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An Obligatory Role for Spinal Cholinergic Neurons in the Antiallodynic Effects of Clonidine after Peripheral Nerve Injury

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Background: **Indirect evidence supports a role of spinal cholinergic neurons in tonically reducing response to noxious mechanical stimulation and in effecting analgesia from α_2-adrenergic agonists. This study directly assessed the role of cholinergic neurons in regulating the level of mechanical allodynia and in participating in the antiallodynic effect of the clinically used α_2-adrenergic agonist, clonidine, in an animal model of neuropathic pain.**

Methods: **Allodynia was produced in rats by ligation of the left L5 and L6 spinal nerves. Rats received a single intrathecal injection of saline or one of three different doses of the cholinergic neurotoxin, ethylcholine mustard aziridinium ion (AF64-A; 2, 5, and 15 nmol). Seven days later, allodynia was assessed before and after intrathecal injection of 15 μg clonidine. The spinal cord was removed, and spinal cord acetylcholine content, cholinergic neuron number and distribution, and α_2-adrenergic receptor expression were determined.**

Results: **AF64-A administration reduced both the number of cholinergic cells and the acetylcholine content of the lumbar dorsal spinal cord by 20–50% but did not affect level of mechanical allodynia. AF64-A did, however, completely block the antiallodynic effect of clonidine. AF64-A did not reduce α_2-adrenergic ligand binding in dorsal lumbar cord.**

Conclusions: **These data suggest that spinal cholinergic tone does not affect the level of mechanical allodynia after peripheral nerve injury. There is a quantitative reliance on spinal cholinergic neurons in the allodynia relieving properties of intrathecal clonidine, and this reliance does not depend on α_2-adrenergic receptors colocalized on spinal cholinergic interneurons.**

THERE is a high density of cholinergic as well as α_2-adrenergic ligand binding sites in the most superficial layers of the dorsal spinal cord, the site of entry of nociceptive signals.[1,2] Cholinergic ligands produce antinociception after intrathecal injection,[3] and intrathecal injection of atropine decreases threshold to response from noxious mechanical stimulation,[4] suggesting a role for tonic release of acetylcholine to determine perception of pain from mechanical stimulation. In the abnormal setting of perceived pain from a normally nonpainful stimulus (allodynia) that accompanies peripheral nerve injury, it is conceivable that the level of spinal cholinergic neuronal activity may also determine the response to mechanical stimulation. One purpose of this study is to determine the role of ongoing spinal cholinergic activity on allodynia after peripheral nerve injury.

α_2-Adrenergic agonists produce behavioral analgesia both in humans and animals after intrathecal administration. Clonidine, the clinically available α_2-adrenergic agonist, is more effective against neuropathic than acute nociceptive pain in humans[5] and is more effective against hypersensitivity states induced by peripheral nerve injury than against acute nociceptive stimulation in normal animals.[6]

Indirect evidence suggests a spinal cholinergic interaction in α_2-adrenoceptor–mediated antinociception. Thus, antinociception to acute noxious thermal stimulation from intrathecal clonidine is partially blocked by atropine and potentiated by physostigmine.[7] Similarly, the reduction in mechanical allodynia after peripheral nerve injury by intrathecal clonidine is reduced by muscarinic and nicotinic antagonists.[6] Finally, intrathecal clonidine increases cerebrospinal fluid concentrations of acetylcholine in animals[8] and humans,[9] and local administration of clonidine into the spinal cord dorsal horn *via* microdialysis increases efflux of acetylcholine in the microdialysate.[10]

Although these studies are consistent with a cholinergic link in spinal α_2-adrenergic agonist–induced analgesia, their interpretation is complicated by the nonspecific effects of antagonists at high concentrations, presynaptic and postsynaptic receptor actions, and the lack of localization of site of action. Because there is no descending cholinergic innervation of the spinal cord (see Nuseir *et al.*[11]), the source of spinal acetylcholine release from α_2-adrenergic agonists is presumably from spinal cholinergic interneurons. Another purpose of the current study was to test the hypothesis that there is a direct relation between the antiallodynic effect of intrathecal clonidine after peripheral nerve injury and the number and function of spinal cholinergic interneurons. To do so, we used a highly specific cholinergic neurotoxin that has been used extensively for studies in the brain.[12–14] A secondary purpose was to determine if there was a direct link between α_2-adrenergic receptors and spinal cholinergic neurons by assessing the influence of cholinergic neuron destruction on specific α_2-adrenergic receptor binding in the spinal cord.

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Methods

Animals

After obtaining approval from the Animal Care and Use Committee of Wake Forest University School of Medicine (Winston-Salem, NC), male rats (Harlan Sprague-Dawley), weighing 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 (2–3% in 100% O_2). The left L5 and L6 spinal nerves were isolated under a surgical microscope and ligated tightly with 5-0 silk suture, as previously described.[15] Animals were allowed to recover for 6–8 days before intrathecal catheterization (PE-10 tubing) as previously described.[16] Intrathecal catheters were advanced 7.5 cm caudal 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 catheterization were studied. All animals received saline or the cholinergic toxin 18–21 days after initial L5–L6 spinal nerve ligation.

Ethylcholine Mustard Aziridinium Ion Synthesis

The cholinergic neurotoxin monoethylcholine mustard aziridinium ion (AF64-A) was synthesized as previously described.[17] Briefly, acetylethylcholine mustard hydrochloride (Research Biochemicals, Inc., Natick, MA) at a concentration of 0.4 nM, was brought to pH 11.5 by addition of 10 N NaOH and maintained at this pH for 30 min while stirring at a constant temperature of 25°C. The pH was then lowered to 7.4 with concentrated hydrochloric acid and stirred for 20 min, and this solution was stored on ice until use (always within 1 h). As previously recommended,[13] 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%.

Experimental Protocol

Rats were randomly assigned to four groups receiving a single intrathecal injection of 5 μl saline or 2, 5, or 15 nmol AF64-A in 5 μl. Because intrathecal administration of AF64-A has not been previously reported, pilot studies were performed to determine the appropriate dose range and tolerability of this agent. These pilot studies demonstrated that the aforementioned doses reduced spinal cord acetylcholine content without behavioral dysfunction. Higher doses resulted in motor dysfunction or paralysis. The threshold to withdrawal from mechanical stimulation (see below) was assessed after nerve ligation to confirm development of hypersensitivity, then saline or AF64-A was administered. Seven days later, threshold to withdrawal from mechanical stimulation was determined before and 60 min after a probe dose (15 μg) of intrathecal clonidine. The investigator involved in the mechanical threshold assessment was blinded to the drug given to the animals until the end of the experiments.

At the end of testing, animals were deeply anesthetized with halothane (5% in 100% O_2 for 5 min), and the spinal cord was quickly removed. Lumbar spinal cord was dissected and separated into dorsal and ventral parts and then frozen in liquid nitrogen and stored at −70°C until analysis of the acetylcholine concentration (n = 5–9 per group) by high-pressure liquid chromatography. Four or five additional animals in each group were used for spinal cord immunocytochemistry, and 6–17 per group (except for the 15-nmol AF64-A group) were used for radioligand binding.

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.

Behavioral Testing

Behavioral testing was always performed between 9 and 12 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 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. Motor dysfunction was assessed by testing the animal's ability to stand and ambulate in a normal posture and to step with the hind paw.

Acetylcholine Extraction and Measurement

Fifty to 150 mg of tissue were homogenized with 400 μl ice-chilled 0.1 M perchloric acid with ethylhomocholine at 2-μM concentration as internal standard by an ultrasonic cell disrupter, then centrifuged at 1,000*g* for 20 min at 4°C. The supernatant was collected and filtered with a 0.2-μm nylon-66 syringe filter. Twenty microliters of each sample was loaded on to high-pressure liquid chromatography. High-pressure liquid chromatog-

raphy was conducted in 15 min by a C18 column (ESA acetylcholine 3, 150 × 3 mm, 5 μM) and 3-cm ESA acetylcholine reactor at 35°C using an electrochemical detector with a flow rate of 0.35 ml/min and a pH 8, mobile phase (35 mM sodium phosphate, 5 mM 1-octanesulfonic acid, 0.1 mM EDTA, 0.1 mM tetramethylammonium chloride) delivered by a high-pressure dual piston pump. Sodium phosphate was purchased from Sigma Chemical Co. (St. Louis, MO), EDTA was purchased from Fisher Scientific Co. (Pittsburgh, PA), 1-octanesulfonic acid was purchased from ACROS Co. (Los Angeles, CA), and tetramethylammonium chloride was purchased from Aldrich Chemical Company, Inc. (Milwaukee, WI). Ethylhomocholine was prepared in the laboratory as described above.

Immunocytochemistry

Four or five animals in each group were deeply anesthetized with 50 mg/kg pentobarbital intraperitoneally and perfused *via* the left ventricle with ice-cold 0.9% saline, 400 ml, followed by perfusion with 400 ml ice-cold 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The spinal cords of the rats were removed, postfixed in 4% paraformaldehyde at 4°C for 2 h, then cryoprotected in 10% and then in 30% sucrose in 0.1 M phosphate buffer, each for 48 h. Only the lumbar spinal cord was used for the study. The specimens were sectioned with a freezing-sliding microtome at a thickness of 50 μM and collected in the wells of an enzyme-linked immunosorbent assay 24-well plate in a cryoprotectant solution and stored at 4°C.

All of the immunocytochemical procedures were performed on free-floating sections at room temperature in 0.1 M phosphate-buffered saline pH 7.4, unless otherwise specified. Sections were washed for 40 min, incubated for 15 min in a blocking solution containing 2% normal goat serum, and then incubated for 96 h at 4°C in a polyclonal antibody against choline acetyltransferase (ChAT; Chemicon, Temecula, CA), diluted at 1:2,000 followed by a 90-min incubation with biotinylated rabbit anti-rat immunoglobulin G (Vector Laboratories, Burlingame, CA; dilution 1:250) and a 75-min incubation with ABC Elite complex (Vector Laboratories). Sections were rinsed for 20 min in 0.1 M phosphate-buffered saline and for 10 min in a sodium actate solution, pH 7.0. Then they were incubated for 3 min in sodium acetate, pH 7.0, containing 0.04% 3,3 diaminobenzidine and 0.06% nickel chloride, and 0.006% H_2O_2 was added, and the sections were incubated an additional 6–8 min. After washing, the sections were mounted, air dried, and coverslipped after dehydration in permount (Sigma). Between successive steps, sections were washed four times for 10 min in 0.1 M phosphate-buffered saline, pH 7.4. For each run, control sections were prepared separately by incubating with no primary and/or secondary antibodies and also with rabbit immunoglobulin G at the same dilution (1:2,000) as used for the primary antibody.

Sections were viewed using a Zeiss Axioplan 2 microscope (Carl Zeiss, Inc., Thornwood, NY) at the magnification of 10× and 20×. Five or six sections of the L4 spinal level were analyzed for each animal, and the average number of ChAT-positive neurons from these sections was used for data analysis. Images from sections were digitized at 10× magnification and stored for later analysis. ChAT-immunoreactive cells were manually drawn on a layer from the previously digitized images, and the number of cells was counted on these layers. Spinal sections were divided into four areas corresponding to the right and left dorsal and ventral horns. The L4 lumbar level was chosen to be included in the volume of diffusion of AF64-A and to be just above the level of the spinal nerve ligation (L5 and L6). The investigator performing the counting of ChAT-immunoreactive cells was blinded to treatment group.

[^{3}H]-Rauwolscine Binding

Examination of α_2-adrenergic receptor density in spinal cord was performed with radioligand binding, using [^{3}H]-rauwolscine.[19] Preliminary experiments were performed to optimize binding conditions for pH, buffer components, time, temperature, and protein concentration. Spinal cords from rats from each group were quickly collected after euthanasia and stored at −70°C until used. Cords from three to six animals per group were prepared together. All tissue was stored for 1–8 weeks. Tissue was thawed and quickly chopped, then suspended in buffer containing 50 mM Tris, 10 mM NaCl, 2 mM EDTA, at pH 7.4, 4°C, wt/vol 1:10. Tissue was homogenized (Polytron, setting 7 for 5 s) then disrupted by sonication (5–7 s). The suspension was centrifuged at 1,000*g*, 4°C, for 20 min. The supernatant was then centrifuged at 48,000*g* for 30–40 min (4°C). The pellet was resuspended in the same buffer and disrupted by sonication for 5 s.

Radioligand binding experiments were performed with 200-μl aliquots of tissue (final volume milliliter incubation), increasing concentrations of [^{3}H]-rauwolscine from 100 pM to 4 nM. Nonspecific binding was defined with the presence of 10 μM phentolamine. Serotonin receptors were blocked by addition of 100 nM 8-OH-DPAT and 100 nM TFMPP[20] (Sigma Chemical Co.). Tissues were incubated at room temperature for 1 h and then terminated by vacuum filtration over GF/B filters on a cell harvester (Brandel, Gaithersburg, MD) with three washes of 4 ml cold 50 mM Tris buffer. All experiments were performed in duplicate.

Radioactivity bound was quantified by immersion of filters in 10 ml scintillation fluid counted in a scintillation counter. Saturation curves and Scatchard-Rosenthal transformation plots were constructed. Protein concentrations were determined by the method of Bradford.[21]

Statistical Analysis

Data are presented as median and interquartile or mean ± SE as appropriate. Behavioral analysis, acetylcholine concentrations, and number of ChAT-immunoreactive cells comparisons were performed using Kruskal-Wallis or analysis of variance followed by Dunn or Bonferroni tests, respectively. Correlation between acetylcholine concentration and the number of ChAT-positive cells was determined by linear regression. Correlation between these variables and the effect of intrathecal clonidine on withdrawal threshold was determined by multiple linear regression. $P < 0.05$ was considered statistically significant.

Results

Intrathecal administration of 2 and 5 nmol AF64-A produced no effect on food and water intake, general behavior, or motor function. Two animals in the 15-nmol AF64-A group presented a delayed paraplegia 2 days after the administration of AF64-A and were not studied. The remaining animals in the 15-nmol AF64-A group showed normal behavior.

Behavior

All animals developed allodynia after spinal nerve ligation. Withdrawal thresholds were comparable among all groups before surgery and 7 days after intrathecal catheterization, before injection of the study drug (fig. 1). Neither intrathecal administration of saline nor AF64-A altered allodynia, which remained unchanged in all groups for 7 days after intrathecal injection (P = ns). In the saline-treated group, 15 μg intrathecal clonidine increased the withdrawal threshold to 80% of the presurgical value (fig. 1). In contrast, pretreatment with AF64-A dose-dependently reduced the antiallodynic effect of clonidine at 60 min (fig. 1).

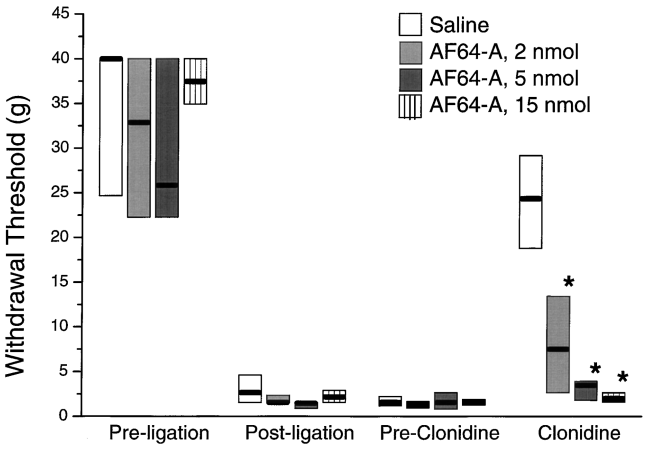

Fig. 1. Withdrawal threshold to mechanical stimulation of the left hind paw before spinal nerve ligation surgery (Preligation), 18–21 days after surgery and before intrathecal administration of saline or AF64-A (Postligation), 7 days after saline or AF64-A (Preclonidine), and 60 min after 15 μg intrathecal clonidine (Clonidine) in groups receiving saline (open bars) or AF64-A (2 nmol in light gray, 5 nmol in dark gray, 15 nmol in striped bars). Data presented as median (dark lines) and 25th and 75th percentiles (vertical extent of bars) of 5–17 animals. *$P < 0.001$ *versus* saline group.

Immunocytochemistry

Choline acetyltransferase immunoreactivity was distributed in the spinal cord as previously described,[22] with greatest density in laminae II and III, but also in the deeper parts of the dorsal horn, surrounding the central canal and in the ventrolateral area corresponding to motoneurons (fig. 2). Many neurons in deeper dorsal laminae were triangular or bipolar, oval shaped cells sending projections to lamina II and III, which were rich in small varicosities. After left L5 and L6 spinal nerve ligation, there was no difference in the number of ChAT-immunoreactive cells between the left and right sides of either dorsal or ventral horn (fig. 2).

Choline acetyltransferase–immunoreactive staining was reduced in both dorsal and ventral horns after AF64-A treatment. All three doses of AF64-A reduced the number of ChAT-immunoreactive cells in dorsal and ventral horn (fig. 3).

Acetylcholine Concentration

There was no difference between dorsal and ventral spinal cord in the acetylcholine tissue content in any group (table 1). AF64-A treatment resulted in a reduction in acetylcholine content in both dorsal and ventral spinal cord (table 1).

Correlation Analysis

There was a close correlation between the number of ChAT-positive cells in the dorsal horn and the content of acetylcholine in the spinal cord ($P < 0.01$, $R^2 = 0.92$).

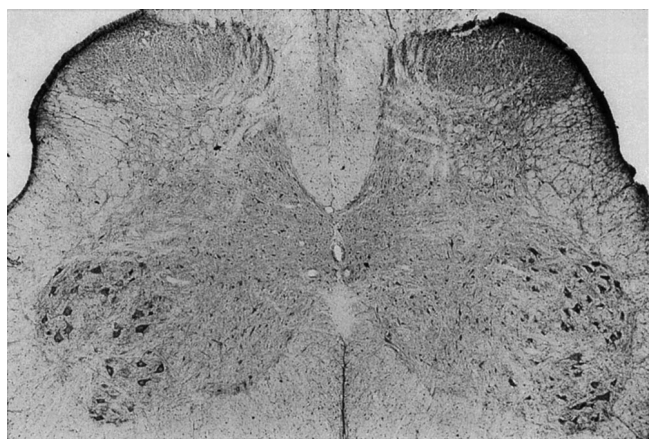

Fig. 2. Low-power photomicrograph of L4 spinal cord section immunostained for choline acetyltransferase, demonstrating motoneuron staining in ventral horn and multiple stained cells and processes in superficial and deep dorsal horn. Note the lack of unilateral changes in this cord from an animal after spinal nerve ligation.

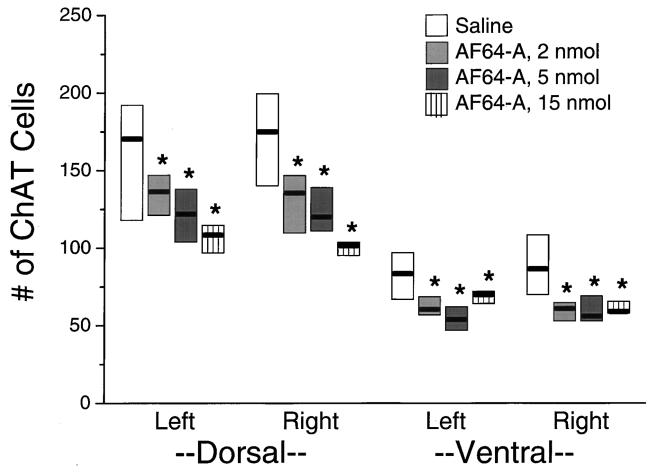

Fig. 3. Number of choline acetyltransferase (ChAT)-immunostained neurons from the L4 spinal level from animals receiving intrathecal saline (open bars) or AF64-A (2 nmol in light gray, 5 nmol in dark gray, 15 nmol in striped bars). Data are presented as median (dark lines) and 25th and 75th percentiles (vertical extent of bars) of 4–5 animals. *$P < 0.001$ *versus* saline group.

Multiple linear regression analysis revealed a significant ($P < 0.001$) effect of either acetylcholine content or number of ChAT-positive dorsal horn neurons on the effect of intrathecal clonidine, and a combination of these variables closely predicted ($R^2 = 0.98$) the effect of intrathecal clonidine. In other words, the more acetylcholine content or ChAT-immunopositive cells were reduced by AF64-A treatment, the more the antiallodynic effect of clonidine was diminished.

[^{3}H]-Rauwolscine Binding

Preliminary experiments with [^{3}H]-rauwolscine demonstrated 75–85% specific binding. Serotonin blocking agents were used to prevent significant rauwolscine binding to these receptors. Equilibrium of binding was present from 40–120 min of incubation. Binding was linearly related to protein concentration from 50–500 μg protein.

Saturation and Rosenthal plot analysis revealed a range of K_D and B_{max} values. Data from normal and spinal nerve–ligated animals were compared and collapsed when no difference in [^{3}H]-rauwolscine binding was found (table 2). Radioligand binding was not performed in the 15-nmol AF64-A group. [^{3}H]-Rauwolscine does not differentiate binding between α_2-adrenergic receptor subtypes. Best fit curves revealed a predominance of one receptor subtype. B_{max} was increased 15% in the 5-nmol AF64-A group compared with the other groups, indicating an apparent increase in α_2-adrenergic receptor expression (fig. 4).

Table 1. Concentration of Acetylcholine in the Dorsal and Ventral Parts of the Lumbar Spinal Cord

	Dorsal Hemicord (pmol/mg Tissue)	Ventral Hemicord (pmol/mg Tissue)
Saline	3.09 ± 0.18	3.98 ± 0.55
2 nmol AF64-A	2.47 ± 0.12	2.46 ± 0.36
5 nmol AF64-A	2.68 ± 0.26	1.93 ± 0.28*
15 nmol AF64-A	1.84 ± 0.36*	1.30 ± 0.19*

Results are mean ± SE. Acetylcholine concentration in the dorsal and ventral parts of the lumbar spinal cord was measured by high-pressure liquid chromatography. Placebo: nine animals; 2 nmol AF64-A: six animals; 5 nmol AF64-A: seven animals; 15 nmol AF64-A: five animals.

* $P < 0.05$ *versus* saline.

Table 2. [^{3}H]Rauwolscine-specific Binding in Lumbar Spinal Cord Homogenates

	K_D (nmol)	B_{max} (fmol/mg Protein)
Saline	2.54	124 ± 11
2 nmol AF64-A	2.47	119 ± 10
5 nmol AF64-A	1.97	140 ± 11*

Results are from three experiments (4–7 animals per group) performed in duplicate. Data are calculated by nonlinear regression analysis. All data fit a single binding site better than multiple site fit.

* $P < 0.05$ *versus* saline.

Discussion

The present study provides unique data concerning the role of tonic acetylcholine release in the spinal cord on the maintenance of mechanical allodynia after spinal nerve ligation, the strong dependence on spinal cholinergic function for the antiallodynic action of intrathecal clonidine, and the lack of presence of α_2-adrenergic receptors on spinal cholinergic neurons themselves. Each of these carries important implications regarding

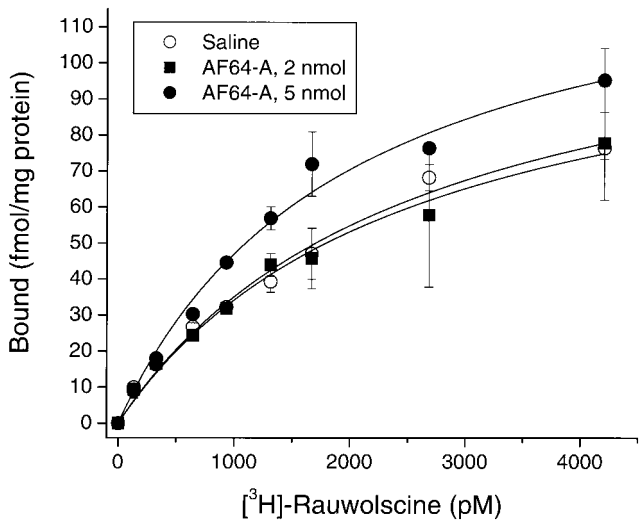

Fig. 4. Saturation curve of specific [^{3}H]-rauwolscine binding. Control group includes data of both normal and spinal nerve ligation animals (n = 4–7 per group). Data presented are mean ± SE of three experiments performed in duplicate. Where SE bars are not seen, SE bars are smaller than symbol size. No apparent difference between the control and 2-nmol AF64-A groups is seen. The 5-nmol AF64-A group shows a 15% increase in binding.

the maintenance and mechanisms of effective treatment of allodynia in this animal model of neuropathic pain. We previously demonstrated an increase in acetylcholine concentration in cerebrospinal fluid in animals and humans after intraspinal clonidine administration[8,23] and a spinal source in the dorsal horn for clonidine-induced acetylcholine release, as determined in microdialysis experiments.[10] Although a positive correlation exists between cerebrospinal fluid acetylcholine concentrations and the degree of analgesia from intraspinal clonidine in humans,[23] this association does not prove causality.

We previously demonstrated that intrathecal atropine produced a complete blockade, and intrathecal mecamylamine a partial blockade, of the antiallodynic effect of intrathecal clonidine after spinal nerve ligation.[6] Interpretation of antagonist studies can be clouded by nonspecific effects, uncertain distribution after intrathecal administration, activation of opposing actions at several sites, and presynaptic receptor actions. Of course, use of a chemical to destroy a class of neurons also has limitations—nonspecific toxicity, local inflammation resulting in altered physiology and pharmacology, and drug distribution affecting neuronal populations affected. However, neurotoxin experiments provide important complementary evidence for the relevance of a neuronal class to pharmacologic action. The current study provides the first evidence that the antiallodynic effect of intrathecal clonidine is directly related to the presence of functioning spinal cholinergic interneurons.

Methodologic Issues

The cholinergic neurotoxin, AF64-A, has not previously been administered intrathecally. In the brain, this agent is known to induce chronic cholinergic hypofunction.[12–14] When administered intraventricularly or intracerebroventricularly in rats, AF64-A produces an irreversible inhibition of ChAT[13,24] and of other enzymes of choline metabolism. Direct hippocampal injection of 2 nmol AF64-A in rats reduces acetylcholine concentrations by 43% after 5 days.[12] In the current study, using a different method of measurement, we observed a reduction of the acetylcholine concentration of the same order of magnitude (approximately 40 and 60% in the dorsal and ventral parts of the lumbar spinal cord, respectively) 7 days after intrathecal administration of 5 nmol AF64-A.

The behavioral alterations induced by intracerebroventricular AF64-A mainly involve acquisition and learning tasks.[14] Doses up to 8 nmol of intracerebroventricular AF64-A induce neither mortality nor motor deficit in rats.[12,14] We observed no toxicity from this agent administered intrathecally at doses of 2 and 5 nmol, but 2 of 10 animals presented delayed paraplegia 2 days after intrathecal injection of 15 nmol, a timing similar to that of mortality observed with doses greater than 25 nmol administered directly into the hippocampus[12] and likely reflecting spinal motoneuron destruction. Thus, it is highly unlikely that the inhibition of the effects of intrathecal clonidine from the lower doses of AF64-A reflects a nonspecific toxicity.

It has been suggested that AF64-A also displays toxicity against noradrenergic neurons, since norepinephrine concentrations in hippocampus decrease after direct local injection of AF64-A.[25] However, this decrease is transient[26] and is usually attributed to a denervation phenomenon secondary to the reduction of the tonic cholinergic output.[27,28] Noradrenergic neurotoxicity as an explanation for the blockade by AF64-A of the effect of intrathecal clonidine is unlikely, first because there are no noradrenergic neurons in the spinal cord, and second because noradrenergic denervation of the spinal cord increases, not decreases, the analgesic effect of intrathecal clonidine.[29]

Anatomic Changes

The location of ChAT neurons observed in the current study is similar to that previously described.[22,30] The high level of ChAT immunoreactivity in the most external laminae of the dorsal horn containing many varicosities of small size (0.2 μm) probably corresponds to cholinergic axon terminals.[22,31] As compared with the number of ChAT-immunoreactive neurons found at the cervicothoracic junction previously described,[32] we observed slightly more ChAT-immunoreactive neurons in the lumbar cord, although we observed a similar dorsal-to-ventral ratio, and this minor discrepancy could result from use of different antibodies.

Peripheral nerve injury or inflammation results in multiple plastic changes in neurophysiology and neuroanatomy in sensory afferents and in the spinal cord. Although most attention has focused on sprouting of noradrenergic fibers in the dorsal root ganglion[33] and Aß fibers from deep to superficial dorsal horn,[34] it is conceivable that part of the increased reliance of intrathecal clonidine on cholinergic mechanisms observed after peripheral nerve injury could reflect altered spinal cholinergic anatomy. This study found no evidence at the light microscope level to support such an anatomic basis for this plasticity in analgesic pharmacology. Thus, the number of cholinergic cells at the L4 level was unaffected by spinal nerve ligation, and there was no asymmetry of cell number after unilateral spinal nerve ligation.

Spinal Cholinergic Activity and Allodynia

It has been suggested in normal animals that there is a tonic spinal release of acetylcholine that elevates the nociceptive threshold to mechanical stimulation[4] and that the effect of acetylcholine depends on nitric oxide synthesis.[35] Furthermore, increases in threshold to cutaneous stimulation after noxious visceral stimulation are considered to partly be a result of increased spinal acetylcholine release.[36] Because allodynia after spinal nerve ligation depends on ongoing afferent activity from the

injury site,[37] it is conceivable that spinal acetylcholine could increase after spinal nerve ligation, thereby reducing the amount of allodynia.

In contrast to normal animals, we observed no reduction in withdrawal threshold to a mechanical stimulus in animals rendered allodynic after spinal nerve ligation from blockade of spinal muscarinic receptors by intrathecal atropine[6] or by destruction of cholinergic interneurons (current report). Whereas one could argue that the ability to observe even more drastic allodynia than is present in this model is limited, these two studies suggest that either tonic acetylcholine release is suppressed after spinal nerve ligation or tonic acetylcholine release does not affect allodynia in this model of peripheral nerve injury.

Location of α_2-Adrenergic Receptors Involved in Stimulating Acetylcholine Release

α_2-Adrenergic receptors were first localized in the superficial dorsal horn of the rat using standard radioligand binding techniques.[38] Since that time, three subtypes of the α_2-adrenergic receptor have been described. Whereas the RNA message for all three subtypes (α_{2A}, α_{2B}, and α_{2C}) exist in human cord, there appears to be relatively little α_{2B} message in rat cord.[39] More recently, use of subtype selective antibodies have suggested that most of the α_{2A} receptors exist on the central terminals of primary afferents, whereas α_{2C} receptors are on neurons or processes intrinsic to the cord.[40] Extracellular recordings from dorsal horn neurons that respond to nociceptive stimulation have demonstrated excitatory as well as inhibitory response to α_2-adrenergic receptor stimulation.[41] Because local administration of clonidine increases acetylcholine release in the spinal cord,[42] one possibility is that there are excitatory α_2-adrenergic receptors on cholinergic interneurons. The current study does not support this hypothesis, since there was a dose-dependent reduction in the antiallodynic effect of intrathecal clonidine, correlating tightly with the reduction in acetylcholine content and number of cholinergic neurons, but no decrease in total α_2-adrenergic receptor number. We cannot exclude the possibility that a small fraction of total spinal α_2-adrenergic receptors exist on cholinergic neurons, and hence their destruction would be missed in examination of total binding.

In conclusion, there is a close association between the amount of spinal cholinergic neuronal destruction from intrathecal AF64-A and the decrease in efficacy of intrathecal clonidine to reduce allodynia after spinal nerve ligation. These data suggest that tonic cholinergic activity is either reduced or unimportant in the degree of allodynia after this injury. Furthermore, the efficacy of intrathecal clonidine, currently approved for treatment of neuropathic pain, may depend exclusively on cholinergic function in the spinal cord.

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