RESEARCH ARTICLE

Localization of Endogenous Morphine-Like Compounds in the Mouse Spinal Cord

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ABSTRACT

Morphine, codeine, morphine-6-glucuronide, and morphine-3-glucuronide are synthesized de novo in mammalian cells and in the central nervous system. Knowledge on endogenous morphine-like compound distribution in the adult mouse brain has been recently improved, and new hypotheses have been suggested about the potential implications in brain physiology. Endogenous morphine-like compounds have been shown to be synthesized in the spinal cord, but their localization is unknown. Here we describe the distribution of endogenous morphine-like compounds (morphine and/or its glucuronides and/or codeine) in the adult mouse spinal cord using a well-validated antibody. By using different microscopy approaches, we found the presence of morphine, codeine, or morphine glucuronides in γ-aminobutyric acid (GABA)-ergic neurons and astrocytes of the spinal cord. Whereas GABAergic neurons containing endogenous morphine-like compounds were located primarily in the ventral horn, astrocytes that were labeled for morphine-like compounds were found throughout the gray matter and the white matter. Our study demonstrates the possibility that endogenous morphine-like compounds in the central nervous system have other functions beyond their analgesic functions. J. Comp. Neurol. 520:1547–1561, 2012.

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INDEXING TERMS: morphine; morphine-6-glucuronide; morphine-3-glucuronide; codeine; astrocytes; GABA; spinal cord; mouse

Endogenous alkaloids, which are structurally identical to vegetal alkaloids, are present in various mammalian tissues and cells (Donnerer et al., 1986, 1987; Gintzler et al., 1978; Glattard et al., 2010; Goldstein et al., 1985; Goumon et al., 2009; Hazum et al., 1981; Herbert et al., 2000; Laux et al., 2011; Muller et al., 2008; Neri et al., 2004; Stefano et al., 2000; Weitz et al., 1986; Zhu et al., 2001). Endogenous morphine biosynthesis pathways have been demonstrated in the SH-SY5Y human cell line and shown to be derived from dopamine (Boettcher et al., 2005; Muller et al., 2008; Neri et al., 2008; Poeaknapo, 2005; Poeaknapo et al., 2004). However, several reports have noted that endogenous alkaloids can be synthesized by nondopaminergic or noncatecholaminergic cells (i.e., pancreatic cells, hepatocytes, and placenta-derived cells; Molina et al., 1995; Poeaknapo et al., 2004; Weitz et al., 1987), suggesting that nondopaminergic cells may internalize dopamine or intermediate metabolites to finalize morphine biosynthesis (Charron et al., 2011; Goumon et al., 2009; Laux et al., 2011).

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Our recent studies have focused on the functional role of endogenous alkaloids in neuronal cells. Using the SH-SY5Y model, production of dopamine, morphine, and morphine-6-glucuronide (M6G) was found to be restricted to large dense-core vesicle-like organelles and to be released via a Ca^{2+}-dependent mechanism (Muller et al., 2008). However, with the mouse brain, we recently found that morphine-like compounds were located in nondopaminergic cells (mainly in γ-aminobutyric acid [GABA]-ergic neurons and astrocytes), and we characterized the

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TABLE 1.
Primary Antibodies Used[1]

Antigen	Immunogen	Raised in	Manufacturer data	IHC dilution
Morphine	BSA-morphine conjugate	Mouse monoclonal	Aviva System Biology (AVAMM16002-4)	1:1,000
ChAT	Human placental enzyme	Goat polyclonal	Chemicon-Millipore (AB144P)	1:250
GFAP	GFAP isolated from cow spinal cord	Rabbit polyclonal	DAKO (Z0334)	1:1,000
TH	Denatured TH from rat pheochromocytoma	Rabbit polyclonal	Chemicon-Millipore (AB152)	1;1,000
GABA	BSA-GABA conjugate done with glutaraldehyde	Rabbit polyclonal	Sigma-Aldrich (A2052)	1:500
MAP2	Purified MAP2 from rat brain	Rabbit polyclonal	Chemicon-Millipore (AB5622)	1:500

[1]BSA, bovine serum albumin; ChAT, choline acetyltransferase; GABA, γ-aminobutyric acid; GFAP, glial fibrillary acidic protein; IHC, immunohistochemistry; MAP2, microtubule-associated protein type 2; TH, tyrosine hydroxylase.

presence of morphine, codeine, M6G, and morphine-3-glucuronide (M3G) in the adult mouse brain (Laux et al., 2011). The presence of M3G and M6G in the brain is explained by the presence of enzymes of the UDP-glucuronosyltransferase 1A and 2B families (UGT1A, UGT2B). These enzymes convert morphine to M3G and M6G and are expressed in neurons and/or astrocytes (Heurtaux et al., 2006; King et al., 1999; Nagano et al., 2000; Suleman et al., 1998).

Interestingly, our recent immunomapping of morphine-like immunoreactivity throughout the entire brain showed the presence of endogenous alkaloids in various structures, including the hippocampus, olfactory bulb, band of Broca, basal ganglia, and cerebellum. Morphine-like immunoreactivity was mainly present in GABAergic neuronal and astrocytic cell bodies and processes. In neurons, endogenous alkaloids are present in presynaptic terminals in the cerebellum and postsynaptic terminals in other regions of the mouse brain (Laux et al., 2011). This further suggests that endogenous morphine and its derivatives may act as neuromodulators of brain functions.

Endogenous morphine has been detected in the spinal cord of many species, including the dog (Meijerink et al., 1999), the rat (Donnerer et al., 1987; Meijerink et al., 1999), and the mouse (Charlet et al., 2010). However, its precise localization and cellular distribution have not been established in the spinal cord, so no indications of potential roles in spinal cord physiology have been described.

The goal of the present study was to determine the structural and cellular distribution of morphine/M3G/M6G/codeine immunoreactivity in the adult mouse spinal cord. A detailed immunohistochemical description of endogenous morphine-like compounds (i.e., morphine, M6G, M3G, and codeine) in the adult mouse spinal cord was performed using conventional, confocal, and electron microscopy to localize and characterize the immunoreactive cells and to determine the subcellular localization of endogenous morphine-like compounds.

MATERIALS AND METHODS

Animals

Experiments were performed on six 45-day-old, laboratory-bred, adult male C57BL/6 mice weighting 30 ± 3 g. Animals were given free access to food and water, with a 12-hour light–dark cycle at a temperature of 22°C ± 2°C. All procedures were performed in accordance with European directives (86/609/EEC) and were approved by the regional ethics committee and the French Ministry of Agriculture (license No. 67-116, to P.P.).

Antibody characterization

Antibodies used in the present study are commercially available and have been used in previous studies. Table 1 provides informations about these antibodies, including immunogens, species, commercial sources, and dilutions used in the present study.

GABA antibody

Affinity-purified anti-GABA antiserum (Sigma-Aldrich, St. Louis, MO; ref: A2052) was raised against GABA conjugated to bovine serum albumin (BSA) via a glutaraldehyde treatment and evaluated for specificity with a dot-blot immunoassay (Ito et al., 2007; Ligorio et al., 2000), and it was blocked by preincubation with either GABA or GABA-BSA in mouse spinal cord (Heinke et al., 2004). The antiserum specifically labeled electrophysiologically identified GABAergic hippocampal neurons in cultures prepared from GAD67-GFP mice (Ikeda et al., 2008). As previously described, this antiserum labels GABAergic neurons after paraformaldehyde fixation without addition of glutaraldehyde (Heinke et al., 2004; Ikeda et al., 2008).

Choline acetyltransferase antibody

Choline acetyltransferase (ChAT) was labeled with an affinity-purified polyclonal antiserum raised in goat against ChAT enzyme purified from human placenta (Chemicon-Millipore, Molsheim, France; ref: AB144P). It stains a single band of 68–70 kDa on a Western blot from rat (see manufacturer's data sheet) and human brain extracts (Gill et al., 2007). This antiserum was previously used to label spinal cord motor neurons in humans (Gill et al., 2007) and mice (Matsumoto et al., 2007).

Glial fibrillary acidic protein antibody

The rabbit anti-glial fibrillary acidic protein (GFAP) polyclonal antiserum (Dako, Glostrup, Denmark; ref: Z0334)

was produced using GFAP isolated from cow spinal cord. The staining pattern by immunohistochemistry matched the expected distribution of GFAP in the mouse auditory brainstem (Abraira et al., 2007). With cultured mouse primary astrocytes, the antibody staining demonstrated a filamentous pattern, which was consistent with the GFAP presence in intermediate filaments. On Western blotting, this antibody recognized a band of approximately 55 kDa from mouse brain extracts (Arantes et al., 2009). This antiserum was used in our previous study on mouse brain and displayed a specific labeling of astrocytes cell bodies and processes (i.e., astrocytic foot around blood vessels; Laux et al., 2011).

Tyrosine hydroxylase antibody

The rabbit anti-tyrosine hydroxylase (TH) polyclonal antiserum (Chemicon-Millipore; ref: AB152) was produced using denatured full-length TH isolated from rat pheochromocytoma. Its specificity has been corroborated by Western blot analysis in rat, mouse, ferret, cat, and *Aplysia* (see manufacturer's data sheet), and it selectively labels a single band at approximately 62 kDa. In addition, a selective labeling of dopaminergic neurons in the substantia nigra pars compacta has been observed (Laux et al., 2011; Martin-Ibanez et al., 2006). The results presented in this study reveal a labeling and distribution of dopaminergic innervations in the spinal cord similar to those previously described (Brumovsky et al., 2006).

Microtubule-associated protein 2 antibody

The rabbit anti-microtubule-associated protein 2 (MAP2) antibody (Chemicon-Millipore, ref: AB5622) was generated using purified MAP from rat brain. The antibody recognizes all isoforms of MAP2 (MAP2a, MAP2b, MAP2c, and MAP2d). However, it has the strongest immunoreactivity for MAP2a and MAP2b. In analyzing adult rat brain soluble extract by Western blot, the antibody recognizes a 280-kD double band that corresponds to MAP2a and MAP2b, as well as a 70-kD doublet that corresponds to MAP2c (see manufacturer's data sheet).

Morphine antibody

The mouse anti-morphine antibody (6D6; Aviva System Biology, San Diego, CA; ref: 16002) was produced using a morphine-BSA conjugate. This antibody has been used previously and extensively controlled (Charron et al., 2011; Glattard et al., 2010; Laux et al., 2011; Muller et al., 2008), demonstrating the specificity of the labeling. 1) In ELISA, the 6D6 antibody recognized morphine, codeine, M3G, and M6G, without any cross-reactivity with other morphine precursors or related compound (i.e., dopamine or THP). 2) Control for the cross-linking of morphine, M6G, M3G, and codeine to proteins by paraformaldehyde (PFA) has been performed in vitro and in vivo, showing that endogenous alkaloids are covalently bound to protein after PFA treatment. 3) An increase in immunoreactivity in the striatum was observed after intracerebroventricular injection of morphine, codeine, M6G, and/or M3G, and a gradient in the morphine immunoreactivity (MIR) density of cells, extracellular space, and process labeling was dependent on the distance from the injection site. 4) Finally, immunoprecipitation experiments using the 6D6 antibody performed on mouse brain extracts led to endogenous alkaloid recovery (Laux et al., 2011).

Three specificity controls were performed in the present study: 1) the morphine antibody was omitted to control for nonspecific binding of the secondary antibody in the spinal cord sections, and the omission of the primary antibody resulted in no peroxidase label in the tissue sections; 2) immunoreactivity was absent when the 6D6 antibody was preadsorbed for 1 hour with 3 μM morphine (Sigma-Aldrich), codeine (Sigma-Aldrich), M6G (Sigma-Aldrich), or M3G (Sigma-Aldrich) before immunohistochemistry; and 3) Western blotting of mouse spinal cord extracts with the 6D6 antibody was performed and showed no cross-reactivity of the antibody with other proteins (Laux et al., 2011).

The MIR observed with the 6D6 antibody may correspond to endogenous morphine, codeine, M6G, and/or M3G. The results observed in the present study were consistent with previous studies (Laux et al., 2011) and showed that MIR was located primarily in astrocytes and GABAergic cells.

Immunohistochemistry

Tissue preparation for immunohistochemistry studies

Mice were deeply anesthetized by intraperitoneal injection of 0.1 ml of a 5.6% (w/v) pentobarbital sodium solution (CEVA Santé Animale, Libourne, France) and perfused transcardially with fixative solution using a peristaltic pump. For conventional and electron microscopy, a fixative solution of 4% formaldehyde (EMS, Hatfield, PA) in a 0.1 M NaCl/Pi buffer (disodium hydrogen phosphate dihydrate, 1.28% [w/v], sodium dihydrogen phosphate monohydrate, 0.38% [w/v], pH 7.4) with 0.25% (v/v) glutaraldehyde (VWR, Fontenay sous bois, France) was used. The same fixative solution was used for laser confocal microscopy, except that glutaraldehyde was omitted. Fixative solutions were chilled and then perfused for 10 minutes with a peristaltic pump at a flow rate of 10 ml/minute. The spinal cord was quickly removed and incubated for 2 hours at 4°C in the same fixative solution. Sections of spinal cord (50 μm thick) were cut with a vibratome (Leica VT 1000 S, Nanterre, France) and collected in Tris-buffered saline (TBS; 50 mM Tris-HCl, 0.9% NaCl, pH 7.4).

Slice immunostaining

Immunostaining was performed on slides free-floating in TBS, as previously described (Hedou et al., 2000). Tissue slices were washed in TBS and incubated for 1 hour in BSA (Euromedex, Mundolsheim, France) diluted in TBS (5%, w/v) to saturate nonspecific immunoreactive sites. After saturation, sections were incubated overnight with mixture of primary antibodies raised in different species in TBS containing 0.1% (v/v) Triton X-100 (Sigma-Aldrich). Triton was omitted for electron microscopy. Primary antibodies were used as described in Table 1. After incubation with the primary antibody, tissue slices were washed six times with TBS (5 minutes each), and specific secondary antisera were added for 2 hours at room temperature (RT), followed by six TBS washes (5 minutes each). These secondary antisera were 1) horseradish peroxidase (HRP)-conjugated donkey anti-mouse IgG (P.A.R.I.S., Paris, France; ref: BI 2413C; dilution 1:400), 2) Alexa Fluor 488-conjugated donkey anti-rabbit IgG (Molecular Probes-Invitrogen, Cergy Pontoise, France; ref: A21206; dilution 1:1,000), 3) Alexa Fluor 488-conjugated donkey anti-goat IgG (Jackson Immunoresearch, Suffolk, England; ref: 705-486-147; dilution 1:500), and 4) Cy3-conjugated donkey anti-mouse IgG (Jackson Immunoresearch; ref: 715-165-150; dilution 1:1,000). Several controls were carried out to assess antibody specificity and nonspecific immunoreactivity. Primary antibodies were omitted, and each secondary antibody was tested individually or in a mixture in the presence of tissue sections. Each antibody was also tested with the secondary antibody used for the second immunolabeling to determine whether interspecies cross-reactivity exists.

Light microscopy

Peroxidase activity was revealed after a 10-minute incubation in a freshly prepared solution of 4-chloro-1-naphtol (0.2 mg/ml) in TBS containing 0.006% (w/v) hydrogen peroxide. After washing with TBS, the sections were mounted in glycerol/TBS (1:1, v/v) before analysis with a Leica DMRB microscope equipped with a digital camera (Axiocam; Zeiss, Le Pecq, France).

Electron microscopy

Peroxidase activity was detected with a freshly prepared solution of 0.025% (w/v) 3,3-diaminobenzidine tetrahydrochloride in TBS containing 0.006% (w/v) hydrogen peroxide. After washing with NaCl/Pi buffer, sections and slides were postfixed for 30 minutes with 2.5% glutaraldehyde in 0.1 M NaCl/Pi buffer (pH 7.4), then with 1% (w/v) osmium tetroxide in 0.1 M NaCl/Pi buffer (pH 7.4) at 4°C for 1 hour. Sections were dehydrated in ethanol and embedded in Araldite resin (Hedou et al., 2000). Ultrathin sections were observed with a Hitachi H 7500 electron microscope, without additional staining. Pictures were acquired with a Hamamatsu digital camera (C 4742-95).

Confocal microscopy observations

Immunofluorescent staining was analyzed with a Zeiss laser scanning microscope (LSM510 inverted; Zeiss) equipped with a plan apo ×63 oil immersion and a ×40 oil immersion lens. Tissue sections were subjected to optical serial sectioning to produce images in the X-Y plane. Each optical section was scanned eight times to obtain an average image. Pictures were recorded digitally in a 1,024- × 1,024-pixel format. A lookup table (range indicator; Zeiss) ensured that the full dynamic range of the photomultipliers was used. Before each measurement, a series of sections was acquired through the vertical axis in order to choose the equatorial section.

Anatomical and cellular distribution

The anatomical structures were identified under direct observation using the atlas and nomenclature of the Allen brain atlas (Allen Spinal Cord Atlas, http://mousespinal.brain-map.org/). Cartography was performed on four animals, and the staining was identical for each experiment.

Photomicrographs

Figures including photomicrographs were made with Adobe Photoshop software without changing brightness, contrast, or exposure. For all series of experiment using microscopy, images were captured with the same parameters and settings of brightness, contrast, and exposure.

RESULTS

Endogenous morphine-like immunoreactivity distribution at the different levels of the adult mouse spinal cord

Light microscopy was used to analyze morphine, morphine glucuronides, and codeine immunoreactivity (MIR) present in the cervical, thoracic, and lumbar mouse spinal cord using the 6D6 antibody.

MIR in neurons of the spinal cord

A strong morphine-like immunoreactivity (MIR) was observed throughout the gray matter of the spinal cord, whereas lower levels of MIR was found in the white matter of the spinal cord (Fig. 1). The MIR distribution in the gray matter was similar through the cervical (Fig. 1A), thoracic (Fig. 1B), and lumbar (Fig. 1C) sections of the spinal cord. However, a stronger MIR was observed in the dorsal horn superficial layers (I and II). Representation of the distribution of MIR neuronal cells (Fig. 1A–C, dots on the right side) showed no difference among the different levels of the spinal cords. No MIR neurons were found in

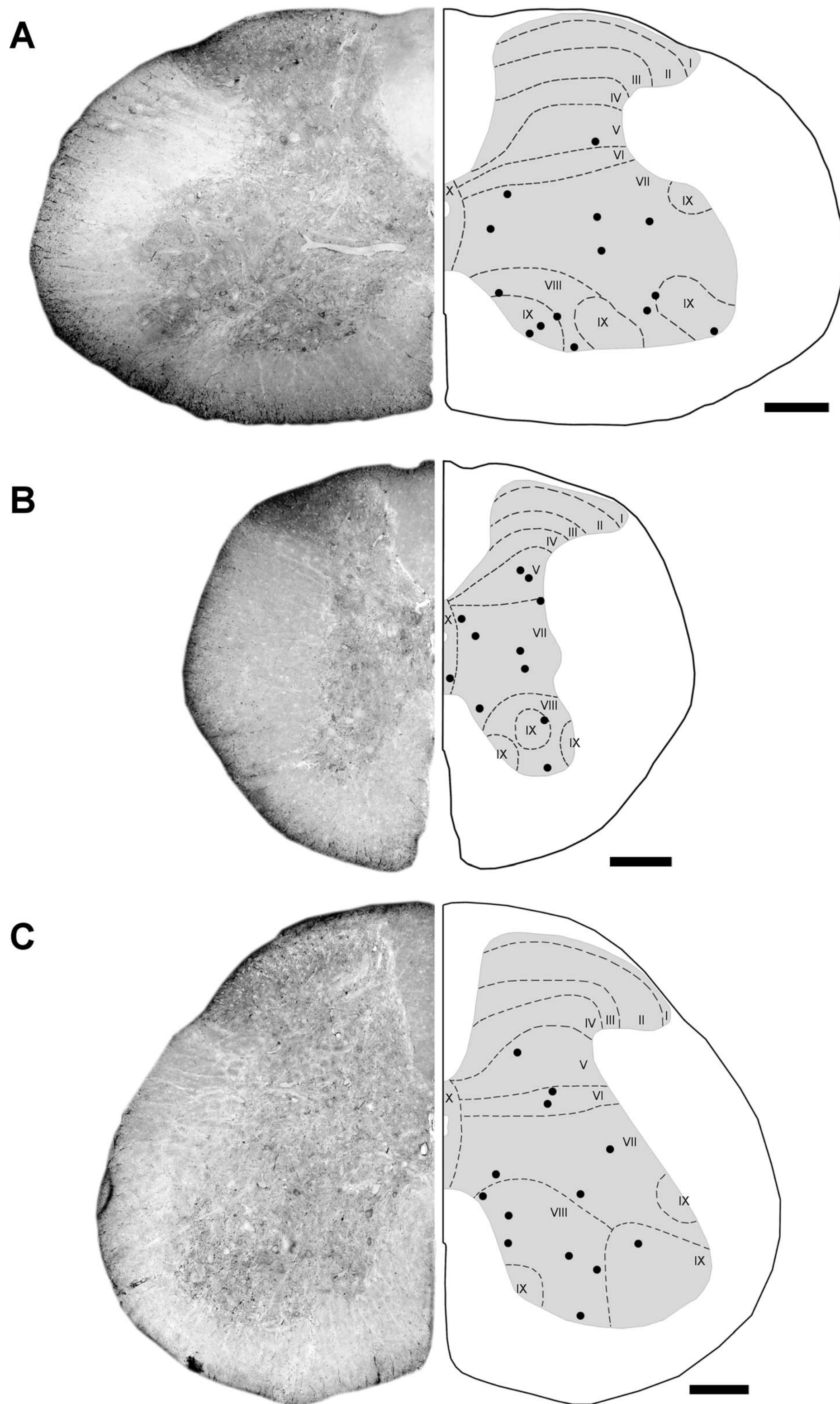

Figure 1. Distribution of MIR in the cervical (**A**), thoracic (**B**), and lumbar (**C**) spinal cord. The left side has representative ×10 magnification microscopic pictures, whereas the right side shows a schematic of the anatomic representation of the section. The black dots represent labeled neuronal cells observed on the picture presented at left. The presented sections are representative of the localization and the number of neuronal cells observed throughout the entire spinal cord (n = 4). Laminae are named in roman numerals. Scale bars = 200 μm.

laminae I–IV of the dorsal horn, but labeled neurons were present in the other gray matter areas. The neuronal MIR-positive cells were localized primarily to laminae V–X. The sections presented are representative of the localization and the number of neuronal cells observed throughout the entire spinal cord (n = 4).

MIR in the dorsal horn

Strong MIR was found in the processes of superficial layers (I and II) of the dorsal horn, and this staining was similar between the different levels of the spinal cord (Fig. 2A–C). No labeled neuronal cells were found in the superficial layers, whereas intense MIR was present in numerous processes and astrocytic cells bodies (Fig. 3A,B, arrows). In the deeper laminae (V and VI), only few labeled neurons were observed (Fig. 3C, arrow).

In the white matter of the dorsal horn, strong MIR astrocytes were found on the edge of the spinal cord (Fig. 3D). The MIR astrocytes in the white matter were distributed homogeneously, so no correlation was detected between the distribution of MIR astrocytes and anatomical or functional fasciculus of the white matter. After preadsorbtion of the 6D6 antibody for 1 hour, with 3 μM of morphine, no immunoreactivity was detected in the dorsal horn gray matter (Fig. 3E) or in the edge of the white matter (Fig. 3F).

MIR in the ventral horn

In the ventral horn, MIR displayed an identical pattern of distribution in the different levels of the spinal cord. However, numerous labeled neuronal cells were observed in the cervical and lumbar ventral horn. In all cervical, thoracic, lumbar, and sacral levels of lamina IX, MIR was observed in small, round cells but not in the motor neurons (Fig. 4A,B, asterisks). The labeled nonmotor neurons were also observed in the lateral motor neuronal pool from the cervical level (Fig. 4C, asterisks). The MIR processes were present around the motor neurons and may correspond to astrocytic or neuronal processes (Fig. 4A–C, white arrows). In lamina VII (Fig. 4D), VIII and X, MIR was found in round cells throughout the spinal cord.

MIR was also found in the whole gray matter of the ventral horn in astrocytic cell bodies (Fig. 4A–D, black arrows) and processes and in the astrocytic foot around blood vessels (Fig. 4E arrow). In the dorsal horn, astrocytes were the only MIR-positive cells in the white matter. Numerous labeled astrocytes were observed at the edge of the spinal cord (Fig. 4F).

Characterization of MIR cells in the adult mouse spinal cord

In the mouse brain, morphine-like compounds were found in GABAergic cells and astrocytes (Charron et al., 2011; Laux et al., 2011). To characterize the cells and processes containing morphine-like compounds in the mouse spinal cord, we used confocal microscopy with different cell markers.

In the superficial laminae of the dorsal horn, with colabeling of morphine-like compounds and MAP2, a dendritic and neuronal cell body marker (Dehmelt and Halpain, 2005; Gritti et al., 2009; Tan et al., 2010), we found no evidence for colocalization of MIR-labeled neurons and MAP2 in the dorsal horn (Fig. 5A,B). However, it cannot be excluded that other neurons (i.e., immature neurons) also express endogenous alkaloids. MAP2 localization in the spinal cord neurons was in agreement with previous studies (Vercelli et al., 2008; M. Zhang et al., 2006). However, in lamina V, colabeling with an anti-MAP2 antibody indicated that large, round cells corresponded to neurons (Fig. 5C, arrow). Moreover, by using an anti-GABA antibody, we demonstrated that MIR was present in GABAergic neurons of lamina V (Fig. 5D, arrow).

In the motor neuron pool of the ventral horn, the cell bodies displaying MIR (Fig. 5E) did not colocalize with ChAT, which is present in motor neurons. This suggests that the motor neurons do not contain endogenous morphine, codeine, or morphine glucuronides. However, immunolocalization of MIR within GABA-positive cells showed that some GABAergic interneurons that contain MIR were present in the motor neuron pools of lamina IX (Fig. 5F, arrow). ChAT localization in our experiment was in agreement with previously described localization in the ventral horn (Matsumoto et al., 2007; Park et al., 2010) and in lamina XI (Tortarolo et al., 2006).

We previously described how, in the mouse brain, MIR was absent from dopaminergic cells and processes (Laux et al., 2011). A similar result was found in the spinal cord; TH processes did not colocalize with the MIR processes, suggesting that MIR-labeled processes do not correspond to dopaminergic innervations (Fig. 5G). TH-immunoreactive processes in the spinal cord were as previously described (Brumovsky et al., 2006; Mizukami, 2004).

Endogenous morphine-like compounds are present in astrocytic cell bodies and processes in the mouse brain (Laux et al., 2011). With a light microscopic approach, we observed the presence of MIR in astrocytes throughout the mouse spinal cord. Double-label immunofluorescence with GFAP antibody, an astrocyte marker (Bramanti et al., 2011), confirmed the presence of MIR in numerous astrocytes in the spinal cord (Fig. 5H,I) and in the astrocytic foot processes around blood vessels (Fig. 5I, arrow). GFAP labeling (i.e., astrocytes) in the spinal cord is similar to what was previously described (Tawfik et al., 2008). Colabeling of GFAP and MIR confirmed the presence of endogenous morphine,

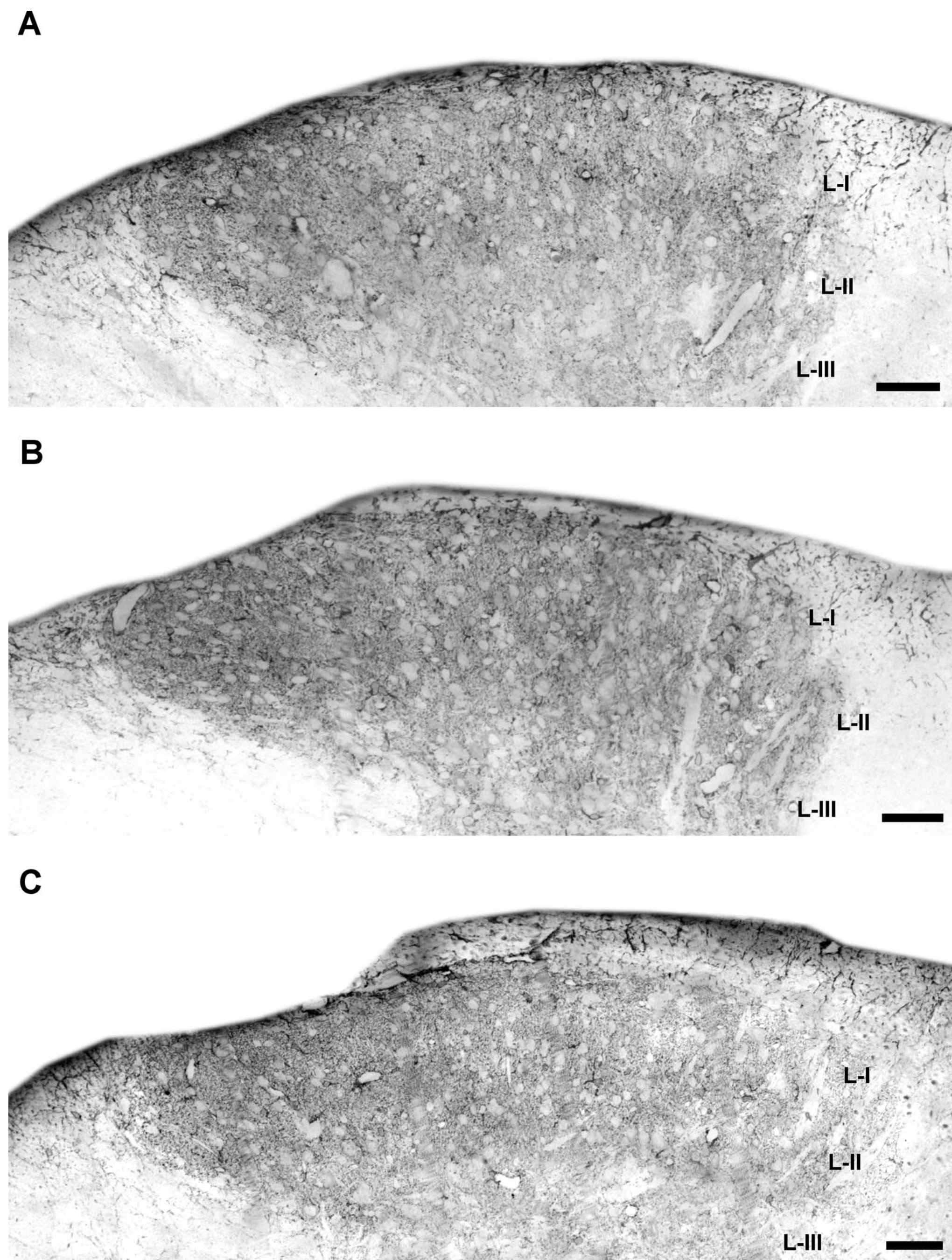

Figure 2. Distribution of MIR in the dorsal horn at different levels of the adult mouse spinal cord. **A:** Cervical. **B:** Thoracic. **C:** Lumbar. The distribution of MIR was similar between the different levels of the spinal cord; no MIR-positive neurons were present in the superficial laminae of the dorsal horn, whereas MIR was observed in astrocytes and processes. L-I, lamina I; L-II, lamina II; L-III, lamina III. Scale bars = 50 μm.

codeine, and/or morphine glucuronides in the astrocytes localized to the edge of the white matter (Fig. 5J).

Ultrastructural localization of morphine-like immunoreactivity in the adult mouse spinal cord

To assess whether MIR elements were from a glial or neuronal origin, we performed electron microscopic immunohistochemistry on spinal cord sections. Similar results were found for each spinal cord section. As in the dorsal and ventral horn of the cervical spinal cord, a strong MIR was present in astrocytic cell bodies (Fig. 6A) and processes. We also observed labeling in the astrocytic processes around blood vessels (Fig 6B, arrow), whereas vascular epithelium was not labeled. For astrocytes, MIR was localized in the cytoplasm of some neuronal cells present in the gray matter

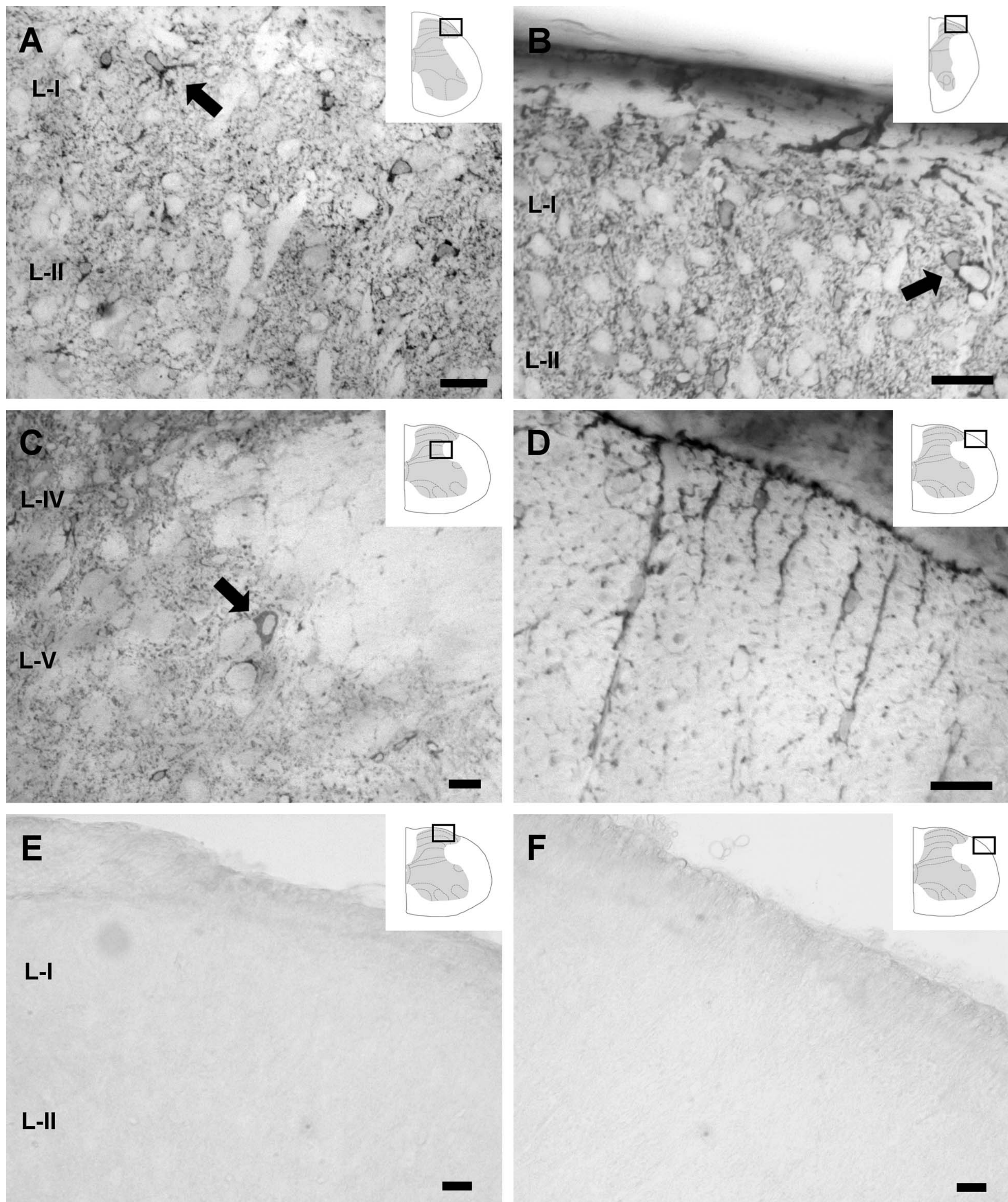

Figure 3. Higher magnifications of MIR distribution in the dorsal horn of the adult mouse spinal cord. **A:** High magnification of laminae I and II from a lumbar section. **B:** High magnification of thoracic laminae I and II. Both pictures in A and B show no MIR in dorsal horn neurons, whereas astrocytes (arrows) show high MIR. **C:** Labeled neuronal cell (arrow) in the lamina V of a cervical section of adult mouse spinal cord. **D:** MIR observed in astrocytes was found at the edge of the dorsal white matter of the spinal cord. **E,F:** No MIR is detected when the 6D6 antibody was preadsorbed for 1 hour with 3 μM morphine, in the dorsal horn gray matter (E) and in the edge of the white matter (F), confirming the specificity of the labeling (experiment done in parallel with nonpreadsorbed antibody). In the upper right corners, schematic representations of a cervical, thoracic, or lumbar spinal cord indicate the localization of the picture with a box. L-I, lamina I; L-II, lamina II; L-III, lamina III; L-V, lamina V. Scale bars = 20 μm.

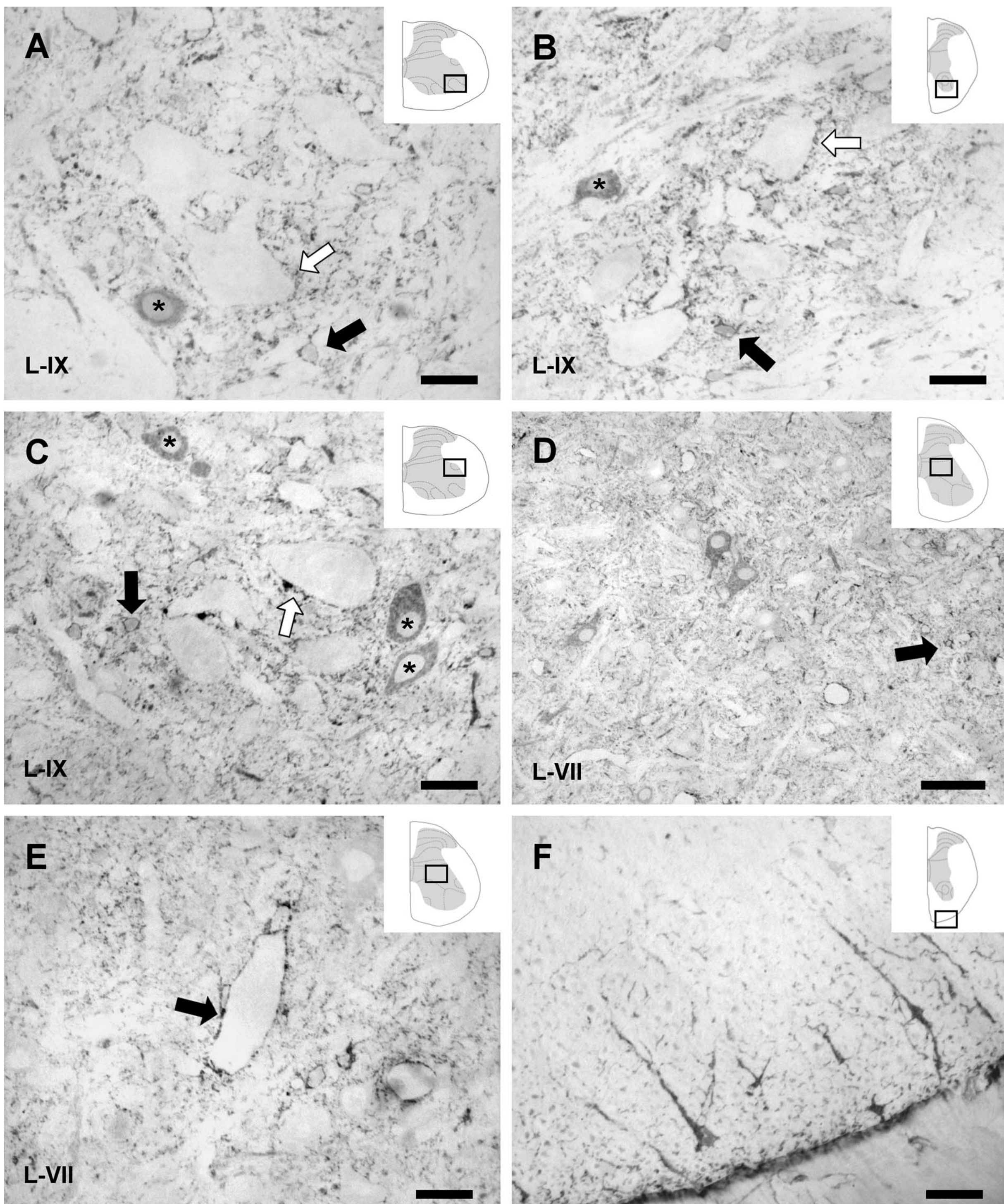

Figure 4. Distribution of MIR in the ventral horn of the adult mouse spinal cord. **A,B:** MIR in the motor neuronal pool present in the lamina IX of a cervical (A) and thoracic (B) section of the spinal cord. *MIR positive neurons. **C:** MIR present in neurons (asterisk), astrocytes (black arrow), and labeled astrocytic or neuronal processes around the motor neuronal soma (white arrow) in the lateral motor neuronal pool of the cervical spinal cord. **D:** MIR present in neuronal cells in the lamina VII of lumbar spinal cord section. The arrow shows an MIR astrocyte. **E:** MIR present in the astrocytic foot around blood vessels (arrow) in the lamina VII of a lumbar section. **F:** MIR observed in astrocytes present at the edge of the ventral white matter of the spinal cord. In the upper right corners, schematic representations of a cervical, thoracic, or lumbar spinal cord display the localization of the picture with a box. L-VII, lamina VII; L-IX, lamina IX. Scale bars = 20 μm in A-C,E,F; 50 μm in D.

of the spinal cord (Fig. 6C). Furthermore, neuronal postsynaptic elements, recognizable by the typical postsynaptic density, contained MIR (Fig. 6D, arrow). However, MIR was not observed in the neuronal presynaptic endings.

DISCUSSION

Morphine-like alkaloids (i.e., morphine, M6G, M3G, and codeine) are found in the CNS, and their concentration and localization have been described in the brain (Bianchi

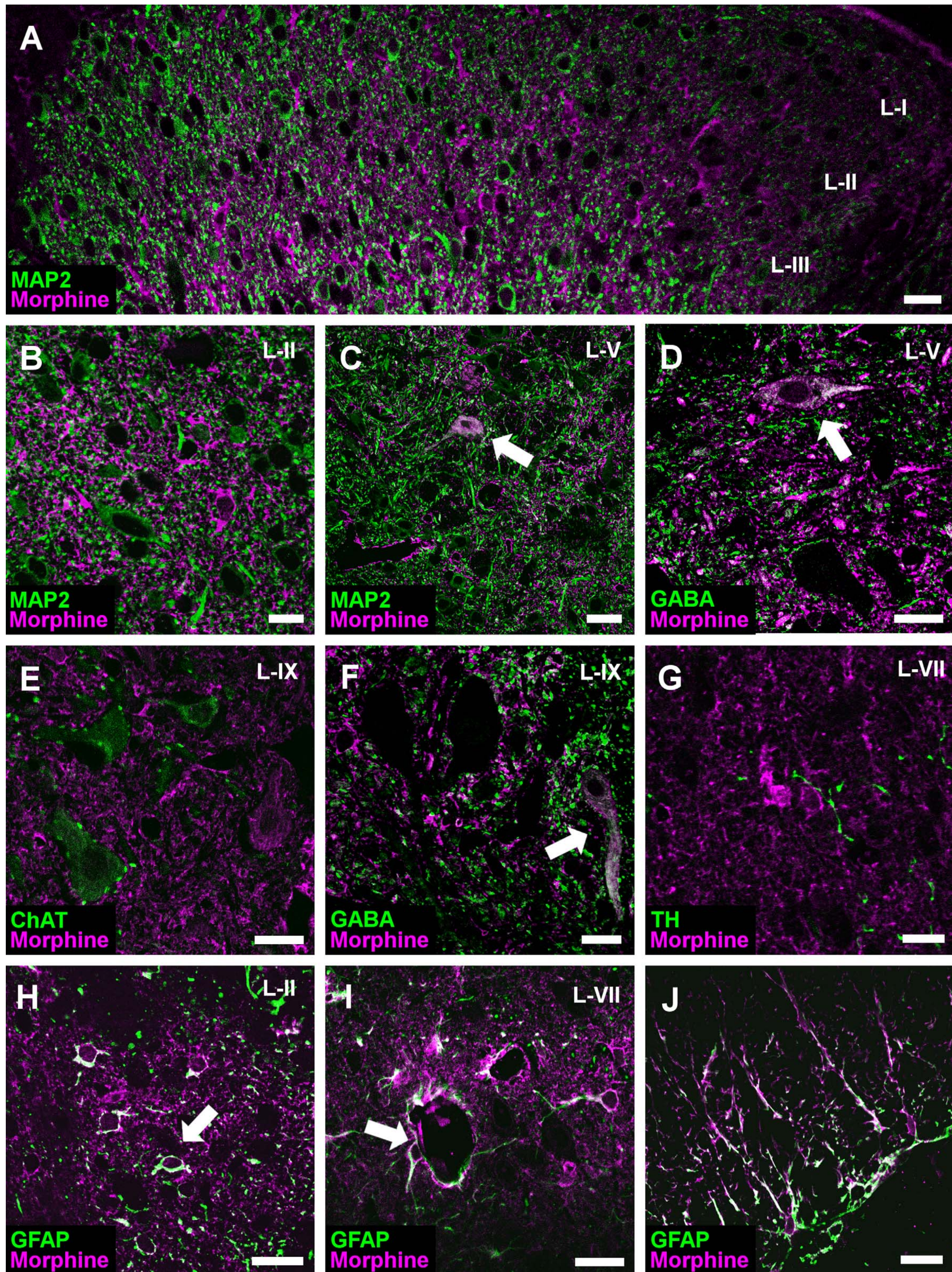

Figure 5

et al., 1993, 1994; Charron et al., 2011; Laux et al., 2011; Meijerink et al., 1999; Muller et al., 2008). However, few studies have examined their presence in the spinal cord, although endogenous morphine presence has been characterized in the spinal cord (Charlet et al., 2010; Donnerer et al., 1987; Meijerink et al., 1999). However, its localization has never been studied in the spinal cord.

The goal of the present study was to determine the localization of endogenous morphine, codeine, M6G, and M3G in the adult mouse spinal cord by using classical, confocal, and electron microscopy to discover the function of endogenous alkaloids in the spinal cord's physiology.

In the adult mouse spinal cord, endogenous morphine, codeine, M3G, and M6G were observed in nondopaminergic cells. Indeed, some of the GABAergic neurons of the spinal cord and astrocytic cell bodies and processes displayed an MIR that was homogeneously distributed at different levels of the spinal cord. The present mapping of MIR represents a key step in designing future experiments aimed at understanding better the role of morphine and morphine derivatives in spinal cord function.

MIR in nondopaminergic cells

Endogenous morphine is believed to be derived from dopamine biosynthesis, and its synthesis pathway has been characterized in SH-SY5Y, a tumor-derived catecholaminergic cell line (Boettcher et al., 2005; Poeaknapo et al., 2004). Moreover, a recent publication showed the absence of endogenous morphine in mice lacking TH, suggesting that dopamine is necessary for endogenous morphine biosynthesis in vivo (Neri et al., 2008). However, two recent articles revealed that, in the mouse and rat brains, endogenous alkaloids are absent from dopaminergic neurons of the substantia nigra and their processes in the striatum (Charron et al., 2011; Laux et al., 2011). Dopaminergic processes have been shown to be present in the spinal cord (Brumovsky et al., 2006), and, as observed in the brain (Charron et al., 2011; Laux et al., 2011), morphine-like immunoreactivity was absent from these neurons. These data are supported by the fact that endogenous morphine can be synthesized by nondopaminergic cells (Molina et al., 1995; Poeaknapo et al., 2004; Weitz et al., 1987).

These results, together with our brain studies (Charron et al., 2011; Laux et al., 2011), suggest that the biosynthesis of morphine is not completed in the dopaminergic neurons of the CNS, implying that the peripheral and central endogenous morphine biosynthesis pathways are different. In the CNS, endogenous morphine, codeine and/or morphine glucuronides may be taken up from the extracellular space by active or passive transport or may result from an uptake of a morphine precursor, such as tetrahydropapaveroline (THP; Charron et al., 2011; Laux et al., 2011). Furthermore, it has been hypothesized that THP, a dopamine metabolite found in the brain extracellular space, may be used to synthesize morphine-like compounds (Boettcher et al., 2005). THP injection in mice leads to morphine production and excretion (Grobe et al., 2010). This hypothesis implies cooperation between different cell types to ensure complete endogenous codeine/morphine/glucuronides biosynthesis.

MIR in GABAergic neurons

Here we described the localization of morphine-like compounds in some GABAergic cells in the spinal cord. The MIR-positive GABAergic neurons were present in all regions of the spinal cord and were located primarily in the gray matter of the ventral horn. MIR corresponded to

Figure 5. Characterization of MIR-positive cells in the adult mouse spinal cord by confocal microscopy. **A:** Colabeling of MIR (magenta) with MAP2 (green) in the dorsal horn of a cervical section, which was similar for all spinal cords regions. No colocalization between the two markers was observed either in the cells bodies or in the processes, suggesting that neurons do not contain morphine-like compounds in the dorsal horn. **B:** High magnification of a colabeling of MIR (magenta) and MAP2 (green) in the dorsal horn of a lumbar section. No colocalization was found in neurons present in this spinal cord area. **C:** Colabeling of MIR (magenta) with MAP2 (green) in the lamina V of a thoracic section, showing that MIR-positive cells in this lamina correspond to neurons. The arrows point to a colabeled cell. **D:** Colabeling of MIR (magenta) with GABA (green) in the lamina V of a lumbar section, showing that the MIR-positive neurons in this lamina correspond to GABAergic neurons. **E:** Colabeling of MIR (magenta) and ChAT (green) in a motor neuronal pool of lamina IX of a lumbar section, showing that motor neurons ($ChAT^{+}$) do not contain morphine-like compounds. **F:** Colabeling of MIR (magenta) and GABA (green) in a motor neuronal pool of lamina IX of a cervical section, showing that MIR neuronal cells are GABAergic. The arrows point to a double-labeled cell. **G:** Colabeling of MIR (magenta) and TH (green) in lamina VII of the lumbar spinal cord. No MIR was observed in dopaminergic endings. **H:** Colabeling of MIR (magenta) and GFAP (green) in lamina II of the thoracic spinal cord, showing the presence of MIR in astrocytes in the dorsal horn. **I:** Colabeling of MIR (magenta) and GFAP (green) in lamina VII of the cervical spinal cord, demonstrating the presence of MIR in astrocytes and astrocytic feet around blood vessels (arrow; white label). **J:** Colabeling of MIR (magenta) and GFAP (green) in the ventral white matter of a lumbar spinal cord section, showing a colocalization of the two markers in astrocytes at the edge of the spinal cord (white label). ChAT, choline acetyltransferase; GABA, γ-aminobutyric acid; GFAP, glial fibrillary acidic protein; MAP2, microtubule-associated protein 2; TH, tyrosine hydroxylase; L-I, lamina I; L-II, lamina II; L-III, lamina III; L-V, lamina V; L-VII, lamina VII; L-IX, lamina IX. Scale bars = 20 μm in A,C–J; 10 μm in B.

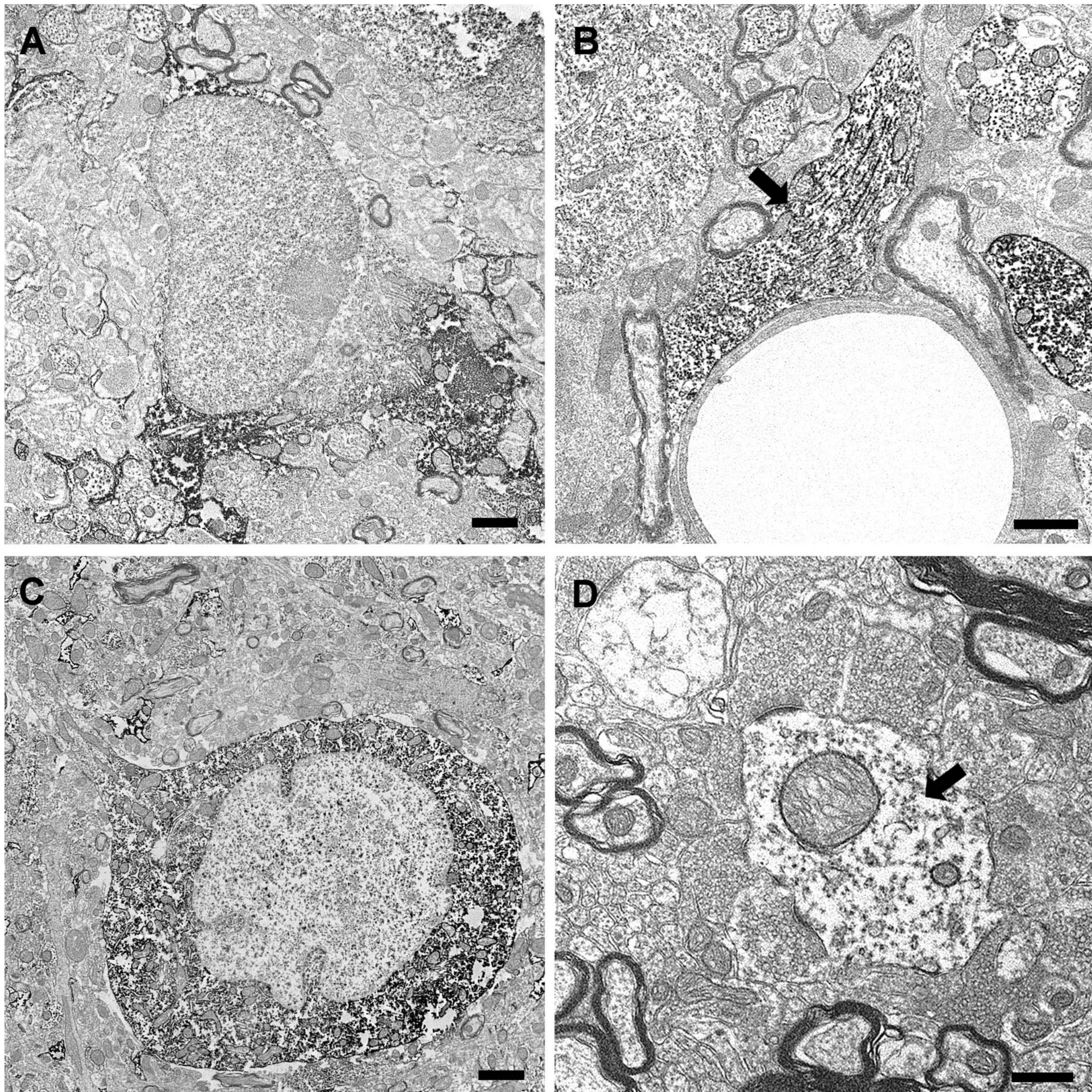

Figure 6. Electron microscopic micrographs of the MIR present in the gray matter of the adult mouse spinal cord. **A:** MIR present in an astrocyte of the ventral horn of the cervical spinal cord, recognizable by its bipolar cell body and small soma. **B:** Label of an astrocytic foot around a blood vessel (arrow). **C:** MIR-labeled neuronal cell present in the gray matter of the spinal cord. The invaginated nucleus and the large, round soma correspond to a neuron's structural characteristics. **D:** An MIR dendrite (arrow) recognizable by numerous post-synaptic densities. Presynaptic endings were not labeled in the spinal cord. Scale bars = 1 μm in A,B; 2 μm in C; 500 nm in D.

the possible presence of morphine, M6G, M3G, or codeine. The presence of UGT1A6 in neurons of the rat brain (Brands et al., 2000; Suleman et al., 1998) suggests that, after morphine synthesis or uptake, some neurons are able to convert morphine into its glucuronides. Our electron microscopic study allowed us to describe the presence of morphine-like immunoreactivity only in postsynaptic elements in the spinal cord (no presynaptic MIR has been observed). This conclusion supports our results obtained in the mouse brain, in which MIR was present in the postsynaptic elements in all regions except for the cerebellum, where presynaptic MIR was observed in GABAergic basket cells. In addition, the absence of morphine-like compounds in some GABAergic cells present in the dorsal horn was consistent with previous studies that showed that not all of the GABAergic cells contained MIR (Charron et al., 2011; Laux et al., 2011). We can hypothesize that morphine-like compounds may be synthesized by a subpopulation of GABAergic neurons and that this synthesis may be dependent on a specific transporter or enzyme expression.

Interestingly, endogenous morphine/GABAergic neurons were restricted to the anterior horn of the spinal cord (or ventral horn), an area where the cell bodies of alpha motor neurons receive input from a number of sources, including sensory neurons and interneurons. GABA plays a role in this area via an inhibitory effect on alpha motor neuron activity. These cells are affected in the so-called anterior horn diseases, namely, amyotrophic lateral sclerosis, spinal muscular atrophy, and progressive muscular atrophy, pathologies characterized by altered GABAergic system (Abdipranoto et al., 2008). Morphine has the highest affinity for the μ opioid receptors (MORs) which are often found at postsynaptic locations as well as presynaptically. Presynaptic MORs inhibit neurotransmitter release; through this mechanism, endogenous morphine might modulate the release of the inhibitory neurotransmitter GABA or regulate GABA synthesis. Further studies are necessary to establish the functional relevance and interdependence of GABA/ morphine-containing neurons in spinal ventral horn, particularly in muscle activity and its related pathologies.

MIR in astrocytes

As previously described for the adult mouse brain (Charron et al., 2011; Laux et al., 2011), our results showed the presence of endogenous morphine-like compounds in a large number of astrocytes throughout the spinal cord. Astrocytes do not express TH (Jaeger, 1985) and, thus, are unable to synthesize dopamine and endogenous morphine and its derivatives de novo. As postulated above, astrocytes may achieve morphine biosynthesis by taking up the precursors. However, another hypothesis suggests that, because astrocytes are known to be involved in the uptake of neurotransmitters from the extracellular space (Iversen, 2006), they may be able to take up morphine via specific transporters (Charron et al., 2011; Laux et al., 2011). In addition, the presence of UGT1A6 in primary rat astrocyte cultures (Heurtaux et al., 2006; Suleman et al., 1998) suggests that astrocytes can catabolize morphine into morphine glucuronides. The present description of MIR in the astrocytic foot processes around blood vessels might also suggest a possible uptake by or release from the blood (Brix-Christensen et al., 1997, 2000; Glattard et al., 2010). Finally, we postulate that astrocytes may be responsible for the morphine catabolism in the spinal cord, which could lead to further studies in the fields of opiate addiction and tolerance processes.

MIR and the μ opioid receptor

Morphine and its derivative, M6G, preferentially bind the MOR and the δ opioid receptor (DOR; Matthes et al., 1996; Trescot et al., 2008). In the spinal cord, the highest density of MORs was found in the processes of the superficial layers I and II of the dorsal horn (Abbadie et al., 2000; Y. Zhang et al., 2006). MORs were also found in the processes of others layers of the spinal cord but with less intensity. In addition, motor neuronal cell bodies have been shown to express the MORs (Abbadie et al., 2000; Y. Zhang et al., 2006). The presence of MORs in the same area as MIR, such as lamina I and II and in the motor neuron pool, suggests a role for endogenous alkaloids in these areas. However, several endogenous peptides are also able to bind MORs, including β-endorphin (Reisine, 1995), endomorphin-1, and endomorphin-2 (Zadina et al., 1997). Because multiple MOR-binding peptides exist in addition to morphine-like compounds (e.g., in the superficial layer I and II; Martin-Schild et al., 1999; Pierce and Wessendorf, 2000), it is difficult to speculate on the individual effects of endogenous alkaloids. However, such colocalization strongly suggests that endogenous morphine or its derivatives could act as novel high-affinity endogenous MOR agonists.

CONCLUSIONS

Although no definitive role has been attributed to endogenous morphine and its derived compounds found in nervous system tissue, their presence in GABAergic neurons and astrocytes in the spinal cord should be studied further. Endogenous morphine has been implicated in the regulation of pain and in weakening the memory of a nociceptive experience, which plays a similar role as its exogenous counterpart (Charlet et al., 2010; Guarna et al., 2002, 2004, 2005). However, the mechanism of action of endogenous morphine in the CNS remains unknown, and the presence of endogenous morphine-like compounds in nondopaminergic cells and in regions of the spinal cord not usually involved in pain modulation allows for exciting future studies to examine and extend the role of these endogenous opiates beyond their analgesic functions.

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