Type-1 Cannabinoid Receptor Expression in the Frog, *Rana esculenta*, Tissues: A Possible Involvement in the Regulation of Testicular Activity

ROSARIA MECCARIELLO,[1] ROSANNA CHIANESE,[2] GIOVANNA CACCIOLA,[2] GILDA COBELLIS,[2] RICCARDO PIERANTONI,[2]* AND SILVIA FASANO[2]

[1]*Dipartimento di "Studi delle Istituzioni e dei Sistemi Territoriali," Università di Napoli "Parthenope," Napoli, Italy*
[2]*Dipartimento di Medicina Sperimentale, Seconda Università di Napoli Costanopoli, Napoli, Italy*

ABSTRACT Endogenous cannabinoids and type-1 cannabinoid receptor (CB1) are widely produced and distributed in the central nervous system (CNS) and peripheral nerves in mammals. In addition, the detection of endocannabinoids and corresponding receptors in non nervous peripheral tissues indicates an involvement of the system in the control of a wide range of physiological activities, including reproduction. Recently, the existence of CB1 was also observed in lower vertebrates and in urochordate suggesting that the endocannabinoid system is phylogenetically conserved. Using RT-PCR, CB1 mRNA expression profiles were characterized in a wide range of tissues of the anuran amphibian, the frog, *Rana esculenta*. Besides a strong expression in the CNS, CB1 was also present in testis, kidney, liver, ovary, muscle, heart, spleen, and pituitary. The CB1 expression pattern has been characterized in both testis and CNS during the annual sexual cycle. In testis, CB1 is poorly expressed during the winter stasis of the spermatogenesis rising during the breeding season and resumption period. An expression profile mismatching to that observed in testis was detected in whole-brain preparations during the sexual cycle; in particular in the diencephalon, the encephalic area mainly involved in the control of reproductive functions. Furthermore, fluctuations inside isolated encephalic areas and spinal cord were observed all over the reproductive cycle. In conclusion, CB1 receptor is expressed in *R. esculenta* CNS and testis. As far as the gonad it concerns, our results suggest the involvement of the endocannabinoids in the control of reproductive function. *Mol. Reprod. Dev. 73: 551–558, 2006.* © 2006 Wiley-Liss, Inc.

Key Words: cannabinoid; CB1 receptor; anuran amphibian; expression analysis; testis; brain

INTRODUCTION

Endocannabinoids are a novel class of lipidic mediators derived from arachidonic acid that bind and activate specific membrane receptor subtypes (CB1 and CB2). The main compounds classified as "endocannabinoids" are arachidonoylethanolamide (AEA, anandamide), 2-arachidonoyl glycerol (2-AG), 2-arachidonyl glyceryl ether (2-AGE, noladin ether), and *o*-arachidonoylethalamine (virodhamine) (Lutz, 2002). These molecules have different affinity for and activate to different extent the CB1 and CB2 receptors. The effect of AEA via CB1 and CB2 receptors depends on its extracellular concentration, which is controlled by uptake and degradation, respectively due to a specific AEA membrane transporter (AMT) and to a fatty acid amide hydrolase (FAAH). Generally, endogenous cannabinoids, cannabinoid receptors, FAAH, and AMT are designed as "endocannabinoid system" (for review Elphick and Egertovà 2001; Fride, 2002; Lutz, 2002; Pertwee and Ross, 2002).

Indeed, a part from the classical CB1 and CB2 receptors, AEA interacts with potassium and calcium channels as well as 5-HT3 receptors and vanilloid receptor (VR_1). In vitro and in vivo studies, the latter conducted using $CB1^{-/-}$ mice, support the existence, almost in mice, of additional non-CB1, non-CB2, and non-VR1 receptors (CBx) not yet identified (Wiley and Martin, 2002, for review).

CB1 is a classical G-protein coupled receptor with seven transmembrane spanning regions (Matsuda et al., 1990). Signaling aspects of this receptor have taken advantage of synthetic and natural (Δ^9THC: Δ^9-tetrahydrocannabinol, the primary psychoactive constituent of the marijuana plant, *Cannabis sativa)* cannabinoids. In vivo, cannabinoids are responsible for a characteristic tetrad of pharmacological effects in mice, consisting in suppression of locomotor activity, hypothermia, antinociception, and catalepsy (Martin et al., 1991). Dramatic effects at central and peripheral level, especially

Grant sponsor: MIUR-Pierantoni 2002; Grant sponsor: ex60%-Fondi Ateneo 2004; Grant sponsor: Regione Campania L.5.

*Correspondence to: Riccardo Pierantoni, Dipartimento di Medicina Sperimentale, sez "F. Bottazzi," Via Costanopoli n. 16, 80138 Napoli, Italy. E-mail: riccardo.pierantoni@unina2.it

Received 29 July 2005; Accepted 5 October 2005
Published online 16 February 2006 in Wiley InterScience (www.interscience.wiley.com).
DOI 10.1002/mrd.20434

on reproduction, in marijuana smokers are widely reported (Habayeb et al., 2002 and reference there in). Cellular consequences, specifically linked to CB1 receptor activation, include inhibition of adenylyl cyclase and modulation of Ca^{2+} and K^{+} ionic channels and activation of mitogen-activated protein kinase (Mukhopadhyay et al., 2002).

CB1 receptor is widely expressed in the nervous system and in some peripheral tissues, including pituitary gland, immune cells, reproductive tissues, gastrointestinal tissues, sympathetic ganglia, heart, lung, urinary bladder, and adrenal gland (Galiegue et al., 1995; Shire et al., 1995). Splice variants of CB1 receptor, CB1a and CB1b, with differential pharmacological effects and expression rate, have been described in human, but their functional role is still unknown (Shire et al., 1995; Ryberg et al., 2005). CB2 receptor is expressed almost exclusively in the immune system, particularly into B-cells and natural killer cells; in rat, CB2 receptor is scantly expressed also in testis and lung (Brown et al., 2002).

CB1 receptor has been cloned in several vertebrates, from fish to amphibians and mammals (Elphick and Egertovà, 2001; Cottone et al., 2003, 2005). Also in the urochordate *Ciona intestinalis* CB1 receptor and FAAH have been cloned and their tissue distribution have been analyzed (Elphick et al., 2003; Matias et al., 2005). At present, high affinity cannabinoid binding sites have been detected in several deuterostomian and protostomian invertebrates but a part from *C. intestinalis*, no functional cannabinoid receptor was found in invertebrates (Salzet and Stefano, 2002 for review; McPartland and Glass, 2003). Overall, the above quoted results indicate that the cannabinoid system is highly conserved.

In this work, we analyze CB1 expression in the anuran amphibian, the frog, *Rana esculenta* tissues, providing evidence for a differential expression in brain and gonads during the annual sexual cycle. A putative relationship between cannabinoid and testicular function at both central and peripheral levels is reported.

MATERIALS AND METHODS

Animals and Tissue Collection

Forty male frogs, *R. esculenta*, were collected monthly from September until July in the neighborhood of Naples (Italy) for 2 years; 4 additional female frogs were captured in June. The animals were killed under anesthesia with MS222 (Sigma-Aldrich Corp., St. Louis, MO). Brain, spinal cord, nerves, testis, kidney, muscle, liver, spleen, heart, and ovary were removed and appropriately stored at −80°C, until used for total RNA extraction. Furthermore, four brains and four spinal cords from June, July, September, October, December, March, and April animals were respectively dissected in telencephalon, diencephalon, mesencephalon, romboencephalon, and cervical, thoracic, lumbar tracts.

This research was approved by the Italian Ministry of University and Scientific and Technological Research.

Total RNA Preparation

Total RNA was extracted from *R. esculenta* tissues using TRIZOL® Reagent (Invitrogen Life Technologies, Paisley, UK). In brief, the sample was homogenized in Trizol Reagent (1 ml TRIZOL® Reagent/50–100 mg of tissue); after homogenization, the sample was incubated for 5 min at RT to allow the complete dissociation of nucleoprotein complexes. Moreover, 0.2 ml of chloroform/ml TRIZOL® Reagent was added and the sample was centrifuged at 12,000*g* for 15 min at 4°C. The aqueous phase was transferred to a fresh tube and total RNA was precipitated by mixing with isopropyl alcohol (0.5 ml/TRIZOL® Reagent). After centrifugation at 12,000*g* for 10 min at 4°C, RNA pellet was washed with 75% ethanol, centrifuged at 7,500*g* for 5 min at 4°C, and then dissolved in an appropriate volume DEPC-treated water. Total RNA purity and integrity were determined by spectrophotometry analysis at 260/280 nm and by electrophoresis.

CB1 mRNA Detection by RT-PCR

Total RNA, previously treated for 30 min at 37°C with *Dnase*I (10 U/sample) (Amersham Biosciences Europe GmbH, Freiburg, Germany) to eliminate genomic DNA contaminations, was reverse transcribed to prepare cDNA. For testis, brain, and spinal cord preparations, 2 μg RNA/month were pooled and processed as single sample. The reverse transcription was carried out using 5 μg total RNA, 0.5 μg oligo $d(T)_{18}$, 10 mM dNTP, 0.01 M DTT, 1 × first strand buffer, 40 U RNase Out, 200 U SuperScript-III RnaseH^{-} Reverse Transcriptase (Invitrogen Life Technologies, UK) in a final volume of 20 μl, following the manufacturer's instructions. As negative control, total RNA not treated with Reverse Transcriptase was used.

PCR reaction was carried out using 1 μl of diluted (1:5) cDNA and 10 pMol oligonucleotide primers specific for frog CB1 mRNA, (sense: 5′-attggggtaaccagtgttct-3′; antisense: 5′-accagggtctttgctaacct-3′; amplificate predicted size: 201 bp) in PCR mix [0.2 mM dNTP, 1 × PCR buffer, 1.5 mM $MgCl_2$, 1.25 U Taq Polymerase (Invitrogen Life Technologies)], using an Applied Biosystem Thermocycler Apparatus. PCR conditions were: 94°C for 5 min, 1 cycle; 94°C for 30 sec, 56°C for 30 sec, 72°C for 30 sec, 30 cycles for brain samples, and 35 cycles for testis and tissues; lastly 72°C for 7 min, 1 cycle. In order, to normalize the signals, 1 μl of diluted cDNA was mixed with 10 pMol specific primers for the *R. esculenta* ribosomal protein P1 mRNA (*fp1*) (sense 5′-tacgagcgtccatcacacac-3′; antisense 5′-agaccaaagcccatgtcatc-3′; amplificate predicted size: 356 bp) with the previously described PCR mix. PCR conditions were: 94°C for 5 min, 1 cycle; 94°C for 30 sec, 56°C for 30 sec, 72°C for 30 sec, 22 cycles; lastly 72°C for 7 min, 1 cycle. Possible contaminations among samples were evaluated using as negative controls samples prepared without cDNA.

Finally, 25 μl of PCR amplification mixture were analyzed by electrophoresis on 1.2% agarose gel in 1 × Tris-borate (TBE) buffer and stained with 0.5 μg/ml ethidium bromide.

Statistics

Analysis of mRNA levels was carried out by GELDOC 1,00-UV fluorescent gel documentation system (BioRAD, Hercules, CA). The relative amounts of the signals are expressed as fold increase of the ratio *cb1* mRNA/*fp1* mRNA ± SEM. ANOVA followed by Duncan's test for multi-group comparison was carried out to assess the significance of differences. Observations range from 3 to 8.

RESULTS

As we have recently cloned CB1 receptor cDNA in the frog *R. esculenta* brain (accession number in GenBank AM113546), to assess CB1 expression and distribution, we have preliminarily used a semiquantitative RT-PCR approach on different frog tissue-derived cDNA's. Specific primers were designed upon frog brain CB1 sequence and an amplificate of the predicted size of 201 bp was analyzed by electrophoresis. Expression of the housekeeping gene, *fp1*, encoding the frog ribosomic protein P1 was also evaluated as an amplificate of 356 bp and used to normalize CB1 signals.

Tissue Distribution of CB1 Receptor mRNA

CB1 is expressed in all the tissues tested (Fig. 1), although the levels in heart, pituitary, and spleen appeared to be extremely low (Fig. 1B). Significant amount of CB1 mRNA is detected in kidney, liver, and muscle. Higher levels of CB1 are detected in gonads ($P < 0.01$), in both testis and ovary. In the central nervous system, highest CB1 expression is observed in total brain. In the spinal cord, CB1 is significantly lower than in brain ($P < 0.01$), but comparable to kidney, liver, and muscle.

CB1 Expression in Testis During the Annual Sexual Cycle

The anuran amphibian, *R. esculenta*, is a seasonal breeder whose gonads undergo precise morphological modifications during the sexual cycle. Spermatogenesis proceeds slowly and occurs in cysts containing cluster of germ cells at the same maturational stage. In this respect, each period of the year is characterized by a well defined germ cell population. In particular, the annual sexual cycle can be divided in four phases: resumption (September–November), when spermatogenesis proceeds and spermiogenesis events are massive; winter stasis (December–February), when proliferation of spermatogonia does not occur and testis progressively becomes empty; breeding season (March–May), when spermatogenesis resumes, spermatogonia proliferate, and spermatozoa are released; post reproductive period (June–July) when spermatogenesis proceeds with appearance of meiotic stages (Rastogi et al., 1976; Pierantoni et al., 2002).

During the annual sexual cycle, fluctuations of CB1 mRNA are observed in mature testis from September until July (Fig. 2). CB1 levels are high in September with a peak in November (September *vs*. November, $P < 0.01$); thereafter they decrease reaching the nadir during the winter stasis (December, January, and

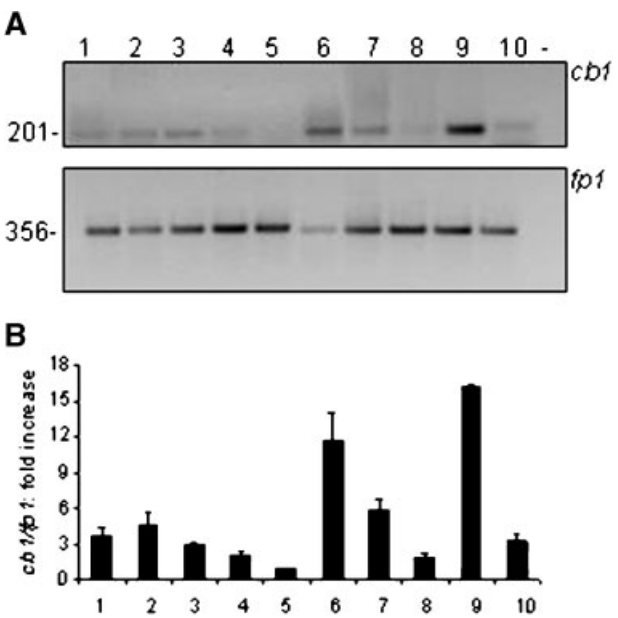

Fig. 1. **A**: *cb1* and *fp1* expression in frog tissues. 1, kidney; 2, liver; 3, muscle; 4, heart; 5, spleen; 6, ovary; 7, testis; 8, pituitary; 9, brain; 10, spinal cord; –, negative control. **B**: Normalization of the signals observed by RT-PCR. Data are representative of three separate experiments at least and are expressed as fold increase of the minimal value observed. Observations range: 3–8.

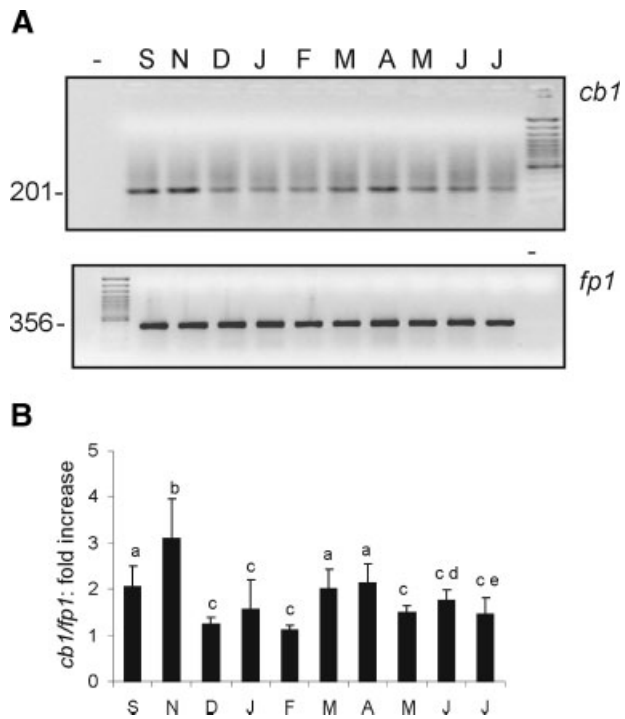

Fig. 2. **A**: *cb1* and *fp1* expression in frog testis during the annual sexual cycle. –, negative control. **B**: Normalization of the signals observed by RT-PCR. Data are representative of three separate experiments at least and are expressed as fold increase of the minimal value observed. Observations range: 3–8. (a *vs*. b, a *vs*. c, and b *vs*. c $P < 0.01$; c = d; d *vs*. e $P < 0.05$).

February *vs*. November and September, $P < 0.01$). As soon as testicular activity resumes during the breeding season, CB1 expression level quickly increases (March-April *vs*. December, January, and February, $P < 0.01$); after that, it decreases in May (March-April *vs*. May, $P < 0.01$) and remains still low in post reproductive period (May *vs*. June N.S.; June *vs*. July, $P < 0.05$).

CB1 Expression in CNS During the Annual Sexual Cycle

To assess CB1 expression in the brain, we have collected samples during the annual cycle, from September until July and processed them as "whole brain" (Fig. 3).

CB1 expression is at comparable levels in September and October, it reaches a minimal value in November ($P < 0.01$), gradually it increases from December until January (December *vs*. January $P < 0.05$), and it remains at the same high levels during March and April. After that, CB1 expression gradually decreases in May-June period ($P < 0.01$), peaking again in July ($P < 0.01$ July *vs*. November, December, May, June).

To gain further information on CB1 mRNA production into the CNS, mainly in relationship to the reproductive function, we have collected and processed an additional series of samples dissecting encephalic areas and spinal cords (Fig. 4).

Generally, CB1 expression in the forebrain and midbrain is higher than in the hindbrain ($P < 0.01$) in all the considered periods, except in September and October. In fact, in September CB1 expression levels in both diencephalon and rombencephalon are comparable and significantly lower than in telencephalon and mesencephalon ($P < 0.01$); in October, CB1 expression is significantly higher only in the mesencephalon ($P < 0.01$) (Fig. 5). In June, high expression levels are detected in mesencephalon ($P < 0.01$); in July, CB1 expression gradually increases from telencephalon to diecephalon ($P < 0.05$) to mesencephalon ($P < 0.01$); in September, a peak is observed in telencephalon and mesencephalon ($P < 0.01$ *vs*. diencephalon); in December, CB1 expression is strong in the diencephalon ($P < 0.01$); in March, CB1 mRNA peak is observed in telencephalon and mesencephalon ($P < 0.01$ *vs*. diencephalon); in April, higher expression is registered in mesencephalon ($P < 0.05$ *vs*. telencephalon and diencephalon).

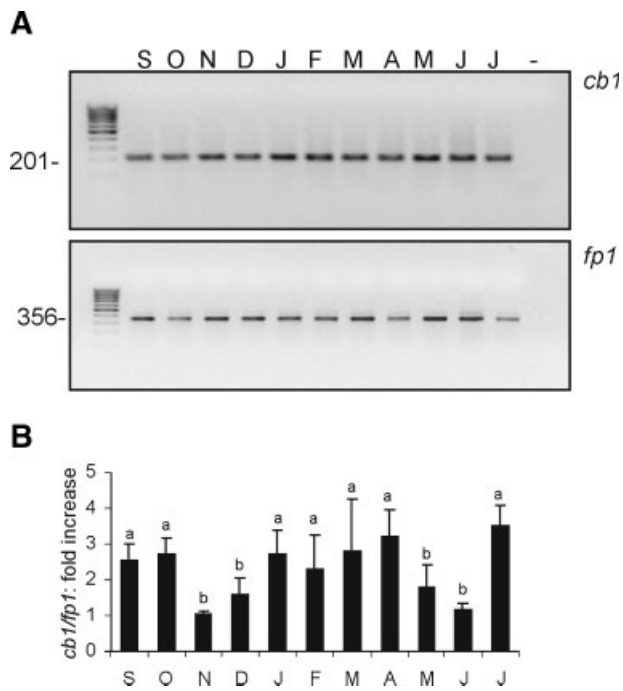

Fig. 3. A: *cb1* and *fp1* expression in frog whole brain during the annual sexual cycle. − = negative control. **B**: Normalization of the signals observed by RT-PCR. Data are representative of three separate experiments at least and are expressed as fold increase of the minimal value observed. Observations range: 3–8. (a *vs*. b $P < 0.05$ at least).

Also in the single encephalic areas, CB1 expression fluctuates during the annual reproductive cycle and a specific profile/area is observed (Fig. 5).

In the telencephalon, where the olfactory system resides, CB1 mRNA levels are minimal in June, it progressively increases until September (June *vs*. July and September $P < 0.01$; July *vs*. September $P < 0.01$) decreasing again at minimal value in October. During the winter stasis, CB1 expression increases (October *vs*. December $P < 0.01$), reaching maximal levels in March ($P < 0.01$) and decreasing in April ($P < 0.01$).

In the diencephalon, the encephalic area mainly involved in reproductive function control, CB1 expression peaks in December ($P < 0.01$), and it is maintained at higher values during the breeding season ($P < 0.01$). Low CB1 mRNA levels are detected during the post reproductive and resumption periods, with minimal values observed in June and in October (June and October *vs*. July and September $P < 0.01$; July *vs*. September $P < 0.01$).

In the mesencephalon, CB1 expression increases from June until September ($P < 0.01$), reaching a minimal value in October ($P < 0.01$ except *vs*. June). Then it peaks in March ($P < 0.01$), and it remains still high in April ($P < 0.01$).

In the rombencephalon, CB1 expression is minimal in June, it gradually increases until September (June *vs*. July $P < 0.05$, June and July *vs*. September $P < 0.01$). Then it decreases in October (September *vs*. October $P < 0.01$), peaking in December and April.

In the cervical tract of the spinal cord, minimal levels of expression are detected in June ($P < 0.01$); then CB1 mRNA gradually increases until April, reaching the maximal expression level (at least $P < 0.05$).

In the thoracic tract of the spinal cord, CB1 expression is constant from June until December, peaking in March ($P < 0.01$), and being minimal in April ($P < 0.01$).

In the lumbar tract of the spinal cord, low CB1 expression levels are observed in June and in April ($P < 0.01$). After that mRNA peaks in July ($P < 0.01$), decreasing until the minimal value in September ($P < 0.01$); then it rises again in October, remaining constant until March.

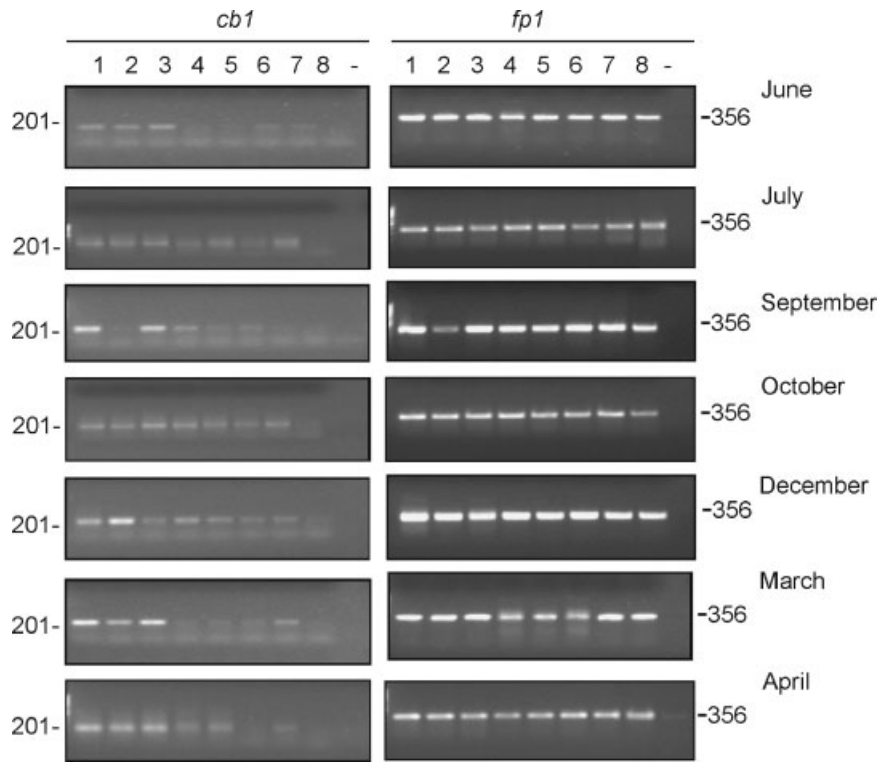

Fig. 4. *cb1* and *fp1* expression in frog-isolated encephalic areas during June, July, September, October, December, March, and April. 1, telencephalon; 2, diencephalon; 3, mesencephalon; 4, rombencephalon; 5, cervical tract of the spinal cord; 6, thoracic tract of the spinal cord; 7, lumbar tract of the spinal cord; 8, nerves of the lumbar plexus; –, negative control.

Scanty expression of CB1 was also detected in the lumbar plexus nerves during the year, except in April.

DISCUSSION

In the present work, we demonstrate the expression of CB1 receptors in the anuran amphibian, the frog, *R. esculenta*. Besides the CNS, in which the strong CB1 expression is expected, RT-PCR approach has allowed the detection of modest CB1 expression also in a wide array of peripheral tissues, mimicking the expression pattern already described in mammals (Galiegue et al., 1995; Shire et al., 1995). In particular, in gonads (ovary

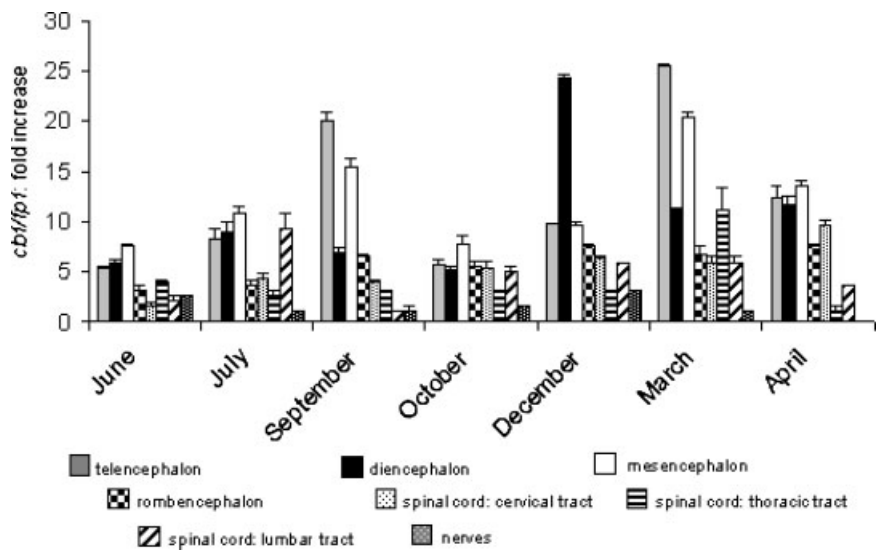

Fig. 5. Normalization of the signals observed by RT-PCR in the isolated encephalic areas and isolated tracts of the spinal cord and nerves. Data are representative of the separate experiments at least and are expressed as fold increase of the minimal value observed. Observations range: 3–8.

and testis), CB1 expression is significantly higher than in other tissues (except brain). Also in the sea squirt *C. intestinalis* CB1 is differentially expressed in tissues in that higher mRNA levels are observed in cerebral ganglion, heart, pharynx, and testis, while lower levels are observed in intestine, ovary, and stomach (Matias et al., 2005). Interestingly, in mouse, the *N*-acyl-phosphatidylethanolamines (NAPE)-hydrolyzing phospholipase D (NAPE-PLD), the enzyme responsible for the release, on demand, of AEA from membrane NAPEs (NAPE-PLD) is highly expressed in brain and also in kidney and testis; lower expression level are reported in other peripheral tissues such as liver, spleen, lung, and small intestine (Okamoto et al., 2004). In mammals, the role of endocannabinoid systems in embryo implantation is well documented (Paria et al., 1996; Yang et al., 1996; Schmid et al., 1997). In addition, alterations of the hypothalamus-pituitary-gonadal axis activity in marijuana smokers and in animal models have also been reported (for review Wenger et al., 2001; Habayeb et al., 2002). Few informations are available on endocannabinoid effects on testicular function. In this respect, our experimental model has the advantage of a slow progression of the spermatogenetic stages; in fact clusters of germ cells at the same maturative stage develop in a particular environment, named germinal cyst, and appear in specific periods of the annual sexual cycle (Rastogi et al., 1976; Pierantoni et al., 2002). The analysis of CB1 expression in the testis reveals high expression level in September-November and March-April periods. In September-November, massive formation of post-meiotic stages occurs; in March-April, the breeding season, sperm cells are released and spermatogenetic activity resumes. Western blot and immunocytochemical studies conducted in the same experimental animals reveal that in September also CB1 protein level is efficiently produced and that CB1 immunoreactivity is restricted to elongated spermatids and spermatozoa (Cobellis et al., 2005, submitted for publication). Effects of anandamide on sperm motility and fertilizing capability are reported in both human and sea urchin (Schuel et al., 1994, 2002); in addition, in humans, CB1 is expressed in spermatozoa (Rossato et al., 2005). Interestingly, CB1 mRNA profile here reported well correlates with plasmatic testosterone levels observed during the annual sexual cycle (d'Istria et al., 1974; Cobellis et al., 1997). In this respect, further investigation will be addressed to assess a possible steroid-dependent expression of CB1 receptor in frogs.

Fluctuations of CB1 mRNA inside the CNS during the annual sexual cycle in total brain, encephalic dissected areas, and spinal cord are described here. Generally, the main sites of CB1 expression in the brain are the forebrain and the midbrain. Similar expression patterns are observed in vertebrates from fish (immunological observations by Cottone et al., 2005) to mammals (in situ hybridization analysis by Matsuda et al., 1993; immunological observations by Tsou et al., 1998), and in the anuran amphibia *Xenopus laevis* (in situ hybridization analysis by Cottone et al., 2003; immunological observations by Cesa et al., 2001). Endocannabinoids control the feeding process in invertebrates. AEA accelerates mouth closures in the protostomian *Hydra vulgaris*, a coelenterate (De Petrocellis et al., 1999); after mechanical stimulation, HU-210, a cannabinoid receptor agonist inhibits the buccal siphon reopening in the deuterostomian *C. intestinalis* (Matias et al., 2005). In mammals, the cannabinoid system modulates several activities in the central nervous system, such as olfactory system, memory, movement control, food intake, analgesia (for review Elphick and Egertova, 2000) and some of these functions are conserved among vertebrates. In *Pelvicachromis pulcher*, *Xenopus laevis*, and rat, a potential action of endocannabinoids at different levels on the olfactory system has been postulated (Tsou et al., 1998; Cesa et al., 2001; Cottone et al., 2003). In the urodele amphibian, the newt, *Taricha granulosa*, high expression of CB1 in the brain has been reported and the effects of cannabinoids on spontaneous locomotor activity and courtship clasping behavior have been described (Soderstrom et al., 2000). Similarly, in both *Xenopus laevis* and rodents, expression and distribution of CB1 into the spinal cord are correlated to nociception (Salio et al., 2002 and references there in). At present, it is not excluded that similar involvement of the cannabinergic system might be, in the next future, extended also to the *R. esculenta*.

Interestingly, a mismatching profile to that observed in testis is described in whole brain preparations. Focusing on the reproductive activity control, negative regulation of gonadotropin releasing hormone (GnRH) secretion, the main controller of gonadal activity, is exerted by cannabinoids in rat and in immortalized GnRH secreting neurones (GT1-7) (Fernandez-Ruiz et al., 1997; de Miguel et al., 1998; Gammon et al., 2005). In *R. esculenta* diencephalon, the encephalic area mainly involved in the release of GnRH, CB1 expression peaks in December. Low levels of GnRH molecular forms in male *R. esculenta* brain have been detected from December until April (Fasano et al., 1993; Di Matteo et al., 1996; Meccariello et al., 2004), matching the increase of gonadotropin release (Polzonetti-Magni et al., 1998) and testicular androgen production (Fasano et al., 1993). In testis, minimal expression levels of CB1 are reported in December, during the winter stasis. Since in *Xenopus laevis* nerve terminal immunoreactivity for CB1 has been observed in the median eminence and in pituitary CB1 colocalizes with LH-releasing cells (Cesa et al., 2002), an involvement of amphibian cannabinergic system in the neuro-endocrine hypothalamic control of adenohypophysis is postulated.

In conclusion, we provide evidence for CB1 expression in the frog, *R. esculenta*, confirming that the cannabinergic system is phylogenetically conserved. In addition, the fluctuations of CB1 mRNA at both central and testicular levels strongly indicate a possible involvement of the cannabinoid system in the regulation of gonadal activity.

REFERENCES

Brown SM, Wager-Miller J, Mackie K. 2002. Cloning and molecular characterization of the rat CB2 cannabinoid receptor. Biochim Biophys Acta 1576:255–264.

Cesa R, Mackie K, Beltramo M, Franzoni MF. 2001. Cannabinoid receptor CB1-like and glutamic acid decarboxylase-like immunoreactivities in the brain of *Xenopus laevis*. Cell Tissue Res 306:391–398.

Cesa R, Guastalla A, Cottone E, Mackie K, Beltramo M, Franzoni MF. 2002. Relationships between CB1 cannabinoid receptors and pituitary endocrine cells in *Xenopus laevis*: An immunohistochemical study. Gen Comp Endocrinol 25:17–24.

Cobellis G, Pierantoni R, Fasano S. 1997. *c-fos*- and *c-jun*-like mRNA expression in frog (*Rana esculenta*) testis during the annual reproductive cycle. Gen Comp Endocrinol 106:23–29.

Cobellis G, Cacciola G, Scarpa D, Meccariello R, Chianese R, Franzoni MF, Mackie K, Pierantoni R, Fasano S. 2005. Endocannabinoid system in frog and rodent testis: type cannabinoid receptor and fatty acid amiole hydrodase activity in male germ cells (in press).

Cottone E, Salio C, Conrath M, Franzoni MF. 2003. *Xenopus laevis* CB1 cannabinoid receptor: Molecular cloning and mRNA distribution in the central nervous system. J Comp Neurol 464:487–496.

Cottone E, Forno S, Campantico E, Guastalla A, Viltono L, Mackie K, Franzoni MF. 2005. Expression and distribution of CB1 cannabinoid receptors in the central nervous system of the African cichlid fish *Pelvicachromis pulcher*. J Comp Neurol 485:293–303.

d'Istria M, Delrio G, Botte V, Chieffi G. 1974. Radioimmunoassay of testosterone, 17beta-oestradiol and oestrone in the male and female plasma of plasma of *Rana esculenta* during sexual cycle. Steroids Lipids Res 5:42–48.

de Miguel R, Romero J, Munoz MR, Garcia-Gil L, Gonzalez S, Villanua MA, Makriyannis A, Ramos JA, Fernandez-Ruiz JJ. 1998. Effects of cannabinoids on prolactin and gonadotrophin secretion: Involvement of changes in hypothalamic gamma-aminobutyric acid (GABA) inputs. Biochem Pharmacol 56:1331–1338.

De Petrocellis L, Melck D, Bisogno T, Milone A, Di Marzo V. 1999. Finding of the endocannabinoid signalling system in Hydra, a very primitive organism: Possible role in the feeding response. Neuroscience 92:377–387.

Di Matteo L, Vallarino M, Pierantoni R. 1996. Localization of GnRH molecular forms in the brain, pituitary, and testis of the frog, *Rana esculenta*. J Exp Zool 274:33–40.

Elphick MR, Egertovà M. 2001. The neurobiology and evolution of cannabinoid signalling. Philos Trans R Soc Lond B Biol Sci 356:381–408.

Elphick MR, Satou Y, Satoh N. 2003. The invertebrate ancestry of endocannabinoid signalling: An orthologue of vertebrate cannabinoid receptors in the urochordate *Ciona intestinalis*. Gene 302:95–101.

Fasano S, Goos HJTh, Janssen C, Pierantoni R. 1993. Two GnRHs fluctuate in correlation with androgen levels in the male frog *Rana esculenta*. J Exp Zool 266:277–283.

Fernandez-Ruiz JJ, Munoz RM, Romero J, Villanua MA, Makriyannis A, Ramos JA. 1997. Time course of the effects of different cannabimimetics on prolactin and gonadotrophin secretion: Evidence for the presence of CB1 receptors in hypothalamic structures and their involvement in the effects of cannabimimetics. Biochem Pharmacol 53:1919–1927.

Fride E. 2002. Endocannabinoids in the central nervous system-an overview. Prostaglandins Leukot Essent Fatty Acids 66:221–233.

Galiegue S, Mary S, Marchand J, Dussossoy D, Carriere D, Carayon P, Bouaboula M, Shire D, Le Fur G, Casellas P. 1995. Expression of central and peripheral cannabinoid receptors in human immune tissues and leukocyte subpopulations. Eur J Biochem 232:54–61.

Gammon CM, Freeman GM, Jr., Xie W, Peterson S, Westel WC. 2005. Regulation of Gonadotropin-Releasing Hormone Secretion by Cannabinoids. Endocrinology 146:4491–4499.

Habayeb OMH, Bell SC, Konje JC. 2002. Endogenous cannabinoids: Metabolism and their role in reproduction. Life Sci 70:1963–1977.

Lutz B. 2002. Molecular biology of cannabinoid receptors. Prostaglandins Leukot Essent Fatty Acids 66:123–142.

Martin BR, Compton DR, Thomas BF, Prescott WR, Little PJ, Razdan RK, Johnson MR, Melvin LS, Mechoulam R, Ward SJ. 1991. Behavioural, biochemical, and molecular modelling evaluations of cannabinoid analogs. Pharmacol Biochem Behav 40:471–478.

Matias I, McPartland JM, Di Marzo V. 2005. Occurrence and possible biological role of the endocannabinoid system in the sea squirt *Ciona intestinalis*. J Neurochem 93:1141–1156.

Matsuda LA, Lolait SJ, Brownstein MJ, Young AC, Bonner TI. 1990. Structure of a cannabinoid receptor and functional expression of the cloned cDNA. Nature 346:561–564.

Matsuda LA, Bonner TI, Lolait SJ. 1993. Localization of cannabinoid receptor mRNA in rat brain. J Comp Neurol 327:535–550.

McPartland JM, Glass M. 2003. Functional mapping of cannabinoid receptor homologs in mammals, other vertebrates, and invertebrates. Gene 312:297–303.

Meccariello R, Mathieu M, Cobellis G, Vallarino M, Bruzzone F, Fienga G, Pierantoni R, Fasano S. 2004. Jun localization in cytosolic and nuclear compartments in brain-pituitary system of the frog, *Rana esculenta*: An analysis carried out in parallel with GnRH molecular forms during the annual reproductive cycle. Gen Comp Endocrinol 135:310–323.

Mukhopadhyay S, Shim JY, Assi AA, Norford D, Howlett AC. 2002. CB(1) cannabinoid receptor-G protein association: A possible mechanism for differential signalling. Chem Phys Lipids 121:91–109.

Okamoto Y, Morishita J, Tsuboi K, Tonai T, Ueda N. 2004. Molecular characterization of a phospholipase D generating anandamide and its congeners. J Biol Chem. 279:5298–5305.

Paria BC, Deutsch DD, Dey SK. 1996. The uterus is a potential site for anandamide synthesis and hydrolysis: Differential profiles of anandamide synthase and hydrolase activities in mouse uterus during the pre-implantation period. Mol Reprod Dev 45: 183–192.

Pertwee RG, Ross RA. 2002. Cannabinoid receptors and their ligands. Prostaglandins Leukot Essent Fatty Acids 66:101–121.

Pierantoni R, Cobellis G, Meccariello R, Palmiero C, Fienga G, Minucci S, Fasano S. 2002. The amphibian testis as model to study germ cell progression during spermatogenesis. Comp Biochem Physiol B Biochem Mol Biol 132:131–139.

Polzonetti-Magni AM, Mosconi G, Carnevali O, Yamamoto K, Hanaoka Y, Kikuyama S. 1998. Gonadotropins and reproductive function in the anuran amphibian, *Rana esculenta*. Biol Reprod 58:88–93.

Rastogi RK, Iela L, Saxena PK, Chieffi G. 1976. The control of spermatogenesis in the green frog, *Rana esculenta*. J Exp Zool 169:151–166.

Rossato M, Ion Popa F, Ferigo M, Clari G, Foresta C. 2005. Human sperm express cannabinoid receptor CB1, the activation of which inhibits motility, acrosome reaction and mitochondrial function. J Clin Endocrinol Metab 90:984–991.

Ryberg E, Vu HK, Larsson N, Groblewski T, Hjorth S, Elebring T, Sjogren S, Greasley PJ. 2005. Identification and characterization of a novel splice variant of the human CB1 receptor. FEBS Lett 579:259–264.

Salio C, Cottone E, Conrath M, Franzoni MF. 2002. CB1 cannabinoid receptors in amphibian spinal cord: Relationships with some nociception markers. J Chem Neuroanat 24:153–162.

Salzet M, Stefano GB. 2002. The endocannabinoid system in invertebrates. Prostaglandins Leukot Essent Fatty Acids 66 :353–361.

Schmid PC, Paria BC, Krebsbach RJ, Schmid HH, Dey SK. 1997. Changes in anandamide levels in mouse uterus are associated with uterine receptivity for embryo implantation. Proc Natl Acad Sci USA 94:4188–4192.

Schuel H, Goldstein E, Mechoulam R, Zimmerman AM, Zimmerman S. 1994. Anandamide (arachidonylethanolamide), a brain cannabinoid receptor agonist, reduces sperm fertilizing capacity in sea urchins by inhibiting the acrosome reaction. Proc Natl Acad Sci USA 91:7678–7682.

Schuel H, Burkman LJ, Lippes J, Crickard K, Mahony MC, Giuffrida A, Picone RP, Makriyannis A. 2002. Evidence that anandamide-signalling regulates human sperm functions required for fertilization. Mol Reprod Dev 63:376–387.

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.

Soderstrom K, Leid M, Moore FL, Murray TF. 2000. Behavioural, pharmacological, and molecular characterization of an amphibian cannabinoid receptor. J Neurochem 75:413–423.

Tsou K, Brown S, Sanudo-Pena MC, Mackie K, Walker JM. 1998. Immunohistochemical distribution of cannabinoid CB1 receptors in the rat central nervous system. Neuroscience 83:393–411.

Wenger T, Ledent C, Csernus V, Gerendai I. 2001. The central cannabinoid receptor inactivation suppresses endocrine reproductive functions. Biochem Biophys Res Commun 284:363–368.

Wiley JL, Martin BR. 2002. Cannabinoid pharmacology: Implications for additional cannabinoid receptor subtypes. Chem Phys Lipids 121:57–63.

Yang Z-M, Paria BC, Dey SK. 1996. Activation of brain-type cannabinoid receptors interferes with pre-implantation mouse embryo development. Biol Reprod 55:756–761.