Plasticity in Action of Intrathecal Clonidine to Mechanical but Not Thermal Nociception after Peripheral Nerve Injury

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Background: Intrathecal clonidine reduces tactile allodynia in animal models of neuropathic pain, and this effect is blocked by atropine. However, the role of tonic spinal cholinergic activity and its interaction with α2-adrenergic systems in normal and neuropathic conditions and to different sensory methods has not been systematically examined. The authors examined cholinergic receptor involvement in thermal and mechanical sensitivity in normal and neuropathic animals and its interaction with intrathecal clonidine.

Methods: Normal rats and rats that received L5/L6 spinal nerve ligation were tested with acute radiant heat, paw pressure, and punctate mechanical stimulation before and after the intrathecal administration of saline, the muscarinic receptor antagonist, atropine, or a toxin to destroy cholinergic neurons, and then after intrathecal clonidine.

Results: Atropine, the cholinergic neuronal toxin, and saline did not alter baseline withdrawal thresholds. In nerve-injured rats, neither saline nor atropine altered antinociception from clonidine to a thermal stimulus, but atropine reduced the effect of clonidine to von Frey filament withdrawal threshold (34 ± 5.6 *vs.* 14 ± 5.8 g [mean $\pm$ SEM], saline *vs.* atropine; $P < 0.05$) and to withdrawal threshold to paw pressure after clonidine (174 ± 18 g *vs.* 137 ± 16 g, saline *vs.* atropine; $P < 0.05$).

Conclusions: These data suggest that after nerve injury, mechanical but not thermal antinociception from intrathecal clonidine relies on a muscarinic interaction, because only mechanical antinociception was antagonized by atropine. These results do not favor a regulation of nociceptive transmission by a tonic release of acetylcholine in nerve-injured rats.

α2-ADRENERGIC agonists produce behavioral analgesia both in humans and animals after intrathecal administration. Some data suggest that the α2-adrenergic agonist clonidine is more potent against neuropathic than acute nociceptive pain in humans,[1] and more effective against hypersensitivity states induced by peripheral nerve injury than against acute nociceptive stimulation in normal animals.[2] However, direct comparisons in animals by means of the same testing methods have not been performed. One goal of this study was to perform such a comparison.

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Recent studies have suggested that a spinal cholinergic system is important in spinal nociceptive modulation. Further, several indirect lines of evidence suggest that spinal α2- adrenoceptor-mediated antinociception after nerve injury from intrathecal clonidine relies on a cholinergic interaction.[3–6] Antinociception to acute noxious thermal stimulation from intrathecal clonidine is partially blocked by atropine and potentiated by physostigmine.[7] Intrathecal clonidine increases cerebrospinal fluid concentrations of acetylcholine in animals[5,6] and humans,[8] and local administration of clonidine into the spinal cord dorsal horn *via* microdialysis increases efflux of acetylcholine in the microdialysate.[6] Finally, both cholinergic muscarinic and α2 adrenergic receptors are localized within the same superficial laminae of the spinal cord dorsal horn.[9,10] In hypersensitivity states induced by peripheral nerve injury, the reduction in mechanical allodynia by intrathecal clonidine is reversed by muscarinic receptor antagonists.[3] In the model of spinal nerve ligation, the selective reduction of cholinergic cells in the lumbar spinal cord by the cholinotoxin monoethylcholine mustard aziridinium ion (AF64-A) reduces both the spinal cord content of acetylcholine and the antiallodynic effect from intrathecally administered clonidine.[4]

In normal animals, intrathecal administration of the muscarinic receptor antagonist atropine reduces the withdrawal threshold to mechanical stimulation in a dose-dependent manner without affecting response to thermal stimulation.[11] Hence, it has been suggested that in normal animals, a tonic spinal release of acetylcholine elevates the nociceptive threshold to mechanical stimulation. In contrast, intrathecal atropine has no effect on withdrawal threshold to a mechanical stimulus after nerve injury,[3] nor does destruction of spinal cholinergic interneurons alter allodynia to a mechanical stimulus in such animals.[4] However, intrathecal atropine antagonizes the effect of intrathecal clonidine to reduce tactile allodynia in nerve-injured animals.[3] Examination of the role of spinal muscarinic receptors in antinociception remains incomplete. We therefore systematically examined, in both normal and neuropathic animals, the role of tonic cholinergic receptor activity and its interaction with intrathecal clonidine to acute thermal, punctate mechanical, and deep pressure noxious stimulation. Our secondary purpose was to compare the effect of atropine on the antinociceptive effect from intrathecal clonidine in these three types of stimuli between normal and nerve-ligated animals.

Materials and Methods

Animals

After obtaining approval from the Animal Care and Use Committee of Wake Forest University School of Medicine, Winston-Salem, North Carolina, male rats (Harlan Sprague- Dawley) that weighed 150–180 g at the time of surgery were studied. Animals were housed at 22°C and under a 12 h–12 h light–dark cycle, with free access to food and water.

Surgery

All surgical procedures were performed with inhalational halothane anesthesia (1–3% in 100% oxygen). For sciatic nerve ligation (SNL) the left L5 and L6 spinal nerves were isolated under a surgical microscope and ligated tightly with 5–0 silk suture, as previously described.[12] Animals were allowed to recover for 6–8 days before intrathecal catheterization, as previously described.[13] Intrathecal catheters in both SNL and normal animals were advanced 7.5 cm caudal through an incision in the cisternal membrane and secured to the musculature at the incision site. Only animals without evidence of neurologic deficit after catheterization were studied. To confirm correct placement of the catheters, 10 μl of lidocaine, 2%, 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 limbs were included in the study.

Intrathecal Administration of Drugs

All intrathecally administered drugs were diluted in 5 μl sterile saline and injected using a hand-driven Hamilton syringe. Immediately after injection of the drug, 15 μl saline was administered to flush the dead space of the catheter. The total volume of 20 μl was administered during a period of 25–30 s.

Ethylcholine Mustard Aziridinium Ion Synthesis

The cholinergic neurotoxin AF64-A was synthesized as previously described.[14] Briefly, acetylethylcholine mustard hydrochloride (Research Biochemicals, Inc., Natick, MA) at a concentration of 0.4 nM, was brought to pH 11.5 by the addition of 10 N NaOH and maintained at this pH for 30 min while being stirred at a constant temperature of 25°C. The pH was then lowered to 7.4 with concentrated hydrochloric acid and was stirred for 20 min, and this solution was stored on ice until use (always within 1 h). As previously recommended,[15] AF64-A was always freshly prepared, and close attention was paid to temperature, pH, and acetylethylcholine mustard hydrochloride concentration to have a percentage of cyclization between 60 and 80%.

Behavioral Testing

Behavioral testing was always performed between 9 and 12 AM. The investigator involved in the behavioral testing was blinded to treatment group.

Noxious Heat. Rats were placed in individual plastic boxes on the glass surface of the testing apparatus, and were allowed to acclimate for 30 min. Paw withdrawal latency (PWL) was determined using an intense light focused on the hind paw, as previously described.[16] Animals were acclimated to the testing apparatus and procedures until stable PWLs were obtained, and the glass surface on which the animals rested was maintained at 30°C during all testing. Light intensity was adjusted so that baseline latency was between 9 and 11 s in all animals. A cutoff of 30 s was established to avoid tissue damage during periods of analgesia, but no animals reached this cutoff point.

Paw Pressure. Withdrawal threshold to paw pressure was measured using a Randall–Sellito method.[17] Rats were acclimated to the testing device on several occasions. The hind paw was inserted on a metal surface and a blunt plinth was pressed on the paw with a linearly increasing force until the animal withdrew the paw, using a commercially available device (Analgesymeter, Ugo Basile, Italy). A cutoff point of 250 g was not exceeded to avoid tissue injury, but no animals reached this cutoff point.

Punctate Allodynia. Rats were placed in individual plastic boxes on a mesh floor, which allowed access to their hind paws, and were allowed to acclimate for 30 min. A series of calibrated von Frey filaments (Stoelting Co., Wood Dale, IL) were applied perpendicularly to the plantar surface of the left hind paw with enough force to bend the filament for 6 s. 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.[18] Each trial was repeated two to three times at approximately 2-min intervals, and the mean value was used as the force to produce withdrawal responses.

Experiments

Thermal Nociception. Two approaches were used to test the cholinergic dependency of the antinociception to noxious heat from intrathecal clonidine in normal animals. First, we previously showed that the cholinotoxin AF64-A, 5 nM, nearly abolishes the effect of intrathecal clonidine in rats with SNL to withdrawal threshold to von Frey filament testing, and that this effect correlates closely with the reduction in spinal cord acetylcholine content.[4] Using this approach, PWL was determined before the intrathecal administration of the cholinotoxin AF64-A 5 nM (n = 5) or saline (n = 5).

Seven days later, paw withdrawal latency was determined before and after the cumulative administration of 5, 15, and 50 μg of intrathecal clonidine, with doses separated by 15 min. Second, other normal rats were randomly assigned to receive either a single intrathecal injection of saline (n = 6) or atropine 30 μg (n = 6) 60 min before intrathecal injection of clonidine in a dose producing a just-maximal response in this test, 50 μg. PWL was determined before and after saline or atropine pretreatment, then at 15 min intervals for 60 min after clonidine.

To test the role of spinal muscarinic receptors in the response to intrathecal clonidine after nerve injury, rats with SNL received either intrathecal saline (n = 4) or atropine 30 μg (n = 4) and 60 min later received the intrathecal administration of clonidine, 50 μg. PWL was determined before and after pretreatment and after clonidine as described for the normal animals.

Paw Pressure Nociception. Normal rats (n = 5) and rats with SNL (n = 5) were randomly pretreated with either intrathecal saline (n = 5) or atropine, 30 μg (n = 5), followed 15 min later by intrathecal clonidine in a dose producing a just-maximal response in this test, 30 μg. Clonidine-induced antinociception to paw pressure was assessed thereafter using the Randall–Selitto device every 15 min for 90 min after intrathecal clonidine administration.

Von Frey Filament-Measured Allodynia. Rats with SNL were randomly pretreated with either intrathecal saline (n = 4) or atropine, 30 μg (n = 4), and 60 min later they received intrathecal clonidine, 50 μg. Withdrawal threshold to probing with von Frey filaments was determined before saline or atropine pretreatment, 60 min later before clonidine administration, then 30 min thereafter for 90min. Normal animals were not studied with von Frey filaments.

Statistical Analysis

Data are presented as mean ± standard error (SE). Behavioral analysis comparisons were performed using t tests or ANOVA for repeated measures followed by the Bonferroni test. $P < 0.05$ was considered significant.

Results

Intrathecal administration of 5 nM AF64-A produced no effect on general behavior. All animals with SNL developed allodynia after spinal nerve ligation. Intrathecal catheterization did not affect withdrawal thresholds, which were comparable among all animals in the normal groups and among all animals in the SNL groups, although normal animals differed from SNL animals in some withdrawal thresholds, as described below.
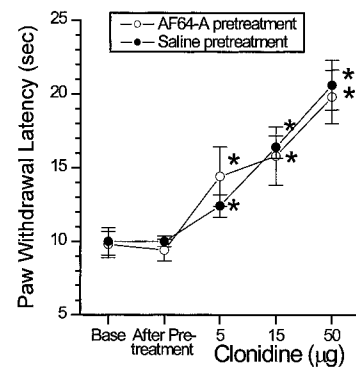

Fig. 1. Antinociception in normal animals from intrathecal clonidine to thermal stimulation in animals treated with intrathecal saline (●) or the cholinergic neuronal toxin AF64-A (○) 1 week previously. Clonidine produced dose-dependent antinociception that was similar in both groups. Each symbol represents the mean ± standard error of five animals. *$P < 0.05$ compared with after treatment.

Thermal Nociception

Intrathecal clonidine produced antinociception to noxious heat in normal animals, as evidenced by increased PWL, in a dose range of 5–50 μg (fig. 1). Pretreatment 1 week earlier with AF64-A did not affect PWL before the administration of clonidine and did not reduce clonidine's antinociception (fig. 1). Consistent with this observation, antinociception from intrathecal clonidine was unaffected by pretreatment with atropine in normal animals and the effect of clonidine was not diminished by atropine in SNL animals (fig. 2).

Two observations differed between normal and SNL animals to testing with noxious heat. First, PWL after SNL was reduced compared with normal animals (fig. 2), consistent with previous reports or thermal hypersensitivity in this model. Second, despite starting from a more sensitive baseline, the PWL after intrathecal administra-
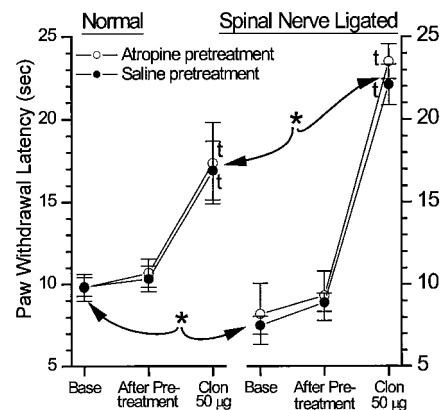

Fig. 2. Antinociception in normal animals and animals with spinal nerve ligation (SNL) from intrathecal clonidine to thermal stimulation in animals treated with intrathecal saline (●) or the muscarinic receptor antagonist atropine (○). There was no difference between saline- and atropine-treated animals in either group. Each symbol represents the mean ± standard error of four to six animals. *$P < 0.05$ between normal and spinal nerve ligated animals. t indicates $P < 0.05$ compared with preclonidine value.
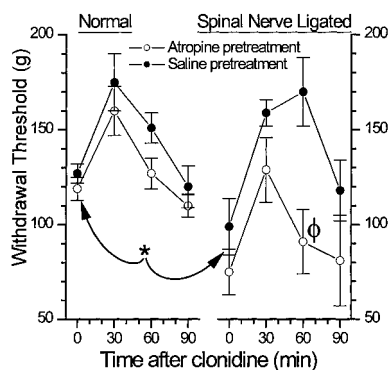
Fig. 3. Antinociception in normal animals and animals with spinal nerve ligation (SNL) from intrathecal clonidine to paw pressure stimulation in animals treated with intrathecal saline (●) or the muscarinic receptor antagonist atropine (○). There was no difference between saline and atropine treatment in normal animals, but there was in SNL animals ($\phi P < 0.05$ between saline and atropine). Withdrawal threshold was reduced by spinal nerve ligation (*$P < 0.05$ between normal and spinal nerve ligated animals). Each symbol represents the mean ± standard error of six animals.

tion of clonidine was significantly greater in SNL than in normal animals (fig. 2), consistent with an increased potency or efficacy of clonidine in this condition.

Paw Pressure Nociception

Intrathecal clonidine, 30 μg, increased withdrawal threshold to paw pressure in normal animals; as with noxious thermal testing, this was unaffected by pretreatment with intrathecal atropine in normal animals (fig. 3). As with thermal stimulation, withdrawal threshold to paw pressure mechanical stimulation was reduced by SNL injury (fig. 3). Intrathecal clonidine increased withdrawal threshold to paw pressure in SNL animals, but, unlike with thermal stimulation, this effect was significantly reduced by intrathecal atropine (fig. 3).

Von Frey–Filament–Measured Allodynia

Withdrawal threshold prior to SNL surgery was 30–40 g, which was significantly reduced at the time of testing to less than 2 g (fig. 4). As with mechanical stimulation from deep paw pressure, intrathecal clonidine increased withdrawal threshold to punctate stimulation with von Frey filaments, which was significantly reduced by pretreatment with atropine (fig. 4).

Discussion

Several observations suggest a tonic release of acetylcholine in the spinal cord to affect sensitivity to noxious input in some conditions, an increase in potency and efficacy of α2-adrenergic agonists for analgesia in chronic compared with acute pain states, and a spinal α2-adrenergic–cholinergic interaction for analgesia after nerve injury. However, most of these observations are indirect, compare one sensory method with another, or
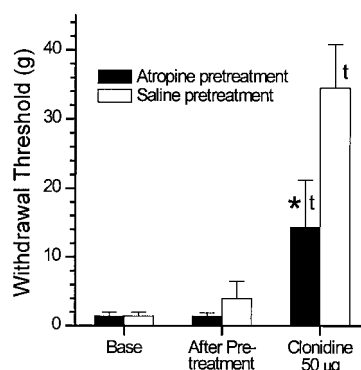

Fig. 4. Antinociception in animals with spinal nerve ligation from intrathecal clonidine to von Frey filament testing in animals treated with intrathecal saline (*filled bars*) or the muscarinic receptor antagonist atropine (*open bars*). Atropine reduced the effect of clonidine (*$P < 0.05$ compared with saline treatment). Each symbol represents the mean ± standard error of four animals. *t* indicates $P < 0.05$ compared with pre-clonidine value.

compare results from different investigators who used different methods of stimulation and drug administration. We believe the current study, which used both thermal and mechanical stimulation and the same mechanical stimulation for both normal and neuropathic conditions, fills an important gap in our knowledge and helps to clarify these issues.

Tonic Spinal Cholinergic Activity and Nociceptive Threshold

Intrathecal injection of acetylcholine itself or muscarinic receptor agonists produces antinociception to either thermal or mechanical stimulation,[19,20] and intrathecal injection of a cholinesterase inhibitor produces analgesia in humans,[21] consistent with the notion that muscarinic receptors, located primarily in the superficial dorsal horn, are inhibitory to nociceptive input. Whether these muscarinic receptors are tonically activated by acetylcholine to modulate resting pain threshold is controversial. One group reported a reduction in tonic receptor activation by intrathecal injection of the muscarinic antagonist atropine, which was shown to reduce withdrawal threshold to heat but not pressure on the rat tail,[11] an effect mediated by nitric oxide synthesis from muscarinic receptor stimulation.[22] Others, as in the current report, observed no effect of intrathecal atropine on withdrawal thresholds to radiant heat or pressure.[19] Consistent with this observation, we also noted that spinal cholinergic neuronal destruction by AF64-A failed to alter withdrawal thresholds in normal animals.

There may be several reasons for the divergent results with regard to tonic cholinergic activity and its effect on nociceptive threshold. First, Zhuo *et al.* stimulated the tail,[11,22] whereas in the current study we stimulated the hind paw, and others have shown that the pharmacology of antinociception in the spinal cord can differ between these structures.[23] Second, animals were not assessed as

early in the current study as in those of Zhuo *et al.,* in which rats were tested within minutes after the intrathecal administration of atropine.[11] In the current study, within minutes after intrathecal atropine injection the animals developed intense exploratory behavior that lasted approximately 30 min before it regressed. Therefore, we were unable to measure the withdrawal threshold during this period in our freely moving animals; however, measurement was possible when studying the tail because the animals were acutely restrained. Third, restraint itself could have induced stress-related changes, such as spinal acetylcholine release,[24] leading to the appearance of tonic activity in the study of the tail but not the hind paw. Interestingly, although acute noxious stimulation induces spinal acetylcholine release,[24] we observed no effect in this or previous studies of intrathecal atropine or AF64-A on withdrawal threshold in animals after SNL, a presumably chronic pain condition,[3,4] which suggests that this model of chronic nerve injury does not result in increased basal release of acetylcholine in the spinal cord.

Potency and Efficacy of α2-Adrenergic Agonists in Acute versus Chronic Pain

Clinical studies suggest that the dose of clonidine, either epidurally or intrathecally, required to treat chronic pain is less than that required to treat acute pain,[1,25] although direct comparisons are difficult because of a lack of matching of intensity of pain and a lack of dose response studies. Epidural clonidine is more potent and effective in reducing the area of allodynia from intradermal capsaicin injection than in reducing pain to acute noxious heat,[26] although in this case one is comparing one sensory method with another. The current study demonstrates by use of the same sensory method (radiant heat) that the effect of intrathecal clonidine is greater in SNL than in normal animals, which is remarkable because the baseline latency to withdrawal was reduced in the SNL animals. A similar increase in effect of clonidine was not observed in the paw pressure test across normal to SNL groups, although full dose responses were not obtained.

Spinal α2-Adrenergic–Cholinergic Interaction for Analgesia after Nerve Injury

The interaction between α2-adrenergic and cholinergic systems in analgesia varies with route of administration, condition, and sensory method, although up to the current investigation, systematic studies using the same stimulus across conditions have not been performed. Atropine potentiates clonidine antinociception to thermal stimulation after systemic administration,[27] partially reverses intrathecal clonidine antinociception to tail withdrawal from heated water in acutely restrained animals,[7] or has no effect on the antinociceptive effect of intrathecal clonidine in either normal or SNL

animals to noxious heat applied to the hind paw in the unrestrained animal (current study). Most previous studies have observed a reversal of intrathecal clonidine's antinociception to thermal testing by α2-adrenergic receptor antagonists but not atropine, and our data suggest that this remains true after induction of thermal hypersensitivity with peripheral nerve injury.

In contrast to this lack of cholinergic interaction to the effect of clonidine to thermal stimulation, we observed plasticity in the cholinergic interaction to the effect of clonidine to mechanical stimulation after nerve injury. As previously noted,[3,4] the antiallodynic effects of intrathecal clonidine to punctate stimulation with von Frey filaments were antagonized by intrathecal atropine. Use of this method to study normal animals is problematic, because withdrawal thresholds to this method increase in normal animals by analgesics only in doses that produced intense sedation. For this reason, we used the same stimulus (Randall–Selitto paw pressure method) to compare the reversal effects of atropine on clonidine between normal and SNL conditions. The results agree with previous reports that clonidine's antiallodynic effects to mechanical stimulation are antagonized by atropine in the nerve-injured animal. Moreover, the results support that this reflects neuroplasticity in that atropine failed to reverse the effect of clonidine effect on normal animals in this test.

The cause of this method-specific plasticity in the mechanism of antinociception from spinal α2-adrenergic receptor stimulation after nerve injury is unknown and was not the subject of the current investigation. We have, however, suggested that there is a shift in the α2-adrenergic receptor subtype activated by clonidine to reduce mechanical allodynia after nerve injury from the α2A to the α2C subtype.[28] Nerve injury reduces α2A-adrenergic receptor immunolabeling in the spinal cord but not α2C-immunolabeling, and α2C-adrenergic receptors are located in the deep dorsal horn, the normal termination of large diameter fibers that subserve mechanical input.[29] We therefore speculate that the method-specific interaction observed in the current study between α2-adrenergic and cholinergic systems reflects an increased or novel interaction in the deep dorsal horn on α2C-adrenergic receptors.

In summary, spinal cholinergic interneurons and muscarinic receptors are not involved in antinociception from intrathecal clonidine in normal rats subjected to acute noxious heat or pressure stimuli. In contrast, although spinal muscarinic receptors are also not involved in antinociception from intrathecal clonidine in SNL rats subjected to thermal stimulation, the receptors are important to clonidine's action to mechanical stimulation. These data suggest that manipulation of the α2-adrenergic mechanism of pain relief after nerve injury by cholinergic modulators may selectively affect mechanical allodynia and hyperalgesia.

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