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ORIGINAL ARTICLE

Localization, distribution and expression of growth hormone in the brain of Asian Catfish, *Clarias batrachus*

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Abstract

Intense immunoreactivity was observed in several neurons of the nucleus preopticus (NPO) located in the preoptic area (POA), in addition to several GH cells in the proximal pars distalis (PPD) of the pituitary gland of *Clarias batrachus*. The immunoreactive cells were located in the paraventricular as well as supraoptic subdivisions of the NPO. GH immunoreactive fibers projecting from the neurons were traced caudally to the pituitary gland via the conspicuous preoptico-hypophysial tract (PHT) in the ventral tuberal area to the neurohypophysis of the pituitary. Apart from these immunoreactive fibers in the preoptico-hypophysial tract, some fine caliber fiber probably arising from the neurons located dorsally in the NPO also showed GH immunoreactivity, and these fibers constituted an independent tract. Bilaterally, it extended caudally through the dorsal hypothalamus almost as far as the saccus vasculosus where it curved sharply to descend into the caudal tuberal region and finally entered into the pituitary gland. The fibers of this tract were mainly distributed in the rostral pars distalis (RPD). This tract is quite distinct from the preoptico-hypophysial tract and herein called as the accessory preoptico-hypophysial tract (APHT). Expression of GH mRNA in the NPO was found 65-fold more than that of the control region, rostral cerebellum. These results altogether suggest that GH secreted by NPO neurons might serve as a neuro-modulatory role in the brain of *C. batrachus*. Transportation of GH to the pituitary via two independent tracts may represent the duality of neuroendocrine function. The present study underscores the possibility that GH in the brain of vertebrates may be a phylogenetically conserved phenomenon and provide clues to our understanding of the evolutionary course taken by the hormone.

Keywords Growth hormone · mRNA · Pituitary · Preoptico-hypophysial tract · Hypothalamus

Introduction

Classically, growth hormone (GH) is produced primarily in the somatotrophs of the pituitary gland of all vertebrates and is known to serve as an important regulator of growth and metabolism. However, several other studies have demonstrated the presence of GH transcript and/or immunoreactive (ir) GH protein in many extrapituitary tissues in mammals (Wu et al. 1996; Yoshizato et al. 1998; Slominski et al. 2000; Recher et al. 2001), particularly in neural tissues (Mustafa et al. 1995; Donahue et al. 2002, 2006; Harvey et al. 2003; Sun et al. 2005). Date back in 1993 Harvey et al. (1993), have reviewed such studies reporting the existence of ir-GH protein and/or GH mRNA in the mammalian brain (telencephalon, diencephalon, mesencephalon, metencephalon, and myelencephalon) with greater GH reactivity in the amygdala, hippocampus, and hypothalamus. Abundant GH immunoreactivity is also reported in the brain of some birds like chicken, Turkey hens, ring doves. Render et al. (1995) have measured considerable amount of ir-GH in the mediobasal hypothalamus and extra hypothalamic regions of the adult chicken brain through radioimmunoassay and western blotting. Nevertheless, the GH immunoreactivity is shown in the whole brain of embryonic chicken immunohistochemically (telencephalon, diencephalon, mesencephalon, metencephalon, and myelencephalon) (Harvey et al. 2001; Murphy and Harvey 2001). A dense ir-GH perikarya and fibers are detected in the hypothalamus (periventricular, paraventricular, inferior hypothalamic and infundibular hypothalamic nuclei), hippocampus, medial and lateral septal areas,

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and median eminence of the Turkey hen and ring dove brain (Ramesh et al. 2000).

ir-GH is also demonstrated in neural tissues other than brain. The gray matter and ventral horn of the thoracic and lumbar spinal cord exhibit GH immunoreactivity in mammals (Lechan et al. 1983; Harvey et al. 1993). GH immunoreactivity is observed in the otic and optic vessels (Harvey et al. 2001; Murphy and Harvey 2001), neural retina, particularly in the retinal ganglion cells (Harvey et al. 2003; Baudet et al. 2003, 2008; Harvey 2010), trigeminal and vagal nerves, extensor nerve of the limb bud, and the ethmoid nerves in the embryonic chickens (Murphy and Harvey 2001).

The extrapituitary expression of GH is reported in some fishes too. GH transcript has been detected in the gonads of rainbow trout (Yang et al. 1999; Biga et al. 2004), Pejerrey, *Odontesthes bonariensis* (Sciara et al. 2006), and *Epinephelus coioides* (Li et al. 2005). Yang et al. (1999) have detected GH mRNA in mature oocytes of the rainbow trout. Li et al. (2005) have noted PCR signal for GH mRNA expression in the unfertilized eggs of groupers, suggesting that GH may be involved in the promotion of the oocyte cell growth and in the embryo development in fish. A low level of GH mRNA has also been detected in the gill, heart, kidney, liver, pyloric caeca of the rainbow trout (Yang et al. 1999). However, to our knowledge, there is no information on the GH expression in the brain of anamniotic vertebrates including fish. In the present study, therefore, attempts were made to examine the neuroanatomy of GH-containing system in the hypothalamus, if any, and the expression of GH mRNA in the freshwater catfish *Clarias batrachus*, which is consumer preferred and widely cultured catfish in Asia.

Materials and methods

Animals and tissue processing

Adult female catfish *C. batrachus* (100–120 g) was collected during the months of April–May, a period that coincides with the recrudescence phase of its reproductive cycle. The fish was maintained for 1 week under normal photoperiodic conditions and fed small pieces of goat liver once daily. Experiments were performed according to the guidelines of the Institutional Animal Ethics Committee (IAEC) under the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India. Twelve fishes were anesthetized with 2-phenoxyethanol (1:2000; Sigma, USA) and perfused transcardially with 100 ml ice-cold phosphate-buffered saline (PBS, pH 7.45) followed by an equal volume of ice-cold 4% paraformaldehyde in phosphate buffer (pH 7.45). Brain along with the pituitary was dissected out, postfixed in the same fixative overnight at 4 °C, cryoprotected in 10% (2 h), 20% (2 h), and 30% (overnight at 4 °C) sucrose solution, frozen with expanding CO_2, and serially cut on a cryostat (Leica CM 1850) at 15 µm thickness in transverse or sagittal planes. Sections were mounted on poly-L-lysine (Sigma) coated slides and processed for immunocytochemical labeling with antiserum against GH according to the details given below.

Immunocytochemistry

Streptavidin–biotin–peroxidase method of immunocytochemistry was employed to localize GH in the pituitary sections according to the protocol already described (Mazumdar et al. 2006). Briefly, the sections were washed in PBS for 15 min and treated with blocking solution containing 2% bovine serum albumin (BSA) and 0.3% Triton X 100 in PBS. Sections were then incubated for 2 h at 25 °C in a humid atmosphere with homologous antisera against GH (1:5000) of *C. batrachus*. The specificity of the GH antiserum was thoroughly checked and validated before its use and is described elsewhere (Lal and Singh 2005; Mazumdar et al. 2006).

Following incubation in primary antisera, the sections were rinsed in PBS for 10 min and incubated with biotinylated anti-rabbit IgG (Vector Laboratories, Burlingame, CA, USA) for 40 min, followed by ExtrAvidin-peroxidase conjugate (Sigma, EXTRA-3). 3-Amino-9-ethyl carbazole was used as a chromogen to visualize the reaction product. Sections were washed in distilled water and mounted in glycerol gelatin.

The immunofluorescence technique was also employed to visualize GH in sections, for which sections were rinsed in PBS, soaked in 2% BSA in PBS, incubated overnight at 4 °C in primary antibodies (1:2000) followed by incubation in biotinylated anti-rabbit IgG (Vector, 1:100) for 40 min at room temperature. Sections were then rinsed in PBS and incubated in ExtrAvidin-conjugated TRITC (Sigma; 1:100) for 1 h. Then after, sections were rinsed in PBS and mounted in Vectashield mounting medium (Vector) and observed under Leitz Laborlux-S epifluorescence microscope (Leica, Germany). The desired fields from the sections were photographed and the images were adjusted for size, contrast, and brightness using Adobe Photoshop 7.0 and Corel Draw 11, and panels were prepared using the same software.

Sample collection for GH mRNA analysis in the preoptic area of *C. batrachus*

Catfish were anesthetized with 2-phenoxyethanol (1:2000) and their heads decapitated. Following decapitation, 1–2 mm thick slices from the preoptic area of the brain from eight to ten female catfish were excised under Stereozoom Microscope (Leica, Model No. MZ3), and collected aseptically

in tubes and snap frozen on powdered dry ice and stored at − 80 °C until processed further. Similarly, 1 mm slices from the rostral part of the cerebellum of the same fish were collected as a control sample.

RNA isolation and reverse transcription

Total RNA was extracted from the pooled brain slices (≈ 100 mg) from preoptic region and cerebellum separately using TRI REAGENT™ RNA Isolation protocol (Sigma-Aldrich, USA, #T9424), based on phenol/chloroform extraction method. The RNA concentrations were quantified spectrophotometrically (260 nm) and the purity of the samples was assessed by the $A_{260/280}$ nm ratio (Sambrook et al. 2001) and integrity was also checked using gel electrophoresis followed by ethidium bromide staining. RNA samples from each brain area were adjusted to 1 µg in each reaction. From total RNA, first strand cDNA was synthesized using 'Omniscript Reverse Transcriptase kit for first-strand cDNA synthesis' (Qiagen, USA, #205111). About 1 or 2 µg total RNA was incubated with 1 µM random hexamer primer. Reverse transcriptase (RT) mix (containing 1× RT buffer, 0.5 mM dNTPs, 10U RNase inhibitor, and 4U RTase) was added to the sample. Prior to reverse transcription (RT), contaminating genomic DNA was removed by treating with RNase-free DNase-I (Ambion, Austin, Tx, USA #2222) at 37 °C for 30 min, and then the RT reactions were stopped by adding 5 mM EDTA. First strand cDNA was synthesized by incubating the above mix at 37 °C for 60 min and stored at − 20 °C until further use.

Design of primers and probes for real-time PCR and real-time quantification (RT-qPCR)

Based on the mRNA sequence of *C. batrachus* GH gene (Gene Accession no. AF416485) qPCR–Gene-specific primers and probes were commercially designed by 'Assays-by-Design Gene Expression Service' (PE-Applied Biosystems, USA, Part no. 4332079). The following primer pair and probe were synthesized by Applied Biosystems: GH forward 5′-tgggcatcagcgtgcttat-3′, reverse 5′-cgtccaggctggcttgt-3′, probe 5′-atcctcgcatccctcg 3′; The TaqMan probes were labeled at the 5′ end with reporter dye 6-carboxyfluorescein (FAM™- TaqMan® MGB probes). Commercially available TaqMan Gene expression Assay system for endogenous control i.e., 'Eukaryotic 18S rRNA Endogenous Control' (ABI, USA, Part no. 4319413E) was selected. The 18 s rRNA probe was labeled at the 5′ end with reporter dye VIC™—TaqMan® MGB probes. Specificity of each primer pair was analyzed by 2.5% agarose gel electrophoresis stained by ethidium bromide and confirmed to give a single amplified band. Quantitative PCR was carried out using an ABI Prism 7000 Sequence Detection System (Applied Biosystems) in a 25 µl reaction mixture prepared in 0.2 ml thin walled, optical-grade PCR tubes using the manufacturer's protocol after full optimization (PE-Applied Biosystems) containing 5 µl of cDNA as template, 900 nM of each primer (forward and reverse primers) and 200 nM of probe/1× primer–probe mix (FAM-labeled probe for the target, GH gene, and VIC-labeled probe for reference gene, 18S rRNA, were used), and 1× Taqman universal master mix. TaqMan PCR assays for GH and 18S rRNA were run from the same samples in duplicate. The thermal cycling protocol included 2 min at 50 °C for optimal AmpErase UNG activity, 10 min of AmpliTaq polymerase activation at 95 °C, and 40 cycles of amplification, consisting of 15 s of denaturation at 95 °C and 60-s annealing/extension at 60 °C. CT determinations were performed by the instrument for each reaction using default parameters. All qPCR reactions were analyzed using the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) following the standard procedures for the operation of the ABI Prism 7000 system.

Validation of target and reference amplification

To ensure that two amplicons (GH/18SrRNA) possess almost the same amplification efficiency, a prerequisite to adopting $\Delta\Delta$CT method, the method was validated by plotting CT against the logarithm of the template dilution factors, and the slope was determined. The relationship between the CT value and the logarithm of the dilution factor of cDNA template for the target genes GH was 0.99, while 18S rRNA was 0.99. The slope of the CT versus log dilution factor plot for GH/18S rRNA was − 3.4/− 3.5, which is as per the recommendation and in an acceptable range. Thus, this method for quantification of GH/18S rRNA amplification was reliably adopted.

Analysis of data

The relative GH mRNA expression levels were calculated using the arithmetic comparative method ($\Delta\Delta$CT method), which involves normalization of the CT values for the GH mRNA to the 18S rRNA CT values. Threshold cycle (CT) numbers were established using SDS 1.1 RQ software (Applied Biosystems). In this method, real-time PCR results were analyzed by subtracting the mean of 18S rRNA threshold cycle (CT) values from the mean CT values of GH for both samples; preoptic area (POA) as well as rostral part of the cerebellum (control sample) to obtain ΔCT values. The ΔCT values of the control samples were then subtracted from the ΔCT values of the POA samples to obtain the $\Delta\Delta C$T values. The fold induction in levels of GH in POA samples as compared to the control samples was obtained by the formula $2^{-\Delta\Delta CT}$. This method has been successfully applied for the quantitative detection of expression levels of

IGF-I and IGF-II in common carp (Vong et al. 2003) and channel catfish (Peterson et al. 2004).

Statistical analyses

Data related to GH gene expression (GH mRNA) is denoted as Mean ± SEM of the three times separately conducted experiments, and Student '*t*' test was employed to compare it with the control level of GH expression in rostral part of the cerebellum.

Results

GH immunoreactivity in the forebrain and pituitary of *C. batrachus*

The ir-GH positive cells were observed in the brain (extending from the dorsal hypothalamus to as far as caudal hypothalamus and tuberal area) and pituitary gland. ir-GH cells were also detected in the neurohypophysis (Fig. 1). The forebrain revealed intensely stained GH-containing neurons in the nucleus preopticus (NPO) located in the preoptic area (POA) and cell in the proximal pars distalis (PPD) of the pituitary in *C. batrachus*. Distinct immunoreactivity was also seen in the preoptico-hypophysial tract (PHT) issued by the NPO neurons which extended to the pituitary.

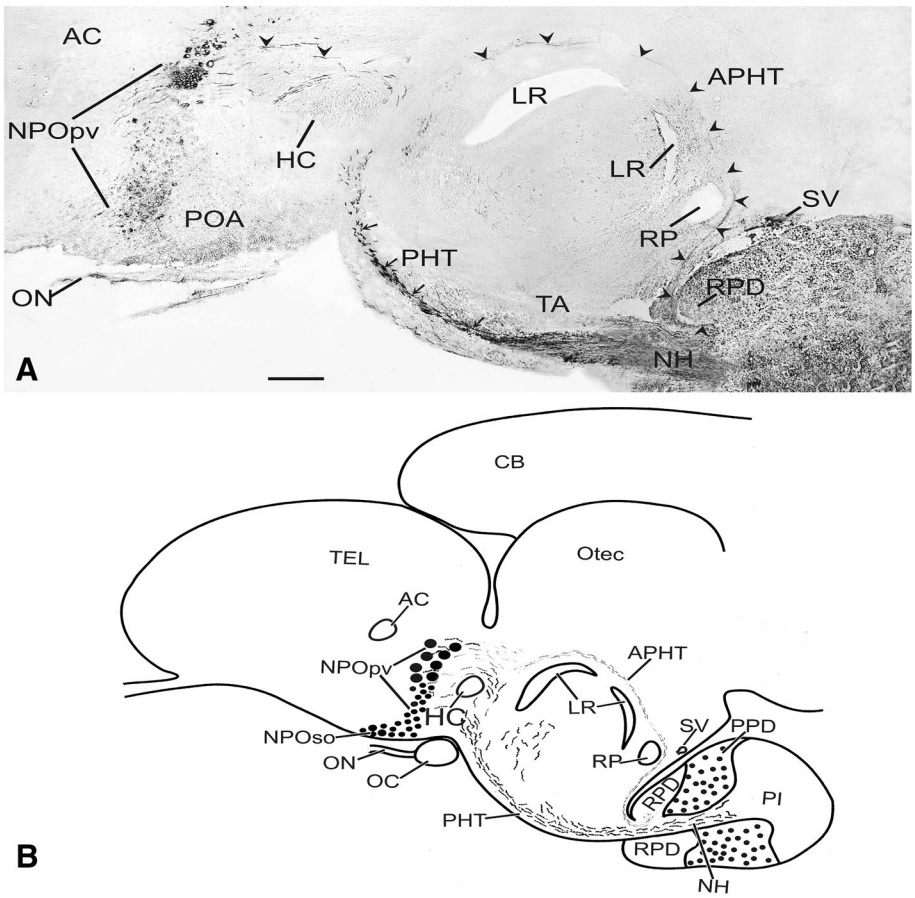

Fig. 1 Sagittal section through the preoptic area, hypothalamus and pituitary (**a**) and schematic drawing of the parasagittal view of the brain (**b**) of *C. batrachus* showing GH immunoreactive system. Note the presence of GH immunoreactive cells in the paraventricular subdivision of the NPO (NPOpv) (**a**) and in the preoptico-hypophysial tract (PHT) as they extend through the neurohypophysis (NH) of the pituitary gland. Note the presence of accessory PHT (APHT) (arrowheads, **a**) which is traceable through the dorsal hypothalamus as far as the caudal hypothalamus where it descends to the tuberal area, penetrates into the pituitary gland and terminates in the rostral pars distalis (RPD). GH cells are seen in the PPD. *AC* anterior commissure, *CB* cerebellum, *HC* horizontal commissure, *NPO* nucleus preopticus, *OC* optic chiasma, *ON* optic nerve, *PI* pars intermedia, *PPD* proximal pars distalis, *RP* recessus posterioris, *NPOso* supraoptic subdivision of NPO, *SV* saccus vasculosus, *TA* tuberal area, *TEL* telencephalon, *Otec* optic tectum. (Scale bar = 200 µm)

The structure of the NPO of *C. batrachus*, has already been described earlier using classical neurosecretory stains (Subhedar et al. 1990). NPO consists of two subdivisions; the neurons located on either side of the preoptic recess are called as the paraventricular neurons, while others located, ventrolaterally just above the optic tract were called as the supraoptic group. Several neurons of the paraventricular group showed intense GH immunoreactivity in the bright field as well as fluorescence microscopy (Figs. 1a, 2b–e). GH immunoreactive fibers from these neurons extend ventrolaterally (Fig. 2d, e), then turned medially and ran around the horizontal commissure, aggregating as the preoptico-hypophysial tract in the midsagittal plane at the base of the tuberal area which finally entered into the pituitary gland

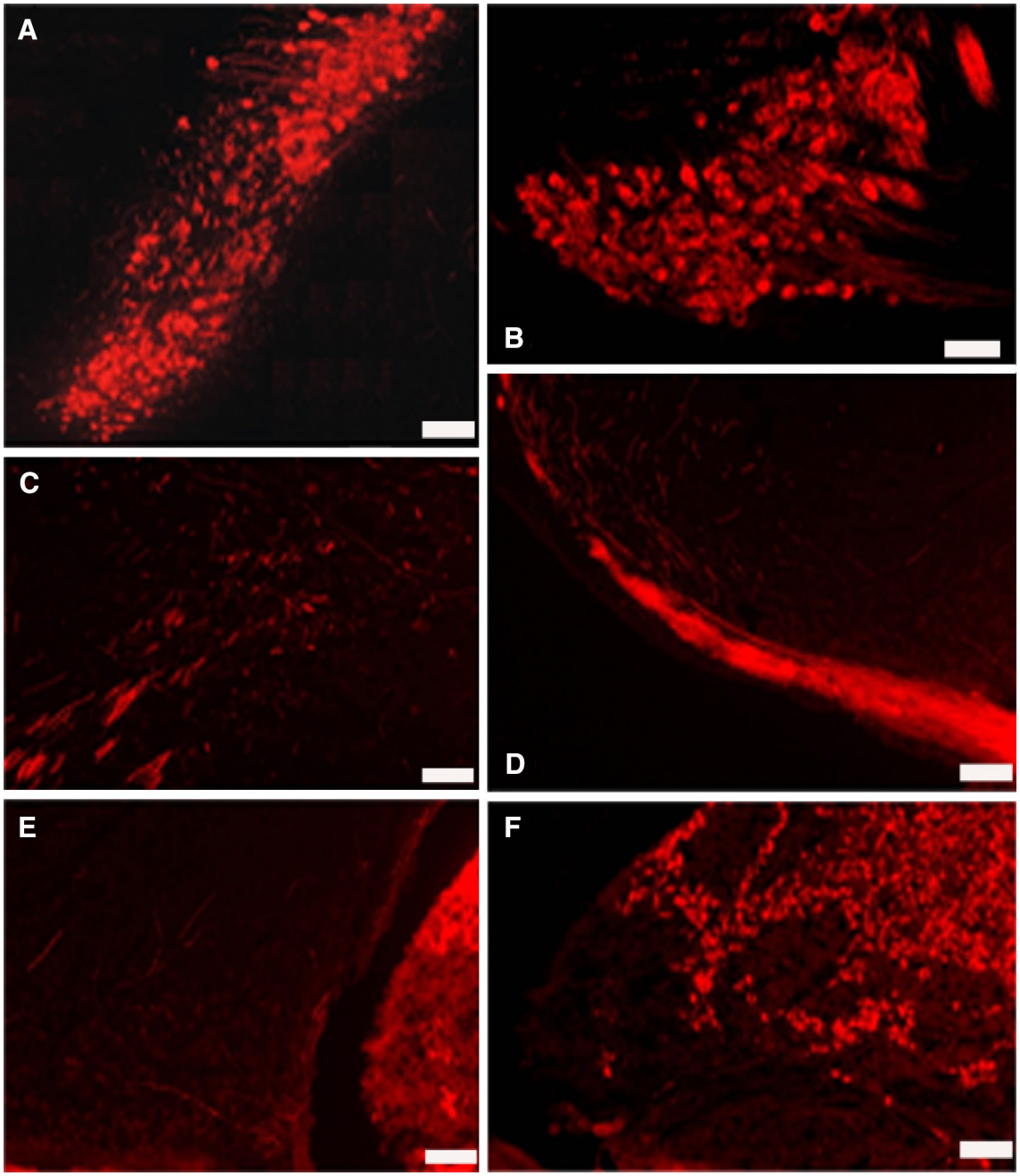

Fig. 2 Sagittal sections through the hypothalamus and pituitary of *Clarias batrachus* stained with GH antibody observed under an epi-fluorescence microscope (Leica, Germany) showing GH immunoreactivity. **a** Entire—NPO part (scale bar = 50 µm), **b** ventral part of NPO region (scale bar = 200 µm), **c** fibers from NPO running caudally (scale bar = 100 µm), **d** hypothalamo-hypophysial tract (scale bar = 50 µm), **e** stray fibers in the hypothalamus (scale bar = 100 µm), **f** GH cells in the PPD of pituitary (scale bar = 50 µm)

(Fig. 1a). Several intensely stained GH fibers were traceable into the neurohypophysis. Some neurons of the supraoptic subdivision of the NPO also showed intense GH immunoreactivity (Fig. 1a). Further, in addition to the aforesaid preoptico-hypophysial tract, a less conspicuous, yet distinct pathway of GH-containing fibers was identified. It was composed of fine caliber GH immunoreactivity fibers that arose from the dorsal NPO neurons (Fig. 1a) and extended caudally through the dorsal hypothalamus, on either side of the third ventricle, over the lateral recesses, and reached almost as far as the saccus vasculosus (Fig. 1). The fibers then curved in ventral direction and descended into the caudal tuberal region (Fig. 1) turning sharply caudally and penetrated the pituitary gland as a delicate fascicle that is quite distinct from the preoptico-hypophysial tract. The fibers of this tract were mainly distributed in the rostral pars distalis of the pituitary gland. This GH immunoreactive fascicle has been called as 'the accessory preoptico-hypophysial tract (APHT)' in the present catfish. In addition to the GH-positive fibers, the fascicle also showed some GH-negative fibers. The GH immunoreactive system in the brain seems to be confined to the NPO neurons and their fiber extensions to the pituitary, and no GH immunoreactive cells or fibers were seen anywhere else in the brain of *C. batrachus*.

Expression of GH mRNA in the preoptic region of the brain

The integrity of isolated RNA from the POA and cerebellum is given in Fig. 3. Agarose gel electrophoresis revealed all RNA samples to be intact, exhibiting 28s and 18s rRNA. Figure 4 depicts the representative amplification plot of the GH and reference gene (universal eukaryotic 18s rRNA) in real-time PCR. Approximately 65-fold more expression of GH mRNA was observed in the brain slices containing a preoptic region of the brain as compared to that from the rostral part of the cerebellum of the catfish, *C. batrachus* (Fig. 5).

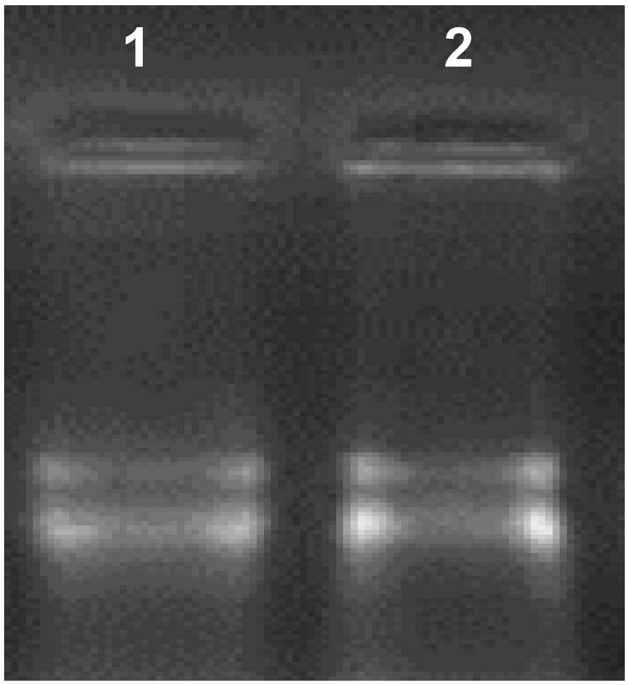

Fig. 3 Total RNA extracted from pituitary of *Clarias batrachus* on agarose gel (0.8%) electrophoresis. Lane 1—control (cerebellum), Lane 2—preoptic area (POA)

Discussion

The present immunohistochemical observations suggest the presence of GH-like protein in the hypothalamic neurons of the NPO and their fibers extending to the pituitary gland in teleost fish. The real-time PCR quantitation of GH mRNA amplicons in NPO slices further substantiates the above notion that NPO neurons transcribe GH gene in the present catfish. NPO is the major neuroendocrine center in the brain of teleost fishes. Axons originating from these neurons are known to directly innervate the pituitary cell types since the median eminence of the tetrapods is absent in teleosts (Peter et al. 1990). Some earlier workers have described the neurosecretory atlas of the hypothalamo-neurohypophyseal system of fishes using the Gomori's aldehyde fuchsin staining technique (Sathyanesan 1969; Haider and Singh 1979). The axonal fibers from the NPO form the preoptico-hypophysial tract which penetrates into the pituitary from its anterior pole, then it passes through the rostral and proximal pars distalis of the pituitary gland and arborizes in the pars intermedia (Prasada Rao and Dabhade 1973). The NPO neurons of teleosts are known to contain a range of peptides such as isotocin, vasotocin, CRF and growth hormone-releasing hormone and regulate the secretions of pituitary gland (Olivereau et al. 1990; Ohya and Hayashi 2006).

The intense immunoreactive GH-NPO perikarya and their ensuing fibers traceable to the pituitary gland is the noteworthy observation in the present catfish. This observation clearly suggests that the GH protein might be produced in the NPO and is transported anterogradely to the pituitary gland, where it may exercise a regulatory influence on, as yet undefined, pituitary cell type. Although, one study of Yang et al. (1999) reports the expression of GH gene in the brain of the rainbow trout, but these authors have analyzed GH mRNA through RT-PCR in the homogenate of whole rainbow trout brain which does not provide any idea of neuroanatomical localization of such GH-expressing neurons in the brain. Thus, the absence of any report on spatial localization of GH-expressing neurons and their ensuing fibers in the fish brain makes the comparison of present findings

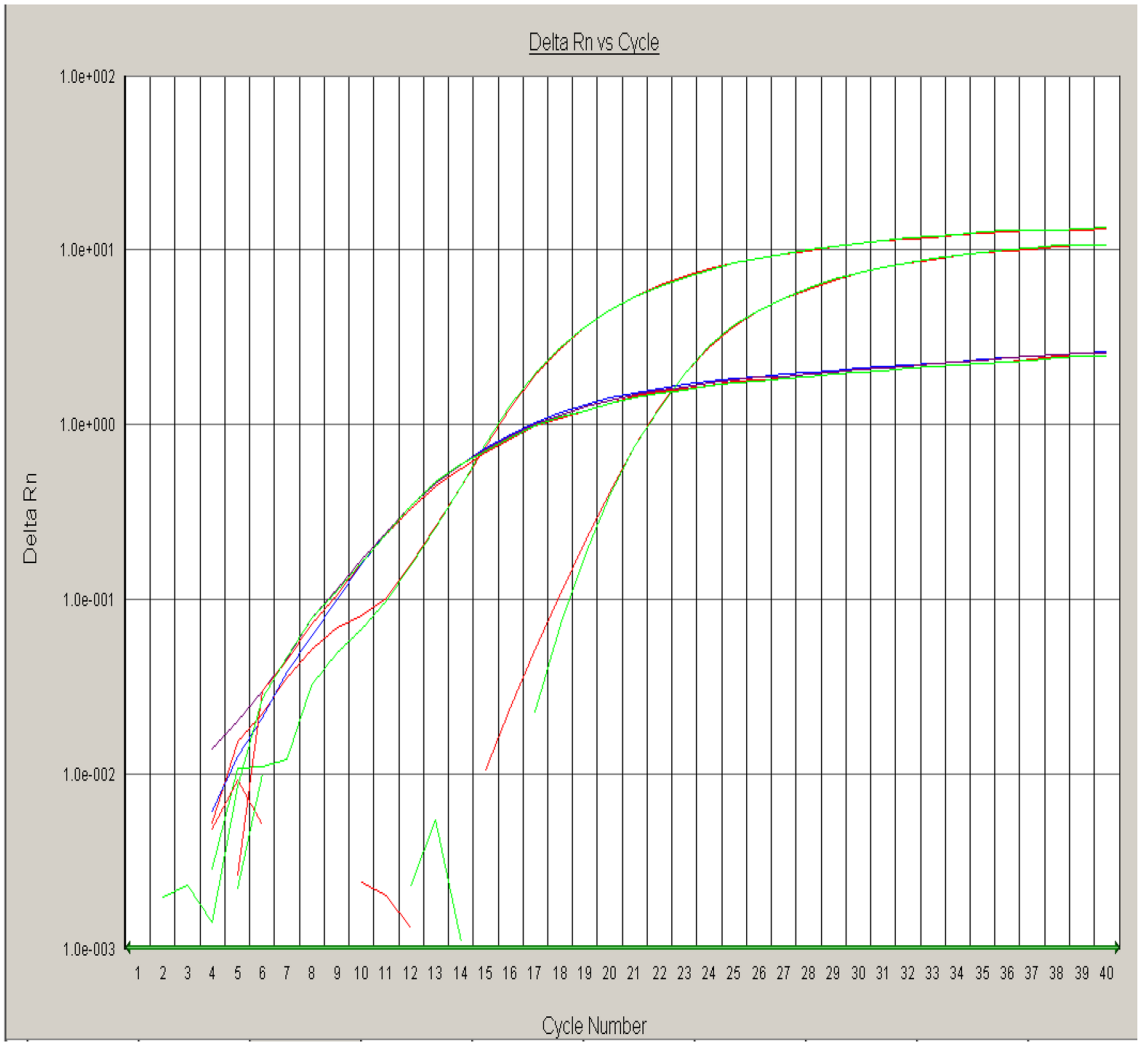

Fig. 4 Representative amplification plot of GH and 18 s rRNA genes in the brain of female catfish, *Clarias batrachus* during the recrudescence phase

on expression of GH in the NPO cells of POA area of forebrain in *C. batrachus* difficult. Nevertheless, the expression of GH gene in many other extra brain-pituitary tissues like gonad, liver, spleen, kidney, gill, heart is well reported in fish (Yang et al. 1999; Biga et al. 2004; Li et al. 2005; Sciara et al. 2006).

However, a good number of studies are available in amniotes, demonstrating not only the existence of GH transcript on ir-GH in different brain regions, but also on the physiological variations in the levels of GH transcript and/or ir-GH in amniotes brain. A considerable amount of GH has been measured through radioimmunoassay in the central nervous system of rodent and primate (Hojvat et al. 1982a). Lechan et al. (1983) have reported depletion of ir-GH in the median eminence following electrical lesioning of the paraventricular nucleus in the rat. The presence of ir-GH cells in the rat brain following hypophysectomy further suggests brain as a site of GH synthesis. It has also been reported that there is a continuous release of ir-GH from dispersed rat hypothalamic cells cultured over a 30-day period (Hojvat et al. 1982a; Lechan et al. 1981). Expression of the GH gene in large cell bodies of the periventricular nucleus indicates further that these cells are the site of GH synthesis (Martinoli et al. 1991). The presence of ir-GH in the fetal brain of rats (10th day of gestation), prior to its appearance in the pituitary gland, is also an indication of its brain expression (Hojvat et al. 1982b). The abundance of GH mRNA in the hypothalamus is markedly increased by growth hormone-releasing hormone (GHRH) and suppressed by stress, under conditions that induce minimal changes in pituitary GH mRNA levels (Yoshizato et al. 1998). Render et al. (1995) have reported that GH cDNA in the chicken brain is identical to that in the pituitary.

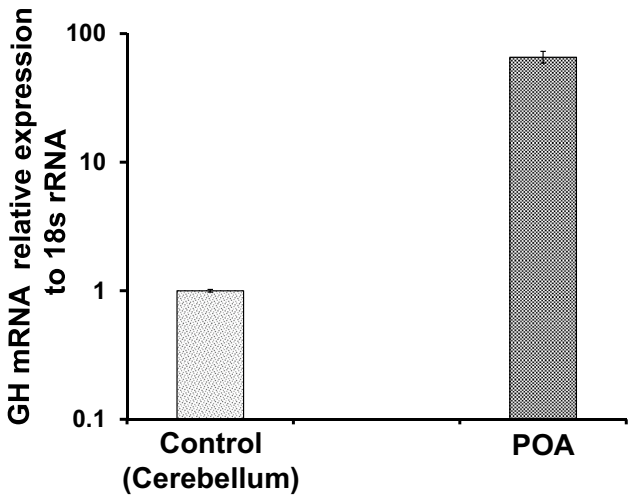

Fig. 5 Expression of GH gene in the preoptic area (POA) and rostral part of the cerebellum (control) of the brain of female catfish, *Clarias batrachus*. GH mRNA level, as measured by real-time PCR, was normalized with 18s rRNA. Results are expressed in fold induction

Thus, the detection of GH mRNA and ir-GH neurons in the NPO cells of POA area of the present catfish brain may be a valuable observation suggesting that GH expression in brain is evolutionarily conserved across the vertebrate phyla.

Further, the occurrence of the accessory preoptico-hypophysial tract is yet another interesting finding of this study. Some of the neurons located in the dorsal paraventricular component of NPO seem to send their axons to the pituitary via this pathway, which may selectively innervate in the rostral pars distalis. Rostral pars distalis in the pituitary of *C. batrachus* is known to have ACTH and prolactin-containing cells (Prasada Rao et al. 1983). In this background, we speculate that the neurons located in the dorsal paraventricular component of NPO might contribute to the accessory preoptico-hypophysial tract that may selectively regulate the ACTH and/or prolactin cells of the RPD. On the other hand, the GH fibers from the rest of the NPO may pass via the preoptico-hypophysial tract and influence other parts of the pituitary. The anatomical course taken by the accessory preoptico-hypophysial tract suggests that the flow of information over this pathway might be influenced by some dorsal hypothalamic neuronal groups. However, additional studies are essential to understand the significance of this pathway.

Thus, the present study explores out the three interesting aspects of neuronal organization and functions of fish brain. First, the GH may be produced in the brain of teleost fish. Second, the GH may serve as a neuroendocrine factor that regulates the pituitary gland. Third, in addition to being a part of the 'classical' preoptico-hypophysial tract that runs along the ventral tuberal area, the GH neuronal system of the NPO may take a different course via the dorsal hypothalamus to the pituitary gland and constitute the "accessory preoptico-hypophysial tract".

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Compliance with ethical standards

Conflict of interest All authors declare that they have no conflict of interest.

Research involving human participants and/or animals In the present study, no human participants were involved. However, experiments on all fish were performed according to the guidelines of the Institutional Animal Ethics Committee (IAEC) under the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India. Care was taken to minimize the number of animals used in these experiments.

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