ORL-1 and Mu Opioid Receptor Antisera Label Different Fibers in Areas Involved in Pain Processing

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ABSTRACT

Mu opioid receptors (MOR) mediate the analgesic effects of opioid drugs such as morphine. The opioid receptor-like (ORL-1) receptor is structurally related to opioid receptors and the ORL-1 receptor agonist, orphanin FQ/nociceptin, induces analgesia at the spinal level, but appears to recruit different circuitry than that used by mu opioids. When administered intracerebroventricularly, orphanin FQ/nociceptin produces hyperalgesia and/or reverses opioid analgesia. The functionally distinct actions elicited by MOR and ORL-1 receptors, which activate similar intracellular signaling systems and show similar regional distributions, could be explained by their differential cellular localization. By using double label immunohistochemistry and confocal microscopy, the present study investigates the distribution of MOR and ORL-1 receptors in regions of the rat nervous system that are involved with nociceptive processing. In general co-localization of MOR and ORL-1 receptor immunore-activity was not observed in either perikarya or neuropil in the dorsal root ganglia, nor in the Lissauer's tract and superficial laminae of the spinal cord. Likewise, there was no evidence for co-localization of these receptors within the periaqueductal gray, the nucleus raphe magnus, the gigantocellular reticular nucleus, and the nucleus of the solitary tract. These observations indicate that MOR and ORL-1 receptors are expressed predominantly on different fiber systems in these regions. This differential distribution is consistent with the distinct pharmacology of ORL-1 and MOR receptor agonists and suggests that the antisera to MOR and ORL-1 receptors may provide useful markers for further investigations of analgesic and counteranalgesic pathways modulating pain perception. J. Comp. Neurol. 399:373–383, 1998.
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Activation of opioid receptors leads to a variety of physiological effects mediating modulation of nociception, locomotion, respiration, and motivation (Pasternak, 1993). Three major types of opioid receptors have been described and are referred to as the mu, delta, and kappa opioid receptors. These receptors are widely expressed throughout the central nervous system (CNS), and each receptor type has a distinct but overlapping distribution (Mansour et al., 1988) and pharmacological profile (Goldstein, 1987). At the structural level, the mu, delta, and kappa opioid receptors belong to the family of G protein-coupled receptors with seven transmembrane domains. Opioid receptors have been reported to modulate adenylyl cyclase, mitogen-activated protein kinase, and phosphoinositide turnover,

in addition to inhibiting calcium channels and activating potassium conductance (Childers, 1991; Jin et al., 1994; Li and Chang, 1996).

Mu opioid receptors (MOR) are of critical clinical importance because of their high affinity for morphine, a potent and widely used alkaloid analgesic. Recent studies on mu

receptor-knockout mice have confirmed the essential role of MOR in the mediation of morphine-induced antinociception (Matthes et al., 1996; Sora et al., 1997). Immunohistochemical studies (Arvidsson et al., 1995; Mansour et al., 1995b; Ding et al., 1996) have reported a wide distribution of MOR protein throughout the rat central and peripheral nervous system. Immunoreactive perikarya and neuropil are observed in many areas, including those involved in pain processing, such as neocortex, thalamus, periaqueductal gray, raphe nuclei, reticular formation, and spinal cord, as well as in primary sensory neurons in dorsal root ganglia. This pattern of distribution correlates reasonably well with MOR binding sites detected by receptor autoradiography (Pert et al., 1976; Goodman et al., 1980; Quirion et al., 1983; Gouarderes et al., 1991) and the distribution of MOR mRNA (Delfs et al., 1994; Mansour et al., 1994; Minami et al., 1994).

Recently, a new member of the opioid receptor family, the opioid receptor-like-1 receptor (ORL-1), has been cloned from various species including the mouse (Pan et al., 1995), rat (Bunzow et al., 1994; Chen et al., 1994; Fukuda et al., 1994; Marchese et al., 1994; Wick et al., 1994; Lachowicz et al., 1995), and human (Keith et al., 1994; Mollereau et al., 1994). ORL-1 is highly homologous with opioid receptors, particularly in its transmembrane and intracellular domains. This similarity suggests that ORL-1 might mediate its pharmacological effects through the same signaling systems as opioid receptors, and a number of recent studies have confirmed this assumption (Meunier et al., 1995; Reinscheid et al., 1995; Conner et al., 1996; Vaughan and Christie, 1996).

Unlike opioid receptors, the ORL-1 receptor does not bind any of the classical peptide or alkaloid opioid ligands with high affinity (Mollereau et al., 1994). However, an endogenous peptide agonist for ORL-1 receptors has been identified and named nociceptin (N; Meunier et al., 1995) or orphanin FQ (OFQ; Reinscheid, et al., 1995); it is partially homologous to opioid peptides derived from proenkephalin and prodynorphin. Pharmacological studies in rats have shown a dual activity of OFQ/N in pain modulation, depending on whether the peptide is administered at the spinal (intrathecally) or supraspinal (intracerebroventricularly) level. Intrathecal injection of OFQ/N produces a potent spinal antinociceptive effect (Xu et al., 1996) and potentiates morphine-induced analgesia (Tian et al., 1997a). However, when administered intracerebroventricularly, OFQ/N antagonizes the analgesic effect elicited by either morphine injection or acupuncture (Zhu et al., 1996; Tian et al., 1997a,b). When OFQ/N was administered to ORL-1 receptor-knockout mice, no pharmacological effects were observed (Nishi et al., 1997), implying that the in vivo actions of this peptide are mediated via ORL-1 receptors.

A role for ORL-1 receptors in the modulation of pain perception was first suggested by in situ hybridization studies in rats (Bunzow et al., 1994; Wick et al., 1994; Lachowicz et al., 1995). High levels of ORL-1 receptor mRNA are found in many areas involved in pain processing such as the dorsal root ganglia, the dorsal horn of the spinal cord, the raphe nuclei, and the periaqueductal gray. Immunolocalization of ORL-1 receptors is in agreement with the reported mRNA distribution, and the majority of staining is found in fiber processes (Anton et al., 1996).

MOR and ORL-1 receptors show an overlapping anatomical distribution in regions of the nervous system involved in pain perception, which is consistent with the involvement of both receptors in the modulation of pain perception. However, co-localization of the two receptors would be surprising given that the spinal analgesic effects of MOR and ORL-1 receptor agonists appear to involve distinct circuitries (Xu et al., 1996), and that OFQ/N is an antiopioid at the supraspinal level (Grisel et al., 1996; Mogil et al., 1996a). To address this issue, we have used a monoclonal antibody against the ORL-1 receptor protein and a rabbit polyclonal antiserum against MOR receptors to investigate the cellular distribution of both receptors by dual wavelength confocal microscopy. We have focused our investigations on areas involved in primary nociceptive transmission (i.e., dorsal root ganglia and dorsal horn of the spinal cord) and in some of the brainstem regions implicated in nociceptive processing, such as the periaqueductal gray, the nucleus raphe magnus, the gigantocellular reticular nucleus, and the nucleus of the solitary tract.

MATERIALS AND METHODS
ORL-1 and MOR receptor antibodies

We have generated specific antibodies in two different species against the MOR and ORL-1 receptors. Mab ORL-1 N-144 is a murine IgG1, which recognizes a peptide sequence at the N-terminus of the murine ORL-1 receptor (MESLFPAPFWEVLYGSHF, also referred to as M-18-F). This antibody has been extensively characterized and used for a complete mapping of ORL-1 receptors in adult Sprague-Dawley rats (Anton et al., 1996). MOR-C12 is a polyclonal rabbit antiserum directed to the C-terminus of the rat MOR protein (LENLEAETAPLP, also referred to as L-12-P; Sternini et al., 1996; Keith et al., 1998; Unterwald et al., 1998). The MOR-C12 antiserum was antigen affinity-purified as previously reported (Sternini et al., 1996).

Animals and tissue preparation

The present study was carried out on five male Sprague-Dawley rats (220–250 g; Harlan, Madison, WI). All procedures for the use of rodents were approved by the Institutional Animal Care Committees at UCLA, and conform to National Institutes of Health guidelines.

Rats were anesthetized with sodium pentobarbital (60 mg/kg body weight) and transcardially perfused at 4°C as previously described (Anton et al., 1996). Briefly, animals initially received 100 ml of 5 mM phosphate-buffered 0.9% saline (PBS; pH 7.4) containing 20 µg/ml of heparin, then 900 ml of 4% phosphate-buffered paraformaldehyde (pH 7.0). Brains, spinal cords, and dorsal root ganglia were removed and immersed in 4% phosphate-buffered paraformaldehyde (pH 7.0) for up to 4 hours at 4°C. Tissue was cryoprotected in a solution of PBS (pH 7.4) containing 30% sucrose at 4°C for 48 hours and then embedded in Tissue Tek OCT compound (Baxter, McGaw Park, IL) and stored at −70°C until use. Coronal tissue sections were generated on a Reichter Jung 2300 cryostat. Brain and spinal cord sections (40-µm-thick) were stored in PBS, 0.1% thimerosal at 4°C for up to 1 month without any significant loss in receptor immunoreactivity. Dorsal root ganglia sections (10-µm-thick) were directly slide-mounted on Superfrost slides (Fisher Scientific, Pittsburgh, PA) and stored at −70°C.

Horseradish peroxidase immunohistochemistry

Adjacent coronal sections (40-μm-thick) of rat brainstem and spinal cord (cervical, thoracic, lumbar, and sacral segments) were processed free-floating in 24-well plates (10 sections/well) as previously described (Anton et al., 1996).

Endogenous peroxidase activity was blocked by incubating the tissue in a solution of 0.3% hydrogen peroxide in methanol for 20 minutes. MOR-C12 and Mab ORL-1 N-144 were both used at a 1:100 dilution. Secondary antibodies, biotinylated affinity-purified goat anti-rabbit IgG (H+L; Vector Laboratories, Burlingame, CA) and biotinylated affinity-purified horse anti-mouse IgG (H+L; Vector), were diluted 1:400 and 1:200, respectively. Sections were incubated with Vectastain Elite ABC (Vector) for 45 minutes and the peroxidase reaction was performed with 0.004% 3,3′ diaminobenzidine tetrahydrochloride (DAB; Sigma, St. Louis, MO), 0.003% H_2O_2, 0.3% $NiSO_4$ in PBS. After washing in distilled water, sections were air dried and permanently mounted in Permount.

Adjacent slide-mounted sections of dorsal root ganglia (10-μm-thick) were immunostained for MOR and ORL-1 receptors using the same protocol. All incubations were performed in Probe-Clip PC200 chamber coverslips (Grace Bio-Labs, Sunriver, OR). MOR-C12 and Mab ORL-1 N-144 were both used at a 1:30 dilution.

Double-immunofluorescence staining of spinal cord and brainstem sections

Coronal tissue sections were processed free-floating in 24-well plates (10 sections/well) and stained simultaneously for ORL-1 and MOR receptors. Sections were washed in PBS (pH 7.4) and preblocked by incubating at room temperature for 2 hours in blocking solution (5% whole goat serum, 5% whole horse serum, 1% bovine serum albumin, 0.3% Tween 20, PBS, pH 7.4). Sections were then incubated for 16–24 hours at 4°C with the primary antibodies (Mab ORL-1 N-144 and rabbit MOR-C12 antigen affinity-purified fraction) both diluted 1:20 in the blocking solution. After extensive washing in PBS, sections were incubated for 3 hours at room temperature in the dark with Texas Red-conjugated goat anti-rabbit IgG (Molecular Probes, Eugene, OR) and fluorescein isothiocyanate (FITC)-conjugated donkey anti-mouse IgG (H+L; Jackson ImmunoResearch, West Grove, PA) diluted 1:300 and 1:500, respectively, in 5% whole goat serum, 5% whole horse serum, 0.1% Tween 20, PBS (pH 7.4). After extensive washes in 0.1% Tween 20/ PBS (pH 7.4) and a final wash in PBS, sections were mounted in Prolong Antifade (Molecular Probes).

For immunospecificity controls, primary antibodies were preincubated with 100 μM of the synthetic peptides used as immunogens to generate the antisera, and then stained as above. Specificity of secondary antibodies was tested by incubating MOR-C12 with the FITC-conjugated donkey anti-mouse IgG (H+L; Jackson ImmunoResearch) and Mab ORL-1 N-144 with the Texas Red-conjugated goat anti-rabbit IgG (Molecular Probes).

Immunostaining analysis

Anatomical areas of the rat CNS were identified and named according to the nomenclature of Paxinos and Watson (1986).

Micrographs of horseradish peroxidase/DAB-immunostained sections were taken on a Wild MZ10 Leica stereomicroscope at 1–2× magnification, or on an Olympus BX60 microscope at 2–20× magnification.

Simultaneous dual wavelength laser confocal microscopy was employed to assess the cellular localization of both markers (LSM 410; Zeiss, Göttingen, Germany). For each of the areas of brainstem and spinal cord examined, three to five sections from five animals were analyzed. Single plane confocal images (approximately 0.8 μm thick) were acquired simultaneously with a krypton/argon laser using Zeiss 40×/1.25 NA or 100×/1.3 NA oil immersion objectives and magnification zoom of 1–3.5×, and then pseudocolored: Texas Red was assigned red pixels and FITC green pixels. Yellow pixels represent areas displaying both red and green pixels.

All micrographs and images were processed and labeled using Adobe Photoshop 4.0.1 (Adobe, Mountain View, CA). Comparable images in Figure 2 (normal staining and absorption control) were altered identically, with the same adjustments to brightness and contrast.

RESULTS
MOR-C12 and Mab ORL-1 N-144 immunolocalization in the rat CNS

Immunostained sections using Mab ORL-1 N-144 and MOR-C12 are shown on adjacent coronal sections through representative areas of the rat CNS (Fig. 1). The neuroanatomical distribution of Mab ORL-1 N-144 immunostaining is similar to that previously described (Anton et al., 1996). The distribution of MOR-C12 immunoreactivity correlates well with the distribution of mu opioid receptors in the rat CNS reported by other groups (Arvidsson et al., 1995; Mansour et al., 1995a; Ding et al., 1996). For example, dense MOR-C12 immunoreactivity was observed in striatal patches (Fig. 1A), central gray (Fig. 1C), dorsal raphe (Fig. 1C), locus coeruleus (Fig. 1D), nucleus of the solitary tract (Fig. 1F), ambiguus nucleus (Fig. 1F), and laminae I and II of the spinal cord (Fig. 1H–K). Moderate to weak MOR-C12 staining was found in cortex (Fig. 1A), thalamus (Fig. 1B), hypothalamus (Fig. 1B), hippocampus (mostly in stratum pyramidale, Fig. 1B), reticular formation (Fig. 1F,G), and deeper laminae of the spinal cord. No MOR-C12 immunoreactivity was found in the cerebellar cortex (Fig. 1E) or in the corpus callosum (Fig. 1A). In general, the anatomical distribution of both MOR-C12 and Mab ORL-1 N-144 was very similar in many regions of the brainstem and the spinal cord (Fig. 1C–K).

Specificity of MOR-C12 and Mab ORL-1 N-144

We evaluated the specificity of MOR-C12 and Mab ORL-1 N-144 staining in the superficial layers of the dorsal horn (Fig. 2A,B), a region of very intense immunoreactivity for both antibodies. No receptor immunoreactivity was found when primary antibodies (MOR-C12 and Mab ORL-1 N-144) were preabsorbed with the synthetic peptide antigens (L-12-P and M-18-F, respectively; Fig. 2C,D). Additionally, no staining was observed when FITC-conjugated donkey anti-mouse IgG was used in conjunction with MOR-C12 , or when Texas Red-conjugated goat anti-rabbit IgG was used to detect Mab ORL-1 N-144, demonstrating the specificity of the secondary antibodies
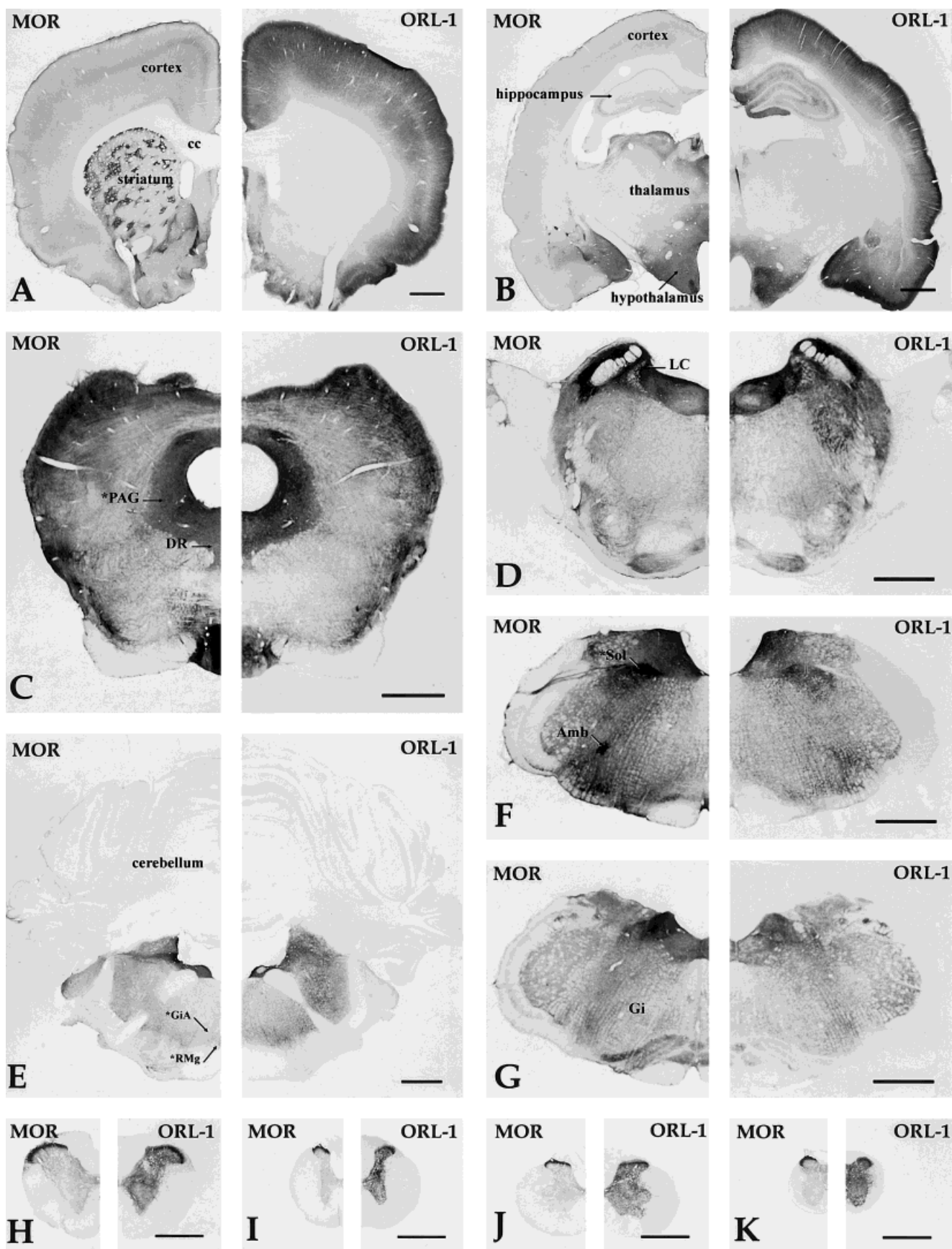
Fig. 1. Distribution of mu opioid receptors (MOR) and opioid receptor-like-1 (ORL-1) horseradish peroxidase/DAB immunostaining in various regions of the rat central nervous system (CNS). Brightfield images compare MOR and ORL-1 immunolocalization on adjacent coronal sections (40 μm thick) through diencephalon (**A,B**), mesencephalon (**C,D**), and brainstem (**E–G**), as well as through cervical 1 (**H**), thoracic 11 (**I**), lumbar 4 (**J**), and sacral 2 (**K**) segments of the spinal cord. Amb, ambiguus nucleus; cc, corpus callosum; PAG, periaqueductal gray; DR, dorsal raphe nucleus; Gi, gigantocellular reticular nucleus; GiA, gigantocellular reticular nucleus, pars alpha; LC, locus coeruleus; RMg, raphe magnus nucleus; Sol, nucleus of the solitary tract. Asterisk indicates areas analyzed with confocal microscopy. Scale bars = 1 mm.
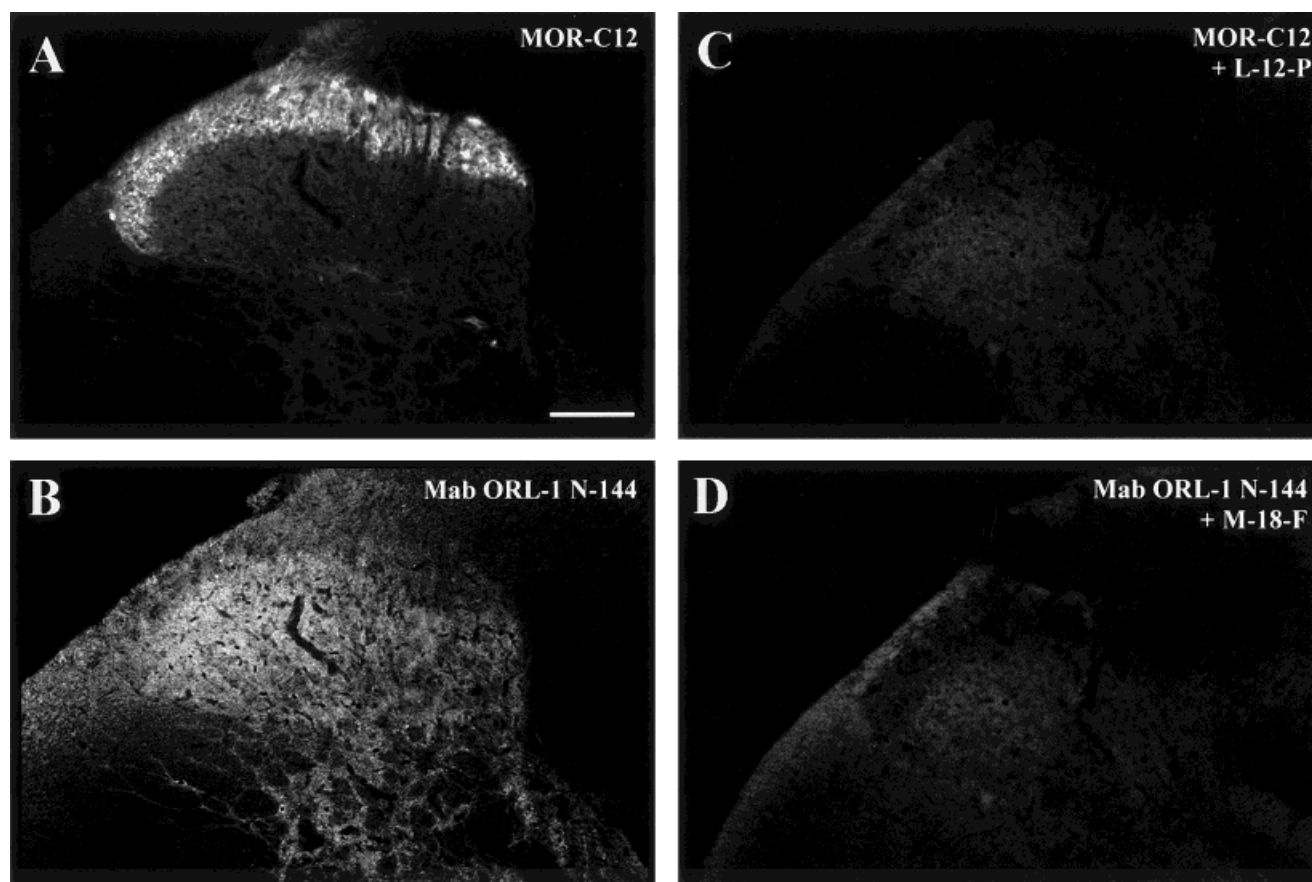
Fig. 2. Conventional microscopic images of MOR (**A**) and ORL-1 (**B**) immunofluorescence in the rat dorsal horn. No significant staining was observed when MOR-C12 and Mab ORL-1 N-144 were preabsorbed with their respective synthetic peptide antigens, L-12-P (**C**) or M-18-F (**D**), prior to the immunostaining. Scale bar = 100 μm.

(not shown). Furthermore, we tested for possible interference between the two labels in adjacent spinal cord sections. The intensity of sections labeled with either MOR-C12 or Mab ORL-1 N-144 alone was indistinguishable from sections that were double labeled with both antibodies (not shown).

Localization of MOR and ORL-1 receptors in dorsal root ganglia and spinal cord

In the dorsal root ganglia, moderate to intense MOR-C12 immunoreactivity was observed in many medium-to large-sized perikarya, as well as in processes exiting the ganglia and projecting to either the dorsal horn or peripheral tissues (Fig. 3A–C). In contrast, no Mab ORL-1 N-144 staining was found in any processes and only weak staining was detected in relatively few medium-sized somata within this area (Fig. 3D–E). Double immunofluorescence staining was not used to investigate co-localization of MOR and ORL-1 receptors in the dorsal root ganglia because of the very low level of ORL-1 immunoreactivity. The data suggest, however, that the dense immunostaining of MOR and ORL-1 in the dorsal horn originates from different fiber systems. We examined this in detail.

In the spinal cord, MOR and ORL-1 receptors were differentially localized throughout the laminae of the dorsal horn. This differential staining pattern was observed in the cervical, thoracic, lumbar, and sacral segments of the spinal cord (Fig. 1H–K). In the Lissauer's tract (Fig. 4A), MOR-C12-immunoreactive fibers did not co-localize with the few scattered processes stained with Mab ORL-1 N-144. In lamina I (Fig. 4B), there was very intense MOR receptor staining of the neuropil and occasional somata at the border of lamina II, whereas ORL-1 staining was weak and mainly detected in puncta and short fibers. No apparent co-localization of MOR and ORL-1 receptor staining was found in lamina I. In lamina II (Fig. 4C,D), high levels of immunoreactivity for both receptors were detected in fibers and puncta, although confocal analysis of multiple sections failed to reveal apparent co-localization of receptor labeling. In lamina III (Fig. 4E), MOR immunostaining was weak and predominantly observed in fine puncta, whereas ORL-1 labeling was intense and detected in many fiber processes. Again, no co-localization was observed within this lamina.

Localization of MOR and ORL-1 receptors in supraspinal areas involved in nociception

We also examined the detailed pattern of MOR and ORL-1 receptor expression in brainstem areas that have been implicated in pain processing: periaqueductal gray, nucleus raphe magnus, gigantocellular reticular nucleus, and nucleus of the solitary tract. Both MOR-C12 and Mab
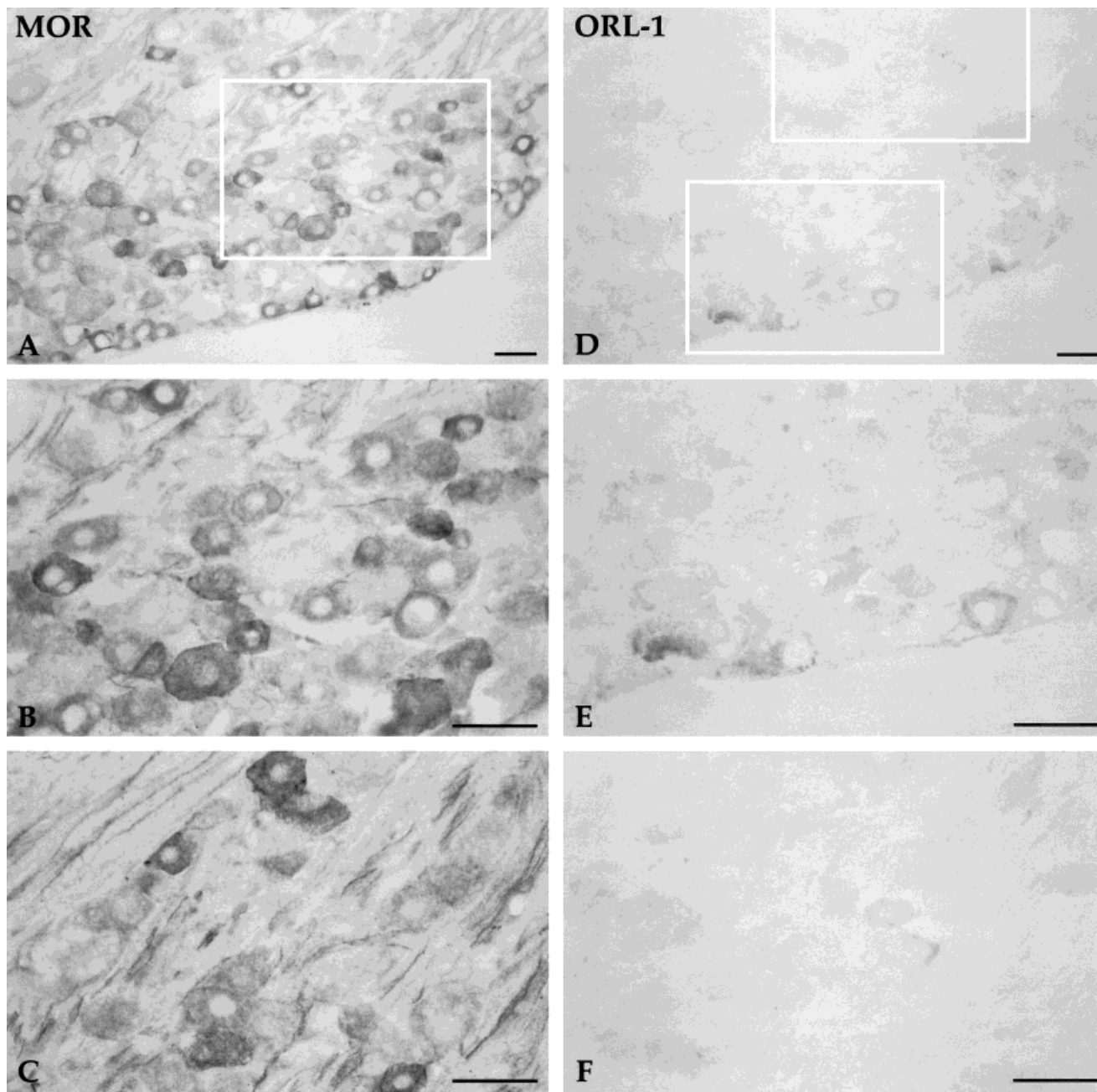
Fig. 3.   Brightfield images of MOR and ORL-1 horseradish peroxidase/DAB immunostaining in adjacent sections (10 µm) through the lumbar dorsal root ganglia. MOR staining was observed in many small- and medium-sized cell bodies (**A–C**) and many processes (C), whereas ORL-1 immunoreactivity was detected in scattered soma (**D–F**). Boxed areas in A and D are magnified in B, and E,F, respectively. Scale bars = 50 µm.

ORL-1 N-144 immunoreactivities were detected in all these supraspinal areas, and their staining patterns were superficially similar (Fig. 1C–G). However, confocal analysis at higher magnification failed to reveal co-localization of MOR and ORL-1 receptors in these brainstem nuclei.

In the ventrolateral periaqueductal gray (Fig. 5A,B), moderate MOR-C12 immunoreactivity was observed in sparsely scattered small soma and processes, as well as in fine puncta. In contrast, Mab ORL-1 N-144 staining was detected in a dense and evenly distributed network of thin fiber processes and fine puncta that were distinct from those labeled with MOR-C12.

In the nucleus raphe magnus (Fig. 5C,D) and in the gigantocellular reticular nucleus (not shown), MOR staining was weak and observed in only a few scattered processes, whereas intense ORL-1 immunoreactivity was detected in a loose network of long fiber processes. In addition to specific staining, large cell bodies and their proximal processes in this area exhibited a high level of red-orange autofluorescence. These intensely fluorescent
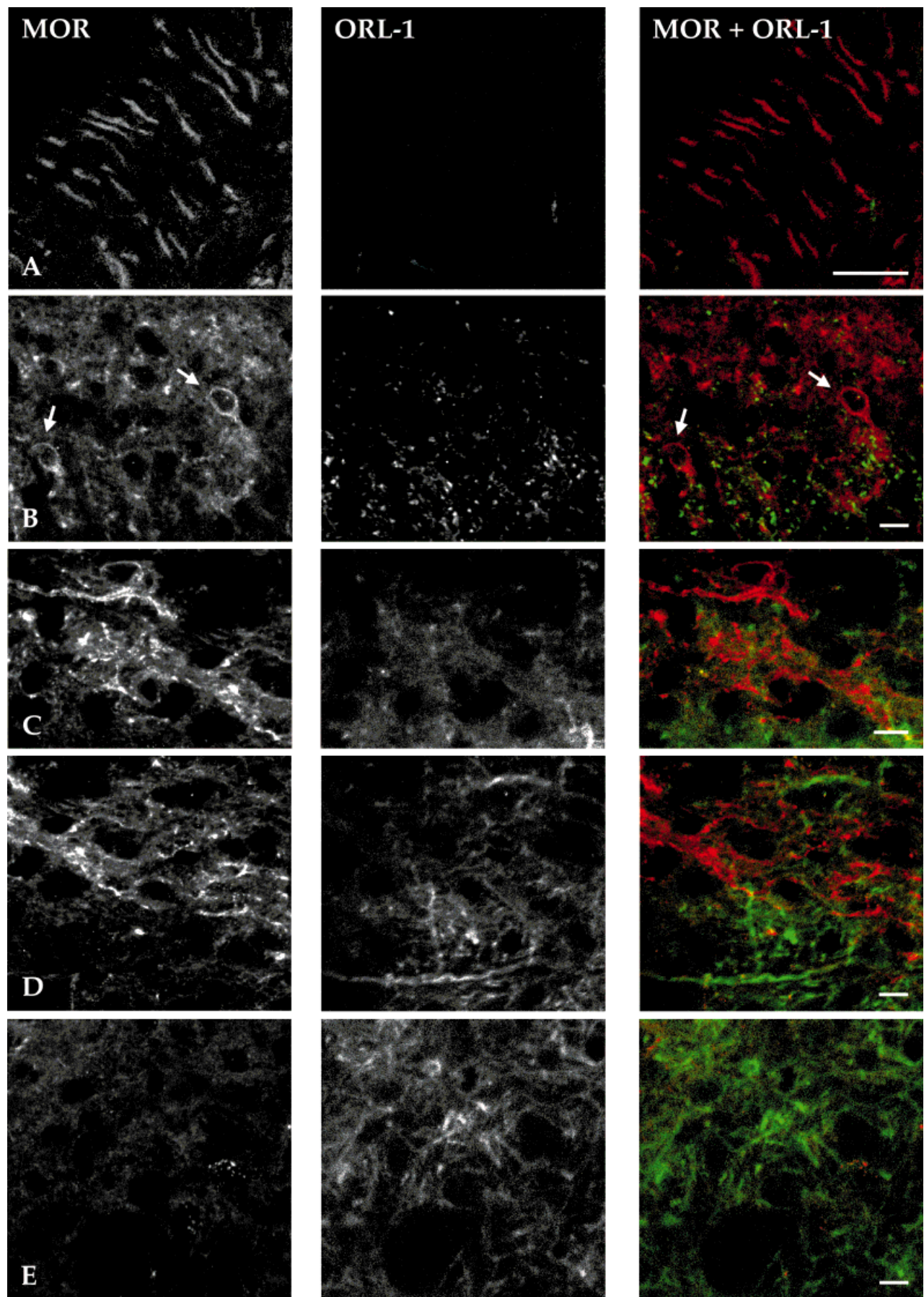
Fig. 4. Confocal microscopic images of MOR and ORL-1 immunofluorescence in the cervical dorsal horn. After superimposition and pseudocoloration of the confocal images, no co-localization (yellow) of MOR (red) and ORL-1 immunostaining (green) was observed in the Lissauer's tract (**A**), lamina I (**B**), lamina II (**C,D**) and lamina III (**E**) of the spinal cord. Images are single plane confocal scans (0.8 μm thick). Arrows show probable cell bodies in lamina I. Scale bars = 10 μm.
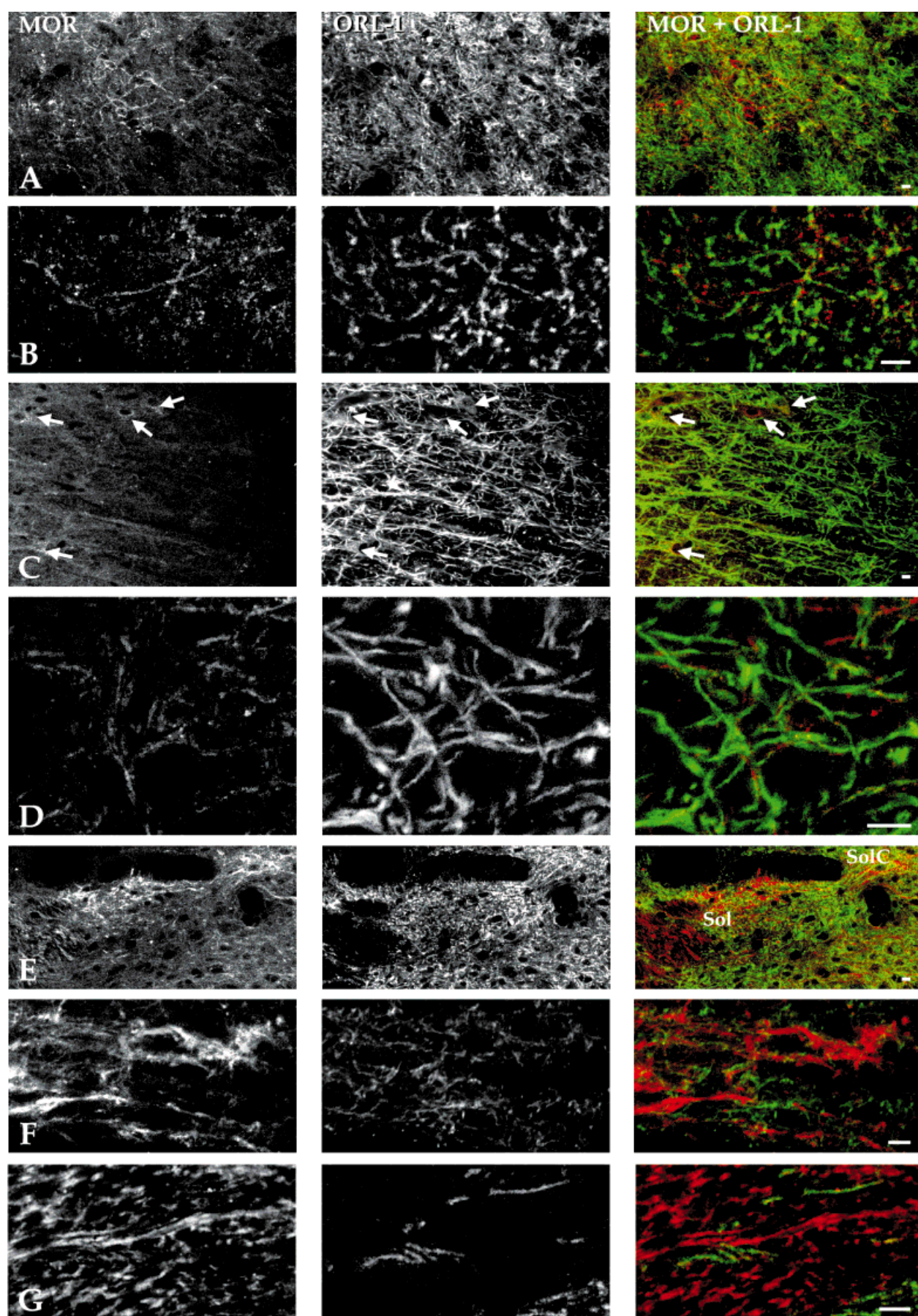
Figure 5

vesicle-like structures (presumably lipofuscin vesicles) were resistant to any attempts at quenching (e.g., various fixatives, the use of 1-month-old rats, 1% sodium borohydride treatment). Therefore, we were unable to use confocal analysis to determine whether MOR and ORL-1 receptors were co-localized in cell bodies of these two regions. In the many stained processes, autofluorescence was not a problem, and confocal analysis did not reveal any apparent MOR and ORL-1 receptor co-localization on the immunoreactive processes within the raphe magnus and the gigantocellular reticular nuclei.

In the nucleus of the solitary tract (Fig. 5E–G), MOR-C12 immunoreactivity was observed in many thick fiber processes in the medial, lateral, and commissural regions. In contrast, Mab ORL-1 N-144 staining was predominantly observed in fine puncta in the medial aspect of the nucleus and in sparse fiber tracts in its lateral and commissural aspect. Many of the Mab ORL-1 N-144-labeled processes showed a different spatial orientation compared with those labeled by MOR-C12. No apparent co-localization of MOR and ORL-1 receptor immunoreactivity was observed in the nucleus of the solitary tract.

DISCUSSION

This study has focused on the cellular distribution of both MOR and ORL-1 receptors in the brainstem, spinal cord, and dorsal root ganglia of the rat. The principal finding is that, although both MOR and ORL-1 receptors have been reported in many of the same regions associated with nociception (Arvidsson et al., 1995; Mansour et al., 1995a; Anton et al., 1996; Ding et al., 1996), antisera to these closely related receptors appear to label different fiber processes. This would suggest that either the receptors are expressed in different cells within these regions or, that the receptors are trafficked to different compartments of the same cell. Since MOR and ORL-1 receptors appear to trigger the same second messenger systems, the differential actions of MOR and ORL-1 agonists on pain perception at both the supraspinal and spinal level would be consistent with a differential cellular distribution of these receptors.

Concordant with the action of mu opiate drugs on primary nociceptive afferents, intense MOR staining was observed in the cells and processes within the dorsal root ganglia, the Lissauer's tract, and laminae I and II of the dorsal horn. This finding parallels other MOR receptor immunolocalization studies in the spinal cord (Arvidsson et al., 1995; Mansour et al., 1995a; Anton et al., 1996; Ding et al., 1996) and is consistent with the high proportion of dorsal root ganglia cells expressing MOR receptor mRNA (Mansour et al., 1994). The dorsal root ganglia is also reported to express mRNA for ORL-1 receptors (Wick et al., 1994); however, our ORL-1 immunostaining revealed only scattered and weakly labeled cell bodies and no staining of processes in this area. The mismatch between ORL-1 protein and ORL-1 mRNA in the dorsal root ganglia could result from ORL-1 receptor protein being trafficked to sensory neuronal terminals in the dorsal horn and/or peripheral sites. Within the dorsal horn, intense ORL-1 immunostaining was indeed observed in laminae II and III, which receive projections from cells of the dorsal root ganglia. However, there are a number of possible sites for the synthesis for ORL-1 receptors in the spinal cord other than the dorsal root ganglia. In situ hybridization (Bunzow et al., 1994; Wick et al., 1994; Lachowicz et al., 1995) reveals ORL-1 mRNA in the dorsal horn itself as well as in brainstem nuclei projecting to the dorsal horn (i.e., the nucleus raphe magnus). Additional studies, such as specific lesions, will be required to investigate the intrinsic or extrinsic origin of ORL-1-immunoreactive spinal processes.

Although both MOR and ORL-1 receptor mRNAs are reported to be expressed in the dorsal root ganglia, in the spinal cord, and in brainstem nuclei projecting to the spinal cord, there was a striking lack of co-localization between MOR and ORL-1 receptor staining on fibers in laminae I–III of the spinal cord or in the Lissauer's tract. Despite this differential localization, activation of spinal ORL-1 receptors, like activation of spinal MOR receptors, results in analgesia (Xu et al., 1996). Furthermore OFQ/N potentiates morphine-induced analgesia (Tian et al., 1997a). The OFQ/N-induced analgesia is not blocked by opiate antagonists and shows no cross-tolerance with morphine (Hao et al., 1997), suggesting that OFQ/N is not acting via stimulation of the endogenous opioid system. The spinal analgesic actions of MOR and ORL-1 receptor agonists can be clearly differentiated by their interactions with other neurotransmitter systems that modulate primary antinociception. Both alpha$_2$-adrenergic (Ramana Reddy et al., 1980) and γ-aminobutyric acid A (GABA$_A$) receptor agonists (Ramana Reddy et al., 1980; Moreau and Pieri, 1988) potentiate morphine-induced antinociception. In contrast, OFQ/N-induced analgesia was not affected by antagonists of either α_2-adrenergic or GABA$_A$ receptors (Xu et al., 1996). This functional disparity and the lack of co-localization of MOR and ORL-1 staining that we observed in the spinal cord suggests that ORL-1 receptors mediate spinal analgesia through a neuronal mechanism distinct from that of MOR receptors.

Several supraspinal areas of the CNS are also implicated in pain processing. Among them, the periaqueductal gray, the nucleus raphe magnus, and the gigantocellular reticular nucleus have been reported to participate in the modulation of nociception. These regions receive direct or indirect nociceptive inputs from the dorsal horn and send inhibitory projections that modulate the transmission of pain sensation in the spinal cord (Basbaum and Fields, 1984). The involvement of the nucleus of the solitary tract in the control of pain transmission is less well documented. This region, rich in mu opioid receptors and enkephalins, receives many projections from the nucleus raphe magnus and sends inputs to the spinal cord (Du and Zhou, 1990). Although both MOR and ORL-1 receptors are expressed in the periaqueductal gray, the nucleus raphe magnus, the gigantocellular reticular nucleus, and the nucleus of the solitary tract, confocal microscopic analysis demonstrated

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Fig. 5. Confocal microscopic images of MOR and ORL-1 immunofluorescent labeling in supraspinal areas involved in nociception. After computerized superimposition and pseudocoloration of black and white pairs of confocal images, MOR (red) and ORL-1 (green) were found in distinct processes within the periaqueductal gray (**A,B**), raphe magnus nucleus (**C,D**) and nucleus of the solitary tract (**E**–**G**). A,C, and E are three-dimensional projections from ten optical sections (0.8 µm thick) acquired at 1 µm intervals; B,D,F, and G are single plane confocal images (0.8 µm thick). Arrows in C point to large lipofuscin-containing cell bodies responsible for artifactual co-localization (yellow). Sol, nucleus of the solitary tract; SolC, nucleus of the solitary tract, commissural part. Scale bars = 10 µm.

a lack of co-localization in any fiber processes within these nuclei. The results obtained in the brainstem thus parallel those obtained at the spinal level.

In contrast to intrathecal administration, the intracerebroventricular (i.c.v.) injection of OFQ/N does not produce antinociception. The original studies in mice (Meunier et al., 1995; Reinscheid et al., 1995; Rossi et al., 1996) suggested that OFQ/N was hyperalgesic. However, subsequent studies indicate that hyperalgesia induced by i.c.v. administration of OFQ/N may, in certain assay protocols, reflect a reversal of stress-induced analgesia (Mogil et al., 1996a). Additionally, there is now increasing evidence of a functional anti-opioid activity of OFQ/N following i.c.v. administration in rats (Tian et al., 1997a) and mice (Grisel et al., 1996; Mogil et al., 1996b). These studies point to differential, yet interactive roles of MOR and ORL-1 receptors in the supraspinal circuitry modulating nociception.

A role of the ventral periaqueductal gray in OFQ/N anti-opioid effects has recently been proposed, based on the inhibition of morphine- and kainic acid-induced antinociception by microinjection of OFQ/N in this area (Morgan et al., 1997). This indicates that OFQ/N may exert its anti-opioid activity by inhibiting periaqueductal gray output neurons that are activated by opioids. Such opposite modulatory effects of OFQ/N and morphine is consistent with the differential localization of their receptors in the periaqueductal gray. It is likely that such opposite modulatory roles may occur in other supraspinal areas involved in pain processing, because there is also no co-localization of MOR and ORL-1 receptors in the nucleus raphe magnus, the gigantocellular reticular nucleus, and the nucleus of the solitary tract.

Very little is currently known about the ORL-1 receptor/OFQ/N system regarding the neuronal circuitries it regulates and its interactions with other neurotransmitters in the CNS. There is however, substantial pharmacological evidence that both ORL-1 and MOR receptors are important modulators of pain perception. This immunostaining study suggests a lack of cellular co-localization of ORL-1 and MOR receptors on processes in areas of the nervous system involved with pain processing. The antisera to MOR and ORL-1 receptors may provide useful markers to differentiate neuronal circuitry involved in either the facilitation or the inhibition of pain perception. Although it remains to be determined whether MOR-1B, the minor isoform of the MOR receptor, also shows no co-localization with ORL-1 receptors, it appears likely that mu agonists can modulate fiber processes distinct from those affected by ORL-1 receptor agonists in many areas of the brainstem and spinal cord.

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