Intrathecal Neostigmine, but Not Sympathectomy, Relieves Mechanical Alldynia in a Rat Model of Neuropathic Pain

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Introduction: Pain resulting from a usually nonpainful stimulus (alldynia) is a common characteristic of neuropathic pain. Among animal models of alldynia, tight ligation of lumbar spinal nerves has been of special interest because it has been reported to be relieved by sympathectomy. The purpose of this study was to determine whether spinal analgesic agents, which have opposite effects on sympathetic nervous system activity (clonidine decreases it and neostigmine increases it), have differing efficacy in this model.

Methods: Male Sprague-Dawley rats were anesthetized, and the left L5 and L6 spinal nerves were ligated. At least 2 weeks later, a lumbar intrathecal or jugular intravenous catheter was inserted. Withdrawal threshold to mechanical stimulation of the hind paw was determined by application of von Frey filaments before surgery; after surgery; after intrathecal injection of clonidine, neostigmine, or their combination; after intravenous injection of phentolamine or guanethidine; and after surgical sympathectomy.

Results: Spinal nerve ligation reduced withdrawal threshold ipsilateral to the lesion. This alldynia was relieved by clonidine (50% block of alldynia at 20 ± 1.2 μg) and neostigmine (50% block of alldynia at 2 ± 0.1 μg) and they interacted synergistically to block alldynia. Neither chemical nor surgical sympathectomy altered alldynia.

Discussion: These results disagree with previous observations that mechanical allodynia in this animal model depends on sympathetic nervous system activity. Therefore, intrathecally administered analgesic agents, one that reduces sympathetic outflow from the spinal cord (clonidine) and one that increases it (neostigmine), were similarly effective in this model. (Key words: α₂-Adrenergic agonist; analgesia; clonidine.)

THREE characteristics of chronic pain result in failure of therapy and have promoted considerable fundamental research into their mechanism(s) and treatments. First is the development of altered responses to sensory stimuli—exaggerated pain to a painful stimulus (hyperalgesia) and pain to a normally nonpainful stimulus (alldynia). Alldynia to light touch is a common and distressing complaint in many patients with chronic pain and is considered a characteristic of neuropathic pain. Second is the reliance of pain and alldynia in many patients on activity of the sympathetic nervous system, because sympathetic blockade often leads to pain relief. Several animal models have been developed recently with the goal of reproducing neuropathic pain characteristics, including a reliance on sympathetic nervous system activity. Among these, spinal nerve ligation in rats produces clear and stable mechanical alldynia, which has been demonstrated to be relieved by sympatholytic treatments.

A third aspect of chronic pain is a loss of sensitivity to opioid agents such that they are either ineffective or limited by side effects at large doses. For this reason, alternative, nonopioid analgesic agents have been sought. Among these, intraspinal injection of clonidine, an α₂-adrenergic agonist, has been reported to relieve neuropathic pain in animal models and in patients with intractable cancer pain and regional complex pain syndrome type 1. One explanation for this efficacy may lie in the reduction of sympathetic outflow from the spinal cord produced by intraspinal clonidine. α₂-Adrenergic agonists are thought to produce analgesia in part via stimulation of spinal cholinergic neurons, and intrathecally administered neostigmine has been examined recently in humans and demonstrated to produce analgesia to experimental pain in volunteers, postoperative patients, and patients with chronic pain.
This study further examined the effects of intrathecal injection of clonidine and neostigmine in the spinal nerve ligation model of mechanical allodynia. Both drugs produce analgesia to an acute noxious stimulus\textsuperscript{15,16}; however, they have opposite effects on sympathetic outflow, because neostigmine increases sympathetic outflow.\textsuperscript{15} and we reasoned that the potency of neostigmine should be greatly diminished in this spinal nerve ligation model. Our initial results of increased potency of intrathecally injected neostigmine and its combination with clonidine in this model led us to further examine the sympathetic nervous system dependency of this model, using chemical and surgical sympa-tholytic approaches.

Materials and Methods

Animal Preparation

After approval by the Animal Care and Use Committee, 25 male Sprague-Dawley rats (Harlan, Indianapolis, IN) weighing 150–200 g underwent spinal nerve ligation during halothane-induced anesthesia as described by Kim and Chung.\textsuperscript{16} The L5 and L6 spinal nerves were isolated adjacent to the vertebral column and tightly ligated with 6-0 silk sutures distal to the dorsal root ganglion. After surgery, the animals were housed individually with free access to food and water and allowed to recover for at least 1 week before study. The first 14 rats were anesthetized at least 2 weeks later with halothane, and an intrathecal catheter (PE-10 tubing) was inserted through a small hole in the cisterna magna and advanced caudad 8 cm such that the tip lay in the intrathecal space around the lumbar enlargement.\textsuperscript{17} Rats showing neurologic deficits were killed immediately with an overdose of pentobarbital. Experiments were performed in these animals between 1 and 2 weeks after implantation of the intrathecal catheter. The second 11 rats were anesthetized with halothane at least 2 weeks after spinal nerve ligation surgery, and a PE-10 catheter was inserted in the jugular vein. At least 2 days after experiments with phenolamine and guanethidine (described later), these rats were anesthetized with halothane, and a surgical sympathectomy was performed as previously described.\textsuperscript{17} Using the renal artery as a landmark for the L2 ganglion, the left sympathetic chain was identified and cut from the L2 to the L6 level, located close to the bifurcation of the descending aorta. Experiments were performed in these animals on the day after surgery and again 1 week later.

Withdrawal Threshold Testing

Rats were placed in individual plastic cages on a plastic mesh bottom floor, which permitted access to their hind paw. After accommodation to the cage, mechanical threshold was assessed by application of standard von Frey filaments (Stoelting, Wood Dale, IL). Each filament was applied perpendicularly to the paw for 5–6 s. Brisk withdrawal or paw flinching were considered as positive responses, in which case the next filament tested was the next lower force. In the absence of such responses, the next filament tested was the next greater force. Withdrawal threshold was calculated as described by Chaplan et al.\textsuperscript{18} using Dixon's nonparametric test.

Experimental Groups

First, the effect of neostigmine and clonidine were determined. In these studies, rats received intrathecal injections of neostigmine (1, 2, 7, and 10-μg cumulative doses; n = 7), clonidine (15, 30, and 45-μg cumulative doses; n = 5), or saline (n = 6) at 15-min intervals, with withdrawal thresholds determined before and after administration of drug or saline.

Combination studies also were performed to test the hypothesis that neostigmine might counteract the effect of clonidine on reduction in allodynia, because these agents have opposing actions on activity of the sympathetic nervous system. In these studies, the effect of cumulative doses of clonidine and neostigmine (in a 10:1 ratio of clonidine:neostigmine; n = 6) on withdrawal threshold was determined. Experiments were separated by a minimum of 3 days.

Next, the effects of sympatholytic treatments were determined in separate animals. In these studies, rats received an intravenous injection of phenolamine (4 mg/kg), guanethidine (30 mg/kg), or saline, and withdrawal thresholds were determined before and 10, 30, 60, 120, and 240 min after intravenous injection (n = 11 in each treatment). Experiments were separated by a minimum of 4 days. These rats then underwent surgical sympathectomy, and withdrawal thresholds were tested 1 and 7 days after surgery. The acute effects of intravenously administered phenolamine and guanethidine on arterial blood pressure was determined in a separate group of lightly anesthetized rats without spinal nerve ligation surgery.

Drugs

Intrathecal injections were performed in a 5-μl volume followed by a 10-μl flush with saline, and intrave-
ous injections were performed in a 0.3-ml volume followed by a 0.3-ml saline flush. All drugs were dissolved in saline for injection. Clonidine was obtained from Boehringer Ingelheim (Ridgefield, CT). Guanethidine monosulfate, neostigmine bromide, and phentolamine hydrochloride were obtained from Sigma Chemical Co. (St. Louis, MO).

Statistics
Data are presented as median (25th–75th percentiles) or mean (± SD) as appropriate. Effect of drug or surgical treatment was determined by a nonparametric one-way repeated-measures analysis of variance. Dose–response data were analyzed for 50% maximal effect using linear regression for each animal. Isobolographic analysis was performed as previously described. A probability value <0.05 was considered significant.

Results
Paw withdrawal threshold decreased from a median of 40.5 g (28.3–46.5 g) before surgery to a median of 2.8 g (1.9–4.2 g) after spinal nerve ligation. Intrathecal injection of saline did not affect withdrawal threshold in rats after spinal nerve ligation (fig. 1). Intrathecally administered clonidine produced a dose-dependent increase in withdrawal threshold after spinal nerve ligation surgery, returning withdrawal threshold to presurgery levels at a cumulative dose of 30–45 μg (fig. 2, bottom). The dose of clonidine that produced a 50% return to presurgery withdrawal threshold was 20 ± 3.1 μg. In doses >15 μg, clonidine produced sedation and diuresis, as has been described previously.20

Intrathecally administered neostigmine also produced a dose-dependent increase in withdrawal threshold after spinal nerve ligation surgery, returning withdrawal threshold to presurgery levels at a cumulative dose of 2–7 μg (fig. 2, top). The dose of neostigmine that produced a 50% return to presurgery withdrawal threshold was 2.0 ± 0.34 μg. In doses >2 μg, neostigmine produced licking and grooming behavior, shaking, and biting of the tail, as has been described previously after intrathecal injection of cholinesterase inhibitors.21 These agitation behaviors were followed in 30 min by sedation and urination in most animals.

Combination of neostigmine and clonidine in a constant ratio of the 50% maximum antiallodynic effect also resulted in a dose-dependent reversal of allodynia (fig. 3, top). The combination dose producing a 50% return to presurgery withdrawal threshold was 4.3 ± 1.7 μg. Isobolographic analysis revealed this interaction to represent an enhancement that was synergistic (fig. 3, bottom), rather than the hypothesized antagonism. The observed dose of clonidine plus neostigmine required to produce a 50% return to presurgery withdrawal threshold was significantly less (39%) than that calculated as necessary for a purely additive interaction. Agitation was not observed with the neostigmine–clonidine mixture.

Intravenous injection of phentolamine, guanethidine, or saline had no effect on withdrawal threshold after spinal nerve ligation (fig. 4). In anesthetized rats, phentolamine decreased mean arterial blood pressure from 87 ± 6 to 42 ± 3 mmHg (n = 3), and guanethidine decreased mean arterial blood pressure from 84 ± 5 to 30 ± 3 mmHg. Surgical sympathectomy also failed to alter withdrawal threshold (fig. 5), either in the immedi-

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either the etiology, characteristics, or responsiveness of subgroups of patients with chronic pain. One assumes that the closer the model approximates the clinical characteristics, the more likely it is that understanding the pathophysiology and pharmacology of the model will lead to a better understanding and treatment of patients.

Considerable effort has been expended on study of the spinal nerve ligation rat model, not because of its

tate postoperative period (24 h after surgery) or 1 week later.

Discussion

Patients with chronic pain vary considerably in the etiologies of their pain (e.g., mechanical, ischemic or chemical peripheral or central nerve injury), the characteristics of their pain (e.g., ongoing, movement evoked, thermal or mechanical hyperalgesia or allodynia), and their responsiveness to treatment (e.g., opioid agents, $\alpha_2$-adrenergic agonists, sympathectomy). Several animal models have been developed in an attempt to mimic

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etiology (surgical ligation of spinal nerves) but because of its mechanical allodynia, response to sympathetic, and poor response to intrathecally administered opioid agents.\textsuperscript{2,22} Its usefulness as a model depends on these characteristics, which are present in many patients with chronic pain.

Intrathecal or epidural injection of clonidine produces relief of pain in placebo-controlled trials in patients with neuropathic cancer pain and in patients with reflex sympathetic dystrophy.\textsuperscript{6,8} The results of the current study agree with previous results demonstrating dose-dependent reductions in tactile alldynia from intrathecally administered clonidine in rats after spinal nerve ligation surgery.\textsuperscript{5} The apparent lesser potency of clonidine in the current study (effective dose in 50% of subjects (ED\textsubscript{50}), 20 \mu g) compared with the previous one (ED\textsubscript{50}, 3 \mu g) can be explained by the use of a low cutoff point (15 g) in the previous study.\textsuperscript{5}

Intrathecal injection of neostigmine produces pain relief to acute noxious stimulation in humans and rats\textsuperscript{10,14} and relieves allodynia after spinal nerve ligation in rats.\textsuperscript{23} Although it has been recognized recently that stimulation of the nicotinic cholinergic receptor produces antinociception,\textsuperscript{24} the antiallodynic effect of neostigmine is attributable to muscarinic stimulation.\textsuperscript{23} Intrathecally administered neostigmine enhances analgesia from clonidine in rats and humans.\textsuperscript{14,25} Unlike clonidine, however, intrathecally administered neostigmine increases sympathetic efferent outflow.\textsuperscript{15} We reasoned, therefore, that the potency of neostigmine alone would be reduced in rats after spinal nerve ligation surgery and that it would be less effective at enhancing the antiallodynic effect of clonidine or perhaps might even antagonize the effect of clonidine. Just the opposite was observed — the potency of neostigmine in rats after spinal nerve ligation surgery in the current study (ED\textsubscript{50}, 2 \mu g) was similar to that observed previously for thermal antinociception in normal animals (ED\textsubscript{50}, 1.2 \mu g).\textsuperscript{14} In addition, neostigmine and clonidine still interacted synergistically in rats after spinal nerve ligation surgery, with a combination dose of only 39% of the theoretical additive compared with 58% of the theoretical additive for thermal antinociception in

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Fig. 4. Withdrawal threshold to stimulation by von Frey filaments before surgery (filled circles); after spinal nerve ligation (SNL); and for 240 min after intravenous injection of saline (filled squares), phenotamine (open circles), or guanethidine (open triangles). Data expressed as median ± 25th and 75th percentiles of 11 rats. No effect of time was found for any of the intravenous treatment groups.

Fig. 5. Withdrawal threshold to stimulation by von Frey filaments before surgery (Base), after spinal nerve ligation (SNL), and 1 day (P-Sx Day 1) or 7 days (P-Sx Day 7) after surgical sympathectomy. Data are expressed as median ± 25th and 75th percentiles of 11 rats. * P < 0.05 versus baseline condition. No effect of surgical sympathectomy compared with the SNL condition was found.
normal animals.\textsuperscript{14} This synergy argues strongly against clonidine-induced sympatholysis as its primary mechanism of antiallodynia in this model and suggests that such a combination could prove useful in reducing side effects from each agent in patients with neuropathic pain.

The clinical implications of these observations are unclear for at least two reasons. First, although apparent side effects were absent with the low doses of clonidine and neostigmine used in this study, the clinically relevant side effects of neostigmine (nausea) and clonidine (hypotension) either do not occur in rats or were not evaluated in the current study. Second, the underlying assumption that allodynia in rats undergoing spinal nerve ligation was sympathetically dependent appears to be incorrect, so these results may not apply to patients with sympathetically maintained pain.

The lack of reliance of allodynia in these spinal nerve-ligated rats on sympathetic nervous system activity is puzzling. The primary pharmacologic probes, clonidine and neostigmine, have opposite effects on sympathetic outflow in rats but similar antiallodynic effects, as discussed earlier. Phentolamine and guanethidine, in doses producing hypotension and previously used in rats after spinal nerve ligation surgery,\textsuperscript{16} had no effect on allodynia, nor did surgical sympathectomy. Rat strains are reported to differ in their electrophysiologic response to nerve injury,\textsuperscript{26} but we used the same strain and vendor as in previous reports. The etiology of the discrepancy between this report and the original observations\textsuperscript{16} is therefore unresolved, although it is noteworthy that others also have failed to observe a sympathetic dependence to allodynia in this model.\textsuperscript{§}

Intrathecally administered neostigmine and clonidine each produce dose-dependent blockade of mechanical allodynia in rats after spinal nerve ligation surgery, and they interact in a synergistic manner for this effect. We were unable to reproduce experiments suggesting that this model relies on local sympathetic nervous system activity, so the relevance of these observations to patients with a sympathetic nervous system component of chronic pain is uncertain.


References

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