review of the time course of 100 μg intrathecal neostigmine in male volunteers and female volunteers [1,3] and in postoperative patients[7] demonstrates an increase in both potency and duration in females. Intrathecal sampling in 10 of the volunteers in the phase I trial of intrathecal neostigmine[1] demonstrated no difference between the sexes in pharmacokinetics of intrathecal neostigmine in cerebrospinal fluid. Although these suggestive data are being formally tested in a randomized, blinded clinical trial, we began examination in these laboratory studies of a basis for such a pharmacodynamic sex difference.

Intrathecal neostigmine was clearly more potent in normal female than male rats in the current study. Several explanations are possible. Female animals were smaller than males and therefore received a larger dose on a weight-adjusted basis than did males. However, there is minimal evidence in adult humans for a correlation between weight and response to intrathecally administered drugs.[26] Lamp intensity was decreased in females to obtain the same baseline latency, and increased apparent potency of neostigmine may have simply reflected a smaller stimulus. This was not supported by the difference in some drugs (neostigmine, RJR-2403), but not in others (bethanechol) and in the differing pharmacology of reversal observed. In addition, studies

in anesthetized animals suggest that the female paw heats more quickly than the male paw, and that if anything, a greater stimulus was present in the female than in the male, as reflected in higher temperatures at the time of withdrawal. Although one could argue that sex differences could reflect unstable responses during repeated testing, previous studies with the same repeated testing[27] showed an SD over time of $< 10\%$ maximum possible effect during a 90-min period. We believe that the difference in potency to intrathecal neostigmine most likely reflects a true sex-related pharmacodynamic difference.

Recently, nAChR agonists have been demonstrated to produce antinociception after systemic administration in a wide variety of acute and chronic pain models in rats.[14] At the spinal level, however, analgesia from cholinergic agents has been traditionally considered to reflect actions on muscarinic receptors, although previous studies were all performed in male rats.[13] We confirmed that intrathecal injection of a muscarinic agonist produces antinociception, with no difference in dose–response curves between male and female animals. Thus, the large sex difference in response to the cholinesterase inhibitor is not a result of actions of acetylcholine at muscarinic receptors.

Of the subunits that can form subtypes of nAChRs, the most prevalent in spinal cord show binding properties of an $\alpha 4\beta 2$-containing receptor.[28] Intrathecal injection of a highly selective agonist for the $\alpha 4\beta 2$ nAChR[19] produced significantly greater antinociception in female than in male rats. Indeed, only at the highest-tolerated dose was there any antinociception in male rats, which was rather small (15% maximum possible effect). Antinociception from RJR-2403 was diminished by pretreatment with a nicotinic but not a muscarinic antagonist, confirming its specificity. These data suggest that there is a nicotinic component of cholinergic analgesia at the spinal level that is more important in females than in males.

To test further the relevance of a nicotinic mechanism for the sex difference in antinociception, a chronic exposure experiment was performed. Previous work has demonstrated a sex difference in the paradoxic increase in nAChR number and response to agonist from chronic nAChR agonist exposure in the brain: male rats have fewer brain nAChRs and a smaller response to acute nAChR agonist exposure in a learning/memory task than do females. With chronic exposure to a nAChR agonist, there is an increase in nAChR number and behavioral response in males, but no change in females.[29] We reasoned that a similar sex difference in response to chronic nAChR agonist exposure might also occur at the spinal level. A 2-week exposure to repeated intrathecal injection of RJR-2403 resulted in increased antinociception in males but not females, supporting this hypothesis. These results suggest that analgesia from nAChR agonists may be sustained with prolonged treatment in women and that they may be enhanced with prolonged treatment in men.

The aforementioned studies with agonists suggest that the sex difference in analgesia could result from a difference in nicotinic but not muscarinic mechanisms in the spinal cord, and this hypothesis was confirmed by studies with antagonists. Thus, intrathecal injection of an nAChR antagonist did not affect antinociception from intrathecal neostigmine in male rats but reduced antinociception in female rats, abolishing the sex difference. This lack of sex difference in the presence of a nicotinic antagonist is consistent with the aforementioned data showing no sex difference with intrathecal injection of a pure muscarinic agonist, bethanechol, and suggests that the importance of the muscarinic component to antinociception is similar between the sexes. However, this muscarinic component acts alone in males, because a muscarinic antagonist completely abolishes antinociception from intrathecal neostigmine in males, whereas only combined muscarinic and nicotinic antagonism abolishes antinociception in females. A nicotinic component is capable of being induced in males, as evidenced by the chronic exposure experiment, but must be minor under normal conditions in the absence of such stimulation.

We also present evidence that the female-specific nicotinic component of analgesia acts in part *via* stimulation of spinal norepinephrine release, which produces analgesia by an α-adrenergic mechanism. Thus, pretreatment with phentolamine had no effect on antinociception from intrathecal neostigmine in male rats but inhibited neostigmine's antinociception in female rats. Similarly, antinociception from intrathecal RJR-2403 was inhibited by phentolamine in female rats. It is unlikely that the difference in response to neostigmine represents a difference in sensitivity to neostigmine-stimulated norepinephrine release, because phentolamine had no effect on neostigmine's antinociception in males. In addition, analgesia from epidural injection of the α_2-adrenergic agonist, clonidine, does not differ in terms of dose–response between men and women.[30]

These data suggest that spinally released acetylcholine produces analgesia in males *via* stimulation of muscarinic receptors, whereas in females, analgesia is the result of an interaction between muscarinic receptor activation and

nicotinic receptor–stimulated norepinephrine release. This accounts for the increased potency of intrathecal neostigmine in female rats and may underlie the mechanism for neostigmine's greater potency in women.

Studies in rats did not predict the presence of nausea, the therapy-limiting side effect of intrathecal neostigmine in humans. Similarly, the agitation behavior produced by spinal nicotinic stimulation in rats could be predictive of pain or dysesthesia on injection. Therefore, the ultimate clinical utility of these observations in animals is uncertain. However, these studies carry two implications for future research. First, the multiple selective $\alpha4\beta2$ nAChR agonists under preclinical and clinical development for the treatment of cognitive and other neurodegenerative disorders may be potent analgesics after intrathecal and systemic administration in women. Second, many chronic neuropathic pain syndromes are more common in women than men,[31] and traditional agents such as opioids may be ineffective to treat this type of pain.[32] Intrathecal injection of α_2-adrenergic agonists is effective in animal models of neuropathic pain that are resistant to opioids.[33] Epidural injection of the α_2-adrenergic agonist clonidine produces pain relief in patients with neuropathic pain who have experienced no relief with opioid therapy,[34] but also produces unwanted sedation and hypotension. The noradrenergic interaction from spinal cholinergic activation in females demonstrated in this study suggests that such nAChR agonists may provide a novel and important therapy for neuropathic pain syndromes in women at lower doses than those necessary in men. The companion article in this issue by Lavan'homme and Eisenach[35] tested this hypothesis in rat models of allodynia.

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