

Endocannabinoids and amphibian reproduction: an immunohistochemical study in the green frog

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Riassunto. *I cannabinoidi, componenti psicoattivi della Cannabis sativa, e i cannabinoidi endogeni o endocannabinoidi, sono in grado di regolare svariate funzioni legandosi a recettori specifici di membrana accoppiati a proteine G, i recettori dei cannabinoidi CB1 e CB2. I recettori CB1 sono abbondanti nel sistema nervoso dei mammiferi e sono stati caratterizzati anche nel SNC di pesci, anfibi e uccelli (oltre che di invertebrati), suggerendo che gli endocannabinoidi siano neuromodulatori filogeneticamente antichi.*

Nel nostro studio, utilizzando tecniche immunoistochimiche per evidenziare i recettori dei cannabinoidi CB1, abbiamo dimostrato che nell'encefalo dell'Anfibio anuro Rana esculenta è presente una ricca innervazione cannabinergica e che, in particolare, neuroni e fibre nervose CB1 immunopositivi sono abbondanti negli emisferi telencefalici, nel diencefalo e nel deuterencefalo. Nel setto (telencefalo basale), nell'area preottica e nell'ipotalamo, centri di controllo della riproduzione, abbiamo ricercato possibili correlazioni morfofunzionali tra endocannabinoidi e molecole coinvolte nella regolazione neuroendocrina dell'attività riproduttiva, quali la gonadoliberina o GnRH e la dopamina. I nostri risultati sono in accordo con dati riportati in letteratura sui mammiferi e suggeriscono un ruolo del sistema cannabinergico nel controllo della funzione riproduttiva degli anfibi.

Abstract. *Cannabinoids, psychoactive constituents of Cannabis sativa, and their endogenous counterpart endocannabinoids are able to control various functions, through their binding to specific G-protein-coupled receptors, known as the CB1 and CB2 cannabinoid receptors. CB1 receptors are very abundant in mammalian CNS and recently were*

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also characterized in the CNS of fish, amphibians, birds and also in many invertebrates, suggesting that the endocannabinoid signalling system does represent an important neuromodulatory system, highly conserved during phylogeny.

In the present paper, by means of immunohistochemistry for the CB1 cannabinoid receptor, we have demonstrated that a cannabinergic innervation is distributed throughout the brain of the anuran Amphibian *Rana esculenta*. In particular, CB1 immunostained neurons and nerve fibers are abundant in the telencephalic hemispheres, the diencephalon and a number of hindbrain areas. By focussing a major attention to the basal telencephalon, preoptic area and hypothalamus proper, which are key areas for the control of reproduction, we have investigated possible morphofunctional relationships between endocannabinoids and signalling molecules known to be involved in reproductive neuroendocrine regulation, such as gonadoliberin and dopamine. Our results support a role for the cannabinoids in the control of amphibian reproduction, as already reported in mammals.

1. Introduction

Cannabinoids, the pharmacologically active constituents of *Cannabis sativa*, produce a multiplicity of behavioral and physiological effects in humans, including alterations in mood, perception, cognition, memory, psychomotor activity as well as analgesia, antiemesis and immunosuppression [1]. The major psychoactive principle of marijuana smoke, the Δ^9 -tetrahydrocannabinol (THC) was identified and characterized in 1965 by MECHOULAM and GAONI [2] and its therapeutic potential in many disorders was recognized even before the mechanism of its pharmacological actions was fully understood. These effects are mediated by receptor activation as demonstrated after cloning and pharmacological characterization of cannabinoid receptors CB1 [3] and CB2 [4]. Furthermore, the discovery of some endogenous ligands such as anandamide [5] and 2-arachidonoylglycerol [6], named endocannabinoids, has prompted for the existence in the brain of a whole system of endogenous ligands, receptors and enzymes for ligand biosynthesis and inactivation [7].

The CB1 receptor, almost exclusively localized in the nervous system (being the CB2 mainly distributed in the immune system and peripheral organs) was found to be involved in mammalian brain in a number of physiological and pathological conditions, suggesting new therapeutic possibilities [8].

The first neuroanatomical study on CB1 receptor in a non-mammalian vertebrate was the immunohistochemical investigation by CESA et al. [9] in an amphibian (the anuran *Xenopus laevis*) brain. It was shown that the cen-

tral cannabinergic system of this species was almost comparable to that described in rat [10]. Moreover a possible involvement of CB1 in the modulation of brain sensory, integrative [9] and neuroendocrine functions [11], as well as in nociception [12], was postulated.

In the present paper the distribution of the CB1-Like-Immunoreactivity (CB1-LI-IR) was studied by means of specific primary anti-CB1 antibodies in the brain of the green frog *Rana esculenta* (anuran Amphibians). Since in mammals a participation of CB1 in reproductive functions was reported [13], our study has been mainly focussed to those forebrain areas which play a key role in vertebrate reproduction, such as septum (medio-basal telencephalon), preoptic area and hypothalamus. Furthermore, by using immunohistochemistry, we have investigated in the above forebrain areas, the possible morpho-functional relationships between the cannabinergic innervation and neural systems characterized by signalling molecules known to be involved in reproductive neuroendocrine mechanisms, such as gonadoliberein (GnRH) and dopamine. For this purpose, consecutive brain sections were alternatively processed with primary antibodies raised against CB1, mammalian GnRH (the prevalent molecular form in the amphibian prosencephalon) and tyrosine hydroxylase (TH, the key enzyme in catecholamine biosynthesis, assumed as a marker for dopaminergic innervation).

2. Materials and methods

The experiments were performed under the guidelines established by the Italian law for animal welfare. Adult specimens of *Rana esculenta* (n=30) of both sexes, collected during all the year, were deeply anesthetized with 1:1000 tricaine methanesulphonate (MS222, Sandoz Ltd., Basel, Switzerland). CNS was removed from the brain case, fixed overnight (O/N) in 4% paraformaldehyde, embedded in Killik medium (Bio-Optica, Milan, Italy) and frozen. Two series of cryostat coronal and sagittal sections of the brains (12 μ m thick) were mounted on 3-aminopropyl-triethoxysilan (TESPA)-coated slides and stored at 4°C until use. For molecular biology experiments, the frog CNS was rapidly dissected out and immediately frozen at -80°C.

Western-blotting. Brain total proteins (100 μ g) were loaded on a 10% polyacrilamide gel and separated by SDS-PAGE. Western-blotting was performed by using, as a primary antibody, an affinity-purified polyclonal antiserum raised against the N-terminus or, alternatively, the C-terminus of the rat CB1 (1:500 dilution; kind gifts of Dr. Ken Mackie, University of Washington, Seattle, USA). As a control, the primary antisera were pre-adsorbed O/N, RT, with the corresponding immunogens (10 μ g/ml). After incubation with an anti rabbit IgG HRP-linked antiserum, the reaction was revealed

with the ECL Plus Western blotting detection reagent and Hyperfilm-ECL autoradiography film (Amersham Biosciences, Little Chalfont, UK).

Immunohistochemistry (BAS) and Indirect Immunofluorescence (IFL). The brain sections were alternatively incubated with the two primary polyclonal antibodies raised against the N- or C-terminus of the rat CB1 receptor (1:800 dilution). Immunoreactivity, revealed by biotin-avidin complex (BAS) system and H₂O₂/DAB (3,3'-diaminobenzidine-tetrahydrochloride) as substrate/chromogen, was observed under a light microscope (Zeiss Axioskop). The specificity of the antisera was evaluated by incubating the sections with the antisera pre-adsorbed O/N, RT with the corresponding immunizing proteins (5 µg/ml). The specificity of the method was assessed by omitting the primary antibody. For the IFL technique, the sections were incubated with the anti CB1 antibody (C-terminus; 1:500 dilution) and with a secondary anti rabbit IgG CY3-linked antiserum. The labelings were detected under a fluorescence microscope (Zeiss Axioskop) and photographed. Images were processed with the software Adobe PhotoShop 7.0 (Adobe Systems Incorporated, Mountain View, CA).

For double labelings, consecutive longitudinal and coronal sections (at the levels of the septum, basal telencephalon, preoptic area and hypothalamus) were alternatively immunostained with one of the above cited anti CB1 antibodies, with anti mGnRH (SW1, 1:3000 dilution; kind gift of Dr. Susan Wray, NIH, Bethesda, USA) or anti TH (1:1000 dilution; Institut Jacques Boy, Reims, France) antisera and processed with BAS technique.

3. Results

In the present paper we have analysed the distribution of the CB1 cannabinoid receptor in *Rana esculenta* brain by means of two primary antibodies raised against the CB1 N- or C-terminals.

The specificity controls by employing the anti CB1 antisera pre-adsorbed with the corresponding immunizing proteins or omitting the primary antibodies, resulted in the complete absence of immunostaining (Fig 1f).

The Western-blotting analysis, performed using the same antisera, revealed CB1-like receptor as a protein with an apparent molecular weight of about 51 kDa. The specific bands were not detected when the blots were incubated with the antibodies pre-adsorbed with the immunogens (data not shown).

Although our observations have concerned all the frog brain, in the present paper we describe the distribution of the CB1-LI-IR through the prosencephalic areas, mainly involved in the control of amphibian reproduction.

3a. CB1-LI-IR

Telencephalon. Apart from some small CB1-LI-immunopositive periglomerular neurons and fibers occurring in the olfactory bulbs, the most important prosencephalic staining is shown by the telencephalic hemispheres. A thick network of immunopositive nerve terminals together with a number of stained neurons are observed in the dorsal and dorsomedial pallial regions. Nerve terminals are varicose or punctuate and frequently surround immunonegative cell bodies especially in the dorsal and dorsolateral pallium (Fig. 1a). Proceeding caudally, a number of CB1-LI immunoreactive neurons are seen in the dorsal pallium (Fig. 1b), in the septum (Fig. 1g), in the bed nucleus of the anterior commissure, as well as in the amygdala and accumbens nuclei.

Preoptic area and hypothalamus. In the Anurans the chiasmatic ridge divides the ventral diencephalon in a rostral preoptic part and a caudal or infundibular hypothalamus, regions both functionally related to the pituitary gland. The preoptic area grey matter, characterized by a laminated organization, shows CB1 immunopositive medium-large perikarya organized in subependymal rows together with a dense network of nerve fibers and processes ventro-laterally directed (Fig. 1c, d). The stained cell bodies, 15-20 μm in diameter, seemingly belong to the magnocellular preoptic nucleus. The immunostaining of these neurons is particularly intense in sections incubated with the anti CB1 antiserum raised against the C-terminus of the molecule.

In the tuberal hypothalamus positive nerve terminals are very abundant, while immunostained cell bodies are sparse.

Immunopositive nerve fibers and terminals, closely arranged in proximity of the blood capillary walls (Fig. 1d insert), are seen within the neurohypophysis. CB1 immunostained cell bodies and fibers are observed in other diencephalic areas such as the habenular nuclei (Fig. 1e).

3b. CB1-LI-, GnRH-LI- and TH-LI-IRs

In order to investigate the topographic relationships of CB1-LI-, GnRH-LI- and dopamine-LI-immunoreactivities through the basal telencephalon (septum) and the preoptic area of the green frog we used consecutive brain sections alternatively processed with primary antibodies raised against the CB1, the mammalian GnRH (the preminent molecular form in the amphibian prosencephalon) and the tyrosine hydroxylase (TH), the catecholamine biosynthetic enzyme assumed as a marker for the dopaminergic innervation.

In the septum, CB1 immunostained nerve cells and terminals (Fig. 1g) were distributed close to both GnRH-containing neurons and fibers (Fig. 1h) and TH positive nerve fibers (Fig. 1i).

4. Discussion

We have first described, by using the same anti N-terminal CB1 antibody used in the present work the distribution of the CB1 receptor in a non mammalian brain, the anuran amphibian *Xenopus laevis* [9], and by comparing our results with those reported in the rat brain [10] we suggested that endocannabinoids may have implemented their functions through different mechanisms during phylogeny.

In the present paper we have stained the brain of the green frog with two primary antibodies respectively raised against the N-terminal and the C-terminal of the CB1 receptor. The specificity of these antibodies was assessed by performing both immunohistochemistry control reactions and Western-blotting experiments. A widely extended CB1 positive innervation was observed through all the forebrain and, more sparsely, within the hindbrain. The labelling patterns obtained with the two antibodies are not substantially dissimilar, although in the pallium of the telencephalon and in the preoptic area they seem to be almost complementary. The antibody raised against the C-terminal tail of CB1 stains both small cell clusters in the dorsal pallium and medium-large neurons in the preoptic area, while the other one finely decorates plentiful punctuate nerve terminals which frequently surround immunonegative perikarya through the pallium.

Briefly, comparing the present observations with the data already obtained in *Xenopus laevis* [9], we can confirm that the endocannabinoids probably play a role as neuromodulators/transmitters. Furthermore, the numerous positive cells in the frog preoptic area and hypothalamus suggest an involvement of CB1 receptors in neuroendocrine hypothalamic mechanisms. Since the participation of cannabinoids in the reproduction control has been postulated in mammals at both pituitary [14] and gonadal [13], [15] levels, we have looked for morphofunctional relationships among CB-LI-, mGnRH-LI- and TH-LI-IRs in the basal telencephalon/hypothalamus, important brain areas controlling reproduction in all vertebrates. Our results show a close contiguity of the three neural systems investigated, indicating possible interactions among them in the central modulation of reproduction. GAMMON et al. [16] have indeed demonstrated that immortalized hypothalamic GnRH neurons are capable of releasing endocannabinoids (anandamide and 2-arachidonoylglycerol) and possess CB1 and CB2 receptors, postulating the existence of a novel mechanism for regulating GnRH secretion in mammals,

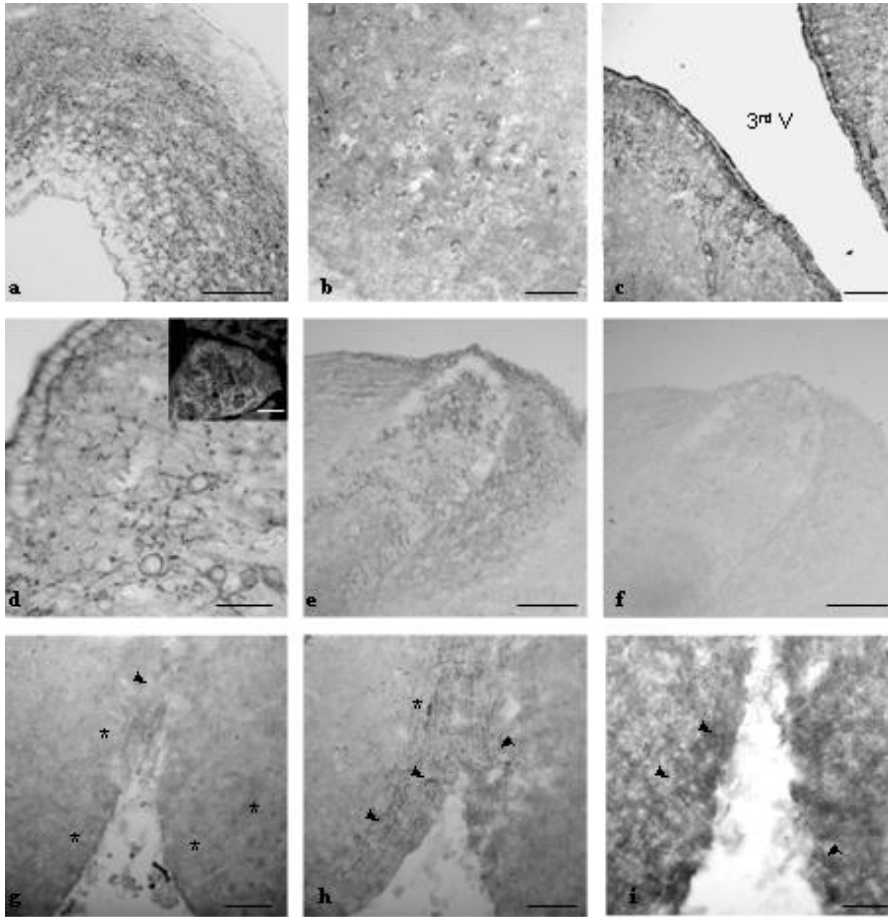


Fig. 1: **a)** Dorsolateral pallium of the telencephalon. CB 1 (N-ter) immunopositive nerve terminals surrounding immunonegative cell bodies. Coronal section, BAS technique. Calibration bar: 50 μ m; **b)** Dorsal pallium of the telencephalon. Immunopositive cell bodies stained with the antibody anti CB1 (C-ter). Coronal section; BAS technique. Calibration bar: 100 μ m; **c)** Preoptic area. Numerous CB1 immunopositive cell bodies and fibers in the periventricular grey of the preoptic area. 3rd V = third ventricle. Coronal section; BAS technique. Calibration bar: 100 μ m; **d)** The CB1 immunopositive neurons of Fig. 1c, at a higher magnification. Coronal section; BAS technique. Calibration bar: 50 μ m. Insert: the CB1 innervation of the neurohypophysis. Sagittal section; IFL technique. Calibration bar: 50 μ m; **e)** CB1 immunopositive cell bodies and nerve terminals in the habenular nucleus. Sagittal section; BAS technique. Calibration bar: 100 μ m; **f)** Sagittal section consecutive to Fig. 1e, stained with anti CB1 (C-ter) antibody pre-adsorbed with the immunogen, shows the complete absence of immunostaining. Sagittal section; BAS technique. Calibration bar: 10 μ m; **g) h) i)** Three consecutive coronal sections of the telencephalic septum. CB1- (g), mGnRH- (h) and TH- (i) immunopositive cell bodies (*) and fibers (▶) are closely distributed in the same area. BAS technique. Calibration bar: 100 μ m.

whose reproductive functions are so complex. Interactions between hypothalamic dopaminergic neurons, gonadal steroids and gonadotropic function are well known in amphibian brain [17], [18], [19]. The co-distribution of the CB1-LI- and TH-LI-IRs described in the present paper might suggest that the endogenous cannabinoids participate to the multiple neuroendocrine control of the amphibian reproduction also through an interaction with dopamine.

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