Endocannabinoid system in *Xenopus laevis* development: CB1 receptor dynamics

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Received 28 November 2005; revised 8 February 2006; accepted 21 February 2006

Available online 2 March 2006

Edited by Takashi Gojobori

Abstract This study investigates for the first time the dynamics of endocannabinoid system appearance during low vertebrate *Xenopus laevis* development. We observed that the CB1 gene started to be expressed during the organogenesis period (±1 dpf, st. 28) and expression persisted throughout the three further stages analyzed. Attention was focused on the localization of the CB1 messenger that was found both at the central level (in romboencephalon and in olfactory placods) and at the peripheral level (in the gastrointestinal tract) at ±3 dpf (st. 41), ±4 dpf (st. 46) and ±12 dpf (st. 49). We also considered the synthesis of CB1 protein that occurred from st. 41 onwards and, from this stage, we tested the receptor functionality in response to anandamide using cytosensor microphysiometry. CB1 functionality increased with development at both central and peripheral level. These data provide sufficient evidence to encourage further analysis on endocannabinoid physiological roles during embryonic and larval *X. laevis* growth.

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Keywords: CB1 receptor; *Xenopus laevis*; Development; Anandamide

1. Introduction

Cannabinoid receptor CB1 was first characterized in 1988 \(^1\) and cloned in 1990 \(^2\). To date, most studies have been performed on mammals where CB1 was found to be densely present, at central nervous system (CNS) level, in several brain areas \(^3\). CB1 has also been detected in sensory neurons of the dorsal root ganglia of the peripheral nervous system \(^4\), and expression persists during the organogenesis period \(^5\) and expression persisted throughout the three further stages analyzed. Attention was focused on the localization of the CB1 messenger that was found both at the central level (in romboencephalon and in olfactory placods) and at the peripheral level (in the gastrointestinal tract) at ±3 dpf (st. 41), ±4 dpf (st. 46) and ±12 dpf (st. 49). We also considered the synthesis of CB1 protein that occurred from st. 41 onwards and, from this stage, we tested the receptor functionality in response to anandamide using cytosensor microphysiometry. CB1 functionality increased with development at both central and peripheral level. These data provide sufficient evidence to encourage further analysis on endocannabinoid physiological roles during embryonic and larval *X. laevis* growth.

In mammals the endocannabinoid system has been shown to play a role in synaptic regulation. This system is involved in many physiological functions, such as control of food intake \(^15\), \(^16\), \(^17\), \(^18\). CB1 agonist administration seems to stimulate the intake of sweet food. At the hepatic level, it has also been demonstrated that hepatocytes express CB1 inducing the expression of enzymes involved in lipid metabolism and de novo fatty acid synthesis \(^6\).

In non-mammalian animals the involvement of CB1 in food intake has been studied in *Carassius auratus* \(^19\), but nothing is known about the endocannabinoid system during embryo development and its role in first feeding.

This study aimed to show the appearance and the functionality of the endocannabinoid system during the growth of a lower vertebrate. *Xenopus laevis* was chosen as an experimental model because of the wide knowledge of its developmental biology and because “amphibian has found to represent key group in CB1 evolution through vertebrates” \(^20, 21\).

2. Materials and methods

2.1. Animals maintenance and reproduction

*X. laevis* embryos were generated using standard methods \(^22\). Embryos and larvae obtained were staged according to Nieuwkoop and Faber’s tables \(^23\). For the analysis the following developmental stages were selected: early gastrula stage, 9 hpf (st. 10), late organogenesis stage, ±1 dpf (st. 28), yolk sac absorption stage, ±3 dpf (st. 41), first hindlimb bud stage, ±4 dpf (st. 46), advanced larval stage, ±12 dpf (st. 49).

2.2. RNA extraction and analysis of CB1 gene expression by RT-PCR

Total RNA was extracted from stages 10, 28, 41, 46, 49 and from whole adult brain, used as positive control, with RNeasy Mini Kit (250) (Qiagen) and first strand cDNA synthesis was performed as already described in \(^24\). cDNA was amplified with 5 U of Taq DNA polymerase (Dynazyme) in 20 μl of total volume containing 1× PCR buffer, 1.5 mM MgCl₂, 2.5 mM dNTPs, and primers (F/CB1: 5'-TCTTACCACTTCAATGTCC-3', R/CB1: 5'-TCCATGGCATGCGTCTGTTCC-3'). CB1 amplification was carried out with the following profile: 5 min at 94 °C, 30 cycles at 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min. In order to semi-quantify the CB1 gene expression, β-actin was used as an internal standard (F/β-actin: 5'-TTCTTCGGTGATAGGTCC-3', R/β-actin: 5'-TGCGATATGGTACCTTCC-3') using the following profile: 20 s at 94 °C, primer annealing at 56 °C for 30 s and primer extension at 72 °C for 30 s.

2.3. CB1 cloning and sequencing

The PCR product obtained with CB1 specific primers was purified using the PCR purification kit (QIAJEN) and then cloned into the p-GEM T easy vector (Promega), following the manufacturer’s protocol. The plasmid was transformed into DH5α cells by the

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TransformAid™ kit (MBI Fermentas). Several positive clones were analyzed by PCR, in order to verify the presence of the insert. Only the clones showing CB1 insert were then grown in liquid broth with ampicillin (1:1000 in LB broth). The insert was then sequenced using an ABI model 310 DNA sequencer (Perkin-Elmer, Oak Brook, IL, USA).

2.4. cRNA probes synthesis

The antisense cRNA probe for CB1 was generated by linearizing the vector with NcoI and transcribing the template using SP6 polymerase. The sense probe was synthesized by using of PstI as restriction enzyme and T7 as polymerase.

2.5. Whole-mount in situ hybridization

The whole mount in situ hybridization let to analyze the localization of CB1 mRNA in all the developmental stages where CB1 gene was evidenced by RT-PCR. Embryos or larvae from each stage mentioned before were fixed in 4% paraformaldehyde during 24 h at 4 °C, then rinsed with serial concentrations of methanol and PBT (25%, 50%, 75% and 100% MeOH) and stored at −20 °C. Prehybridization, hybridization, washing and detection procedures were conducted according to Roche protocols except for the final staining reaction which was not applied [25]. Negative controls were performed using the antisense probe preadsorbed with the sense probe in excess (200/400 ng/μl). Autofluorescence was analyzed in all developmental stages. Images were obtained by Confocal Microscopy.

2.6. Western blot analysis

To study the presence of CB1 receptor, embryos and larvae samples were homogenised and treated as described by Tsou et al. [26]. CB1 was detected with the antibody [anti-cannabinoid receptor CB1, Rat (Rabbit) Calbiochem] diluted 1:1000.

2.7. Cell preparation and functional assay by cytosensor microphysiometry

The activation of CB1 receptor was assessed in whole embryos and larvae in st. 41, st. 46 and st. 49, where the presence of CB1 protein was demonstrated by Western blot. X. laevis embryos and larvae were analyzed in toto and with head and trunk separately. Cells were isolated by mechanical dissociation and filtration through nylon mesh (40 μm) to remove cellular aggregates and then centrifuged, washed and resuspended in Dulbecco’s modified Eagle’s medium 4500 mg/ml glucose supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. The stimulation with CB1 agonist, (Anandamide, (AEA) Calbiochem) and antagonist (AM 251, Cayman, [27]) dissolved in DMSO (10⁻² M as stock solutions), and the acidification rate analysis performed by cytosensor microphysiometry were conducted as already described by Pihlavisto and Scheinin [28] and Gentili et al. [29].

2.8. Statistical analysis

Values of pEC50 and the extent of maximal response (E_max) were calculated from the dose response curves using the program GraphPad Prism (Graph-Pad Software, San Diego, CA). The results are expressed as means SEM of three separated experiments.

3. Results and discussion

At CNS level, the involvement of the endocannabinoid system in several pathways is quite well known [30,21]. Currently there is a lot of interest in CB1 peripheral pathways, especially concerning food intake and metabolism. This study aims to provide basic evidence on CB1 receptor dynamics during amphibian development both at central and peripheral levels.

3.1. CB1 appearance during X. laevis development

In the first stage (st. 10) analyzed by RT-PCR (Fig. 1) and Western blot (Fig. 3), there was no trace of CB1 receptor mRNA expression and protein synthesis, thus suggesting that the endocannabinoid system is not involved in biological processes until the gastrula period.

CB1 mRNA expression was first detected in the whole embryos at st. 28 (Fig. 1), but at a very low level. The amplification provided a single band of 603 bp corresponding to the brain CB1 previously sequenced and used as positive control. The localization of CB1 mRNA was not possible because of the high autofluorescence of the sample that did not allow the signal of the messenger to be recognised (Fig. 2, a', a'').

Western blot analysis demonstrated that in the latter stage mentioned (st. 28) the expression of the CB1 gene was not followed by synthesis of the protein: no reaction with CB1 antibody was observed (Fig. 3). CB1 mRNA expression (Fig. 1), protein synthesis (Fig. 3) as well as the activation of the receptor by AEA (Fig. 4) were first observed at st. 41 in whole embryos and larvae. The protein detected by Western blot was 63 kDa, the same as the CB1 detected in the adult Xenopus brain which was used as positive control. This size also corresponds to CB1 described in the rat [26] (Fig. 3).

The same evidence found at st. 41 persisted in st. 46 and st. 49 (Figs. 1–4), with an increase in both mRNA expression and CB1 receptor affinity to AEA during development (Table 1). These data induced us to focus the attention on central and peripheral CB1 dynamics separately in order to determine CB1 functionality at both central and peripheral levels.

3.2. CB1 at CNS level

Using whole mount in situ hybridization it was possible to localize the CB1 mRNA, previously detected by RT-PCR in whole embryos and larvae.

Three days after fertilization (st. 41) CB1 mRNA appeared in the roomboencephalon (Fig. 2b’), where it was also found in adults [21].

Fig. 1. Temporal CB1 gene expression during X. laevis development. β-Actin was used as internal standard. Brain and water were used as positive and negative controls, respectively.
In the following stage (st. 46) CB1 mRNA persisted in the romboencephalon and appeared in olfactory placodes (Fig. 2c). This finding is supported by Buckley et al. [31], who showed the same localization in rat embryos, and by similar findings in adult X. laevis brain [21] and in adult rat brain [32].
In the last stage studied (st. 49), because of the fusion of the olfactory bulbs, the presence of the CB1 was more concentrated than in the romboencephalon area (Fig. 2d).

The functional analysis performed in the head showed an increasing CB1 affinity for AEA during development, indicating that the specificity of the endocannabinoid system grows with the complexity of the organism. In particular, in the stages analyzed in this study, these data could be due to the first feeding period (st. 41 and st. 46), when the appetite starts to be stimulated at CNS level because of yolk sack absorption [23]. CB1 is known to be involved in this kind of physiological mechanism in mammals: it is involved in suckling activity [32]. Such a hypothesis should however be confirmed through further specific analysis on the experimental model studied here.

3.3. CB1 presence and functionality at peripheral level

It was interesting to observe what occurred at peripheral level during development. After st. 41, the yolk sack is completely absorbed [23], involving many physiological changes regarding first feeding and food metabolism.

The appearance of CB1 mRNA at st. 41 (Fig. 1) and its localization in the digestive tract (Fig. 2b) were supported by the functionality assay performed with AEA on the trunk of the larvae (Fig. 4). CB1 was already active at st. 41 (Fig. 4), thus suggesting that the endocannabinoid system starts to work when the yolk sack is completely re-absorbed and the larvae start to feed. The yolk sack contains proteins, lipids and enzymes as lipases: its disappearance provokes several physiological changes.

Food first appears in the intestinal tract at st. 46 [23]; larvae start to eat on their own and metabolic processes are in progress. In this study, at st. 46 and st. 49, the presence of CB1 mRNA persisted in the digestive tract (Fig. 2c and d) suggesting its possible involvement in lipid metabolism by increasing lipoprotein lipase activity, as described by Cota in mammals [12]. The functionality analysis performed on the trunk showed that, at this stage, CB1 affinity for AEA was higher than at the previous stage (Fig. 4), suggesting that the CB1 lipase pathway could fill the gap arising from yolk sack absorption. All these hypotheses need further investigation. The possible involvement of CB1 in such biological processes, underlines the possibility of investigating items previously studied only in mammals.

This study provides a clear picture of endocannabinoid system localization and functionality at central and peripheral level during X. laevis development.

Acknowledgments: This work has been supported by COFIN 2003 awarded to Prof. Oliana Carnevali. The author thank Dr. Simone Bellagamba for the assistance in Confocal Microscopy analysis.

References


