

Peripheral Nerve Injury Alters the α_2 Adrenoceptor Subtype Activated by Clonidine for Analgesia

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Background: Previous studies suggest that the α_{2A} adrenoceptor subtype is the target for spinally administered α_2 -adrenergic agonists, i.e., clonidine, for pain relief. However, ST 91, a preferential $\alpha_{2\text{NON-A}}$ adrenoceptor subtype agonist, induces antinociception, and intrathecally administered α_{2C} antisense oligodeoxynucleotide decreases antinociception induced by clonidine in the rat, suggesting non-A sites may be important as well. Therefore, the authors examined the subtype of α_2 adrenoceptor activated by clonidine and ST 91 in normal rats and those with nerve injury–induced hypersensitivity.

Methods: The same mechanical stimulus was applied to normal rats and those following spinal nerve ligation, and the effect of intrathecal clonidine and ST 91 on withdrawal threshold to the stimulus was determined. To further examine subtypes, animals were spinally pretreated with vehicle, BRL 44408 (an α_{2A} subtype–preferring antagonist), and ARC 239 (an $\alpha_{2\text{NON-A}}$ subtype–preferring antagonist).

Results: In normal animals, clonidine's effect was diminished by pretreatment with either antagonist, whereas ST 91's antinociceptive effect was solely blocked by pretreatment with ARC 239. In nerve-injured animals, the antihypersensitivity action of both clonidine and ST 91 was blocked by administration of ARC 239, whereas BRL 44408 was ineffective.

Conclusions: These data agree with previous studies supporting that the α_{2A} adrenoceptor is important to the antinociceptive effect of clonidine in normal animals. Nerve injury alters this and results in a total reliance on $\alpha_{2\text{NON-A}}$ adrenoceptors.

INJURY to peripheral nerves may result in neuropathic pain, which is characterized by spontaneous persistent pain and hypersensitivity to both mechanical and thermal stimuli, often resistant to treatment with conventional analgesics or with traditional agents, such as opioids.¹ Spinal administration of α_2 adrenoceptor agonists,

such as clonidine, produces antinociception both in animals and humans.² Thus, the use of the α_2 adrenoceptor agonists has become an interesting alternative to currently used analgesics because they lack respiratory depressant effects and addictive liability. Epidural clonidine for cancer pain was the first analgesic specifically approved for the treatment of neuropathic pain. Epidural or intrathecal administration of clonidine is limited because of side effects, mainly hypotension, bradycardia, and sedation, reflecting actions on the α_{2A} adrenoceptors. Unfortunately, initial molecular biologic or radioligand binding studies suggested that the α_{2A} adrenoceptor is also the target for analgesia from spinally administered α_2 agonists.^{3,4} Moreover, analgesic effects are lost in α_{2A} but not $\alpha_{2\text{NON-A}}$ knockout mice,^{5,6} and by α_{2A} knockdown by intrathecal administration of specific antisense oligodeoxynucleotide (ODN) to this subtype.⁷

Other work supports a potential role for $\alpha_{2\text{NON-A}}$ adrenoceptor subtypes in some circumstances. For example, α_{2A} adrenoceptor expression is decreased, but α_{2C} is maintained after peripheral nerve injury in rats.^{8,9} In addition, a recent study showed that spinally administered, α_{2C} antisense (ODN) administration decreased mechanical antinociception induced by clonidine in rats.¹⁰ A problem with previous studies comparing normal to nerve-injured animals is that different test stimuli are applied to each group. In an attempt to clarify the involvement of α_2 adrenoceptor subtypes in normal rats and after peripheral nerve injury, we exposed both groups to the same mechanical stimulus and used subtype-preferring agonists and antagonists to define the subtypes involved.

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Materials and Methods

All surgical preparations and experimental protocols were approved by the Animal Care and Use Committee of Wake Forest University School of Medicine (Winston-Salem, North Carolina).

Surgical Preparation

Male rats (Harlan Sprague-Dawley) weighing 250–300 g were used in this study. Under halothane anesthesia, the left L5 and L6 spinal nerves were isolated and ligated tightly with 4-0 silk sutures, as previously described.¹¹ Animals recovered for 5–7 days; then, intrathecal catheters were inserted under halothane anesthesia as previously described.¹² Catheters were advanced 8 cm caudally through an incision in the cisternal membrane and

secured to the musculature at the incision site. Only animals with no evidence of neurologic deficit after catheter insertion were studied. All the pharmacologic experiments were conducted between 3 and 4 weeks after spinal nerve ligation, a time of stable hypersensitivity to mechanical stimuli. Normal animals had intrathecal catheters but did not undergo spinal nerve ligation.

Behavioral Testing

The nociceptive flexion reflex was quantified with a Ugo Basile Analgesymeter (Stoelting, Chicago, IL), which applies a linearly increasing force to the hind paw of the lightly restrained animal. During the week preceding experiments, rats were habituated to the device and tested at 5-min intervals for 1 h each day. This adaptation procedure reduces variability, produces a more stable baseline paw withdrawal threshold, and enhances the ability to detect effects of treatments.¹³ On the day of the experiment, rats were exposed to the test stimulus at 5-min intervals for 1 h. The baseline threshold was defined as the mean of the last six determinations before injection of test agents. A cutoff of 250 g was used to avoid tissue damage.

Drugs and Their Administration

α_2 Adrenoceptor agonists used in this study were clonidine hydrochloride (non-subtype selective; Sigma Chemical, St. Louis, MO) and ST91 (α_2 NON-A -preferring; Boehringer Ingelheim, Ridgefield, CT). Animals received cumulative dosing, at 40-min intervals, of intrathecal clonidine (19, 75, 190 nmol) or, at 60 min intervals, of intrathecal ST 91 (10, 40, 100 nmol). Timing of cumulative injections was determined by pilot experiments with either drug. Dose-response curves were constructed from percent maximum possible effect.

Percent maximum possible effect was defined as: $100 \times (\text{postdrug response} - \text{baseline}) / (\text{cutoff threshold or pre-nerve injury threshold} - \text{baseline})$. Agonists were administered intrathecally in volumes of 10 μl , and thresholds for withdrawal were determined at 40 min after clonidine administration and 60 min after ST91 injection.

α_{2A} Adrenoceptor subtype antagonists were BRL 44408, a selective α_{2A} adrenoceptor subtype antagonist, and ARC 239, a selective α_2 NON-A adrenoceptor subtype antagonist (both from Tocris Cockson Inc., Ballwin, MO). Antagonists or vehicles were injected spinally in volumes of 10 μl prior to agonists. Based on pilot experiments, we used probe doses of clonidine of 56 nmol (15 μg) and ST 91 of 40 nmol (20 μg). Doses of antagonists were 0.1, 1, or 5 times the equimolar dose of agonist ($n = 6$ or 8 in each dose). All studies were conducted with the investigator blinded to drug administered. Drugs were dissolved in normal saline or, when necessary, in 2-hydroxypropyl- β -cyclodextrin (Sigma Chemical).

Statistical Analysis

Data are represented as mean \pm SD. Paw withdrawal thresholds in response to mechanical stimulation before and after nerve ligation were compared using a paired Student t test. Effects of individual drugs on paw pressure threshold withdrawal were determined using a two-way analysis of variance for repeated measures followed by the Bonferroni correction for appropriate multiple comparisons. $P < 0.05$ was considered significant.

Results

Paw pressure withdrawal threshold before spinal nerve ligation was 130 ± 14.6 g. The mechanical threshold decreased significantly (67 ± 11.3 g) within 2 weeks after surgery and was stable thereafter.

Antinociceptive Effect of Clonidine and ST 91 in Normal Rats

Clonidine and ST 91 produced antinociception to mechanical pressure stimulus in a equipotent manner with a significantly greater efficacy of ST 91 (fig. 1, upper panel). Larger doses of clonidine could not be studied due to intense behavioral sedation.

Antagonism of Clonidine and ST 91 in Normal Rats

Intrathecal injection of 56 nmol clonidine increased the withdrawal threshold significantly 40 min after injection (figs. 2A and B). Intrathecal injection of BRL 44408 (5.6 and 56 nmol) or ARC 239 (5.6 and 56 nmol) significantly inhibited the antinociceptive effect of intrathecal clonidine in a dose-dependent manner (figs. 2A and B).

Intrathecal injection of 40 nmol ST 91 significantly increased the withdrawal threshold 60 min after injection (figs. 3A and B). Intrathecal injection of BRL 44408 (40 and 200 nmol) did not influence the antinociceptive effect of ST 91 (fig. 3A). In contrast, intrathecal injection of ARC 239 (40 and 400 nmol) inhibited the antinociceptive effect of intrathecal ST 91 in a dose-dependent manner (fig. 3B). Intrathecal injection of vehicle or either antagonist failed to alter the withdrawal threshold (control time points: figs. 2 and 3).

Antinociceptive Effect of Clonidine and ST 91 after Peripheral Nerve Injury

Clonidine and ST 91 reduced antihypersensitivity to mechanical pressure stimulus testing in an equipotent manner (fig. 1, lower panel). ST 91, but not clonidine, was able to return paw withdrawal threshold to pre-nerve injury values.

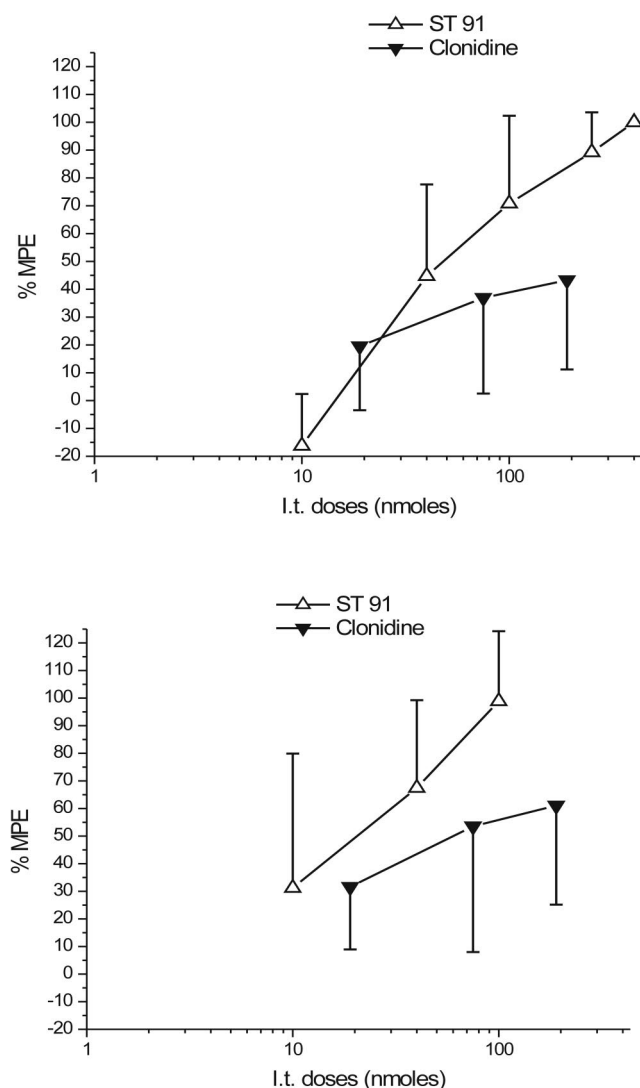


Fig. 1. Dose-response curves for the effects of intrathecally administered clonidine and ST 91 on the pressure nociceptive threshold in normal animals (upper panel) and in nerve-injured animals (lower panel). The response is presented as percent maximum possible effect (% MPE) versus dose in nanomoles. Each point on the graph represents the mean \pm SD of 7–9 animals.

Antagonism of Clonidine and ST 91 in Animals after Peripheral Nerve Injury

Intrathecal injection of 56 nmol clonidine increased the paw pressure withdrawal threshold significantly 40 min after injection (figs. 4A and B). Intrathecal injection of BRL 44408 (56 and 280 nmol) failed to inhibit the effect of intrathecal clonidine in a dose-dependent manner (fig. 4A). In contrast, intrathecal injection of ARC 239 (5.6 and 56 nmol) significantly inhibited the antihypersensitivity effect of intrathecal clonidine in a dose-dependent manner (fig. 4B). Greater doses of BRL 44408 were not possible to evaluate due to transient excitability.

Intrathecal injection of 40 nmol ST 91 significantly increased the withdrawal threshold 60 min after injection (figs. 5A and B). Intrathecal injection of BRL 44408

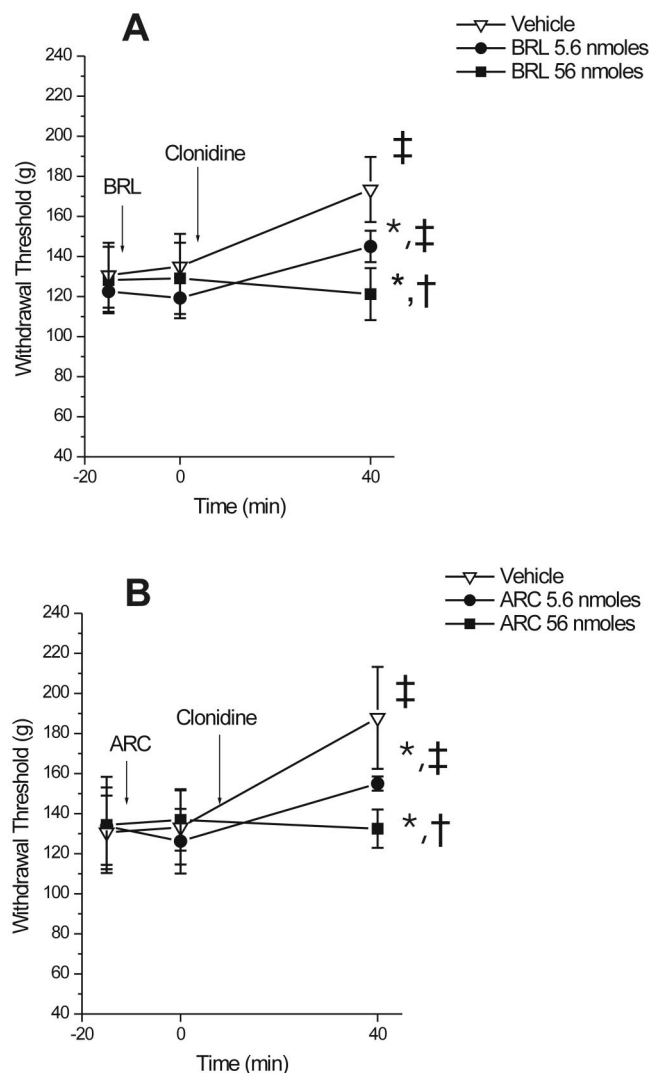


Fig. 2. Effect of intrathecal pretreatment with vehicle, BRL 44408 (α_{2A} subtype-preferring antagonist, A, upper panel), and ARC 239 ($\alpha_{2 \text{ NON-A}}$ subtype-preferring antagonist, B, lower panel) on the antinociceptive effect of intrathecal injection of 56 nmol clonidine in normal animals. Data are mean \pm SD of 6–8 animals. * P < 0.05 versus vehicle. † P < 0.05 versus 5.6-nmol dose of BRL (α_{2A} subtype-preferring antagonist, A, upper panel) or 5.6-nmol dose of ARC ($\alpha_{2 \text{ NON-A}}$ subtype-preferring antagonist, B, lower panel). ‡ P < 0.05 versus baseline and time 0.

(40 and 200 nmol) failed to alter the antinociceptive effect of ST 91 (fig. 5A). In contrast, intrathecal injection of ARC 239 (40 and 400 nmol) significantly inhibited the antinociceptive effect of intrathecal ST 91 in a dose-dependent manner (fig. 5B). Intrathecal injection of vehicle or either antagonist failed to alter the withdrawal threshold (control time points: figs. 4 and 5).

Discussion

The current study, the first to examine the effect of α_2 adrenoceptor agonists using the same noxious mechanical stimulus in both normal animals and nerve-injured

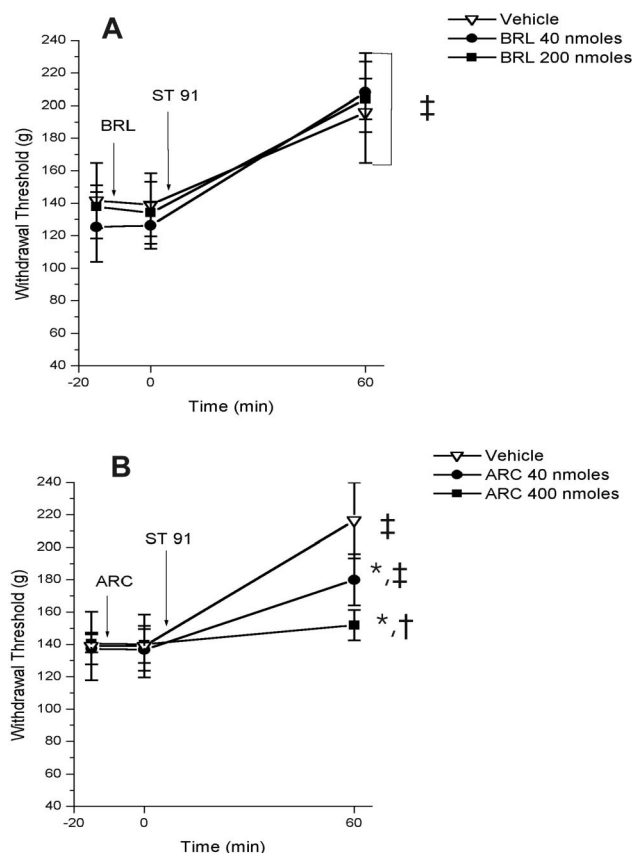


Fig. 3. Effect of intrathecal pretreatment with vehicle, BRL 44408 (α_{2A} subtype–preferring antagonist, *A*, upper panel), and ARC 239 ($\alpha_{2\text{NON-A}}$ subtype–preferring antagonist, *B*, lower panel) on the antinociceptive effect of intrathecal injection of 40 nmol ST 91 in normal animals. Data are mean \pm SD of 6–8 animals. * P < 0.05 versus vehicle. † P < 0.05 versus 40-nmol dose of ARC. ‡ P < 0.05 versus baseline and time 0.

animals, provides new experimental evidence indicating that the antihypersensitivity effect produced by intrathecally administered clonidine in neuropathic pain depends primarily on its interaction with $\alpha_{2\text{NON-A}}$ adrenoceptor subtypes in the spinal cord, whereas in normal rats, the antinociceptive action of clonidine is mediated by both α_{2A} and $\alpha_{2\text{NON-A}}$ adrenoceptor subtypes. Should these data apply to humans, this suggests that it may be possible to separate analgesia in patients with neuropathic pain from unwanted side effects due to α_{2A} adrenoceptor activation (sedation, cardiovascular depression).

We chose a pharmacologic approach to define roles of α_2 adrenoceptor subtypes in mediating analgesia in normal and nerve-injured animals. We are aware that resolution of the functions specific to each α_2 adrenoceptor subtype is difficult due to lack of perfectly selective pharmacologic tools. However, the antagonists we employed distinguish reasonably well between α_{2A} and $\alpha_{2A\text{NON-A}}$ adrenoceptor subtypes.¹⁴

The current study suggests that intrathecal clonidine produces antinociception in normal rats by interacting with both α_{2A} and $\alpha_{2A\text{NON-A}}$ adrenoceptor subtypes. This

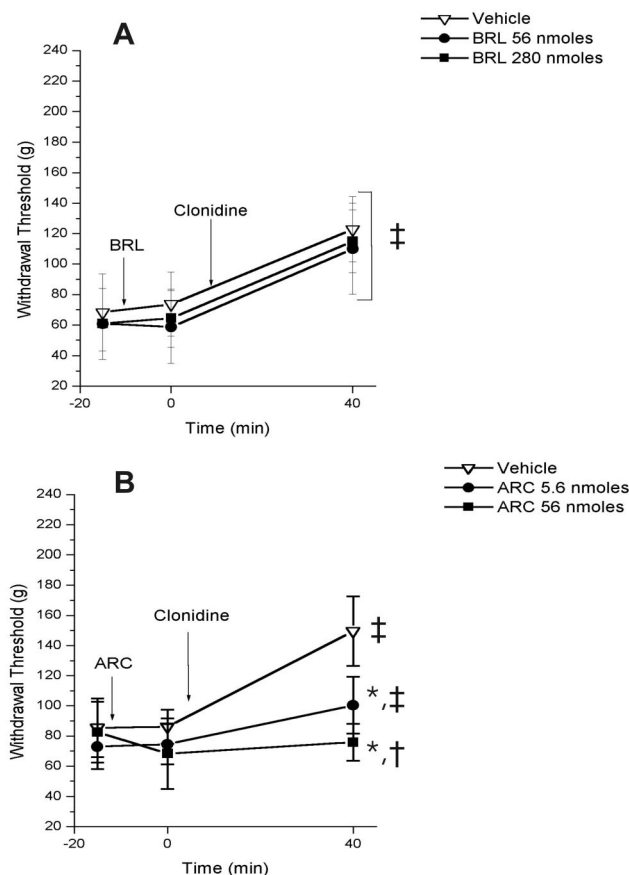


Fig. 4. Effect of intrathecal pretreatment with vehicle, BRL 44408 (α_{2A} subtype–preferring antagonist, *A*, upper panel), and ARC 239 ($\alpha_{2\text{NON-A}}$ subtype–preferring antagonist, *B*, lower panel) on the antihypersensitivity effect of intrathecal injection of 56 nmol clonidine in injured animals. Data are mean \pm SD of 6–8 animals. * P < 0.05 versus vehicle. † P < 0.05 versus 5.6-nmol dose of ARC. ‡ P < 0.05 versus baseline and time 0.

is somewhat discrepant from previous work. For example, α_{2A} adrenoceptor mRNA or immunoreactivity predominates in the spinal cord of normal rats and is mostly located in the superficial dorsal horn, whereas the α_{2C} adrenoceptor subtype immunoreactivity is mostly present in the ventral horn area.^{4,15–17} Other pharmacologic studies in mice support an exclusive role of α_{2A} adrenoceptor subtypes to produce antinociception.¹⁸ Finally, studies using transgenic mice or antisense ODN injections in rats support an analgesic action of α_2 adrenoceptor agonists primarily mediated by the α_{2A} adrenoceptor subtype under normal conditions.^{5–7,19} In those studies, α_2 adrenoceptor agonists lost their analgesic action in knockout mice lacking the α_{2A} adrenoceptor subtype or in animals treated with α_{2A} adrenoceptor subtype antisense ODN, whereas this effect was not reduced in the mice lacking the α_{2C} adrenoceptor subtype or in animals treated with α_{2C} adrenoceptor subtype antisense ODN. Several factors could account for the apparent discrepancy between these results and ours in normal animals. The amount of mRNA in tissue need

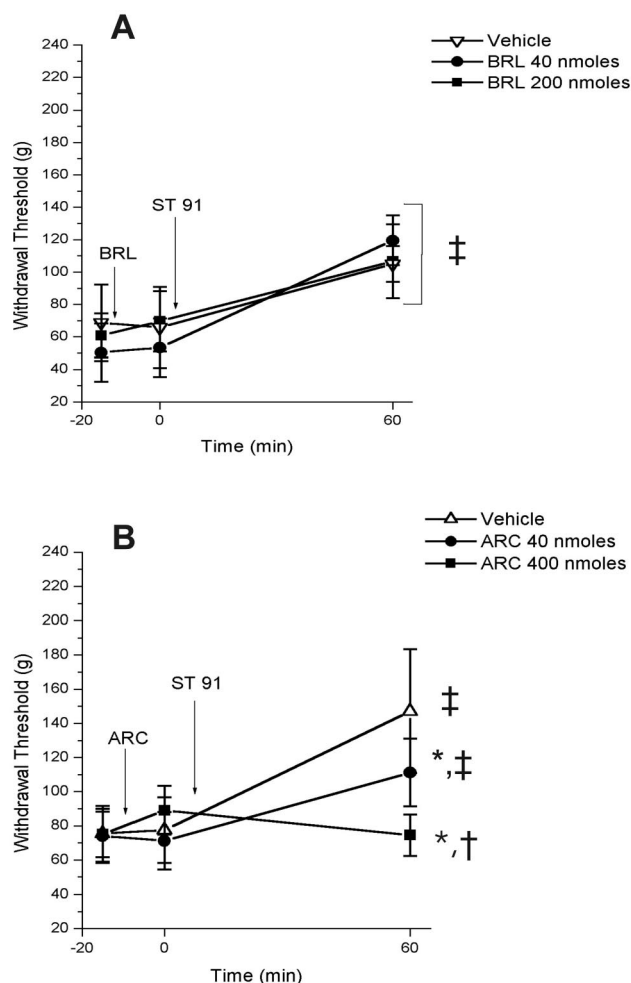


Fig. 5. Effect of intrathecal pretreatment with vehicle, BRL 44408 (α_{2A} subtype–preferring antagonist, *A*, upper panel), and ARC 239 ($\alpha_{2 \text{ NON-A}}$ subtype–preferring antagonist, *B*, lower panel) on the antihypersensitivity effect of intrathecal injection of 40 nmol ST 91 following peripheral nerve injury. Data are mean \pm SD of 6–8 animals. * $P < 0.05$ versus vehicle. † $P < 0.05$ versus 40 nmol ARC 239. ‡ $P < 0.05$ versus baseline and time 0.

not reflect the relative amount of functional receptor protein. Moreover, previous behavioral studies have evaluated the effect of α_2 adrenoceptor agonists by using hot-plate or tail-flick tests, studying thermally evoked C-fiber activity. Although these techniques are validated in assessing antinociception to thermal stimuli, they may not reflect results from mechanical stimuli, such as the paw pressure used in our normal animals. Studies of genetically modified reagents may also provide misleading information. First, the knockout animals had the opportunity to adapt to the deficiency during their development, and mice have different spinal noradrenergic anatomy than other species, such as rats or humans (Weiya Ma, Ph.D., Assistant Professor of Anesthesiology, Wake Forest University School of Medicine, personal oral communication and submitted data, January 2002). Second, in the case of the studies using antisense ODNs,

the authors failed to prove that they had selectively reduced expression of the cognate protein in the spinal cord (e.g., Western blot), while the administration of sense ODN resulted in altered behavioral function.⁷

Other previous work supports the concept that antinociception could be mediated by $\alpha_{2 \text{ NON-A}}$ adrenoceptor subtypes. Prazosin, which is not only a highly selective α_1 antagonist but also a preferential $\alpha_{2 \text{ NON-A}}$ adrenoceptor subtype antagonist, inhibits the effect of clonidine on the release of substance P.²⁰ In addition, ST 91 induces antinociception in rats when administered spinally,²¹ and this effect is blocked by prazosin. Subsequently, dense and similar immunoreactivity for both α_{2A} and α_{2C} adrenoceptor subtypes was recently demonstrated in the superficial layers of the rat spinal cord, suggesting that both α_{2A} and α_{2C} adrenoceptor subtypes are present in spinal cord regions related to nociception.^{8,9}

Our data from injured animals suggest that the antihypersensitivity action of clonidine is mediated by interacting with $\alpha_{2A \text{ NON-A}}$ adrenoceptor subtypes solely. This is not entirely consistent with a previous study suggesting a predominant role of α_{2A} adrenoceptor subtype in inhibition after nerve injury.²² In that study, the agonist structure–activity relationship suggested an α_{2A} adrenoceptor subtype, and clonidine's effect was not reversed by the $\alpha_{2A \text{ NON-A}}$ -preferring antagonist, prazosin. However, the effect of specific α_2 adrenoceptor antagonist subtypes (i.e., ARC 239 or BRL 44408) on the α_2 adrenoceptor agonists, or antagonist dose responses, were not examined. Later, Malmberg *et al.*²³ have recently studied the contribution of the α_2 adrenoceptor subtypes to the development of neuropathic pain after partial sciatic nerve ligation in genetically altered mice. The authors showed that dexmedetomidine reduced mechanical hypersensitivity in $\alpha_{2 \text{ NON-A}}$ adrenoceptor knockout mice, but its action was preserved in α_{2A} adrenoceptor subtype knockout animals. However, as noted above, mice have distinctly different spinal cord anatomy of noradrenergic systems, and these authors did not evaluate the action of ST 91.

Several observations support the current study to suggest a plasticity in α_2 adrenoceptor subtype inhibition after peripheral nerve injury. First, α_{2A} adrenoceptor subtype immunoreactivity, likely located on C-fibers terminals, decreases dramatically in the rat spinal cord ipsilateral to the injury following sciatic nerve transection, chronic constriction injury of the sciatic nerve, or L5–L6 spinal nerve ligation.⁸ Of course, one would not expect mechanical hypersensitivity to be transduced by C-fibers to begin with. Second, in the same study, there was a significant increase in dorsal spinal cord α_{2C} adrenoceptor subtype immunoreactivity ipsilateral to injury following nerve ligation compared with sham animals. Also, Khasar *et al.*²⁴ previously showed that in acute inflammatory pain models, $\alpha_{2 \text{ NON-A}}$ adrenoceptor subtype antagonists but not α_{2A} adrenoceptor subtype an-

tagonists were able to reverse the inhibitory effect of clonidine using the paw pressure stimulus. In support of these findings, more subtype-specific approaches showed that intrathecally administered, α_C adrenoceptor subtype ODN significantly attenuated clonidine's antinociceptive effect following hind paw inflammation.²⁵ Finally, in α_{2C} knockout mice, an $\alpha_{2 \text{ NON-A}}$ subtype-prefering agonist spinally administered was not able to produce analgesia after intrathecal injection of substance P.¹⁰ In this case, the cognate protein (*i.e.*, $\alpha_{2 \text{ NON-A}}$ adrenoceptor subtype) was shown to be reduced after ODN treatment.

In summary, the current pharmacologic study shows that intrathecally administered clonidine produces antinociception in normal rats through interaction with both α_{2A} and $\alpha_{2 \text{ NON-A}}$ adrenoceptor subtypes. In contrast, after nerve injury, clonidine acts solely *via* $\alpha_{2 \text{ NON-A}}$ adrenoceptor subtypes. This result, plus the efficacy of ST 91, which is devoid of hypotensive or sedative side effects,^{26,27} could lead the way to a better use of α_2 adrenoceptor agonists for analgesia in patients with chronic pain.

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