CB1 cannabinoid receptors in amphibian spinal cord; relationships with some nociception markers.

CB1 cannabinoid receptors in amphibian spinal cord: relationships with some nociception markers

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Abstract
The role of cannabinoids in spinal analgesia has so far been investigated in mammals and the interactions between cannabinoid receptors and markers involved in nociception have been described in the rat spinal cord. An endocannabinoid system is well developed also in the amphibian brain. However, the anatomical substrates of pain modulation have been scarcely investigated in anamniotes, neither is there reference to such a role for cannabinoids in lower vertebrates. In the present paper we employed multiple cytochemical approaches to study the distribution of CB1 cannabinoid receptors and their morphofunctional relationships with some nociception markers (i.e. Substance P, nitric oxide synthase, GABA and m opioid receptors) in the spinal cord of the anuran amphibian Xenopus laevis . We found a co-distribution of CB1 receptors with the aforementioned signaling molecules, as well as a more limited cellular co-localization, in the dorsal and central fields of the spinal cord. These regions correspond to the mammalian laminae I-IV and X, respectively, areas strongly involved in spinal analgesia. Comparison of these results with those previously obtained in the mammalian spinal cord, reveals a number of similarities between the two systems and suggests that cannabinoids might participate in the control of pain sensitivity also in the amphibian spinal cord.

Keywords: Double labeling; Immunohistochemistry; Indirect immunofluorescence; In situ hybridization; Histochemistry; Comparative neuroanatomy; Xenopus laevis

Abbreviations: cc, central canal; cf, central field; df, dorsal field; lf, lateral field; Lt, Lissauer's tract; vf, ventral field.

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1. Introduction
Since Adrian's classic investigations (Adrian, 1926, 1928), providing the first electrophysiological support to the morphological studies correlating frog skin free nerve endings with painful sensations, a number of reports on peripheral pain receptors in anurans have been published (for a review, see Spray, 1976). Nevertheless, there is little information on the mechanisms of analgesic agents in amphibians. Pharmacological and behavioural studies have demonstrated a spinal site of opioid analgesia in amphibians (Stevens, 1996) and, recently, a unique opioid receptor, mediating the action of m, k and d opioid agonists, has been hypothesized (Newman et al., 2000). However, the chemical neuroanatomy of the analgesic spinal circuitry in these vertebrates remains largely unexplored. Since the characterization and cloning of the G protein coupled CB1 cannabinoid receptor in the mammalian brain (Devane et al., 1988; Matsuda et al.,
1990) and the discovery of endogenous ligands (endocannabinoids) such as anandamide (Devane et al., 1992) and 2-arachidonoylglycerol (Bisogno et al., 1997), much information has been gathered on the brain cannabinoid organization in both mammals (Ge´rard et al., 1990, 1991; Chakrabarti et al., 1995; Abood andMartin, 1996; Gebremedhin et al., 1999; Murphy et al., 2001) and non mammalian vertebrates (Howlett et al., 1990; Van der Kloot, 1994; Yamaguchi et al., 1996;

Soderstrom et al., 2000; Cesa et al., 2001), suggesting a high degree of conservation of this system during phylogeny. The role of cannabinoids in mammalian pain modulation at the spinal level was demonstrated by different approaches (behavioural studies: Buxbaum, 1972; Bloom et al., 1977; Moss and Johnson, 1980; Martin et al., 1993, 1995; Edsall et al., 1996; pharmacological and physiological investigations: Smith and Martin, 1992; Tsou et al., 1996; neuroanatomical studies: Ong and Mackie, 1999; San˜udo-Pen˜a et al., 1999; Farquhar-Smith et al., 2000). Moreover, in the dorsal horn of the rat spinal cord, CB1 is expressed in pre- and post-synaptic sites
(San˜udo-Pen˜a et al., 1999; Farquhar-Smith et al., 2000; Salio et al., 2002), the latter being interneurons coexpressing GABA and/or nitric oxide (NO; Salio et al., 2002), and m opioid receptors (MOR) (Salio et al., 2001a).
While the occurrence of CB1 receptors in the brain of the clawed toad Xenopus laevis (Anuran, Amphibian) has been already demonstrated by Cesa et al. (2001), data on their expression and distribution in the spinal cord are still lacking. Since the scarce differentiation of the cellular groups, the anuran spinal cord has been subdivided into primary afferent terminal fields (Ten Donkelaar, 1998) instead of applying the higher vertebrate lamination (Rexed, 1952). The dorsal field, a projection site of cutaneous afferences (Jhaveri and Frank, 1983; Sze`kely and Antal, 1984), roughly corresponds to laminae I_/IV of the mammalian dorsal horn (although a substantia gelatinosa is difficult to delimit). On entering the spinal cord, dorsal root fibres segregate into a medially placed component directed to the dorsal funiculus and a lateral bundle situated in the dorsal part of the lateral funiculus which, for its position, presumably represents the anuran homologue of the mammalian Lissauer's tract (Nikundiwe et al., 1982). The central field surrounding the central canal, corresponding to mammalian lamina X, is composed of ependymal cells and fusiform/pyriform neurons which send their dendrites into the adjacent fields and contribute to ascending spinal cord projections (Ten Donkelaar, 1998).
In the present paper, the distribution of CB1 cannabinoid receptors was analyzed in the dorsal and central fields of the spinal cord of the anuran amphibian X. laevis . Moreover, the morphological relationships between CB1 receptors and some molecules known in mammals as nociception markers were investigated. In particular, co-distribution and co-localization of CB1 with: (1) Substance P (SP), (2) nitric oxide synthase (NOS), the biosynthetic enzyme of NO, (3) GABA and its biosynthetic enzyme glutamic acid decarboxylase (GAD), and (4) MOR were analyzed. Double labeling experiments, such as double simultaneous indirect immunofluorescence (IFL) (for CB1/SP and CB1/GABA), as well as NADPH diaphorase (NADPHd) histochemistry to detect NOS plus immunohistochemistry for CB1, were performed on the same tissue sections. In order to improve the resolution of colocalization studies, selected double IFL labelings were examined with a confocal laser-scanning microscope (CLSM). Since CB1 mRNA distribution in the spinal cord, as revealed by in situ hybridization (ISH), was found to overlap CB1 immunoreactivity (Cottone, personal communication), in some experiments the CB1 ISH procedure was coupled to the immunohistochemistry for SP, GAD and MOR.

2. Materials and methods

The experiments were performed on five adult female X. laevis , in agreement with the Italian law for animal welfare. Animals deeply anaesthetized with 1/1000 tricaine methanesulphonate (MS222; Sandoz, Switzerland) were intracardially perfused with 20 ml of Ringer solution followed by 80 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4. Spinal cords were rapidly dissected out, post-fixed for 2 h in the same fixative at 4 8C, cryoprotected and then frozen in liquid isopentane. Coronal sections (12 mm thick), cut with a cryostat at different levels of the spinal cord, were mounted on gelatine- or 3-aminopropyl-triethoxysilan (TESPA)-coated slides and stored at -20 8C until use.

2.1. CB1 immunohistochemistry (BAS)

The CB1 immunostaining was performed by using an affinity-purified polyclonal anti-CB1 antibody which was 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). Spinal cord sections were washed in 0.01 M phosphate buffer saline (PBS), pH 7.4, then preincubated 30 min in PBS containing 0.1% Triton-X-100 (TX) and normal goat serum (1/100). Sections were incubated

overnight (O/N) with the rabbit anti-CB1 receptor antibody diluted (1/1000) in PBS-TX. After washing in PBS, sections were incubated with a biotinylated goat anti-rabbit IgG (1/200) (Vector, Burlingame, USA) for 1 h. Sections were then washed in PBS and incubated for 45 min with the avidin-biotinylated-peroxidase complex (Vector), diluted (1/100) in PBS. After washing in 0.05 M Tris-HCl, pH 7.4, sections were incubated (5-7 min, 20 8C) in the same buffer supplemented with 0.03% (w/v) 3,3?-diaminobenzidine (DAB, Sigma, France) and 0.01% (v/v) hydrogen peroxide. Sections, washed in PBS and mounted in an aqueous mounting medium, were then observed with a light microscope Axioskop Zeiss and photographed.

2.2. Double labeling experiments

2.2.1. Double simultaneous IFL

Spinal cord sections were incubated O/N in a mixture of rabbit polyclonal anti-CB1 antibody and mouse monoclonal anti-SP antibody (Couraud et al., 1987) or mouse monoclonal anti-GABA antibody (Sigma), each one diluted 1/1000 in PBS TX. After washing in PBS, sections were incubated 1 h in a mixture of anti-rabbit IgG secondary antisera conjugated to cyanin 3 (CY3, 1/500; Sigma) and anti-mouse IgG conjugated to fluorescein isothiocyanate (FITC, 1/40; Chemicon International, USA). Sections were then rinsed twice in PBS, postfixed 10 min in 4% paraformaldehyde, washed in PBS and mounted in an aqueous mounting medium. The observation with a CLSM was performed by using a TCS-4D confocal imaging system (Leica Instrument, Germany), equipped with an argon-krypton ion laser. Sections were observed with a Leica plan apochromat X 40 oil immersion objective with a numerical aperture of 1.4.

2.2.2. ISH plus immunohistochemistry (BAS)

Sections were previously treated for ISH procedure and then processed for immunohistochemistry. Sections were rehydrated in sterile PBS 5 min, incubated 5 min, 37 8C, with 5 mg/ml Proteinase K, washed 10 min in a 0.1 M Tris-HCl, 0.1 M glycine solution, pH 7.5, then acetylated 10 min in 0.25% acetic anhydride, 0.1 M triethanolamine, pH 8 and neutralized 10 min in 0.1 M Tris-HCl, 0.1 M glycine, pH 7.5. Afterwards, sections were washed in 2X SSC, then incubated O/N at 42 8C in the hybridization buffer (50% deionized formamide, 2X SSC, 250 mg/ml yeast tRNA, 100 mg/ml heat-denatured herring sperm DNA, 5X Denhardt's reagent, 20 U RNase inhibitor), containing 150 ng/ml digoxigenin-labeled RNA probe. CB1 antisense RNA probe, complementary to a cloned region (650 bp) of X. laevis CB1 mRNA (GenBank accession number AF484157), was produced by digestion of pGEM-CB1Xen recombinant plasmid with NotI restriction enzyme and in vitro transcription using T7 RNA polymerase and the non-radioactive, digoxigeninbased system DIG RNA Labeling kit (SP6/T7) (Roche, Germany). An antisense RNA probe, suitable as positive control for ISH, was produced from pSP65-Ribo, containing the cDNA complementary to rat 28S ribosomal RNA, by digestion with BamHI and in vitro transcription with SP6 RNA polymerase. An antisense RNA probe to be used as a negative control was produced from pSPT18-Neo, containing the bacterial Neomycin phosphotransferase gene, by cutting with PvuII and in vitro transcription with T7 RNA polymerase.

After hybridization, sections were washed 15 min in 2/ SSC, room temperature (RT), then treated 30 min, 37 8C, with 20 mg/ml RNase A in 10 mM Tris-HCl, 0.5 M NaCl, pH 8; they were washed 30 min, 37 8C, in 10 mM Tris-HCl, 0.5 M NaCl, pH 8, then 10 min, 65 8C, in 0.1/ SSC, and finally 10 min in 0.1 SSC, RT. Labeling was revealed using DIG Nucleic Acid Detection kit (Roche). Briefly, the sections were washed, RT, in Buffer 1 (0.1 M maleic acid, 0.15 M NaCl, pH 7.5) containing 0.3% Tween-20, incubated 30 min in Buffer 2 (1% blocking reagent in Buffer 1), then incubated 30 min with anti-digoxigenin Fab fragments conjugated with alkaline phosphatase (1/5000 in Buffer 2). After washing in Buffer 1 (2_/15 min) and then 2 min in Buffer 3 (100 mM Tris_/HCl, 100 mM NaCl, 50 mM MgCl2, pH 9.5), the sections were incubated with freshly prepared color-substrate solution (NBT/BCIP in Buffer 3) and color development was stopped, after about 2.5 h by washing in Buffer 4 (10 mM Tris_/HCl, 1 mM EDTA, pH 8). Sections were then washed inPBS and incubated O/Nin one of the following primary antisera, diluted 1/1000 in PBS-TX: mouse monoclonal anti-GAD antibody (Chemicon International), mouse monoclonal anti-SP antibody (Couraud et al., 1987) or rabbit polyclonal anti-MOR antibody (DiaSorin, USA). Sections were processed following the BAS protocol, as described above.

2.2.3. Histochemistry for NADPHd plus CB1 immunohistochemistry (BAS)

Sections were washed in PBS and then incubated 60-90 min, 37 8C in PB 0.1 M, pH 7.4, containing 0.3% TX, 0.1 mg/ml nitro blue tetrazolium (NBT) and 1 mg/ ml bNADPH. Sections were then washed in PBS and incubated with the rabbit anti-CB1 receptor antibody, diluted 1/1000 in PBS-TX and processed following the BAS protocol described above.

2.3. Controls

Preabsorption of the diluted anti-CB1 antibody (1/1000) with 1 mg/ml of the immunizing fusion protein for 3 h at RT before addition onto the sections, totally abolished the immunostaining.

Omission of the primary antibodies (CB1, SP, GABA, GAD, MOR) in the incubation medium resulted in a complete lack of immunostaining. Procedure accuracy of ISH was confirmed by the presence of a cytoplasmic labeling in all cell bodies in sections incubated with the antisense probe complementary to 28S rRNA, while the specificity of the reaction was confirmed by the absence of any labeling when using the antisense probe complementary to bacterial Neomycin phosphotransferase mRNA.

3. Results

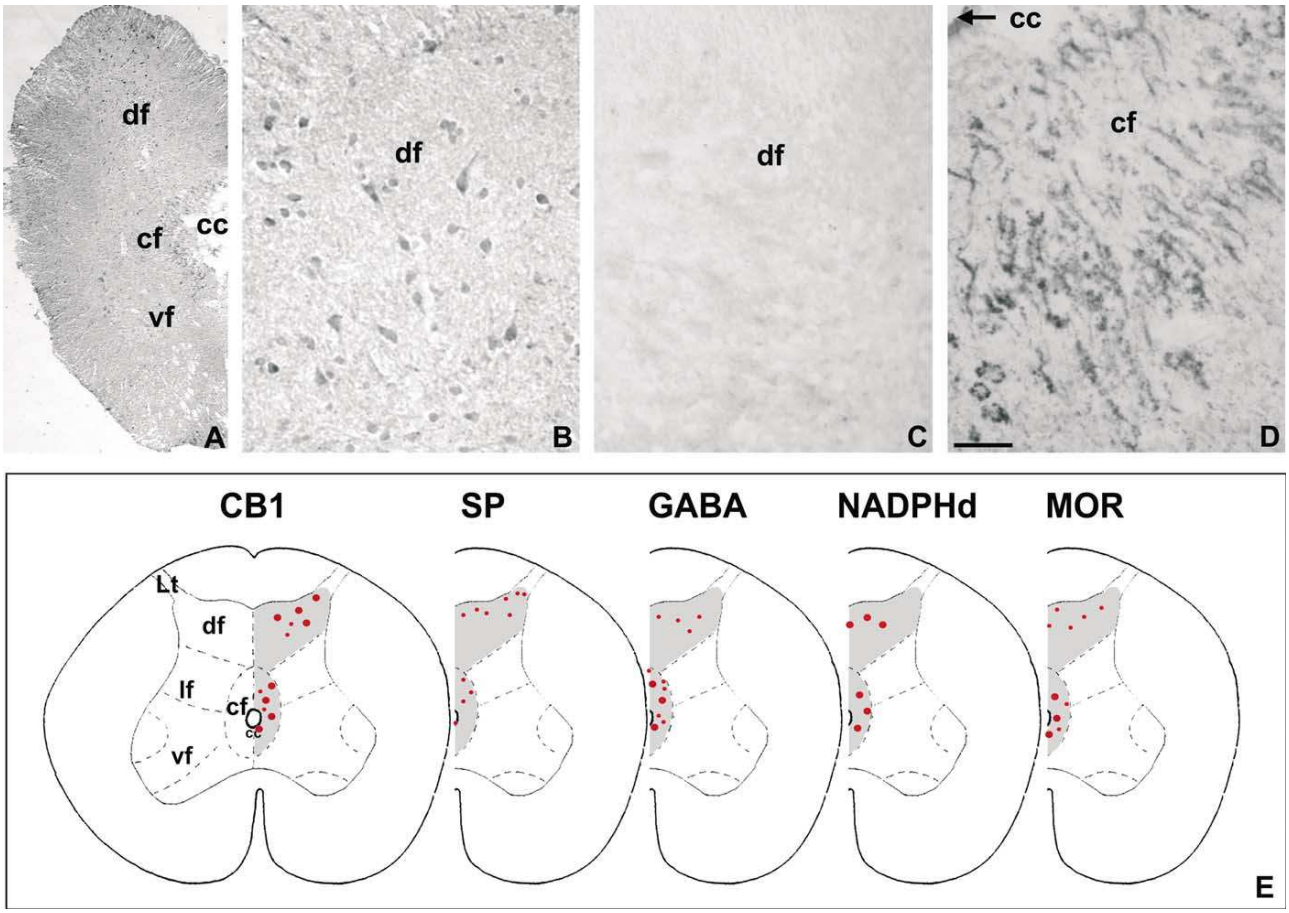

Fig. 1. (A, B, C, D) CB1-LI-IR in X. laevis spinal cord. (A) A low magnification of a transverse emisection of the spinal cord, showing numerous CB1-LI immunoreactive cell bodies dispersed throughout the dorsal and the central fields. Some positive cell bodies are also seen in the ventral field. (B) CB1-LI immunolabeled perikarya in the dorsal field. (C) A section adjacent to B, processed for specificity control protocol with preabsorbed primary antibody does not show any labeling. (D) CB1-.LI immunopositive neurons in the central field. Calibration bar: 280 mm in A, 100 mm in B, C, D. (E) Schematic representation of CB1, SP, GABA, NOS (NADPHd) and MOR distributions, restricted to the dorsal and central fields (shadowed), in five consecutive coronal sections of Xenopus spinal cord. Large dots: neurons; small dots: nerve terminals.

A number of CB1-like immunoreactive neurons were distributed throughout the grey matter of the spinal cord (Fig. 1A). In the dorsal field, small and medium sized (5-10 mm), unipolar 'tufted' or bipolar neurons, showing CB1_/LI_/IR of variable intensity, were observed. These neurons sent their dendritic processes in ventral and medial directions (Fig. 1B). In the central field numerous CB1-immunostained, generally pearshaped neurons (15-20 mm) were arranged in rows surrounding the central canal (Fig. 1D). The immunoreactive material contained in their perikarya and dendritic stumps included very fine dots or granules (well appreciable after both BAS, Fig. 1D, and IFL techniques, Fig. 2E) and their dendrites were directed toward the adjacent fields. By contrast, no immunolabeling was present in the dorsolateral fasciculus or Lissauer's tract (Fig. 1A). Tissue sections processed for specificity control procedures did not show any labeling (Fig. 1C). The distributions of CB1, SP, GABA, NOS (NADPHd) and MOR, restricted to the dorsal and central fields, are schematically represented in five consecutive coronal sections of the Xenopus spinal cord (Fig. 1E). A semiquantitative evaluation of the neural structures positive for the different nociception markers is reported in Table 1.

3.1. CB1/SP

Serial transverse sections treated with double simultaneous IFL showed a partial co-distribution of CB1 and SP immunolabelings in both the superficial dorsal field and the central field surrounding the central canal. In the entry zone of the dorsal root fibres, where the SP-LI fibres were abundant

(Fig. 2A), CB1-LI-IR was scarce or absent (Fig. 2B).

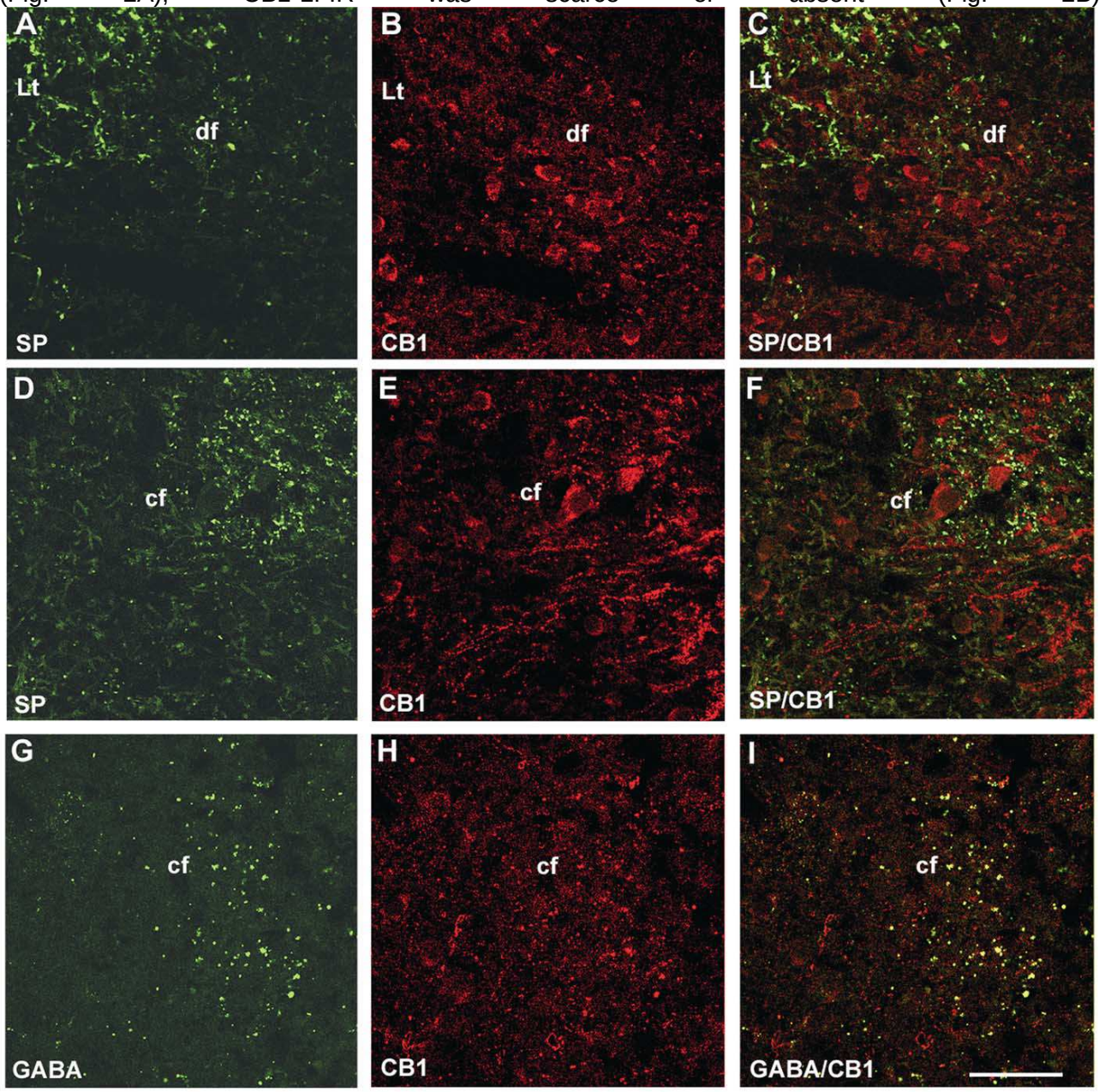

Fig. 2. (A_/I) Double simultaneous IFL in Xenopus spinal cord, analyzed with a CLSM. (A) SP-LI nerve fibres and terminals in the Lissauer's tract and superficial dorsal field (FITC). (B) CB1-LI cell bodies and terminals in the same section as A (CY3). (C) The two molecules co-distribute only in the superficial dorsal field. (D) A cluster of SP-LI terminals in the central field (FITC). (E) CB1-LI cell bodies and nerve processes in the same section as D (CY3). (F) The two molecules do not co-localize but a number of SP-LI punctuated terminals are in close contact with CB1-LI neurons. (G) GABAergic nerve terminals in the central field (FITC). (H) CB1-LI nerve terminals and processes in the same area as G (CY3). (I) Numerous varicose terminals co-contain the two molecules (yellow). (D, E, F, G, H, I) The central canal, at right in the picture, is not shown. Calibration bar: 70 mm.

(Fig. 2C). In the central field, too, some SP-immunos-In the superficial dorsal field, numerous SP-LI-positive varicose fibres were seen by CLSM to closely contact CB1-LI containing neurons tained terminals (Fig. 2D) were seen in contiguity to CB1 neurons (Fig. 2E and F). Some sections, previously treated following ISH procedure to reveal CB1 mRNA, were stained with the anti-SP antibody (BAS technique). SP positive beaded primary afferences projecting to the superficial dorsal field were seen to contact 'en passant' some CB1 mRNA containing neurons (Fig. 3A). In the

central field, numerous SP-immunolabeled buttons on CB1 positive neurons were observed (Fig. 3B).

Table 1
Semiquantitative evaluation of the neural structures positive for the different nociception markers in X. laevis spinal cord

Spinal distribution		Nociception markers				
		CB1	SP	GABA	NADPHd	MOR
Dorsal field	Nerve terminals	++	+++	++	+++	-
	Cell bodies	++++	-	++	++	-
Central field	Nerve terminals	+++	++	++	+	++
	Cell bodies	++	-	+	+	++

3.2. CB1/GABA (OR GAD)

Using double simultaneous IFL technique, nerve fibres positive for both anti-GABA and anti-CB1 antibodies were found to co-distribute in the dorsal (data not shown) and central fields (Fig. 2G and H). By CLSM we confirmed that numerous nerve terminals surrounding the central canal were simultaneously GABA- and CB1/LI-immunopositive (Fig. 2I). CB1 mRNA detection, followed by anti-GAD immunohistochemistry, revealed that some of the CB1 expressing neurons in the central field are also GAD□/LI-immunoreactive (Fig. 3C). By contrast, no co-localizations were observed in the dorsal field, where, on the other hand, the two innervations were largely co-distributed (data not shown).

3.3. CB1/MOR

Since an anti-MOR antibody raised in a heterologous species in respect to CB1 antibody was not available, spinal cord sections, previously treated to detect CB1 mRNA, were labeled with an anti-MOR antibody (BAS technique). A number of CB1 mRNA containing cells co-expressed MOR in the central field (Fig. 3D), while no co-localizations were found in the dorsal field (data not shown).

3.4. CB1/NADPHd

In the deepest dorsal field a number of medium sized, pear shaped neurons positive for NADPHd, were also immunostained for CB1 (Fig. 3E). Some neurons positive for both NADPHd and CB1 were also observed in the lateral part of the grey matter surrounding the central canal (Fig. 3F).

4. Discussion

In order to gain insight into the neuroanatomical substrates underlying pain transmission in the amphibian spinal cord, in the present paper we have studied the relationships between CB1 receptors and a number of markers involved in nociception, specifically SP, GABA, NOS and MOR, in X. laevis spinal cord. This study was carried out by coupling different cytochemical techniques, such as immunohistochemistry (BAS), IFL, histochemistry and ISH. The specificity of the antibody directed against the N terminus of the CB1 receptor used in these experiments was previously assessed in rat (Tsou et al., 1998) and Xenopus (Cesa et al., 2001) brains. In addition, the complete lack of immunostaining in the spinal cord of Xenopus after the preabsorption of the CB1 antibody with the immunizing protein has confirmed that the immunoreaction was specific for the CB1 receptor. The specificity of the other primary antibodies was previously demonstrated in amphibians (SP: Salio et al., 2001b; NOS: Artero et al., 1995; GABA: Barale et al., 1996) except for the anti-MOR antisera, whose specificity was assessed in mammals (Arvidsson et al., 1995). Moreover, after omission of the above mentioned primary antibodies, no immunostaining was present (data not shown). On the basis of the 98% correspondence between NADPHd histochemistry and NOS immunohistochemistry (Bru¨ning and Mayer, 2001) and because an anti-NOS antisera not raised in rabbit was not available, NAPDHd was assumed as NOergic innervation marker. The probe used in the ISH reaction for CB1 is complementary to a cloned region of the Xenopus CB1 mRNA (GenBank accession number AF484157) and nucleotide alignments with other known Xenopus sequences have not revealed significative homologies. In addition, ISH negative controls did not show any labeling (data not shown). Immunohistochemistry, autoradiography and histochemistry, combined in the same tissue section, or used as a single technique, have provided data on the distributions of various neuropeptides (e.g. Substance P, Met-enkephalin, somatostatin, galanine, corticotropin-releasing factor, calcitonin gene-related peptide, cholecystokinin; see for example Lorez and Kemali, 1981; Adli et al., 1988; Pieribone et al., 1994), as well as classic and putative neurotransmitters (e.g. GABA, serotonin, NOS; see for example Barale et al., 1996; Pieribone et al., 1994; Bru¨ning and Mayer, 2001) in the amphibian spinal cord.

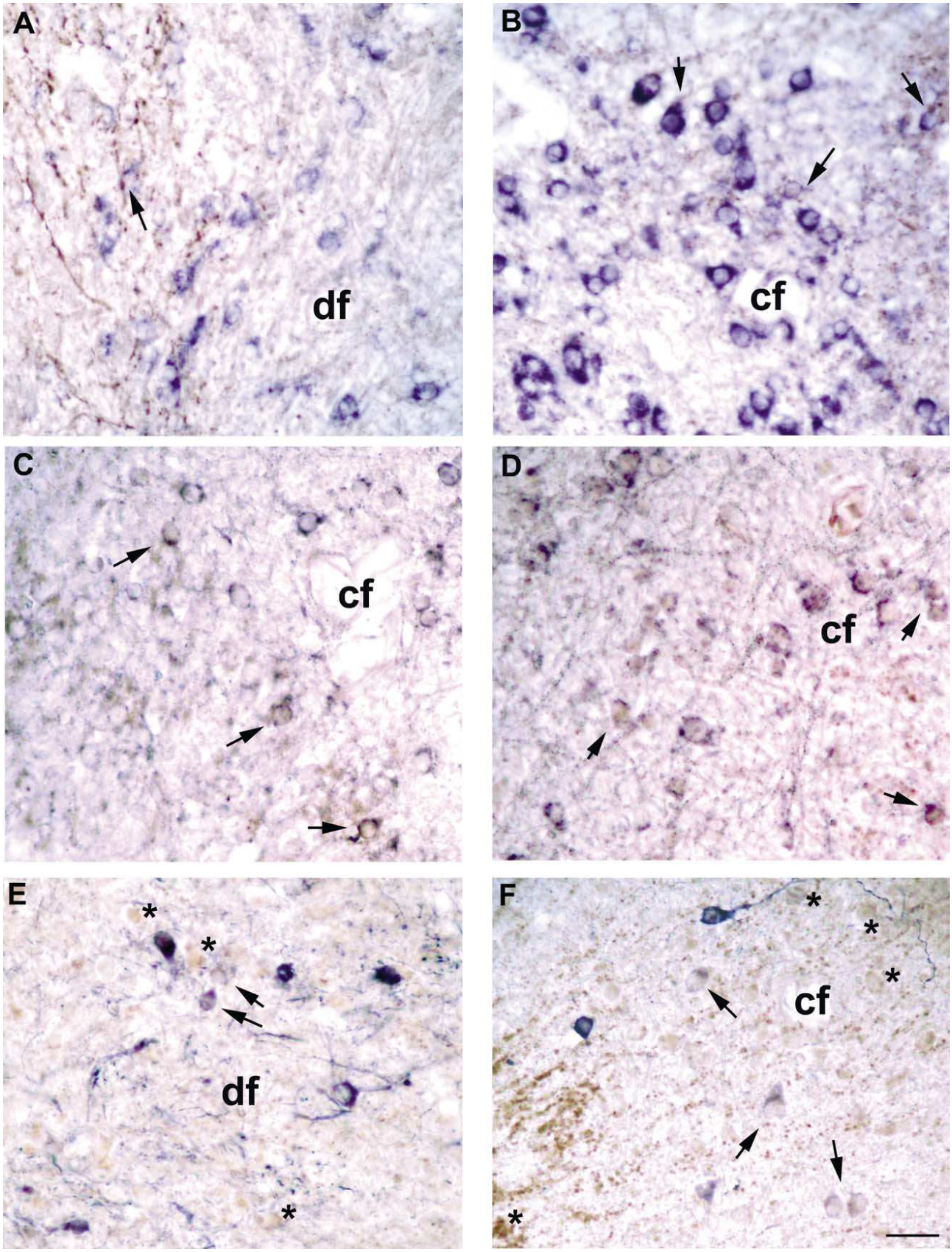

Fig. 3. (A, B) Double labeling CB1 mRNA/SP/LI/IR. (A) CB1 positive neurons (blue) are in contact with SP/LI varicose fibres and terminals (brown) in the superficial dorsal field (arrow). The Lissauer's tract, here not shown, is at top left. (B) Some neurons expressing CB1 mRNA and numerous SP/LI terminals are found in close contact (arrows). The central canal, at right, is not shown. (C) Double labeling CB1 mRNA/GAD/LI/IR. In the central field some neurons (arrows) co-contain CB1 mRNA and GAD/LI/IR. The central canal, at right, is not shown. (D) Double labeling

CB1 mRNA/MOR/LI/IR. Neurons (arrows) which co-express CB1 mRNA and MOR are found in the central field. The central canal, at right, is not shown. (E, F) Double labeling NADPHd/CB1□/LI□/IR. (E) NOS and CB1 co-localize in some neurons (arrows) of the dorsal field. A NOS positive nerve fibre (blue) seems to contact 'en passant' one of the CB1□/LI neurons (light brown, asterisks). (F) Some neurons co-containing NOS and CB1□/LI□/IR (arrows) are seen in the central field. Small CB1□/LI cell bodies are indicated by asterisks. The central canal, at bottom left, is not shown. Calibration bar: 50 mm.

Nevertheless, interactions between the different signaling systems and their functional implications have been scarcely analyzed, with the exception of the co-existence of serotonin with SP and galanine in Xenopus (Pieribone et al., 1994). Our results reveal that, in Xenopus spinal cord, CB1 receptors are expressed by small/medium sized cell bodies scattered throughout the dorsal field, as well as by numerous neurons in the central field. Such observations are in keeping with the description of CB1-LI-IR in spinal interneurons of rat (San˜udo-Pen˜a et al., 1999; Farquhar-Smith et al., 2000; Salio et al., 2002) and primates (Ong and Mackie, 1999). Since no labeling was observed in Xenopus neither in the spinal dorsal roots nor in the Lissauer's tract, it can be suggested that CB1 receptors are prevalently expressed by intrinsic neurons, in contrast with mammalian spinal cord, where CB1-LI-IR was described in both pre-synaptic (Tsou et al., 1998; San˜udo-Pen˜a et al., 1999; Salio et al., 2002) and post-synaptic sites (San˜udo-Pen˜a et al., 1999; Ong and Mackie, 1999; Farquhar-Smith et al., 2000; Salio et al., 2002). Nevertheless, discrepancies concerning mammalian spinal CB1-LI-IR (see for example Farquhar-Smith et al., 2000) cannot be disregarded, suggesting an heterogeneity of cannabinoid binding sites.

4.1. CB1/SP

The SP-LI immunolabelings were carried out by using an antibody directed against the C-terminal part of SP (Couraud et al., 1987) that should recognize most amphibian tachykinins. The SP distribution found with our studies is in agreement with that previously described in Xenopus by Pieribone et al. (1994) and with recent observations in the green frog (Salio et al., 2001b). In the Xenopus dorsal and central fields, SP-LI nerve terminals are in close contact with CB1-LI cell bodies, pointing out functional sites for interactions between the SP-LI innervation and the endocannabinoid system. On the other hand, it was demonstrated that SP, mostly contained in primary afferent fibers, is released in mammalian dorsal horn upon nociceptive stimulation (Otsuka and Yoshioka, 1993; Quartara and Maggi, 1998; Saria, 1999). The present results suggest that, in amphibian spinal cord, cannabinoids may postsynaptically inhibit nociceptive information brought by SP afferent fibers.

4.2. CB1/GABA and CB1/NOS

The GABA spinal system has been already described in Xenopus (Barale et al., 1996) and is characterized by numerous dispersed neurons in the spinal grey matter and a well developed innervation in both dorsal and central fields. In the present co-localization experiments, nerve terminals and few cell bodies, localized in the central field, co-express CB1 and GABA. Correlations between CB1 and GABA were investigated in mammalian brain by using both immunohistochemical (e.g. hippocampus, Katona et al., 1999; Tsou et al., 1999; Katona et al., 2000) and pharmacological (e.g. hippocampus, Katona et al., 1999; medulla, Vaughan et al., 1999) approaches. Moreover, CB1 nerve terminals in tight apposition to GABAergic cells and few interneurons doubly labeled for CB1 and GABA were previously described in Xenopus olfactory bulbs (Cesa et al., 2001). In agreement with observations by Vaughan et al. (1999), Salio et al. (2002), carried out in rat medulla oblongata and spinal cord respectively, our present data seem to suggest for the amphibians a participation of cannabinoids in nociception through an inhibition of GABAergic transmission, although restricted to the central field. Since co-localization experiments in rat have evidenced a number of GABAergic interneurons, presumably islet cells (Barber et al., 1982), which are CB1 immunopositive (Salio et al., 2002) and also contain NOS (Valtschanoff et al., 1992), we have investigated interactions between CB1 and NO by performing NADPHd histochemistry, followed by CB1 immunohistochemistry. Recently, NO has been reported as a widely used messenger molecule in Xenopus spinal cord (Bru¨ning and Mayer, 2001) where it was found in many, possibly second order, ascending neurons of the dorsal and central fields,

suggesting an important involvement of NO in the processing of sensory informations. In our co-localization experiments, NOergic neurons containing CB1 were observed in both the deepest dorsal field and central grey matter, suggesting that endocannabinoids could control sensory inputs through both propriospinal interneurons and cells projecting to supraspinal targets.

4.3. CB1/MOR

Since cannabinoids enhance the analgesic action of opioids in mammals (for a review, see Howlett, 1995), another nociception marker investigated in the present paper was MOR. Comparative studies (see Stevens, 1988, for a review) indicate that opioid-mediated antinociception systems first arose in amphibians (Buatti and Pasternak, 1981) and that the spinal administration of opioids in low doses increases pain threshold in unanaesthetized frogs (Stevens et al., 1987; Stevens, 1996). Recently, a pharmacological study on opioid binding in amphibian spinal cord (Newman et al., 2000) has indicated a unique opioid receptor which mediates the action of selective m, k and d opioid agonists. Although ascending nociception pathways are not demonstrated in amphibians (Simpson, 1976), physiological studies have shown that electrical stimulation of the frog sciatic nerve produces evoked potentials in posterior thalamic nuclei and hypothalamus (Vesselkin et al., 1971). Indeed, in our experiments, cellular CB1/MOR co-localizations are found in the central field of the Xenopus spinal cord, where most neurons contribute to the ascending pathways (Ten Donkelaar, 1998).

5. Conclusions

Our results indicate that the spinal components for pain transmission are present in Xenopus and the anatomical substrates, as well as their neurochemistry, show similarities to those of mammals. Furthermore, the coexpression of CB1 with some nociception markers, more restricted than in rat and mainly concentrated in the central field, might indicate that, during phylogeny, endogenous cannabinoids implement their interactions in pain control through different mechanisms.

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