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Co-localization of endomorphin-2 and substance P in primary afferent nociceptors and effects of injury: a light and electron microscopic study in the rat

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Abstract

Endomorphin-2 (EM2) is a tetrapeptide with remarkable affinity and selectivity for the mu-opioid receptor. In the present study, we used double-fluorescence and electron microscopic immunocytochemistry to identify subsets of EM2-expressing neurons in dorsal root ganglia and spinal cord dorsal horn of adult rats. Within the lumbar dorsal root ganglia, we found EM2 immunoreactivity mainly in small-to-medium size neurons, most of which co-expressed the neuropeptide substance P (SP). In adult rat L4 dorsal root ganglia, 23.9% of neuronal profiles contained EM2 immunoreactivity and ranged in size from 15 to 36 μm in diameter (mean 24.3 ± 4.3 μm). Double-labelling experiments with cytochemical markers of dorsal root ganglia neurons showed that approximately 95% of EM2-immunoreactive cell bodies also label with SP antisera, 83% co-express vanilloid receptor subtype 1/capsaicin receptor, and 17% label with isolectin B4, a marker of non-peptide nociceptors. Importantly, EM2 immunostaining persisted in mice with a deletion of the preprotachykinin-A gene that encodes SP. In the lumbar spinal cord dorsal horn, EM2 expression was concentrated in presumptive primary afferent terminals in laminae I and outer II. At the ultrastructural level, electron microscopic double-labelling showed co-localization of EM2 and SP in dense core vesicles of lumbar superficial dorsal horn synaptic terminals. Finally, 2 weeks after sciatic nerve axotomy we observed a greater than 50% reduction in EM2 immunoreactivity in the superficial dorsal horn. We suggest that the very strong anatomical relationship between primary afferent nociceptors that express SP and EM2 underlies an EM2 regulation of SP release via mu-opioid autoreceptors.

Introduction

Endomorphin-1 (EM1; Tyr-Pro-Trp-Phe-NH_2) and endomorphin-2 (EM2; Tyr-Pro-Phe-Phe-NH_2) are two recently isolated tetrapeptides that have received considerable attention for their remarkable affinity and selectivity for the mu-opioid receptor (Zadina *et al*., 1997). Immunocytochemical studies have shown that the endomorphins are widely distributed in the nervous system, in areas relevant not only to pain, including spinal cord dorsal horn, parabrachial nucleus, periaqueductal grey, medial thalamus and amygdala, but also to autonomic, neuroendocrine and homeostatic functions, and in the limbic system where they can influence affective states (Martin-Schild *et al*., 1997, 1999; Pierce & Wessendorf, 2000; Whitten *et al*., 2001; Wang *et al*., 2002). Both peptides show potent pain-relevant activity *in vivo*, as spinal antinociceptive agents (Zadina *et al*., 1997; Wang *et al*., 1999), as antiallodynic agents (Przewlocka *et al*., 1999) and as inhibitors of substance P (SP)-induced biting and scratching behaviours (Stone *et al*., 1997). Although EM2 produces antinociception via both spinal and supraspinal routes of administration, it is more potent at the spinal level in mice and rats (Zadina *et al*., 1997; Goldberg *et al*., 1998).

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Noxious stimuli are transmitted from the periphery to the central nervous system via activation of nociceptor A delta and C primary afferent fibres, the cell bodies of which reside in the trigeminal and dorsal root ganglia (DRG). These afferents activate second-order 'pain' transmission neurons, via release of excitatory amino acid and peptide neurotransmitters, and are subject to a variety of controls. Opioid inhibition of nociceptive transmission at the spinal level is primarily attributed to an action on mu-opioid receptors in the dorsal horn. Two possible mechanisms have been proposed: presynaptic inhibition of neurotransmitter release from primary afferent terminals that express mu-opioid receptors and/or postsynaptic hyperpolarization of mu-opioid receptor-expressing excitatory interneurons. In support of a presynaptic site of action, mu ligands decrease Ca^{2+} conductance in small-diameter DRG neurons (Taddese *et al*., 1995) and inhibit the release of SP from primary afferents *in vitro* and *in vivo* (Jessell & Iversen, 1977; Yaksh *et al*., 1980; Hirota *et al*., 1985; Aimone & Yaksh, 1989; but see Mauborgne *et al*., 1987; Lang *et al*., 1991; Trafton *et al*., 2000).

Because the gene that encodes the endomorphins has not yet been identified, studies of its localization have relied on immunocytochemical approaches. The presence of EM2 in primary afferent fibres, however, has been inferred not from immunostaining of DRG neurons with EM2 antisera, but from the dramatically reduced immunolabelling within the superficial dorsal horn following dorsal rhizotomy or

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neonatal destruction of C fibres with the neurotoxin capsaicin (Martin-Schild *et al*., 1998; Pierce *et al*., 1998). Furthermore, electrical stimulation of A delta/C afferents *in vitro* evokes a significant release of an EM2-like substance from the isolated spinal cord (Williams *et al*., 1999).

Despite the consensus as to the primary afferent origin of spinal EM2 immunoreactivity, the paucity of immunocytochemical data in DRG means that little is known about the relationship between EM2 and other molecules present in different populations of sensory neurons. In the present study, we used light and electron microscopic immunocytochemical methods to address this question. We also studied the regulation of EM2 expression after transection of the sciatic nerve. A preliminary report of this work has appeared in abstract form (Sanderson Nydahl *et al*., 2002).

Materials and methods

Tissue preparation

All experiments were reviewed and approved by the University of California at San Francisco Institutional Animal Care and Use Committee. Tissues were obtained from male Sprague–Dawley rats (Bantin and Kingman, Fremont, CA, USA; weighing 250–350 g) after perfusion fixation. Animals were deeply anaesthetized with an intraperitoneal injection of pentobarbital (100 mg/kg) and perfused transcardially with 150 mL of 0.1 M phosphate-buffered saline (PBS) followed by 500 mL of ice-cold 4% paraformaldehyde in 0.1 M phosphate buffer (PB) pH 7.4 over 30 min. After the perfusion, the lumbar spinal cord and L4/L5 DRG were removed, postfixed in the same fixative for 1 h and cryoprotected in 30% sucrose in PBS overnight at 4 °C. In separate groups of rats, we denervated the hindpaw by tightly ligating and then transecting the common sciatic nerve at the mid-thigh level; the denervation was made under isofluorane anaesthesia. A 5.0-mm segment of the distal part of the nerve was resected to prevent regeneration. In another (sham) group of rats we exposed the sciatic nerve but did not cut it. The rats in the denervation study ($n = 3$ for all groups) were perfused as described above at either 1, 2 or 8 weeks after the transection.

Immunocytochemistry

L4/L5 DRG were cut on a cryostat at 14 μm, thaw-mounted onto Superfrost microscope slides (Fisher Scientific, Pittsburgh, PA, USA), dried overnight in the dark and stored at −80 °C until processed for immunostaining. The lumbar spinal cord was cut transversely at 40 μm on a freezing microtome and immunostained free-floating. For double-immunofluorescence labelling, sections were incubated in a blocking solution consisting of 10% normal goat serum in 0.1 M PBS containing 0.3% Triton-X for 1 h, followed by primary antisera (diluted in 1% normal goat serum in 0.1 M PBS containing 0.3% Triton-X) for 48 h at 4 °C. After several washes in 1% normal goat serum in 0.1 M PBS containing 0.3% Triton-X, sections were incubated with appropriate secondary antisera for 2 h at room temperature. Following final washes in 0.1 M PB, the slides were dried and coverslipped with Fluoromount-G (Southern Biotechnologies, Birmingham, AL, USA). For labelling with primary antisera raised in goat, normal horse serum replaced the goat serum in all incubations steps described above. We used the following dilutions of primary antisera for fluorescence labelling: rabbit anti-EM2 (1 : 100; Neuromics, Minneapolis, MN, USA), guinea-pig anti-SP (1 : 20,000; kindly provided by Dr J.E. Maggio), goat anti-calcitonin gene-related peptide (CGRP; 1 : 300; Santa Cruz Biotechnology, Santa Cruz, CA, USA), guinea-pig anti-vanilloid receptor subtype 1/capsaicin receptor (TRPV1; 1 : 2000; generated by D.J.), mouse anti-NF200 (clone N52; 1 : 1000; Sigma, St. Louis, MO, USA), goat anti-peripherin (1 : 600; Santa Cruz Biotechnology), rabbit anti-neuropeptide FF (NPFF; 1 : 1000; Chemicon, Temecula, CA, USA). Secondary antibodies were used at the following dilutions: cyanine 3.18-conjugated goat anti-rabbit or donkey anti-rabbit IgG (1 : 600; Jackson ImmunoResearch Laboratories, West Grove, PA, USA), Alexa Fluor488-conjugated goat anti-guinea-pig, goat anti-mouse or donkey anti-goat IgG (1 : 1000; Molecular Probes, Eugene, OR, USA). The FITC-conjugated lectin, isolectin B4 (IB4; Sigma) was used at a 1 : 100 dilution and applied together with the secondary antisera. Tissue sections were viewed with a Nikon Eclipse fluorescence microscope and images were collected with a Spot camera and processed with Adobe Photoshop, version 6.0.

Tissue from axotomized animals was immunostained for EM2 or SP according to the avidin–biotin–peroxidase method of Hsu *et al*. (1981). After a 1-h incubation in blocking solution (see above), sections were placed in primary antisera and gently agitated for 48 h at 4 °C followed by several washes in 1% normal goat serum (NGST) in 0.1 M PBS containing 0.3% Triton-X. The sections were then incubated in a biotinylated secondary antibody for 2 h and the avidin–biotin–peroxidase complex (Vector Laboratories, Burlingame, CA, USA) for 1 h. To localize the horseradish peroxidase immunoreaction product, we used a nickel-intensified diaminobenzidine protocol with glucose oxidase. Finally, the sections were washed three times in 0.1 M PB, mounted on gelatinized slides, dried and coverslipped with DPX (Electron Microscopy Science, Fort Washington, PA, USA).

Specificity of immunolabelling

To test the specificity of the immunostaining, several control experiments were performed. Controls for non-specific binding of the secondary antisera, where the primary antisera was omitted, were always negative. In controls for the specificity of the primary antisera, the EM2 antiserum (diluted to its working concentration) was preabsorbed with 10 μM of its cognate peptide. Additional preabsorption controls were performed in the presence of the following peptides (all at 10 μM): CGRP, EM1, Met-enkephalin, neuropeptide F (NPFF) and SP. After rotating for 24 h at 4 °C, the antibody/peptide solutions were centrifuged at 14,000 ***g*** for 5 min at 4 °C, and the supernatant was used as preabsorbed antisera under otherwise identical staining conditions as described above. An unabsorbed aliquot of the EM2 antiserum (at its working dilution) was rotated overnight at the same time as the antibody/peptide solutions and used as a positive control. Because a small degree of cross-reactivity with SP has been previously reported for other endomorphin antisera (Martin-Schild *et al*., 1997), in addition to preabsorption with 10 μM SP peptide we tested the EM2 antibody on tissue obtained from mice with a deletion of the preprotachykinin A (PPT-A) gene, which encodes SP and neurokinin A.

Quantification of immunoreactivity

To determine the proportion of EM2-immunoreactive DRG cells, we counted the number of immunoreactive cell bodies in sections of DRG as well as the total number of cell bodies, using a monoclonal anti-NeuN antibody (1 : 2000, Chemicon). To this end we sectioned at 14 μm, in their entirety, L4 DRG from three rats. For each ganglion we immunostained every eighth section (six sections per ganglion, 18 sections total). To estimate cell diameters we multiplied the length and width of immunolabelled cell bodies and took the square root of those values. (This calculation corrects for sections that contained elliptical rather than round cells.) Only cells with a visible nucleus were included.

To estimate the extent of co-localization of EM2 immunoreactivity with CGRP, SP, TRPV1, N52, peripherin and IB4, we selected

three–five 14-μm L4 DRG sections for each pair of markers and for each animal (10–15 sections total for each marker pair). In every section, we counted the number of EM2-positive cell bodies, the number of cell bodies immunolabelled for the other marker (CGRP, SP, TRPV1, N52, peripherin or IB4), and the number and percentage of cell bodies that expressed both markers.

To assess changes in density of immunoreactivity following sciatic nerve injury, we used a densitometric approach on 8–10 sections from the L4–L5 segments of the spinal cord from each rat. This method has been described in detail previously (Abbadie *et al*., 1996). Briefly, using a Spot camera, the dorsal horn of the spinal cord was digitized into an 8-bit image (255 grey levels). Quantitative image analysis was performed with publicly available software (NIH Image; http://rsb.info.nih.gov/nih-image/). For each image we defined identically sized rectangular areas on both dorsal horns, which encompassed the medial half of the superficial laminae I and II, both ipsilateral and contralateral to the treated hindpaw. The medial edge of the dorsal horn was used as a landmark for the medial edge of the rectangle. We then set a single threshold grey value, and the number of pixels within each rectangle whose grey values were greater than threshold was counted. All values are expressed as the ratio of the density (number of pixels above threshold in the rectangle) of the ipsilateral to the contralateral side. Ratios were calculated for each section and then a mean ratio was determined for each rat. Using this approach it was possible to normalize the differences between the two sides for each section of the spinal cord. We recognize that this approach may be distorted if contralateral effects of the treatment are produced; however, it is an objective approach to quantifying the immunoreactivity and comparing results across animals.

Electron microscopic immunocytochemistry

For electron microscopy, an adult Sprague–Dawley rat was deeply anaesthetized and perfused first with heparin saline and then with 4% paraformaldehyde and 1% glutaraldehyde in PB. After postfixation for 3 h, 200-μm transverse sections of lumbar spinal cord were cut on a Vibratome. From these sections, disks that encompassed the dorsal horn were cut with a biopsy punch and placed into a copper planchette of the same size. These were immediately frozen at high pressure in an EM PACT apparatus (Leica, Vienna, Austria) and then transferred to an Automatic Freeze Substitution apparatus (Leica) for freeze-substitution over 3 days at −90 °C in 0.1% osmium tetroxide, 0.1% uranyl acetate in acetone. The solutions were changed to anhydrous acetone, and the temperature was raised slowly to −40 °C. After brief rinses in absolute ethanol, the tissues were infiltrated in the Automatic Freeze Substitution apparatus with Lowicryl Monostep HM20 (Electron Microscopy Sciences), and polymerized by ultraviolet light at −40 °C (2 days), 0 °C (2 days) and 25 °C (1 day).

For post-embedding immunocytochemistry, thin sections were mounted on nickel mesh grids and labelled by a modification of the procedure of van Lookeren Campagne *et al*. (1991). Sections were etched with sodium ethanolate for 2 s, washed and dried. They were then treated for 10 min with 0.1% sodium borohydride and 50 mM glycine in 0.05 M Tris-buffered saline containing 0.1% Triton X-100, and blocked in 2% human serum albumin in Tris-buffered saline containing 0.1% Triton X-100. Grids were exposed to a mixture of primary antibodies (guinea-pig anti-SP, 1 : 1000 and rabbit anti-EM2, 1 : 100) in blocking solution for 2–3 h at room temperature. After washing, the sections were blocked again and exposed to secondary antibodies, donkey anti-guinea-pig conjugated to 6 nm gold (Jackson ImmunoResearch), and goat anti-rabbit conjugated to 15 nm gold (Amersham) in blocking solution containing 0.5 mg/mL polyethylene glycol (MW 15,000–20,000, Sigma P-2263). The sections were rinsed, counterstained with uranyl acetate and lead citrate, and examined in a Jeol electron microscope.

Preabsorption control sections were treated with EM2 antibody and 0.5 or 5.0 μM of EM2 peptide. The grids and the outline of the tissue were sketched and the labelled axonal boutons were counted in every grid square that supported the region of superficial dorsal horn. We considered that a labelled axonal bouton was positively labelled if it contained two or more gold-labelled dense core vesicles. In sections immunostained with antisera that had been preabsorbed with EM2 peptide, the staining was completely abolished. There was no reduction in EM2 label in the dorsal horn after incubation of EM2 antibody with SP peptide, indicating that the EM2 antibody does not cross-react with SP.

Results

Distribution of EM2 immunoreactivity in rat DRG and spinal cord

As previously reported (Martin-Schild *et al*., 1997; Pierce *et al*., 1998), we found dense EM2 immunolabelling in the superficial dorsal horn in normal, untreated rats (Fig. 1A). High-magnification analysis showed this labelling to consist of immunoreactive fibres and varicosities concentrated in laminae I and outer II. We also observed a few EM2-labelled terminals in deeper laminae of the dorsal horn and around the central canal. We never observed EM2-immunoreactive cell bodies in the lumbar spinal cord. EM2 immunostaining in the dorsal horn was completely abolished when the antibody had been preabsorbed with 10 μM of EM2 peptide (Fig. 1B). A concentration-dependent reduction in staining was observed when the EM2 antiserum was preabsorbed with EM1 peptide (10 μM−1 nM). Immunolabelling for EM2 did not change when the antibody was preabsorbed with 10 μM CGRP, met-enkephalin or SP (data not shown), but we noted a slight decrease in the intensity of labelling for EM2 after preabsorption of the antibody with 10 μM NPFF; 1.0 μM NPFF had no effect.

To further investigate the possibility of cross-reactivity between EM2 and NPFF, we stained alternate sections with EM2 and NPFF antisera. Consistent with previous reports (Roumy & Zajac, 1998), we observed robust NPFF immunostaining in superficial dorsal horn, in patches of deeper dorsal horn and around the central canal (data not shown). The pattern of EM2 immunoreactivity was similar, albeit much more limited, especially in deeper laminae and around the central canal. Finally, we stained tissue from mice lacking the PPT-A gene. In wild-type mice, SP staining was concentrated in the superficial laminae of the dorsal horn (Fig. 1C). As expected, immunostaining for SP was completely absent in the PPT-A mutant mice (Fig. 1D); however, the EM2 immunostaining in the wild-type (Fig. 1E) did not differ from that in the mutant (Fig. 1F).

In the DRG, many cells and axons were EM2 immunoreactive, with an apparently even distribution throughout the ganglion (Fig. 1G). The EM2 immunoreactivity was completely abolished in adjacent DRG sections stained with antiserum that had been preabsorbed with EM2 peptide (see Materials and methods; Fig. 1H). A similar pattern of EM2 immunolabelling was seen in trigeminal ganglia, and in a population of small-diameter cell bodies from the nodose ganglia (data not shown). We never observed DRG neurons that stained with NPFF antisera under the same conditions where we found robust EM2 immunolabelling in adjacent sections. This was consistent with previous reports that did not find NPFF immunoreactivity or NPFF mRNA expression in sensory ganglion cells (Panula *et al*., 1996; Vilim *et al*., 1999), and points to an intrinsic origin of spinal cord NPFF.

Analysis of L4 DRG sections colabelled with a marker of neuronal cell bodies (NeuN) showed that 23.9% of the DRG neurons contained

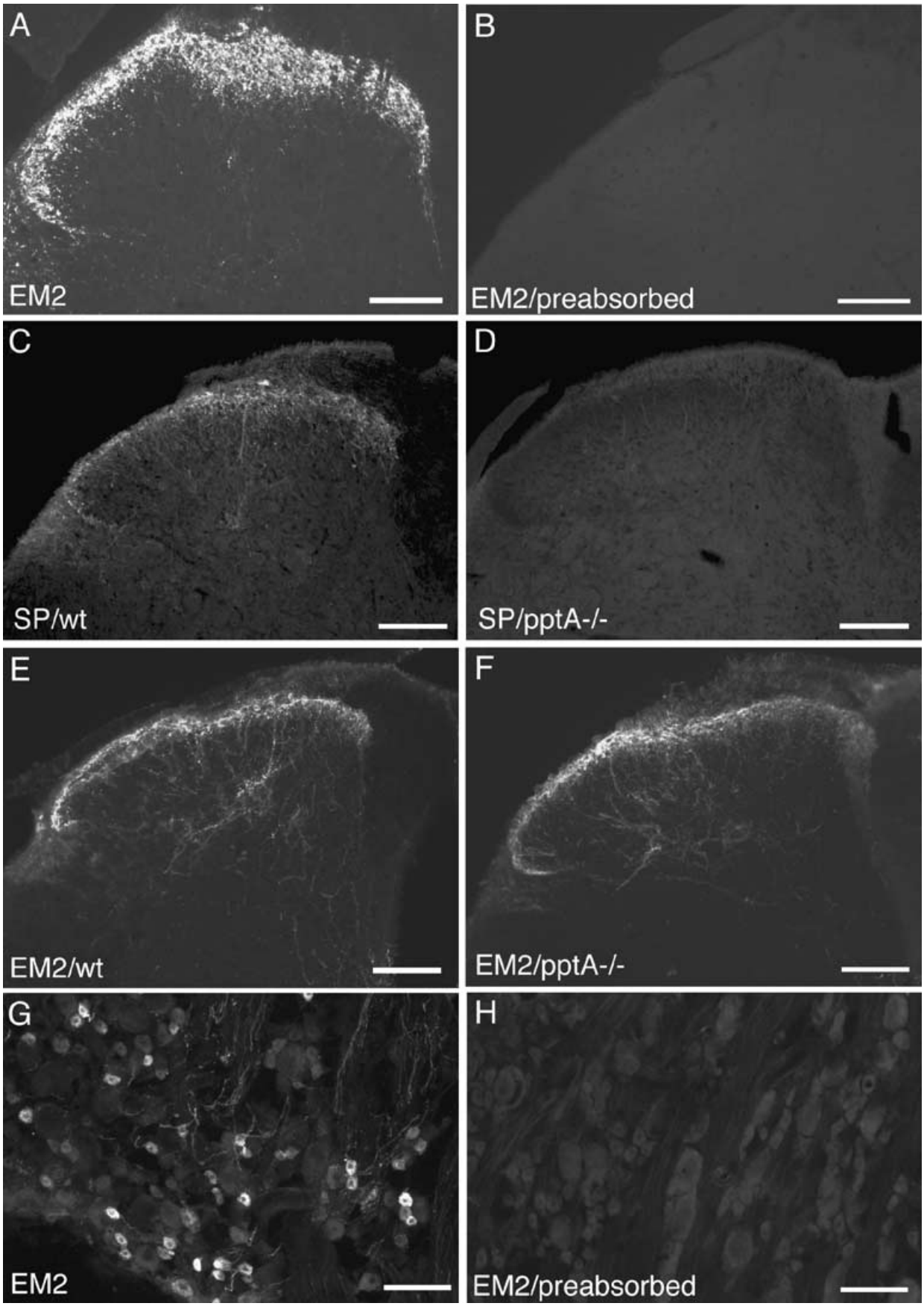

A
EM2
B
EM2/preabsorbed
C
SP/wt
D
SP/pptA-/-
E
EM2/wt
F
EM2/pptA-/-
G
EM2
H
EM2/preabsorbed

TABLE 1. Quantification of the co-localization of EM2 with various markers

Marker Pair	Cells (n_1/n_2)	Double-labelled Cells (n)	Percentages (A of B)	(%)
NeuN/EM2	1234/295	295	EM2 of NeuN	23.9
SP/EM2	448/464	444	EM2 of SP	99.1
			SP of EM2	95.7
IB4/EM2	797/384	65	EM2 of IB4	8.2
			IB4 of EM2	16.9
CGRP/EM2	570/437	436	EM2 of CGRP	76.5
			CGRP of EM2	99.8
TRPV1/EM2	911/437	365	EM2 of TRPV1	40.1
			TRPV1 of EM2	83.5
Peripherin/EM2	1268/488	464	EM2 of Peripherin	36.6
			Peripherin of EM2	95.1
N52/EM2	904/332	32	EM2 of N52	3.5
			N52 of EM2	9.6

EM2 immunoreactivity (Table 1). Figure 2 shows the results from the quantitative analysis of the size distribution of cell profiles immunostained for EM2. Most of the EM2-labelled cells were small to medium in size, ranging from 15 to 36 μm in diameter (mean 24.3 ± 4.3 μm).

Co-localization of EM2 immunoreactivity with cytochemical markers of DRG neurons

We examined EM2 immunoreactivity in relation to several widely used phenotypic markers of subsets of DRG neurons (Snider & McMahon, 1998), including CGRP and SP, to label peptide-containing DRG neurons and TRPV1 to identify capsaicin-sensitive nociceptors (Caterina *et al*., 1997). We used IB4 from *Bandeiraea simplicifolia* to label the 'peptide-poor' subpopulation of DRG cells. We also co-labelled with peripherin, the C fibre marker, or N52, an NF200 antibody (clone N52 that is directed against the 200-kDa, high-molecular weight neurofilament protein subunit), that labels cell bodies with myelinated axons. Table 1 summarizes the quantification of the co-localization of EM2 with these different markers.

We found an extensive overlap of EM2 and SP labelling in the L4 DRG. Almost all EM2-positive cell bodies immunostained with SP antibody (95.7%), and an even higher percentage (99.1%) of the SP-labelled cell bodies were EM2 positive (Fig. 3A–C). Interestingly, when we noted an EM2-immunoreactive neuron that was not double-labelled for SP, it was often of much larger diameter. In contrast to the high degree of co-localization with peptidergic neurons, few EM2-positive cells also labelled with the lectin IB4 (Fig. 3D–F). In fact, EM2 labelling was observed in less than 10% of IB4 cell bodies, and only 16.9% of all EM2-positive cell bodies were labelled by the lectin.

A large fraction (76.5%) of CGRP-labelled cell bodies also contained EM2 immunoreactivity, and almost all (99.8%) EM2-positive profiles were CGRP positive (Fig. 3G–I). Double-labelling with antisera against EM2 and the capsaicin receptor, TRPV1, showed that 83.5% of EM2-positive cell bodies also contained TRPV1 immunoreactivity (data not shown).

Not surprisingly, most EM2-positive cells (95.1%) labelled with peripherin antiserum and many (~65%) peripherin-positive cells were EM2 negative (Fig. 3J–L). Finally, we found a very limited overlap between EM2 and N52 (Fig. 3M–O), a marker of cell bodies with myelinated axons. Of the N52-positive cell bodies counted, only 3.5% were labelled with the EM2 antibody.

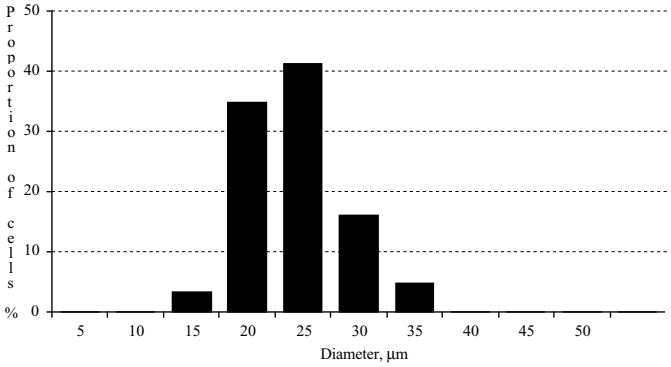


FIG. 2. Size distribution of EM2-immunostained cells in L4 DRG. A total of 150 cell bodies were measured in seven sections taken from three adult rats.

Because it is well known that the central projections of SP- and IB4-expressing neurons are anatomically distinct, it was not surprising that double-labelling of terminals in the spinal cord revealed a high degree of terminal overlap between EM2 and SP (Fig. 4A–C), in regions distinct from the characteristic IB4 labelling in inner lamina II (Fig. 4D–F).

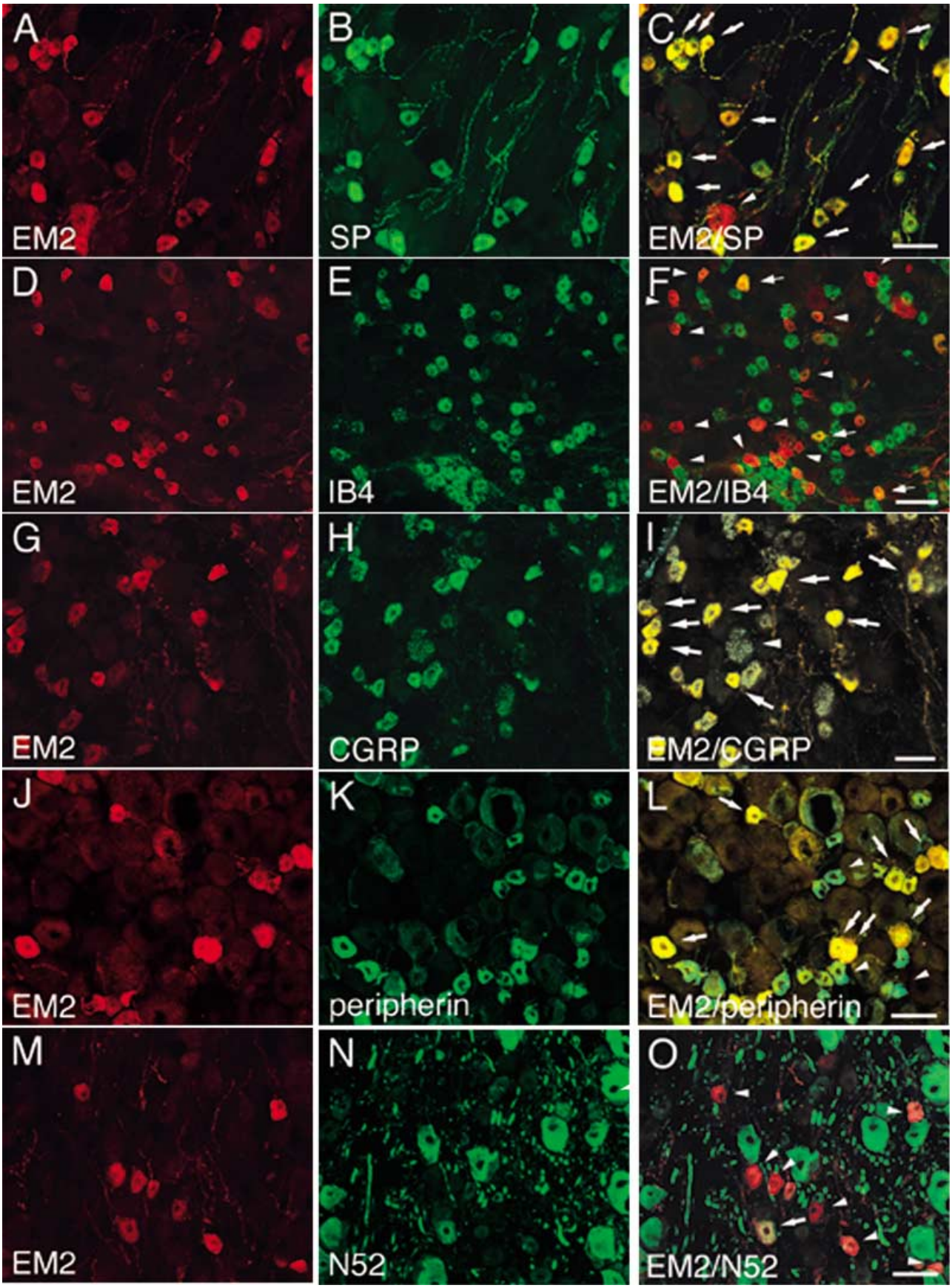
Subcellular distribution of EM2 immunoreactivity in the dorsal horn

To assess the nature of the co-localization of EM2 and SP immunoreactivity in synaptic terminals in the lumbar dorsal horn, we performed additional investigations at the electron microscopic level. Using a post-embedding immunogold double-labelling protocol, we found that both EM2 and SP immunoreactivity are concentrated in dense core vesicles of axon terminals (Fig. 5A). A higher magnification view of the dense core vesicles (Fig. 5B) confirmed that EM2 and SP localized within the same vesicle.

Effect of sciatic nerve section on EM2 immunoreactivity

We found no significant difference in the magnitude of labelling between the left and right side in untreated (control) rats, or between the ipsilateral and contralateral side of rats that underwent sham surgery (sciatic nerve was exposed, but not cut; data not shown). However, we observed a significant reduction in EM2 immunoreactivity in the ipsilateral dorsal horn after sciatic nerve section, at all time points investigated (up to 8 weeks after surgery). The decrease was focused in the medial part of the dorsal horn in the L4 and L5 segments, which receive the majority of input from the afferents of the hindpaw. One week after sciatic nerve lesion we observed a 22.1% reduction in EM2 immunolabelling on the ipsilateral side compared with control and contralateral dorsal horns. The decrease in staining increased with time, peaking about 2 weeks post-lesion. Figure 6 illustrates the decrease in EM2 (Fig. 6A and B) and SP (Fig. 6C and D) immunolabelling observed at 2 weeks post-axotomy. The EM2 immunoreactivity was reduced by 51.4% on the ipsilateral side compared with the contralateral side, and this decrease persisted for at least 8 weeks after the lesion (last time point investigated). In the case of SP, we observed

FIG. 1. Endomorphin-2 (EM2) expression in spinal cord dorsal horn and DRG. (A) EM2 immunoreactivity is concentrated in the superficial dorsal horn. (B) Immunostaining of dorsal horn with primary antibody absorbed with 10 μM EM2 peptide. (C and D) Substance P (SP) immunoreactivity in section of the lumbar spinal cord from wild-type (C) and preprotachykinin-A (PPT-A) mutant (D) mouse. (E and F) EM2 expression in lumbar dorsal horn in wild-type (E) and PPT-A mutant (F) mouse. (G) EM2 immunoreactivity in L4 DRG of the adult rat. (H) Absorption control with 10 μM EM2 peptide in DRG. Scale bars, 100 μm (A–H).


A
EM2
B
SP
C
EM2/SP
D
EM2
E
IB4
F
EM2/IB4
G
EM2
H
CGRP
I
EM2/CGRP
J
EM2
K
peripherin
L
EM2/peripherin
M
EM2
N
N52
O
EM2/N52

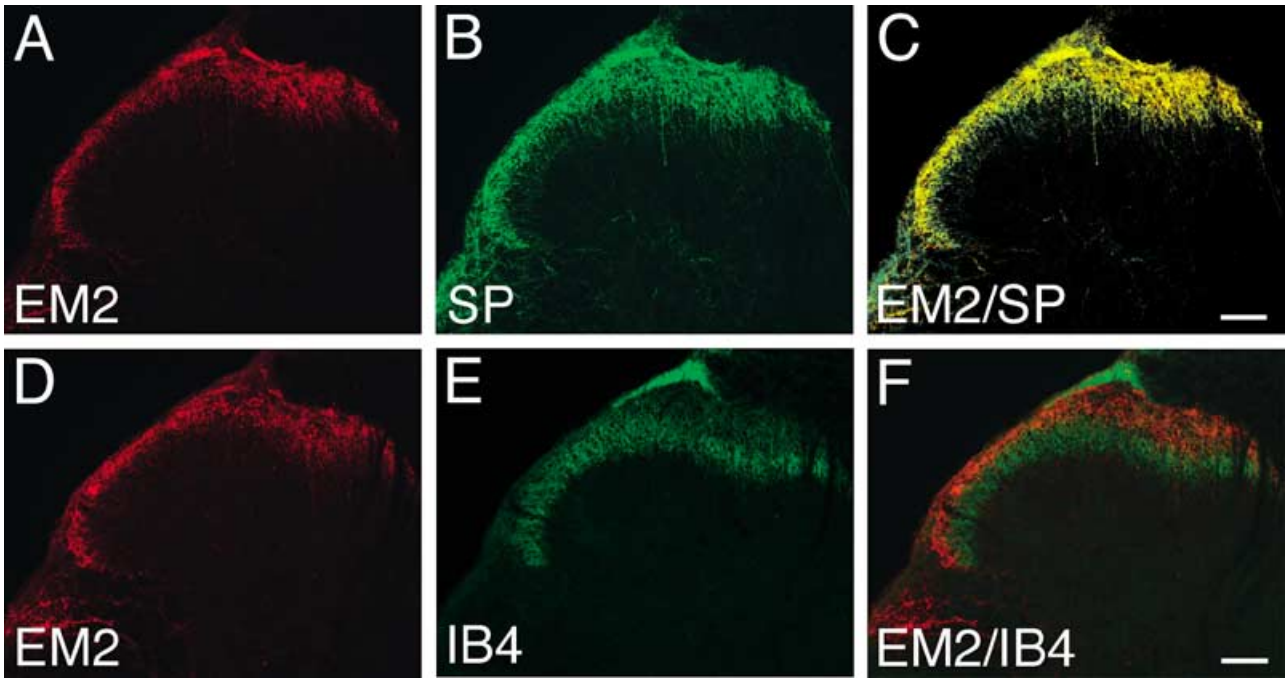


FIG. 4. (A–C) Double-labelling of endomorphin (EM2; A; red) and substance P (SP; B; green) in the spinal cord dorsal horn. The merged image (C) reveals extensive overlap of EM2 and SP in terminals of laminae I and outer II. Co-localization appears yellow. (D–F) Double-staining for EM2 (D; red) and the lectin isolectin B4 (IB4; E; green) in the dorsal horn shows a limited overlap between these markers as illustrated by the merged image (F). Scale bars, 100 μm (A–F).

a more modest, but still significant (28.1%), decrease in SP immunoreactivity 2 weeks after the lesion. Sciatic nerve transection also differentially affected NPFF and EM2 immunoreactivity. Staining of alternate sections taken from three rats at 2 weeks following axotomy showed that while there was a marked decrease in EM2 immunoreactivity in the medial dorsal horn of lumbar segments ipsilateral to the injury, immunolabelling for NPFF was unchanged (data not shown). The latter result is in line with a previous report showing that spinal nerve ligation did not change the level of NPFF expression at the time of maximal allodynia (Vilim *et al*., 1999).

Discussion

In the present study, we used light and electron microscopic immunocytochemistry to examine the distribution of EM2 in adult rat DRG and spinal cord before and after peripheral nerve injury. First, we found that EM2-like immunoreactivity is localized to a subpopulation of small- to medium-sized DRG cell bodies and to their central terminals in the spinal cord dorsal horn. Second, we examined the neurochemistry of the subpopulation(s) of DRG neurons that express EM2 immunoreactivity. We found a remarkably close association of EM2 and the peptidergic (CGRP, SP) subpopulation of DRG neurons. Almost all EM2-positive cells co-labelled for CGRP and SP. At the ultrastructural level we found that EM2 and SP co-localized in dense core vesicles of superficial dorsal horn synaptic terminals. Finally, 2 weeks following sciatic nerve axotomy, we observed a dramatic reduction in EM2 immunoreactivity in the superficial dorsal horn, ipsilateral to the injury. Based on the anatomical location of EM2 immunoreactivity, we suggest that upon release from primary afferent terminals, EM2 could regulate SP release via presynaptic opioid autoreceptors.

Specificity of EM2 immunostaining

We used a commercially available rabbit polyclonal EM2 antibody that, based on preabsorption controls, did not cross-react with CGRP, met-enkephalin or SP. Preabsorption with higher concentrations (>1 μM) of EM1 or NPFF (both of which contain a C-terminally amidated phenylalanine, as does EM2), reduced, but did not eliminate, EM2 staining. This suggests that the antibody does not completely distinguish between EM2 and EM1/NPFF in their native peptide form. On the other hand, because immunostaining is performed on fixed tissue (while absorption controls use 'unfixed' peptide) we cannot establish with certainty what the antibody recognizes in fixed tissue. This, of course, reflects the fact that antibodies recognize epitopes, not molecules (Burry, 2000).

NPFF is an amidated octapeptide that interacts with opioid systems both at spinal and supraspinal levels (Roumy & Zajac, 1998). NPFF is found in highest density in the hypothalamus, caudal brainstem and in

FIG. 3. Double-immunofluorescence labelling of endomorphin-2 (EM2), various cytochemical markers in rat DRG. (A–C) In the lumbar DRG, we found that the great majority of EM2 (A; red)-immunoreactive DRG cell bodies also express substance P (SP; B; green). In the merged image (C), co-localization appears yellow (arrows in C). A few larger EM2-positive cells do not express SP (arrowhead in C). (D–F) Double-labelling of EM2 (D; red) with the lectin isolectin B4 (IB4; E; green) in the DRG reveals a limited overlap between these markers as illustrated in the merged image (F) where arrowheads point to single-labelled EM2-positive cells bodies and arrows show examples of double-labelled neurons. (G–I) Double-staining for EM2 (G; red) and calcitonin gene-related peptide (CGRP; H; green) in L4 DRG. In (I), arrows show examples of double-labelled neurons (yellow); arrowheads show CGRP-positive cells that are not EM2 immunoreactive. (J–L) Double-labelling of EM2 (J; red) and peripherin (K; green). The merged image (L) shows that almost all EM2-immunoreactive cells are also peripherin-positive (yellow, at arrows). Arrowheads in (L) point to examples of peripherin-labelled cells that are not EM2 immunoreactive. (M–O) Double-labelling of EM2 (M; red) and N52 (N; green) in L4 DRG. In (O) the merged image illustrates a limited overlap between EM2 and N52. Co-localization appears yellow (at arrow). Arrowheads show single-labelled EM2-immunoreactive cell bodies. Scale bars, 100 μm (A–O).

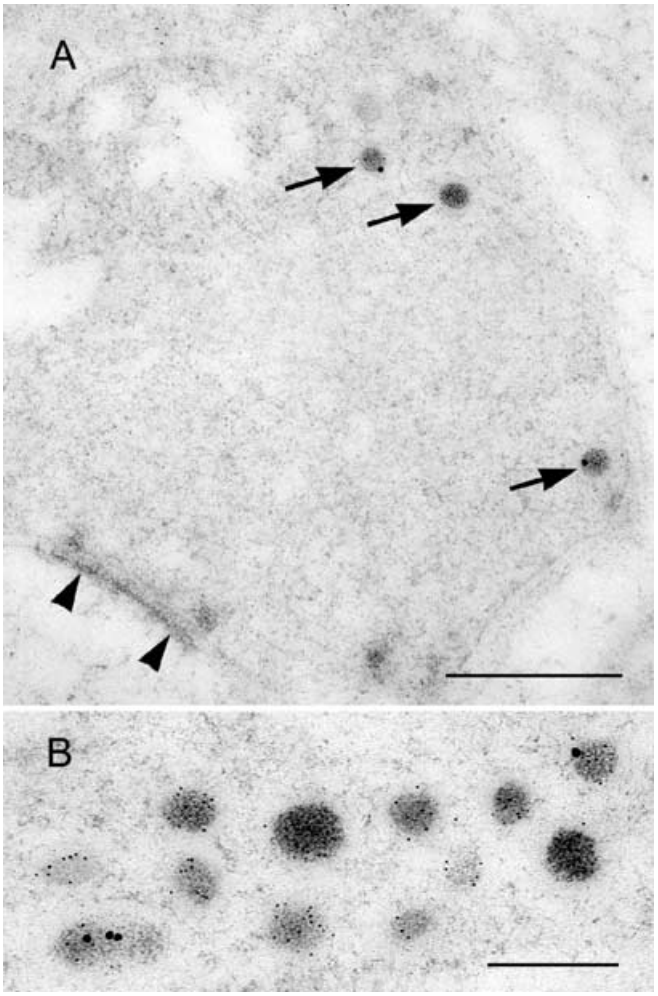


FIG. 5. EM2 and SP are localized in the same dense core vesicles. (A) Electron microscopic double-labelling, as in this lamina I axonal bouton (synaptic density at arrowheads), revealed gold particles localizing immunoreactivity (black dots, at arrows) in the dense core vesicles. A magnified view of the dense core vesicles (B) shows that EM2 (large dots) and SP (small dots) are contained within the same structures. Scale bars, 0.5 μm (A) and 0.25 μm (B).

the superficial dorsal horn of the spinal cord (Roumy & Zajac, 1998), i.e. in several regions where EM2 is concentrated. However, there are many areas where the two peptides do not overlap. For example, colchicine treatment, which is used to enhance the level of peptide in cell bodies, revealed NPFF cells in the spinal cord dorsal horn (Kivipelto & Panula, 1991), but EM2-immunoreactive cell bodies have yet to be demonstrated in the spinal cord, even after colchicine (Wang *et al*., 2002). Furthermore, although spinal cord transection reduces NPFF immunoreactivity caudal to the lesion (implicating a supraspinal origin), dorsal rhizotomy does not (Majane *et al*., 1989). This argues against the DRG as the source of spinal cord NPFF. Consistent with this view we never observed DRG immunostaining with NPFF antisera. In sharp contrast, however, dorsal rhizotomy or destruction of C fibres by neonatal capsaicin treatment abolished EM2 immunostaining in the dorsal horn (Martin-Schild *et al*., 1998; Pierce *et al*., 1998). These studies indicate therefore that NPFF immunoreactivity derives from local interneurons and descending fibres, but that dorsal horn EM2 immunoreactivity originates predominantly from primary afferents.

Finally, we found no reduction of EM2 staining in tissue obtained from mice lacking the PPT-A gene, which codes for SP and neurokinin A. Because a small degree of cross-reactivity with SP was previously reported using a different EM2 antibody (Martin-Schild *et al*., 1997), this is a critical control. Taken together with the fact that preabsorption with SP peptide did not alter EM2 immunostaining, the studies in the PPT-A mutant mice provide very strong evidence that the EM2 antibody that we used does not cross-react with SP in tissue.

EM2 immunostaining in DRG and spinal cord dorsal horn

We observed intense EM2-like immunoreactivity in DRG cell bodies and terminals of the superficial dorsal horn, where primary afferent nociceptors arborize. We found EM2 immunoreactivity in 23.9% of DRG cells, ranging in size from 15 to 36 μm in diameter (mean 24.3 ± 4.3 μm), which likely includes both C and A delta afferents. These results are consistent with previous reports (Martin-Schild *et al*., 1998; Pierce *et al*., 1998), which found that dorsal rhizotomy or neonatal treatment with capsaicin depletes EM2 immunoreactivity in the superficial dorsal horn. Although Martin-Schild *et al*. (1997) observed EM2-immunoreactive fibres and cell bodies within rat DRG and trigeminal ganglia, their preabsorption controls (EM2, EM1 or SP) did not abolish the immunostaining of cell bodies. In the present study, preabsorbing the EM2 antibody with its cognate peptide completely abolished DRG staining, and as described above we could rule out cross-reactivity with CGRP, SP or NPFF.

Co-localization of EM2 with cytochemical markers of DRG neurons

The small diameter population of DRG neurons, most of which are nociceptors, can be divided broadly into two, largely non-overlapping populations (Lawson, 1992). The so-called peptidergic group expresses the neurotrophin receptor, TrkA, contains peptide neurotransmitters (e.g. CGRP and SP) and is responsive to nerve growth factor. The remaining C fibre population, the 'peptide-poor' group, can be selectively labelled with the *Griffonia simplicifolia* IB4 and expresses c-Ret, the target of glial-derived neurotrophic factor (Snider & McMahon, 1998; Julius & Basbaum, 2001). We found that the great majority of EM2-positive neurons were of small to medium size, expressed peripherin, a marker of unmyelinated axons and that there was an almost 100% overlap of EM2-immunoreactive neurons with markers of the peptide class of primary afferent nociceptors. Thus, almost all SP-positive neurons were EM2 immunoreactive and 96% of the latter were SP positive. In agreement with others (Martin-Schild *et al*., 1997, 1998; Pierce *et al*., 1998; Spike *et al*., 2002), we also found almost complete overlap of EM2 and SP terminals in laminae I and outer II of the superficial dorsal horn.

The EM2-positive neurons that did not express SP were of larger diameter and probably belong to the subpopulation of CGRP-positive cells that do not co-label with SP. Not surprisingly, almost 100% of EM2-immunoreactive profiles were labelled by the CGRP antibody, but almost 25% of the CGRP-positive cells were not EM2 immunoreactive. The expression of EM2 in the IB4-positive population was also much more limited. Only 17% of EM2-immunoreactive cells showed IB4 immunoreactivity in the L4 DRG. This likely corresponds to the fraction of IB4-positive neurons that are SP-positive (Kashiba *et al*., 2001; Price *et al*., 2002). Finally, as predicted from our previous analysis (Tominaga *et al*., 1998), we found that the great majority of EM2-expressing cells are TRPV1 positive, and thus likely to be capsaicin-sensitive nociceptors. Finally, based on the localized distribution of EM2 terminals in the dorsal horn, we suggest that the few N52-positive DRG neurons that express EM2 correspond to lightly myelinated, A delta nociceptors.

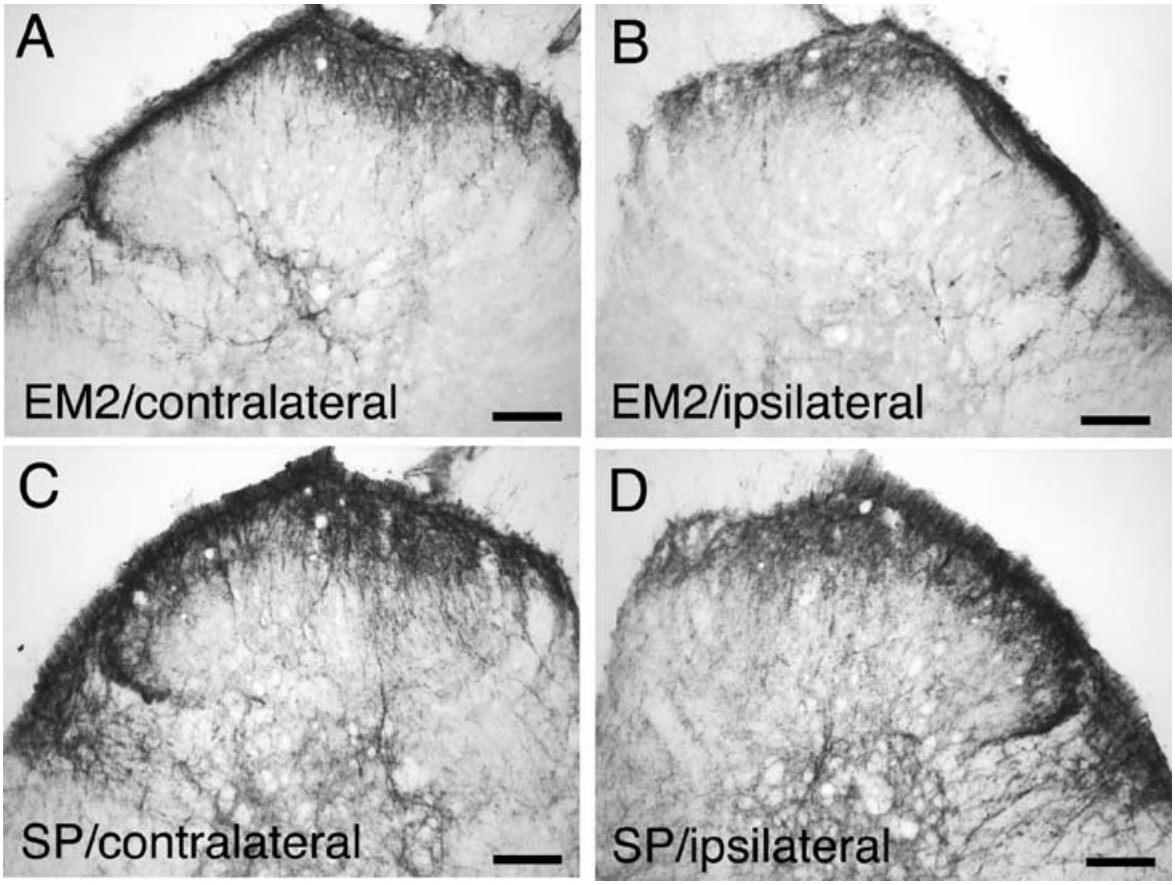


FIG. 6. Transection of the sciatic nerve results in a significant decrease of endomorphin-2 (EM2) immunoreactivity in the dorsal horn. These photomicrographs illustrate the density of EM2 and substance P (SP) immunoreactivity in the lumbar spinal segment contralateral (A and C) and ipsilateral (B and D) to the sciatic lesion. Two weeks after axotomy, there was a 51.4% decrease in EM2 immunoreactivity in the medial half of the dorsal horn, ipsilateral to the injury (compare A and B) and a 28.1% decrease in SP immunoreactivity (compare C and D). The decrease in staining was restricted to the region of termination of afferents from the hindpaw ($n = 3$ for all groups).

EM2 is co-expressed with SP in dense core vesicles in dorsal horn primary afferent terminals – functional significance

The co-occurrence of SP and EM2 raised the possibility that noxious stimulus-evoked release of endomorphin can regulate nociceptive processing by a presynaptic inhibitory action on opioid receptor-laden primary afferents. Our finding that EM2 and SP are co-stored in dense core vesicles, in fact, suggests that there is coincident release of excitatory and inhibitory neurotransmitters from the same nociceptor. If the endomorphin were to act back on the terminal from which it is released, i.e. in an autoreceptor function, this arrangement would serve to limit the duration of transmitter release from the terminal. In this regard, it is of interest that CGRP is also co-stored in dense core vesicles that contain SP. Because CGRP enhances the action of SP, in part by regulating the endopeptidase that degrades SP (Le Greves *et al*., 1985), it appears that the dense core vesicle contains a complete store of molecules that regulate output and the excitability of the terminal.

Although there is good evidence that enkephalin and dynorphin target the delta- and kappa-opioid receptors, respectively, the endogenous ligand for the mu-opioid receptor is less clear. The enkephalins can target both mu- and delta-opioid receptors, but the remarkable selectivity of the endomorphin for the mu receptor suggested that it is the predominant endogenous mu ligand (Zadina *et al*., 1997). Because the mu-opioid receptor is located both presynaptically on nociceptor terminals and postsynaptically on interneurons and projection neurons in the dorsal horn, there are multiple mechanisms through which endomorphin release from primary afferents could regulate nociceptive processing. In this regard, the recent ultrastructural co-expression studies of EM2 and mu-opioid receptors in the trigeminal (Aicher *et al*., 2003) and cervical (Wang *et al*., 2003) dorsal horn are significant. Specifically, Aicher and co-workers showed that mu-opioid receptor immunoreactivity was frequently localized to dendrites contacted by EM2-immunoreactive axon terminals. To a lesser extent, they co-localized mu-opioid receptor and EM2 immunoreactivity in presynaptic profiles, providing an anatomical substrate for an EM2 regulation of transmitter release by an action on mu-opioid autoreceptors.

In a previous study, we monitored internalization of the mu-opioid receptor and showed that exogenous administration of endomorphins and other opioid peptides clearly act at postsynaptic receptors. However, we found no evidence that stimuli of strength sufficient to evoke the release of endogenous opioids, which would include the endomorphins, resulted in postsynaptic opioid receptor internalization. Based on those results, we hypothesized that noxious stimulus-evoked release of endogenous opioids likely exerts antinociceptive effects by targeting presynaptic opioid receptors (Trafton *et al*., 2000). Spike and co-workers recently showed that although many mu-opioid receptor-expressing neurons in lamina II receive contacts from SP-containing primary afferents, these contacts were mostly non-synaptic (Spike *et al*., 2002). In addition, supporting the results of Trafton *et al*., they showed that very few mu-opioid receptor-containing neurons

expressed Fos after mechanical or chemical (formalin) noxious stimuli. Taken together with the results of the present study, we conclude that there are likely both interneuronal (enkephalin) and primary afferent nociceptor (endomorphin) sources of this presynaptic opioid inhibitory control.

Effect of injury on EM2 immunoreactivity

Consistent with previous studies that found a significant plasticity of the neurochemistry of primary afferent nociceptors after tissue or nerve injury (Hökfelt *et al*., 1994), we observed that sciatic nerve section produces a long-lasting (at least 8 weeks) decrease in EM2 immunoreactivity in the superficial dorsal horn ipsilateral to the injury. The magnitude and time course of the reduction was similar to that previously reported after sciatic nerve constriction in the mouse (Smith *et al*., 2001) and paralleled the changes observed for SP. Although it is reasonable to propose that the relative unresponsiveness of nerve injury-induced pain conditions to opioid therapy reflects nerve injury-induced down-regulation of mu- and delta-opioid receptors in DRG and dorsal horn (Zhang *et al*., 1998), the significance of the loss of EM2 is not clear. One possibility is that this underlies a reduced opioid inhibitory tone, which could contribute not only to the spontaneous pains associated with nerve injury, but also to the exacerbated responses to non-noxious and noxious stimulation. On the other hand, because there is a concurrent loss of SP, an excitatory peptide, from the same presynaptic terminal, this should at least partly balance the loss of inhibitory controls that would result from endomorphin down-regulation. For these reasons, the functional relevance of these nerve injury-induced changes in peptide content is not clear.

In conclusion, our light and electron microscopic analysis of endomorphin immunoreactivity in the rat dorsal horn has provided strong evidence not only that this mu-opioid agonist is expressed by a highly defined subset of primary afferent nociceptors, but that its release is also likely to influence subsequent flow of nociceptive information via an inhibitory action on the synaptic terminals from which it is released. These observations, taken together with the fact that endomorphin content in nociceptors is dynamically regulated by injury, underscores the importance of identifying the gene that encodes endomorphin so that the molecular basis for this regulation can be better studied.

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Abbreviations

CGRP, calcitonin gene-related peptide; DRG, dorsal root ganglia; EM1, endomorphin-1; EM2, endomorphin-2; IB4, isolectin B4; NPFF, neuropeptide FF; PB, phosphate buffer; PBS, phosphate-buffered saline; PPT-A, preprotachykinin-A; SP, substance P; TRPV1, vanilloid receptor subtype 1/capsaicin receptor.

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