Anesthesiology 2003; 98:748–53

Clonidine-induced Neuronal Activation in the Spinal Cord Is Altered after Peripheral Nerve Injury

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Background: **α_2 Adrenoceptor agonists produce antinociception in normal animals and alleviate mechanical allodynia in animals with nerve injury, although their mechanism of action may differ in these situations. The purpose of this study was to examine the location and number of cells in the spinal cord activated by intrathecal clonidine in these two circumstances and to test whether one class of interneurons, cholinergic, express α_2 adrenoceptors.**

Methods: **Intrathecal saline or clonidine, 10 and 30 μg, was injected in normal rats or those with mechanical allodynia following partial sciatic nerve section. Two hours later, animals were anesthetized and pericardially perfused. The number of cells in superficial and deep dorsal horn laminae at the L4–L5 level immunostained for phosphorylated cAMP response element binding protein (pCREB) were quantified. In separate studies, the authors colocalized α_{2C} adrenoceptors with cholinergic neurons.**

Results: **Intrathecal clonidine increased pCREB immunoreactive cells in both superficial and deep laminae by 50–100% in normal animals. The number of pCREB immunoreactive cells increased in nerve-injured compared to normal rats. Intrathecal clonidine decreased pCREB immunoreactive cells in the deep dorsal horn of injured animals. α_{2C} Adrenoceptors colocalized with cholinergic neurons in both superficial and deep dorsal horn.**

Discussion: **Previous studies suggest a shift in α_2 adrenoceptor subtype and the involvement of cholinergic interneurons in antinociception in the spinal cord after nerve injury. The current results suggest that intrathecal clonidine, by direct or indirect methods, increases neuronal activation in normal animals, presumably leading to net inhibition of pain signaling, whereas it reduces the increase in neuronal activity induced by nerve injury.**

α_2-ADRENERGIC receptor (α_2-AR) agonists produce analgesia in humans and animals, primarily by actions in the spinal cord. The mechanisms by which they do so appear to differ depending on the cause of pain—acute noxious stimuli in normal subjects compared to ongoing pain and hypersensitivity to innocuous stimuli, termed *allodynia*, in subjects with nerve injury. Several observations support this concept. For example, the potency and efficacy of intrathecal or epidurally administered clonidine increases in patients with neuropathic pain compared to normal subjects with experimental noxious stimuli or patients with acute postoperative pain.[1–3] Similarly, intrathecal clonidine is more potent to reverse allodynia from sciatic nerve section than to cause antinociception in animals.[4]

Not only are α_2-AR agonist potency and efficacy altered by nerve injury, but the α_2-AR subtypes involved and their interaction with cholinergic systems of inhibition are shifted as well. There are three α_2-AR subtypes, termed A, B, and C, and the A and C subtypes predominate in the spinal cord of rats.[5] Sciatic nerve injury associated with allodynia results in a loss of the A but not the C subtype in the lumbar spinal cord.[6] In accordance with this anatomic observation, intrathecal clonidine causes antinociception that is inhibited by either α_2-ARA or C subtype antagonists in normal animals but only by an α_2-ARC subtype antagonist after nerve injury.[7] Finally, there is a shift in the importance of a cholinergic interaction induced by α_2-AR agonists with nerve injury. Intrathecal clonidine increases acetylcholine concentrations in cerebrospinal fluid of humans without chronic pain,[8] and intrathecal atropine partially reverses clonidine antinociception in normal animals.[9] In contrast, intrathecal atropine totally reverses clonidine's effect on allodynia after nerve injury, as does destruction of spinal cholinergic interneurons.[10,11]

The purpose of the current study was to apply two methods to further our understanding of this plasticity in mechanisms of α_2-ARs in alleviating allodynia after nerve injury. First, we compared the pattern of spinal cord neuronal activation from intrathecal clonidine in normal to nerve-injured animals, using immunostaining for the phosphorylated (activated) gene transcription factor, cAMP response element binding protein (pCREB). We chose this marker because of its importance in regulation of many proteins related to nociceptive neurotransmission in the spinal cord and because of its long-lasting and stable expression after sciatic nerve injury.[12] There is controversy over the inhibitory *versus* excitatory effect of α_2-ARs after nerve injury,[13] and we used this approach to ask whether overall cellular activation in the spinal cord was increased or decreased with exposure to clonidine after nerve injury. Second, others have observed cellular immunostaining for α_{2C}- but not α_{2A}-ARs in the spinal cord,[5] and we sought to determine whether some of these α_{2C}-ARs were directly expressed on spinal cholinergic interneurons, as a mechanism for clonidine-induced cholinergic activation.

Materials and Methods

After obtaining approval from the Animal Care and Use Committee of Wake Forest University School of Medi-

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Received from the Department of Anesthesiology and Center for the Study of Pharmacologic Plasticity in the Presence of Pain, Wake Forest University School of Medicine, Medical Center Boulevard, Winston-Salem, North Carolina. Submitted for publication August 14, 2002. Accepted for publication November 13, 2002. Supported in part by grant Nos. GM35523 and NS41386 from the National Institutes of Health, Bethesda, Maryland.

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cine (Winston-Salem, North Carolina), 18 male rats (Harlan Sprague-Dawley), weighing 250–300 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.

Surgical Procedures

All surgical procedures were performed with halothane anesthesia (2–3% in oxygen). First, a 32-gauge polyurethane catheter was inserted intrathecally to the level of the lower lumbar space as previously described.[14] Only animals without evidence of neurologic deficit after catheterization and demonstrating bilateral hind limb weakness after injection of 500 μg intrathecal lidocaine 1 day later were studied. In 9 rats, the right sciatic nerve was exposed at mid-thigh level, and half of the diameter was transected in a ventrocranial direction, as previously described.[15]

Experimental Protocol

The study was divided into two parts. The first part included three groups without sciatic nerve surgery that received 10 or 30 μg intrathecal saline or clonidine (n = 3 per group). The second part comprised the three groups with injured sciatic nerve. These animals also received, 3 weeks after nerve injury, 10 or 30 μg intrathecal saline or clonidine (n = 3 per group). Injections and behavioral testing were always performed between 9:00 and 12:00 AM. For testing, rats were placed in individual plastic boxes on a mesh floor, which allowed access to their hind paws, and were allowed to accommodate for 30 min. A series of calibrated von Frey filaments (Stoelting Co., Wood Dale, IL) were applied perpendicularly to the plantar surface of the hind paws 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.[16] Each trial was repeated twice at approximately 2-min intervals, and the mean value was used as the withdrawal threshold. A third group of normal animals was prepared without testing only for immunohistochemistry for α_{2C}-AR immunoreactivity.

pCREB Immunohistochemistry

Animals were deeply anesthetized with 170 mg/kg pentobarbital and were intracardially perfused with cold phosphate-buffered saline (PBS) containing 1% sodium nitrite and subsequently with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The L4–L5 spinal cord was removed and postfixed in the same fixative for 2–3 h. Then, all tissues were transferred to 30% sucrose in 0.1 M phosphate buffer at 4°C for cryoprotection. Spinal cord tissue was then frozen and cut on a cryostat at a 40-μm thickness. The free-floating sections were collected in PBS. After being pretreated with 0.3% hydrogen peroxide and 10% normal goat serum (NGS; Vector, Burlingame, CA), sections were incubated for 36 h at 4°C in a rabbit polyclonal anti-pCREB (phosphoserine 133) antibody (1:1,000; Sigma, St. Louis, MO) diluted in PBS containing 0.3% Triton X-100 (PBS+T) and 3% NGS. Subsequently, the sections were incubated in biotinylated goat antirabbit immunoglobulin G (Vector) and further processed using an Elite Vectastain ABC kit (Vector) according to the instructions of the manufacturer. Between each incubation, sections were washed in PBS+T (15 min × 2). Finally, the immunoprecipitates were developed by 3,3'-diaminobenzidine, and the chromogen was enhanced by the glucose oxidase–nickel–3,3'-diaminobenzidine method.[17]

Five L4–L5 spinal cord sections randomly selected from each rat were used. Images of both ipsilateral and contralateral dorsal horns of nerve-injured rats or from the dorsal horn of normal rats were captured at ×250 magnification using a charge-coupled device camera. In a given area (390 × 246 mm) that covered laminae I and II or laminae III and IV and was located in the middle one third of the mediolateral extent of the dorsal horn, the number of pixels occupied by pCREB cells on each side was measured automatically using image analysis software (SigmaScan; Jandel Scientific Inc., San Rafael, CA). All sections were processed simultaneously using the same brightness and contrast settings and threshold for image analysis.

Double Immunofluorescent Staining of α_{2C} Immunoreactivity and ChAT Immunoreactivity

Normal rats were used to examine the colocalization of α_{2C} immunoreactivity and ChAT immunoreactivity in the spinal dorsal horn. Forty-micrometer-thick spinal cord sections were incubated in 10% NGS in 0.1 M PBS+T for 2 h and then in a mouse monoclonal anti-ChAT (1:500; Chemicon, Temecula, CA) diluted in PBS+T containing 10% NGS. The incubation lasted for 18 h at 4°C. After thorough rinsing, sections were then incubated in a goat antimouse immunoglobulin G conjugated with Alexa Fluor 488 (1:400; Molecular Probes, Eugene, OR) diluted in PBS+T containing 10% NGS for 1 h. After rinsing thoroughly with PBS+T, sections were further incubated in guinea pig polyclonal antibody raised against $-\alpha_{2C}$ (1:500; Neuromics, Minneapolis, MN) for 18 h at 4°C and then in biotinylated goat anti–guinea pig immunoglobulin G (Vector) for 1 h. Finally, sections were incubated in StrepAvidin conjugated with Alexa Fluor 568 (1:400; Molecular Probes). Between each incubation, sections were rinsed thoroughly with PBS+T. Eventually, sections were coverslipped with antifading mounting material (Vector) and observed under a laser confocal microscope (LSM 510; Carl Zeiss Microscopy,

Jena, Germany). Controls included immunostaining with the absence of one or both primary antibodies.

Drugs

Undiluted lidocaine (Xylocaine, 50 mg/ml; AstraZeneca, Westborough, MA) was injected intrathecally in 10 μl. Clonidine (Sigma) was diluted in saline and was injected intrathecally in a 15-μl volume followed by 15 μl saline to flush the catheter.

Statistical Analysis

Data are presented as mean ± SE. Behavioral analysis comparisons were performed using Kruskal-Wallis followed by the Dunn test. To compare the mean number of pixels occupied by pCREB immunoreactive cells between two locations of the dorsal horn, a paired *t* test or a one-way analysis of variance followed by Tukey *post hoc* test were performed. $P < 0.05$ was considered statistically significant.

Results

Withdrawal threshold was 24 ± 2.7 g in animals without sciatic nerve section. Withdrawal threshold decreased from 23 ± 4.5 g before surgery to 5.5 ± 2.7 g after sciatic nerve injury ($P < 0.05$). Intrathecal clonidine increased withdrawal threshold 60 min after injection in animals with sciatic nerve injury to 13 ± 6.4 g after 10 μg and 22 ± 4.5 g after 30 μg ($P < 0.05$).

pCREB immunoreactivity was localized in nuclei in the dorsal horn (fig. 1). In normal animals, 10 μg intrathecal clonidine increased the number of pCREB-immunoreactive nuclei in both superficial and deep laminae of the lumbar dorsal horn (fig. 2). The effect of the larger dose, 30 μg, was numerically similar to the 10-μg dose in both areas but differed from intrathecal saline control only in the deep laminae (fig. 2). Sciatic nerve injury resulted in an increase in the number of pCREB-immunoreactive nuclei bilaterally in the deep lumbar dorsal horn and a numerical increase that was only statistically significant on the contralateral side in the superficial dorsal horn (fig. 2). In contrast to normal animals, intrathecal clonidine did not increase the number of pCREB-immunoreactive nuclei in the spinal cord of animals after sciatic nerve section. Clonidine had no effect on pCREB immunoreactivity in the superficial laminae and decreased, in a dose-independent fashion, pCREB immunoreactivity in deep laminae (fig. 2).

In the spinal dorsal horn of normal rats, ChAT-immunoreactive neuronal profiles were localized throughout all laminae (fig. 3, top). These ChAT-immunoreactive neuronal profiles in the superficial laminae were all of small size, while those in deeper laminae were of relatively large size. α_{2C}-immunoreactive axons with varicosities were abundantly distributed in the superficial laminae, whereas α_{2C}-immunoreactive neuronal profiles were only seen in the deeper laminae (IV–V) of the dorsal horn (fig. 3, middle). Numerous ChAT-immunoreactive neuronal profiles in the superficial dorsal horn were apposed by abundant α_{2C}-immunoreactive axons in the superficial layers, while some ChAT-immunoreactive neuronal profiles in the deeper layers also coexpressed α_{2C} immunoreactivity (fig. 3, bottom).

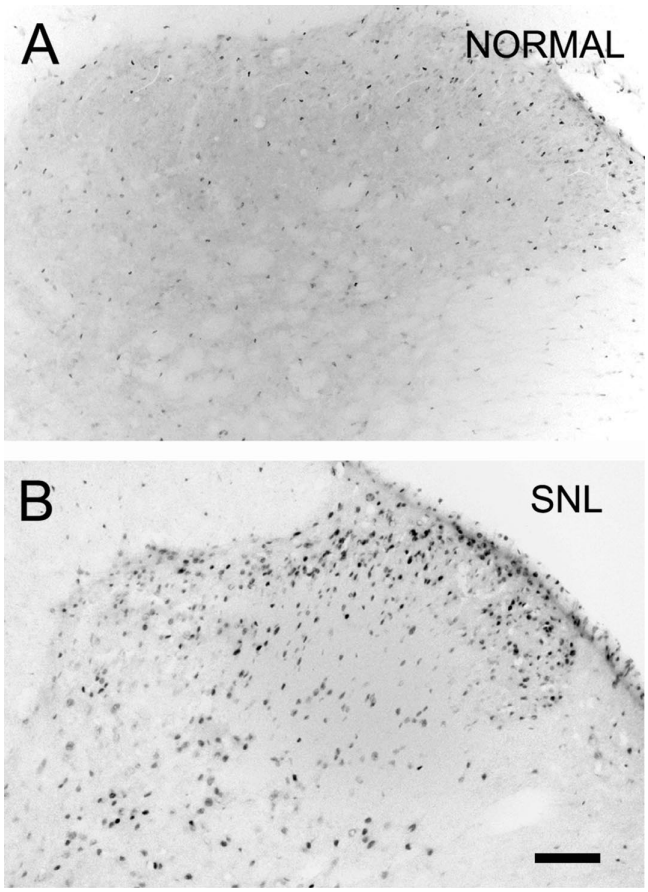

Fig. 1. Phosphorylated cAMP response element protein (pCREB) immunostaining in dorsal horn of normal and partial sciatic nerve–injured (PSNL; ipsilateral to injury) spinal cord. Note dense nuclear staining, in both superficial and deep dorsal horn, and increase in number of immunostained elements in the injured animal. Bar = 50 μm.

Discussion

Clonidine is the most commonly used adjunct to or replacement for morphine for intrathecal infusion in the treatment of chronic pain[18] but is rarely used in the perioperative period because of side effects associated with the larger doses required in this setting. The current study suggests that the pattern of spinal neuronal activity induced by clonidine is altered in the setting of peripheral nerve injury as a model of chronic neuropathic pain and adds to a series of observations to explain this shift in clonidine's potency and efficacy.

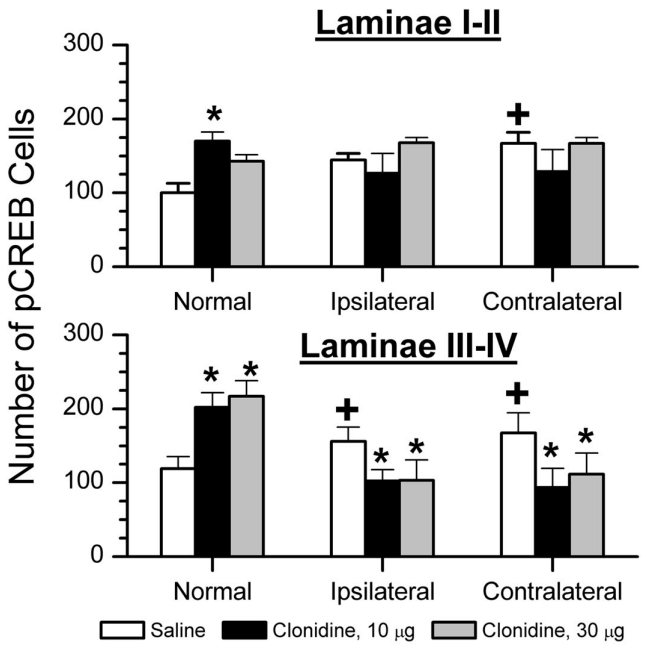

Fig. 2. Number of immunostained nuclei for phosphorylated cAMP response element protein (pCREB) per section in the superficial laminae (*top*) or deep dorsal horn laminae (*bottom*) of L4 and L5 lumbar cord in tissue from normal animals or that ipsilateral or contralateral to sciatic nerve injury. Each bar represents the mean ± SE of three animals. *$P < 0.05$ compared to intrathecal saline. +$P < 0.05$ compared to normal animal.

Utility of the Study of pCREB Immunoreactivity to Spinal Nociceptive Neurotransmission

There are two reasons to use pCREB immunoreactivity to study effects of analgesics. First, neuronal activation in the spinal cord, either by drug or noxious stimuli, results in intracellular Ca^{2+} entry and altered gene transcription. For this reason, the intermediate-early gene, cfos, which controls transcription of several genes, increases expression in the spinal cord during noxious stimuli, and drug-induced reduction in cfos expression occurs at dose ranges similar to those resulting in behavioral antinociception.[19,20] Increased intracellular Ca^{2+}, through calmodulin and other mechanisms, also results in phosphorylation of CRE,[21] as measured in the current study. pCREB regulates expression of multiple peptides important to nociceptive neurotransmission in the spinal cord, including somatostatin,[22] dynorphin,[23] and enkephalin.[24] Second, pCREB expression increases not only after acute noxious stimulation,[25] but also with manipulations known to induce central sensitization, such as chronic morphine exposure,[26] opioid withdrawal,[27] and sciatic nerve injury.[12] As opposed to cfos, which only transiently increases its expression after nerve injury, pCREB expression is maintained for weeks during the period of chronic allodynia, and is reduced by manipulations that reduce allodynia during this period.[12,28] Thus, pCREB expression in the spinal cord can reflect acute neuronal excitation as well as chronic sensitization processes important to allodynia.

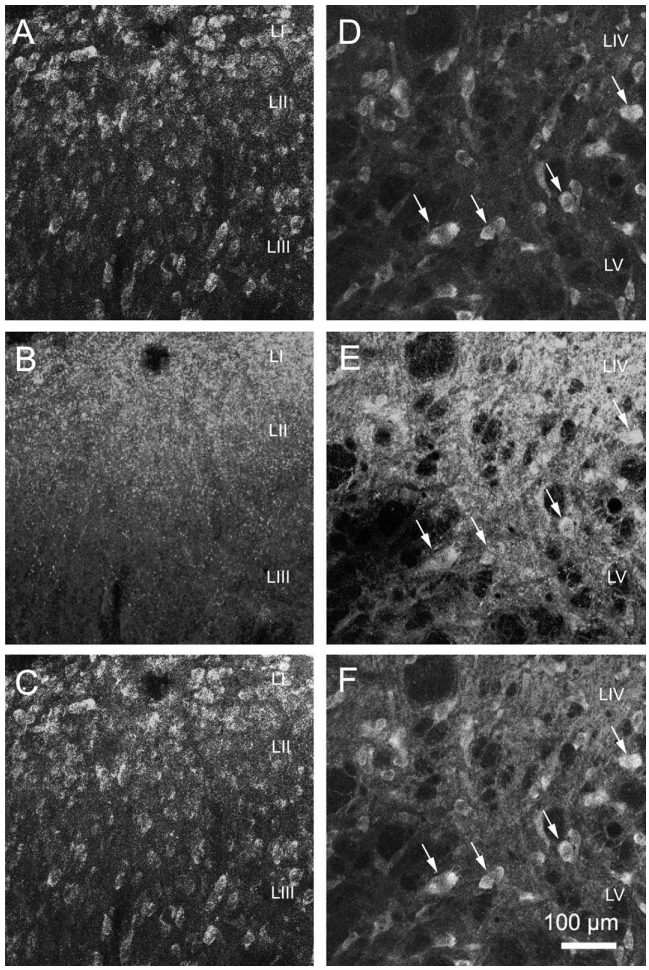

Fig. 3. Confocal photomicrographs of double immunofluorescent staining of ChAT (*A* and *D*) and α_{2C} (*B* and *E*) in the dorsal horn of a normal rat. Abundant ChAT-immunoreactive neurons were observed in the superficial laminae (*A*) and deeper laminae (*B*). α_{2C}-immunoreactive axons were concentrated in the superficial laminae, but no obvious cell profiles were observed there (*B*). Some α_{2C}-immunoreactive neurons were seen in the deeper laminae of the dorsal horn (*E*). ChAT-immunoreactive axons were apposed by abundant α_{2C}-immunoreactive axons in the superficial laminae (*C*). ChAT immunoreactivity and α_{2C} immunoreactivity were colocalized in some neurons in the deeper laminae (*F*, arrows). Scale bar = 100 μm. L = laminae.

Clonidine-induced Changes in pCREB Immunoreactivity

pCREB expression is regulated primarily by increases in intracellular Ca^{2+}, reflecting neuronal activation. A strength of the method is its ability to capture a snapshot of neuronal activity as it is influenced by drug exposure and, in this case, nerve injury. A weakness is that the functions of these neuronal profiles is unknown. They most likely represent a mixture of populations of cells with inhibitory, excitatory, and unrelated actions on nociceptive transmission and regulation. Colocalization studies can indicate the neurotransmitters synthesized by these cells, but, given the large number and diffuse location of these cells (more than 100 per section in specified areas of the superficial and deep laminae),

quantification of degree of colocalization would be extremely difficult. We therefore chose to restrict our study to the overall pattern or neuronal activation, as has been most commonly performed in the past.

In normal animals, intrathecal clonidine increased pCREB immunoreactivity in both superficial and deep laminae in a dose-independent manner in a dose range known to be antinociceptive to acute noxious heat stimuli.[29] We speculate that some of these cells are inhibitory neurons. For one reason, they are associated with antinociception, as noted. For another, intrathecal clonidine reduces spinal cord metabolism and blood flow,[30] as one would expect with net inhibition. Indeed, intrathecal bupivacaine, which is associated with reduced spinal cord metabolism, increases cfos expression in the spinal cord, consistent with increased activity of inhibitory neurons.[31] However, the nature of the neurons that were activated, as measured by CREB phosphorylation, is not determined by the current study.

In contrast, intrathecal clonidine decreased pCREB-immunoreactive nuclei in the spinal cord of animals with sciatic nerve injury. We have previously shown that pCREB immunoreactivity increases bilaterally in lumbar spinal cord after unilateral partial sciatic nerve lesion,[12] as confirmed in the current study. The easiest interpretation is that clonidine stimulates a population of inhibitory neurons and that the inhibition of neuronal activation in this amplified state outweighs the number of neurons activated by clonidine, leading to a net decrease in pCREB immunoreactivity. As in the normal condition, a weakness of using this anatomic approach to identify activated neurons does not distinguish between inhibitory or excitatory interneurons or projection neurons.

Importance of α_{2C}-AR and ChAT Colocalization

Spinal α_2-AR activation stimulates a circuit that results in spinal acetylcholine release, and this effect is essential for clonidine's relief of allodynia after nerve injury.[11] In addition, there is a shift in the potency of α_2-AR subtype–preferring antagonists to reverse clonidine's effect after nerve injury, consistent with anatomic changes showing a reduction in α_{2A}- but not α_{2C}-ARs.[6,7] Although α_{2C}-AR immunoreactivity was previously noted on some cells in the spinal cord dorsal horn,[5] this is the first colocalization study to determine the nature of these neurons. Our results clearly indicate that a proportion of them are cholinergic.

α_2-ARs are classically thought to be inhibitory, due to negative coupling with adenylyl cyclase. Under certain agonist concentrations or G protein receptor density conditions, however, they can also stimulate this enzyme and be excitatory.[32] Interestingly, the α_{2C}-AR subtype induces this excitation in a wider variety of conditions and to a larger extent than the α_{2A}-AR subtype.[33] In addition, iontophoretically applied clonidine excites many dorsal horn cells in electrophysiologic studies.[34] Thus, we hypothesize that intrathecal clonidine stimulates α_{2C}-ARs on cholinergic interneurons, leading to direct excitation and acetylcholine release. Technical factors precluded us in the current study from triple labeling with pCREB, α_{2C}-AR, and ChAT to directly test this hypothesis.

In summary, the pattern of intrathecal clonidine–induced cellular activation in the spinal cord, as measured by pCREB immunoreactivity, in normal animals, where especially deep laminar increases in expression occur, differs from that after nerve injury–associated allodynia, where decreases in expression occur. In addition, at least some cholinergic interneurons directly express the α_{2C}-AR subtype. These differences in the pattern of spinal neurons influenced by intrathecal clonidine reinforce previous observations regarding a shift in efficacy and potency of this agent for treatment of neuropathic pain and suggest that direct activation of cholinergic neurons by clonidine may occur.

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