General and Comparative Endocrinology 159 (2008) 214–225

Contents lists available at ScienceDirect

General and Comparative Endocrinology

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

Immunocytochemical localization, HPLC characterization, and seasonal dynamics of vasotocin in the brain, blood plasma and gonads of the catfish *Heteropneustes fossilis*

V. Singh, K.P. Joy *

Center of Advanced Study, Department of Zoology, Banaras Hindu University, Varanasi, Uttar Pradesh 221005, India

ARTICLE INFO

Article history:
Received 25 June 2008
Revised 30 August 2008
Accepted 11 September 2008
Available online 19 September 2008

Keywords:
Brain
Catfish
Gonads
HPLC
Immunoassay
Seasonal changes
Sex differences
Vasotocin

ABSTRACT

Immunocytochemical distribution and dynamics of vasotocin (VT) were studied in the air-breathing catfish *Heteropneustes fossilis* in relation to the reproductive cycle. Vasotocin was localized in the brain and ovary by streptavidin–biotin immunocytochemistry. The immunoreactivity was found throughout the hypothalamo–hypophysial neurosecretory system consisting of the magnocellular and parvocellular neurons of the nucleus preopticus, neurosecretory axonal tract and neurohypophysis (NH). The VT neurons showed seasonal changes; they were numerically less in resting phase but increased during the recrudescent phase. The neurons were hypertrophied and degranulated in pre-spawning phase and heavily degranulated and vacuolated in spawning phase. In the NH, the density of VT fibers increased up to the pre-spawning phase and decreased thereafter. In the ovary, VT immunoreactivity was noticed in the follicular layer and varied with the growth of the follicles. Vasotocin was characterized and quantified by a high performance liquid chromatography with UV detection method in the brain, plasma and ovary. Brain and plasma VT concentrations were also assayed with an EIA method, which was more sensitive than the HPLC method with values about 2-fold higher. Vasotocin levels showed significant seasonal and sexual differences with higher concentrations in females in the recrudescent (preparative, pre-spawning and spawning) phase. Brain VT recorded the highest concentration in the preparative phase (both sexes) while plasma (both sexes) and ovarian VT in the spawning phase. The ovarian concentration of VT was 15- and 25-fold higher in the pre-spawning and spawning phases (when expressed per mg protein), respectively, than plasma but lower than brain levels. In testis, VT concentration was relatively low and apparently did not show any significant seasonal variation. The seasonal activity patterns and gonadal distribution of VT indicate a reproductive function of the peptide.

1. Introduction

In teleosts, vasotocin (VT) is the basic neurohypophysial nonapepetide, as in other non-mammalian vertebrates, and isotocin (IT) the neutral peptide (Acher, 1996; Urano et al., 1994). These peptides are secreted by the hypothalamo–hypophysial neurosecretory (HN)) system consisting of the nucleus preopticus (NPO) and neurohypophysis (NH) and released into the circulation for peripheral actions. But recent studies show that they are secreted/distributed in other parts of the brain (Foran and Bass, 1998; Goodson and Bass, 2000; Urano et al., 1994). The brain apart, nonapeptides like oxytocin (OT), vasopressin (VP) and VT are also present in peripheral organs such as ovary, uterus, testis, adrenal, thymus and sympathetic nervous system of higher vertebrates (Barth et al., 1997; see review by Clements and Funder, 1986; Saito et al., 1990). In the ovary, the nonapeptide levels varied with the maturity of the gonad, physiological state and species, and were implicated in various ovarian functions. The occurrence of the peptides in the ovary of lower vertebrates like fish has not been investigated, though in a recent study Bobe et al. (2006) characterized the genes encoding for VT and IT, which were up-regulated during oocyte maturation in the rainbow trout *Oncorhynchus mykiss*.

Vasotocin is demonstrated to have a role in the regulation of diverse physiological activities such as cardiovascular function, osmoregulation, stress response, metabolism, reproduction, behavior and circadian and seasonal activities (see review by Balment et al., 2006). The VT system has been demonstrated to show sexual dimorphism in the number and size of neurons, immunoreactivity and gene expression (Boyd and Moore, 1992; Foran and Bass, 1998; Moore et al., 2000; Moore and Lowry, 1998; Ota et al., 1996). The administration of VT has been shown to influence or modify reproductive/seasonal behavioral activities (spawning, courtship, egg laying, clasping and song production) in a variety of vertebrates like fish, amphibians, reptiles and birds (Guillette and Jones, 1982;

* Corresponding author. Fax: +91 542 2368174.
E-mail address: kpjoybhu@gmail.com (K.P. Joy).

0016-6480/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.ygcen.2008.09.003

Moore, 1992; Pickford and Strecker, 1977; Salek et al., 2002; Seth et al., 2004).

The HN system of the catfish was described earlier (Sathyanesan and Haider, 1971) but no attempt was made to characterize the neurohypophysial peptides and their dynamics. An osmoregulatory involvement of VT was demonstrated in the catfish and VT was implicated in osmotic homeostasis in the catfish along with prolactin and cortisol (Goswami et al., 1983) but no reports are available on its reproductive role. Therefore, the present study was undertaken to localize, characterize and quantify VT in the brain, plasma and gonads of the catfish *Heteropneustes fossilis* during different phases of the reproductive cycle to demonstrate its possible involvement in reproduction.

2. Materials and methods

2.1. Fish collection and acclimatization

The reproductive cycle of the catfish in Varanasi can be divided into five phases: resting (November–January), preparatory (February–April), pre-spawning (May–June), spawning (July–August) and post-spawning (September–October) (Senthilkumaran and Joy, 1994). Adult female (40–60 g) and male (30–50 g) catfish were purchased from local fish markets in Varanasi in each phase for two consecutive years (2004 and 2005) between February–December. The fish were acclimated in flow-through aquarium tanks under normal photoperiod and ambient temperature (13L: 11D; 28 ± 2 °C) for 48 h before sampling. During the maintenance, the fish were fed daily minced goat liver *ad libitum*.

2.2. Chemicals

Synthetic arg8-vasotocin acetate salt, 4-Ser, 8-Ileu oxytocin (IT), oxytocin acetate salt hydrate and 3, 3-diamino benzidine tetrahydrochloride (DAB) were purchased from Sigma Chemicals, St. Louis, USA. Vasopressin (VP) (Cpressin-P, Samarth Life Sciences Pvt. Ltd., Solan, India) was purchased from local a drug store. Arg8-vasotocin enzyme immunoassay kit [EIA kit, catalogue No S-1239) (EIAH8121] was purchased from Bachem Penisula Laboratories, California, USA. HPLC grades of acetonitrile and methanol, and glacial acetic acid, trifluoroacetic acid (TFA) and acetone were purchased from E. Merck, Mumbai, India. Solid phase extraction (SPE) C18 cartridge was purchased from Ranbaxy Fine Chemicals Ltd. Ghaziabad, India. Vasotocin antiserum was a generous gift of Dr. Daisuke Saito (through Dr. A. Urano), Division of Biological Sciences, Graduate School of Sciences, Hokkaido University, Sapporo, Japan. Biotinylated IgG and Streptavidin–biotin horseradish peroxide kit were purchased from Bangalore Genei, Bangalore, India. Other chemicals were of analytical grade and purchased locally.

2.3. Seasonal study

The experiments were conducted in accordance with the guidelines of the national/local ethical committee for research in animals.

Male and female catfish were sampled in the middle of each phase of the reproductive cycle, weighed and blood samples were withdrawn from caudal vein with a heparinized syringe and kept for 30 min at 4 °C. The fish were decapitated and brain along with pituitary was removed and dropped into cold anhydrous acetone. The acetone-dried samples were stored at −80 °C for a week, before extraction of the peptides (Perrott et al., 1991). The ovaries and testes of the catfish were removed from the peritoneal cavity and were immediately transferred to −80 °C. For plasma separation, the blood samples were centrifuged at 2800*g* at 4 °C for 15 min and clear plasma was collected in separate tubes and stored at −80 °C until extracted within a week. As tissue controls, liver and ventral muscle were sampled in the preparatory phase and processed similar to the ovarian tissue.

For immunocytochemical study, brains of either sexes, ovary and testis ($n = 5$) were sampled in different seasons and fixed in Bouin's fluid, without acetic acid. Sagittal and transverse paraffin sections were taken at 8 μm thick, spread on gelatin-coated slides and stored.

2.3.1. Immunocytochemistry

Immunocytochemistry (ICC) was performed according to the method of Hsu et al. (1981) with some modifications, as mentioned below. The hydrated sections were immersed in 0.5% H_2O_2 in methanol for 30 min at room temperature to remove endogenous peroxidase. For differential staining, primary antiserum (anti-VT pre-adsorbed with heterologous peptides) was applied to the sections (1:2500) and incubated at 37 °C for 2 h in a humid chamber. The sections were rinsed with PBS and were then incubated with biotinylated goat anti-rabbit IgG (1:200) for 60 min at room temperature, followed by a wash in PBS for 20 min and were incubated with streptavidin–biotin horseradish peroxidase complex for 60 min at room temperature. The sections were stained with 0.05% DAB containing 0.03% H_2O_2 in Tris-buffer for 10 min or till the brown color appeared, further processed and mounted in DPX. A detailed control study was conducted by omitting the primary antiserum (anti-VT) or using the antibodies pre-adsorbed with VT, IT or OT. For pre-adsorption, the antiserum was treated with homologous or heterologous peptide in a concentration of 100 μg/ml at 4 °C in a shaker bath for overnight (Saito et al., 2004). The mixture was centrifuged and the supernatant was used for the incubation.

2.3.2. HPLC assay

The acetone-dried brains were weighed and homogenized in a glass homogenizer in 0.5 ml of 0.5 M acetic acid. The homogenate was heated for 5 min in a boiling water bath, cooled at room temperature and centrifuged at 2800*g* at 4 °C for 15 min (Perrott et al., 1991). Clear supernatant was collected and processed for extraction of the hormones. The samples of ovary, testis, muscle and liver were processed according to the method of Walsh and Niall (1980). Protein content in the tissue was determined by the method of Lowry et al. (1951).

The peptides were extracted from the samples by reverse phase chromatography using a C18 SPE cartridge of 3 ml capacity (pore size 60 A°, particle diameter 40 μm) according to the method of Kulczykowska (1995). Since the peptide concentration was low, plasma and supernatants of brain, ovary and testis were pooled from 5 fish each to make samples (group size = 5). Muscle and liver were pooled from ten fish each to make samples (group size = 5). Just before the assay, the samples were reconstituted with acidified ethanol (plasma, testis—100 μl; brain—300 μl; ovary, liver and muscle—200 μl) for complete dissolution. Twenty μl samples were injected into the HPLC column for analysis.

Chromatography was performed according to the method of Kulczykowska (1995) with a Shimadzu (Kyota, Japan) system with two pumps (LC-10 ATVP), system controller (SCL-10 AVP) and digital UV detector (SPD-10AVP). The system was operated with Shimadzu Class VP series software. Chromatographic separation was carried out on a Phenomenex Luna (2) C18-reverse phase column (150 × 4.5 mm ID; 5 μm particle diameter, 80 A° pore size) connected to a Phenomenex guard column filled with the same material. The system was run at a flow rate of 1 ml/min and the eluate was monitored at 215 nm. The column temperature was maintained at 22 °C and a gradient elution system was applied. Solvent A was 0.1% TFA in water and solvent B 0.1% TFA in acetonitrile: water (3:1). A linear gradient from 20% to 40% solvent B in