Neurokinin-1 Projection Cells in the Rat Dorsal Horn Receive Synaptic Contacts from Axons That Possess α_{2C} -Adrenergic Receptors

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The α_{2C} subclass of adrenergic receptor (α_{2C} -AR) mediates some of the antinociceptive actions of norepinephrine in the spinal cord. Axon terminals, which possess this receptor, are concentrated in the superficial dorsal horn and originate from spinal interneurons. We performed a series of combined tract-tracing and immunocytochemical studies to determine whether α_{2C} -AR-immunoreactive axons target projection neurons that possess the neurokinin-1 (NK-1) receptor because such cells are likely to transmit nociceptive information to the brain. Spinomedullary neurons were labeled by stereotaxic injection of the B-subunit of cholera toxin (CTb) into the caudal ventrolateral medulla of three anesthetized adult rats. After 3 d, the animals were anesthetized again and fixed by perfusion. Sections were cut from midlumbar segments and reacted with antibodies to reveal α_{2C} -ARs, CTb, and NK-1 receptors. Retrogradely labeled neurons possessing the NK-1 receptor ($n = 45$) were examined with confocal microscopy to investigate their relationship with α_{2C} -AR-immunoreactive axons. Numerous α_{2C} -AR axons were apposed to cell bodies and proximal dendrites of cells in lamina I and also to distal dendrites that originate from labeled cell bodies in lamina III/IV. A combined confocal and electron microscopic method confirmed that these appositions were synaptic. Additional experiments showed that virtually all α_{2C} -AR terminals in contact with labeled cells are also immunoreactive for the vesicular glutamate transporter 2 and therefore are glutamatergic.

These data suggest that norepinephrine can modulate excitatory synaptic transmission from spinal interneurons to projection cells by acting at α_{2C} -ARs. This could be one of the mechanisms that underlie the antinociceptive actions of norepinephrine.

Key words: α_{2C} -adrenergic receptor; vesicular glutamate transporter 2; neurokinin-1 receptor; spinomedullary neurons; noradrenergic antinociception; immunocytochemistry

Introduction

Norepinephrine (NE) modulates the transmission of nociceptive information in the dorsal horn of the spinal cord and is known to induce antinociception. Pharmacological evidence indicates that this action is mediated principally through α_2 -adrenergic receptors (α_2 -ARs) (Howe et al., 1983; Yaksh, 1985; Proudfit, 1988); these are coupled to G-proteins (Hoehn et al., 1988), which induce membrane hyperpolarization by decreasing the Ca^{2+} influx and increasing the K^+ efflux (Surprenant et al., 1990; Shen et al., 1992). Both the α_{2A} - and α_{2C} -subclasses of adrenergic receptor are involved in NE-induced antinociception (Stone et al., 1997; Li and Eisenach, 2001; Fairbanks et al., 2002), and immunoreactivity for both receptor subclasses is concentrated in the superficial dorsal horn. Experiments using antibodies specific for the α_{2A} - and α_{2C} -subclasses show that they are associated with different axonal populations (Stone et al., 1998). The α_{2A} -AR is found in axons that contain substance P and calcitonin gene-related pep-

tide (Stone et al., 1998), which are likely to be terminals of nociceptive primary afferents (Levine et al., 1993), whereas the α_{2C} -AR is present in axon terminals of spinal origin (Stone et al., 1998; Olave and Maxwell, 2003). Most (84%) α_{2C} -AR terminals are immunoreactive for the vesicular glutamate transporter 2 (VGLUT2) (Olave and Maxwell, 2003) and therefore are likely to have an excitatory action; however, a small proportion (11%) of terminals are inhibitory and contain glutamate decarboxylase (Olave and Maxwell, 2003). Ultrastructural observations of α_{2C} -AR-immunoreactive axon terminals show that they form multiple synapses with large dendritic profiles in lamina I and indicate that they may target certain cells in this region (Olave and Maxwell, 2002). The identity of these target cells is not known, but if they prove to be projection neurons that are activated by nociceptive stimuli, NE could specifically inhibit excitatory polysynaptic input to such cells. This type of arrangement could be particularly important, because the α_{2C} -AR is a potentially interesting target for selective analgesics considering that sedative effects mediated via α_{2A} -ARs could be avoided (Guo et al., 1999; Fairbanks et al., 2002).

The aim of the present study was to test the hypothesis that axons of excitatory interneurons possessing α_{2C} -ARs target nociceptive projection neurons. We retrogradely labeled spinomedullary projection neurons with the B-subunit of cholera toxin

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(CTb) and used triple immunofluorescence to examine the relationship of projection neurons that possess the substance P (NK-1) receptor and axons that possess α_{2C} -ARs. Neurons of this type are likely to be involved in the transmission of nociceptive information (Naim et al., 1997; Todd et al., 2002). Furthermore, mice lacking NE display a substance P-dependent chronic thermal hyperalgesia (Jasmin et al., 2002), which suggests that NK-1 neurons are components of the circuitry involved in NE antinociception. We also used a combined confocal and electron microscopic method to determine if α_{2C} -AR terminals make synapses with this type of neuron and a sequential immunocytochemical method with a VGLUT2 antibody to determine if the α_{2C} -AR terminals that contact NK-1 projection cells are likely to have an excitatory action on these cells.

Materials and Methods

Confocal microscopy. Three male Wistar rats (250 gm; Harlan, Loughborough, UK) were deeply anesthetized (ketamine/xylazine mixture, 7.33 and 0.73 mg/100 gm, i.p.) and an aqueous solution of the CTb (1%, 200 nl; Sigma, Poole, Dorset, UK) was injected stereotactically within the left caudal ventrolateral medulla (CVLM; anteroposterior, -4.8 ; dorsoventral, -0.6 ; mediolateral, $+2.1$) (Paxinos and Watson, 1997). After 3 d, the animals were anesthetized with sodium pentobarbitone (1 ml, i.p.) and perfused through the left ventricle with saline, followed by a fixative containing 4% formaldehyde in phosphate buffer, pH 7.6. The L4 lumbar segment was removed from each animal and postfixed in the same solution for 8 hr. Parasagittal sections (70 μ m thick) were cut with a Vibratome. Sections were treated with 50% ethanol (30 min) to enhance antibody penetration, which was followed by blocking in 10% normal donkey serum for 1 hr. Triple-labeling immunofluorescence was performed with a guinea pig anti- α_{2C} -AR antiserum (1:500; Neuromics, Minneapolis, MN; see Stone et al., 1998, for details), rabbit anti-NK-1 antiserum (1:10,000; Sigma), and goat anti-CTb antiserum (1:5,000; List Laboratories, Campbell, CA). After a 48 hr incubation period, sections were rinsed and incubated for 3 hr in solutions containing species-specific secondary antibodies coupled to the following fluorophores (all raised in donkey and diluted 1:100): rhodamine-red to identify α_{2C} -AR immunoreactivity; fluorescein isothiocyanate (FITC) to identify NK-1 receptor immunoreactivity; and cyanine 5.18 to identify CTb (all obtained from Jackson ImmunoResearch, Luton, UK). Antibodies were diluted in PBS containing 0.3% Triton X-100 and 1% normal donkey serum. The sections were mounted in anti-fade medium (Vectashield; Vector Laboratories, Peterborough, UK) and stored in a freezer at -20°C . Double-labeled cells (i.e., retrogradely labeled cells with NK-1 receptor immunoreactivity) contralateral to the side of the injection were systematically scanned using a Bio-Rad (Hemel Hempstead, UK) MRC 1024 confocal laser scanning microscope with a $40\times$ oil-immersion lens at 0.5 μ m intervals in the z-axis and a zoom factor of 2. Thirty lamina I and 15 lamina III/IV neurons were reconstructed with Neurolucida for Confocal software (MicroBrightField, Colchester, VT), and appositions formed by α_{2C} -AR axon terminals were plotted on the reconstructions. A Sholl analysis was performed to study the pattern of distribution of contacts for the two populations of neurons; numbers of contacts per 100 μ m unit length of dendrite contained within concentric spheres with radii that increased at 25 μ m intervals from the center of the cell body were estimated.

To determine if α_{2C} -AR contact densities on NK-1 projection cells were greater than would be expected by chance, we compared them with protein kinase C γ (PKC γ)-immunoreactive cells, which are also found in lamina I and II within the dense plexus of α_{2C} -AR immunoreactive axons. PKC γ cells are predominantly excitatory interneurons (Polgár et al., 1999); therefore, they would be expected to be very different functionally from NK-1 projection neurons. Triple-labeling immunofluorescence was performed as described above, except that rabbit anti-NK-1 antiserum was used in place of the rabbit anti-PKC γ antiserum (1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA). Contact densities per 100 μm^2 of dendritic surface area were calculated for lamina I and lamina

Figure 1. Injection of the retrograde tracer in the CVLM. *a*, Photomicrograph illustrating the CTb-injection site in the left CVLM. 4v, Fourth ventricle; cu, cuneate nucleus; cc, central canal; XII, hypoglossal nucleus; spV, spinal trigeminal nucleus; ml, medial lemniscus; LRT, lateral reticular nucleus; vsc, ventral spinocerebellar tract; py, pyramidal tract. *b*, Reconstruction of an injection site indicating the interaural coordinate (Paxinos and Watson, 1997). The spread of the tracer is represented by the dark gray area.

III/IV NK-1 projection cells and for PKC γ -immunoreactive cells using the Neurolucida program. The average contact density was calculated for each animal ($n = 3$ on each occasion; 10 cells from each animal for lamina I and 5 cells from each animal for lamina III/IV and PKC γ), and the overall mean ($\pm$ SD) for three animals was calculated. Statistical comparisons were made using one-way ANOVA and a *post hoc* Tukey's pairwise comparison. *P* values <0.05 were considered to be significant.

Combined confocal and electron microscopy. A second set of three male Wistar rats (250 gm; Harlan) were deeply anesthetized with ketamine and xylazine and received unilateral 200 nl stereotaxic injections of 1% CTb (Sigma) in the left CVLM as described above. The combined confocal and electron microscopic method we used is a modification of that described by Todd (1997). After a 3 d survival period, the animals were deeply anesthetized with sodium pentobarbitone (1 ml, i.p.) and perfused with saline followed by a fixative containing 4% formaldehyde, 0.2% glutaraldehyde, and 0.2% of saturated picric acid in phosphate buffer, pH 7.6. L4 segments were removed, placed in the same fixative for 8 hr and cut into 50 μ m horizontal sections with a Vibratome. The sections were treated with 50% ethanol for 30 min to improve antibody penetration and also with 1% sodium borohydride for 30 min to counteract the effects of glutaraldehyde. Sections were incubated for 3 d at 4°C in guinea pig anti- α_{2C} -AR antiserum (1:500; Neuromics), rabbit anti-NK-1 antiserum (1:10,000; Sigma), and a goat anti-CTb antiserum (1:5,000; List Laboratories). Sections were then rinsed in PBS and placed for 1 d in a cocktail of donkey secondary antibodies, which consisted of rhodamine-red-anti-guinea pig IgG, FITC-anti-rabbit IgG, cyanine 5.18-anti-goat IgG (all 1:100), and biotinylated anti-guinea pig IgG (1:500; Jackson ImmunoResearch). Primary and secondary antibodies were diluted in detergent-free PBS. After rinsing in PBS, sections were incubated in avidin-biotin-HRP complex (1:1000; Vector Laboratories) for 1 d. Once the sections were mounted, they were scanned with the confocal microscope and lamina I cells were selected for analysis. Six NK-1 receptor-immunoreactive CTb-labeled neurons (two from each animal), which received multiple contacts from α_{2C} -AR-immunoreactive terminals, were examined. Optical sections were gathered sequentially to avoid bleedthrough. Multiple scans were performed with a $60\times$ oil-immersion lens at 0.5 μ m intervals in the z-axis and a zoom factor of 1.5 to produce a montage of each selected neuron. In addition, scans with a $40\times$, $20\times$, $10\times$, and $4\times$ lenses were performed to gather progressively lower power images that would serve as a frame of reference for identification of each cell with the electron microscope.

Sections containing scanned cells were removed from the slides and processed for electron microscopy. After rinsing, they were reacted with

Figure 2. Confocal microscopic images of immunoreactivity for CTb, the NK-1 receptor, and α_{2C} -AR in a horizontal section. *a*, Merged image of a horizontal section of lamina I (blue, CTb; green, NK-1 receptor; red, α_{2C} -AR) illustrating the general pattern of triple labeling at a low magnification (made from 20 projected confocal images gathered at 1 μ m steps with a 20 $\times$ lens). *b–d*, Projected images of three retrogradely labeled neurons at high magnification (built from 15, 10, and 5 single optical sections for *b*, *c*, and *d*, respectively). Optical sections were gathered at 0.5 μ m steps with a 40 $\times$ lens). Cells shown in *b* and *c* are multipolar, whereas the cell shown in *d* is fusiform. Colors are keyed as in *a*. All three neurons receive multiple contacts from α_{2C} -AR-immunoreactive terminals. *b'–d'*, α_{2C} -AR immunoreactivity corresponding to *b–d*, respectively. Note that α_{2C} -AR terminals delineate the cell bodies and dendrites of these neurons. Scale bars: *a*, 100 μ m; (in *b*) *b–d*, *b'–d'*, 10 μ m.

3,3'-diaminobenzidine (DAB) in the presence of hydrogen peroxide. They were then placed in a 1% solution of osmium tetroxide for 30 min, dehydrated in acetone, stained en bloc with uranyl acetate and finally flat-embedded in Durcupan resin (Fluka, Buchs, Switzerland) between cellulose acetate sheets. Sections were examined with a light microscope to establish the location of each cell; DAB-positive α_2 C-AR-immunoreactive terminals surrounding cells and landmarks such as blood vessels were used to identify their location within sections. Sections were mounted onto blocks of cured resin, which were trimmed to include the region containing the cell. Ultrathin sections were cut serially with a diamond knife, collected on Formvar-coated grids, and viewed with an electron microscope (model CM100; FEI Company, Eindhoven, The Netherlands).

Sequential immunocytochemistry. A third set of three male Wistar rats (250 gm; Harlan) was used for this part of the study. The initial procedure applied was identical to that described in the section above for confocal microscopy. When scanning of selected double-labeled neurons with contacts from α_2 C-AR-immunoreactive terminals was complete, sections were retrieved from the slides and re-incubated in a fourth anti-serum: rabbit anti-VGLUT2 antiserum (1:5000; Synaptic Systems, Göttingen, Germany) for 48 hr. They were then rinsed and incubated for 3 hr in a solution containing donkey anti-rabbit IgG coupled to FITC (1:100, Jackson ImmunoResearch). Finally, sections were remounted and the same neurons that had been scanned previously were identified and scanned again. The same secondary antibody was used to reveal the rabbit anti-NK-1 and rabbit anti-VGLUT2 primary antibodies, but by comparing the FITC labeling before and after the re-incubation in the VGLUT2 antiserum, we could detect the additional FITC labeling, which represents immunoreactivity for VGLUT2. No additional immunoreactivity was observed in control experiments when the VGLUT2 antibody was omitted in the sequential reaction. Equally, the pattern of immunostaining was not altered when performing the sequential incubation in a reverse order for the NK-1 and VGLUT2 labeling (i.e., first incubation containing the rabbit anti-VGLUT2 antiserum in the cocktail of primary antisera followed by sequential incubation in rabbit anti-NK-1 antiserum).

Results

NK-1 spinomedullary neurons are innervated by axons that possess the α_2 C-AR

Spinomedullary neurons were labeled by injection of the retrograde tracer CTb in the left CVLM. Figure 1 shows the injection site for one of the experiments and an example of the extent of the tracer spread in another experiment. As predicted, numerous retrogradely labeled neurons were found in lamina I and lamina III/IV of the spinal dorsal horn, especially contralateral to the injection site (Todd et al., 2000). A large proportion of CTb-labeled neurons was present in lamina I along with immunoreactivity for the NK-1 receptor and axon terminals possessing the α_2 C-AR (Figs. 2*a*, 3*a*). At high magnification it was possible to identify NK-1 projection cells by the presence of CTb within them and to study their relationship with α_2 C-AR-immunoreactive profiles. Cell bodies and proximal dendrites of lamina I cells frequently received large numbers of contacts from α_2 C-AR-immunoreactive structures (Figs. 2*b–d*, 3*c*), as did distal dendrites of labeled cells in lamina III/IV, which extended dorsally into lamina I (Fig. 3*b,d–f*). More than half of the NK-1 projection cells were found to receive contacts from α_2 C-AR terminals (average $\pm$ SD, 57.3 $\pm$ 5.26%).

Distribution and density of α_2 C-AR contacts on NK-1 projection neurons

The distribution of α_2 C-AR contacts was studied in the two populations of NK-1 projection neurons. The neurons were sampled from parasagittal sections that were contralateral to the injection

Figure 3. Labeling of CTb, the NK-1 receptor, and α_2 C-AR in a parasagittal section. *a*, Projected image of a parasagittal section built from 30 confocal images, which were gathered at 1 μ m steps with a 20 $\times$ lens (colors as in Fig. 2). The locations of laminae I to IV are indicated on the right side of the image. A cell that is labeled with CTb and the NK-1 receptor can be observed in lamina III. This cell has three dorsally oriented dendrites that extend into lamina I. *b*, Projected image at high magnification showing the boxed area in *a*, which includes one of the dorsal dendrites from the lamina III cell (made from 20 optical sections, gathered at 0.5 μ m steps with a 60 $\times$ lens). *c*, Single optical section showing that α_2 C-AR terminals form contacts on the lamina I cell seen in *b*, which contains CTb and is labeled for the NK-1 receptor. *d*, Projected image of the boxed area in *b* (built from 7 optical sections, gathered at 0.5 μ m steps with a 60 $\times$ lens); seven boutons, indicated by the numbered arrows, form appositions with the NK-1-positive dendrite, which belongs to the lamina III cell seen in *a*. Appositions are shown in single optical sections: 1 and 3 in *e*, and 2, 4–7 in *f*. Scale bars: *a*, 100 μ m; *b*, *c*, 20 μ m; (*d–f*, 10 μ m).

site. A sample of 30 lamina I neurons was scanned (10 from each of the three experiments), whereas the sample of lamina III/IV neurons was reduced to 15 (5 from each experiment) because they were found less frequently. The intensity of immunostaining for the NK-1 receptor varied from cell to cell, and although some neurons were very strongly labeled, others were weakly labeled. Neurons showing any evidence of NK-1 receptor immunoreac-

Figure 4. Distribution of α_2c -AR contacts on NK-1 projection cells. Left, Two examples of NK-1 projection cells reconstructed with NeuroLucida. Contacts (filled circles) from α_2c -AR-immunoreactive puncta were mapped on the reconstructions. A lamina I cell is shown above and a lamina III/IV cell below. Scale bars, 100 μm . Right, Histograms summarizing the Sholl analysis of the distribution of α_2c -AR contacts on both populations of cells (lamina I, $n = 15$; lamina III/IV, $n = 30$). The number of contacts per 100 μm of dendritic length is plotted against the distance from the soma, where 0 μm represents the cell body and the consecutive numbers represent the radii of the concentric shells, which increase at 25 μm intervals.

tivity were included in the analysis. On average ($\pm$ SD) $83 \pm 16.7\%$ of the retrogradely labeled neurons were NK-1-positive. Once the neurons were scanned they were reconstructed and the α_2c -AR contacts were mapped (examples in Fig. 4). Sholl analysis of the distribution of α_2c -AR contacts revealed that NK-1 projection neurons with somata in lamina I receive numerous α_2c -AR contacts on cell bodies and proximal dendrites, whereas NK-1 projection neurons with somata in lamina III/IV receive most α_2c -AR contacts on distal dendrites that extend into lamina I–II. The average number of contacts per 100 μm of dendritic length was higher for lamina I neurons than for lamina III/IV neurons (average $\pm$ SD, 109.3 ± 38.1 and 69.3 ± 20.7 , respectively) (Fig. 4, histograms). We also calculated average densities of α_2c -AR contacts per unit area ($100 \mu\text{m}^2$) of dendritic surface for lamina I and lamina III/IV NK-1 projection cells to compare them with a population of PKC γ -immunoreactive interneurons (Fig. 5). Dendrites of lamina I cells had approximately seven times the density of contacts associated with PKC γ cells (average $\pm$ SD contacts per $100 \mu\text{m}^2$, 1.09 ± 0.07 and 0.14 ± 0.01 , respectively) and lamina III/IV cells were associated with three times the density (0.51 ± 0.09 contacts per $100 \mu\text{m}^2$). Statistical comparisons confirm that these differences are significant ($p < 0.001$, ANOVA; individual differences between all three groups were significant at $p < 0.05$, Tukey's *post hoc* pairwise comparison).

Axon terminals containing the α_2c -AR form synapses with NK-1 projection neurons in lamina I

The combined confocal and electron microscopic method allowed us first to visualize α_2c -AR terminals forming appositions on NK-1 spinomedullary neurons, and second, after processing of the tissue for electron microscopy, to determine if such terminals formed synapses with the neuron (Fig. 6). In total, six neurons were examined (two from each animal), which received 45 appositions from α_2c -AR terminals. Electron microscopic analysis confirmed that 42 of these formed synapses with the neurons. Most of these synapses could be classified as asymmetric, but occasionally it was difficult to define the type of synapse. The remaining three appositions were not observed to form synapses. Synaptic boutons contained circular agranular vesicles and often granular vesicles also (Fig. 6).

α_2c -AR axon terminals that innervate NK-1 spinomedullary projection neurons are excitatory

Sequential immunocytochemistry with a fourth antibody against VGLUT2 enabled us to determine if α_2c -AR-immunoreactive profiles forming appositions with NK-1 projection cells were immunoreactive for VGLUT2 (Takamori et al., 2000; Varoqui et al., 2002). A sample of 30 NK-1 retrogradely labeled lamina I neurons was scanned (10 from each of the three animals) from the side contralateral to the injection. An average ($\pm$ SD) of 43 ± 12

Figure 5. Labeling of PKC γ and the α_{2C} -AR in a parasagittal section. *a*, Projected image of a parasagittal section showing immunoreactivity for PKC γ and the α_{2C} -AR (green and red, respectively) at low magnification (made from 20 single optical sections, gathered at 0.5 μ m steps with a 20 $\times$ lens). The location of laminae I–III is indicated on the right side of the image. A PKC γ -immunoreactive cell can be seen in lamina I, dorsal to the main PKC γ plexus in lamina II. Note that α_{2C} -AR immunoreactivity is dense in the superficial dorsal horn, in which dorsal PKC γ cells are found. *b*, Projected image of the dorsal PKC γ cell at high magnification (made from 15 optical sections, gathered at 0.5 μ m steps with a 40 $\times$ lens). *b'*, Corresponding α_{2C} -AR immunoreactivity; note that α_{2C} -AR terminals do not delineate the outline of the cell. *c*, Reconstruction of the dorsal cell illustrated in *a* and *b*; only one α_{2C} -AR terminal was found to form a contact (red circle) with the cell. *d*, *e*, Single optical sections extracted from the z-series in *b*, illustrating the paucity of contacts formed by α_{2C} -AR terminals with the cell body and dendrites of the PKC γ cell. Scale bars: *a*, 40 μ m; (in *b*) *b*, *b'*, *d*, *e*, 20 μ m; *c*, 20 μ m.

α_{2C} -AR appositions per neuron was recorded. Of these appositions, 100% were found to be VGLUT2-positive when the selected neurons were rescanned after the sequential incubation in VGLUT2 antiserum (Fig. 7).

Discussion

The major finding of this study is that axon terminals containing the α_{2C} -AR densely innervate NK-1 spinomedullary neurons that project to the CVLM. Both lamina I and lamina III/IV projection neurons were associated with α_{2C} -AR contact densities that were significantly greater than contact densities on interneurons possessing PKC γ immunoreactivity; we conclude that this represents a specific type of arrangement that has not arisen merely by chance. Terminals were concentrated around cell bodies and proximal dendrites of lamina I neurons, whereas in lamina III/IV neurons they were apposed predominantly to distal dendrites, which extended dorsally into laminae I and II. We were able to demonstrate that axon terminals possessing the α_{2C} -AR form synapses with NK-1 projection neurons in lamina I, and that they are likely to have an excitatory action because they also contained VGLUT2, which is a marker for glutamatergic axon terminals (Takamori et al., 2000; Varoqui et al., 2002; Todd et al., 2003). On this basis, we conclude that our hypothesis is correct and that axons of excitatory interneurons possessing α_{2C} -ARs do indeed target nociceptive projection neurons.

Identification of NK-1 projection neurons

Our quantitative analysis was confined to neurons contralateral to the CVLM injection site that were NK-1-positive because neurons of this type are very likely to be involved in the transmission of nociceptive information (Naim et al., 1997; Todd et al., 2002). Stereotaxic injections were made into the CVLM because this region of the brainstem is known to receive substantial input from lamina I (Lima et al., 1991; Craig, 1995) and also because lamina I neurons labeled from the CVLM are more numerous than those labeled from other projection targets (i.e., dorsal reticular nucleus, periaqueductal gray, or thalamus; Marshall et al., 1996; Todd et al., 2000). Most spinomedullary neurons labeled from the CVLM project contralaterally, and only a minority project ipsilaterally. Neurons retrogradely labeled from the CVLM also include a population of lamina III/IV cells, which have dorsally directed dendrites that terminate in lamina I. Double-labeling studies indicate that most CVLM neurons also have collateral projections to the lateral parabrachial area (Todd, 2002); therefore, many of the cells analyzed in this study are likely to project to this area in addition to the CVLM. Both these regions of the brainstem are known to be targets of nociceptive neurons and are intimately involved in nociceptive processing (Gauriau and Bernard, 2002; Lima et al., 2002).

Figure 6. Combined confocal and electron microscopy of α_{2C} -AR contacts. *a*, Single optical section of a NK-1 projection cell in lamina I, which is apposed by three α_{2C} -AR-immunoreactive terminals (arrows numbered 1–3; blue, CTb; green, NK-1 receptor; red, α_{2C} -AR). *b*, Electron micrograph of the same cell. The α_{2C} -AR-immunoreactive terminals can be recognized by the dark DAB-reaction product. The three terminals indicated by the arrows (numbered 1–3) correspond to those indicated in *a*. The areas delineated by the purple and blue boxes in *b* are shown at higher magnification in *c* and *f*, respectively. *c*–*e*, Progressively magnified images of the axon terminal indicated by arrow 1. This forms an asymmetric synapse with a proximal dendrite of the cell. *f*–*h*, Progressively magnified images of the terminal indicated by arrow 3. This bouton forms a long asymmetric synapse. Boutons contained circular agranular vesicles and often also granular vesicles (red arrowheads in *g*). Scale bars: *a*, *b*, 10 μ m; *c*, *f*, 1 μ m; *d*, *g*, 0.5 μ m; *e*, *h*, 0.25 μ m.

Origin of α_{2C} -AR axons

It is probable that the majority of α_{2C} -AR axons in the spinal cord originate from interneurons. Three lines of evidence support this view. First, α_{2C} -AR immunoreactivity is not reduced after rhizotomy or capsaicin treatment, and it is not associated with markers that identify primary afferent terminals (Stone et al., 1998; Olave and Maxwell, 2002, 2003). Second, many α_{2C} -AR-

immunoreactive terminals also contain peptides such as enkephalin, somatostatin, neurotensin, and neuropeptide Y, which are mainly or exclusively found in spinal interneurons (Stone et al., 1998; Olave and Maxwell, 2002). Third, VGLUT2 is found in the terminals of most α_{2C} -AR-axons (Olave and Maxwell, 2003), and it is predominantly associated with spinal interneurons (Todd et al., 2003).

Figure 7. Sequential immunocytochemistry for VGLUT2. *a*, A single optical section of a NK-1 projection cell from lamina I that receives numerous contacts from α_{2C} -AR terminals. Immunoreactivity for the NK-1 receptor (NK1), CTb (CTb), and α_{2C} -AR (α_{2C}) are shown independently. A merged image formed from the previous three is shown on the right. *b*, A single optical section of the same cell that has been rescanned after sequential incubation with a fourth antibody against VGLUT2. The extra-green labeling present in *b*, which was absent in *a*, corresponds to the additional VGLUT2-immunostaining (see NK1 + VGLUT2). Note that all α_{2C} -AR-immunoreactive terminals, which form appositions on the NK-1 projection cell, are immunoreactive for VGLUT2 (yellow profiles in *b*, merged image on the right) and hence can be considered to be excitatory glutamatergic terminals. Scale bars, 20 μ m.

Functional significance of α_{2C} -AR-innervation of NK-1 projection neurons

Our findings indicate that NE can influence NK-1 projection neurons through a presynaptic action on axon terminals that possess α_{2C} -ARs. Both lamina I and lamina III/IV NK-1 projection cells receive dense innervation from small-diameter primary afferent fibers that contain a combination of substance P and glutamate (De Biasi and Rustioni, 1988; Naim et al., 1997; Todd et al., 2002). Glutamate is undoubtedly involved in acute pain signaling mechanisms, but the role of substance P in nociceptive transmission is subtle. Ablation of lamina I NK-1 cells attenuates the development of thermal and mechanical hyperalgesia (Mantyh et al., 1997; Khasabov et al., 2002), and mice that lack the NK-1 receptor do not display the characteristic amplification and intensity coding of nociceptive reflexes (De Felipe et al., 1998). Such “knock-out” animals also have reduced descending inhibition evoked by peripheral noxious stimuli (Bester et al., 2001), indicating that NK-1 neurons are components of an ascending–descending antinociceptive loop. The NK-1 receptor is implicated directly in the mediation of NE antinociception because genetically engineered mice that lack NE show a substance P–dependent chronic hyperalgesia (Jasmin et al., 2002). These lines of evidence suggest that NK-1 neurons are likely to be components of the circuitry that underlies NE antinociception. NE descending inhibitory systems could be recruited by NK-1 spinomedullary neurons, because these cells terminate in a region close to the lateral reticular nucleus that contains dopamine β -hydroxylase-immunoreactive neurons, which in turn, project to the spinal dorsal horn (Lee et al., 2001).

It is well established that the antinociceptive action of NE is mediated by α_2 -ARs (Howe et al., 1983; Yaksh, 1985; Proudfit, 1988), but it has been shown only recently that both α_{2A} -AR and α_{2C} -AR subtypes are involved in this process (Stone et al., 1997;

Li and Eisenach, 2001; Fairbanks et al., 2002). Budai et al. (1998) reported that periaqueductal gray neurons inhibit nociceptive dorsal horn cells by a presynaptic action on α_2 -ARs. Our findings also support this proposal, because we have shown that α_{2C} -ARs are located on axon terminals that are presynaptic to nociceptive cells. In addition, virtually all of these terminals are likely to be glutamatergic because they are immunoreactive for VGLUT2 and therefore would be expected to have an excitatory action on their target cells. This finding is also in agreement with studies reporting that activation of α_2 -ARs can reduce the release of glutamate. Pan et al. (2001) showed that the α_2 -AR agonist clonidine inhibits glutamatergic synaptic input to spinal neurons in outer lamina II by a presynaptic action on α_2 -ARs. The α_{2A} and α_{2C} -ARs differ not only in their cellular location in the dorsal horn, but also in their affinity for NE and deactivation kinetics. The α_{2C} -AR has a greater affinity for NE than the α_{2A} -AR and the α_{2C} -AR shows slower deactivation after NE stimulation (Bunemann et al., 2001). These differences suggest that each subclass has distinct physiological functions, even if both types of receptor are implicated in NE antinociception. Our results indicate that part of the antinociceptive action of NE could be mediated via α_{2C} -ARs present on terminals of excitatory spinal interneurons that are presynaptic to nociceptive neurons in lamina I and distal dendrites of presumed nociceptive neurons in lamina III/IV.

Both presynaptic actions on primary afferent terminals (Kuraishi et al., 1985; Kamisaki et al., 1993) and postsynaptic actions on dorsal horn cells (Davies and Quinlan, 1985; Fleetwood-Walker et al., 1985) have been proposed as mechanisms for NE modulation of nociceptive transmission through α_2 -ARs. In this study we suggest that a third mechanism can also operate (i.e., that NE modulates transmission at terminals of interneurons). We propose that these three mechanisms operate in a complementary manner. It is likely that both α_{2A} -ARs located

Figure 8. A model of a possible mechanism of noradrenergic antinociception mediated by α_{2C} -ARs. 1, NE acts by volume transmission on α_{2C} -ARs. 2, NE induces hyperpolarization of the excitatory interneuron axon terminal by acting on α_{2C} -ARs. 3, Reduction in the efficacy of synaptic transmission results in 4, inhibition of supraspinal transmission of nociceptive information.

on primary afferent terminals and α_{2C} -ARs on interneuron terminals and dorsal horn cells (Rosin et al., 1996; Stone et al., 1998) are involved in these modulatory processes. In the superficial dorsal horn, there is a dense plexus of NE fibers (Rajaoetra et al., 1992) that widely overlaps with areas of intense α_{2C} -AR immunoreactivity. Noradrenergic axons do not form axo-axonic synapses in this region (Doyle and Maxwell, 1991a,b); indeed, axo-axonic synapses have been found only on primary afferent terminals and are not formed with terminals of interneurons (Alvarez, 1998). Therefore, NE cannot act on interneuron axons through a classic synaptic mechanism, and any interaction with terminals possessing adrenergic receptors must occur via volume transmission.

The NE descending system, along with the serotonergic system, performs a major role in the regulation of nociceptive transmission in the dorsal horn. However the mechanisms of action of these two monoamines on NK-1 neurons are likely to be different. Many projection cells that possess the NK-1 receptor in lamina I and laminae III/IV are heavily targeted by serotonin-immunoreactive axons, which form numerous contacts with their cell bodies and proximal dendrites (Stewart and Maxwell, 2000; Polgár et al., 2002) but NE contacts on these cells are very sparse indeed (Stewart and Maxwell, 2000; Stewart, 2001). This evidence suggests that serotonin operates directly on NK-1 cells via a postsynaptic action, whereas the effect of NE is more likely to be diffuse and is consistent with our contention that NE operates via volume transmission on interneuron axon terminals.

We propose a model of the possible mechanism of antinociception mediated through α_{2C} -ARs in Figure 8. The action of NE on the α_{2C} -AR would be predicted to induce membrane hyperpolarization (Surprenant et al., 1990; Shen et al., 1992), which would lead to a reduction in the efficacy of synaptic transmission between α_{2C} -AR-axon terminals of excitatory interneurons and projection neurons. This would selectively suppress excitatory polysynaptic input to these neurons, which, in turn, would attenuate the transmission of nociceptive information to supraspinal structures.

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