

α_2 -Adrenoceptor Activation by Clonidine Enhances Stimulation-evoked Acetylcholine Release from Spinal Cord Tissue after Nerve Ligation in Rats

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Background: Spinally administered clonidine produces analgesia via α_2 -adrenergic receptors. The analgesic potency of clonidine and its dependency on muscarinic acetylcholine receptors increase in rats after nerve injury. The authors hypothesized that these changes reflect greater acetylcholine release from the spinal cord by clonidine, either through direct or indirect effects.

Methods: Male Sprague-Dawley rats were divided into two groups: no surgery or left L5 and L6 spinal nerve ligation (SNL). All experiments were performed 3 weeks after SNL. Crude synaptosomes were prepared from the spinal enlargement and loaded with [3 H]choline. Samples were incubated with clonidine in the absence or presence of KCl depolarization. The authors also examined the effect of clonidine on KCl evoked acetylcholine release using perfusion of spinal cord slices, in which some spinal circuitry is maintained.

Results: In synaptosomes, clonidine alone induced minimal acetylcholine release, which was actually greater in tissue from normal rats than in tissue from SNL rats. In the presence of KCl depolarization, however, clonidine enhanced acetylcholine release in tissue from SNL rats but inhibited release in tissue from normal rats. Similarly, in spinal cord slices, clonidine enhanced KCl evoked acetylcholine release in tissue from SNL animals but inhibited such release in tissue from normal animals. The α_2 -adrenoceptor antagonist idazoxan inhibited the effects of clonidine in slices from SNL rats.

Conclusion: These results suggest that clonidine enhances depolarization-induced acetylcholine release in neuropathic but not in normal spinal cord tissue. Interestingly, this enhanced acetylcholine release by clonidine occurs in a synaptosomal preparation, consistent with a direct effect on α_2 adrenoceptors on cholinergic terminals. Enhanced release of acetylcholine by clonidine could contribute to increased analgesia of clonidine in neuropathic pain.

CLONIDINE, an α_2 -adrenergic agonist, produces analgesia in humans and animals after intrathecal administration. Previous data suggest that clonidine is more potent in treating neuropathic pain than acute nociceptive pain in humans¹ and is more effective against hypersensitivity states induced by peripheral nerve injury than against

acute nociceptive stimulation in normal animals.^{2,3} The goal of the current study was to probe one possible mechanism for this shift in potency and efficacy of intrathecal clonidine in neuropathic pain.

It has been suggested that spinal acetylcholine release has an important role in α_2 adrenoceptor-mediated antinociception in the spinal cord. Intrathecal clonidine increases cerebrospinal fluid concentrations of acetylcholine in humans,⁴ and intrathecal or local administration of clonidine into the spinal cord dorsal horn increases efflux of acetylcholine in spinal cord dorsal horn extracellular fluid, as measured by microdialysis.^{5,6} Several lines of evidence suggest that α_2 adrenergic-mediated antinociception after nerve injury from intrathecal clonidine relies heavily on a cholinergic interaction. For example, in hypersensitivity states induced by spinal nerve ligation (SNL) in rats, the reduction in mechanical allodynia by intrathecal clonidine is reversed by muscarinic receptor antagonists.⁷ In SNL rats, the selective destruction of cholinergic cells in the spinal cord by the cholinotoxin monoethyl-choline mustard aziridium ion (AF64-A) reduces both the spinal cord content of acetylcholine and the antiallodynic effect from intrathecally administered clonidine.⁸ In contrast, antinociception to short-term noxious heat and mechanical stimulation from intrathecal clonidine in normal rats is not blocked by intrathecal atropine.²

These observations suggest that the muscarinic cholinergic receptor dependency of spinal α_2 adrenoceptor-mediated analgesia increases progressively from acute nociception in normal animals to nerve injury-induced chronic hypersensitivity. We speculated that one mechanism for this shift might be increased acetylcholine release in the dorsal horn of the spinal cord by clonidine after nerve injury compared with normal rats. To test this hypothesis, we compared acetylcholine release from spinal cord tissue in response to clonidine application in SNL with normal rats using two different methods of neurotransmitter release *in vitro*: synaptosomes, which test direct effects on synaptic release,^{9,10} and spinal cord slices, in which some neuronal circuits remain in place.¹¹

Materials and Methods

Animal Preparation

After approval was obtained from the institutional Animal Care and Use Committee (Wake Forest University School of Medicine, Winston-Salem, North Carolina),

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male Sprague-Dawley rats weighing 200–280 g at the time of purchase (Harlan, Indianapolis, IN) were studied. Animals were housed under a 12-h light-dark cycle, with access to food and water *ad libitum*. Animals were separated into two groups: no surgery (n = 35) or SNL (n = 66). We chose no surgery as a control rather than sham surgery with exposure of nerves without ligation, because we were interested in changes within the dorsal root ganglia cells during the neuropathic state. We recognize that we therefore could not exclude an effect of inflammation or surgery itself as opposed to nerve injury.

Spinal nerve-ligated rats weighing 200–220 g were anesthetized with halothane (2–3% in oxygen), and the left L5 and L6 spinal nerves were isolated and ligated tightly with 6-0 silk sutures distal to the dorsal root ganglia as previously described.¹² After surgery, animals were housed individually with free access to food and water and were allowed to recover for at least 14 days. Left paw tactile allodynia was confirmed at this time by measuring the hind paw withdrawal threshold in response to application of von Frey filaments, using a previously described up-down method.¹³ Only animals with a withdrawal threshold less than 4 g were used. All *in vitro* studies were performed 3 weeks after SNL surgery, at a time of allodynia to von Frey filament testing.

Synaptosomes

After induction of anesthesia with 2–3% halothane, animals were killed by decapitation, and the lumbar enlargement (approximately L2–L5) of the spinal cord was quickly removed and placed in oxygenated (with 95% oxygen–5% carbon dioxide) ice-cold modified Krebs buffer containing 125 mM NaCl, 3 mM KCl, 1.2 mM MgSO₄, 1.2 mM CaCl₂, 1 mM KH₂PO₄, 22 mM NaHCO₃, and 11.5 mM glucose (pH = 7.35). The left dorsal quadrant of the spinal cord ipsilateral to nerve injury was removed from SNL rats and homogenized in 8 ml ice-cold 0.32 M sucrose. To decrease the number of rats, the entire dorsal half of the spinal cord was used in normal rats. Each synaptosome preparation contained tissue of four SNL rats or two normal rats. A crude synaptosomal pellet was prepared from mechanically homogenized tissue by differential centrifugation at 2,000g followed by 20,000g as previously described.¹⁴

Acetylcholine release from synaptosomes and tissue slices was done after loading with [³H]choline according to standard methods, which results in rapid uptake of choline, acetylation to acetylcholine, and release of [³H]acetylcholine with depolarization.^{15,16} We did not, however, confirm in the current experiments that released radioactivity was completely in the form of [³H]acetylcholine. For the synaptosome experiments, the crude pellet was resuspended into 4 ml modified Krebs buffer, loaded with [³H]choline, and incubated at 37°C for 20 min. Free [³H]choline was then removed by

centrifugation at 20,000g for 5 min. The synaptosomal pellet was again suspended into 4.5 ml modified Krebs buffer, and 150 μl of the suspension was aliquoted into each test tube with 850 μl modified Krebs buffer containing different concentrations of clonidine. Each tube also contained hemicholinium-3 at a final concentration of 10 μM to prevent reuptake of acetylcholine. For KCl stimulation, the concentration of KCl in the test tube was increased to 12 mM. Tubes were then incubated for 10 min at 37°C in a total volume of 1 ml. At the end of incubation, the amount of [³H] remaining in synaptosomes was determined by rapid filtration through GF/C glass filters presoaked for 30 min in 0.1% polyethylene to reduce nonspecific binding. This was followed by three times 4-ml washes with ice-cold buffer in which glucose was substituted for NaCl. The bound (retained) radioactivity was determined 24 h later by scintillation counting. The effects of clonidine at doses from 10⁻⁸ to 10⁻⁴ M on [³H]acetylcholine release were determined by the amount of tritium remaining in the synaptosomes incubated in buffer without high KCl concentration (control) compared with treatment with each concentration of clonidine with physiologic (3 mM) or depolarizing (12 mM) concentrations of KCl. Fractional release was calculated in each sample from the retained radioactivity, and percentage change in fractional acetylcholine release by clonidine defined as (control – clonidine)/control × 100. Each experiment was performed in duplicate for each concentration, and the mean value was included.

To determine whether another α₂-adrenoceptor agonist would mimic the effects of clonidine, synaptosomes from normal and SNL rats (n = 7 each) were prepared, and the effect of KCl alone and in the presence of 10 nM dexmedetomidine was examined.

Spinal Cord Slices

Rats were anesthetized with halothane and killed by decapitation, and the lumbar enlargement of the spinal cord was quickly removed. The left dorsal semihalf was chopped in 0.6-mm slices. The slices were preincubated with [³H]choline for 40 min at 37°C in oxygenated (with 95% oxygen–5% carbon dioxide) modified Krebs buffer containing 125 mM NaCl, 3 mM KCl, 1.2 mM MgSO₄, 1.2 mM CaCl₂, 1 mM KH₂PO₄, 22 mM NaHCO₃, and 11.5 mM glucose (pH = 7.35). Four to five spinal cord slices were put into each chamber surrounded by a temperature-controlled water bath maintained at 33°C.

The slices were superfused continuously with a multichannel pump at 0.5 ml/min with oxygenated modified Krebs buffer containing 10 μM hemicholinium-3. After an initial perfusion of 40 min, 5-min fractions were collected. At 15 min and again at 50 min after beginning of fraction collection, the slices were depolarized for 5 min by increasing the concentration of KCl in the superfusion fluid to 25 mM. The total tritium overflow in re-

sponse to 25 mM KCl was usually between 150 and 200% of the baseline release.

The test drugs (clonidine and idazoxan) were applied beginning 40 min after the beginning of fraction collection until 55 min. This coincided to the time beginning 10 min before the second KCl depolarization to the end of this depolarization. The radioactivity of each sample was determined 24 h later by scintillation counting. The KCl-evoked [^3H]acetylcholine release was calculated by subtracting the values of the basal release from the total release during the stimulation period. For each sample, the ratio of fractional release value during the second to the first KCl depolarization was calculated. In control slices, this ratio was 1.01 ± 0.05 . The effects of clonidine concentrations from 10^{-6} to 10^{-4} M on KCl-evoked [^3H]acetylcholine release were determined and compared to release in the presence of buffer only before KCl depolarization.

Materials

[^3H]Choline was purchased from Perkin Elmer (Boston, MA). Bio Safe II scintillation cocktail was obtained from Research Product International Corp. (Mount Prospect, IL). MgSO_4 , KH_2PO_4 , and NaHCO_3 were obtained from Fisher Scientific (Fair Lawn, NJ). The remaining chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

Statistics

Experiments were replicated four to five times for synaptosomes and five times for slices. All data are expressed as mean \pm SEM. For concentration-response studies, groups were compared by one-way or two-way analysis of variance. For antagonist studies, the clonidine-treated group and other groups were compared by one-way analysis of variance. A P value less than 0.05 was considered significant.

Results

Synaptosomes

In normal rats, 10^{-8} to 10^{-4} M clonidine alone (before KCl depolarization) increased [^3H]acetylcholine release compared with vehicle. In contrast, the highest concentration of clonidine inhibited [^3H]acetylcholine release in SNL rats compared with vehicle in the absence of KCl depolarization (fig. 1). The effect of clonidine alone on [^3H]acetylcholine release differed significantly in synaptosomes between normal and SNL animals.

KCl, 12 mM, provoked [^3H]acetylcholine release by $17 \pm 0.9\%$ in synaptosomes from normal rats and $17 \pm 1.2\%$ in synaptosomes from SNL rats, compared with Krebs buffer control. The effect of clonidine on KCl-evoked [^3H]acetylcholine release in synaptosomes from SNL rats was greater than that in synaptosomes in normal

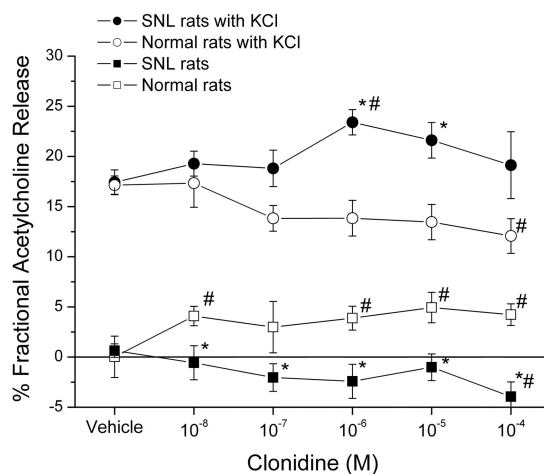


Fig. 1. Effect of clonidine on acetylcholine release from lumbar dorsal spinal cord synaptosomes from spinal nerve-ligated (SNL; closed symbols) or normal (open symbols) rats, with (circle) or without (square) 12 mM KCl stimulation. Each point represents the mean \pm SEM of four to five experiments. * $P < 0.05$ versus normal (P value for analysis of variance = 0.012). # $P < 0.05$ versus vehicle (P value for analysis of variance = 0.008).

rats (fig. 1). In a separate analysis within normal and SNL animals, 1 μM clonidine increased KCl-evoked acetylcholine release compared with control in synaptosomes from SNL animals, whereas 100 μM clonidine inhibited KCl-evoked acetylcholine release compared with control in synaptosomes from normal animals (fig. 1).

Qualitatively similar results were obtained with the highly selective α_2 -adrenoceptor agonist dexmedetomidine. In spinal cord synaptosomes from normal animals, 10 nM dexmedetomidine inhibited KCl-evoked [^3H]acetylcholine release, whereas in spinal cord synaptosomes from SNL animals, dexmedetomidine enhanced KCl-evoked [^3H]acetylcholine (fig. 2; $P < 0.005$ for each effect).

Spinal Cord Slices

Perfusion of spinal cord slices for 5 min with 25 mM KCl provoked [^3H]acetylcholine release at $164 \pm 3\%$ of 3 mM KCl control. The second KCl exposure resulted in less fractional release than the first, with a ratio of 0.65 ± 0.019 ($n = 45$). Compared with vehicle control within each group, clonidine did not alter the response to the second KCl exposure, although there were strong trends ($P < 0.1$ for main analysis of variance) toward enhanced release from slices from SNL animals and reduced release in slices from normal animals. Comparing the effect of clonidine between the groups demonstrated significantly greater KCl-evoked acetylcholine release in slices from SNL animals than in slices from normal animals, with a peak effect at 10^{-5} M (fig. 3).

Simultaneous perfusion of 10^{-5} M idazoxan with 10^{-5} M clonidine in spinal cord slices from SNL animals significantly reduced KCl-evoked release. Interestingly, ida-

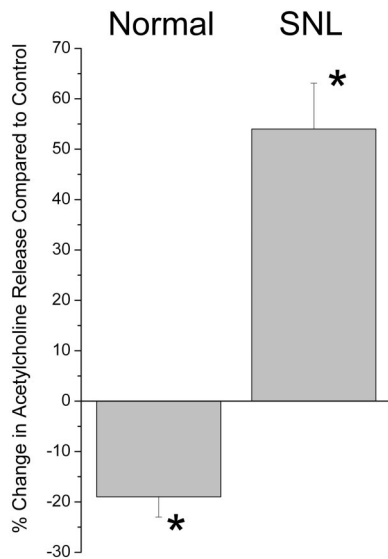


Fig. 2. Effect of 10 nM dexmedetomidine on acetylcholine release from lumbar dorsal spinal cord synaptosomes from normal (left) or spinal nerve-ligated (SNL; right) rats. Each bar represents the mean \pm SEM of four experiments. * $P < 0.005$ compared with control.

zoxan alone produced a small reduction in response to the second depolarization with KCl (fig. 4), consistent with tonic α_2 -adrenoceptor tone in this preparation.

Discussion

Spinally released acetylcholine produces analgesia primarily by actions on muscarinic cholinergic receptors.¹⁷ Activation of muscarinic receptors facilitates γ -aminobutyric acid release onto primary afferents in lamina II to inhibit the release of glutamate.^{18,19} In addition, evoked activity of projecting neurons in deeper laminae of the spinal cord is inhibited *in vivo* by muscarinic receptors.²⁰

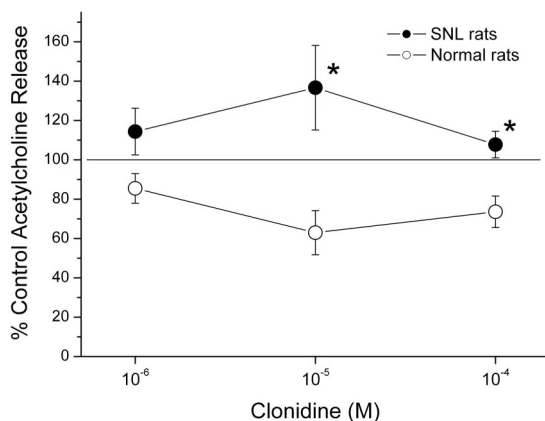


Fig. 3. Effect of clonidine on KCl-evoked acetylcholine release from lumbar dorsal spinal cord slices from spinal nerve-ligated (SNL; closed circles) or normal (open circles) rats. Data are expressed as percent of fractional release without drug exposure. Each point represents the mean \pm SEM of five experiments. * $P < 0.05$ versus normal (P value for analysis of variance = 0.011).

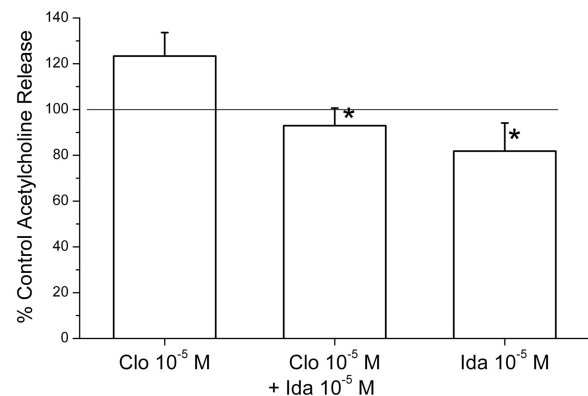


Fig. 4. Effect of idazoxan (Ida) on clonidine (Clo)-induced facilitation of acetylcholine release from KCl exposure in lumbar dorsal spinal cord slices from spinal nerve ligated rats. Data are expressed as percent of fractional release without drug exposure. Each point represents the mean \pm SEM of five experiments. * $P < 0.05$ versus clonidine-treated group (P value for analysis of variance = 0.018).

Spinal acetylcholine release is increased by analgesics, including systemically administered morphine²¹ and intrathecally administered carbachol²² and clonidine.²³

The purpose of the current study was to better understand the mechanisms that underlie the increase in potency and efficacy of α_2 -adrenoceptor agonists for analgesia in chronic compared with acute pain states. The focus on acetylcholine follows from observations that intrathecal clonidine increases concentrations of this neurotransmitter in patients with pain⁴ and that intrathecal clonidine-induced relief of hypersensitivity to mechanical stimuli in animals with peripheral nerve injury is blocked by intrathecal atropine, whereas atropine does not block the effect of clonidine in normal animals.² The selective reliance of clonidine on muscarinic cholinergic receptor activation in animal models of neuropathic pain could reflect increased clonidine-induced acetylcholine release. Alternatively, clonidine induced acetylcholine release could be similar in normal and SNL animals, but there may be a change in cholinergic receptor expression or location. The current study provides evidence that clonidine stimulates spinal acetylcholine release after peripheral nerve injury.

All previous studies examining acetylcholine release by α_2 -adrenoceptor agonists were performed *in vivo*, by either dorsal horn microdialysis or sampling of cerebrospinal fluid. The current study sought to determine whether clonidine-induced acetylcholine release reflected an action on nerve terminals themselves, which we tested using synaptosomes, or acted on local spinal circuits, which we tested using spinal cord slices. Synaptosomes are commonly used to examine regulation of neurotransmitter release in the absence of indirect effects from disinhibition or activation of excitatory neuronal inputs to the terminals.^{9,10,24}

Clonidine alone produced a small and concentration-independent release of acetylcholine from spinal cord

synaptosomes in normal animals and none from synaptosomes from SNL animals. We did not anticipate that clonidine would induce acetylcholine release in synaptosomes, because α_2 adrenoceptors are classically inhibitory, coupling with G proteins, which decrease cyclic adenosine monophosphate concentrations and result in reduced voltage-gated calcium channel opening.²⁵ Because α_2 adrenoceptors colocalize with cholinergic neurons in the spinal cord,²⁶ we anticipated that clonidine would, if anything, reduce acetylcholine release and that clonidine-induced acetylcholine release *in vivo* reflected activation of circuits rather than direct effects on cholinergic terminals.

In contrast to the minimal effect of clonidine alone on acetylcholine release in synaptosomes, clonidine clearly augmented acetylcholine release in synaptosomes depolarized by KCl only in tissue from SNL animals. We chose KCl to nonselectively stimulate all synaptosomes to examine the influence of clonidine on evoked release. As with release from drug alone, we anticipated that clonidine would, if anything, reduce evoked acetylcholine release, not increase it, but this occurred only in tissue from normal animals. The current results, in contrast to our expectations, are consistent nonetheless with reports that α_2 adrenoceptors can shift from inhibitory to excitatory mechanisms in neurons after peripheral nerve injury²⁷ or inflammation,²⁸ possibly because of a shift in G-protein species, because α_2 adrenoceptors can couple with excitatory, Gs species when these are present in adequate numbers.²⁹

Perfusion of spinal cord slices *in vitro* also demonstrated enhancement of evoked acetylcholine release only in tissue from SNL animals. In both synaptosome and slice studies, the effect of clonidine was reduced at higher concentrations, potentially because of actions of clonidine on α_1 adrenoceptors.³⁰ At a clonidine concentration producing a peak effect, however, this was completely reversed by the selective α_2 -adrenoceptor antagonist idazoxan. The peak effect of clonidine synaptosomal experiments was an order of magnitude less (10^{-6} M) than that in slices (10^{-5} M), possibly reflecting reduced tissue penetration in the slices.

The cause of this plasticity in evoked acetylcholine release from spinal cord after nerve injury is unknown. Previous studies suggest that clonidine inhibits stimulation-evoked neurotransmitter release *via* actions on an α_{2A} -adrenoceptor subtype.^{31,32} However, nerve injury reduces α_{2A} - but not α_{2C} -adrenoceptor immunolabeling in the spinal cord, and α_{2C} -adrenoceptors are located in the deep dorsal horn, the normal termination of large-diameter fibers that subserve mechanical input³³ and the site of cholinergic cell bodies, which coexpress the α_{2C} adrenoceptor.²⁶ The α_2 -adrenoceptor subtype activated by clonidine to reduce mechanical allodynia after nerve injury shifts from the α_{2A} - to α_{2C} -adrenoceptor subtype,³⁴ and it is possible that increased α_{2C} -adrenoceptor

number or coupling efficiency on spinal cholinergic neurons after nerve injury is responsible for increased evoked acetylcholine release by clonidine.

In summary, clonidine enhances K^+ -evoked acetylcholine release in spinal cord tissue from SNL, but not normal rats, using two different preparations to measure neurotransmitter release: synaptosomes, which only measure direct action on terminals, and spinal cord slices, in which some circuits are preserved. The facilitating effect of clonidine in slices is blocked by the α_2 -adrenoceptor antagonist idazoxan. These data suggest that, after nerve injury, activation of spinal α_2 adrenoceptors induces more acetylcholine release from cholinergic neurons when these are stimulated. This neuronal plasticity may contribute to the increased potency of clonidine for analgesia and its dependency on cholinergic neuronal activation in the neuropathic pain state.

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