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RESEARCH ARTICLE

CYP17 mRNA EXPRESSION IN DIFFERENT REGIONS OF BRAIN OF AN INDIAN MAJOR CARP ESPECIALLY *CIRRHINUS MRIGALA*

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ABSTRACT

Steroid genesis is regulated by several enzymes. P450c17 is the single enzyme mediating both 17α-hydroxylase and 17,20lyase activities in the synthesis of steroid hormones. P450c17 is a universal enzyme which converts progesterone and pregnenolone to their 17α-hydroxylated products, and then to Dehydroepiandrosterone (DHEA) and androstenedione respectively P450c17 is needed for final maturation of oocytes, which is necessary for fertilization. Present attempt made to find out the expression of Cyp17 mRNA of different region of brain in the *Cirrhinus mrigala*. The gene was observed by PCR amplification with a specific primer for Cyp17. Our results confirm the presence of the gene cyp17 that codes for the enzyme p450c17 in the various regions of brain of an Indian major carp, especially *C. mrigala* by catalyzing the gene expression through PCR techniques. The further study was require to analyse characterization of Cyp17 gene in these fish species along with different classes of vertebrates and with fishes to know its similarity, evolutionary distance and relationship with other organisms.

INTRODUCTION

The reproductive cycle of the Indian major carp *C. mrigala* is highly coordinated by pituitary activity under the control of the hypothalamus and higher brain centre's. Neurotransmitters and neurosteroids play a vital role in reproduction in both males and females. The synthesis of steroid hormones requires the expression of several steroidogenic enzymes. P450c17 is a steroidogenic enzyme that catalyzes both steroid 17α-hydroxylase and 17,20-lyase activities. 17α-hydroxylase is necessary for the synthesis of cortisol, and 17,20-lyase, which cleaves the C17-20 bond converting C21, steroids, and form dehydroepiandrosterone (DHEA) and androstenedione which are the precursors of the sex hormones. The steroids also identified as neurosteroids which was synthesizing in the brain and inturn controls the neuroendocrine mechanism through the hypothalamic regulation of pituitary functions. Steroid hormones exert important functions in the control of growth, maturation and differentiation of the central and peripheral nervous systems. They actions have long been attributed exclusively to steroid hormones secreted by endocrine glands, ie. Adrenal, ovary and testis.

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However, during the last decade, it has been shown that nerve cells (both neurons and glial cells)are capable of synthesizing bioactive steroids, now called neurosteroids, which also participate in the control of various functions in the CNS. One of the major criteria supporting the concept neurosteroidogenesis is based on the occurrence of steroidogenic enzymes in the nerve cells.

The first report of P450c17 expression in the central and peripheral nervous system was done by Compagnone *et al.* (1995). They reported that P450c17 is found in most of the tissues desired from the spinal neural crest, including the peripheral nervous system (PNS), but is not found in melanocytes and adrenal medulla. Within the PNS, P450c17 is mainly expressed in sensory structures. In tissues derived from the cranial neural crest, P450c17 is expressed only in neural tissues. Nakajin *et al*. (1981) first isolated cyp17 gene from neonatal Pig testis and reported it to have two distinct enzymic activities (17α- hydroxylase and 17, 20- lyase). The cortisol produced by the hydroxylase activity of P450c17 in the internal cells of the head kidney is essential for the osmoregulation and energy metabolisms in fish, Gallo and Civinini (2003). Cortisol synthesis is in a vital to the existence of vertebrates including fish. In the brain of adult mammals, the regional distribution of the mRNA encoding protein P450c17 has been studied by RT-PCR, Stromstedt and

Waterman (1995); Kochi *et al*. (1998) but the localization of the mRNA at the cellular levels remains unknown. The studies of Do Rego *et al.* (2007) revealed that the morphological characterisation and the distribution of immunoreactive cells suggested that in the brain, P450c17 is primarily expressed in neurons. P450c17 immnoreactivity was widely distributed in the frog telencephalon, diecephalon, mesencephalon and metencephalon. The occurrence of P450c17-immunoreactivity cells bodies and fibres in there hypothalamic hypophysiotropic centres suggests that nerosteroids may control neruoendocrine release of hypophysiotropic neruohormones. In support of this hypothesis, it has already been shown that neuroactive steroids regulate the activity of neurons producing vasopressin, Patchev *et al*. (1996), gonadotropin-releasing hormone, Sullivan and Moentec (2003); El-Etr *et al*. (2006) and corticotrophin – releasing hormone, Patchev *et al*. (1994).

An immunological study conducted by Do Rego *et al*. (2007), indicates that P450c17 like immunoreactivity was widely distributed in the frog telecephalon, diencephalon, mesencephalon and metencephalon. The Do Rego *et al*. (2007), is first report of describing the expression of P450c17 in the pituitary of any vertebrate, although C17- hydroxylase and C17,20-lyase activities have already been detected in the cat pituitary, Ficher and Baker, (1978). Do Rego *et al*., (2007), reported the first exhaustive mapping of P450c17-immunoreactive cells in the brain and pituitary of a vertebrate. The anotamical distribution and cellular localization of P450c17- immunoreactive structures in the CNS and pituitary of the Frog was reported by Tremblay *et al*. (1994). Western blot analysis of a microsomal preparation from frog brain and pituitary extracts revealed that the P450c17 antibodies recognized a single band with an apparent molecular weight of 58KDa, consistent with the predicted molecular weight of bovine P450c17, Gyomorey *et al*. (2000), Pigs, Guthrie *et al*. (1994) seals and sea lions, Ishinazaka *et al*. (2001), sheep and cows, Conley *et al*. (1995).

In the steroidogenic pathway of the adrenal cortex 17α-hydroxylase (17α-OH) cytochrome P450 (P450c17α) is a major regulatory enzyme, Zuber *et al*. (1986). P450c17 is unique among the other enzymes in that it apparently catalyzes two distinct catalytic activities and as such represents a branch point between mineralocorticoids or corticosterone on one hand and between cortisol and sex hormone production on the other. Thus, changes in the levels of 17α-OH activities can regulate the pattern of steroids produced. However, the steroidogenesis is reported previously in several species and many of the vertebrates, but not yet known to identify the steroidogenic enzymes and proteins encoding genes expression in the brain of *C. mrigala*. Hence, the present study focussed to identify the genes in the fish *C. mrigala.*

MATERIALS AND METHODS

Sample Collection

Brain samples of an Indian Major Carp, *C. mrigala* were collected from the Nathaipettai Lake located at Kanchipuram in the month of July. Fishes were captured in live condition they were dissected out to get brain samples. Brain samples were kept in a sterilized eppendrop tube containing RNA later and keep in ice to transport to laboratory and they were stored at -70ºC until analysis.

Total RNA isolation

Total RNA was isolated by homogenizing the different regions of brain sample (200mg) with 500 µl of Tri Reagent (Sigma) and 200 µl of DEPC water. After homogenizing the sample, it was then incubated at -20˚C for 5 minutes. 0.2ml of chloroform was added and incubated at -20˚C for 5 minutes. It was then centrifuged at 12,000rpm (4˚C) for 15 minutes. Supernatant was collected in a fresh tube. Total RNA was precipitated by adding equal volume of isopropanol and it was stored at -20˚Cfor 45 minutes. The sample was centrifuged again at 12,000 rpm (4˚C) for 15 minutes. Total RNA was obtained as a pellet and added 75% ethanol (7.5 ml of Ethanol was mixed with 2.5ml of DEPC water) it was centrifuged at 12,000 rpm for 5 minutes and air dried the RNA pellet, and dissolved by adding 40 µl of DEPC water. It was then freezed and stored at -20˚C for half-an-hour. It was then tested with 1.2% agarose gel for its purity.

Synthesis of first strand cDNA

Total RNA was isolated from the brain of *C. mrigala*. The separated RNA has been reverse transcribed into cDNA using RT-PCR method. A clean PCR tube was taken to this 1 µl of the sample, 1 µl of Oligo (dT) 18 primer, 12 µl of de-ionized water was added and it was spinned gently for few seconds in a micro centrifuge. This mixture was incubated at 70˚C for 5minutes. After incubation 4 µl of 5X reaction buffer, 0.5 µl of Ribonuclease inhibitor (40 µl/dl), 2 µl of 10mM dNTP mix were added. It was mixed gently and centrifuged. This mixture was incubated at 42˚C for 5 minutes. After the period of incubation it was added with M-MuLV reverse transcriptase (20U/µl) to make it around 20 µl volumes. It was incubated finally at 42˚C for 60 min and 25˚C for 10 min. The reaction was stopped by heating at 70˚C for 10 min and chilled on ice. PCR products were then tested with agarose gel electrophoresis. cDNA was quantified to measure the concentration of DNA using the Smart spec plus spectrophotometer.

Polymerase Chain Reaction

2 µl of cDNA was taken in a sterilized PCR tube with 1 µl of CYP17 primers of sense and antisense. The following primers were used.

Cyp17 Sense- GACAGCCTGGTGGACATCTT
Cyp17 Antisense- GATCTCTCTGCACGTGGTCA

25 µl PCR master mix consists of all basic components: Taq DNA Polymerase, dNTPs and reaction buffer (1.5mM Magnesium chloride) were added. The PCR amplification was used to check the expression of the mRNAs of Cyp17 enzyme in the brain sample. The temperature followed in the amplification is as follows: 94ºC for 2 minutes in 1 cycle. 95ºC for 30sec, 48ºC for 30sec and 72ºC for 1 minute in 35cycles finally holding temperature is 4ºC. The PCR products of gene specific primer of Cyp17 along with 100bp DNA Ladder were then subjected to 1.2% agarose gel electrophoresis. After running the gel, the images of specific bands were captured using UV transillumination under Gel documentation system.

RESULTS

Total RNA from the different regions of brain of an Indian major carp, *C. mrigala* isolated and checked the purity of the RNA separation (Fig.1.). The isolated RNA was reverse transcribed into cDNA using RT-PCR Medox kit protocols. The cDNA was used to catch the specific P450c17 gene with the gene specific primer designed for Cyp17 genes along with the PCR master mix by using PCR amplifications at specific annealing temperature to find out the gene expression. The gel was loaded with 100bp DNA Ladder and amplified PCR product with the primer of both P450c17 sense and antisense. The showing the bands were represented in (Fig. 2) L1 and L11- shows the 100bp DNA ladder, L2- L4 shows the bands for P450c17 mRNA in Pituitary, Olfactory lobe and Optic lobe respectively. L5 and L10 show the negative control for PCR amplification without cDNA products were used. L6-L9 shows the bands for P450c17 Hypothalamus, Cerebrum, Cerebellum and Medulla oblongata respectively.

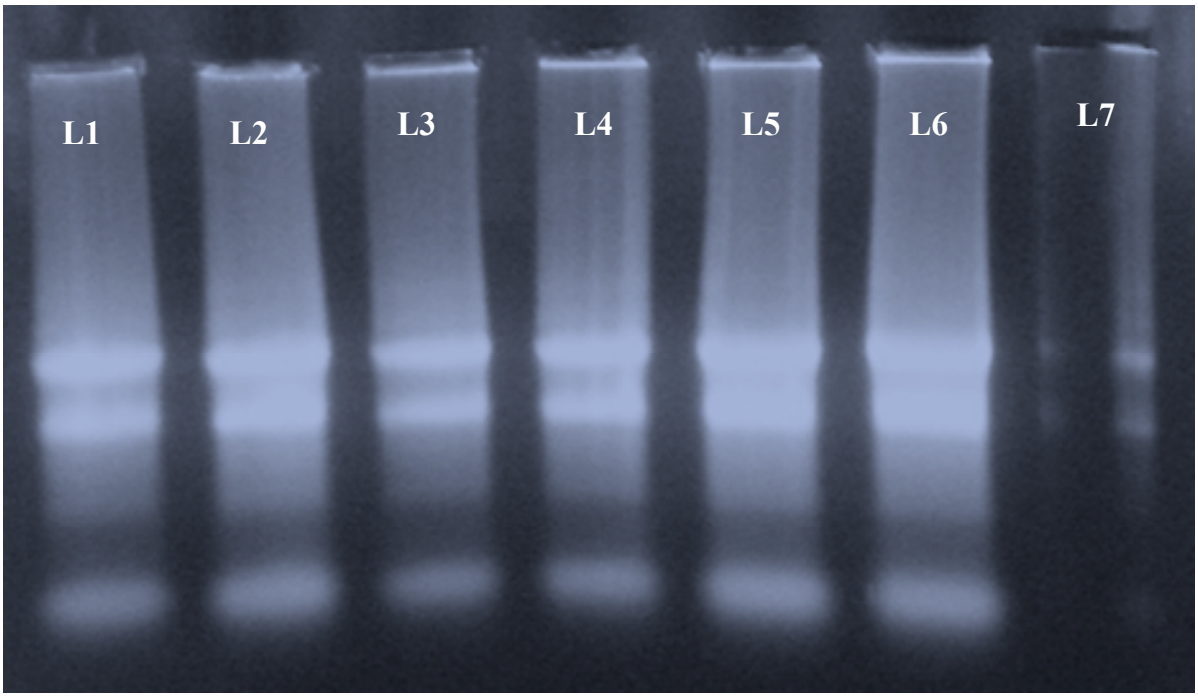

Fig. 1. The total RNA bands in the different regions of brain samples in the Cirrhinus mrigala, (L1- Pituitary, L2- Olfactory lobe, L3- Optic lobe, L4- Hypothalamus, L5- Cerebrum, L6- Cerebellum and L7- Medulla oblongata

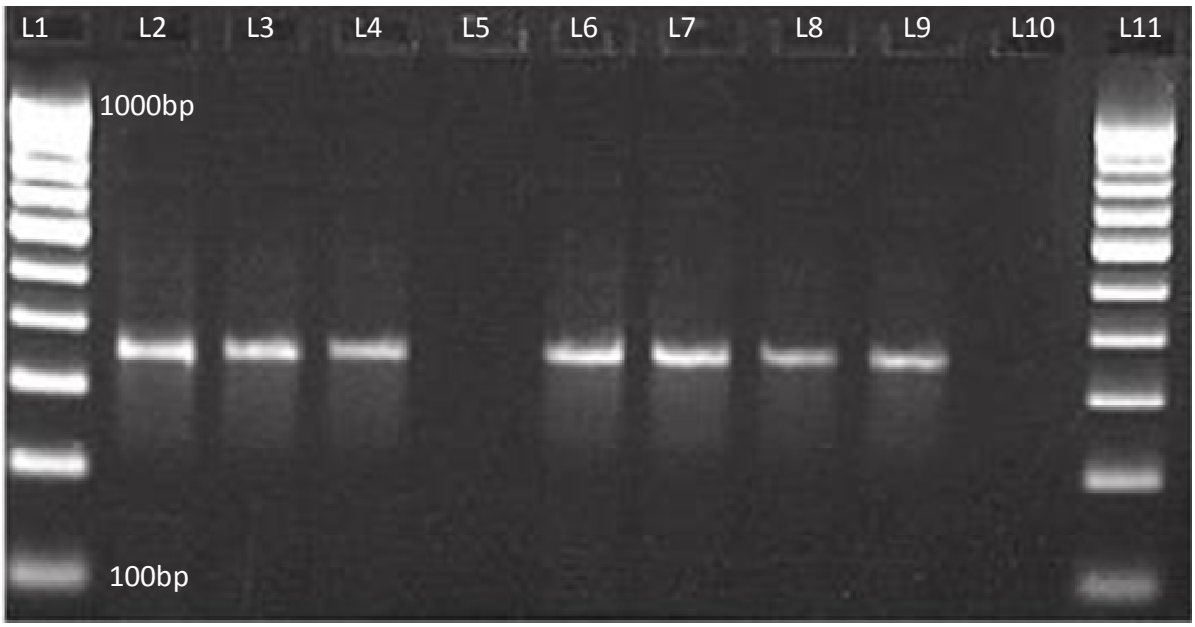

Fig. 2. The expression of Cyp17 mRNA in the different regions of brain in the Cirrhinus mrigala, L1 and L11 shows the 100bp maker DNA, L2-L4 shows the Pituitary, Olfactory lobe and Optic lobe respectively, L5 and L10 Shows the Negative control without cDNA products. L6-L9 shows the PCR amplified products of Hypothalamus, Cerebrum, Cerebrum, Cerebellum and Medulla Oblongata of brain of these fish

DISCUSSION

Steroidogenic pathway is regulated by several steroidogenic enzymes and proteins, the P450c17 enzyme expression has been reported in different tissues in vertebrates. P450c17 expression in the CNS and PNS has been proposed first by Compagnone *et al*. (1995). In the brain of adult mammals, the regional distribution of the mRNA encoding P450c17 has been studied by RT-PCR, Stromstedt and Waterman, (1995); Kohchi *et al*. (1998) but the localization of the mRNA at the cellular level remains unknown. The presence of other steroidogenic enzymes, including 17β-HSD, 5α-R and aromatase, has already been detected in human pituitary of rat, Celotti *et al*. (1992) and Gold fish (Pasmanik and Callard, 1985, 1988). In our laboratory previously reported in steroidogenic pathway regulating steroidogenic protein and enzymes encoding mRNA expression in the brain of Indian major carps, *Labeo rohita* (Saravanan *et al*., 2011, 2013 and Uma *et al*., 2013). Previous reports have revealed the ability of cat brain and pituitary tissues, Ficher and Baker (1978), rat neurons and astrocytes, Zwain and Yen (1999), rat hippocampal tissues, Hojo *et al*., (2004) and quail brain tissues, Matsunaga *et al*. (2001, 2002) to convert Δ5P and P into DHEA and Δ4 through a P450c17 – dependent mechanisms. The zonal distribution of P450c17 has been studied in the adrenal cortex of the gunea pig, Shinzawa *et al*. (1991), Pig, Sasano *et al.* (1989), Rhesus monkey, Mesiano *et al*. (1993) and the human fetus, Mesiano *et al*. (1993); Breault *et al*. (1996).

Immuno histochemical observations of P450scc, 3β-HSD, P450c17 and P450arom have been reported in the corpus luteum of numerous species, including humans, Suzuki *et al*., (1993), bears, Tsubota *et al*. (1994, 2001), horses, Albercht and Daels (1979); Albercht *et al*. (1997); Albercht *et al*. (2001), Dogs, Nishiyama *et al*. (1999), Pigs, Guthrie *et al*. (1994) seals and lions, Ishinazaka *et al*. (2001), sheep and cows, Conley *et al*. (1995). In the medulla, it is in the basal plate suggesting that it is in motor neurons. Furthermore, P450c17 expressing neurons are observed in the pentene nucleus, which has been reported to be related to the motor compartment of the trigeminal tract, Paxinos (1994). Hamsters differ from other rodents in that they express adrenal P450c17 (Cloutier *et al*., 1997) and produce cortisol as their major corticosteroid (Le Houx *et al*., 1992). Le Goascogne *et al.* (1991), reported immunoreactive P450c17 in rat gonads but not in the adrenals, thus confirming the absence of expression of this cytochrome in this species. Immunological studies of Weng *et al.* (2005), proposed that immunostaining for P450c17 and P450arom was observed in syncytiotrophoblast of the placenta in pregnancy (120 days). Do Rego *et al*. (2007) reported the first exhaustive mapping of P450c17-immunoreactive cells in the brain and pituitary of a vertebrate. The anatomical distribution and cellular localization of P450c17- immunoreactive structures in the CNS and pituitary of the frog was reported by Tremblay *et al*. (1994). Zhou *et al*., 2007 reported the two P450c17 types encoded by two different genes in Fugu, tetraodon, stickleback, tilapia (*Percomorpha*), medaka (*Atherinomorpha*) and zebrafish (*Ostaruiohysi*), which belong to three different clades in the phylogenetic tree (Nelson, 1994). They did an *in silico* search on the genomes of *Xenopus*, chicken, mouse, rat, and human but failed to obtain any P450c17-II-like sequences. They have reported from the sequences and structure of the medaka P450c17 type I and II genes that the two are completely different. Many report explaining the expression of the enzyme P450c17 in different organisms in different tissues.

Zhou *et al*. 2007 constructed a phylogenetic tree based on the amino acid sequences of vertebrate P450c17 type I and type II. P450c17 type II of Fugu, Medaka, Tetraodon, Stickleback, Tilapia and Zebra fish clustered into one clade, which was distinct from the P450c17 type I. The isolation of P450c17 type II sequences from six species of teleosts, especially from Zebra fish, which is phylogenetically far from Fugu, Medaka, and tilapia indicates that the duplication of P450c17 is not restricted to single clad of fish. The tree demonstrates that this duplication is a phenomenon unique to the teleost fishes (ray-finned fish Actinopterygii). This experiment is the first attempt to report all the regions of the brain of an Indian carp particularly *C. mrigala*. Our results suggest the brain of *C. mrigala* is mainly play important role in the synthesis of brain steroidogenesis. Further, study requires knowing the other steroidogenic enzymes expression and the characterization of these enzymes in the brain of an Indian major carp, especially *C. mrigala*.

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