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Calcitonin Receptor-Like Receptor and Receptor Activity Modifying Protein 1 in the rat dorsal horn: localization in glutamatergic presynaptic terminals containing opioids and adrenergic α_{2C} receptors

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Abstract

Calcitonin-gene related peptide (CGRP) is abundant in the central terminals of primary afferents. However, the function of CGRP receptors in the spinal cord remains unclear. CGRP receptors are heterodimers of calcitonin receptor-like receptor (CRLR) and receptor activity modifying protein 1 (RAMP1). We studied the localization of CRLR and RAMP1 in the rat dorsal horn using well-characterized antibodies against them, which labeled numerous puncta in laminae I–II. In addition, RAMP1 was found in cell bodies, forming patches at the cell surface. The CRLR- and RAMP1-immunoreactive puncta were further characterized using double and triple labeling. Colocalization was quantified in confocal stacks using Imaris software. CRLR did not colocalize with primary afferent markers, indicating that these puncta were not primary afferent terminals. CRLR- and RAMP1-immunoreactive puncta contained synaptophysin and vesicular glutamate transporter-2 (VGLUT2), showing that they were glutamatergic presynaptic terminals. Electron microscopic immunohistochemistry confirmed that CRLR immunoreactivity was present in axonal boutons that were not in synaptic glomeruli. Using tyramide signal amplification for double labeling with the CRLR and RAMP1 antibodies, we found some clear instances of colocalization of CRLR with RAMP1 in puncta, but their overall colocalization was low. In particular, CRLR was absent from RAMP1-containing cells. Many of the puncta stained for CRLR and RAMP1 were labeled by anti-opioid and anti-enkephalin antibodies. CRLR and, to a lesser extent, RAMP1 also colocalized with adrenergic a2C receptors. Triple label studies demonstrated three-way colocalization of CRLR-VGLUT2-synaptophysin, CRLR-VGLUT2-opioids, and CRLR-opioids-a2C receptors. In conclusion, CRLR is located in glutamatergic presynaptic terminals in the dorsal horn that contain a2C adrenergic receptors and opioids. Some of these terminals contain RAMP1, which may form CGRP receptors with CRLR, but in others CRLR may form other receptors, possibly by dimerizing with RAMP2 or RAMP3. These findings suggest that CGRP or adrenomedullin receptors modulate opioid release in the dorsal horn.

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Keywords

Adrenergic receptor; calcitonin gene-related peptide; calcitonin receptor-like receptor; CGRP; CGRP receptor; colocalization; confocal microscopy; dynorphin; electron microscopy; enkephalin; immunohistochemistry; neuropeptide; opioid; pain; presynaptic terminal; rat; receptor activity modifying protein; release; spinal cord; synaptophysin; tyramide signal amplification; vesicular glutamate transporter

A large number of C and Ad primary afferent fibers contain calcitonin gene-related peptide (CGRP) (Gibson et al., 1984; Wiesenfeld-Hallin et al., 1984; Franco-Cereceda et al., 1987). Many of these CGRP-containing fibers also contain substance P (Tuchscherer and Seybold, 1989; Ribeiro-da-Silva, 1995), a neuropeptide that contributes to the induction of hyperalgesia by acting on dorsal horn neurons that express its receptor, the neurokinin 1 (NK1) receptor (Traub, 1996; Mantyh et al., 1997; De Felipe et al., 1998). CGRP release from the peripheral terminals of primary afferents produces vasodilatation, an important component of the inflammatory response (Van Rossum et al., 1997). CGRP is also released into the dorsal horn from the central terminals of primary afferents (Malcangio and Bowery, 1996; Garry et al., 2000). However, its effects in the spinal cord are still unclear. There is evidence that CGRP, like substance P, contributes to the induction of hyperalgesia. Thus, CGRP8–37, an antagonist of CGRP receptors, produced antinociception to thermal and mechanical stimuli (Yu et al., 1994). CGRP8–37 also decreased allodynia in rats with spinal hemisection, a model of central pain (Bennett et al., 2000). Moreover, a CGRP antiserum injected intrathecally decreased hyperalgesia associated with inflammation (Kawamura et al., 1989), and CGRP knock out mice showed reduced hyperalgesia in a model of arthritic pain (Zhang et al., 2001). Still, the mechanisms by which CGRP contributes to hyperalgesia remain unknown.

One important step in elucidating the central actions of CGRP is to determine the location and function of its receptors in the dorsal horn. An initial attempt to localize CGRP receptors in the rat spinal cord was made by Ye et al. (Ye et al., 1999) using monoclonal antibodies against a CGRP-binding protein isolated from pig cerebellum, which was a monomer of ~66 KDa (Wimalawansa et al., 1993). This is consistent with the molecular weight of CGRP receptors isolated from human brain (Foord and Craig, 1987; Stangl et al., 1991). The CGRP receptor is now believed to be a heterodimer of calcitonin receptor-like receptor (CRLR) and receptor activity modifying protein 1 (RAMP1), two proteins of molecular weights of 66 KDa and 17 KDa, respectively (McLatchie et al., 1998). Therefore, it is possible that the antibodies used by Ye et al. recognized CRLR. However, CRLR can also heterodimerize with RAMP2 to form a receptor recognizing adrenomedullin, and with RAMP3 to form a mixed CGRP and adrenomedullin receptor (McLatchie et al., 1998; Husmann et al., 2003; Roh et al., 2004). Recently, commercially available antibodies against CRLR, RAMP1, RAMP2 and RAMP3 were used to label the rat dorsal horn (Ma et al., 2006), resulting in the staining of cell bodies in laminae I–III. However, these antibodies have not been fully characterized, and no attempt was made to study the colocalization of these antibodies with each other and with cellular markers. To determine where CGRP receptors are present in the spinal cord, it is crucial to study the colocalization of CRLR with RAMP1. It is also important to characterize the structures containing these proteins using cellular markers to obtain clues on the function of these receptors.

In a previous study (Cottrell et al., 2005), we characterized two antibodies raised against CRLR (antibody RK11) and RAMP1 (antibody 9892). This characterization provided ample evidence that these antibodies specifically recognized their target proteins, and that they label functional CGRP receptors both at the cell surface and when they are internalized in endosomes. This evidence was collected mainly by using human embryonic kidney (HEK) cells stably

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transfected with CRLR and RAMP1 to make them express functional CGRP receptors. It is summarized as follows (Cottrell et al., 2005). 1) In Western blots from these cells, the CRLR and RAMP1 antibodies recognized single proteins with masses consistent with those predicted for glycosylated CRLR and RAMP1. 2) Immunofluorescence using the CRLR and RAMP1 antibodies stained the surface of these cells. 3) The Western blot bands and immunofluorescence staining disappeared after preabsorption of the antibodies with their immunizing peptides, or in HEK cells expressing the vector without the CRLR and RAMP1 inserts. 4) Incubating these cells with CGRP led to the internalization of CRLR and RAMP1 immunoreactivities in endosomes. 5) Incubating these cells with a fluorescent CGRP peptide led to the internalization of the peptide in endosomes, where it colocalized with immunoreactivity for these CRLR and RAMP1 antibodies.

In addition to these experiments in transfected cells, the CRLR and RAMP1 antibodies were used for immunofluorescence labeling of a variety of tissues, including the spinal cord, dorsal root ganglia, gastrointestinal tract and arteries (Cottrell et al., 2005). This labeling was abolished after preabsorption of the antibodies with their immunizing peptides. In particular, CRLR and RAMP1 immunoreactivities in the spinal cord were found mostly in puncta in the superficial dorsal horn (laminae I–II). Although CRLR (and RAMP1) was detected at low levels in dorsal root ganglion (DRG) neurons, the CRLR immunoreactive puncta in the dorsal horn were not primary afferent terminals, because CRLR did not colocalize with the primary afferent markers CGRP, isolectin B4 or neurofilament of 200 KDa.

The goal of this study was to further characterize these CRLR- and RAMP1-immunoreactive puncta in the dorsal horn. We hypothesized that they may be presynaptic terminals of dorsal horn neurons. In addition, we wanted to determine whether CRLR and RAMP1 were present in the same structures where they could form functional CRGP receptors.

Experimental Procedures

Animal procedures were approved by the Institutional Animal Care and Use Committee of the Veteran Affairs Greater Los Angeles Healthcare System, and conform to NIH guidelines. Efforts were made to minimize the number of animals and their suffering.

Double and triple label immunohistochemistry

Adult male Sprague Dawley rats (250–300 g, Harlan, Indianapolis, IN) were euthanized with pentobarbital (100 mg/kg) and fixed immediately by aortic perfusion of 100 ml phosphate buffer (0.1 M sodium phosphate, pH 7.4) and 400 ml of ice-cold fixative (4 % paraformaldehyde, 0.18 % picric acid in phosphate buffer). The spinal cord was extracted and cut into the L3, L4 and L5 segments based on root identification. These segments were post-fixed, cryoprotected in 20 % sucrose, embedded in a drop of Tissue-Tek (Sakura Finetek USA, Inc., Torrance, CA), frozen on dry ice, and cut in a cryostat into 25 μm sections (coronal and sagittal). Sections form different segments were pooled for processing. Free-floating sections were washed twice with PBS, incubated for 30 min in 50 % ethanol to increase antibody penetration, and washed twice with PBS, 0.5 % Triton X-100, 0.01% thimerosal (PBS/Triton) containing 1 % donkey or goat normal serum (NS, Jackson ImmunoResearch, West Grove, PA). Sections were then incubated overnight with a mixture of two or three primary antibodies in PBS/Triton with 5 % NS. The source, characteristics, and dilutions of the primary antibodies are given in Table 1. After three washes with PBS, sections were incubated for 2 hr with a mixture of secondary antibodies diluted 1:2000 in 1% NS, PBS/Triton. Secondary antibodies were raised in goat or donkey against the species of the primary antibody (guinea pig, mouse or rabbit) and were coupled to Alexa Fluor 488, Alexa Fluor 568 or Alexa Fluor 633 (Molecular Probes, Eugene, OR). Sections were washed four more times with PBS and mounted in Prolong

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Gold (Molecular Probes) or Vectashield Hard Mount (Vector Laboratories, Burlingame, CA) to reduce photobleaching. All incubations were done at room temperature.

Double-label immunohistochemistry using antibodies from the same species

To double-label spinal cord sections with the CRLR and RAMP1 antibodies, which were both raised in rabbit, we used the method of Shindler and Roth (1996). This method consists in sequentially labeling the sections with one of the primary antibodies at high dilution, using tyramide signal amplification (TSA) to increase its signal, and then with the other antibody at low dilution using conventional immunofluorescence. The procedure was similar to the one described above, except that the sections were incubated with the antibodies in the following sequence: 1) overnight with the first primary antibody (CLRL or RAMP1, 1:75,000 or 1:100,000); 2) two hours with donkey anti-rabbit biotinylated secondary antibody (Fab fragment) diluted 1:500; 3) overnight with the second primary antibody (RAMP1 1:1,000 or CRLR 1:2,000); 4) two hours with donkey anti-rabbit Alexa Fluor 488 (1:3,000); 5) 30 min with streptavidin-HRP (1:100); 6) 7 min with tyramide-tetramethyl rhodamine (1:100). Sections were washed three times in PBS between each step. All incubations were done at room temperature in PBS/Triton (with 5% NS for the secondary antibodies). In some experiments, triple labeling was obtained by adding a third primary antibody (raised in mouse or guinea pig) together with the second primary antibody in step 3, and the corresponding secondary antibody coupled to Alexa Fluor 633 in step 4. It proved important to perform the incubations with streptavidin-HRP and tyramide-tetramethyl rhodamine at the end; otherwise labeling with the second primary antibody was occasionally hindered. Streptavidin-HRP, tyramide-tetramethyl rhodamine and amplification buffer were purchased as a kit from PerkinElmer (Wellesley, MA). Biotinylated Fab fragment secondary antibodies (Jackson ImmunoResearch) were raised in donkey against rabbit antiserum, and were absorbed to give minimal species cross-reactivity. We used a monovalent antibody consisting of the Fab fragment of the IgG, because using a divalent secondary antibody may produce an artifact in which the free attachment site of this antibody binds the second primary antibody, resulting in false colocalization.

Controls for the TSA procedure included the following. Control 1: second primary and secondary antibodies present, but no first primary antibody and TSA reagents. This control should produce green (Alexa Fluor 488) label and no red (TSA) label, and was used to see whether the green label was detected in the red channel. Control 2: second primary and secondary antibodies and all TSA reagents present, but no first primary antibody (for TSA). This control should also produce the green label only, and was used to see whether the TSA reagents produced non-specific staining or cross-staining of the second primary antibody. Control 3: first primary antibody and all TSA reagents present, but no second primary antibody. This control should produce only the red (TSA) label, and served to see whether the TSA label was detected in the green channel (bleed-through). Control 4: all reagents present except tyramide-tetramethyl rhodamine. This control should produce only the green label, and was used to further confirm that the signal in the red channel was not an artifact.

Microscopy

Sections were visually examined with a Zeiss AxioImager A1 fluorescence microscope (Carl Zeiss, Inc., Thornwood, NY) outfitted with objectives of 20x (numerical aperture [NA] 0.8), 63x oil (NA 1.4) and 100x oil (NA 1.4) and narrow bandwidth filter cubes. Confocal images were acquired at UCLA's Carol Moss Spivak Cell Imaging Facility with a Leica TCS-SP confocal microscope (Leica Microsystems GmbH, Wetzlar, Germany). For this study we used a pinhole of 1.0 Airy units and objectives of 10x (0.4 NA) and 100x oil (NA 1.4), resulting in an estimated optical section thickness (full width at half maximum [FWHM]) of 8.13 µm and 0.62 µm, respectively. Excitation for the green, near red and far red signals, respectively, was

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provided by argon (488 nm), diode (568 nm) and HeNe (633 nm) laser lines. Emission windows were: green, 500–530 nm; near-red, 600–650 nm; far-red, 650–710 nm. Controls with one label omitted (see above) confirmed that the bleed-through between channels with these setting was minimal. The near-red images are presented as red in the figures, and the far-red images are color-coded blue. Most images were acquired with the 100x objective as confocal stacks with optical section separation (z-interval) of 0.488 μm, but a z-interval of 0.203 rm was used to image cell somata. Optical sections were averaged 3–6 times to reduce noise. Images were acquired at a digital size of 1024x1024 pixels (no digital zoom was used) and were later cropped to the relevant part of the field (512x384 pixels) without changing the resolution. Image processing to make the figures was done using Adobe Photoshop 5.5 (Adobe Systems Inc., Mountain View, CA). The “adjustment layer / curves” feature of the program was used to adjust the contrast and balance the intensity of the red, green and blue channels, but only minor adjustments were necessary. In addition, the color hue of the single-color blue panels (but not the color-merged panels) in the figures was shifted slightly to the cyan to make them more visible when printed.

Electron microscopy

Three adult male Wistar rats (230–300 g) were deeply anaesthetized and perfused with fixative consisting of 1% glutaraldehyde/1% formaldehyde. Lumbar spinal cord segments were removed and post-fixed overnight. Spinal cord blocks were cryoprotected in 30% sucrose, frozen rapidly and then thawed to enhance antibody penetration. Transverse sections 60 μm thick were cut with a Vibratome, treated for 30 min each in 50% ethanol and 1% sodium borohydride, then rinsed and incubated for 72 h at 4°C in antibody against CRLR (Table 1) diluted 1:50,000 in PBS. They were incubated for 24 h each in biotinylated donkey anti-rabbit IgG (Jackson ImmunoResearch, 1:500) and Extravidin®-peroxidase conjugate (Sigma, catalogue number E2886, 1:1000), before being reacted with 3,3′-diaminobenzidine in the presence of hydrogen peroxide to reveal peroxidase activity. The sections were osmicated (1% OsO4 for 20 min), dehydrated in acetone and flat-embedded in Durcupan resin. Ultrathin sections through laminae I–III from one dorsal horn were cut with a diamond knife, stained with lead citrate and viewed with a Philips CM100 electron microscope. For each rat, one or two ultrathin sections were examined and at least 100 immunoreactive boutons were observed.

Colocalization measures

Colocalization of two labels means that they are present close enough in the tissue that they cannot be resolved optically. The extent of colocalization of two labels was measured using the “Colocalization” module of Imaris 5.0.2., 64-bit version (Bitplane AG, Saint Paul, MN, www.bitplane.com). This program analyzes stacks of confocal sections acquired in two channels. Each confocal section consists of an array of square elements called pixels. A voxel is defined from a pixel as a prism in which the base is the pixel and the height is the thickness of the confocal section. Imaris Colocalization analyzes the entire confocal stack by measuring the intensity of each label in each voxel. The program uses an iterative procedure (Costes et al., 2004) to determine an intensity threshold (in the 0–255 scale of pixel intensity) for each of the two labels. Voxels with intensities above this threshold are considered to be above the background. A voxel is defined as having colocalization when the intensities of both labels are above their respective thresholds. To avoid investigator bias in setting the thresholds, the program has an automatic thresholding feature (Costes et al., 2004).

The extent of colocalization was expressed by two different measures. 1) “Percentage of material colocalized”, given by the equation:

$$(\sum_{i \in coloc} SA_i / \sum_{i \in objectA} SA_i) \times 100\%$$

where SAi is the intensity of the ith voxel for label A, *i εcoloc* are the voxels of label A with colocalization with label B, and *i εobjectA* are the voxels above threshold for label A. A similar equation is used for channel B. Hence, this approach takes into account the number of voxels with colocalization as well as the intensities ("material") of the two labels in each voxel. It produces a colocalization measure for each of the labels. It has the disadvantage that it does not reflect whether the intensities of the two labels increase and decrease together in the same voxels, whereas when there is a high degree of colocalization the intensities of the two labels would be expected to vary together. Because of this, it may overestimate the amount of colocalization. 2) Pearson coefficient in voxels with colocalization. The Pearson coefficient is a number between +1 and -1, with positive values indicating a direct correlation, negative values indicating an inverse correlation, and values near 0 indicating no correlation. Negative values of the Pearson coefficient are seldom encountered in colocalization studies. In this case, the Pearson coefficient measures the correlation between the intensities of the two labels only in the voxels with colocalization. This measure is more stringent than the 'percentage of material colocalized' because it imposes the additional requirement that the intensities of the two labels vary together. Hence, it may underestimate the amount of colocalization.

The following procedure was used to measure colocalization. A computer folder containing the stack of confocal sections for the two labels was generated by the Leica TCS-SP confocal microscope. This folder was opened with Imaris and converted into an Imaris file. A broad region of interest (ROI) was defined as all the voxels in which the intensity of one of the labels was above a pixel intensity of 10 (in the 0–255 scale). Defining this ROI was necessary to avoid the automatic thresholding feature of the program from failing in some cases. Pixel intensity of 10 was well within the background for all images. Then, automatic thresholding was used to calculate the thresholds for each label. Threshold values varied between pixel intensities of 10 and 90, and generally agreed with background levels estimated visually. Once the thresholds were set, the program outputs the four measures of colocalization described above.

Statistical analysis

Data were analyzed and plotted using Prism 5 (GraphPad Software, San Diego, CA). Statistical analyses consisted of one-way and two-way ANOVA, with significance set at 0.05.

Results

CRLR and RAMP1 are present in the superficial dorsal horn

CGRP receptors are heteromers of CRLR and RAMP1, so we used antibodies against these two proteins to localize these receptors in the rat spinal cord. The two antibodies used were extensively characterized in a previous study (Cottrell et al., 2005). CRLR immunoreactivity was abundant in the superficial dorsal horn (laminae I and II) and in the dorsolateral funiculus (Fig. 1 A), consisting mostly of punctate staining. Very few cells were labeled for CRLR. RAMP1 immunoreactivity was also found in the superficial dorsal horn (Fig. 1 B), but it was less abundant than CRLR immunoreactivity in lamina I and the dorsolateral funiculus. Like CRLR immunoreactivity, RAMP1 immunoreactivity was found in puncta, but it was also present in many dorsal horn neurons. In these, RAMP1 staining was found mostly at (or near) the cell surface, forming a patchy, discontinuous label. Fig. 2 shows an example of these RAMP1 neurons; in the right panel (B), a computerized rendition of the staining was used to better illustrate the discontinuous nature of the RAMP1 staining. It is unlikely that this patchy distribution of RAMP1 at the cell surface was an artifact of the immunohistochemistry procedure, because it was not observed in dorsal horn neurons labeled with antibodies against NK1 receptors (Mantyh et al., 1995; Marvizon et al., 1997) or μ-opioid receptors (Song and Marvizon, 2003a,b), which were uniformly distributed at the cell surface.

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CRLR is not present in primary afferent terminals

There was no colocalization of CRLR immunoreactivity with CGRP (Fig. 3 A), which is contained in a large subpopulation of primary afferents including Aδ- and C-fibers. Moreover, we have shown previously (Cottrell et al., 2005) that CRLR immunoreactivity does not colocalize in the dorsal horn with isolectin B4, a marker for a subpopulation of C-fibers largely complementary to CGRP-containing C-fibers, or with neurofilament of 200 KDa, a marker of A-fibers. Therefore, CRLR immunoreactive puncta in the dorsal horn appear not to be primary afferent terminals.

CRLR and RAMP1 partially colocalize

If CRLR and RAMP1 dimerize to form CGRP receptors, these two proteins should be found together in the same compartments. To determine whether this is the case, we double-labeled spinal cord section with these two antibodies. However, because the CRLR and RAMP1 antibodies were both raised in rabbit, we had to use the method of Shindler and Roth (1996) for double-labeling with antibodies of the same species. This method consists of a sequential labeling procedure in which one of the antibodies is used a very low concentrations and then its signal is amplified using TSA to avoid cross labeling (see Experimental Procedures for details). In parallel assays, TSA was used to amplify the signal of low concentrations (1:75,000 or 1:100,000 dilutions) of either the CRLR or the RAMP1 antibodies, adding the other antibody (RAMP1 or CRLR, respectively) at higher concentration in a subsequent step. The staining obtained was similar, independently of which antibody was used for TSA. Controls in which one of the reagents was omitted confirmed that there was no bleed-though. Furthermore, control slides in which only one of the labels was present were used at the confocal microscope to confirm that there was no bleed-through of one of the signals into the other channel. This control was important because TSA produces an intense label that could potentially be detected in the other channel, giving false colocalization. To prevent this, the emission window of the spectral confocal microscope was narrowed down to 30 nm (500–530 nm) for the green channel (488 nm laser excitation). Another precaution was the use of a monovalent (Fab fragment) secondary antibody for TSA to avoid a possible artifact in which the free binding site of a divalent secondary antibody binds the second primary antibody, leading to false colocalization (see Experimental Procedures). Images in Fig. 3 B show an example of the double staining for CRLR and RAMP1 obtained using this method. Although there were instances of clear colocalization of CRLR and RAMP1 in puncta (arrows in Fig. 3 B), a substantial amount of both labels did not colocalize. CRLR immunoreactivity was largely absent from the cells containing RAMP1, and many puncta were labeled by only one of these antibodies.

We wondered whether the CRLR immunoreactivity that did not colocalize with RAMP1 could represent adrenomedullin receptors, formed by the association of CRLR with RAMP2 and RAMP3 (McLatchie et al., 1998; Husmann et al., 2003; Roh et al., 2004). To explore this possibility, we tested antibodies against RAMP2 and RAMP3 supplied by Santa Cruz Biotechnology (Santa Cruz, CA), which have been used by other investigators (Ma et al., 2006). Unfortunately, we could not get any specific staining with these two antibodies in separate attempts made in two of our laboratories (J.C.G. Marvizon's at UCLA and A.J. Todd's at the University of Glasgow). Our attempts included using four different fixatives: 1) 4% paraformaldehyde, 0.18% picric acid; 2) 1% paraformaldehyde; 3) 4% paraformaldehyde, 4% sucrose, 1.4% cacodylate, and 4) Bouin's fixative (Sillevis Smitt et al., 1993). We also tried using TSA to amplify the signal produced by these antibodies. In all these attempts, only diffused, speckled staining of the dorsal horn was observed, which was likely non-specific.

CRLR and RAMP1 are present in glutamatergic presynaptic terminals

We hypothesized that the puncta labeled for CRLR and RAMP1 were presynaptic terminals. To confirm this, we studied the colocalization of CRLR and RAMP1 with synaptophysin, a

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marker of presynaptic terminals. The synaptophysin antibody stained numerous puncta, but this staining faded towards the inside of the histological sections, indicating poor antibody penetration. This occurred even after the sections were permeabilized by incubating them in 50 % ethanol for 30 min. To avoid false negatives, colocalization with synaptophysin was studied only in confocal sections close to the surface of the histological sections. Both CRLR (Fig. 4 A) and RAMP1 (Fig. 5 A) showed good colocalization with synaptophysin. CRLR (Fig. 4 A, B, D) and RAMP1 (Fig. 5 B) also colocalized with vesicular glutamate transporter-2 (VGLUT2), a marker of glutamatergic presynaptic terminals that is found throughout the dorsal horn (Fig. 1 E). They did not colocalize, however, with vesicular glutamate transporter-1 (VGLUT1), which produced only sparse staining of the superficial dorsal horn (Fig. 1 D) where most of the CRLR and RAMP1 immunoreactivity was present. In triple label experiments, we found abundant three-way colocalization of CRLR, VGLUT2 and synaptophysin (Fig. 4 A), confirming the idea that the presynaptic terminals that contain CRLR are glutamatergic. In triple label experiments with CRLR, RAMP1 and VGLUT2 we found some instances of 3-way colocalization (arrows in Fig. 4 B). However, many of the puncta stained for CRLR and VGLUT2 did not contain RAMP1, indicating the presence of glutamatergic terminals in which CRLR does not form CGRP receptors with RAMP1. Similar results were obtained using triple labeling of CRLR, RAMP1 and synaptophysin (data not shown).

Electron microscopy confirmed that CRLR is present in presynaptic terminals

To further characterize CRLR-containing puncta, we performed electron microscopic immunohistochemistry using the CRLR antibody. Unfortunately, the RAMP1 antibody did not stain glutaraldehyde-fixed tissue and thus could not be used for electron microscopic immunohistochemistry. Virtually all of the CRLR immunoreactive profiles that were seen in laminae I–II contained synaptic vesicles and were identified as axonal boutons. In the majority of cases, synapses formed by these boutons were not present in the section examined. However, some of the immunoreactive boutons were seen to be presynaptic at a single synapse, which could be either asymmetrical (Fig. 6 A, B) or symmetrical (Fig. 6 C). Although the asymmetrical synapses seemed to be more common, no attempt was made to quantify the two types, since asymmetrical synapses can appear symmetrical when viewed in a single section through the edge of the synaptic specialization. Numerous synaptic glomeruli were encountered in laminae II and III, but immunoreaction product was never seen in either the central axonal boutons of these glomeruli, nor in the peripheral axons which form axo-axonic synapses with the central boutons (Ribeiro-da-Silva and Coimbra, 1982; Todd, 1996).

CRLR and RAMP1 are present in opioid-containing terminals

The spinal cord contains opioid peptides derived from the pre-proenkephalin and the pre-prodynorphin genes (Todd and Spike, 1993), whereas the pro-opiomelanocortin gene encoding for endorphins is not expressed in the dorsal horn (Tsou et al., 1986). Enkephalins and dynorphins are released by intrinsic neurons and possibly by descending axons, but not by primary afferents in naïve rats (Song and Marvizon, 2003b). The monoclonal antibody 3-E7 recognizes peptides with the YGGF N-terminus (Gramsch et al., 1983) common to all naturally-occurring opioids with an identified precursor gene, and therefore can be used as a pan-opioid antibody. This antibody labeled puncta in the superficial dorsal horn (laminae I–III) and the dorsolateral funiculus (Fig. 1 C) (Song and Marvizon, 2003a; Song and Marvizon, 2003b), a pattern similar to the staining produced by the CRLR and RAMP1 antibodies (Fig. 1 A, B). In view of this, we wondered whether the presynaptic terminals containing CRLR and RAMP1 also contained opioids. Indeed, we found extensive colocalization of the staining with the 3-E7 pan-opioid antibody with both CRLR (Fig. 3 C) and RAMP1 (Fig. 5 C). In a triple label experiment (Fig. 4 B) we found extensive 3-way colocalization of immunoreactivities for CRLR, VGLUT2 and opioids (3-E7 antibody). Therefore, the puncta containing CRLR and opioids are glutamatergic presynaptic terminals. We also found some instances of 3-way

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colocalization of RAMP1, CRLR and opioids (Fig. 4 C), indicating that some of the opioid-containing terminals have CGRP receptors formed by CRLR and RAMP1. However, many other terminals contained only opioids and CRLR, perhaps because CRLR forms other receptors in these terminals. There were also terminals that contained opioids and RAMP1, but not CRLR.

To determine whether the opioids in CRLR- and RAMP1-containing terminals are enkephalins or dynorphins, we used another mouse monoclonal antibody that recognizes both Met- and Leu-enkephalin, and a guinea pig antiserum against pro-dynorphin (Table 1). Like the 3-E7 antibody, the enkephalin antibody labeled puncta in the superficial dorsal horn, many of which were immunoreactive for CRLR (Fig. 3 D) and for RAMP1 (Fig. 5 D). However, the extent of colocalization was lower than with the 3-E7 antibody (see below). Pro-dynorphin immunoreactivity was found in the cytoplasm of dorsal horn neurons and in a few puncta, but those were less numerous than the puncta stained with the 3-E7 or anti-enkephalin antibodies. A few of the pro-dynorphin positive puncta also contained CRLR (Fig. 3 E). Therefore, it appears that the opioids present in CRLR-containing terminals are both enkephalins and dynorphins.

CRLR and RAMP1 colocalize with adrenergic α_{2C} receptors

Since opioid peptides colocalize with adrenergic α_{2C} receptors in the spinal cord (Stone et al., 1998; Olave and Maxwell, 2002), we studied the possible colocalization of α_{2C} receptors with CRLR and RAMP1 in the dorsal horn. A guinea pig antiserum against α_{2C} receptors (Table 1) stained the dorsal horn and the dorsolateral funiculus (Fig. 1 F). The staining was somewhat more intense in the superficial dorsal horn, where it was found in puncta (Fig. 4 E) and a few cell bodies (Fig. 5 E). There was also abundant 3-way colocalization between α_{2C} receptors, CRLR and opioids, but there were many more terminals containing CRLR and α2C receptors than CRLR and opioids (Fig. 4 E). Although we did not study the colocalization between α_{2C} receptors and VGLUT2, another study (Olave and Maxwell, 2003) reported that it was quite extensive, as suggested by the large colocalization of CRLR with both VGLUT2 and α_{2C}. Although there was also colocalization of α_{2C} receptors with RAMP1, they colocalized with RAMP1 much less frequently that with CRLR in presynaptic terminals (Fig. 5 E). Interestingly, unlike CRLR, RAMP1 colocalized with α_{2C} receptors at the surface of some dorsal horn neurons (Fig. 5 E). Therefore, there is a large population of glutamatergic presynaptic terminals in the dorsal horn that have adrenergic α_{2C} receptors and CRLR-containing receptors, but many of these terminals do not appear to contain RAMP1.

Colocalization quantification

In order to draw clear conclusions in this study, it was important to measure the extent of the colocalization of the different labels. However, this was complicated by the fact that the structures labeled were small puncta whose size was close to the limits of optical resolution. Moreover, the intensity of the two labels varied between puncta, making it difficult to judge whether there was colocalization. To solve these problems, we used Imaris Colocalization software to analyze whole stacks of confocal sections taken from laminae I–II of double-labeled spinal cord sections. The approach used was developed by Costes and Lockett (Costes et al., 2004), and is explained in detail in Experimental Procedures. We used as measures of colocalization the "percentage of material colocalized" (Fig. 7 A) and the Pearson correlation coefficient in voxels with colocalization (Fig. 7 B). Two values for percentage of material colocalized were obtained, corresponding to each antibody (Fig. 7 A), but a two-way ANOVA yielded no significant differences between these pairs of values. Measures of colocalization were found to be unaffected by the orientation of the tissue sections (coronal or sagittal) or by the second antibody and fluorophore. Accordingly, data obtained changing these variables were pooled together. Because minor variations in staining intensity were observed between

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experiments, we did not attempt to standardize the confocal microscope parameters affecting image intensity, which were optimized for each image. Images were not modified in any way before the analysis, in order to avoid introducing biases in it. Consequently, we relied in the thresholding feature of the program to correct for differences between images.

To validate this method, it was first applied to a positive and a negative control for colocalization. As positive control, we double-labeled spinal cord sections with two antibodies against CGRP, one raised in mouse and the other in rabbit (Table 1). As expected, the two antibodies produced virtually the same staining, and we obtained a high percentage of material colocalized (about 90%, Fig. 7 A) and a high Pearson coefficient in colocalized voxels (0.8, Fig. 7 B). As a negative control, we double-labeled spinal cord sections with antibodies against μ-opioid receptors and NK1 receptors. These two receptors are found in different neurons in the dorsal horn (Spike et al., 2002;Song and Marvizon, 2003b) and hence were not expected to colocalize. In this case, the Pearson coefficient was close to zero (Fig. 7 B), but the percentage of material colocalized was quite large, about 40%. This discrepancy reflects the fact that the two labels may be present in the same voxels ('material colocalized') but their intensities may not vary together (Pearson coefficient). Therefore, the value of material colocalized and the Pearson coefficient need to be considered together to evaluate the extent of the colocalization.

The rest of the data in Fig. 7 provide a quantitative evaluation of the qualitative data shown in Figs. 3–5. For the colocalization of CRLR and CGRP, we obtained a Pearson coefficient of zero and a value of material colocalized lower than the negative control, indicating a lack of colocalization. For the colocalization of CRLR and RAMP1, the Pearson coefficient was quite low, and the value of material colocalized (~40%) was comparable with that of the negative controls. This indicates that the overall extent of colocalization of CRLR and RAMP1 was small despite their clear colocalization in some of the puncta (Figs. 3 B, 4 B). In terms of both Pearson correlation coefficients and percentages of material colocalized, CRLR had very high levels of colocalization with VGLUT2 and a2C receptors and somewhat lower levels with synaptophysin, perhaps due to the poor penetration of the synaptophysin antibody in the tissue sections. In contrast, RAMP1 had relatively low Pearson coefficients for colocalization with synaptophysin, VGLUT2 and α_{2C} receptors, indicating that RAMP1 was present in less glutamatergic terminals than CRLR. This finding may also reflect the fact that a substantial part of the RAMP1 immunoreactivity was present in cell bodies. Regarding colocalization with opioids, the values obtained for CRLR and RAMP1 were comparable, suggesting that RAMP1 may be present in some opioid terminals that do not have CRLR. In terms of material colocalized, the colocalization of CRLR with all opioids (3-E7 antibody) was higher than its colocalization with enkephalins, suggesting that CRLR may be present in some dynorphin-containing terminals. We did not attempt to quantify the colocalization of CRLR with the pro-dynorphin antibody used in Fig. 3 E, because the extensive staining of cell bodies with the pro-dynorphin antibody would inevitably skew the results towards low colocalization values. Finally, the high colocalization of opioids with VGLUT2 was confirmed by this quantitative analysis.

Discussion

Colocalization of CRLR and RAMP1

One critical issue in this study is whether the CRLR and RAMP1 proteins detected in our experiments dimerize to form CGRP receptors (McLatchie et al., 1998). A prerequisite for this is that these two proteins are found in the same cellular compartment in close proximity to each other. Although there were some clear instances of colocalization of CRLR and RAMP1 in dorsal horn puncta, many other puncta contained only one of these proteins. Overall, the colocalization of CRLR and RAMP1 was quite low, as indicated by the low Pearson correlation coefficient of their staining intensities. It is unlikely that this low colocalization was caused by

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the inability of the antibodies to recognize CRLR and RAMP1 when they form CGRP receptors, because they were able to recognize functional CGRP receptors expressed in HEK cells (Cottrell et al., 2005). Therefore, our findings indicate that, whereas some of the CRLR and RAMP1 proteins present in the dorsal horn may indeed heterodimerize to form CGRP receptors, a substantial fraction of these proteins may form part of other receptors or exist as monomers. One interesting possibility to explore in future work is that the colocalization of CRLR with RAMP1 increases in some conditions, for example during hyperalgesic states.

In particular, we found that RAMP1 was present in the surface of many neuron-like cells in the dorsal horn that did not stain for CRLR. Since CRLR was detected mostly in presynaptic terminals, it is possible that it undergoes some modification at the terminals that makes it recognizable by the antibody. Some of the RAMP1 immunoreactive puncta that did not stain for CRLR may represent dendrites of the neurons stained for RAMP1, because the colocalization of the presynaptic markers synaptophysin and VGLUT2 with RAMP1 was quite low.

Conversely, CRLR was found in puncta that did not contain RAMP1. It is possible that CRLR dimerizes with RAMP2 to form adrenomedullin receptors, or with RAMP3 to form a mixed adrenomedullin / CGRP receptor (McLatchie et al., 1998; Hay et al., 2003; Husmann et al., 2003; Roh et al., 2004). Although the receptor formed by CRLR and RAMP3 has a lower affinity for CGRP, the high content of CGRP in primary afferent terminals in the dorsal horn suggests that CGRP may be released in amounts high enough to activate it. On the other hand, adrenomedullin may play a role in nociception as important as CGRP, since it is present in DRG neurons and in primary afferent terminals, and it produced hyperalgesia when injected intrathecally (Ma et al., 2006). Using antibodies different from the ones used by us, Ma et al. (2006) found that CRLR, RAMP1 and RAMP2 stained the superficial dorsal horn, whereas RAMP3 stained all dorsal horn laminae. In contrast to our findings, they reported that CRLR and RAMP1 immunoreactivity was present in cell bodies and not in puncta. These antisera were not characterized in transfected cells, unlike the antibodies described here, RK11 and 9891 (Cottrell et al., 2005). Moreover, they did not determine whether CRLR colocalized with RAMP1 and RAMP2, which would be required for the formation of functional receptors. We could not replicate the staining obtained by Ma et al. with the RAMP2 and RAMP3 antibodies that they used. It would be important to study the colocalization of RAMP2 and RAMP3 with CRLR in the dorsal horn using reliable antibodies.

CRLR and RAMP1 are present in presynaptic terminals

The high degree of colocalization of CRLR with the presynaptic markers synaptophysin (Knaus and Betz, 1990) and VGLUT2 shows that this protein is located mainly in presynaptic terminals. Moreover, the CRLR-containing presynaptic terminals are glutamatergic, since VGLUT2 is a marker for this type of terminals (Li et al., 2003; Todd et al., 2003). Electron microscopic immunohistochemistry confirmed that CRLR was present in presynaptic terminals, since the structures that contained it had synaptic vesicles and were often associated with synaptic clefts and synaptic densities. RAMP1 also colocalized with VGLUT2 and synaptophysin, although to a lesser degree than CRLR, which can be attributed to the presence of RAMP1 in cell bodies and dendrites. Triple label experiments revealed a few instances of three-way colocalization of CRLR, RAMP1 and VGLUT2, indicating that some glutamatergic terminals contain CGRP receptors formed by CRLR and RAMP1.

The presynaptic terminals that contain CRLR are not the central terminals of primary afferents. In a previous study (Cottrell et al., 2005), we showed that CRLR immunoreactivity did not colocalize with the following markers of primary afferents (Stucky and Lewin, 1999; Marvizon et al., 2002): CGRP, a marker of peptidergic C-fibers; isolectin B4, a marker of non-peptidergic C-fibers, and neurofilament of 200 KDa, a marker of A-fibers. In the present study we

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quantified the colocalization of CRLR with CGRP, showing that it yielded a Pearson correlation coefficient of zero (0.014, 95% confidence interval −0.009 − 0.035). We also found that CRLR did not colocalize with VGLUT1, which is present in many A-fiber terminals (Li et al., 2003; Todd et al., 2003). Moreover, the CRLR-containing presynaptic terminals observed with electron microscopy were not in synaptic glomeruli, structures formed in association with some C-fiber terminals (Todd, 1996). Oddly, CRLR and RAMP1 immunoreactivities were detected in DRG neurons and in peripheral fibers (Cottrell et al., 2005). Moreover, mRNAs encoding for CRLR and RAMP1 were also detected in DRG (Cottrell et al., 2005). Hence, it appears that CRLR and RAMP1 are expressed by DRG neurons but are selectively transported to the periphery and not to the central terminals of primary afferents. Further work will be necessary to clarify this issue.

The general distribution of the staining that we obtained with the CRLR and RAMP1 antibodies agrees with that obtained by Ye et al. (1999) with an antibody against CGRP receptors isolated from pig brain. However, at high magnification and using electron microscopy, Ye et al. found that the receptor was located in dorsal horn neurons, dendrites and some primary afferent terminals, in contrast with the presynaptic location that we found. At present, it is not clear whether the CGRP-binding protein isolated from the brain by Wimalawansa et al. (1993) was indeed CRLR.

Given the small size of presynaptic terminals, it was difficult to establish whether CRLR was localized at the membrane or intracellularly. RAMP1 was clearly present at the cell surface of somata, but this was unclear in puncta. Under high magnification confocal microscopy, CRLR and RAMP1 puncta presented a uniform staining consistent with an intracellular localization, but their additional presence at the cell surface cannot be ruled out. Using electron microscopy, CRLR immunoreactivity appeared associated with synaptic vesicles. However, pre-embedding with immunoperoxidase is not entirely reliable for subcellular localization, since the reaction product can translocate (within membrane-delimited spaces) during processing (Spike et al., 1997). Electron microscopic immunohistochemistry using immunogold provides a more reliable method to localize proteins. However, the immunogold technique often does not work and has very limited penetration in the tissue.

CRLR and RAMP1 colocalize with opioid peptides

An important finding is this study was that both CRLR and RAMP1 colocalize in the dorsal horn with opioids. Triple label experiment using antibodies against CRLR, opioids and VGLUT2 showed that CRLR and opioids are present together in glutamatergic presynaptic terminals. This is consistent with a previous study (Todd et al., 2003) suggesting that opioid-containing terminals in the dorsal horn are largely excitatory. Another triple label experiment showed occasional three-way colocalization of CRLR, RAMP1 and opioids. These findings show that glutamatergic terminals that contain opioids in the dorsal horn also contain CRLR, which may form CGRP receptors with RAMP1.

The opioids that colocalize with CRLR and RAMP1 are enkephalins and may also include dynorphins. A selective antibody against enkephalins produced somewhat less colocalization with CRLR than the pan-opioid antibody 3-E7 ($p<0.05$ for material colocalized, Bonferroni's post-test), suggesting that terminals with CGRP receptors are not exclusively enkephalinergic. A pro-dynorphin antibody yielded some colocalization with CRLR in puncta. Products of the pro-opiomelanocortin gene like β-endorphin are largely absent from the dorsal horn (Tsou et al., 1986).

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CRLR and RAMP1 colocalize with adrenergic a2C receptors

Previous studies have shown that opioid-containing terminals in the dorsal horn have adrenergic α_{2C} receptors (Stone et al., 1998; Olave and Maxwell, 2002), and that most of these terminals are excitatory (Olave and Maxwell, 2003). Given the similarities between these terminals with those containing CRLR, we investigated whether CRLR and RAMP1 colocalized with α_{2C} receptors. Indeed, we found that the colocalization of CRLR with α_{2C} receptors was even more extensive than their colocalization with opioids ($p<0.05$ for 'material colocalized' and Pearson coefficient, Bonferroni's post-test). In contrast, the colocalization of α_{2C} receptors with RAMP1 was much less abundant than with CRLR, particularly if measured with the Pearson coefficient ($p<0.001$, Bonferroni's post-test). Many presynaptic terminals that contain CRLR and α_{2C} receptors appear to lack RAMP1, suggesting that CRLR in these terminals may form adrenomedullin receptors with RAMP2 or RAMP3.

Importantly, the glutamatergic presynaptic terminals that contain α_{2C} receptors make synapses with lamina I neurons in that possess NK1 receptors and send axons to the brainstem (Olave and Maxwell, 2003). Our data indicate that these presynaptic terminals also contain CRLR and thus may respond to CGRP and/or adrenomedullin. Therefore, α_{2C} receptors and CGRP/ adrenomedullin receptors may control pain sensations by modulating the excitatory input onto the NK1 receptor projection neurons.

Triple label experiments confirmed that CRLR, α_{2C} receptors and opioids are present together in the same terminals. This suggests that CGRP receptors and α_{2C} receptors control the release of opioids. Indeed, our preliminary results show that CGRP decreases opioid release evoked by electrical stimulation of spinal cord slices, measured with μ-opioid receptor internalization (Marvizon et al., 2005). However, an inhibitory effect of CGRP receptors is surprising, because they signal through α_s G proteins which usually produce stimulatory effects by activating adenylyl cyclase and protein kinase A. In contrast, α_{2C} receptors signal through α_i G proteins, producing inhibition. When they are located presynaptically, α_{2C} receptors inhibit neurotransmitter release by inactivating voltage-gated Ca^{2+} channels through the βγ subunits of G proteins (Adamson et al., 1989; Li and Bayliss, 1998; Dolphin, 2003). Hence, it is possible that CGRP receptors inhibit opioid release indirectly through the α_{2C} receptors. For example, CGRP receptors may induce the phosphorylation of the α_{2C} receptors by protein kinase A, increasing their inhibitory effect on opioid release. The fact that these are also excitatory terminals making synapses with projection neurons (Olave and Maxwell, 2003) may lead to paradoxical effects. On the one hand, inhibition of glutamate release by α_{2C} receptors would diminish the firing of the projection neuron and thus decrease pain. On the other hand, the same α_{2C} receptors would be expected to inhibit opioid release, and thus increase pain. How CGRP/ adrenomedullin receptors present in the same terminals contribute to these effects is unclear. Further studies will be necessary to investigate these issues.

To summarize, we used antisera specifically recognizing RAMP1 and CRLR to localize these components of the CGRP receptor in the rat spinal cord, and found they were present in presynaptic glutamatergic terminals that often contained opioids and α_{2C} receptors. Because of incomplete colocalization between CRLR and RAMP1, some of the CRLR staining may reflect the presence of other CRLR-containing receptors. Their presynaptic location suggests that CGRP receptors may modulate neurotransmitter release from these excitatory terminals.

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Abbreviations

ANOVA
: analysis of variance

CGRP
: Calcitonin Gene-Related Peptide

CRLR
: Calcitonin Receptor-Like Receptor

DRG
: dorsal root ganglion

FWHM
: full width half maximum

NA
: numerical aperture

NS
: normal serum

PBS
: phosphate-buffered saline

PBS/Triton
: phosphate-buffered saline with 0.5 % Triton X-100 and 0.01% thimerosal

RAMP-1
: Receptor Activity Modifying Protein 1

TSA
: tyramide signal amplification

VGLUT1
: vesicular glutamate transporter 1

VGLUT2
: vesicular glutamate transporter 2

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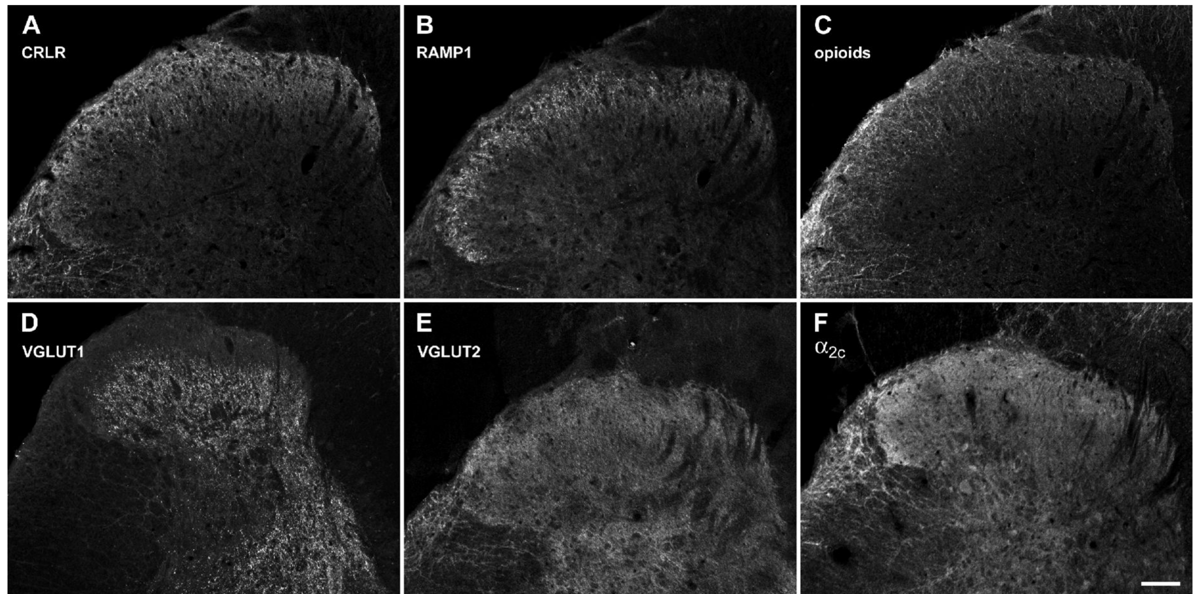

Fig. 1. Dorsal horn staining with antibodies against CRLR, RAMP1, opioids, VGLUT1, VGLUT2 and adrenergic a2C receptors

Spinal cord sections were labeled with antibodies recognizing CRLR (A), RAMP1 (B), opioid peptides (C, monoclonal antibody 3-E7), VGLUT1 (D), VGLUT2 (E) or adrenergic α_{2C} receptors (F). Images A, B and C are from the same histological section triple-labeled using, respectively, an Alexa Fuor 488 secondary antibody, TSA with tetramethyl-rhodamine, and an Alexa Fluor 633 secondary antibody. Images are single confocal sections taken with a 10x objective. Scale bar is 100 µm.

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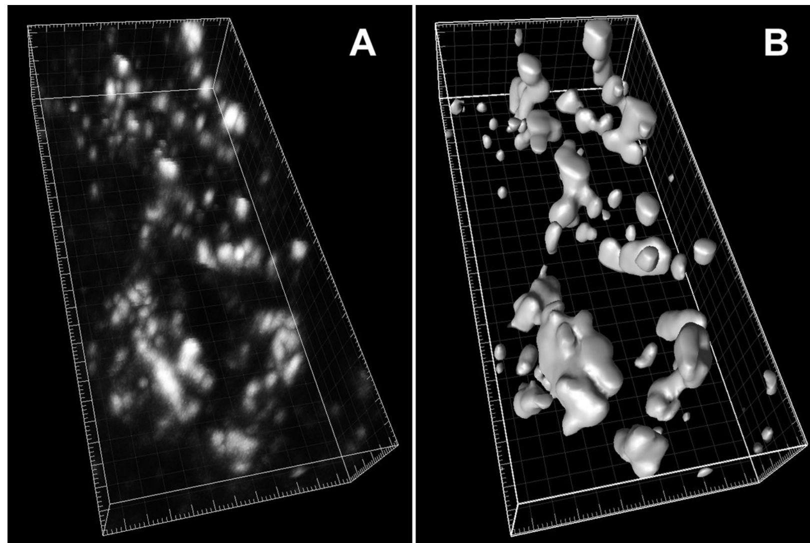

Fig. 2. Dorsal horn neuron labeled with the RAMP1 antibody

Three-dimensional reconstruction of a neuron in the superficial dorsal horn (lamina II) stained for RAMP1. Confocal microscope images were obtained with optical section separation (z-interval) of 0.203 μm and voxel size of 0.049(x)–0.049(y)–0.203(z) rm. **A:** Stack of 27 optical sections from the cell. **B:** Iso-surface rendition obtained from the stack using Imaris. The surfaces enclose voxels of intensities over 40, and were resampled to a voxel size of 0.180(x) –0.180(y)–0.196(z) and smoothed using a Gaussian filter width of 0.200 μm. RAMP1 is present mostly at the cell surface and is distributed forming patches. A few RAMP1 immunoreactive puncta are also present around the cell. Grid units are 1 μm.

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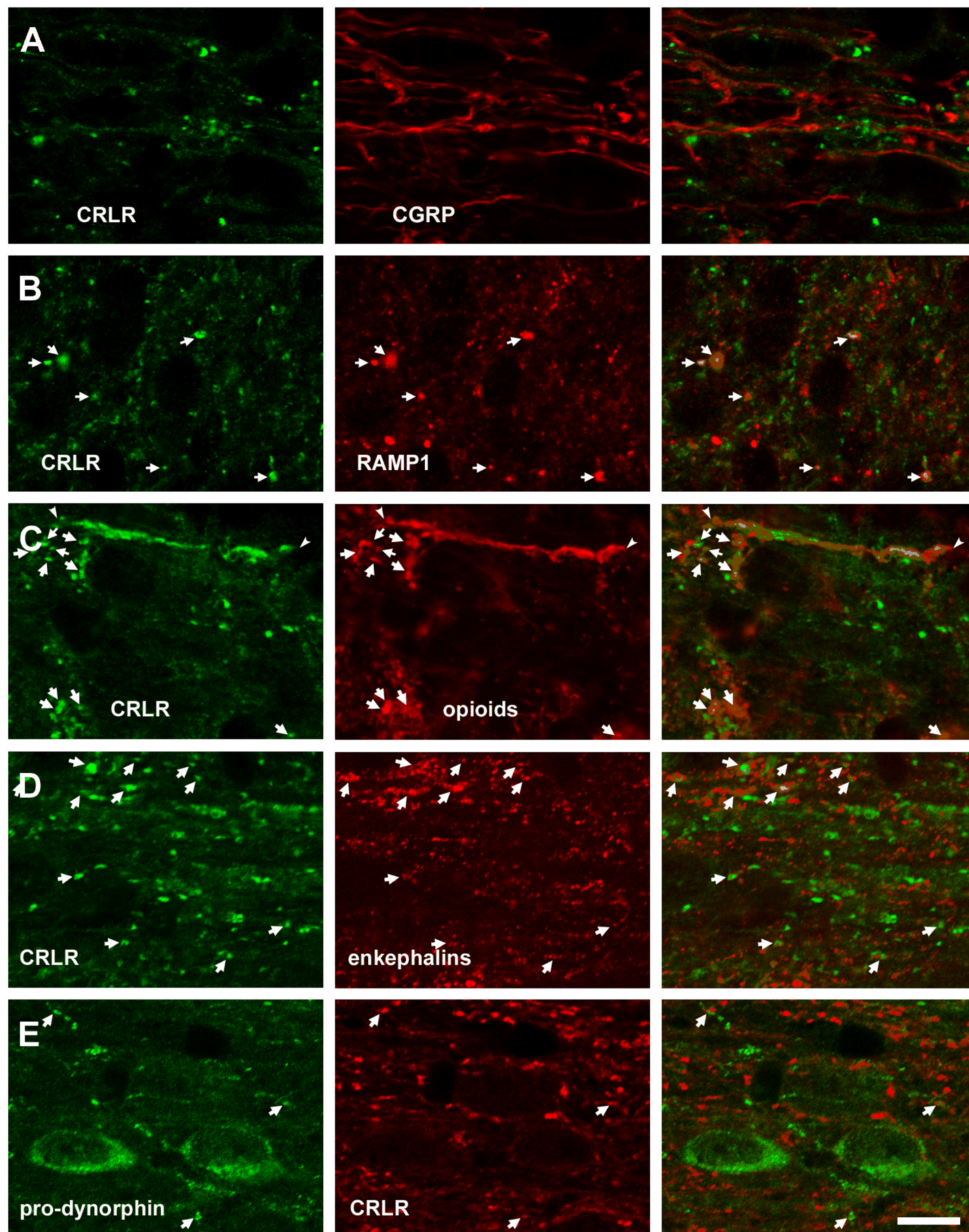

Fig. 3. Double-labeling of spinal cord sections with antibodies against CRLR and CGRP, RAMP1 or opioids

Images are single optical sections taken with a 100x objective (section thickness 0.6 μm). Left and central panels correspond to labels with green (Alexa Fluor-488) and red fluorophores (Alexa Fluor-568 or tetramethyl-rhodamine with TSA), respectively. Right panels are the merged images. **A.** Sagittal section (lamina II) labeled with the CRLR antibody and a mouse anti-CGRP antibody using conventional double-label immunofluorescence; there was no colocalization. **B.** Coronal section (lamina II) labeled for RAMP1 and CRLR using the method of Shindler & Roth; there is colocalization in some of the puncta (arrows) but single label for CRLR or RAMP1 in other puncta. **C.** Sagittal section (lamina I) labeled for CRLR and opioids

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(3-E7 antibody, conventional double-label); the two labels colocalize in one long fiber (bracketed by arrowheads at the top of the panel) and in several puncta (arrows). **D.** Sagittal section (lamina I) labeled for CRLR and enkephalins (conventional double-label); there is colocalization in some puncta (arrows). **E.** Sagittal section (lamina II) labeled for prodynorphin and CRLR (conventional double-label); there is some colocalization (arrows). Scale bar is 10 μm.

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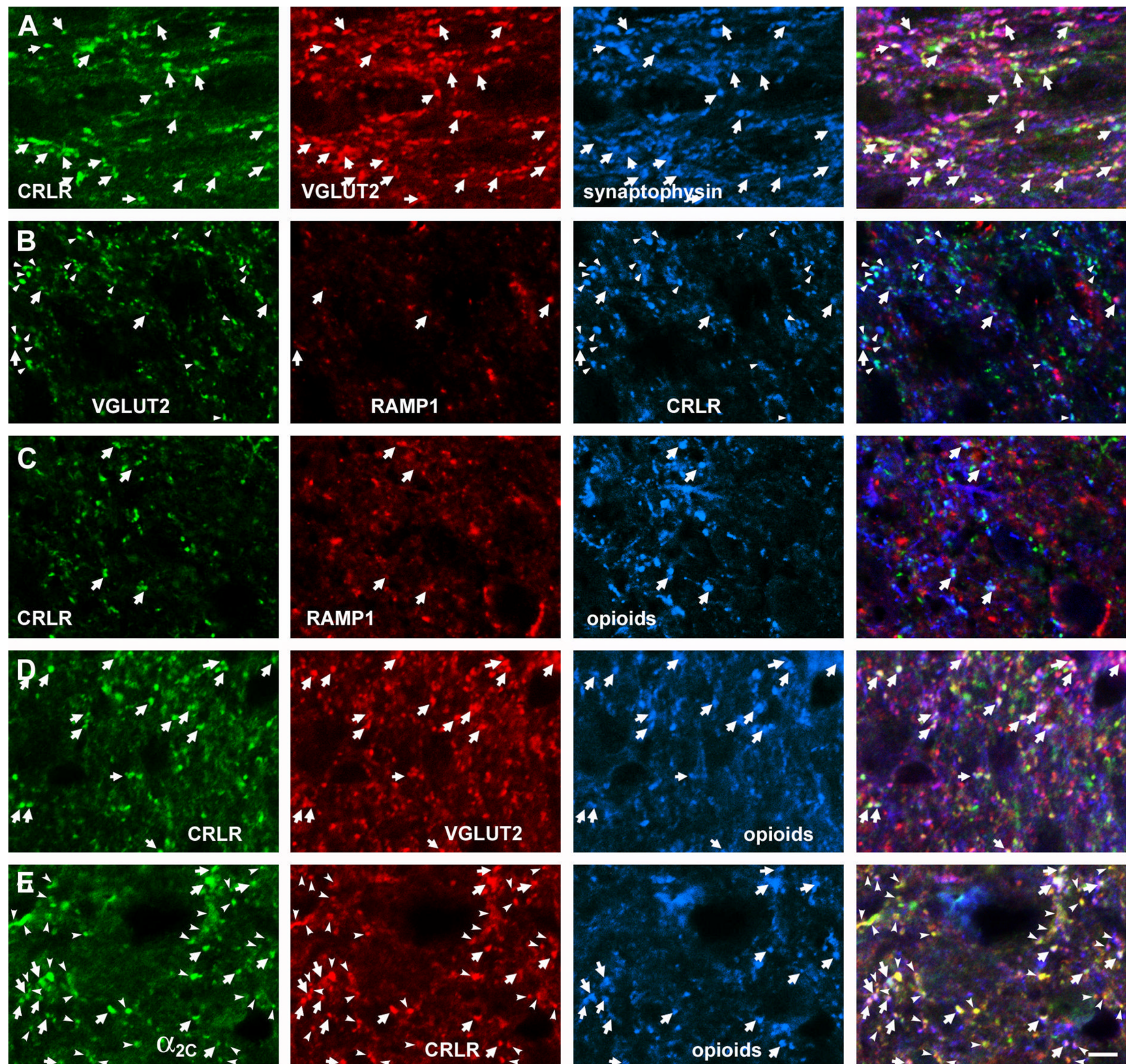

Fig. 4. Triple-labeling of spinal cord sections with antibodies against CRLR, RAMP1, VGLUT2, synaptophysin, opioids and α_{2C} adrenergic receptors

Images are single optical sections (100x objective; section thickness 0.6 μm). The first, second and third panels from the left correspond, respectively, to labels with green (Alexa Fluor-488), near red (Alexa Fluor-568 or tetramethyl-rhodamine) and far red (Alexa Fluor-633) fluorophores. The far red signal was coded blue. Right panels show the merged images. Arrows indicate 3-way colocalization. Conventional double-label immunofluorescence was used except panels A and B, in which the method of Shindler & Roth (1996) was used for CRLR and RAMP1. A. Coronal section (laminae I–II) labeled for VGLUT2, RAMP1 (using TSA), and CRLR; arrowheads indicate colocalization of VGLUT2 and CRLR. B. Coronal section (laminae I–II) labeled for CRLR, RAMP1 (using TSA) and opioids (antibody 3-E7). C. Sagittal section (lamina I) labeled for CRLR, VGLUT2 and synaptophysin; practically all the VGLUT2 puncta are positive for synaptophysin (purple-magenta), and many of them are also positive for CRLR (arrows). D. Coronal section (lateral lamina I) labeled for CRLR, VGLUT2 and opioids (3-E7 antibody); VGLUT2 colocalizes abundantly with opioids (purple/magenta) and many of these puncta are also positive for CRLR (arrows). E. Coronal section (lateral lamina

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I) labeled for α_{2C} adrenergic receptors, CRLR, and opioids (3-E7 antibody); colocalization of CRLR with α_{2C} receptors is almost complete (yellow, arrowheads), and there is also abundant three-way colocalization (arrows). Scale bar is 10 μm.

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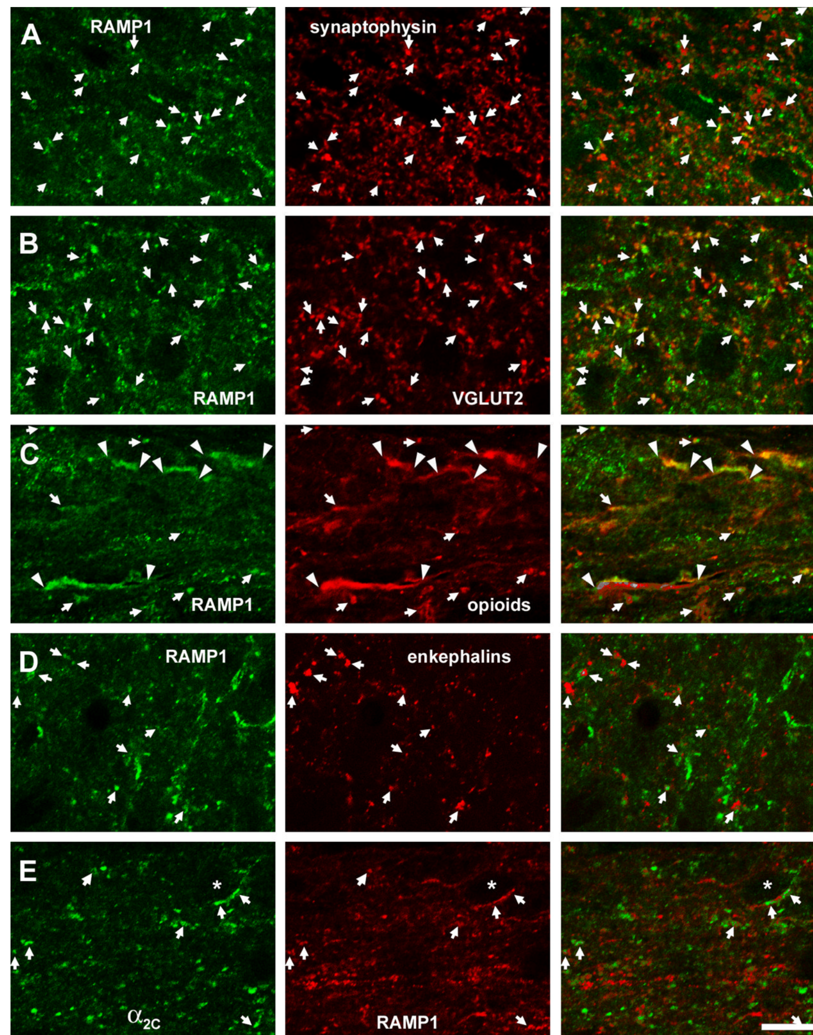

Fig. 5. Double-labeling of spinal cord sections for RAMP1 and synaptophysin, VGLUT2, opioids or α_{2C} adrenergic receptors

Images are single optical sections taken with a 100x objective (section thickness 0.6 µm). Left and central panels correspond, respectively, to labels with green (Alexa Fluor-488) and red (Alexa Fluor-568) fluorophores. Right panels show merged images. Arrows indicate puncta with colocalization. Conventional double-label immunofluorescence was used in all panels. **A.** Coronal section (central lamina I) labeled for RAMP1 and synaptophysin. **B.** Coronal section (central lamina I) labeled for RAMP1 and VGLUT2. **C.** Sagittal section (lamina I) labeled for RAMP1 and opioids (antibody 3-E7); the two labels colocalize in several fibers (bracketed by arrowheads) and in puncta. **D.** Coronal section (lamina II) labeled for RAMP1 and enkephalins. **E.** Sagittal section (lamina II) labeled for adrenergic α_{2C} receptors and RAMP1; the asterisk (*) indicates a cell with both α_{2C} receptors and RAMP1 at the surface. Scale bar is 10 µm.

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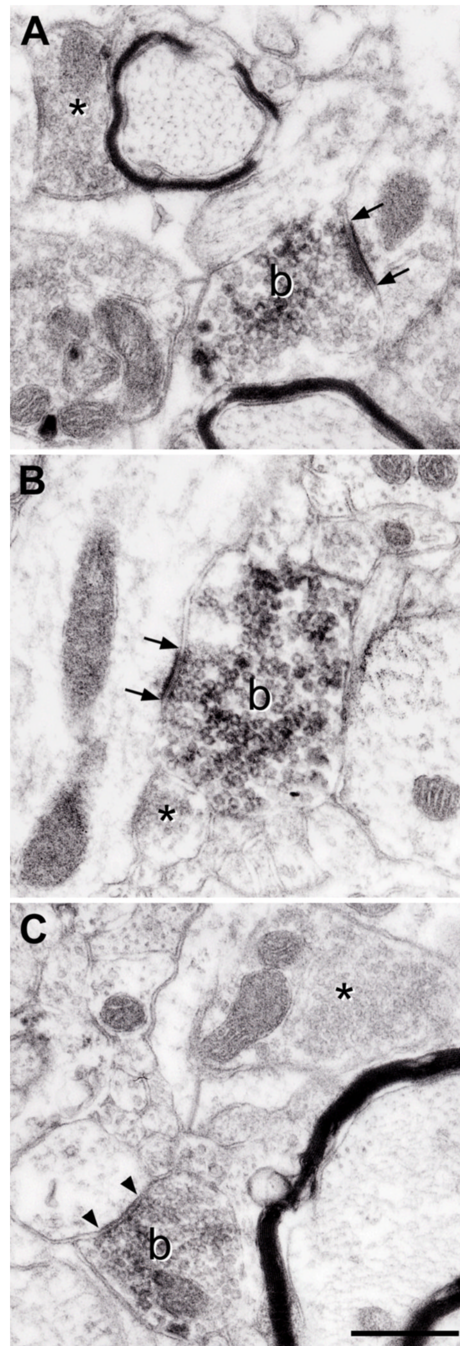

Fig. 6. Electron microscopic immunohistochemistry using the CRLR antibody

Staining was often found in boutons that contained dense-cored vesicles. **A, B:** Immunoreactive boutons (b) that formed asymmetrical synapses (between arrows). **C:** Immunoreactive bouton (b) that forms a symmetrical synapse (between arrowheads). Note that in the immunoreactive boutons many of the synaptic vesicles are coated with immunoreaction product, whereas in non-immunoreactive boutons (asterisks) the vesicles are much paler. Scale bars are 0.5 μm.

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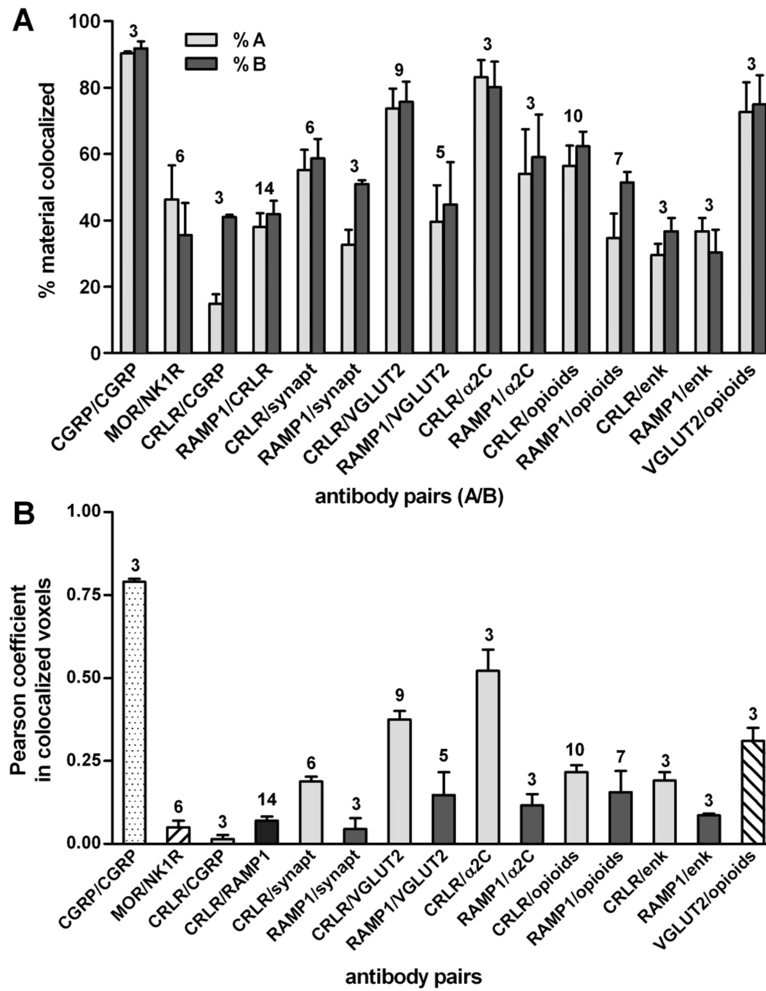

Fig. 7. Colocalization quantification

Confocal images (100x objective) were taken from laminae I–II of sections double-labeled with the antibody pairs indicated in the X-axis (examples are shown in Figs. 3–5). The extent of colocalization was measured in whole stacks of optical sections using Imaris Colocalization (see Experimental Procedures). A voxel was defined to have colocalization when the intensities of both labels are above intensity thresholds calculated by the program. Bars are the mean ± SEM of values from n confocal images (indicated above each bar) obtained from as many histological sections. Data are from four rats. **A:** Amount of colocalization measured as the “percentage of material colocalized”. Each pair of bars indicates the percentage of material A and B (indicated under each bar pair in that order) that are in the colocalized volume relative to the total material of that label above threshold. “Material” refers to a measure taking into account both the number of voxels and their intensities. A two-way ANOVA revealed significant differences between antibody pairs ($p<0.0001$), but not between A and B within pairs ($p=0.09$). **B:** Amount of colocalization measured as the Pearson correlation coefficient in the voxels with colocalization. One-way ANOVA revealed significant differences ($p<0.0001$). Bar patterns: clear bars represent colocalization with CRLR; dark bars represent colocalization with RAMP1; darkest bar is colocalization of CRLR with RAMP1; pointed and hatched patterns correspond to controls or other antibody pairs. Abbreviations: MOR, μ-opioid receptors; NK1R, neurokinin 1 receptor; synapt, synaptophysin; α_{2C}, adrenergic α_{2C} receptors; enk, enkephalins. CGRP/CGRP: two antibodies against CGRP (rabbit and mouse).

Table 1

– Primary antibodies

antibody name	Species and type	source Cat. No.*	dilution used	specificity	Characterization references
3-E7	mouse monoclonal	Gramsch E7-1000	1:1000	Recognizes the YGGF common N-terminus of enkephalins, dynorphins and endorphins	(Gramsch et al., 1983; Song and Marvizon, 2003a)
α_{2C} receptor	guinea pig serum	Neuromics GP-10102	1:300	Raised against C-terminus residues 443-455 of the rat α_{2C} receptor	(Stone et al., 1998)
CGRP	mouse monoclonal	CURE 4901	1:1000	Raised against rat α-CGRP	(Wong et al., 1993)
CRLR	rabbit serum	CURE RK-11	1:2000†	Raised against the C-terminal 18 residues of rat CRLR	(Cottrell et al., 2005)
enkephalin	mouse monoclonal	Chemicon MAB-135	1:200	Recognizes Met- and Leu-enkephalins and Met-enkephalin hexapeptides, but not dynorphin or β-endorphin	(Cuello et al., 1984)
pro-dynorphin	guinea pig serum	Neuromics GP-10110	1:500	Raised against residues 245-248 of rat pro-dynorphin	(Arvidsson et al., 1995)
RAMP1	rabbit serum	CURE 9892	1:1000†	Raised against the C-terminal 9 residues of human RAMP1	(Cottrell et al., 2005)
synaptophysin	mouse monoclonal	Chemicon MAB-5258	1:500	Clone SY38. Raised against pentapeptide in the C-terminus of synaptophysin. Labels presynaptic vesicles.	(Knaus and Betz, 1990)
VGLUT1	guinea pig serum	Chemicon AB-5905	1:5000	Raised against a 19-residue peptide of rat VGLUT1	(Todd et al., 2003)
VGLUT2	guinea pig serum	Chemicon AB-5907	1:2000	Raised against a 18-residue peptide of rat VGLUT2	(Todd et al., 2003)

The table lists the primary antibodies used in this study; the species in which they were raised and whether they were monoclonal or antiserums; their source (commercial or otherwise); the dilution at which they were incubated with histological sections; the immunizing peptides used to raise them or the sequence that they recognize, and references in which the antibodies were characterized.

* Complete names of sources: Chemicon International, Inc., Temecula, CA; CURE: Digestive Diseases Research Center, Antibody/RIA Core, Dept. of Medicine, UCLA, Los Angeles, CA; Gramsch Laboratories, Schwabhausen, Germany; Neuromics, Edina, MN.

† 1:100000 for TSA.

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