General and Comparative Endocrinology 168 (2010) 280–286

Contents lists available at ScienceDirect

General and Comparative Endocrinology

journal homepage: www.elsevier.com/locate/ygcen

Changes in the distribution of corticotropin-releasing factor (CRF)-like immunoreactivity in the larval bullfrog brain and the involvement of CRF in the cessation of food intake during metamorphosis

Kouhei Matsuda [a,*], Noriaki Morimoto [a], Kazumasa Hashimoto [a], Reiko Okada [b], Hiroshi Mochida [b], Minoru Uchiyama [a], Sakae Kikuyama [b,c]

[a] Laboratory of Regulatory Biology, Graduate School of Science and Engineering, University of Toyama, Toyama 930-8555, Japan
[b] Department of Biology, Faculty of Science, Shizuoka University, Shizuoka 422-8529, Japan
[c] Department of Biology, Faculty of Education and Integrated Arts and Sciences, Waseda University, Tokyo 162-8480, Japan

ARTICLE INFO

Article history:
Received 26 October 2009
Revised 18 December 2009
Accepted 3 January 2010
Available online 12 January 2010

Keywords:
Bullfrog larvae
CRF
Metamorphosis
Immunohistochemistry
Food intake
Anorexigenic action
α-Helical $CRF_{(9–41)}$
Intracerebroventricular injection

ABSTRACT

In submammalian vertebrates, corticotropin-releasing factor (CRF) acts as an anorexigenic neuropeptide as well as a potent stimulator of corticotropin and thyrotropin release from the pituitary. As a step for demonstrating the involvement of CRF in the feeding regulation of anuran larvae, which are known to stop feeding toward the metamorphic climax, we studied firstly the changes in the distribution of CRF-like immunoreactivity (CRF-LI) in the brain of metamorphosing bullfrog larvae. Neuronal cell bodies showing CRF-LI were invariably present in the thalamic regions throughout larval development. Cells with CRF-LI were also found in the hypothalamus. The number of cells with CRF-LI in the hypothalamus, but not in the thalamus, showed a significant increase as metamorphosis progressed. Immunoreactive nerve fibers were observed mainly in the median eminence, and became abundant as metamorphosis proceeded. The number of cells showing CRF-LI in the hypothalamus as well as the density of immunoreactive fibers in the median eminence decreased at the end of metamorphosis. Secondly, we examined the effect of intracerebroventricular (ICV) injection of CRF on the food intake in the premetamorphic larvae. ICV injection of CRF at 10 pmol/g body weight (BW) induced a significant decrease of food intake during 15 min. The CRF-induced anorexigenic action was blocked by the treatment with a CRF receptor antagonist [α-helical $CRF_{(9–41)}$] at 100 pmol/g BW. The results suggest the involvement of CRF in the accomplishment of metamorphosis through the pituitary and in the feeding restriction that occurs during the later stages of metamorphosis through the central nervous system.

1. Introduction

Corticotropin-releasing factor (CRF), a neuropeptide comprising 41 amino acids and existing in the brain of the vertebrates, was first isolated and characterized from the ovine hypothalamus (Vale et al., 1981), and then subsequently identified in non-mammalian brains (Lovejoy and Balment, 1999). CRF is a member of a family of related peptides present in vertebrates, including urotensin-I, sauvagine and urocortin/stresscopin (Lovejoy and Balment, 1999; Boorse and Denver, 2006). CRF is known to induce the release of adenohypophysial hormones such as adrenocorticotropic hormone (ACTH), ß-endorphin and α-melanocyte-stimulating hormone (α-MSH) from the pituitary, and there is ample evidence that CRF and its related peptides play multiple roles in animal development and also in physiological and behavioral adaptation to environmental changes (Tonon et al., 1986; Hauger et al., 1988, 2006; Denver, 2009; Papadimitriou and Priftis, 2009).

In submammalian vertebrates, CRF acts as a potent stimulator of corticotropin and thyrotropin release (Carsia et al., 1986; Malagón et al., 1991; Geris et al., 1996; Larsen et al., 1998; Boorse and Denver, 2004, 2006; Ito et al., 2004; Okada et al., 2007). During metamorphosis in anuran amphibians, plasma thyroidal and adrenocortical hormone levels increase almost simultaneously (see Kikuyama et al., 1993). Since adrenal corticoids potentiate the action of thyroid hormone (Kikuyama et al., 1983), the increase of both hormone levels has been considered to facilitate metamorphic changes (Denver, 1993; Krain and Denver, 2004).

CRF acts not only as a hypophysiotropic factor as described above, but also as a regulator of feeding behavior and stress responses in vertebrates (Uehara et al., 1989; Kalra et al., 1999; Bernier and Peter, 2001; Hillebrand et al., 2002; Ohgushi et al., 2001;

* Corresponding author. Address: Laboratory of Regulatory Biology, Graduate School of Science and Engineering, University of Toyama, 3190 Gofuku, Toyama 930-8555, Japan. Fax: +81 76 445 6549.
E-mail address: kmatsuda@sci.u-toyama.ac.jp (K. Matsuda).

0016-6480/$ - see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.ygcen.2010.01.004

Tachibana et al., 2004; Saito et al., 2005). It has been reported that, in the goldfish, intracerebroventricular (ICV) injection of CRF exerts an anorexigenic action (de Pedro et al., 1993; Volkoff et al., 2005). This CRF-induced anorexigenic action is blocked by the treatment with a CRF 1 receptor/CRF 2 receptor antagonist, α-helical $CRF_{(9–41)}$ (de Pedro et al., 1997; Bernier, 2006; Maruyama et al., 2006). In this species, our previous reports indicate that neuromedin U, octadecaneuropeptide and α-MSH exert anorexigenic effects, which are blocked by treatment with α-helical $CRF_{(9–41)}$, suggesting that CRF has a crucial role in the regulation of feeding behavior in goldfish as a potent anorexigenic neuropeptide (Matsuda et al., 2008; Maruyama et al., 2009; Matsuda, 2009).

It is known that as metamorphosis progresses, oropharyngeal parts and intestinal epithelium of anuran larvae are reconstructed (Ishizuya-Oka and Shi, 2007), resulting in the decline of feeding behavior (Larson and Reilly, 2003). Some previous reports also indicate that CRF reduces feeding or foraging behavior in anurans such as *Xenopus* and spadefoot toads (Crespi et al., 2004; Crespi and Denver, 2004, 2005). However, there is no direct evidence that CRF suppresses the food intake by anuran larvae during metamorphosis.

The present study was conducted to study changes of the distribution of neuronal cell bodies and nerve fibers exhibiting CRF-like immunoreactivity (CRF-LI) in the brain of bullfrog larvae during metamorphosis and to obtain direct evidence that CRF suppresses the food intake by the larvae.

2. Materials and methods

2.1. Animals

Bullfrog (*Rana catesbeiana*) larvae weighing 7–20 g at several developmental stages were collected from ponds in the suburbs of Toyama City, Japan. The developmental stages of the larvae were determined according to Taylor and Kollros (1946). Animal experiments were conducted in accordance with the Invasive Alien Species Act of Japan and the University of Toyama's guidelines for the care and use of alien and laboratory animals.

2.2. Antiserum and immunohistochemical staining

The primary antiserum against bullfrog CRF (anti-CRF serum) was raised in a rabbit; details of its production and characterization have been described elsewhere (Ito et al., 2004). Larvae at premetamorphic stages (VII and X), late prometamorphic stage (XVIII) and the end of climax stage (XXV) were anesthetized with MS-222 (3-aminobenzoic acid ethyl ester, Sigma–Aldrich Co., St. Louis, MO, USA) at 2 mM and decapitated, and their whole brains with pituitary glands were collected. Each brain from larvae was immersed in Bouin's fixative at 4 °C for 24 h, trimmed, dehydrated and embedded in paraffin. Six-micrometer-thick cross and sagittal sections were cut, and several sections were stained with cresyl violet. All steps during immunostaining were preceded by washing in 0.1 M phosphate buffered saline (PBS, pH 7.2). The sections were blocked with normal swine serum (diluted 1:50, DAKO A/S, Denmark), then stained with anti-CRF serum (diluted 1:4000) at room temperature for 24 h. Following a 1-h incubation with swine anti-rabbit immunoglobulin (diluted 1:100, DAKO), the sections were treated with rabbit peroxidase–anti-peroxidase complex (diluted 1:100, DAKO) for 1.5 h and allowed to react by incubation with 20 mg 3,3′-diaminobenzidine-4 HCl (Dojin, Tokyo, Japan) and 0.005% H_2O_2 in 100 ml 0.1 M Tris–HCl buffer (pH 7.6) containing 0.06% imidazole (Kanto Chemical Co., Inc., Tokyo, Japan). After immunostaining, the sections were counterstained with hematoxylin, dehydrated, cleared in xylene, and mounted. Specificity of the immunostaining was checked by incubating the sections with normal rabbit serum instead of the primary antiserum, or with 1 ml of anti-CRF serum (diluted 1:4000) preincubated with excess (20 μM) synthetic ovine CRF (Peptide Institute, Inc., Osaka, Japan). In both cases, immunoreactivity for CRF was negative. Immunostained sections were observed using a light microscopy (CH40, Olympus, Tokyo, Japan), and were recorded as digital images by a digital camera (CoolPix 955, Nikon, Tokyo, Japan). The nomenclature of brain nuclei was based on the paper of Mathieu et al. (2001) for the frog, *Rana ridibunda*.

2.3. Changes in the distribution of CRF-LI in the larval brain

Larvae at stages VII, X, XVIII and XXV were anesthetized, decapitated, and the brains with pituitary glands were sectioned and immunostained as described above. Neuronal cell bodies containing CRF-LI were counted manually as described elsewhere (Nakamachi et al., 2006; Matsuda et al., 2007). Immunohistochemical staining for CRF was carried out on every other section, and the other sections were stained with cresyl violet. Individual neuronal cell bodies with CRF-LI could be identified, and only those with a nucleus were quantified. The number of cells with CRF-LI observed in every other section was counted, and 2 times of counting was expressed as total number of neuronal cells containing CRF-LI in the thalamus and hypothalamus.

2.4. Evaluation of food intake by bullfrog larvae

Two types of powder diet with green and red colors, respectively, were obtained from Itosui Co., Tokyo, Japan, and Kyorin Co., Hyogo, Japan. Firstly, the test larvae at stage IX were fed the green-colored diet and kept in the laboratory condition. Then following 48 h starvation, feeding of an adequate amount of the red-colored food [(3% of their body weight (BW)] started. After 15, 30 and 60 min, each specimen was decapitated and the intestine was removed. The weight of the intestinal contents in red color was measured, and expressed as mg food taken in per g BW during 15-, 30- or 60-min period. The experiments were conducted around noon.

2.5. Effect of CRF on food intake by stage IX larvae

Each larva at stage IX which had been fed the green-colored diet was fasted for 48 h. Thereafter, the animal was placed in a stereotaxic apparatus under anesthesia with MS-222. A small area of the parietal skull was carefully removed using a surgical blade (No. 19, Futaba, Tokyo, Japan), and then approximately 0.7–1.0 μl of CRF dissolved in saline (0.6% NaCl and 0.02% Na_2CO_3) at the concentration of 0.1, 1.0 or 10 pmol/g BW was injected into the third ventricle of the brain using a small Hamilton syringe. The gap in the bone was then filled with a surgical bonding agent (Aron Alpha, Sankyo, Japan). The accuracy of the injection site and volume was confirmed after the experiment by examining whether Evans blue dye was present in the ventricle without leakage. Control larvae in each experiment were injected with the same volume of saline in the same way as for the experimental group. Each larva that had received the ICV injection was placed individually in a small experimental tank (diameter 11 cm) containing 700 ml of tap water. After the recovery from anesthesia, each larva was supplied with the red-colored food equivalent to 3% of its BW. After 15 min, the weight of the intestinal contents was measured as described above.