Published in final edited form as:

J Comp Neurol. 2009 November 1; 517(1): 51–68. doi:10.1002/cne.22130.

Enkephalins, dynorphins and β -endorphin in the rat dorsal horn: an immunofluorescence colocalization study

Juan Carlos G. Marvizón^{1,2}, Wenling Chen¹, and Niall Murphy³

¹ Center for the Neurobiology of Stress, Division of Digestive Diseases, Department of Medicine, David Geffen School of Medicine at UCLA, Los Angeles, CA 90095, USA

² Veteran Affairs Greater Los Angeles Healthcare System, Los Angeles, CA 90073, USA

³ Molecular Neuropathology Group, RIKEN Brain Science Institute, Wako, Saitama 351-0198, Japan

Abstract

To characterize neuronal pathways that release opioid peptides in the rat dorsal horn, multiple-label immunohistochemistry, confocal microscopy and computerized colocalization measures were used to characterize opioid-containing terminals and cells. An antibody that selectively recognized β -endorphin labeled fibers and neurons in the ventral horn, fibers in the lateral funiculus and lamina X, but practically no fibers in the dorsal horn. An anti-enkephalin antibody, which recognized Leu-, Met- and Phe-Arg-Met-enkephalin, labeled the dorsolateral funiculus and numerous puncta in laminae I–III and V of the dorsal horn. An antibody against Phe-Arg-Met-enkephalin, which did not recognize Leu- and Met-enkephalin, labeled the same puncta. Antibodies against dynorphin and prodynorphin labeled puncta and fibers in laminae I, II and V, and some fibers in the rest of the dorsal horn. Dynorphin and prodynorphin immunoreactivities colocalized in some puncta and fibers, but the prodynorphin antibody additionally labeled cell bodies. There was no colocalization of dynorphin (or prodynorphin) with enkephalin (or Phe-Arg-Met-enkephalin). Enkephalin immunoreactivity did not colocalize with the C-fibers markers CGRP, substance P and isolectin B4. In contrast, there was some colocalization of dynorphin and prodynorphin with CGRP and substance P, but not with isolectin B4. Both enkephalin and dynorphin partly colocalized with vesicular glutamate transporter 2, a marker of glutamatergic terminals. The prodynorphin-positive neurons in the dorsal horn were distinct from neurons expressing μ -opioid receptors, neurokinin 1 receptors and protein kinase C- γ . These results show that enkephalins and dynorphins are present in different populations of dorsal horn neurons. In addition, dynorphin is present in some C-fibers.

Keywords

Calcitonin gene-related peptide; dorsal root ganglia; μ -opioid receptor; prodynorphin; spinal cord; substance P

Introduction

The strong analgesic effect of opiate drugs takes place in large part at the dorsal horn of the spinal cord, where is mediated by μ , δ and κ opioid receptors (Budai and Fields, 1998; Chen and Pan, 2006; Jensen and Yaksh, 1984; Morgan et al., 1991; Yaksh, 1997; Zorman et al.,

1982). One important, largely unresolved question is what neuronal circuits release the opioid peptides that activate these receptors.

There are close to thirty endogenous opioid peptides, which are encoded by three genes (Costa et al., 1987; Weber et al., 1983). Endorphins, as well as the non-opioid peptides adrenocorticotrophic hormone (ACTH) and melanocyte-stimulating hormone, are encoded by the pro-opiomelanocortin (POMC) gene. The proenkephalin gene contains six copies of Met-enkephalin and one copy of Leu-enkephalin, which can give rise to these two pentapeptides, to the heptapeptide Phe-Arg-Met-enkephalin (FRM-enkephalin) or to longer peptides. The prodynorphin gene encodes dynorphin A, dynorphin B and α -neoendorphin, all of which contain the Leu-enkephalin sequence.

β -endorphin was generally believed not to be present in the spinal cord, except during development (Haynes et al., 1982). Measures of opioid release from the cat or the rat spinal cord (Yaksh et al., 1983) found that β -endorphin was released in much smaller amounts than enkephalins and dynorphins. Later studies (Kohler et al., 1984; Tsou et al., 1986) showed the presence in the spinal cord of fibers immunoreactive to POMC-derived peptides. In particular, Tsou et al. reported the presence of β -endorphin in lamina X and the ventral horn, but it was unclear whether it was present in the dorsal horn, where it could modulate pain. β -endorphin-containing fibers were thought to be of supraspinal origin, but a later study (Gutstein et al., 1992) revealed that about one third of the β -endorphin in the spinal cord persisted below the level of a spinal transection. This suggests that some spinal cord neurons express the POMC gene. However, the method used to measure β -endorphin (radioimmunoassay) did not allow to determine whether β -endorphin was present in the dorsal horn.

The dorsal horn contains in abundance both enkephalins (Aronin et al., 1981; Elde et al., 1976; Glazer and Basbaum, 1981; Hokfelt et al., 1977; Hunt et al., 1980; LaMotte and de Lanerolle, 1983) and dynorphins (Botticelli et al., 1981; Cruz and Basbaum, 1985; Khachaturian et al., 1982; Vincent et al., 1982). Since immunoreactivity for these two peptide families overlaps in the superficial dorsal horn, it is not clear whether enkephalin and dynorphins are contained by the same or different presynaptic terminals. Based on differences in the distribution and morphology of enkephalin-immunoreactive and dynorphin B-immunoreactive cells in the cat spinal cord, Cruz and Basbaum (1985) inferred that they were expressed in different neurons. In another study (Standaert et al., 1986), sequential labeling with enkephalin and dynorphin antibodies suggested that these two peptides are expressed by different neurons.

A related question is whether enkephalins and dynorphins are present in the primary afferent terminals in the spinal cord. In situ hybridization revealed that only 3.5 % of the DRG neurons express the proenkephalin gene, whereas Met-enkephalin immunoreactivity was practically absent from the DRG (Pohl et al., 1994). Some studies reported colocalization of enkephalins with substance P in the dorsal horn (Ribeiro-da-Silva et al., 1991; Senba et al., 1988), which may represent substance P-containing dorsal horn neurons and not primary afferent terminals (Hunt et al., 1981). Still, opioids do not appear to be co-released with substance P (Song and Marvizón, 2003b; Trafton et al., 2000).

Whether dynorphin is present in primary afferents is still unclear. Dorsal rhizotomy was found to decrease dynorphin immunoreactivity in the dorsal horn of the rat (Tuchscherer and Seybold, 1989) and in the sacral (but not lumbar) dorsal horn of the cat (Basbaum et al., 1986). Tuchscherer and Seybold also found that dynorphin A (1–8) and substance P immunoreactivities colocalizes in dorsal horn terminals. However, another study (Botticelli

et al., 1981) reported that rhizotomy did not affect dynorphin immunoreactivity in the rat dorsal horn.

In the present study we used multiple-label immunofluorescence, confocal microscopy and computerized colocalization measures to characterize terminals containing enkephalins and dynorphins in the rat dorsal horn.

Material and Methods

Animals

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.

Transgenic mice with a selective deficiency of β -endorphin were obtained as described (Rubinstein et al., 1996) and used to characterize the Mu(-5)N antibody against β -endorphin. Wild type mice were C57BL/6N from the Jackson Laboratory (Bar Harbor, Maine).

Antibody Characterization

The species, sources, dilutions and immunogen of the primary antibodies are indicated in Table 1.

β -endorphin antibody [Mu(-5)N]—The antibody was raised in rabbit against a peptide encompassing the majority of the β -endorphin sequence except for the N-terminus Tyr-Gly-Gly-Phe-Met sequence (Table 1), which was omitted in order to avoid cross-reactivity with enkephalins and dynorphins. A Cys residue was added for conjugation to keyhole limpet hemocyanine (KLH). The antibody was purified by affinity chromatography. This antibody was characterized in this study using β -endorphin deficient mice (see Results and Fig. 1).

Calcitonin gene-related peptide (CGRP) antibodies—Three different antibodies against CGRP were used in this study (Table 1). One was raised in goat against a 20-residues peptide at the N-terminus of human CGRP, coupled to KHL. A second one was a mouse monoclonal antibody produced from a hybridoma made from spleen cells from mice immunized with rat α -CGRP. In radioimmunoassay, this antibody displays 10-fold selectivity for rat α -CGRP over rat β -CGRP and human α -CGRP (Wong et al., 1993). The third one was a rabbit antisera generated against the synthetic peptide (Tyr⁰) rat CGRP₂₃₋₃₇ coupled to KLH via glutaraldehyde. In radioimmunoassay, this antibody showed high affinity and sensitivity and no cross-reactivity with calcitonin and other unrelated peptides (Sternini and Brecha, 1986). Immunoreactivity to these three antibodies colocalized extensively at the cellular and subcellular levels in dorsal root ganglia (Marvizón et al., 2002). In the present study, triple labeling of spinal cord sections with these three antibodies resulted in a high degree of three-way colocalization of the staining in puncta and fibers in the dorsal horn (Fig. 2). The pattern of staining with these antibodies matches the previously described staining pattern for CGRP in the rat dorsal horn (Cottrell et al., 2005; Garry et al., 2000).

Dynorphin A antibody—This antibody was raised in rabbit against pig dynorphin A₁₋₁₇ conjugated with bovine serum albumin (BSA) (Table 1). The specificity of this antibody for different opioid peptides was studied by measuring its immunoreactivity in the dorsal horn after preadsorption with dynorphin A and several other opioids (Fig. 3C).

Enkephalin antibodies—A monoclonal antibody against enkephalins was generated by immunizing mice with Leu-enkephalin conjugated with BSA. In radioimmunoassay, this antibody did not discriminate between Leu- and Met-enkephalin, and displayed 40 % crossreactivity with enkephalin hexapeptides (Cuello et al., 1984). A polyclonal antibody against FMR-enkephalin was generated by immunizing rabbits with this peptide coupled to bovine thyroglobulin. The selectivity of these antibodies was studied by measuring their immunoreactivity in the dorsal horn after preadsorption with opioids (Fig. 3A, B).

μ -Opioid receptor (MOR) antibody—This antibody was generated against a peptide corresponding to amino acid residues 384–398 predicted from the cloned rat MOR1 (Table 1). The peptide was conjugated with bovine thyroglobulin using glutaraldehyde (Arvidsson et al., 1995a). The specificity of the anti-MOR rabbit antiserum was determined in cell lines by its colocalization with epitope-tagged constructs of MOR-1 (Arvidsson et al., 1995a). The antibody produced a band of a molecular weight of 63–67 kDa by immunoisolation with ^{125}I - β -endorphin in membrane proteins from Neuro2a cells transfected with MOR1. Similarly, it produced a band with a molecular weight of 67–72 kDa in Western blots from the same transfected cell line. Staining of the rat spinal cord with this antibody was eliminated by preadsorption with its immunizing peptide (Marvizón et al., 1999). This antibody was shown to label neurons in the dorsal horn (Spike et al., 2002). MOR immunoreactivity in dorsal horn neurons was present at the cell surface in control rat spinal cord tissue and it internalized in endosomes after application of MOR agonists but not a δ -opioid receptor agonist (Marvizón et al., 1999; Song and Marvizón, 2003a).

Neurokinin 1 receptor (NK1R) antibody—This antiserum was generated in rabbits using a peptide corresponding to the C-terminus of the rat NK1R (Table 1) coupled to KLH (Grady et al., 1996). It labeled by immunofluorescence cells transfected with rat NK1R, and it did not label nontransfected cells. Staining of the transfected cells was eliminated by preadsorption with its immunizing peptide. In Western blots from cells transfected with the NK1R it produced a single band corresponding to a molecular weight of 100 kDa (Grady et al., 1996).

Pan-opioid antibody (3-E7)—This monoclonal antibody was generated against β -endorphin in a clone hybrid myeloma (3-E7) from mouse. It recognizes any opioid with the YGGF N-terminus motif (Gramsch et al., 1983). This includes Leu- and Met-enkephalin, β -endorphin, dynorphin A and α -neoendorphin, but not endomorphins 1 and 2 (Fig. 3D).

Protein kinase C- γ (PKC γ) antibody—This antibody produced two closely spaced bands in Western blots prepared from rat cerebellum and neocortex, with an approximate molecular weight of 80 kDa (Cardell et al., 1998). Labeling in the Western blots was eliminated by preadsorption with the immunizing peptide.

Prodynorphin antibody—This antibody was raised against the C-terminus of the rat prodynorphin peptide (residues 235–248), an epitope distinct from the sequence of any of the opioid peptides (Table 1). Staining of rat brain and spinal cord tissue with this antibody was blocked by preadsorption with its cognate peptide (0.1–1 μM) (Arvidsson et al., 1995b).

Substance P antibodies—Two different antibodies against substance P were used in this study (Table 1). One was raised in goat against substance P coupled to KLH. The other was raised in rabbit against substance P coupled to BSA. Preadsorption controls showed that the rabbit antibody was selective for substance P and did not recognize neurokinin A or neurokinin B. In the present study, double labeling of spinal cord sections with these two antibodies resulted in good colocalization of their staining in puncta and fibers in the dorsal

horn (Fig. 2). The pattern of staining with these antibodies matches the previously described staining pattern for substance P in the rat dorsal horn (De Biasi and Rustioni, 1988; Wiesenfeld-Hallin et al., 1984).

Vesicular glutamate transporter-2 (VGLUT2) antibody—This antibody was raised in guinea pig against amino acids 565–582 of rat VGLUT2 (Table 1) (Montana et al., 2004). Its staining colocalized with that of a rabbit antibody against amino acids 510–582 of rat VGLUT2 in the rat dorsal horn (Todd et al., 2003) and in astrocytes (Montana et al., 2004). In Western blots of rat brain and spinal cord membranes, the VGLUT2 antibodies recognized a single band with the appropriate molecular weight of 65kDa (Montana et al., 2004; Takamori et al., 2001). Staining with these antibodies was eliminated by preadsorption with the immunizing peptides (Takamori et al., 2001; Todd et al., 2003).

Secondary Antibodies

Secondary antibodies were raised in goat or donkey against the species of the primary antibody (goat, guinea pig, mouse or rabbit) and were coupled to Alexa Fluor 488 (AF-488), Alexa Fluor 568 (AF-568), Alexa Fluor 633 (AF-633) or rhodamine red-X (RRX). Secondary antibodies coupled to the Alexa Fluor fluorophores were purchased from Molecular Probes (Eugene, OR), and those coupled to RRX were purchased from Jackson ImmunoResearch.

Immunohistochemistry

Adult male Sprague Dawley rats (300–400 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) containing 0.01% heparin, followed by 400 ml of ice-cold fixative (4 % paraformaldehyde, 0.18 % picric acid in phosphate buffer). The spinal cord was extracted and cut into a L1–L3 segment and a L4–L5 segment based on root identification. Both segments were post-fixed, cryoprotected in 20 % sucrose, embedded in a drop of Tissue-Tek (Sakura Finetek USA, Inc., Torrance, CA) and frozen on dry ice for sectioning in a cryostat. The L1–L3 segment was used to prepare sagittal sections of 20 μ m. The L4–L5 segment was used to prepare transverse sections of 25 μ m. Sagittal and transverse sections were pooled together and processed free-floating. They 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 % normal serum (NS, Jackson ImmunoResearch, West Grove, PA) of the species of the secondary antibodies (donkey or goat). Sections were then incubated overnight with a mixture of two or three primary antibodies (Table 1) in PBS/Triton with 5 % NS. 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. Sections were washed four more times with PBS and mounted in Prolong Gold (Molecular Probes) to reduce photobleaching. All incubations were done at room temperature.

Sections through the arcuate nucleus of wild type and β -endorphin deficient mice were stained using the 3,3'-diaminobenzidine (DAB) staining protocol as described (Anton et al., 1996). Mice were fixed by aortic perfusion of fixative (4% paraformaldehyde). Brains were extracted, post-fixed, cryoprotected with 30% sucrose and frozen. Sections (50 μ m) through the arcuate nucleus were cut with a cryostat. Sections were incubated at room temperature for 10 min with 3% H₂O₂/methanol, and for 1 hr with 5 % goat NS in 0.3 % Tween-20/PBS. Incubation with the primary antibody (Mu(-5)N anti- β -endorphin, 1:1000, Table 1) was done overnight at 4 °C. The secondary antibody was biotinylated anti-rabbit (Vector Laboratories, Burlingame, CA) and was incubated with the sections for 2 hr. Sections were

subsequently incubated with ABC-Elite (Vector Laboratories) for 1 hr. Immunostaining was visualized with DAB.

Microscopy

Confocal images were acquired with a Leica TCS-SP confocal microscope (Leica Microsystems GmbH, Wetzlar, Germany) using and objectives of 10x (numerical aperture [NA] 0.4), 20x (NA 0.70) and 100x oil (NA 1.4). Some images were acquired with a Zeiss LSM-710 confocal microscope (Carl Zeiss, Inc., Thornwood, NY) with objectives of 20x (NA 0.8) and 63x oil (NA 1.4). The pinhole was 1.0 Airy unit. Images were acquired as confocal stacks of 6–22 optical sections of 1024×1024 pixels, and were averaged 2–4 times to reduce noise. Images were taken from the top of the histological sections whenever possible to minimize light scatter and blur, and because some antibodies (CGRP, VGLUT2) did not penetrate well the sagittal sections. Excitation light was provided by the 488 nm line of argon lasers for the AF-488 fluorophore, the 561 nm line of diode lasers for the AF-568 and RRX fluorophores, and the 633 nm line of HeNe lasers for the AF-633 fluorophore. For double-labeled samples, emission windows were 500–550 nm for AF-488 (emission peak 519 nm), 580–700 nm for RRX (emission peak 590 nm) and 600–700 nm for AF-568 (emission peak 603 nm). For triple-labeled samples, emission windows were 500–530 nm for AF-488, 580–640 nm for RRX and 640–710 nm for AF-633 (emission peak 648 nm). Controls with one label omitted were used to confirm that the bleed-through between channels with these setting was minimal.

Deconvolution and image processing

The antibody combinations used in this study produced high quality confocal images, with low background and noise. Nevertheless, the images could be further improved by reducing blur using deconvolution (Cannell et al., 2006; Wallace et al., 2001). In particular, the increase in sharpness and resolution achieved with deconvolution reduced the uncertainty of colocalization measures. The program AutoQuant X2.0.1 (Media Cybernetics, Inc., Bethesda, MD) was used to perform adaptive point spread function (PSF) deconvolution - also called blind deconvolution (Holmes et al., 2006) - of the whole confocal stack, using 5 or 10 iterations. Images from two or three color channels were deconvolved simultaneously. The resulting 32-bit float point three dimensional image file was imported into Imaris (x64, 6.1.5, Bitplane AG, Zurich, Switzerland). To make the figures, Imaris was used to crop the image in three dimensions (without changing the resolution), adjust the brightness and gamma, and select the color for each label channel. Single-channel panels in the figures are shown as grayscale images to make their details more clearly visible. Two-channel images were color-coded in green (AF-488) and magenta (AF-568, RRX or AF-633) instead of the conventional green/red in order to make them visible to colorblind readers. Three channel images were color coded green (AF-488), red (RRX or AF-568) and blue (AF-633). Using Imaris, a two-dimension projection picture of the processed image was obtained, which was imported as a TIFF file into Adobe Photoshop 5.5 (Adobe Systems Inc., Mountain View, CA). Photoshop was used to compose the multi-panel figures, and to add text and arrows.

Peptide block and fluorescence intensity measures

The specificity of some of the antibodies was determined by preadsorbing them with various opioid peptides (1 μ M or 5 μ M), overnight at 4 °C. Then the preadsorbed antibodies were used to label spinal cord sections (transverse and sagittal) as described above. Several (3–5) confocal images of laminae I–II from different histological sections were obtained for each antibody/peptide pair. Identical confocal acquisition parameters were used for the different peptides, including objective (63x, 1.4 NA), laser power, photomultiplier gain and offset, emission window, pinhole (1 Airy unit) and speed. Confocal stacks consisted of seven optical sections separated 0.383 μ m, and were not deconvolved because this would have

changed their relative intensities. For the 3-E7 antibody, images of the entire dorsal horn were obtained using a digital camera (Real-14, Cambridge Research Instrumentation, Cambridge, MA) and a 10x (0.4 NA) objective. The integrated optical intensity was measured in one optical section (the fourth of seven optical sections) or in the digital images of antibody 3-E7, using the program ImageJ 1.41 (National Institutes of Health, USA, public domain) and defining a threshold of pixel intensity above which the measure was made. The integrated optical intensity is a function of both the number of pixels above threshold (“area”) and the intensity of these pixels. The integrated optical intensity with each peptide was then compared to that of a control in which the blocking peptide was omitted, to determine the extent to which the peptide bound to the antibody and hindered its labeling of the tissue.

Colocalization measures

In this study, colocalization of two labels means that they are present in the tissue with enough proximity that they cannot be resolved optically (Hibbs et al., 2006). This was interpreted as indicating that the targets of the antibodies are located in the same small neuronal compartment, for example presynaptic terminals or axons. Colocalization was measured using Imaris Colocalization, which analyzes entire two-channel confocal stacks by measuring the intensity of each label, voxel by voxel. Because the analysis is done in three dimensions, false positives generated in projection images by projecting voxels in different Z positions into the same X-Y position are avoided.

Confocal stacks were deconvolved (see above) before colocalization analysis. The resulting improvement in image sharpness increased the accuracy of the colocalization analysis by eliminating areas of blur that can produce ambiguous colocalization (Hibbs et al., 2006). Deconvolution also decreased the background, making the colocalization measure less dependent on the thresholds set to screen out background voxels. In triple-label images, colocalization was measured in all the three possible two-channel combinations, but no measure of three-way colocalization was attempted.

As primary measure of colocalization we took the Pearson correlation coefficient in all voxels, which indicates the extent to which the intensity of the two labels increases together in the same voxels. It varies between +1 and -1, with positive values indicating a positive correlation, values near 0 indicating no correlation, and negative values indicating an inverse correlation (which is seldom encountered in colocalization studies). Based on the results obtained with negative and positive colocalization controls, Pearson coefficients below 0.1 were interpreted as no colocalization, and Pearson coefficients above 0.5 as near maximal colocalization. Because no background subtraction or any other alteration of the native voxel intensity (other than deconvolution) was performed, Pearson coefficients were independent of investigator bias.

In image stacks with Pearson coefficients above 0.1, the colocalization coefficients MA and MB (Hibbs et al., 2006), or percentage of material colocalized, were also considered. They represent the proportion of voxels in each channel that contribute to the colocalized volume, and take into account the number of voxels with colocalization (“volume”) as well as the intensities (“material”) of the two labels in each voxel. The colocalization coefficient MA for label “A” is given by the equation:

$$MA = \left(\sum_{i \in \text{coloc}} S A_i / \sum_{i \in \text{objectA}} S A_i \right) \times 100\%$$

where SA_i is the intensity of the i th voxel for label A, $i \in \text{coloc}$ are the voxels of label A that also contain some intensity component from label B, and $i \in \text{objectA}$ are the voxels above threshold for label A. A similar equation is used for channel B. For example, $MA = 40\%$ means that 40% of the label A (above its threshold) is present in the same voxels as label B (above its threshold). Unlike the Pearson coefficient, determination of the colocalization coefficients requires the setting of intensity thresholds for each channel, so that voxels with intensities below the threshold are considered to be part of the background and are excluded from the colocalization analysis. Imaris Colocalization has a feature that allows the automatic determination of the thresholds by an iterative procedure (Costes et al., 2004). However, this feature does not work well with the 32-bit floating point images files generated by the deconvolution program, often producing inconsistent threshold values for similar images. In 32-bit floating point images, intensity values are not in the usual 0–255 scale of 8-bit files, but have a wider value range, complicating the calculation of the threshold. We determined the thresholds visually by moving the cursor in the display histogram until all the background pixels in the image were darkened; the intensity value at the position of the cursor was taken as the threshold value for that channel. The procedure was then repeated for the other channel. Because deconvolution produced a nearly black background by eliminating noise, threshold values determined this way were low (10–50 in intensity scales of 0 to 2000). Hence, almost all the colored voxels visible in the image were included in the measurement of the colocalization coefficients.

Further information about the degree of colocalization was obtained from the Pearson coefficient of voxels above thresholds, although this is not given in the figures. The value of this Pearson coefficient roughly paralleled the Pearson coefficient for all voxels described above, but was consistently lower and often took slightly negative values when there was no colocalization.

Statistical analyses

Data were analyzed and plotted using Prism 5 (GraphPad Software, San Diego, CA). Statistical analyses consisted of one-way and two-way ANOVA and Bonferroni's post-hoc tests. Significance was set at 0.05.

Results

β -endorphin is absent from the dorsal horn

To investigate the presence of β -endorphin in the rat spinal cord, we used an antibody [Mu(-5)N] raised in rabbit against the β -endorphin sequence minus the N-terminus, in order to avoid cross-reactivity with enkephalins and dynorphins. In immunoblots, the antibody was shown to recognize rat and camel β -endorphin, but not dynorphin and nociceptin. To determine its specificity for immunohistochemistry, the antibody was tested in arcuate nucleus sections from wild type and β -endorphin deficient mice. In wild type mice, the antibody stained cell bodies and fibers through the arcuate nucleus (Fig. 1A). The staining completely disappeared in β -endorphin deficient mice (Fig. 1B), demonstrating its specificity for β -endorphin.

We used this antibody to label transverse sections from the rat lumbar spinal cord (L5-L6). The antibody produced staining of excellent quality, with unusually low background (Fig. 4). Because of this, we co-stained with an antibody against vesicular glutamate transporter-2 (VGLUT2, not shown) to define the edges of the sections and the limits between gray and white matter. β -endorphin immunoreactivity was found in the ventral horn (Fig. 4C), where the antibody labeled fibers and puncta. Less prominent staining, with a granular appearance, was noted in the cytoplasm and dendrites of large neurons. Immunoreactive fibers were also

found in the lateral funiculus. In the area around the central canal (lamina X, Fig. 4B) the antibody labeled puncta, but no cell bodies. No labeling with this antibody was detected in the dorsal horn. Fig. 4A shows the atypical labeling of a group of fibers in laminae II–III. This section was selected to illustrate that the antibody labeled rare β -endorphin immunoreactive fibers in the dorsal horn.

Opioid peptide specificity of the antibodies against enkephalin, FRM-enkephalin and dynorphin

We used antibodies raised against enkephalin, FRM-enkephalin and dynorphin to study the localization of enkephalins and dynorphins in the dorsal horn. To determine the specificity of these antibodies for different opioid peptides, we performed pre-absorption controls of these three antibodies with seven opioids: Leu-enkephalin, Met-enkephalin, FRM-enkephalin, dynorphin A, dynorphin B and α -neoendorphin. The peptides (at 5 μ M) were incubated with the antibodies at their final dilutions (enkephalin and dynorphin antibodies at 1:1000, FRM-enkephalin antibody at 1:5000) overnight at 4 °C. The preabsorbed antibodies were then used to label spinal cord sections with our standard immunohistochemistry protocol. Controls (“none” in Fig. 3) used antibodies incubated overnight without peptide.

To measure differences in the intensity of labeling of the spinal cord sections, we took confocal images encompassing laminae I and II (with a 63x objective), using identical confocal acquisition parameters. The integrated optical intensity was measured in the fourth confocal section of the seven-section confocal stacks, using the ImageJ program. A pixel intensity threshold was defined to exclude the background, and the integrated optical intensity was calculated for the pixels above this threshold. The integrated optical intensity is a function of both the number of pixels above threshold (“area”) and their intensity, so is a good measure of the extent of antibody labeling of the tissue.

For the enkephalin and the FRM-enkephalin antibodies the threshold was 20 in the 0–255 scale of pixel intensity; therefore only the darkest pixels were excluded. As shown in Fig. 3A, staining with the enkephalin antibody was substantially reduced by its preadsorption with Leu-enkephalin, Met-enkephalin and FRM-enkephalin, indicating that the antibody recognized well these peptides. No reduction in the staining was detected after incubating the enkephalin antibody with dynorphin A and α -neoendorphin, but dynorphin B produced an unexpected, statistically significant decrease. Fig. 3B shows the integrated optical intensity measures obtained with the FRM-enkephalin antibody. FRM-enkephalin itself, but not Met- or Leu-enkephalin, decreased the intensity of the staining, indicating that this antibody recognizes FRM-enkephalin much more efficiently than the enkephalin pentapeptides. Unexpectedly, α -neoendorphin produced a small but statistically significant decrease in the staining with the FRM-enkephalin antibody.

This approach did not work quite as well for dynorphin antibody. This antibody produced a higher background and labeled less fibers and puncta than the enkephalin and FRM-enkephalin antibodies (Fig. 5), which complicated the measurement of the integrated optical intensity. Moreover, fibers were labeled by the antibody at very different intensities, which made the measure dependent on the field chosen for the image. To compensate for the higher background, a high threshold value (100) was chosen. Fig. 3C shows that this antibody was blocked by dynorphin A and α -neoendorphin. Differences from control were not statistically significant in the Bonferroni’s post-hoc test, although the ANOVA was significant overall ($p = 0.027$). Leu- and Met-enkephalin did not decrease the staining, but FRM-enkephalin did. This suggests that the Arg residue in FRM-enkephalin, also present in the dynorphins, is an important motif of recognition by this antibody.

In addition, the specificity of the monoclonal antibody 3-E7 was studied by preadsorbing it with Leu- and Met-enkephalin, β -endorphin, dynorphin A, α -neoendorphin and endomorphin-1 and -2 (all 1 μ M). In this case images of the entire dorsal horn were taken at low magnification (10x objective). Staining with this antibody was blocked by all the opioid peptides except the endomorphins, showing that it recognizes the YGGF N-terminus motif of the opioids (Gramsch et al., 1983).

Enkephalin and dynorphin are present in different terminals

Fig. 5 shows staining of the dorsal horn produced by the antibodies against enkephalin, FRM-enkephalin, dynorphin and prodynorphin. Images in Figs. 5A and 5B are from the same histological section double-labeled with the enkephalin and the dynorphin antibodies, respectively. Enkephalin immunoreactivity (Fig. 5A) consisted of puncta located throughout laminae I-III and lamina V, and was very intense in the dorsolateral funiculus. The FRM-enkephalin antibody (Fig. 5C) labeled puncta with the same distribution. In fact, the image in Fig. 5C is from a double-labeled histological section in which the staining with the enkephalin antibody (not shown) completely overlapped that of the FRM-enkephalin antibody. In contrast, the dynorphin antibody (Fig. 5D) labeled puncta that were most abundant in lamina I but could be found more sparsely throughout the dorsal horn. Prodynorphin immunoreactivity (Fig. 5D) was found in lamina I, outer lamina II, the dorsolateral funiculus and lamina V. Unlike that obtained with the dynorphin antibody, it consisted not only of puncta but also cell bodies.

When the rat dorsal horn (laminae I-II) was double-labeled with the dynorphin and the prodynorphin antibodies (Fig. 6C), a partial colocalization pattern was revealed. Many of the fibers and puncta stained by the dynorphin antibody were also stained by the prodynorphin antibody. However, the prodynorphin antibody also stained cell bodies and dendrites that were not labeled by the dynorphin antibody. This indicates that the dynorphin precursor is synthesized in neuronal somata and cleaved into the dynorphin peptide in the axons and presynaptic terminals. The lack of staining of cell bodies with the dynorphin antibody suggests that its epitope is masked in the prodynorphin peptide.

Staining with the enkephalin antibody did not colocalize with the staining produced by either the dynorphin antibody (Fig. 6A) or the prodynorphin antibody (Fig. 6B), indicating that opioids encoded by the preproenkephalin and preprodynorphin genes are located in different presynaptic terminals.

We also studied the staining produced by the pan-opioid monoclonal antibody 3-E7, which we previously used to label opioid-containing terminals in the dorsal horn (Gramsch et al., 1983; Song and Marvizón, 2003b). In a double-label experiment with the dynorphin antibody, a certain degree of colocalization was found (Fig. 6D). However, there were 3-E7 immunoreactive puncta that were not labeled by the dynorphin antibody, which may be enkephalinergic terminals. We did not attempt double-labeling with 3-E7 and the enkephalin antibody because both antibodies were raised in mouse.

The colocalization of immunoreactivities in these and other experiments was quantified using the program Imaris Colocalization (see Material and Methods). All images were acquired with a confocal microscope at the highest resolution and deconvolved before analysis to avoid false positives caused by blur (Hibbs et al., 2006). As negative control, we used double-labeling of laminae I-II with antibodies against μ -opioid receptors and neurokinin 1 receptors, which are present in different dorsal horn neurons (Song and Marvizón, 2003b). This yielded a low Pearson correlation coefficient of about 0.1 (Fig. 2A), which was taken as defining no colocalization. As positive controls we used double-labels with three CGRP antibodies raised in different species (mouse, goat and rabbit) or with two

substance P antibodies (raised in rabbit and goat). All three possible combinations of the CGRP antibodies were tried, but only the Pearson correlation coefficient for the mouse and goat antibody pair is shown in Fig. 2; other CGRP antibody combinations produced similar values. Note that when using antibodies against the same target, Pearson correlation coefficients were between 0.75 (CGRP) and 0.5 (substance P). This represents the maximum amount of colocalization that is possible to obtain in practice, probably because of small differences in the ability of the antibodies to recognize their targets in different structures. For antibody pairs with Pearson coefficients above 0.1, the percentage of material colocalized, given by the colocalization coefficients MA and MB (Hibbs et al., 2006), was also measured (Fig. 2B).

The dynorphin - prodynorphin double-label yielded a Pearson correlation coefficient of 0.25 (Fig. 2A), because the staining colocalized in puncta but not in cell bodies. Indeed, the colocalization coefficient (Fig. 2B) was low for the prodynorphin antibody and higher for the dynorphin antibody, indicating that few of the prodynorphin-positive voxels were dynorphin-positive, whereas many of the dynorphin-positive voxels were prodynorphin-positive. The enkephalin - FRM-enkephalin double label yielded a high Pearson correlation coefficient (Fig. 2A), comparable to that obtained in the positive controls. Since the FRM-enkephalin antibody does not recognize the Leu- and Met-enkephalin pentapeptides (Fig. 3B), this indicates that the FRM-enkephalin heptapeptide is present in the dorsal horn in the same presynaptic terminals that contain the pentapeptides.

Colocalization measures clearly show a lack of colocalization between enkephalin and dynorphins: Pearson correlation coefficients less than 0.1 were obtained for the antibody pairs enkephalin - dynorphin, enkephalin - prodynorphin and FRM-enkephalin - prodynorphin (Fig. 2A). Note that, although the dynorphin antibody seems to recognize the FRM-enkephalin peptide in the peptide blocking experiment (Fig. 3C), in the actual staining of the tissue there is no evidence of this cross-reactivity, because there was a low degree of colocalization for the dynorphin - enkephalin antibody pair. Likewise, the blockade of the enkephalin antibody by dynorphin B (Fig. 3A) did not seem to translate into a staining of dynorphin terminals by this antibody. These findings support the idea that the products of the proenkephalin and prodynorphin genes are located in different presynaptic terminals.

Enkephalins are not present in primary afferent terminals

Next, we investigated whether some of the enkephalin-immunoreactive puncta could be the central terminals of primary afferents. It is unlikely that A-fiber terminals contain enkephalins, because enkephalinergic terminals are concentrated in laminae I–II, whereas A-fibers terminate deeper in the dorsal horn (Marvizón et al., 2002). For that reason, we studied the colocalization of enkephalin immunoreactivity with three markers of C-fibers: CGRP, isolectin B4 (IB4) and substance P.

We found that enkephalin immunoreactivity did not colocalize with any of those markers. In sagittal sections, where CGRP-containing and IB4-binding axons can be seen coursing rostro-caudally, these axons were clearly different from the enkephalinergic terminals (Fig. 7A–C). In lamina II, we found a high degree of colocalization of CGRP with substance P (Fig. 7B), reflected in a high Pearson correlation coefficient (Fig. 2A) and colocalization coefficients (Fig. 2B). However, we found some substance P immunoreactive puncta that did not contain CGRP (Fig. 7B), specially in lamina III. Like CGRP, substance P did not colocalize at all with enkephalin (Figs. 2A, 7 B). Therefore, enkephalin immunoreactive puncta are not C-fiber terminals.

Enkephalin did colocalize with vesicular glutamate transporter 2 (VGLUT2), a marker of glutamatergic terminals. Although the Pearson correlation coefficient for this antibody pair

was low (Fig. 2A), the corresponding colocalization coefficients were high for enkephalin and low for VGLUT2. This indicates that many of the enkephalin puncta contain VGLUT2, while there are a lot of VGLUT2 puncta without enkephalin. This is to be expected, since there are many more VGLUT2-containing terminals than enkephalinergic terminals. Therefore, our results agree with previous studies showing that a large number of the enkephalinergic terminals in the dorsal horn are glutamatergic (Marvizón et al., 2007; Todd et al., 2003).

Dynorphins are present in primary afferent terminals and dorsal horn neurons

In contrast, some of the dynorphin immunoreactive puncta appear to be C-fiber terminals containing CGRP and substance P. Fig. 7D shows some instances of colocalization between the dynorphin and CGRP labels. In general, puncta showing colocalization with CGRP were stained for dynorphin at lower intensity, whereas the brightest dynorphin-positive puncta did not label for CGRP. Moreover, a triple label experiment showed some colocalization of prodynorphin immunoreactivity with fibers stained for both CGRP and substance P (Fig. 7E). In this case, some fibers were observed to be triple-labeled for some length (double-arrows), indicating that the colocalization was not an artifact caused by the close apposition of axons. This idea was confirmed by the quantitative colocalization study (Fig. 2) which is performed voxel-by-voxel in 3 dimensions. In it, moderately high Pearson coefficients and colocalization coefficients were obtained for four pair of antibodies: dynorphin - CGRP, prodynorphin - CGRP, dynorphin - substance P and prodynorphin - substance P. There was no colocalization of dynorphin immunoreactivity with IB4 (Figs. 2A, 7 F). Like enkephalin, dynorphin showed some colocalization with VGLUT2 (Fig. 2), indicating that some of the prodynorphin-expressing dorsal horn neurons are glutamatergic.

To further investigate the presence of dynorphins in primary afferents, we labeled DRG sections with the dynorphin and prodynorphin antibodies. The dynorphin antibody labeled DRG neurons of both large and small diameter with varying degrees of intensity (Fig. 6E). The prodynorphin antibody produced a diffuse, uniform staining throughout the DRG that we deemed non-specific. Dynorphin immunoreactivity was located in the cytoplasm, and in some cells it was more intense in rings around the nucleus that likely correspond to the Golgi apparatus (Marvizón et al., 2002). Unlike what we observed in the dorsal horn, there was a low degree of colocalization between dynorphin and CGRP immunoreactivities in the DRG. Still, some of the CGRP-positive cell bodies showed a weak staining for dynorphin (Fig. 6E, arrows).

Pro-dynorphin is expressed by a distinct population of dorsal horn neurons

The ability of the prodynorphin antibody to label cell bodies in the dorsal horn allowed us to investigate the relationship of these neurons with other well-known types of dorsal horn neurons. Neurons expressing μ -opioid receptor have characteristic rostro-caudal apical dendrites, and have closely apposed enkephalinergic terminals, as shown in Fig. 8A. The prodynorphin immunoreactive neurons are clearly distinct from the μ -opioid receptor neurons (Fig. 8B). Interestingly, some prodynorphin-positive puncta are also observed in apposition to the μ -opioid receptor neurons. Neurokinin 1 receptor-expressing neurons in lamina I are another major class of dorsal horn neurons which often project supraspinally (Todd et al., 2002); these are also different from the prodynorphin expressing neurons (Fig. 8C). Some of the prodynorphin terminals were found in close apposition to the neurokinin 1 receptor neurons. The prodynorphin neurons were also distinct from PKC γ -expressing neurons of inner lamina II (Fig. 8D).

Discussion

This study elucidates some important facts about the opioid peptides present in the rat dorsal horn (summarized in the diagram in Fig. 9). First, β -endorphin is absent from the dorsal horn. Second, enkephalins and dynorphins are present in different presynaptic terminals. Third, C-fiber terminals do not contain enkephalin, but some of them contain moderate amounts of dynorphins.

β -endorphin is not present in the dorsal horn

The availability of a selective antibody against β -endorphin allowed us to study its distribution in the rat spinal cord. We found β -endorphin immunoreactive fibers in the ventral horn, lateral funiculus and in lamina X, but not in the dorsal horn. These results are in general agreement with the distribution of POMC-derived peptides reported by Tsou et al. (1986), who found that they were most abundant in lamina X and also present in the lateral funiculus and the ventral horn. Unlike them, though, we did not find any β -endorphin immunoreactivity in the deep dorsal horn and the dorsal funiculus. Tsou et al. state that β -endorphin-positive fibers are seen less frequently than ACTH-positive fibers, so is possible that ACTH, but not β -endorphin, is present in the dorsal horn.

It was believed that most of the β -endorphin-positive fibers in the spinal cord are of supraspinal origin. Tsou et al. did not detect any cell bodies immunoreactive for POMC-derived peptides, even after an intrathecal injection of colchicine to inhibit axonal transport. Moreover, they reported that thoracic spinal transection resulted in the disappearance of ACTH immunoreactivity caudal to the section. However, a later study (Gutstein et al., 1992) found that about one third of the β -endorphin persisted in the lumbosacral spinal cord after thoracic spinal transection. Interestingly, we found some β -endorphin immunoreactivity in the cell bodies and dendrites of large ventral horn neurons. Hence, it is possible that these neurons are the ones producing the β -endorphin detected after spinal transection by Gutstein et al., who detected β -endorphin using radioimmunoassay and therefore could not determine its exact location.

The fact that β -endorphin immunoreactivity was practically absent from the dorsal horn is of critical importance. μ -Opioid receptors in the dorsal horn contribute substantially to opioid antinociception (Chen and Pan, 2006; Kline and Wiley, 2008). Our finding indicates that these receptors are activated physiologically by enkephalins and possibly dynorphins, but not by β -endorphin. This idea is consistent with the fact the sciatic nerve stimulation (to mimic noxious signals) released negligible amounts of β -endorphin from the spinal cord (Yaksh et al., 1983). One important aspect of this issue is that enkephalins and dynorphins, but not β -endorphin, are extremely susceptible to inactivation by peptidases (Chen and Marvizón, 2009; Song and Marvizón, 2003a). Therefore, peptidases are capable of limiting the activation of μ -opioid receptors in the dorsal horn by physiologically released opioids. In turn, this supports the concept that inhibitors of opioid-degrading peptidases have valuable analgesic properties (Noble et al., 1997; Roques, 2000).

Enkephalins and dynorphins are present in different presynaptic terminals

We demonstrate that enkephalins and dynorphins are present in different presynaptic terminals in the dorsal horn, probably because the proenkephalin and prodynorphin genes are expressed by different dorsal horn neurons (Fig. 9).

There was a high degree of colocalization between the enkephalin and FRM-enkephalin immunoreactivities. Colocalization of dynorphin and prodynorphin produced only moderate quantitative values because the prodynorphin antibody labeled cell bodies while the

dynorphin antibody did not. However, dynorphin and prodynorphin immunoreactivities clearly colocalized in puncta and fibers.

Puncta labeled with antibodies against enkephalin or FRM-enkephalin were clearly different from puncta labeled with antibodies against dynorphin or prodynorphin. This was apparent both qualitatively in confocal images and quantitatively in colocalization measures performed in these images. In fact, we expected to detect some degree of colocalization in our experiments, for two reasons. First, in peptide preadsorption controls the enkephalin antibody was blocked by dynorphin B and the dynorphin antibody was blocked by FRM-enkephalin, so some cross-reactivity was expected from these antibodies. The fact that we did not observe any colocalization with these antibodies suggests that the cross-reactivity predicted by the peptide absorption controls did not occur in the actual experiments. Second, Leu-enkephalin can also be produced from the processing of prodynorphin (Weber et al., 1983). Since the enkephalin antibody recognizes Leu- and Met-enkephalin equally well, this antibody should have labeled any Leu-enkephalin present in the dynorphin terminals. The fact that it did not suggests that little Leu-enkephalin is produced from the prodynorphin peptide in the dorsal horn.

Our findings confirm what was inferred by Cruz and Basbaum (1985) based on morphological differences between enkephalin and dynorphin immunoreactive dorsal horn neurons, and by Standaert et al. (1986) from sequential labeling with enkephalin and dynorphin antibodies of dorsal horn neurons.

Enkephalins are not present in C-fiber terminals in the dorsal horn

Additional colocalization studies were performed to determine whether some of the enkephalin-positive fibers and puncta could be primary afferent terminals. Because there was little enkephalin immunoreactivity in the deep dorsal horn, where most A-fibers terminate, we did not assess colocalization with A-fibers markers. Using CGRP, substance P and IB4 as markers for different C-fibers populations, we found no colocalization with enkephalin-positive puncta, showing that enkephalin is not present in C-fiber terminals. This is consistent with an in situ hybridization study in DRG (Pohl et al., 1994) showing that the proenkephalin gene is active in only 3.5 % of the DRG somas. The same study found no Met-enkephalin immunoreactivity in DRG.

We found a total lack of colocalization of enkephalins with substance P, using two different substance P antibodies. There is a discrepancy between this finding and three previous immunohistochemistry studies (Kato et al., 1988; Ribeiro-da-Silva et al., 1991; Senba et al., 1988) reporting that enkephalins and substance P coexist in some dorsal horn neurons and presynaptic terminals. The terminals where this colocalization occurred were not primary afferent terminals, but were deemed to originate in dorsal horn neurons (Ribeiro-da-Silva et al., 1991). The most likely explanation for this discrepancy is that all three of these studies used intrathecal injections of colchicine to inhibit axonal transport and thus induce the accumulation of enkephalin and substance P in neuronal somas. As we found in our own experiments, no enkephalin or substance P immunoreactivity is detected in the soma of dorsal horn neurons otherwise. However, colchicine has been shown to alter gene expression in the CNS, in particular the expression of genes encoding neuropeptides (Aguado et al., 1999; Ceccatelli et al., 1991a; Ceccatelli et al., 1991b; Cortes et al., 1990). Therefore, it is likely that the colocalization of substance P with enkephalin was caused by de novo synthesis of substance P in enkephalinergic neurons induced by colchicine. Importantly, we found that several stimuli that induce substance P release in the dorsal horn, including electrical stimulation of the dorsal root, capsaicin and NMDA, do not induced enkephalin release, measured with MOR internalization (Song and Marvizón, 2003b).

In agreement with previous studies (Marvizón et al., 2007; Todd et al., 2003), we found that many of the enkephalin-positive puncta in the dorsal horn were also positive for VGLUT2, a marker of glutamatergic terminals. Therefore, many of the enkephalinergic dorsal horn neurons appear to be excitatory. However, in other dorsal horn neurons enkephalins colocalize with the inhibitory neurotransmitter GABA (Todd et al., 1992).

Dynorphin is present in both C-fiber terminals and dorsal horn neurons

Our results show that there are two different sources of dynorphins in the dorsal horn: a population of intrinsic dorsal horn neurons that immunostain with the prodynorphin antibody, and CGRP- and substance P-containing C-fiber terminals (Fig. 9). Nevertheless, CGRP-positive fibers generally had a weaker staining for dynorphin and prodynorphin, whereas the brighter dynorphin- and prodynorphin-positive fibers and puncta were CGRP-negative. C-fibers that bind isolectin B4 did not contain dynorphins at all. To confirm the observations in the dorsal horn, we labeled DRG sections with the dynorphin and the prodynorphin antibodies. Staining with the prodynorphin antibody appeared non-specific, but the dynorphin antibody labeled some DRG cell bodies, both of large and small diameter. Dynorphin immunoreactivity was found in the cytoplasm, occasionally in structures forming a ring around the nucleus, a pattern of staining noted for other neuropeptides like CGRP (Marvizón et al., 2002). Since dynorphin and CGRP immunoreactivities colocalized in the dorsal horn, we expected that DRG cell bodies stained for CGRP would have a high dynorphin content. Surprisingly, we found almost the opposite: DRG cell bodies that stained brightly for dynorphin were CGRP-negative. Still, faint dynorphin immunoreactivity could be detected in some CGRP-positive cell bodies. Taken together, our findings showing that CGRP-positive fibers in the dorsal horn and CGRP-positive DRG cell bodies stained faintly for dynorphin indicate that dynorphin levels in CGRP-containing C-fibers are generally low. It should be noted that dynorphin levels in the spinal cord increase substantially in a variety of chronic pain models, including inflammation, polyarthritis and peripheral nerve injury (Cho and Basbaum, 1988; Ruda et al., 1988; Todd and Spike, 1993). It is possible that an augmented expression of prodynorphin in primary afferents contribute to this increase.

Our findings are in agreement with those of Tuchscherer and Seybold (1989), who showed that substance P colocalized with dynorphin and that dorsal rhizotomy depleted about one third of the dynorphin immunoreactivity in the rat dorsal horn. Basbaum et al. (1986) also showed that rhizotomy depleted dynorphin in the cat sacral spinal cord. However, an earlier study (Botticelli et al., 1981) reported that dorsal rhizotomy did not affect dynorphin levels in the rat spinal cord. Unlike us, they also found low levels of dynorphin immunoreactivity in the DRG. Differences in the antibodies used may be responsible for this discrepancy.

The prodynorphin antibody labeled a population of dorsal horn neurons that was clearly different from neurons expressing μ -opioid receptors, neurokinin 1 receptors and protein kinase C- γ . Some of them are projection neurons to the parabrachial nucleus (Standaert et al., 1986). The fibers stained most brightly with the dynorphin antibody probably represent the axons of the prodynorphin neurons. Some of them were found in close apposition to μ -opioid receptor neurons, suggesting that they make synapses with them. Although dynorphins have lower affinities for μ -opioid receptors than enkephalins, the higher susceptibility of enkephalins to peptidase inactivation causes the apparent potency of dynorphins to activate μ -opioid receptors to be comparable to or even higher than the potency of enkephalins (Song and Marvizón, 2003a). Therefore, it is possible that both enkephalins and dynorphins activate μ -opioid receptors in physiological conditions, while co-activation of δ - or κ -receptors lead to synergistic interactions that modify their ability to induce antinociception (Chen et al., 2007).

Acknowledgments

Acknowledgement of support

This work was supported by grant R01-DA-012609 from the National Institutes of Health and grant B4766I from the Department of Veteran Affairs to J.C.M.

We thank RIKEN Brain Science Institute Research Resources Center for assistance in raising the β -endorphin antibody, Patrick J. Culhane and Hoa Lam for assistance in its characterization, and Christopher J. Evans and Nigel T. Maidment for advice on antibody production and providing endorphin knockout mice and facilities. We also thank Dan Ha Phan for her technical help in the immunohistochemistry experiments.

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Fig. 1. Lack of β -endorphin immunoreactivity in β -endorphin KO mice

The β -endorphin antibody [Mu(-5)N] was used to stain sections through the arcuate nucleus (ArcN) of wild type mice (WT, A) and β -endorphin deficient mice (β -endorphin KO, B). Note the complete disappearance of β -endorphin immunoreactivity in the β -endorphin deficient mice. Images were taken using a 20x objective and a digital camera.

Fig. 2. Colocalization quantification

Sections from the lumbar spinal cord were double labeled with the antibodies pairs indicated (see Table 2 for abbreviations) or with isolectin B4 (IB4). Confocal images were obtained from laminae I–II with a 100x objective, and were de-blurred using blind deconvolution. Colocalization of the indicated label pairs was measured in all the voxels in a confocal stack with Imaris Colocalization, and expressed as the Pearson correlation coefficient (A) and colocalization coefficients (B). Three anti-CGRP antibodies were used, raised in goat (g), mouse (m) or rabbit (r). Two anti-substance P antibodies were used: one raised in goat (g) and another in rabbit (r). Data obtained with the different CGRP and substance P primary antibodies, with different secondary antibodies, and in transverse and sagittal sections did not differ, and were often combined. Bars represent the mean $\pm$ standard error of n confocal stacks (n is given above each bar in A). **A:** Pearson correlation coefficients. Positive controls were the colocalization of two CGRP antibodies (mouse and rabbit) or two substance P antibodies (goat and rabbit). Negative control was MOR - NK1R. Based on this, Pearson

coefficients below 0.1 (horizontal line) were interpreted as no colocalization. Statistical comparisons (Bonferroni's post-test) are with the negative control. **B:** Colocalization coefficients or percentage of material colocalized (MA and MB), measured in stacks that had Pearson coefficients above 0.1. Statistical comparisons (Bonferroni's post-test) are between MA and MB for each pair. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Fig. 3. Blockade by different opioid peptides of the enkephalin, FRM-enkephalin, dynorphin and 3-E7 antibodies

Each of the four antibodies was incubated overnight with the indicated opioid peptides (5 μ M in A-C; 1 μ M in D), and then used to label sections from the lumbar spinal cord. For each antibody/peptide pair, images of laminae I–II from different histological sections were obtained using identical acquisition parameters. Using ImageJ, a pixel intensity threshold was defined (enkephalin 20, FRM-enkephalin 20, dynorphin 100, 3-E7 90; in the 8-bit pixel intensity scale of 0–255) and the integrated optical intensity above this threshold was measured (in arbitrary units). Bars are the mean and SEM of 3–5 images. ANOVA was performed for each panel: enkephalin antibody, $p < 0.0001$; FRM-enkephalin antibody, $p = 0.0006$; dynorphin antibody, $p = 0.027$; 3-E7 antibody, $p < 0.0001$. Bonferroni's post-hoc test comparing each bar to control ("none"): * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. See Table 2 for abbreviations.

Fig. 4. β-endorphin immunoreactivity in the spinal cord

Coronal sections from the lumbar spinal cord (L5-L6) were labeled with antibodies against β-endorphin [Mu(-5)N] and VGLUT2. Only the β-endorphin staining is shown. The VGLUT2 label was used to define the edge of the gray matter, indicated by the lines. Confocal sections were obtained with a 20x objective at 1.18 μm intervals, and were de-blurred using blind deconvolution. Dorsal side is up. **A**, dorsal horn (10 sections); a rare β-endorphin fiber is shown. **B**, lamina X (9 sections), β-endorphin immunoreactive puncta are present around the central canal. **C**, ventral horn (10 sections); some β-endorphin immunoreactive puncta and fibers are observed in the grey and the white matter; large cell bodies were stained at lower intensity. Scale bars are 50 μm. See Table 2 for abbreviations.

Fig. 5. Labeling of the dorsal horn with antibodies against enkephalin, FRM-enkephalin, dynorphin and prodynorphin

Coronal sections from the lumbar spinal cord (L5) were labeled with antibodies indicated. Each image consists of 3 confocal sections through the middle of the histological section. Images were de-blurred using blind deconvolution. Lines indicate the edge of the dorsal horn and the approximate borders between laminae II–III, and laminae IV–V. In panel D, these borders were determined based on double-labeling for CGRP. **A**, enkephalin immunoreactivity; sections obtained with a 10x objective at 2.60 μm intervals. **B**, dynorphin immunoreactivity in the same histological section as A (double label); sections obtained with a 10x objective at 2.60 μm intervals. **C**, FRM-enkephalin immunoreactivity; sections obtained with a 20x objective at 1.18 μm intervals. **D**, prodynorphin immunoreactivity; sections obtained with a 10x objective at 6.62 μm intervals.

Fig. 6. Double-label of dorsal horn and DRG for enkephalin, dynorphins and prodynorphin Sagittal sections from the lumbar spinal cord (L1–L3) or DRG (L1–L5) were labeled with antibodies against dynorphin (dyn), prodynorphin (pdyn), enkephalin (enk) and all opioids (3E-7). Confocal stacks were de-blurred using blind deconvolution. **A–D**, images from the dorsal horn (laminae I–II, dorsal side is up) obtained with a 100x objective at 0.285 μ m intervals. **A**, dynorphin and prodynorphin (7 sections) showed some colocalization in puncta, but only prodynorphin staining was found in cell bodies. **B**, dynorphin and enkephalin (12 sections) showed no colocalization. **C**, prodynorphin and enkephalin (5 sections) showed no colocalization. **D**, dynorphin and the pan-opioid antibody 3-E7 (4 sections) showed a good degree of colocalization. **E**, image (4 sections) from a DRG stained

for dynorphin and CGRP, taken with a 20x (0.8 NA) objective at 0.85 μm intervals; arrows indicate cells immunoreactive for dynorphin and CGRP, although the brightest dynorphin-positive cells were not CGRP-positive.

Fig. 7. Presence of enkephalins and dynorphins in primary afferent terminals

Sagittal sections from the lumbar spinal cord (L1–L3) were double or triple labeled with antibodies against enkephalin (enk), dynorphin (dyn), prodynorphin (pdyn), substance P (SP) or CGRP, or with isolectin B4 (IB4). The left and middle panels show single labels, and the right panels the merged images. Single labels are shown in grayscale for better visualization. In B and E, the middle panel shows double labeling with substance P and CGRP. The color of the legends in the right and middle panels correspond to the color of the corresponding label in the merged image. Confocal images are from laminae I and II (dorsal side is up). They were obtained with a 100x objective at 0.285 μm intervals, and were de-blurred using blind deconvolution. **A**, enkephalin and CGRP (mouse antibody) do not colocalize; 5 optical sections. **B**, enkephalin, CGRP (rabbit antibody) and substance P (goat

antibody); CGRP and substance P show a high degree of colocalization, but neither colocalize with enkephalin; 9 optical sections. **C**, enkephalin and isolectin B4 do not colocalize; 6 optical sections. **D**, dynorphin and CGRP (mouse antibody) show some colocalization (arrows); 5 optical sections. **E**, prodynorphin, CGRP (mouse antibody) and substance P (rabbit antibody) show some colocalization (arrows, double arrows indicate colocalization along fibers); 4 optical sections. **F**, dynorphin and isolectin B4 do not colocalize.

Fig. 8. Localization of enkephalins and dynorphins relative to MORs, NK1Rs and PKC γ
Sagittal sections from the lumbar spinal cord (L1–L3) were double labeled with antibodies against enkephalin (enk), prodynorphin (pdyn), μ -opioid receptors (MOR), neurokinin 1 receptors (NK1R), and protein kinase C- γ (PKC γ), as indicated. The color of the legend corresponds to the color of the corresponding label in the image. Confocal images were obtained from laminae I and II with a 100x objective at 0.285 μ m intervals, and were de-blurred using blind deconvolution. Images are three-dimensional projections of the confocal stacks (5–9 optical sections). Scale bars and grid units are 5 μ m. Dorsal is side up. Arrows indicate possible synapses between opioid-containing terminals and receptor-expressing neurons. In panel D, asterisks indicate PKC γ immunoreactive neurons.

Fig. 9. Summary diagram

Enkephalins (enk) are present in neurons in laminae II, III and V, and in fibers in the dorsolateral funiculus (DLF). Dynorphin (dyn) is present in neurons in laminae I, II and V, and in the terminals of C-fibers containing substance P (SP) and CGRP. Other neurons in lamina II express μ -opioid receptors (MOR).

Table 1

Primary Antibodies

antibody	species	source	catalog number	dilution	immunogen
β-endorphin	rabbit	RIKEN	Mu(-5)N	1:1000	β-endorphin minus YGGF N-terminus (CTSEKSTQPLVTFLFKNAHKKGQ)
CGRP(γ)	goat	Santa Cruz	sc-8856	1:5000	human CGRP, N-terminus (ACDTATCVTHRLAGLLSRSG)
CGRP(m)	mouse*	CURE	4901	1:10000	rat α-CGRP (SCNTATCVTHRLAGLLSR SGGVVKDNFVPTNVGSEAF-NH ₂)
CGRP(r)	rabbit	Dr. Sternini	--	1:10000	rat CGRP ₂₃₋₃₇ (VKDNFVPTNVGSEAF)
dynorphin A	rabbit	Peninsula	IHC8730	1:200	pig dynorphin A ₁₋₁₇ (YGGFLRRIRPKLKWDNQ)
enkephalin	mouse*	Millipore	MAB135	1:1000	YGGFL
enkephalin, FMR-	rabbit	CSORDA	--	1:5000	YGGFMR
MOR	rabbit	ImmunoStar	24216	1:6000	MOR-1 residues 384-398 (NHQLENLEAETAPLP)
NK1R	rabbit	CURE	94168	1:3000	NK1R residues 393-407 (KTMTSSSFYSNMLA)
pan-opioid (3-E7)	mouse*	Gramsch	3-E7	1:1000	YGGFM
PKC _γ	rabbit	Santa Cruz	sc-211	1:1000	mouse PKC _γ residues 679-697 (DFVHPDARSPTSPVPVPM)
prodynorphin	guinea pig	Neuromics	GPI0110	1:2000	rat pro-dynorphin residues 235-248 (SQENPNTYSEDLDV)
SP(γ)	goat	Santa Cruz	sc-9758	1:2000	substance P (RPKPQQFFGLM-NH ₂)
SP(r)	rabbit	CURE	3801	1:1000	substance P (RPKPQQFFGLM-NH ₂)
VGLUT2	guinea pig	Millipore	AB5907	1:2000	rat VGLUT2 residues 565-582

* monoclonal antibody

Antibody sources: CURE: Digestive Diseases Research Center (CURE), UCLA, Los Angeles, CA; Center for the Study of Opioid Receptors and Drugs of Addiction (CSORDA), UCLA, Los Angeles, CA; Gramsch Laboratories, Schwabhausen, Germany; ImmunoStar, Hudson, WI; Millipore, Billerica, MA; RIKEN Brain Science Institute, Wako, Saitama, Japan; Neuromics, Edina, MN; Peninsula Laboratories Inc. (now Bachem), San Carlos, CA; Santa Cruz Biotechnology, Santa Cruz, CA; Dr. Catia Sternini, Department of Medicine, UCLA, Los Angeles, CA.

Table 2

Abbreviations used in the figures and in Table 1

3-E7	pan-opioid antibody 3-E7
Ab	antibody
α -neoend	α -neoendorphin
CGRP	calcitonin gene-related peptide
DRG	dorsal root ganglia
DLF	dorsolateral funiculus
dyn	dynorphin
enk	enkephalin
FRM-enk	Phe-Arg-Met-enkephalin
(g)	antibody raised in goat
IB4	isolectin B4
KO	knock-out (gene product deficient)
Leu-enk	Leu-enkephalin
(m)	antibody raised in mouse
Met-enk	Met-enkephalin
MOR	μ -opioid receptor
NK1R	neurokinin 1 receptor
pdyn	prodynorphin
PKC γ	protein kinase C- γ
(r)	antibody raised in rabbit
SP	substance P
VGLUT2	vesicular glutamate transporter-2
WT	wild-type