Pre- and postsynaptic localizations of the CB1 cannabinoid receptor in the dorsal horn of the rat spinal cord.

PRE- AND POSTSYNAPTIC LOCALIZATIONS OF THE CB1 CANNABINOID RECEPTOR IN THE DORSAL HORN OF THE RAT SPINAL CORD

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Abstract
Several lines of evidence show that endogenous and exogenous cannabinoids modulate pain transmission at the spinal level through specific cannabinoid-1 (CB1) receptors. Since anatomical data concerning spinal CB1 receptors are rather contradictory, we studied the cellular and subcellular localizations of the CB1 receptors by immunocytochemistry. Results show a dual pre- and postsynaptic localization of CB1 receptors. Presynaptic receptors are evidenced by the labeling of (1) heterogeneous dorsal root ganglion neurons and (2) axons of Lissauer's tract. Postsynaptic receptors are shown by the labeling of numerous interneurons in the outer part of lamina II. Double immunolabelings show that lamina II outer CB1 neurons, probably islet cells, may also contain GABA or nitric oxide synthase. Numerous CB1-containing neurons in lamina X are also immunostained with anti-nitric oxide synthase (NOS) antibody. Under the electron microscope, CB1 immunoreactivity is exclusively localized postsynaptically in both somatic and dendritic compartments. The absence of labeling on primary a¡erent axon terminals is discussed and compared to the absence of labeling on terminals or vesicle-containing dendrites of islet cells, where a presynaptic localization was expected according to data of the literature.

Key words: dorsal root ganglia, nitric oxide synthase, GABA, immunocytochemistry, ultrastructure.

The role of cannabinoids in modulating pain sensitivity was suggested by numerous behavioral studies demonstrating analgesic e¡ects of cannabinoids in rodents (Buxbaum, 1972; Bloom et al., 1977; Moss and Johnson, 1980; Martin et al., 1993, 1995). It was also shown that the selective cannabinoid agonist WIN 55,512-2 induces a suppression of noxious stimulus-evoked activity in wide dynamic range neurons (Hohmann et al., 1995, 1998) and of c-Fos protein immunoreactivity (Tsou et al., 1996) in rat lumbar dorsal horn. Exogenous cannabinoids are analgesic in models of persistent in£ammatory pain (Calignano et al., 1998; Jaggar et al., 1998) or neuropathic pain (Herzberg et al., 1997). Cannabinoids are anti-hyperalgesic (Richardson et al., 1998a,b) and anti-allodynic (Martin et al., 1999b,c) in di¡erent chronic pain models. In addition, cannabinoid-1 (CB1) receptor knockout mice exhibited hypoalgesia in hotplate and formalin tests (Zimmer et al., 1999). This antinociceptive action is mediated partly through spinal mechanisms since a direct action on spinal nociceptive neurons has been demonstrated by both behavioral (Richardson et al., 1998a,b; Welch et al., 1998) and electrophysiological studies (Hohmann et al., 1998). Moreover, cannabinoids remain analgesic after spinal transection (Smith and Martin, 1992). Two genes encoding G protein-coupled receptors that bind cannabinoids were identi¢ed: the CB1 receptor (Devane et al., 1988; Matsuda et al., 1990) expressed in both central and peripheral nervous systems and the CB2 receptor expressed in peripheral tissues and cells of the immune system (Munro et al., 1993; Shire et al., 1996). The cloning of the CB1 receptor allowed the study of its localization in the rat brain and spinal cord by in situ hybridization (Mailleux and Vanderhaeghen, 1992; Matsuda et al., 1993; Hohmann et al., 1999; Hohmann and Herkenham, 2000) and immunocytochemistry (Dove-Pettit et al., 1998; Tsou et al., 1998; Katona et al., 1999; Ong and Mackie, 1999a,b; Egertova and Elphick, 2000; Farquhar-Smith et al., 2000). Moreover, the development of synthetic agonists and antagonists of the CB1 receptors opened the possibility of studying the distribution of cannabinoid binding sites (Mailleux and Vanderhaeghen, 1992; Thomas et al., 1992; Glass et al., 1997; Herkenham et al., 1991; Hohmann and Herkenham, 1999; Hohmann et al., 1999). Nevertheless, the distribution of CB1 receptors in the spinal cord is still far from being completely known. The density of cannabinoid binding sites in the spinal cord appeared rather low compared to otherregions in rat (Herkenham et al., 1991; Mailleux and Vanderhaeghen, 1992) and human brains (Glass et al., 1997). The work of Hohmann et al. (1999) clearly demonstrated the presynaptic localization of about half of the CB1 receptor binding sites on primary a¡erent terminals, part of them being sensitive to capsaicin treatment (Hohmann and

Herkenham, 1998). They have also shown that a subpopulation of dorsal root ganglion (DRG) cells express the CB1 receptor mRNA (Hohmann and Herkenham, 1999a). However, immunocytochemical distribution in the rat dorsal horn remains controversial since the laminar distribution in the study of Tsou et al. (1998) is di¡erent from that observed by Farquhar-Smith et al. (2000). In addition, the latter authors did not confirm the peripheral origin of half of the CB1 receptor binding sites. We have thus reexamined the distribution of the CB1 receptor in the rat spinal cord with immunocytochemistry by using specific antibodies directed against the N-terminal sequence of the CB1 receptor (Tsou et al., 1998; Twitchell et al., 1997) at the light and electron microscope levels focussing on the dorsal horn. To further characterize the chemical nature of CB1 receptorcontaining neurons, double labelings were performed using antibodies directed against markers of spinal interneurons such as GABA and NO.

EXPERIMENTAL PROCEDURES

The experiments were performed in agreement with the European Community Council Directive (86/609/EEC).

Primary antisera

The anti-CB1 receptor antibodies were obtained by immunization of rabbits with the N-terminal 77 amino acids of the cloned rat CB1 receptor fused to glutathione S-transferase (Twitchell et al., 1997). Its specificity has been fully characterized on cell culture and transfected cell lines (Twitchell et al., 1997). Moreover, its speci¢city has also been demonstrated on rat brain membranes and the pattern of staining observed by immunocytochemistry is highly consistent with previous data (Tsou et al., 1998). To perform double immuno£uorescence we used a mouse monoclonal anti-nitric oxide synthase (the biosynthetic enzyme of nitric oxide or NOS) and mouse monoclonal GABA antibodies (Sigma, France).

Tissue preparation

Adult male Wistar rats were deeply anesthetized with 60 mg/kg i.p. of sodium pentobarbital (Sano¢, Libourne, France) and intracardially perfused with 50^100 ml of 9xNaCl containing 0.1% sodium nitrite followed by 800 ml of 4% paraformaldehyde in 0.1 M phosphate bu¡er (PB), pH 7.4. Spinal cords and DRG were dissected out and postfixed overnight in the same fixative at 4³C. Spinal cords were washed in 0.02 M phosphate-bu¡ered saline (PBS) and then cut using a vibratome in 50 Wm thick sections. DRG were cryoprotected by overnight incubation in 20% sucrose and cut using a cryostat in 12-µm sections which were collected on gelatin-coated slides.

Immunocytochemistry

Free-floating spinal cord sections were preincubated in 0.02 M PBS containing 6% normal goat serum (NGS) and 1% bovine serum albumin (PBS-NGS-BSA) for 30 min at room temperature and then incubated with an affinity-purified polyclonal rabbit anti-CB1 receptor antibody (Twitchell et al., 1997), diluted (1/500-1/1000) in PBS-NGS-BSA for 15 h at room temperature. For light microscopy 0.1% Triton X-100 was added to the preincubation and incubation media. After washing in PBSNGS-BSA, sections were incubated in 1/250 biotinylated antirabbit IgG (Vector Laboratories, Burlingame, CA, USA) for 1 h. Sections were then washed in PBS and incubated for 1 h with the avidin-biotin-peroxidase complex (Vector), diluted (1/100) in PBS. After washing in 0.5 M Tris^HCl, pH 7.4, sections were incubated in the same buffer supplemented with 0.03% (w/v) 3,3P-diaminobenzidine (Sigma, France) and 0.01% (v/v) hydrogen peroxide. The reaction proceeded at 20³C and was stopped by washing in Tris buffer after 5-7 min of incubation. Sections were then washed in PBS, transferred onto glass slides and mounted in PBS-glycerol. Immunocytochemistry for DRG sections was performed as described above, directly on gelatin coated slides. Slides were kept in a wet chamber during the immunocytochemical procedure.

Double immunofluorescence

For double immuno£uorescence, spinal cord vibratome sections were preincubated in PBS-NGS-BSA for 30 min, and then incubated overnight in a mixture of two primary antibodies : rabbit anti-CB1 receptor (1/500) and mouse anti-NOS (1/1000) or mouse anti-GABA (1/1000), diluted in PBS-NGS-BSA. After repeated washings in PBS, the sections were incubated for 2 h in a mixture of the two appropriate secondary antisera: 1/200 antirabbit IgG conjugated to cyanin 3 (Institut Pasteur, France) and 1/100 anti-mouse IgG conjugated to fluorescein isothiocyanate (Institut Pasteur). The sections were then rinsed twice in PBS, postfixed for 10 min in 4% PAF, washed in PBS and mounted with Vectashield (Vector). Confocal laser scanning microscopy was performed using a

TCS-4D confocal imaging system (Leica Instrument, Heidelberg, Germany), equipped with an argon-krypton ion laser. Cells were observed with a Leica plan apochromat U40 oil immersion objective with a numerical aperture of 1.4. Image elaboration was done with Adobe Photoshop 5.0.

Controls

Omission of the primary antibodies (CB1, NOS, GABA) in the incubation medium resulted in a complete absence of immunostaining. Preabsorption of the diluted anti-CB1 antibody (1/500) with 1 μg/ml of the immunizing conjugate for 3 h at room temperature before addition of the sections totally inhibited the labeling. The speci¢city of anti-NOS and anti-GABA antibodies has been tested by preincubation of the primary antibody with 1 μg/ml NOS or GABA which resulted in an absence of immunolabeling (NOS and GABA were performed from Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Electron microscopy

Immunoperoxidase staining. Small blocks of the dorsal horn, dissected from sections labeled with immunoperoxidase, were postfixed for 45 min at room temperature with 2% osmium tetroxide in 0.2 M PB. After 10 min washing in 0.1 M PB, blocks were dehydrated in graded alcohol, then incubated successively in alcohol/araldite (v/v), araldite (Fluka, Switzerland) and araldite/accelerator (2%) at 37³C. Finally, blocks were embedded in araldite/accelerator (2 days, at 60³C). Ultrathin sections were obtained using an LKB ultramicrotome. The sections, about 70 nm thick, collected on copper grids, were counterstained with lead citrate for 10 min in a dried chamber at room temperature, and examined with a Jeol electron microscope (JEM 100 CX).

Immunogold staining.

Free-floating sections were preincubated in PBS-NGS-BSA for 30 min at room temperature. The sections were then incubated overnight in rabbit polyclonal anti-CB1 antibody (1/500) in PBS-NGS-BSA at room temperature. They were washed in the same medium supplemented with 0.1% fish gelatin (IGSS quality, Amersham, France) and then incubated for 4 h at room temperature in the same medium containing 1/100 goat anti-rabbit IgG conjugated to 0.8 nm colloidal gold (Aurion, France). After repeated washings in PBS-NGSBSA-gelatin followed by a washing in 0.1 M PB, the sections were post¢xed for 10 min in 2.5% glutaraldehyde in 0.1 M PB. After washing in 0.1 M PB and then in citrate bu¡er pH 4.2, gold was intensi¢ed by the silver intensi¢cation kit (Sigma) under light microscopic control for 5-15 min. After washing in citrate buffer and postfixation in 1% sodium thiosulfate in distilled water, the sections were finally incubated in 1% osmium tetroxide in 0.1 M PB for 15 min and then embedded as previously described.

RESULTS

Light microscopy

In the DRG, numerous cell bodies of large, medium and small size were immunoreactive and no difference could be evidenced between the cervical, thoracic or lumbar levels (Fig. 1). While the large and medium-sized cells appeared more numerous, the most striking observation was the size variability of these immunoreactive cell bodies.

At all levels of the spinal cord, the most intense CB1 receptor immunoreactivity was detected in the superficial layers of the dorsal horn (Figs. 2 and 3). In order to recognize the subdivisions of the dorsal horn, several transverse sections were labeled for substance P which classically labels lamina I and the outer part of lamina II (IIo) (not shown). The heaviest CB1 receptor labeling was observed in laminae I and IIo, while the inner part of lamina II (IIi) and lamina III showed a lower labeling (Figs. 2 and 3). When present, Lissauer's tract showed a marked immunoreactivity (Fig. 2). A moderate immunoreactivity was observed in the dorsolateral funiculus (Fig. 3A) and in lamina X around the central canal (not shown). Other laminae are almost devoid of labeling. Immunoreactive cell bodies were observed in laminae I and II (Fig. 3). They were particularly abundant in lamina IIo (Figs. 2B, 3 and 4), although occasional immunoreactive cell bodies were also observed in laminae I (Fig. 3Ab) and IIi (Fig. 2B). Lamina IIo neurons looked like typical interneurons, being round in shape and small in size (10-12 μm). They were observed in the whole dorsomedial extent of the lamina at all spinal levels. In addition, numerous large fusiform immunoreactive cell bodies might be observed in lamina X around the central canal (Fig. 4, lower panel). No cell bodies were observed in other laminae. In order to further characterize the chemical nature of these cell bodies, double labeling was performed by using antibodies directed against

markers of spinal interneurons such as GABA and NO. The immunolabelings were then observed with a confocal laser scanning microscope. The results clearly showed that many CB1 receptor-like neurons of lamina IIo were double-labeled for GABA (Fig. 4, upper panel).

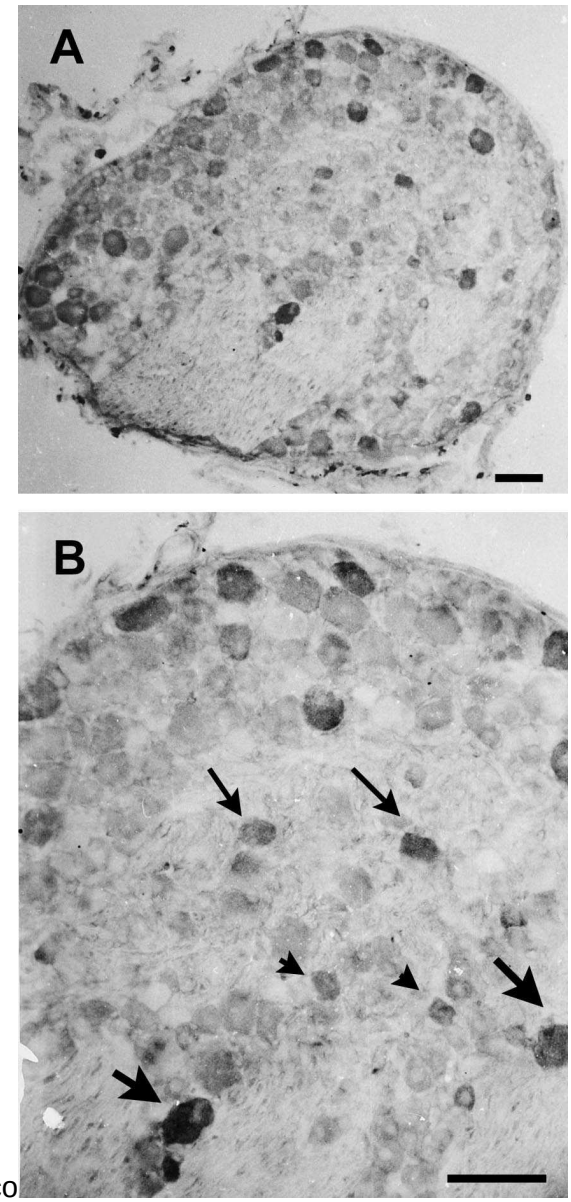

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Fig. 1. CB1 receptor-like immunoreactivity in a DRG at the cervical level. A low power micrograph (A) shows that immunoreactive cell bodies are relatively numerous and very heterogeneous in size. At higher magni¢cation (B), we observe that large (large arrows), medium (thin arrows) and small (arrowheads) cells may be immunoreactive. Scale bars= 100 µm.

They are small (10^12 Wm), round neurons often clustered. In addition, NOS coexisted in most CB1 lamina II neurons (Fig. 4, middle panel). These neurons had the same size and morphology than those double-labeled for CB1 and GABA. Neurons double-labeled for NOS and CB1 were also observed in lamina X (Fig. 4, lower panel).

Electron microscopy

No differences in labeling were found after using either horseradish peroxidase or gold as marker. The labeling was mostly observed in postsynaptic sites such as dendrites (Figs. 5 and 6) and perikarya (Fig. 6A).

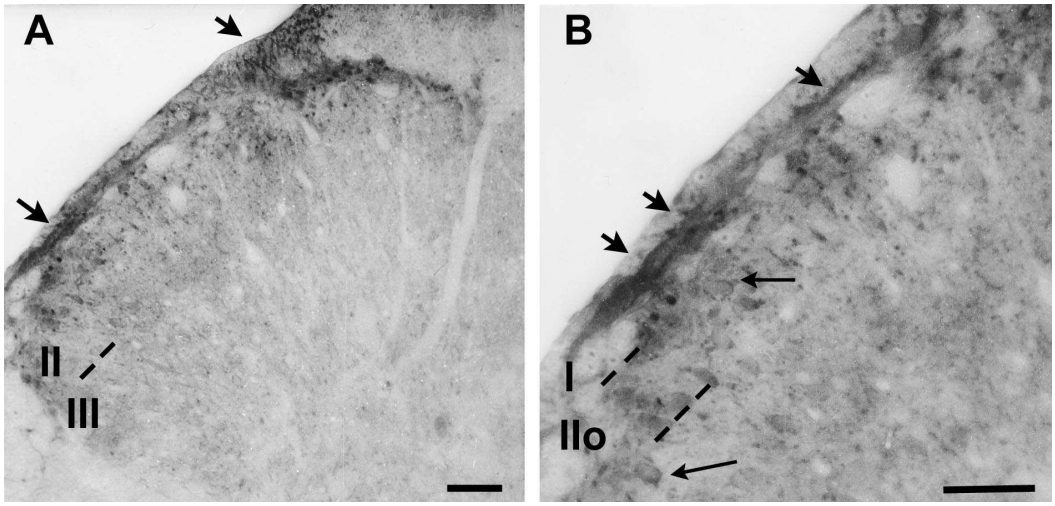


Fig. 2. CB1 receptor-like immunoreactivity in the dorsal horn of the lumbar segment. At lower magnification (A), an important immunoreactivity is observed in Lissauer's tract (arrows) and in the two superficial layers. At higher magnification (B) immunoreactive cell bodies (thin arrows) are observed in lamina II. The approximate boundaries between laminae are indicated by dotted lines. Scale bars =250 μm.

Anyway, a number of thin unmyelinated axons were immunoreactive (Fig. 5A, B). By contrast, labeled axon terminals were never observed. Whatever the marker used, the immunoreactivity was mainly intracytoplasmic and only rarely associated with the plasma membrane. In this latter case, it was generally observed on undi¡erentiated plasma membranes rather than associated with synaptic thickenings. However, the labeling might be occasionally observed in close proximity to both asymmetric (Figs. 5B and 6B) or symmetric (Fig. 6C) synapses. The intracytoplasmic labeling was localized on microtubules (Figs. 5C, D and 6A), the Golgi apparatus (Fig. 6A), and endosomes (Fig. 6B).

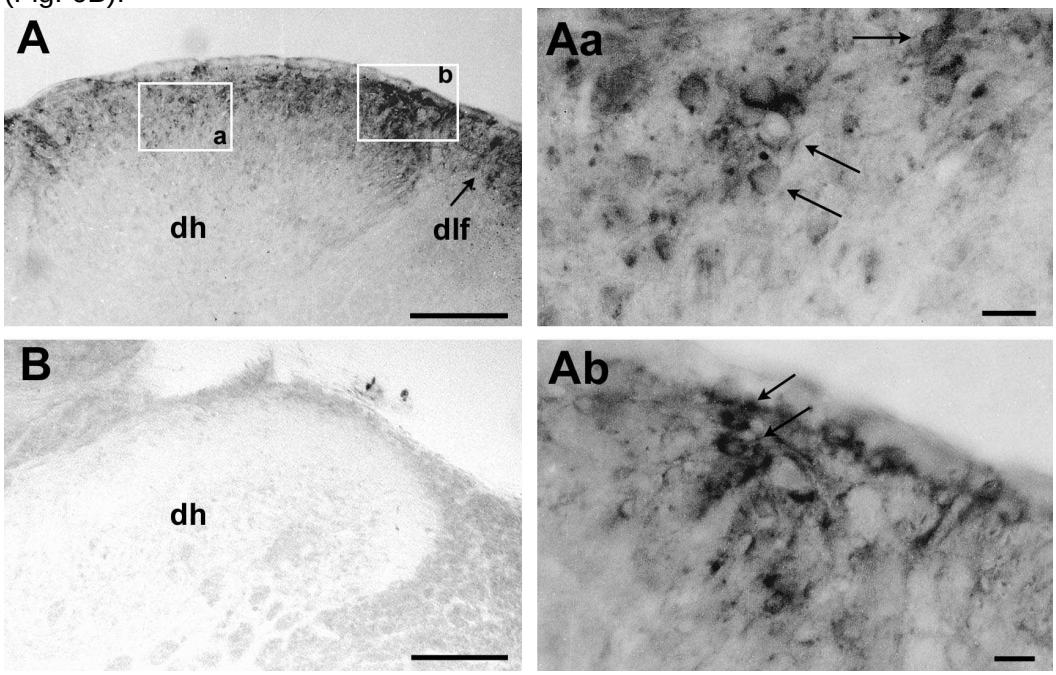


Fig. 3. CB1 receptor-like immunoreactivity in the dorsal horn of the rat spinal cord. At the cervical segment (A) the labeling is very intense in the superficial laminae particularly in the lateral part of the horn (dh). The dorsolateral funiculus (dlf) presents a moderate immunoreactivity. The medial and lateral parts of the dorsal horn, square inserts a and b respectively, are shown at higher magnification (Aa and Ab) to evidence the immunoreactive cell bodies in lamina II (Aa) and lamina

I (Ab). In B, a cervical section stained with preabsorbed antisera (see Experimental procedures) is shown. Scale bars= 250 µm (A, B); 50 µm (Aa, Ab).

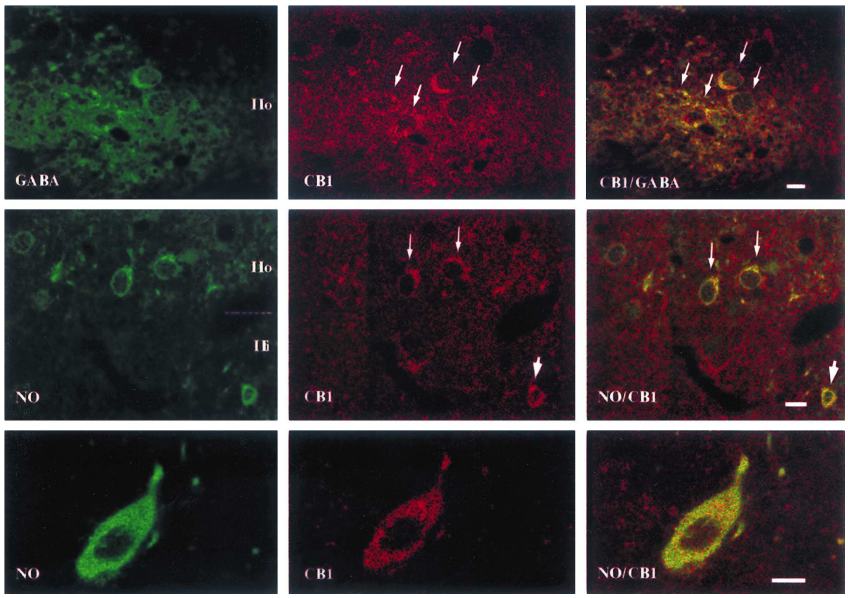


Fig. 4. Double labeling under the confocal laser scanning microscope. CB1 receptor immunoreactivity appears in red (central column), while green fluorescence (left column) represents GABA (upper panel) or NOS (middle and lower panels) immunoreactivity. In the dorsal horn GABA is expressed in all CB1 neurons of the dorsal horn. They are small (10-12 µm) and round neurons. The middle panel shows the colocalization of NOS and CB1 receptors in the dorsal horn. All neurons seem double-labeled in lamina IIo (small arrows) and in lamina IIi. In the lower panel, an example of a neuron double-labeled in lamina X is shown. Scale bars=10 µm (two upper panels); 15 µm (lower panel).

DISCUSSION

Pre- and postsynaptic CB1 receptor localization in the rat dorsal horn

Our results have shown that in the dorsal horn of the rat spinal cord CB1 receptors are localized both pre- and postsynaptically. A presynaptic localization of cannabinoids on primary afferent fibers can be inferred from the observation of several CB1 receptor-like-containing cell bodies in the DRG and of an intense CB1 receptor immunoreactivity in Lissauer's tract. Although at the moment some discrepancies occur in neuroanatomical data concerning a presynaptic localization (see the Introduction), Hohmann and Herkenham have shown a moderate reduction of CB1 receptor binding sites in the dorsal horn after capsaicin treatment (Hohmann and Herkenham, 1998) or after rhizotomy (Hohmann et al., 1999). Moreover, they have observed numerous DRG cells expressing CB1 receptor mRNA (Hohmann and Herkenham, 1999a). These results have been recently confirmed using immunocytochemistry on in vitro DRG cells arising from adult rats (Ahluwalia et al., 2000). In line with these data, we have shown that the CB1 receptor-immunolabeled DRG cells are heterogeneous in size and shape. Since the major excitatory transmitter of primary afferent fibers is glutamate, and since cannabinoids have been shown to modulate synaptic plasticity at glutamatergic synapses and interfere with long-term depression (Auclair et al., 2000), cannabinoids in the spinal cord may play a similar role in controlling glutamatergic afferences.

In the present research, a postsynaptic localization of CB1 receptors was shown in the rat spinal cord at both the light and electron microscope levels. In particular, in agreement with in situ hybridization experiments (Mailleux and Vanderhaeghen, 1992), several CB1 receptor-immunopositive cell bodies were found in lamina II of the dorsal horn, where, at the ultrastructural level, the CB1 receptor immunoreactivity was mainly observed in somatic and dendritic compartments.

CB1 receptors in GABA and NO interneurons

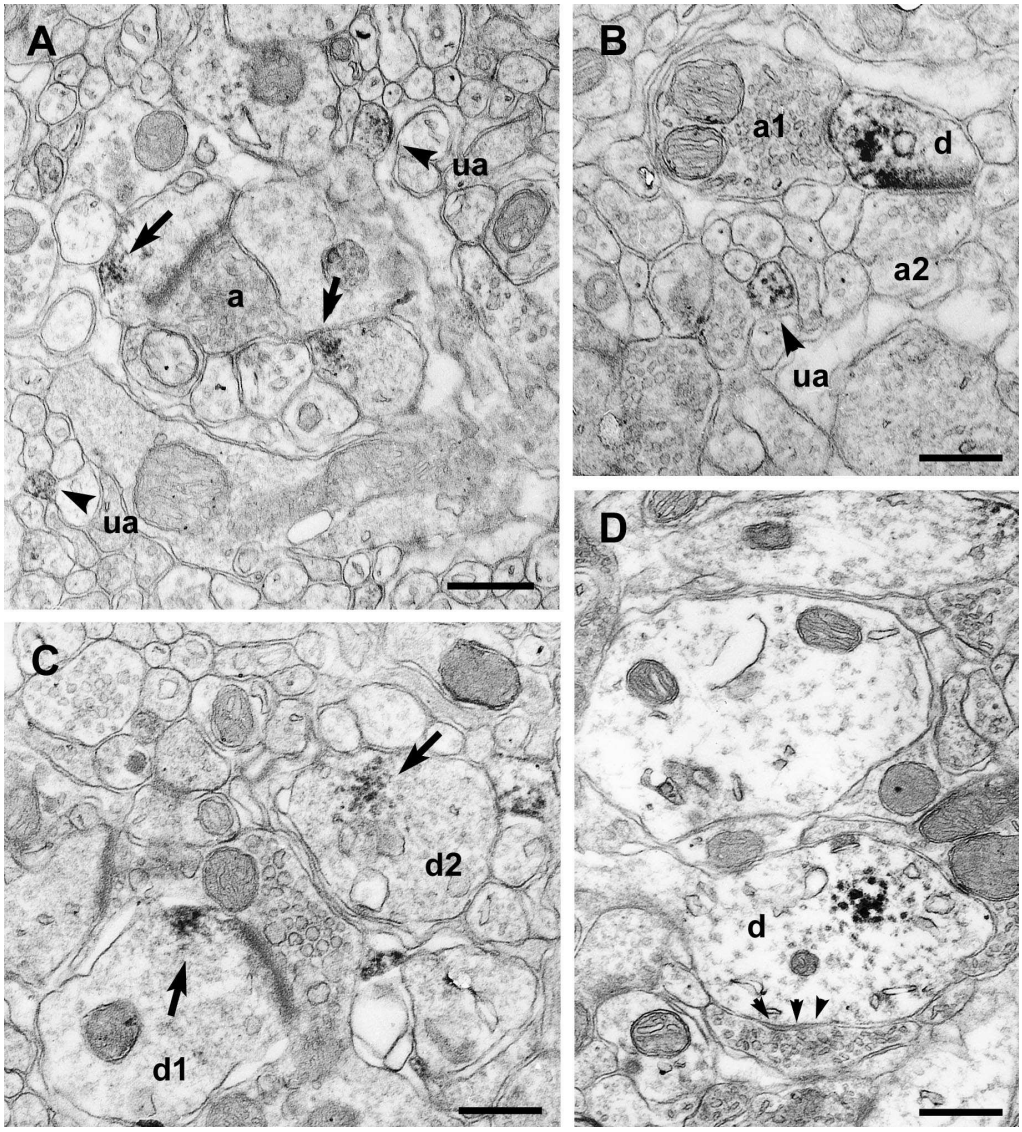
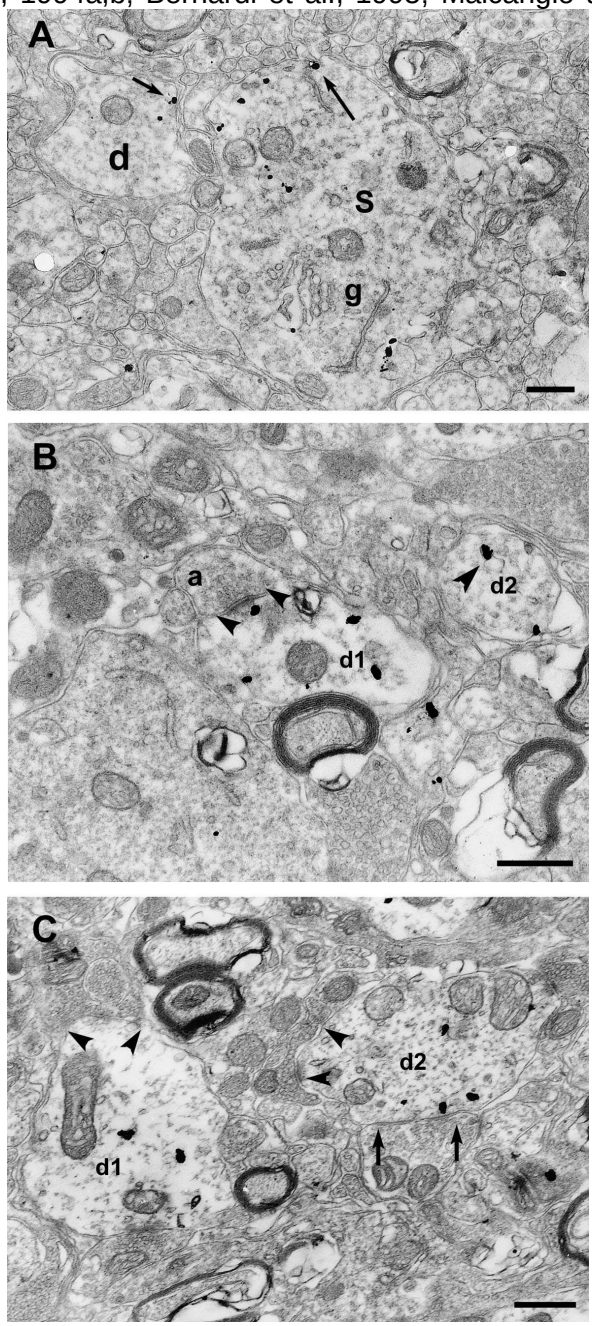


Fig. 5. Ultrastructural CB1 receptor peroxidase labeling in the dorsal horn. (A) The immunoreactivity is localized in dendrites (arrows) postsynaptic to an unlabeled axon terminal (a) and in small neurites (arrowheads), presumably unmyelinated axons (ua). (B) CB1 receptor-like immunoreactivity is observed in a dendrite (d) receiving two synaptic contacts of the symmetric (with axon a1) and asymmetric (with axon a2) types. Immunoreactivity is also observed in unmyelinated axons (ua). (C) The immunoreactivity is observed in two dendrites: in d1, the labeling (arrow) is localized close to the plasma membrane in a region clearly distinct from the synaptic specialization ; in d2, the labeling is associated with microtubules and restricted to a small part of the cytoplasm. (D) Another example of cytoplasmic labeling in a dendrite (d) postsynaptic to an unlabeled axon terminal (arrowheads). Scale bars=500 nm.

The distributions of NO and GABA immunoreactivities are in agreement with those previously described (Aimar et al., 1998; Barber et al., 1982; Bernardi et al., 1995; Todd and McKensie, 1989; Valtschano¡ et al., 1992a,b; Vizzard et al., 1994a,b), attesting to the specificity of the immunoreactions. Double-labeling experiments show that CB1 cell bodies in the rat spinal cord may also contain GABA (lamina II) or NOS (laminae II and X). Since all NO lamina II terminals have been shown to contain GABA (Valtschano¡ et al., 1992a,b), and since we observe that all CB1 neurons of lamina II are also labeled for NOS, CB1 interneurons could contain both NOS and GABA. NO and GABA lamina II neurons belong to the best characterized inhibitory interneurons of the dorsal horn, the islet cells (Barber et al., 1982; Magoul et al., 1987; Valtschanoff et al., 1992a,b;

Vizzard et al., 1994a,b; Bernardi et al., 1995; Malcangio and Bowery, 1996; Aimar et al., 1998).



CB1 lamina II

Fig. 6. Ultrastructural CB1 receptor gold labeling in the dorsal horn. (A) The immunoreactivity is observed in dendrites (d) and soma (s). Only a few grains are localized close to the plasma membrane (arrow), others are seen in the cytoplasm, occasionally on the Golgi apparatus (g). (B) An immunoreactive dendrite (d1) is postsynaptic to an unlabeled axon terminal (a), a grain (arrow) is observed near the postsynaptic di¡erentiation (small arrowheads). Another labeled dendrite (d2) shows a labeled endosome (large arrowhead). (C) Two labeled dendrites (d1 and d2) are contacted by several unlabeled axons by both symmetric (between two thin arrows) and asymmetric (arrowheads) contacts. Scale bars=500 nm.

neurons may thus be islet cells although no immunoreactive vesicle-containing dendrites, the typical morphological feature of islet cells (Gobel et al., 1980; Todd, 1988), were observed. We have found, in addition, a group of CB1 receptor-immunoreactive cell bodies in lamina X and shown that they also contain NO. These neurons may be ascending neurons, very numerous in this region (Nahin et al., 1983). This is further suggested by results of Farquhar-Smith et al. (2000) who observed CB1 receptor-immunoreactive cell bodies in lamina X after spinal transection. The colocalization of CB1 receptors with GABA and/or glutamate has been demonstrated by

immunocytochemistry in the cortex, striatum, hippocampus and cerebellum of rat (Dove-Pettit et al., 1998; Tsou et al., 1998; Egertova and Elphick, 2000) and primate brains (Ong and Mackie, 1999b). Double immunocytochemical or in situ hybridization labelings indeed have largely confirmed the presence of CB1 receptors in GABAergic neurons in rat hippocampus (Tsou et al., 1999; Hajos et al., 2000) and striatum (Hohmann and Herkenham, 2000), and in mouse hippocampus, amygdala and entorhinal cortex (Marsicano and Lutz, 1999). Moreover, in human Huntington's disease, a parallel degeneration pattern was observed in both GABA and CB1 neurons of the basal ganglia (Glass et al., 2000). In lamina II, GABA presynaptically inhibits primary afferent inputs (Malcangio and Bowery, 1996) through vesicle-containing dendrites contacting glomerular afferent terminals (Todd and McKensie, 1989; Bernardi et al., 1995). In contrast, NO synthesis, linked to Nmethyl-D-aspartate receptor activation, has been correlated to the maintenance of hyperalgesia in chronically suffering rats (Moore et al., 1991; Meller et al., 1992). The functional meaning of CB1 receptors in spinal GABA- and NO-containing neurons remains to be determined. In the hippocampus, CB1 receptor agonists presynaptically inhibit GABA release through CB1 receptors, localized on axon terminals of basket cells (Katona et al., 1999). By contrast, the exclusive somatic and dendritic localizations of CB1 receptors in the dorsal horn suggest a postsynaptic action of cannabinoids on NO and GABA interneurons, although the possibility that our antibody may detect CB1 receptors only at synthesis and transport sites cannot be ruled out. Our results are not in agreement with those of Farquhar-Smith et al. (2000), who have shown a different laminar distribution of CB1 receptor immunoreactivity with two bands at lamina I and at the border between laminae IIi and III, separated by a band of lower immunoreactivity at lamina IIo. This discrepancy may be accounted for by different immunocytochemical procedures and/or antibodies used. A spliced variant of the human CB1 receptor (CB1A), with a shorter N-terminal tail (Shire et al., 1995), has been described. Our antibody, directed against the N-terminal tail of the receptor, is unable to recognize the truncated form, while the antibody of Farquhar-Smith et al. (2000), directed against the C-terminal sequence, is able to recognize both CB1 and CB1A receptor isoforms. It would thus be possible that both CB1 receptor and CB1A are expressed in the dorsal horn with a different laminar distribution. Moreover, if these two isoforms exist their different laminar distribution suggests that they would control different primary afferent terminals. Interestingly, anandamide and tetrahydrocannabinol seem to act at different receptor sites to modulate endogenous opioids in a model of chronic pain (Smith et al., 1998), further suggesting a heterogeneity of cannabinoid binding sites in the spinal cord. The reexamination of cannabinoid receptor distribution in the DRG using several antibodies directed against different epitopes of the CB1 and CB2 receptors would shed light to answer this question.

The localization of the epitope recognized by the antibody also has important consequences for the subcellular localization of the receptor using immunocytochemistry. An epitope localized at the N-terminal part of the receptor would be poorly detected when the receptor is expressed at the plasma membrane since this would require the penetration of the antibody in the extracellular space, a very narrow compartment (Zerari et al., 1998). Consequently, the present subcellular study may drastically underestimate the plasma membrane localization of CB1 receptors and may reflect principally synthesis, transport and storage sites. A fast turnover of the receptor may also be responsible for a low detection at the plasma membrane and indeed the spinal cannabinoid system is tonically active (Martin et al., 1999a). Whereas CB1 receptors are synthesized in numerous DRG cells (the present study and Hohmann and Herkenham, 1999a), transported centrally (Hohmann and Herkenham, 1998, 1999b), the number of labeled axon terminals observed here is lower than one might expect. It has to be noted however that a relatively low level of capsaicin-sensitive binding sites (16%) was observed by Hohmann and Herkenham (1998). Ong and Mackie (1999a), using the same antibody, also described an exclusively postsynaptic localization for CB1 receptors in the primate spinal cord. Moreover, recent results on the vanilloid VR1 receptor showed the same discrepancy between cell body (in DRG) and central terminal (in the dorsal horn) expression of the receptor (Valtschanoff et al., 2001). This absence, or at least low immunoreactivity, in central axon terminals could perhaps be explained by a preferential addressing of VR1 receptors to the periphery (Guo et al., 1999).

CONCLUSION

We have shown the presence of CB1 receptor-like immunoreactivity on heterogeneous primary afferent DRG cells suggesting a presynaptic control of different sensory messages, including nociceptive, by cannabinoids. A postsynaptic control of nociceptive information is achieved by cannabinoid modulation of GABA and/or NO lamina II islet cells. Comparison of our results with data in the literature suggests that a CB1A receptor variant with a truncated N-terminal tail exists in the rat dorsal horn.

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REFERENCES

Ahluwalia, J., Urban, L., Capogna, M., Bevan, S., Nagy, I., 2000. Cannabinoid 1 receptors are expressed in nociceptive primary sensory neurons. Neuroscience 100, 685-688.

Aimar, P., Pasti, L., Carmignoto, G., Merighi, A., 1998. Nitric oxide-producing islet cells modulates the release of sensory neuropeptides in the rat substantia gelatinosa. J. Neurosci. 18, 10375-10388.

Auclair, N., Otani, S., Soubrie, P., Cre¨pel, F., 2000. Cannabinoids modulate synaptic strength and plasticity at glutamatergic synapses of rat prefrontal cortex pyramidal neurons. J. Neurophysiol. 83, 3287-3293.

Barber, R.P., Vaughn, J.E., Roberts, E., 1982. The cytoachitecture of GABAergic neurons in rat spinal cord. Brain Res. 238, 305-328.

Bernardi, P.S., Valtschano¡, J.G., Weinberg, R.J., Schmidt, H.H., Rustioni, A., 1995. Synaptic interactions between primary a¡erent terminals and GABA and nitric oxide-synthesizing neurons in super¢cial laminae of the rat spinal cord. J. Neurosci. 15, 1363-1371.

Bloom, J.M., Dewey, W.L., Harris, L.S., Brosius, K.K., 1977. 9-nor-9beta-Hydroxyhexahydrocannabinol, a cannabinoid with potent antinociceptive activity : comparison aith morphine. Physiol. Rev. 67, 67-186.

Buxbaum, D.M., 1972. Analgesic activity of 9-tetrahydrocannabinol in the rat and mouse. Psychopharmacology 25, 275-280.

Calignano, A., La Rana, G., Giu¡rida, A., Piomelli, D., 1998. Control of pain initiation by endogenous cannabinoids. Nature 394, 277-281.

Devane, W.A., Dysarz, F.A., Johnson Melvin, L.S., Howlett, A.C., 1988. Determination and characterization of a cannabinoid receptor in rat brain. Mol. Pharmacol. 34, 605-613.

Dove-Pettit, D.A., Harrison, M.P., Olson, J.M., Spencer, R.F., Cabral, G.A., 1998. Immunohistochemical localization of the neural cannabinoid receptor in rat brain. J. Neurosci. Res. 51, 391-402.

Egertova, M., Elphick, M.R., 2000. Localisation of cannabinoid receptors in the rat brain using antibodies to the intracellular C-terminal tail of CB(1). J. Comp. Neurol. 422, 159-171.

Farquhar-Smith, W.P., Egertova, M., Bradury, E.J., McMahon, S.B., Rice, A.S.C., Elphick, M.R., 2000. Cannabinoid CB1 receptor expression in rat spinal cord. Mol. Cell. Neurosci. 15, 510-521.

Glass, M., Dragunow, M., Faull, R.L., 1997. Cannabinoid receptors in the human brain: a detailed anatomical and quantitative autoradiographic study in the fetal, neonatal and adult human brain. Neuroscience 77, 299-318.

Glass, M., Dragunow, M., Faull, R.L., 2000. The pattern of neurodegeneration in Huntington's disease: a comparative study of cannabinoid, dopamine, adenosine and GABA(A) receptor alterations in the human basal ganglia in Huntington's disease. Neuroscience 97, 505-519.

Gobel, S., Falls, W.M., Bennett, G.J., Abdelmoumene, M., Hayashi, H., Humprey, E., 1980. An EM analysis of the synaptic connections of horseradish peroxidase ¢lled stalked cells and islet cells in the substantia gelatinosa of adult cat spinal cord. J. Comp. Neurol. 194, 781-807.

Guo, A., Vulchanova, L., Wang, J., Li, X., Elde, R., 1999. Immunocytochemical localization of the vanilloid receptor 1 (VR1): relationship to neuropeptides, the P2X3 purinoceptor and IB4 binding sites. Eur. J. Neurosci. 11, 946-958.

Hajos, N., Katona, I., Naiem, S.S., Mackie, K., Ledent, C., Mody, I., Freund, T.F., 2000. Cannabinoids inhibit hippocampal GABAergic transmission and network oscillations. Eur. J. Neurosci. 12, 3239-3249.
Herkenham, M., Lynn, A.B., Johnson, M.R., Melvin, S., de Costa, B.R., Rice, K.C., 1991. Characterization and localization of cannabinoid receptors in the rat brain: a quantitative in vitro autoradiographic study. J. Neurosci. 11, 563-583.
Herzberg, U., Eliav, E., Bennett, G.J., Kopin, I.J., 1997. The analgesic e¡ect of R(+)-WIN 55,212-2 mesylate, a high a¤nity cannabinoid agonist, in a rat model of neuropathic pain. Neurosci. Lett. 221, 157-160.
Hohmann, A.G., Briley, E.M., Herkenham, M., 1999. Pre- and postsynaptic distribution of cannabinoid and mu opioid receptors in rat spinal cord. Brain Res. 822, 17-25.
Hohmann, A.G., Herkenham, M., 1998. Regulation of cannabinoid and mu opioid receptors in rat lumbar spinal cord following neonatal capsaicin treatment. Neurosci. Lett. 252, 13-16.
Hohmann, A.G., Herkenham, M., 1999a. Localization of central cannabinoid CB1 receptor messenger RNA in neuronal subpopulations of rat dorsal root ganglia a double-label in situ hybridization study. Neuroscience 90, 923-931.
Hohmann, A.G., Herkenham, M., 1999b. Cannabinoid receptors undergo axonal £ow in sensory nerves. Neuroscience 92, 1171-1175.
Hohmann, A.G., Herkenham, M., 2000. Localization of cannabinoid CB(1) receptor mRNA in neuronal subpopulations of rat striatum: a double label in situ hybridization study. Synapse 37, 71-80.
Hohmann, A.G., Martin, W.J., Tsou, K., Walker, J.M., 1995. Inhibition of noxious stimulus-evoked activity of spinal cord dorsal horn neurons by the cannabinoid WIN 55,212-2. Life Sci. 56, 2111-2118.
Hohmann, A.G., Tsou, K., Walker, J.M., 1998. Cannbinoid modulation of wide-dynamic range neurons in the lumbar dorsal horn of the rat by spinal administered WIN55,212-2. Neurosci. Lett. 257, 119-122.
Jaggar, S.I., Sellaturay, S., Rice, A.S., 1998. The endogenous cannabinoid anandamide, but not the CB2 ligand palmitoylethanolamide, prevents the viscero-visceral hyper-re£exia associated with in£ammation of the rat urinary bladder. Neurosci. Lett. 253, 123-126.
Katona, I., Sperlagh, B., Sik, A., Kafalvi, A., Vizi, E.S., Mackie, K., Freund, T.F., 1999. Presynaptically located CB1 cannabinoid receptors regulate GABA release from axon terminals of speci¢c hippocampal interneurons. J. Neurosci. 19, 4544-4558.
Magoul, R., Onteniente, B., Ge¡ard, M., Calas, A., 1987. Anatomical distribution and ultrastructural organization of the GABAergic system in the rat spinal cord. Neuroscience 20, 1001-1009.
Mailleux, P., Vanderhaeghen, J.J., 1992. Distribution of neuronal cannabinoid receptor in the adult rat brain: a comparative receptor binding radioautography and in situ hybridization histochemistry. Neuroscience 48, 655-668.
Malcangio, M., Bowery, N.G., 1996. GABA and its receptors in the spinal cord. Trends Pharmacol. Sci. 17, 457-462.
Marsicano, G., Lutz, B., 1999. Expression of the cannabinoid receptor CB1 in distinct neuronal subpopulations in the adult mouse forebrain. Eur. J. Neurosci. 11, 4213-4225.
Martin, W.J., Co¤n, P.O., Attias, E., Balinsky, M., Tsou, K., Walker, J.M., 1999a. Anatomical basis for cannabinoid-induced antinociception as revealed by intracerebral microinjections. Brain Res. 822, 237-242.
Martin, W.J., Gupta, N.K., Loo, C.M., Rohde, D.S., Basbaum, A.I., 1999b. Di¡erential e¡ects of neurotoxic destruction of descending noradrenergic pathways on acute and persistent nociceptive processing. Pain 80, 57-65.
Martin, W.J., Lai, N.K., Patrick, S.L., Tsou, K., Walker, J.M., 1993. Antinociceptive actions of cannabinoids following intraventricular administration in rats. Brain Res. 629, 300-304.
Martin, W.J., Loo, C.M., Basbaum, A.I., 1999c. Spinal cannabinoids are anti-allodynic in rats with persistent in£ammation. Pain 82, 199-205.
Martin, W.J., Patrick, S.L., Co¤n, P.O., Tsou, K., Walker, J.M., 1995. An examination of the central sites of action of cannabinoid-induced antinociception in the rat. Life Sci. 56, 2103-2109.
Matsuda, L.A., Bonner, T.I., Lolait, S.J., 1993. Localization of cannabinoid receptor mRNA in rat brain. J. Comp. Neurol. 327, 535-550.

Matsuda, L.A., Lolait, S.J., Brownstein, M.J., Young, A.C., Bonner, T.I., 1990. Structure of a cannabinoid receptor and functional expression of the cloned cDNA. Nature 346, 561-564.
Meller, S.T., Dykstra, C., Gebhart, G.F., 1992. Production of endogenous nitric oxide and activation of soluble guanylate cyclase are required for N-methyl-D-aspartate-produced facilitation of the nociceptive tail-£ick re£ex. Eur. J. Pharmacol. 214, 93-96.
Moore, P.K., Oluyomi, A.O., Babbedge, R.C., Wallace, P., Hart, S.L., 1991. L-NG-nitro arginine methyl ester exhibits antinociceptive activity in the mouse. Br. J. Pharmacol. 102, 198-202.
Moss, D.E., Johnson, R.L., 1980. Tonic analgesic effects of delta 9-tetrahydrocannabinol as measured with the formalin test. Eur. J. Pharmacol. 61, 313-315.
Munro, S., Thomas, K.L., Abu-Shaar, M., 1993. Molecular characterization of a peripheral receptor for cannabinoids. Nature 365, 61-65.
Nahin, R.I., Madsen, A.M., Giesler, G.J., 1983. Anatomical and physiological studies of the gray matter surrounding the spinal cord central canal. J. Comp. Neurol. 220, 321-335.
Ong, W.Y., Mackie, K., 1999a. A light and electron microscopic study of the CB1 cannabinoid receptor in the primate spinal cord. J. Neurocytol. 28, 39-45.
Ong, W.Y., Mackie, K., 1999b. A light and electron microscopic study of the CB1 cannabinoid receptor in primate brain. Neuroscience 92, 1177-1191.
Richardson, J.D., Aanonsen, L., Hargeaves, K.M., 1998a. Anhyperalgesic e¡ects of spinal cannabinoids. Eur. J. Pharmacol. 345, 145-153.
Richardson, J.D., Aanonsen, L., Hargreaves, K.M., 1998b. Hypoactivity of the spinal cannabinoid system results in NMDA-dependent hyperalgesia. J. Neurosci. 18, 451-457.
Shire, D., Calandra, B., Rinaldi-Carmona, M., Oustic, D., Pessegue, B., Bonnin-Cabanne, O., Le Fur, G., Caput, D., Ferrara, P., 1996. Molecular cloning, expression and function of the murine CB2 peripheral cannabinoid receptor. Biochim. Biophys. Acta 1307, 132-136.
Shire, D., Carillon, C., Kaghad, M., Calandra, B., Rinaldi-Carmona, M., Le Fur, G., Caput, D., Ferrara, P., 1995. An amino-terminal variant of the central cannabinoid receptor resulting from alternative splicing. J. Biol. Chem. 270, 3726-3731.
Smith, F.L., Fujimori, K., Lowe, J., Welch, S.P., 1998. Characterization of delta9-tetrahydrocannabinol and anandamide antinociception in nonarthritic and arthritic rats. Pharmacol. Biochem. Behav. 60, 183-191.
Smith, P.B., Martin, B.R., 1992. Spinal mechanisms of delta 9-tetrahydrocannabinol-induced analgesia. Brain Res. 578, 8-12.
Thomas, B.F., Wei, X., Martin, B.R., 1992. Characterization and autoradiographic localization of the cannabinoid binding site in rat brain using [3H]11-OH-delta 9-THC-DMH. J. Pharmacol. Exp. Ther. 263, 1383-1390.
Todd, A.J., 1988. Electron microscope study of Golgi-stained cells in lamina II of the rat spinal dorsal horn. J. Comp. Neurol. 275, 145-157.
Todd, A.J., McKenzie, J., 1989. GABA-immunoreactive neurons in the dorsal horn of the rat spinal cord. Neuroscience 31, 799-806.
Tsou, K., Brown, M.C., Sanudo-Pena, M.C., Mackie, K., Walker, J.M., 1998. Immunohistochemical distrubution of cannabinoid CB1 receptors in the rat central nervous system. Neuroscience 83, 393-411.
Tsou, K., Lowitz, K.A., Hohmann, A.G., Martin, W.J., Hathaway, C.B., Bereiter, D.A., Walker, J.M., 1996. Suppression of noxious stimulusevoked expression of Fos protein-like immunoreactivity in rat spinal cord by a selective cannabinoid agonist. Neuroscience 70, 791-798.
Tsou, K., Mackie, K., Sanudo-Pena, M.C., Walker, J.M., 1999. Cannabinoid CB1 receptors are localized primarily on cholecystokinin-containing GABAergic interneurons in the rat hippocampal formation. Neuroscience 93, 969-975.
Twitchell, W., Brown, S., Mackie, K., 1997. Cannabinoids inhibit N- and P/Q-type calcium channels in cultured rat hippocampal neurons. J. Neurophysiol. 78, 43-50.
Valtschano¡, J.G., Rustioni, A., Guo, A., Hwang, S.J., 2001. Vanilloid receptor VR1 is both presynaptic and postsynaptic in the superficial laminae of the rat dorsal horn. J. Comp. Neurol. 436, 225-235.
Valtschanoff, J.G., Weinberg, R.J., Rustioni, A., 1992a. NADPH diaphorase in the spinal cord of rats. J. Comp. Neurol. 321, 209-222.

Valtschanoff, J.G., Weinberg, R.J., Rustioni, A., Schmidt, H.H., 1992b. Nitric oxide synthase and GABA colocalize in lamina II of rat spinal cord. Neurosci. Lett. 148, 6-10.
Vizzard, M.A., Erdman, S.L., Erickson, V.L., Stewart, R.J., Roppolo, J.R., de Groat, W.C., 1994a. Localization of NADPH diaphorase in the lumbosacral spinal cord and dorsal root ganglia of the cat. J. Comp. Neurol. 339, 62-75.
Vizzard, M.A., Erdman, S.L., Roppolo, J.R., Forstermann, U., de Groat, W.C., 1994b. Di¡erential localization of neuronal nitric oxide synthase immunoreactivity and NADPH-diaphorase activity in the cat spinal cord. Cell Tissue Res. 278, 299-309.
Welch, S.P., Hu¡man, J.W., Lowe, J., 1998. Differential blockade of the antinociceptive effects of centrally administered cannabinoids by SR141716A. J. Pharmacol. Exp. Ther. 286, 1301-1308.
Zerari, F., Fischer, J., Sagot, M.A., Frobert, Y., Couraud, J.-Y., Conrath, M., 1998. Substance P receptor immunodetection in the spinal cord: comparative use of direct anti-receptor antibody and anti-complementary peptide antibody. Brain Res. Bull. 46, 263-268.
Zimmer, A., Zimmer, A.M., Hohmann, A.G., Herkenham, M., Bonner, T.I., 1999. Increased mortality, hypoactivity, and hypoalgesia in cannabinoid CB1 receptor knockout mice. Proc. Natl. Acad. Sci. USA 96, 5780-5785.