

# Perineural $\alpha_2$ -Adrenoceptor Activation Inhibits Spinal Cord Neuroplasticity and Tactile Allodynia after Nerve Injury

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**Background:** Nerve injury in animals increases  $\alpha_2$ -adrenoceptor expression in dorsal root ganglion cells and results in novel excitatory responses to their activation, perhaps leading to the phenomenon of sympathetically maintained pain. In contrast to this notion, peripheral  $\alpha_2$ -adrenoceptor stimulation fails to induce pain in patients with chronic pain. We hypothesized that  $\alpha_2$  adrenoceptors at the site of nerve injury play an inhibitory, not excitatory role.

**Methods:** Partial sciatic nerve ligation was performed on rats, resulting in a reduction in withdrawal threshold to tactile stimulation. Animals received perineural injection at the injury site of clonidine, saline, or clonidine plus an  $\alpha_2$ -adrenergic antagonist, and withdrawal threshold was monitored. Immunohistochemistry was performed on the sciatic nerve ipsi- and contralateral to injury and on the spinal cord.

**Results:** Clonidine reduced this hypersensitivity in a dose-dependent manner, and this was blocked by an  $\alpha_{2A}$ -preferring antagonist. Perineural clonidine injection had a slow onset (days) and prolonged duration (weeks). Systemic or intrathecal clonidine, or transient neural blockade with ropivacaine, had short lasting or no effect on hypersensitivity.  $\alpha_{2A}$ -adrenoceptor immunostaining was increased near the site of peripheral nerve injury, both in neurons and in immune cells (macrophages and T lymphocytes). Phosphorylated cAMP response element binding protein (pCREB) in lumbar spinal cord was increased ipsilateral to nerve injury, and this was reduced 1 week after perineural clonidine injection.

**Conclusions:** These data suggest that peripheral  $\alpha_2$  adrenoceptors are concentrated at the site of peripheral nerve injury, and their activation receptors produce long-lasting reductions in abnormal spinal cord gene activation and mechanical hypersensitivity.

THIS study addresses a curious paradox regarding the role of peripheral adrenoceptors in pain perception. Whereas all agree that  $\alpha$ -adrenergic stimulation or activation of the sympathetic nervous system has minimal effect on pain perception in the normal condition,<sup>1</sup> these manipulations excite peripheral afferents and induce nocifensive behavior in animals after nerve injury.<sup>2</sup> Sympatholysis reduces pain in some patients with chronic pain, further supporting a pronociceptive role

for peripherally released norepinephrine. In addition, nerve injury causes postganglionic sympathetic efferents to sprout and surround dorsal root ganglion (DRG) cells, these cells increase expression of  $\alpha_2$  adrenoceptors, and they respond to  $\alpha_2$ -adrenergic agonist exposure with excitation rather than inhibition.<sup>3</sup> Based on these studies, it has been proposed<sup>4</sup> that plasticity of sympathetic efferents and increased  $\alpha_2$ -adrenoceptor expression on afferents contribute to pain in patients with causalgia.

In contrast to this hypothesis generated in animal models, there is no evidence to support a pronociceptive action of peripheral  $\alpha_2$ -adrenoceptor activation in patients with chronic pain. Although subcutaneous norepinephrine induces pain in some patients, topical application of the selective  $\alpha_2$ -adrenergic agonist, clonidine reduces rather than exacerbates pain.<sup>5</sup> Epidural injection of clonidine, which bathes the nearby DRG with high concentrations of drug, relieves rather than induces pain in patients with sympathetically maintained pain.<sup>6</sup> These studies suggest that  $\alpha_2$  adrenoceptors on afferent terminals or DRG cell bodies are unlikely to induce pain in humans with peripheral nerve injury and pain.  $\alpha_2$ -Adrenoceptor immunostaining is increased in rats at the site of nerve injury,<sup>7</sup> and the success of perineural clonidine injection to relieve pain in a patient with nerve injury-induced pain (personal communication, Patricia Lavand'homme, M.D., Department of Anesthesiology, Universite Catholique de Louvain, Brussels, Belgium, September, 2000), suggested that this may be an important site of analgesic action of these compounds. We therefore investigated perineural application of clonidine at the site of injury in rats with mechanical hypersensitivity induced by partial sciatic nerve ligation.<sup>8</sup> To examine the effect of perineural clonidine on central plasticity, we examined its effect on expression of phosphorylated cAMP response element binding protein (pCREB), known to increase chronically after nerve injury and to control genes involved in sensory transduction in the spinal cord.<sup>9</sup>

## Materials and Methods

After receiving Animal Care and Use Committee approval at each institution, male Wistar rats were prepared for behavioral (Universite Catholique de Louvain) or immunohistochemical (Wake Forest University) studies. Briefly, under halothane anesthesia, the right sciatic nerve was exposed at the proximal thigh level and liberated from surrounding tissues. One-third to one-half of

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the nerve was then tightly ligated with silicon-treated silk suture (Prolene 7-0) as previously described,<sup>8</sup> then muscle and skin layers were closed. The animals were allowed to recover for 4–6 weeks, at which time hypersensitivity to light touch was clearly evident. To investigate the effect of intrathecal clonidine, lumbar spinal catheters were inserted in some animals through an incision in the cisternal membrane as previously described.<sup>10</sup>

#### *Behavioral Testing*

Sensitivity to mechanical stimulation was assessed by application of calibrated von Frey filaments (Stoelting, Wood Dale, IL). Animals were placed on a plastic mesh floor in individual clear boxes. After accommodation to the environment, von Frey filaments were applied vertically to the plantar surface of the ligated hind paw and pressed to the point of bending 3 to 4 times over approximately 2 s. If no response was elicited, a larger filament was applied in the same manner. The filaments were applied in increasing order until a brisk withdrawal or paw flinching occurred, which was considered a positive response. This withdrawal threshold was determined three times, with testing separated by 5–10 min, and the mean withdrawal threshold was used for data analysis.

#### *Drug Administration*

For perineural injection, rats were briefly anesthetized with 4% halothane in 100% oxygen by inhalation. Percutaneous sciatic nerve block was performed on the injured side by introducing a 25-gauge needle between the greater trochanter and the ischial tuberosity as previously described.<sup>11</sup> A total volume of 0.3 ml was injected in a fanning motion along the path of the sciatic nerve. Animals received either perineural injection of saline ( $n = 6$ ) or clonidine, 3  $\mu\text{g}$  ( $n = 9$ ), 15  $\mu\text{g}$  ( $n = 8$ ), or 30  $\mu\text{g}$  ( $n = 11$ ). Withdrawal threshold to von Frey filament testing was determined 5 h and 1, 3, 7, 10, 14, and 21 days after perineural injection.

Additional studies examined the site and mechanism of action of clonidine. To determine the site of action, both systemic and intrathecal controls were performed. In these studies, animals received perineural saline in addition to either intramuscular clonidine, 30  $\mu\text{g}$  ( $n = 11$ ) or intrathecal clonidine ( $n = 12$ ). Intrathecal clonidine was administered in a cumulative manner, with total doses of 7.5, 15, and 30  $\mu\text{g}$  and injections separated by 30 min. Because the duration of inhibition of mechanical hypersensitivity from intrathecal clonidine after nerve injury has been demonstrated to be approximately 3 h,<sup>12</sup> a 21 day time course was not performed in these animals. To determine the receptor type activated by clonidine, animals received perineural clonidine, 30  $\mu\text{g}$  alone or with  $\alpha_{2A}$ -preferring adrenergic antagonist, BRL44408,<sup>13</sup> 5  $\mu\text{g}$ , 15  $\mu\text{g}$ , and 45  $\mu\text{g}$  ( $n = 6$  in each group). To

determine the role of transient neural blockade, animals received perineural ropivacaine, 1,500  $\mu\text{g}$  ( $n = 11$ ).

#### *Immunohistochemistry*

Four weeks after nerve injury and 1 week after perineural injection of saline or clonidine, 30  $\mu\text{g}$ , rats were anesthetized with sodium pentobarbital and perfused intracardially with cold phosphate buffered saline (PBS) containing 1% sodium nitrite followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). The L4–L5 spinal cord segments and the ipsilateral and contralateral sciatic nerve segments at both proximal and mid-thigh levels were removed and postfixed in the same fixative for 3–6 h. Then all tissues were transferred to 30% sucrose in 0.1 M PB at 40°C for cryoprotection.

The spinal cord segments were cut on a cryostat at a 40  $\mu\text{m}$  thickness. The free-floating sections were collected in PBS. After being pretreated with 0.3% hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) and 10% normal goat serum (NGS, Vector Laboratories, Burlingame, CA), sections were incubated for 36 h at 40°C in a rabbit polyclonal antiphospho-CREB antibody (1:1,000, New England Biolabs, Beverly, MA, USA) diluted in phosphate buffered saline containing 0.3% Triton-X 100 (PBS + T) and 3% NGS. Subsequently, the sections were incubated in biotinylated goat antirabbit IgG (Vector) and further processed using Elite Vectastain ABC kit (Vector) according to the instructions of the manufacturer. Between each incubation, sections were washed in PBS + T (15 min, twice). Finally, the immunoprecipitates were developed by 3, –3' diaminobenzidine and the chromogen was enhanced by the glucose oxidase-nickel-3, –3' diaminobenzidine method.<sup>14</sup>

Four sciatic nerve segments, ipsilateral at proximal thigh level (containing ligation), contralateral at proximal thigh level, ipsilateral at mid-thigh level, and contralateral at mid-thigh level, were mounted on the same block using O.C.T. mounting media. Sections were cut at 10  $\mu\text{m}$  thickness, thaw mounted on precleaned Superfrost-plus slides (Fisher Scientific, Pittsburgh, PA) and kept at –80°C until used. After pretreatment in 0.1%  $\text{H}_2\text{O}_2$  and 10% NGS in 0.1 M Tris buffered saline (TBS), sections were incubated in a polyclonal rabbit antibody directed against  $\alpha_{2A}$ -adrenergic receptor (generously provided by Robert Elde, Ph.D., Dean of Neuroscience, University of Minnesota, Minneapolis, MN). Subsequently, the sections were incubated in biotinylated goat antirabbit IgG (Vector) and further processed using Elite Vectastain ABC kit (Vector) according to the instructions of the manufacturer. Finally, the immunoprecipitates were developed by the glucose oxidase-nickel-diaminobenzidine method described previously. Between each incubation, sections were washed in TBS containing 0.05% + T (15 min, twice).

### Quantification of pCREB-Immunoreactive (IR) Cells in the Dorsal Horn

Four normal rats, four nerve injured rats, and four nerve injured rats 1 week after perineural injection of clonidine, 30  $\mu$ g were used for quantification. Five L4–L5 spinal cord sections were randomly selected from each rat. Images of both ipsilateral and contralateral dorsal horns of nerve injured rats or from the dorsal horn of normal rats were captured at  $\times 250$  magnification using a CCD camera. The number of digitized pixels overlaid pCREB-IR cells and the number of pCREB-IR cells in the fixed area on each side of the dorsal horn was measured automatically using image analysis software (SigmaScan, Jandel Scientific Inc., San Rafael, CA). In a fixed area ( $235 \mu\text{m} \times 235 \mu\text{m}$ ) that covered the entire LI to LIII in depth and was located in the middle one-third of the mediolateral extent of the dorsal horn, a threshold at which all pCREB-IR cells were overlaid by digitized pixels was applied to all sections measured. To examine possible changes in expression of pCREB within cells, the mean optical density of all positive (supra-threshold) objects was compared.

### Double Staining of $\alpha_{2A}$ -Adrenoceptor IR with ED1-IR, CD2-IR, or S100-IR

To determine if  $\alpha_{2A}$ -adrenoceptor IR cells in injured sciatic nerve also coexpress ED1, a marker for macrophages, CD2, a marker for mature T lymphocytes, or S100, a marker for Schwann cells, 10  $\mu\text{m}$  thick sciatic nerve sections were used. Sections were incubated in 10% NGS in 0.1 M TBS for 2 h and then in a rabbit polyclonal anti- $\alpha_{2A}$ -adrenoceptor antibody 1:1,000 diluted in TBS + T containing 10% NGS. The incubation lasted for 18 h at 40°C. After thorough rinsing, sections were incubated in a goat antirabbit IgG conjugated with Alexa Fluor 488 (1:400, Molecular Probes, Eugene, OR) diluted in TBS containing 10% NGS for 1 h. After rinsing with TBS + T, sections were further incubated in either a mouse monoclonal antibody raised against ED1 (1:500, Serotec, Raleigh, NC), against CD2 (1:50, Serotec), or a mouse monoclonal antibody raised against S100A1 (1:100, DAKO Corp., Carpinteria, CA) for 18 h at 40°C and then in biotinylated goat antimouse IgG (Vector) for 1 h. Sections were then incubated in StreptAvidin conjugated with Alexa Fluor 568 (1:400, Molecular Probes). Between each incubation, sections were rinsed thoroughly with TBS + T. Finally, sections were coverslipped with antifading mounting material (Vector) and examined under a laser confocal microscope (LSM 510, Carl Zeiss Microscopy, Jena, Germany).

### Statistical Analysis

Values are expressed as mean  $\pm$  SEM. Groups were compared using two-way analysis of variance (ANOVA) followed by the Dunnett or Fisher LSD test. To compare the mean number of the pixels occupied by pCREB-IR

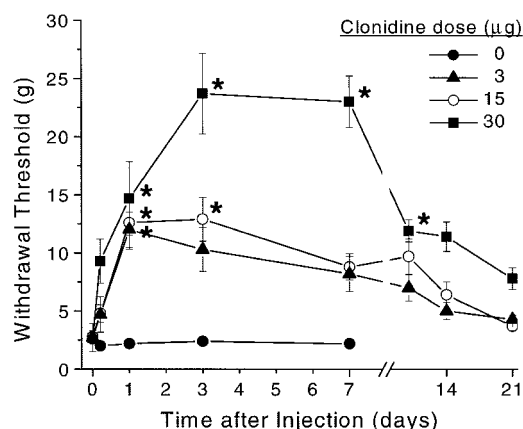


Fig. 1. Effect of perineural clonidine on tactile allodynia after nerve injury. Withdrawal threshold to von Frey filament probing was determined before and after perineural injection, at the site of previous nerve injury, of saline (solid circles) or clonidine, 3  $\mu$ g (solid triangles), 15  $\mu$ g (open circles), or 30  $\mu$ g (solid squares). Each point represents the mean  $\pm$  SE of 6–11 animals. \* $P < 0.05$  compared with preinjection value.

cells between two-side dorsal horn, a paired  $t$  test or one-way ANOVA with Student Newman-Keuls multiple comparison method was used.  $P < 0.05$  was considered significant.

## Results

### Behavioral Testing

Four to six weeks after partial sciatic nerve ligation, animals developed hypersensitivity to von Frey filament testing, with paw withdrawal thresholds reduced from  $29 \pm 9.6$  g before surgery to  $2.9 \pm 1.4$  g after surgery ( $P < 0.01$ ). Perineural saline injection did not affect paw withdrawal threshold (fig. 1). In contrast, perineural clonidine injection produced a dose-dependent increase in paw withdrawal threshold (fig. 1). In terms of percent return to prenerve ligation withdrawal threshold, the peak effect of perineural clonidine was  $34 \pm 5.1\%$  after 3  $\mu$ g,  $41 \pm 6.4\%$  after 15  $\mu$ g, and  $77 \pm 9.2\%$  after 30  $\mu$ g. Peak effect occurred between 1 and 7 days after injection of clonidine. Perineural clonidine injection had no other effect on behavior, and animals ambulated normally immediately after recovery from the brief anesthetic for perineural clonidine injection.

Intramuscular injection of clonidine, 30  $\mu$ g, did not affect withdrawal threshold at any time for up to 21 days after injection (withdrawal threshold was  $2.6 \pm 1.8$  g before clonidine and a maximum of  $7.9 \pm 4.3$  g at 24 h after injection;  $P > 0.4$ ). Intrathecal clonidine dose-dependently inhibited mechanical hypersensitivity (withdrawal threshold  $2.8 \pm 0.4$  g before clonidine and  $6.4 \pm 0.9$ ,  $12 \pm 2.2$ , and  $17 \pm 2.1$  g after 7.5, 15, and 30  $\mu$ g, respectively;  $P < 0.05$  for 15 and 30  $\mu$ g). However, the effect of 30  $\mu$ g intrathecal clonidine was not as great as that produced by perineural clonidine, and was

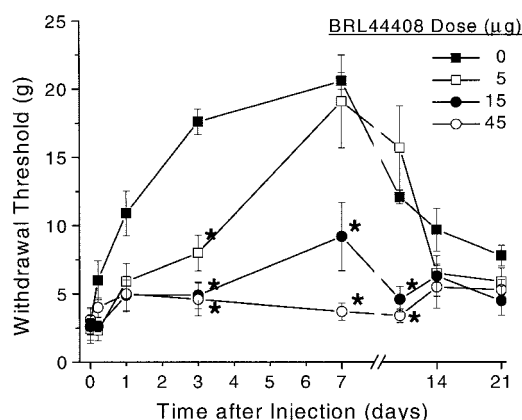


Fig. 2. Reversal of clonidine's effect by BRL44408. Withdrawal threshold to von Frey filament probing was determined before and after perineural injection, at the site of previous nerve injury, of clonidine, 30  $\mu$ g, alone (solid squares) or with the  $\alpha_{2A}$ -adrenoceptor antagonist, BRL44408, 5  $\mu$ g (open squares), 15  $\mu$ g (solid circles), or 45  $\mu$ g (open circles). Each point represents the mean  $\pm$  SE of six animals. \* $P < 0.05$  compared with clonidine alone.

transient, with the withdrawal threshold no different than the control 3 days after injection.

Perineural injection of the  $\alpha_{2A}$ -preferring antagonist, BRL44408, alone did not modify the paw withdrawal

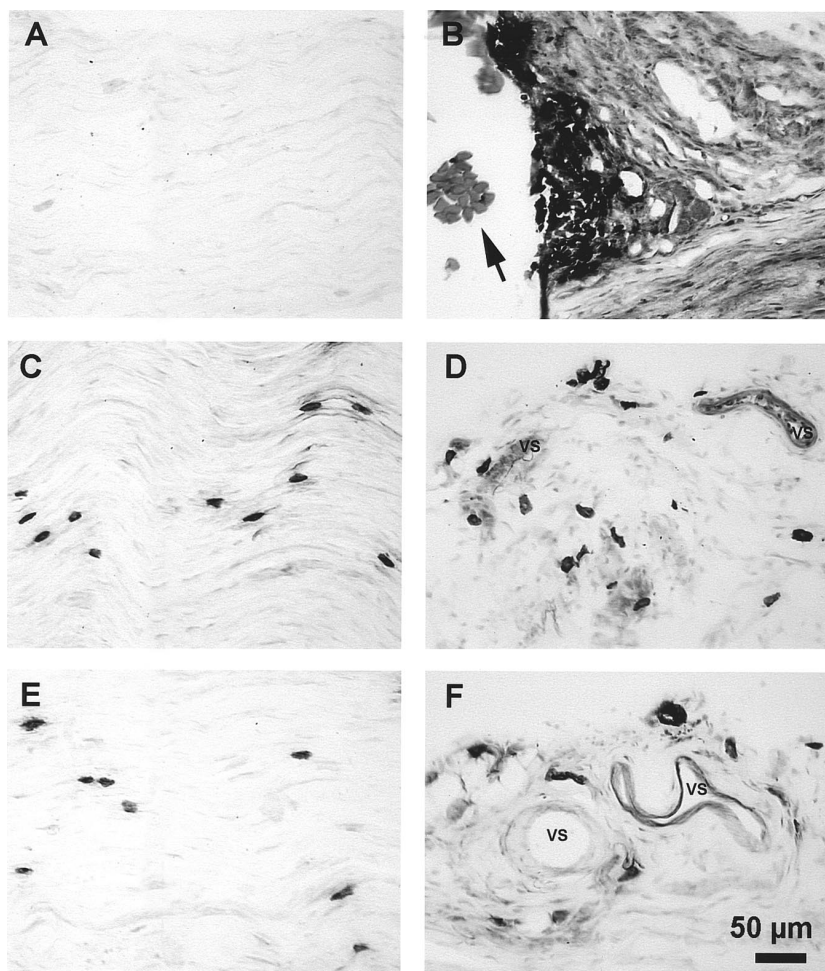
threshold in rats after nerve injury (data not shown). In contrast, BRL44408 produced a dose-dependent blockade of the antihypersensitivity effect of perineural clonidine, 30  $\mu$ g (fig. 2). The lowest dose of BRL44408 studied, 5  $\mu$ g, delayed the onset of clonidine's effect until 7 days after injection, whereas larger doses prevented the effect of clonidine (fig. 2).

Finally, we tested whether transient peripheral nerve blockade would reproduce clonidine's effect. Perineural injection of the local anesthetic, ropivacaine, caused unilateral motor dysfunction in all animals, lasting approximately 1 h, consistent with peripheral nerve blockade. However, the withdrawal threshold was not affected beyond this period (withdrawal threshold was  $3.2 \pm 0.6$  g before ropivacaine injection and a maximum of  $6.6 \pm 0.9$  g at 4 days after injection;  $P > 0.6$ ).

#### $\alpha_{2A}$ -Adrenoceptor Immunoreactivity after Nerve Lesion

In the normal sciatic nerve (fig. 3A), there were no  $\alpha_{2A}$ -adrenoceptor IR profiles in either endoneural or epineural tissues. Two and four weeks after partial sciatic nerve ligation, intensely stained small size  $\alpha_{2A}$ -adrenoceptor IR cell profiles were present at the injury site,

Fig. 3. Photomicrographs of  $\alpha_{2A}$ -adrenoceptor immunoreactivity (IR) cell profiles in the ipsilateral and contralateral sciatic nerve 4 weeks after partial sciatic nerve ligation. (A) No clear  $\alpha_{2A}$ -adrenoceptor IR cell profiles were observed in the sciatic nerve of normal rats. (B) Intensely stained  $\alpha_{2A}$ -adrenoceptor IR cell profiles were accumulated around ligation sutures (arrow). These labeled cells were of small size. Some of these cells were adjacent to small blood vessels. (C)  $\alpha_{2A}$ -adrenoceptor IR cell profiles were also observed in the injured sciatic nerve 1 cm distal to the injury site, distributing relative sparsely among the axons. The nuclei of some cells were clearly discerned. (D)  $\alpha_{2A}$ -adrenoceptor IR cell profiles were also observed in the epineural connective tissues. Note that some cells were close to blood vessels (VS). Interestingly enough, numerous  $\alpha_{2A}$ -adrenoceptor IR cell profiles were also noticed in the contralateral sciatic nerve at proximal thigh level, equivalent to the level of injury site in the contralateral sciatic nerve. (E) similarly, some  $\alpha_{2A}$ -adrenoceptor IR cell profiles were also detected in the epineural connective tissue in the contralateral sciatic nerve at proximal thigh level. (F) some of these cells were closely associated with blood vessels. Scale bar = 50  $\mu$ m.



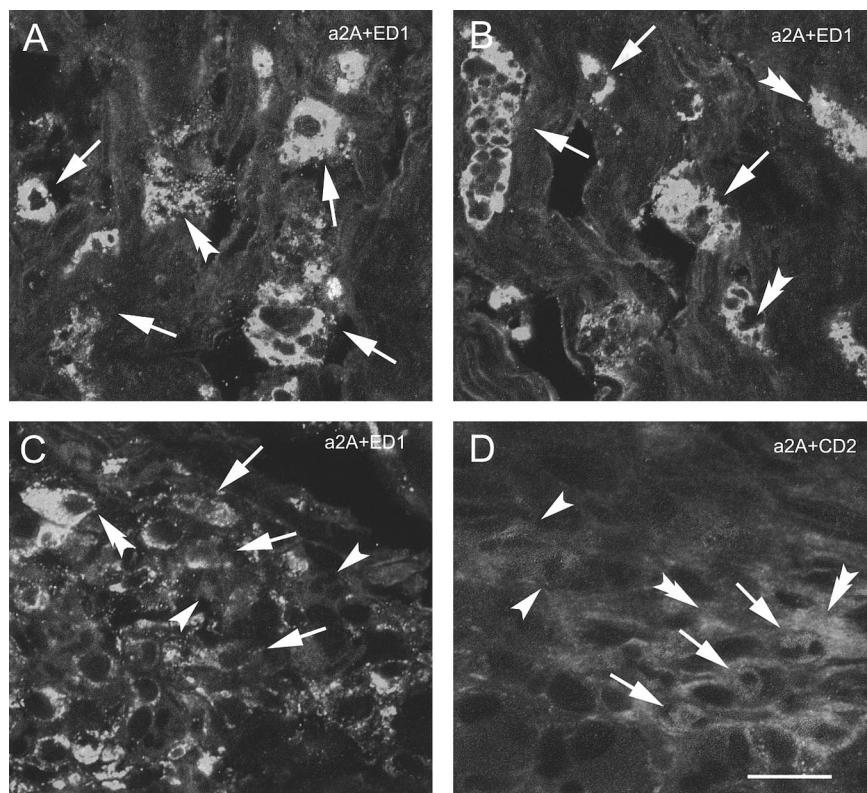


Fig. 4. Color confocal photomicrographs of double immunofluorescent staining for  $\alpha_{2A}$ -adrenoceptors with the macrophage marker ED1 (A, B, C), or with the T lymphocyte marker CD2 (D). Confocal photomicrographs of double immunofluorescent staining of  $\alpha_{2A}$  with the macrophage marker, ED1 (A, B, C), and  $\alpha_{2A}$  with the T lymphocyte marker, CD2 (D). (A and B) show the proximal and distal regions of a partially ligated sciatic nerve. These regions were adjacent to the ligation site. (C) shows the area immediately adjacent to the ligation suture. Note that  $\alpha_{2A}$ -IR was colocalized with ED1-IR in the same cells which exhibit typical macrophage morphology (arrows). Some  $\alpha_{2A}$ -IR (single arrowheads) or ED1-IR (double arrowheads) were singly stained only. (D) shows one area immediately adjacent to the ligation site, in which arrows indicate the coexpression of  $\alpha_{2A}$ -IR and CD2-IR in the same cell populations. Some  $\alpha_{2A}$ -IR (single arrowheads) and CD2-IR (double arrowheads) single-stained cells were also noticed. Scale bar = 20  $\mu$ m.

particularly around the ligation sutures (fig. 3B). Some strongly stained  $\alpha_{2A}$ -adrenoceptor IR cell profiles were also observed in a region of 1 cm proximal and distal to the ligation injury site (fig. 3C). The nuclei of some of these cells could be discerned clearly. However, at the mid-thigh level of the ipsilateral sciatic nerve (2 cm distal to the ligation site), no such strongly stained  $\alpha_{2A}$ -adrenoceptor IR cells were observed (not shown). In the epineural connective tissue, strongly stained  $\alpha_{2A}$ -adrenoceptor IR cells were also observed (fig. 3D). Numerous labeled cells were located around small blood vessels. Interestingly, some intensely stained  $\alpha_{2A}$ -adrenoceptor IR cells also appeared at the proximal thigh level of the contralateral sciatic nerve (fig. 3E). Similarly, some labeled cells were present around the small blood vessels in the epineural tissue (fig. 3F).

To characterize these  $\alpha_{2A}$ -IR cells, we carried out the double immunofluorescent staining of  $\alpha_{2A}$  with the macrophage marker ED1 (fig. 4A–C), with the T lymphocyte marker CD2 (fig. 4D) or with Schwann cell marker S100 (data not shown). In the proximal (fig. 4A) and distal (fig. 4B) areas adjacent to the lesion site, all ED1-IR cells displayed characteristic morphology of macrophages. A majority of these macrophages coexpressed  $\alpha_{2A}$ -IR. In the ligation site (fig. 4C), where both ED1-IR and  $\alpha_{2A}$ -IR cells were most abundant, some cells also coexpress both markers. It is noticeable that some  $\alpha_{2A}$ -IR cells did not coexpress ED1. Interestingly, in the ligation site (fig. 4D) and the proximal and distal areas adjacent to the lesion site, some  $\alpha_{2A}$ -IR cells coexpress T lymphocyte

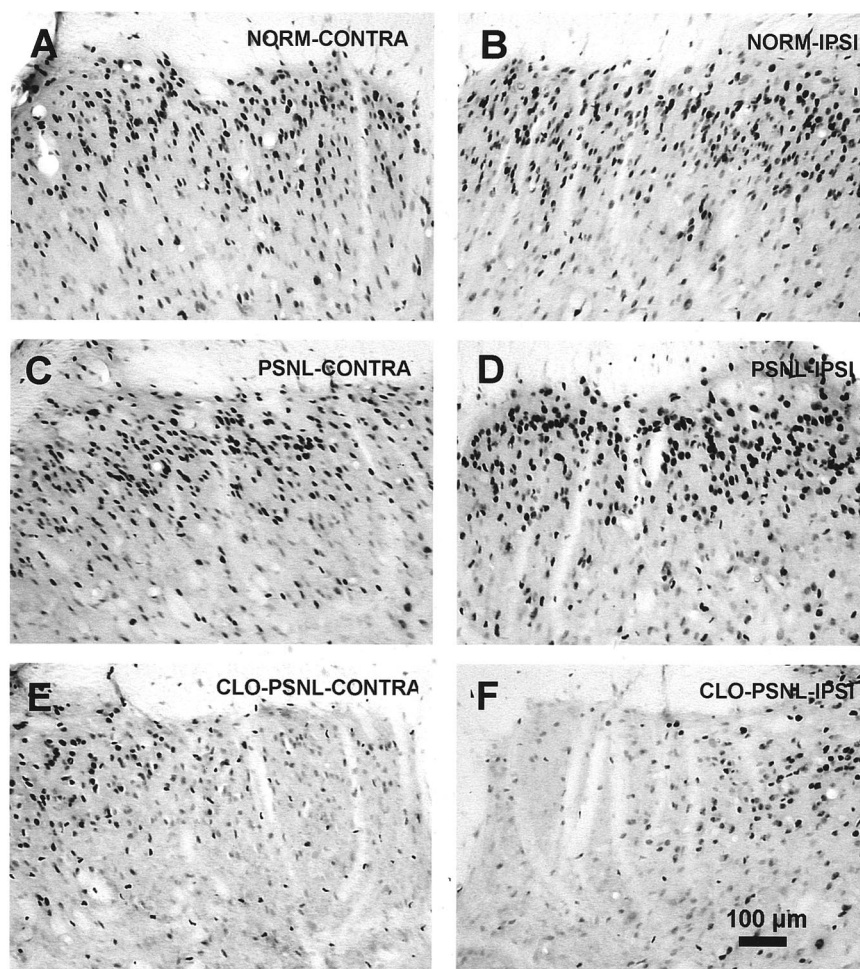
marker CD2-IR, but that majority of  $\alpha_{2A}$ -IR and ED1-IR cells were not colocalized. In contrast, double staining of  $\alpha_{2A}$ -IR and Schwann cell marker S100 revealed that none of S100-IR cells coexpressed  $\alpha_{2A}$ -IR (not shown). Thus, the majority of  $\alpha_{2A}$ -IR cell profiles were likely macrophage (approximately  $\frac{2}{3}$  of costained cells), some of them were T lymphocytes (approximately  $\frac{1}{3}$  of costained cells), and none of them were Schwann cells.

#### *Effect of Perineural Clonidine on Spinal Cord pCREB Expression*

Numerous pCREB-IR cells were observed in the dorsal horn of L4–L6 spinal cord of normal rats (fig. 5A and B). The immunoprecipitates were all localized in the nuclei of these cells. pCREB-IR cells were concentrated in the superficial laminae. Similar to a previous report,<sup>9</sup> compared with the contralateral side (fig. 5C), the number of pCREB-IR cells was significantly increased in the ipsilateral dorsal horn (fig. 5D) 4 weeks after partial sciatic nerve ligation. One week after a single perineural injection of clonidine at the injury site the number of pCREB-IR cells in the contralateral (fig. 5E) and ipsilateral dorsal horn (fig. 5F) was significantly decreased. After a single injection of saline into the injury site of nerve-ligated rats, the number of pCREB-IR cells in the ipsilateral dorsal horn remained significantly higher than the contralateral side (data not shown), similar to nerve-ligated rats without injection.

The mean number of pixels in a fixed area occupied by pCREB-IR cells in the ipsilateral dorsal horn was signifi-

Fig. 5. Photomicrographs of pCREB-immunoreactive (IR) cells in the L4–L6 dorsal horn of a normal rat (A and B), a rat after partial sciatic nerve ligation (C and D) and a rat after partial sciatic nerve ligation and perineural clonidine injection (E and F). In (A and B), numerous pCREB-IR cells were observed in the dorsal horn of L4–6 spinal cord of the normal rat. The immunoreactivities were restricted to the nuclei of these cells. pCREB-IR cells are particularly more abundant in the superficial laminae than in other laminae. Four weeks after nerve injury, the number of pCREB-IR cells in the ipsilateral dorsal horn (D) was markedly increased compared with these in the contralateral side (C). The increase was even more evident in the superficial laminae. Five days after one single injection of clonidine into the injury site of PSNL rats, the number of pCREB-IR cells were dramatically reduced in both ipsilateral (F) and contralateral (E) dorsal horn. The reduction was more prominent in the medial half of the dorsal horn. Scale bar = 100  $\mu$ m.



cantly greater than the contralateral side in perineural saline injected controls or in comparison with normal animals (fig. 6A,  $P < 0.01$ ). However, the mean optical density of pCREB-IR cells in this fixed area was not significantly different between the ipsilateral and contralateral dorsal horn or in comparison with normal animals (fig. 6B). After perineural injection of clonidine in nerve-ligated rats, both the mean number of pixels and the mean optical density in a fixed area occupied by pCREB-IR cells in the dorsal horn were significantly reduced ipsilateral and contralateral to the ligation-injection site compared with controls ( $P < 0.001$ ).

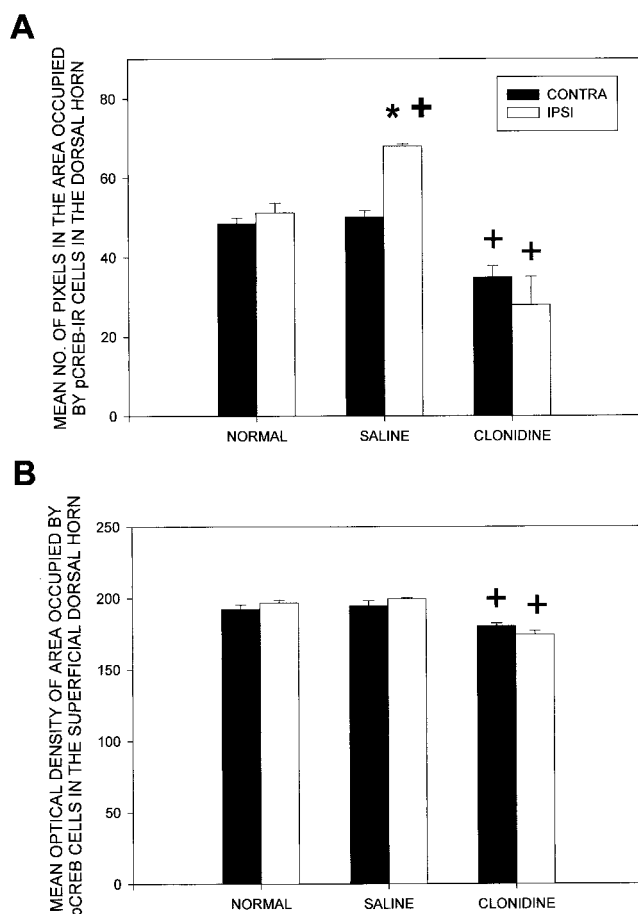
## Discussion

Spinally and epidurally administered  $\alpha_2$ -adrenergic agonists reduce nerve injury-induced hypersensitivity in animals<sup>12</sup> relieve chronic pain in humans,<sup>15</sup> and are commonly used to treat neuropathic pain.<sup>16</sup> Unlike perispinal injection, systemic administration of clonidine provides minimal analgesia in patients with chronic pain,<sup>17</sup> and it is assumed that activation of peripheral  $\alpha_2$ -adrenergic receptors should exacerbate, rather than

alleviate pain.<sup>4</sup> The current studies after nerve injury in rats argue against this assumption, and point to a novel method to treat nerve injury-induced pain.

### *Nerve Injury Site as a Pain Generator*

Many consider the site of nerve injury to be an important "pain generator." Abnormal spontaneous (ectopic) activity originating from this site<sup>18</sup> and in uninjured afferents<sup>19</sup> may drive changes in DRG cells as well as the spinal cord, resulting in ongoing pain and hypersensitivity to sensory stimuli. Blockade of neural activity with perineural injection of local anesthetic reduces mechanical hypersensitivity after spinal nerve ligation,<sup>20</sup> although the effect is brief. It is unlikely that clonidine diminished tactile allodynia in the present study by inhibition of ectopic activity at the site of nerve injury. For one reason, although clonidine is commonly added to local anesthetics for perineural injection, it is inactive alone in the normal animal or human, and prolongs the duration of local anesthetics by a few hours only.<sup>21</sup> For another reason, silencing ectopic activity by local anesthetic injection at the nerve injury site in the current study had only a very transient effect on tactile allodynia.



**Fig. 6.** Quantitative analysis of pCREB-immunoreactive (IR) cells in the dorsal horn of rats with partial sciatic nerve ligation after a perineural injection of saline or clonidine. In (A) the mean number of pixels in an area occupied by pCREB-IR cells in the ipsilateral dorsal horn was significantly higher than in the contralateral side of PSNL rats receiving perineural saline (\*,  $P < 0.01$ ) and than in the normal dorsal horn (+,  $P < 0.01$ ). The value was not significantly different between the contralateral dorsal horn of PSNL rats and normal rats. Five days after a single injection of clonidine into the injury site, the mean number of pixels in an area occupied by pCREB-IR cells in both the ipsilateral and contralateral dorsal horn was significantly decreased compared with their respective counterparts in the dorsal horn of PSNL rats with saline injection or with normal rats (+,  $P < 0.001$ ). In (B), the mean optical density of an area occupied by pCREB-IR cells in the ipsilateral dorsal horn of PSNL rats with saline injection was not significantly different from the contralateral side. However, the mean optical density of an area occupied by pCREB-IR cells in both ipsilateral and contralateral dorsal horn of PSNL rats with clonidine injection was significantly lower than their respective counterparts in the dorsal horn of rats with saline injection or than normal dorsal horn (+,  $P < 0.05$ ). Mean  $\pm$  SEM,  $n = 4$ .

#### Noradrenergic Plasticity and Pain after Nerve Injury

Perl postulates that injury to a mixed peripheral nerve results in increased expression of excitatory  $\alpha_2$  adrenoceptors that maintain hypersensitivity and pain.<sup>4</sup> Approximately 30–50% of C fiber nociceptors are excited by application of norepinephrine after nerve injury in rabbit<sup>2</sup> or rat,<sup>22</sup> whereas few or none are excited in the

normal animal. This excitation is blocked by  $\alpha_2$ , but not by  $\alpha_1$ -adrenergic receptor antagonists.<sup>2</sup>  $\alpha_2$ -Adrenoceptor agonists stimulate DRG cells after nerve injury,<sup>23</sup> but not in normals. Noradrenergic fibers at the site of nerve injury and surrounding DRG cells provide the presumed anatomic substrate for these changes.<sup>3</sup>

However, other data fail to support  $\alpha_{2A}$ -adrenergic receptor mediated excitation. Some  $\alpha_2$ -adrenoceptor agonists stimulate C fiber polymodal nociceptors from rabbit injured nerve, but others, including clonidine, do not.<sup>24</sup> In nerve injured monkeys<sup>25</sup> and Lewis rats,<sup>26</sup> hypersensitivity from norepinephrine is blocked by  $\alpha_1$ -rather than  $\alpha_2$ -adrenoceptor antagonists. Finally, there are no clinical case reports of pain or increased hyperalgesia or allodynia from peripheral or central administration of clonidine in patients with chronic pain.

Only one previous study examined the functional role of  $\alpha$ -adrenergic receptors at the site of nerve injury.<sup>27</sup> In that study, the whole nerve, from injury site to DRG to central projection, was removed and studied *in vitro*. Norepinephrine applied at the nerve injury site excited C and A $\delta$  fibers, and, to a much lesser extent, A $\beta$  fibers, and excitation was blocked by  $\alpha_2$ , but not  $\alpha_1$ -adrenergic receptor antagonists. One would expect that excitation of C and A $\delta$  fibers would be perceived as noxious in the conscious state. In contrast, we observed no evidence of nociception after perineural injection of clonidine after partial sciatic nerve ligation in the current study, and we observed inhibition, rather than excitation of A fiber-mediated mechanical hypersensitivity. These discrepant results could reflect different methods of nerve injury, methods of drug delivery, *in vitro* versus *in vivo* application, or divergent end-points (electrophysiology *vs.* behavior).

#### Sites of Altered $\alpha_2$ -Adrenergic Receptor

##### Immunostaining after Peripheral Nerve Injury

Others have demonstrated increased  $\alpha_2$ -adrenoceptor ligand binding at the site of neuroma formation<sup>28</sup> and a buildup of  $\alpha_{2A}$ -adrenoceptor immunoreactivity at the site of nerve ligation 48 h after injury,<sup>7</sup> but fine anatomic localization, subtype characterization, and identity of adrenoceptor expressing structures have not previously been performed. We demonstrate for the first time that nerve injury results in increased  $\alpha_{2A}$ -adrenergic receptor immunoreactivity at the site of injury several weeks after the insult. Schwann cell origin of the staining can be excluded by lack of colocalization with a specific marker for this cell type. At least some of the immunoreactivity was in nerve fibers, as suggested by staining sandwiched between and parallel to Schwann cell profiles. This is consistent with the increased  $\alpha_{2A}$ -adrenoceptor immunoreactivity observed in DRG cell bodies following this lesion.<sup>29</sup>

In other cases, immunostaining was present on cell profiles with obvious nuclear staining, most likely rep-

representing immune cells. Some of these had foamy vacuole structures resembling macrophages, and this was confirmed by specific labeling with the macrophage marker, ED1. As indicated in figure 5, some of the  $\alpha_{2A}$ -adrenoceptor immunostaining in these macrophages was consistent with ingested cellular debris containing these receptors, and in other cases the receptor staining was on the plasma membrane and likely represents functional receptors. Other cells expressing  $\alpha_{2A}$  adrenoceptors had morphology consistent with lymphocytes, and this was confirmed by specific labeling with the T helper and Natural Killer lymphocyte marker, CD2. Interestingly, these lymphocytes were present not only at the injury site, but both proximal and distal to the site and in the proximal sciatic nerve contralateral to the injury. This is consistent with bilateral changes often observed after unilateral injury (like pCREB expression), and with the known expression of  $\alpha_2$  adrenoceptors by lymphocytes.<sup>30</sup> Sham surgery did not result in new expression of  $\alpha_2$ -adrenoceptor immunostained cells in either contralateral or ipsilateral lesions (Personal Communication, Weiya Ma, Ph.D., Department of Anesthesiology, Wake Forest University School of Medicine, Winston-Salem, NC, March, 2002).

#### *Mechanism of Action of Perineural Clonidine*

Clonidine could reduce hypersensitivity by actions on peripheral nerve terminals, at the injury site, or in the central nervous system. Topical clonidine reduces pain in the surrounding skin in patients with complex regional pain syndrome I,<sup>5</sup> and intrathecal clonidine is analgesic in patients with neuropathic pain, most likely by an action in the spinal cord.<sup>31</sup> In the current study, neither systemic nor intrathecal clonidine mimicked the effect of perineural clonidine on mechanical hypersensitivity. Similarly, perineural injection of a local anesthetic only briefly diminished hypersensitivity. Thus, clonidine potently reduces hypersensitivity after perineural injection by a local action not related to conduction blockade. This is consistent with the observation that the increased potency of  $\alpha_2$ -adrenergic agonists after nerve injury reflects actions outside the central nervous system.<sup>32</sup>

The mechanisms by which clonidine acts at the site of nerve injury are currently uncertain. Clonidine is rapidly absorbed into the systemic circulation following peripheral injection,<sup>33</sup> making a local depot effect unlikely. In addition, the effect of clonidine was reduced in a dose-dependent manner by perineural injection of the  $\alpha_{2A}$ -preferring antagonist, BRL44408, which is most consistent with a local, specific action with delayed consequences.

It is quite possible that perineural clonidine produces a long-lasting and delayed action by affecting immune cell function near the site of nerve injury.  $\alpha_2$ -Adrenergic agonists can reduce cytokine release from monocytes, including TNF- $\alpha$ <sup>34</sup> and such factors sensitize nerve end-

ings and drive central sensitization to innocuous stimuli. T lymphocytes contain norepinephrine, and incubation of lymphocytes with norepinephrine suppresses mitogen-induced proliferation and induces apoptosis.<sup>35</sup> Peripheral and central sensitization and plasticity can also be driven by uptake at the site of nerve injury of nerve growth factors,<sup>36,37</sup> and clonidine could affect either generation, production, or central trafficking of these factors. We propose that clonidine most likely acts by reducing cytokine and growth factor production by immune cells, perhaps in addition inducing apoptosis in some immune cells, thereby reducing centrally transported cytokines and growth factors and reducing central sensitization.

#### *Spinal Cord Consequences of Perineural Clonidine after Nerve Injury*

Phosphorylation of CREB, stimulated primarily by increases in intracellular calcium, alters gene transcription.<sup>38</sup> Nerve injury induces early immediate gene expression *cfos*,<sup>39</sup> as well as somatostatin,<sup>40</sup> dynorphin,<sup>41</sup> and enkephalin,<sup>42</sup> all products important to spinal sensory neurotransmission. However, *cfos* expression wanes in established hypersensitivity, yet pCREB expression remains elevated.<sup>9</sup> For this reason, continued pCREB expression, driven by ongoing abnormal afferent input, may play an important role in maintaining central sensitization after nerve injury. One week after perineural clonidine injection, at the time of maximum reduction in hypersensitivity, pCREB expression was diminished in the spinal cord. Although causality is not proven by these observations, we propose that this central effect on this transcription factor reflects reduced central trafficking of factor(s), which enhance spontaneous activity of afferents and convergent neurons in the spinal cord.

In summary, behavioral and immunohistochemical studies in rats with peripheral nerve injury fail to support the hypothesis that peripheral  $\alpha_2$ -adrenoceptor stimulation causes and maintains pain and hypersensitivity. Rather, they are consistent with a site- and subtype-specific action on novel expressed  $\alpha_{2A}$  adrenoceptors at the site of injury that reduce hypersensitivity. The delayed onset and prolonged duration observed in the patient and in this animal model, accompanied by reduction in a clonidine-induced reduction in a spinal transcription factor, suggest that perineural clonidine acts by altering generation of factors released by immune cells and their central trafficking that regulates and maintains hypersensitivity. These findings support trials of a new approach to treatment of nerve injury-associated pain in humans and investigation of peripherally derived factors altering spinal cord gene expression in models of chronic pain.

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