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Neuronal and astrocytic localization of the cannabinoid receptor-1 in the dorsal horn of the rat spinal cord

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Abstract
Cannabinoids are involved in the control of pain at the spinal level through the cannabinoid receptor-1 (CB1) localized pre- and postsynaptically on primary afferent fibres and dorsal horn interneurones, respectively. Using immunocytochemistry, we show that in addition to its neuronal localization, CB1 is also expressed in numerous astrocytes in laminae I and II of the rat dorsal horn. This ubiquitous localization may account for the complex role played by cannabinoids in antinociception. CB1 receptors in astrocytes may be involved in the anti-hyperalgesic action of exogenous cannabinoids.

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Cannabinoids are involved in pain control, at almost all relays of pain transmission, in particular at the spinal level [6,18] where they act through cannabinoid receptor-1 (CB1) localized in the dorsal horn [1,2,5,7,12,15]. CB1 receptors are expressed in numerous dorsal root ganglion (DRG) cells [1,5] and in primary afferent terminals of the dorsal horn [7,15]. Indeed, intracellular recordings have shown that cannabinoids presynaptically inhibit the response of dorsal horn neurones to capsaicin stimulation [11]. Quantitatively, autoradiography with the synthetic cannabinoid CP 55.940, has shown that about 50% of CB1 binding sites in the dorsal horn at the lumbar enlargement are presynaptic [7], suggesting a major involvement of cannabinoids on postsynaptic sites too. Indeed, several immunocytochemical studies confirmed a postsynaptic localization [1,12]. It has been further shown that CB1 receptors are expressed in numerous spinal neurones [12], in particular, in a number of dorsal horn interneurones containing gamma-aminobutyric acid (GABA) [15]. Recent physiological data show that cannabinoids presynaptically inhibit not only glycinergic neurotransmission but also GABAergic transmission in the superficial medullary dorsal horn [8]. We will show here with immunocytochemistry that CB1 receptors are also expressed in glial cells.

The experiments were performed on seven adult male Wistar rats (200–300 g) in accordance with the European Community Council Directive (86/609/EEC). Under deep pentobarbital (Sanofi, France) anaesthesia (60 mg/kg, intraperitoneally), animals were perfused intracardially with 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4. Lumbar spinal cords were dissected out and postfixed overnight. Fifty micrometer-thick Vibratome sections were preincubated for 30 min at room temperature in 0.02M PB (pH 7.4) containing 0.9% NaCl (phosphate-buffered saline; PBS) and 6% bovine serum albumin (BSA), then incubated for 15 h at 4 8C with 1:2000 specific anti-CB1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) directed against the carboxy terminus of the receptor. After extensive washing in PBS–BSA, sections were incubated for 1 h in 1:500 biotinylated anti-goat IgG (Vector, Burlingame, CA). For light microscopy, preincubation and incubation baths were supplemented with 0.1–0.3% Triton X-100. Sections were then washed in PBS and incubated for 1 h in the avidinbiotinylated-peroxidase complex (Vector, Burlingame, CA) diluted (1:250) in PBS.
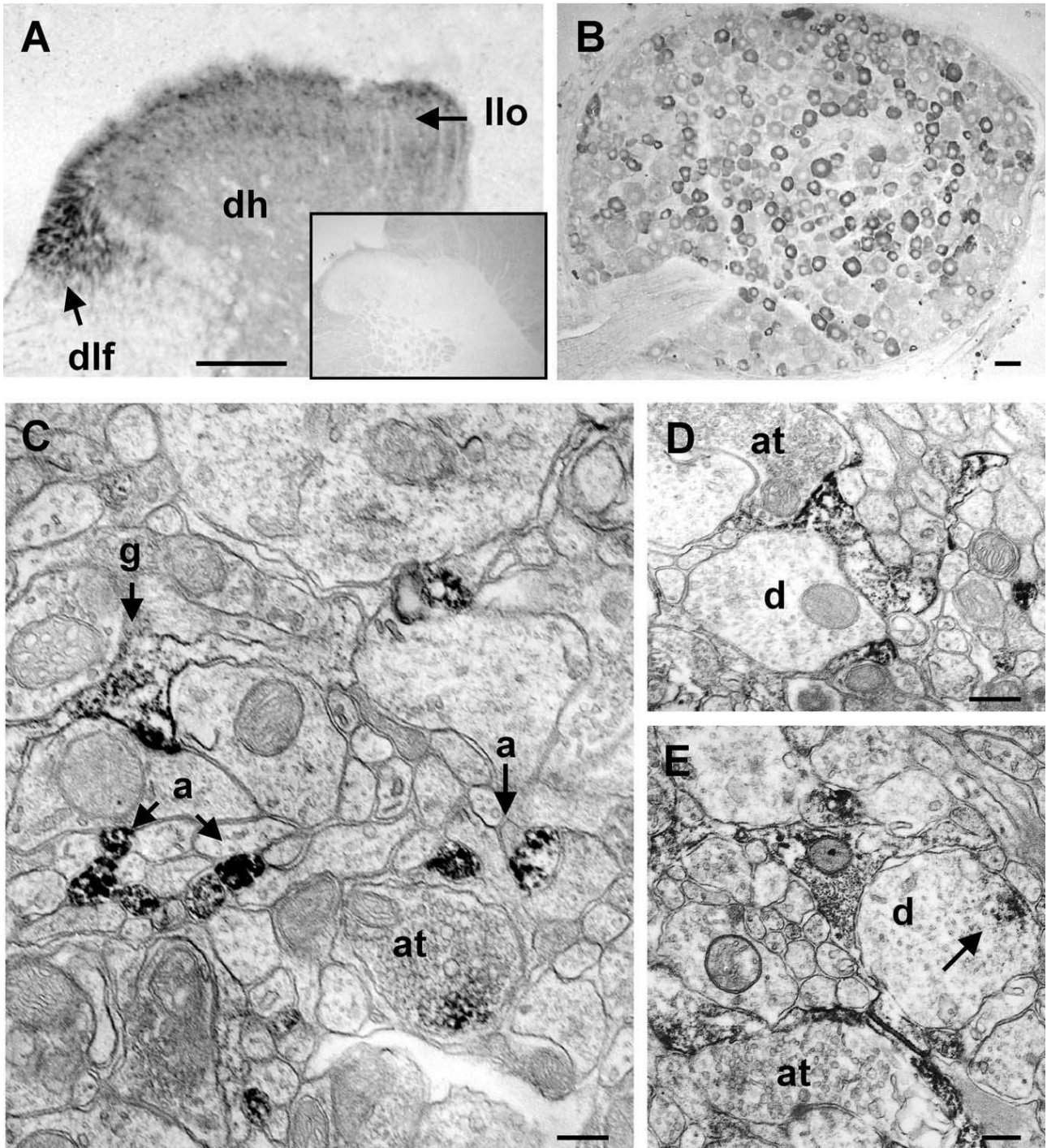
Fig. 1. (A) CB1-immunoreactivity is observed in the dorsal horn (dh) of the spinal cord and in the dorso-lateral funiculus (dlf), with a lower immunoreactivity in lamina IIo. Insert in (A) shows the absence of immunoreactivity when the antibody is preabsorbed with the immunizing peptide. Part (B) shows that immunoreactive DRG cells are heterogeneous (15–40 mm in diameter); larger cells are not labelled. In (C), immunoreactivity is localized in numerous thin unmyelinated axons (a), in an axon terminal (at) and in a large glial process (g) surrounding unlabelled neurites. In (D), an immunoreactive glial process is apposed to unlabelled axon terminal (at), and dendrite (d). In (E), two immunoreactive glial processes are apposed to a labelled dendrite (d) and an unlabelled axon terminal (at). Scale bars: (A), 250 mm; (B), 50 mm; (C–E), 500 nm.

After 10 min washing in 0.5MTris–HCl, pH 7.4, sections were incubated in 50 ml of the same buffer supplemented with 0.04% (w/v) 3,30-diaminobenzidine (Sigma, France) and 0.01% (v/v) hydrogen peroxide.

The reaction proceeded at 20 8C under light microscope control; it was stopped after 5–10 min of incubation, by washing in Tris buffer. Sections were then washed in PBS, transferred onto glass slides and dehydrated in graded alcohol before being mounted in Permount (Fisher, USA). The specificity of the reaction was controlled by preabsorption of antibodies at the working concentration (1:2000) with 1–10 mg/ml immunizing peptide for 3 h at room temperature before addition of the sections. Small blocks of dorsal horn laminae I–II from sections previously immunolabelled were dissected out and postfixed for 1 h at room temperature with 2% osmium tetroxide in PB. After 10 min washing in PB, blocks were dehydrated in graded alcohol, incubated successively in alcohol/araldite (v/v) for 1 h, araldite for 15 h and araldite (Fluka, Switzerland) supplemented with 2% accelerator (Fluka, Switzerland) for 5 h at 37 8C. Blocks were then embedded in araldite/accelerator between two siliconed slides (2 days at 60 8C). Silver ultrathin sections (,70 nm-thick) were obtained using an LKB ultramicrotome and collected on copper grids. They were counterstained with lead citrate for 10 min at room temperature in a dried-chamber and examined using a Jeol electron microscope.

At the lumbar enlargement, CB1-immunoreactivity was very dense in the dorsal horn. Lamina I and the inner part of lamina II (IIi) were highly labelled, while, by contrast, the outer part of lamina II (IIo) was less immunoreactive (Fig. 1A). A dense labelling was also observed in the dorsolateral funiculus (Fig. 1A) and in lamina X (not shown). When preabsorbed with the immunogen (1 mg/ml), no more immunoreaction was observed (insert in Fig. 1A). In the DRG, numerous cell bodies were labelled (Fig. 1B). They were heterogeneous in size from very small cell bodies (about 15 mm) to relatively large cell bodies (40 mm), the largest cell bodies were never labelled.

Under the electron microscope, the labelling was present in neuronal cells either in thin unmyelinated axons, often grouped in bundles (Fig. 1C), in axon terminals (Fig. 1C) or occasionally in dendrites (Fig. 1E). In addition, a marked CB1-immunoreactivity was observed in numerous astrocytic processes (Fig. 1C–E). The immunoreactive glial cells surrounded neuronal elements generally unlabelled (Fig. 1C,D). Occasionally, labelled astrocytes were in close apposition to labelled dendrites (Fig. 1E).

The present regional distribution of CB1-immunoreactivity in the spinal cord quite corresponds to that previously described [1,2,12,15]. CB1 receptors are expressed in heterogeneous DRG cells that mainly project to laminae I and IIi. At the ultrastructural level, CB1 receptors are observed, as expected, in both pre- and postsynaptic neuronal compartments. Unexpectedly, numerous large glial processes, presumably astrocytes, are highly immunoreactive in laminae I and II. They are apposed to either labeled or unlabelled neuronal elements. We have already observed this astrocytic labelling (unpublished observations) with another antibody also directed against the C-terminal tail of the CB1 receptor (kindly provided by Dr Ken Mackie). By contrast, no astrocytic labelling was observed with a N terminal-specific antibody [15]. Whether this discrepancy depends on the antibody used or on technical or physiological considerations, deserves further investigation. It has to be kept in mind that N-terminal antibodies are not able to recognize the spliced variant CB1A which exhibits a truncated N terminus [17]. The possible existence of CB1 receptor subtypes in spinal astrocytes may be of great functional importance.

In other regions of the brain, such as the cortex [10,13], olfactory bulb, striatum and substantia nigra [10], CB1-immunoreactivity has been found in astrocytes. In the cortex, at the ultrastructural level, immunoreactive astrocytes represented until 25% of the total immunoreactive profiles [13]. In the same way, CB1 mRNA and protein have been detected in astrocytic and glioma/astrocytoma cell cultures [16] where cannabinoids control cell death [3]. In the central nervous system, anandamide, one endogenous cannabinoid ligand, inhibits the permeability of gap junctions in cultured mice and rat astrocytes [19]. However, this effect seems to be mediated by a Gi/Go coupled receptor, different from CB1 or CB2 receptors. In addition, no CB1-immunoreactivity was observed by these authors in cultured striatal and cortical astrocytes from embryonic rats and mice [14]. It seems thus that cannabinoids act on brain astrocytes perhaps through different receptors (CB1 or non-CB1). In addition, the variable detection of immunoreactive astrocytes (from total absence to high immunoreactivity) suggests that the level of CB1 receptors in astrocytes may be physiologically regulated.

In the spinal cord, the ubiquitous distribution of CB1 receptors may be a reflection of the complex role played by endogenous and exogenous cannabinoids. They are involved in acute [6] as well as chronic pain of neurogenic [4] or inflammatory origin [9]. Indeed, spinal astrocytes are involved in pathological pain in which a drastic swelling of astrocytes leading to an increase in the release of proinflammatory substances such as nitric oxide and ecosanoids, is observed (for a review, see Ref. [20]). CB1 astrocytic receptors may quite well be involved at this level by reducing glutamatergic transmission.

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