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Transforming Growth Factor β: Cloning and Expression Profiling in the Brain of Catfish Model

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Abstract

Transforming growth factor-beta (*tgf-β*) is implicated as a signalling molecule and considered a vital regulator for neuronal survival, development, ovarian growth, and many other biological processes. The present study was aimed to analyze the expression of *tgf-β* and its significance during the early brain and gonadal development. Initially, cloning of partial cDNA of *tgf-β* from the female brain was performed followed by expression analysis during various developmental stages using quantitative real-time PCR. Cloned *tgf-β* is approximately 210 bp, which encodes a putative protein of 69 amino acid residues, and showed high similarity with other counterparts. The tissue-specific expression of *tgf-β* was ubiquitous but significantly higher in brain as well as gonads of both male and female. Further, in ontogeny analysis, *tgf-β* mRNA levels were significantly greater in 75-, 100- and 125-days post-hatch during development. The pre-spawning stage showed higher expression of *tgf-β* when compared to all other reproductive stages. Immunolocalization study revealed the presence of *Tgf-β* protein in the preoptic area-hypothalamus which was well correlated with the mRNA expression in discrete regions of the catfish brain. Expression of *tgf-β* seems to have a role in the regulation of gonadal function through the brain-pituitary axis.

Keywords: Brain, Development, Expression, Signalling Molecule, *Tgf-β*

1. Introduction

Transforming growth factor-beta (*Tgf-β*) is an essential molecule that acts in an autocrine and/or paracrine manner to regulate neuronal cell survival synergistically with neurotrophic factor steroidogenesis, ovarian follicle growth as well as oocyte maturation in teleost[1,2]. However, it also increases the potency of selected neurotrophin (NT), fibroblast growth factor-2, brain cell-derived neurotrophic factor (BDNF), ciliary neurotrophic factor, and glial cell line-derived neurotrophic factor (GDNF) which control diverse cellular processes during embryogenesis[3,4]. Expression of *tgf-β* in goldfish ovary revealed a decrease in androgen production at two different stages and its vital role in gonadal development[2]. Likewise, prostaglandins that play an essential role in ovulation, whose production level was elevated by *tgf-β*, exhibit their control on brain and gonadal development in teleost[5]. *Tgf-β* superfamily includes growth/differentiation factors and the GDNF subfamily which act in a synchronized manner to regulate the neural development[6] and maturation of ovarian follicle[7]. Moreover, the growth factor *Tgf-β* most likely plays an important role in neuronal survival, spermatogenesis, oocyte proliferation and maturation that may be exerted by interacting with estrogens[8]. Evidence obtained using model neurons shows that chromaffin cells release *Tgf-β* in response to presynaptic neurotransmitters and may be dependent on neuronal activity[9]. *Tgf-β* also regulates the occurrence of ciliary ganglionic neurons in association with another neurotrophic factor and NT[3,10]. The several cell functions of *Tgf-β* include cell cycle control,

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differentiation, regulation of early development and immune functions. Interestingly, *Tgf-β* requires GDNF not only *in vitro*, but also *in vivo* to exert its neurotrophic functions in teleost[3,11].

Tgf-β is a potential factor in maintaining homeostasis, development, and repair in nearly all the tissues from lower to higher organisms, which indicates that its expression is ubiquitous, yet temporal[12–15]. It is involved in the regulation of the neural circuits' assemblage in the retina and indirectly regulates axon specification and growth[1]. *Tgf-β* autocrine characteristic features are well-known for their protagonist nature in cell transformation and were further confirmed by its role in transforming naive neurites into axons in neocortical neurons[17]. In mammals, *Tgf-β* was known to promote outgrowth and survival of neurons on neonatal dorsal root ganglion during neuronal development[18a,18b]. Other studies shown that the *Tgf-β* domain might be liable for the Amh-receptor interaction in common carp[19], which is important for the development of male reproductive system. In addition, the profound significance of *Tgf-β*1 in male and female reproductive physiologies provides a basis for discovering the significance of cytokines in mammal infertility and sexual dysfunction[20]. In consideration of these findings, it is worthwhile to investigate the impact of *Tgf-β* in a seasonally breeding fish due to the modulatory action of a brain-pituitary-gonadal axis. Based on this premise, the present study is aimed to evaluate the significance of *Tgf-β* and its expression profiling to understand the regulation of brain over gonadal function in catfish, which undergoes seasonal gonadal recrudescence.

2. Materials and Methods

2.1 Animal Sampling and Maintenance

The catfish, *Clarias gariepinus,* was used as the model organism. The reproductive cycle of this fish is categorized into four different stages (preparatory, pre-spawning, spawning, and post-spawning). Spawning male and fertile female fishes were bred through *in vitro* fertilization and the fingerling so obtained was maintained strictly in the aquaculture containment facility of the institute. Immediately after hatching, the fingerlings were kept in round plastic tubs and maintained at oxygen level 3 mg/L with the suitable thermal condition. The fingerlings were fed *Tubifex tubifex*, live tube worms, besides commercially available fish feed pellets until adulthood. As described earlier by Raghuveer & Senthilkumaran[21], the sex differentiation in catfish was observed at 50-day post-hatch (dph). The fingerlings of different age groups were grown in freshwater tanks and the gonads were dissected out under a stereo-zoom microscope with fine forceps scissors after animal sacrifice on 50, 75, 100, and 150 dph. Similarly, mature gonads and brain of adult *C. gariepinus* during the spawning phase were obtained after animal sacrifice, as mentioned earlier, following exposure to ethyl 3-aminobenzoate methane sulfonate (MS-222; Sigma; St. Louis, MO, USA)as the anesthetizing agent, were fixed in 4% Para Formaldehyde Solution (PFA) for immunehistochemical analysis and stored at -80°C for total RNA isolation to perform cloning and expression analysis. Animal maintenance and sampling were performed by following the IAEC (Institutional Animal Ethics Committee) guidelines of Hyderabad University (dt.22.07.1999, CPCSEA, Ins. Reg #No#151/1999).

2.2 Cloning of *tgf-β* partial cDNA

Total RNA from catfish brain was extracted using TRI-reagent® by following the manufacturer's protocol and the RNA purity was determined by NanoDrop spectrophotometer, USA. The total RNA, 2 µg, was used to generate the cDNA by reverse transcription using OneScript® Plus cDNA Synthesis kit (Applied Biological Materials Inc. (ABM), Richmond, BC, V6V 2J5, Canada, USA). Degenerate primers were designed using the available nucleotide sequence information of *tgf-β* from NCBI database of different teleost species. The master mix of Taq 2x polymerase (New England Biolabs Inc., Ipswich, MA) was used for PCR amplification by degenerate primers i.e., Fw primer 5'-CTGCTGYRTVMGVMVBCTNTACA-3', and Rv primer 5'-CTTGCARKHVYKSACVAYCAT-3', as per the following thermal cycle conditions: a first crucial step of 94 °C (2 min), 94 °C (1 min), 94 °C (1.15 min), 72 °C (1 min), for 35 cycles, and 72 °C (10 min) final extension. The gel-extracted sample was ligated and the amplified product was purified using pGEM®-T easy vector (Promega, Madison, WI, USA) and bidirectionally sequenced to confirm through BLAST.

2.3 Quantitative Real-time PCR (qRT-PCR)

Expression analysis of *tgf-β* was carried out by qRT-PCR to determine the tissue distribution and brain ontogeny using SYBR green detection process. Total

RNA (1 µg) was prepared, and reverse transcribed as described earlier. Furthermore, one set of gene-specific qRT-PCR primers i.e., RT Fw primer 5'-GAGCAGAGACTGGAGCTGTATCAG-3'; RT Rv primer 5'- CTGCGTCACATCAAACGATAGCC-3', were designed and the reaction was performed at 94 °C (1 min) and 72°C (1 min) using *18S rRNA* as an internal control FW 5'-GCTACCACATCCAAGGAAGGCAGC3'; and RV 5'-CGGCTGCTGGCACCAGACTTG3' reference gene. The experiment was carried out with biological triplicates (n=3) using SYBR green master mix in MicroAmp® 96-Well plates by a 7500 fast thermal cycler (Applied Biosystems, USA) using universal thermal cycling conditions. The intensity of the PCR amplification was determined by analyzing the melting curve of the reaction. The exponential phase of PCR amplification was used to estimate the cycle threshold (Ct) values and ΔCt values were calculated by standardizing the expression of *tgf-β* against *18S rRNA* expression (Ct of target gene -Ct of reference gene). Relative expression was calculated using $2^{-\Delta Ct}$ method.

2.4 Tissue Distribution of *tgf- β*

Tissue distribution of *tgf-β* was determined by qRT-PCR using gene-specific primers in both sexes. The total RNA was isolated from different tissues of adult catfish by practicing the protocol mentioned earlier.

2.5 Differential Expression of *tgf-β* during Brain Ontogeny

qRT-PCR was carried out using SYBR Green detection to estimate the *tgf-β* levels in the brain during various developmental stages. Diverse age groups, i.e., 0, 10, 20, 30, 40, 50, 75, 100, 150, 200, and 250 dph, were taken and samples were pooled till 100 dph, however morphological differences of the gonad arose at nearly 50 dph. Further, ontogeny analysis was carried out in brain of both sexes. All the collected samples (total RNA, cDNA, etc.) were stored temporarily at -80 °C for further experiments.

2.6 Phase-wise Expression

The total RNA was extracted then cDNA synthesis was performed and analyzed by qRT-PCR to analyze the *tgf-β* expression from four different reproductive phases i.e., preparatory, pre-spawning, spawning, and post-spawning were determined as catfish shows seasonal reproductive cycle.

2.7 Expression of *tgf-β* in Different Regions of Brain

To determine the relative expression levels of *tgf-β*, tissues were collected from distinct regions of the brain i.e., thalamus (THA), olfactory bulb, telencephalon (OB + TEL), preoptic area-hypothalamus (POA-HYP), medulla oblongata (MO), and pituitary (P). The tissues were collected as mentioned previously by Mamta and Senthilkumaran[6].

2.8 Immunohistochemistry (IHC)

IHC analysis was performed to examine the localization of *Tgf-β* in the brain as per the procedure described previously[6,22]. In brief, 4% PFA in PBS was used for brain tissue fixation and kept at 4°C. Tissues were washed in PBS several times and a tissue freezing medium (Leica, 140201) was used to embed the tissues for sectioning by cryostat (Leica CM1850 Microsystems). Cryomold brain sections (5µm) were prepared on Poly-L-Lysine-coated glass slides. Then PBS was used to hydrate the sections before blocking with 8% goat serum (Bangalore Genei, Merck) for 60 mins. Later, anti-*Tgf-β* polyclonal antibody (1:500) and pre-adsorbed antibody with the excess antigen of *Tgf-β* were used as a negative control. Tissue sections were washed with PBS and incubated with HRP-conjugated secondary antibody (1: 4000) for 60 min at Room Temperature (RT). Later, avidin-biotinylated horseradish peroxidase (ABC) reagent complex VECTASTAIN® Elite supplied in ABC kit (Vector Lab) was used for half an hour. Sections were adequately washed in PBS and developed using 3′,3′-diaminobenzidine as chromogen and H_2O_2, Vector Laboratories, as a substrate for horseradish peroxidase. The sections were washed in PBS to complete color development and counter-stained using hematoxylin (Qualigens Chemicals) followed by dehydration using a series of graded ethanol and finally mounted using DPX. Olympus Microscope CX41 was used to perform imaging of the sections prepared.

2.9 Western Blot

Western blot with polyclonal antibodies (Biosciences, Seattle, WA, USA) raised against human *Tgf-β* protein in N-terminal regions that exposed~85% similarity with the conserved region of catfish was performed. Adult catfish brain was homogenized with ice-cold homogenization buffer containing NaCl 150 mM, Tris 1 M, pH 7.4, 1 mM dithiothreitol and cocktail of protease inhibitors.

Protein estimation was done using the Bradford assay. One hundred microgram protein was run on a 12% SDS-polyacrylamide gel electrophoresis and then transferred onto a nitrocellulose membrane (Port Washington). The transfer of bands was confirmed by Ponceau S staining. Further, 5% skimmed milk powder in Tris-Buffered Saline (TBS) was used to block the membrane and kept for 1 h at RT. After blocking, sections were adequately washed in TBS containing 0.1% Tween 20 (TBST). Then the membrane was incubated with 1:500 dilution of polyclonal anti-*Tgf-β* antibody at 4°C in 0.5% skim milk powder/TBST solution. The membrane was washed and incubated with alkaline phosphatase-conjugated secondary antibody i.e., Goat anti-rabbit IgG (1:4000; Merk Bangalore Genei, Bengaluru) for 60 min and the blot was developed using BCIP-NBT (Roche Diagnostics GmbH) as substrates. β-tubulin protein was used as a positive control.

2.10 Statistical Analysis

All the data are expressed with mean ± Standard Error of the Mean (SEM). Significance among groups was determined by Analysis of Variance (ANOVA) using Student's *t*-test, and one-way ANOVA was considered statistically significant at $p<0.05$, Student's–Newman–Keuls' test was performed using Sigma Plot 11.0 software (Systats Software, USA).

3. Results

3.1 Cloning Partial cDNA of *tgf-β*

A set of degenerate primers were used to clone a partial fragment of *tgf-β* ~210 bp from *C. gariepinus* brain. The clone was further confirmed by NCBI-BLAST analysis where the aligned sequence coding for *Tgf-β* domain region was revealed (Figure 1).

3.2 Tissue Distribution

tgf-β were ubiquitously expressed in all the tissues (n=3) analyzed (Figure 2). However, the expression level of *tgf-β* was significantly ($p<0.05$) elevated in male and female brains as well as gonads. Moderate expression levels were observed in the liver, kidney, heart, gills, intestine, and spleen whereas low expression was seen in muscles.

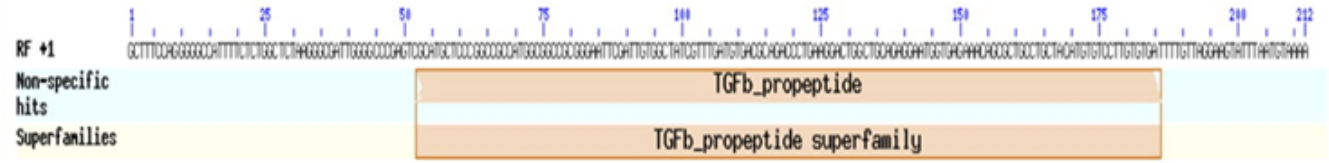

Figure 1. Protein BLAST analysis of the putative conserved domains of Tgf-β. Protein-containing the domain of Tgf-β propeptide, is known as Latency-Associated Peptide (LAP) in TGF-β (52-186).

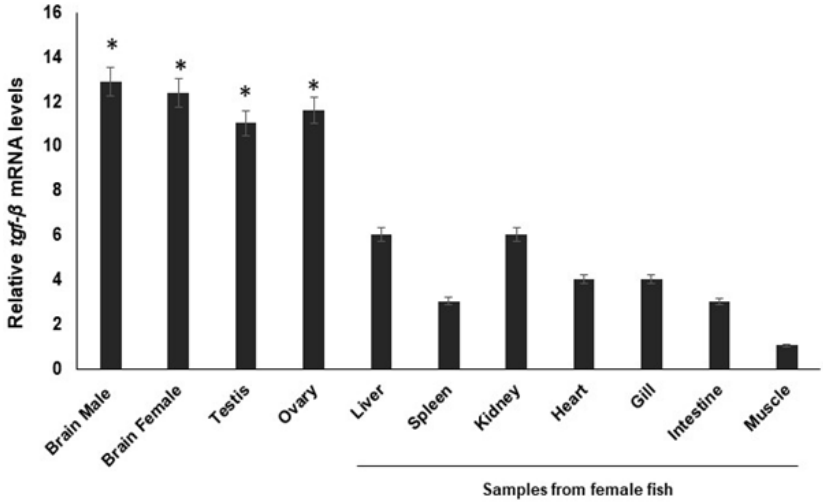

Figure 2. Tissue distribution of *tgf-β* in male and female brains and gonads. The relative expression was normalized against *18S rRNA* in various tissues i.e., the brain, testis, ovary, heart, liver, kidney, spleen, gill, intestine, and muscle. The data were expressed as mean ± SEM (*/$p<0.05$, Student's *t*-test; n = 3).

3.3 Brain Ontogeny

Expression of *tgf-β* in various developmental stages of catfish starting from 0 to 250 dph was analyzed. The expression pattern was seen in the brain of both sexes by qRT-PCR (Figure 3). Increase in *tgf-β* expression from 50 dph to 125 dph during the gonadal sex differentiation was observed. Considerably higher levels ($p<0.05$) of *tgf-β* were observed at 75, 100 and 125 dph in the male and female brain with a minor change.

3.4 Expression of *tgf-β* during Different Phases of Reproduction

In phase-wise expression analysis, the pre-spawning phase showed significantly higher expression levels of *tgf-β* than all other reproductive stages (Figure 4).

3.5 Expression of *tgf-β* in Discrete Regions of Brain

Expression of *tgf-β* was determined in five distinct regions (POA-HYP, THA, MO, OB + TEL and P) of the

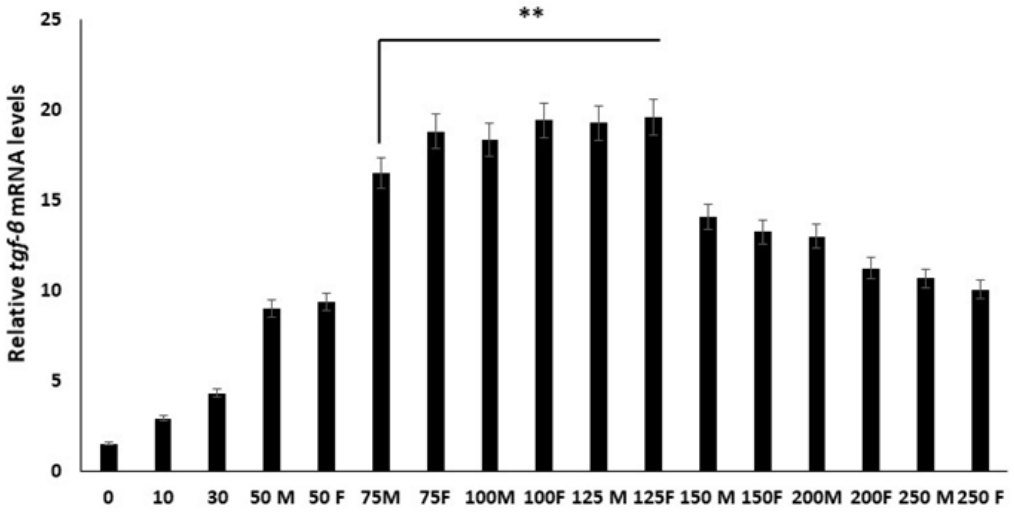

Figure 3. Ontogeny of *tgf-β* expression in male and female brain by qRT-PCR. *tgf-β* expression analysis was carried out to quantify mRNA levels during ontogeny from 0 dph to 250 dph'*' and showed significantly higher *tgf-β* mRNA levels in brains at 75, 100 and 125 dph in both the sexes as compared with other age groups (n = 5 */$p < 0.05$; ANOVA followed by SNK test).

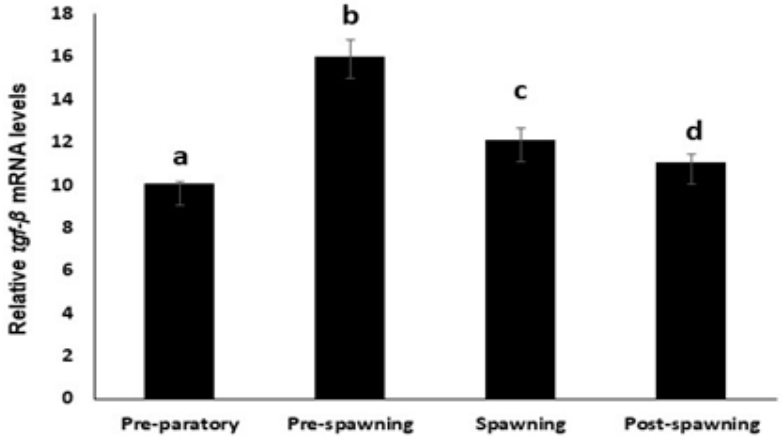

Figure 4. *tgf-β* expression in adult brain by qRT-PCR analysis during reproductive phases. The expression level was normalized with *18Sr RNA*. The values were calculated using comparative $2^{-}\Delta Ct$ method, and expressed as mean ± SEM (*/$p < 0.05$; one-way ANOVA followed by SNK test; n=5). The pre-spawning phase showed significantly higher expression.

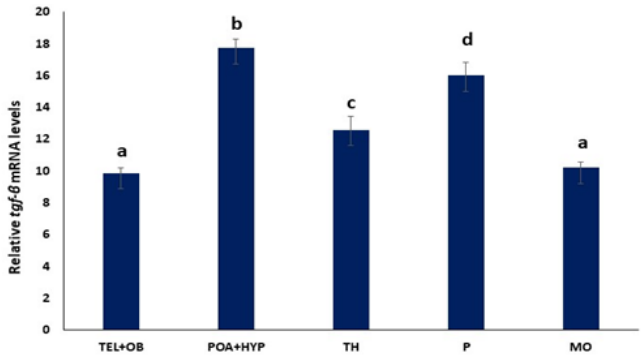

Figure 5. Expression of *tgf-β* levels in distinct regions of adult female brain by qRT-PCR analysis. Mean with '*' indicates significantly high expression of *tgf-β* ($p<0.05$; ANOVA followed by SNK test; n=3). Abbreviation: TEL + OB- telencephalon+olfactory bulb, POA-HYP- preoptic area-hypothalamus, TH-thalamus, MO-medulla oblongata, and P-pituitary.

adult female brain where the expression level was higher in POA+HYP and P in comparison to other brain regions (Figure 5).

3.6 Immunolocalization and Western Blot Analysis of *Tgf-β* Protein

Immunoreactivity of *Tgf-β* protein in the brain was seen in POA-HYP, confirming the antibody specificity (Figure 6C-E). However, no immunoreactivity signals of *Tgf-β* were observed in negative control or pre-adsorbed antibody (Figure 6B) indicating the antigen used. The results were further confirmed by Western blot wherein a single band of ~ 45 kDa of putative *Tgf-β* was observed (Figure 6F).

4. Discussion

In the present study, *Tgf-β* partial cDNA was cloned from the female catfish brain. The differential expression of *tgf-β* in various tissues revealed higher levels of *tgf-β* in

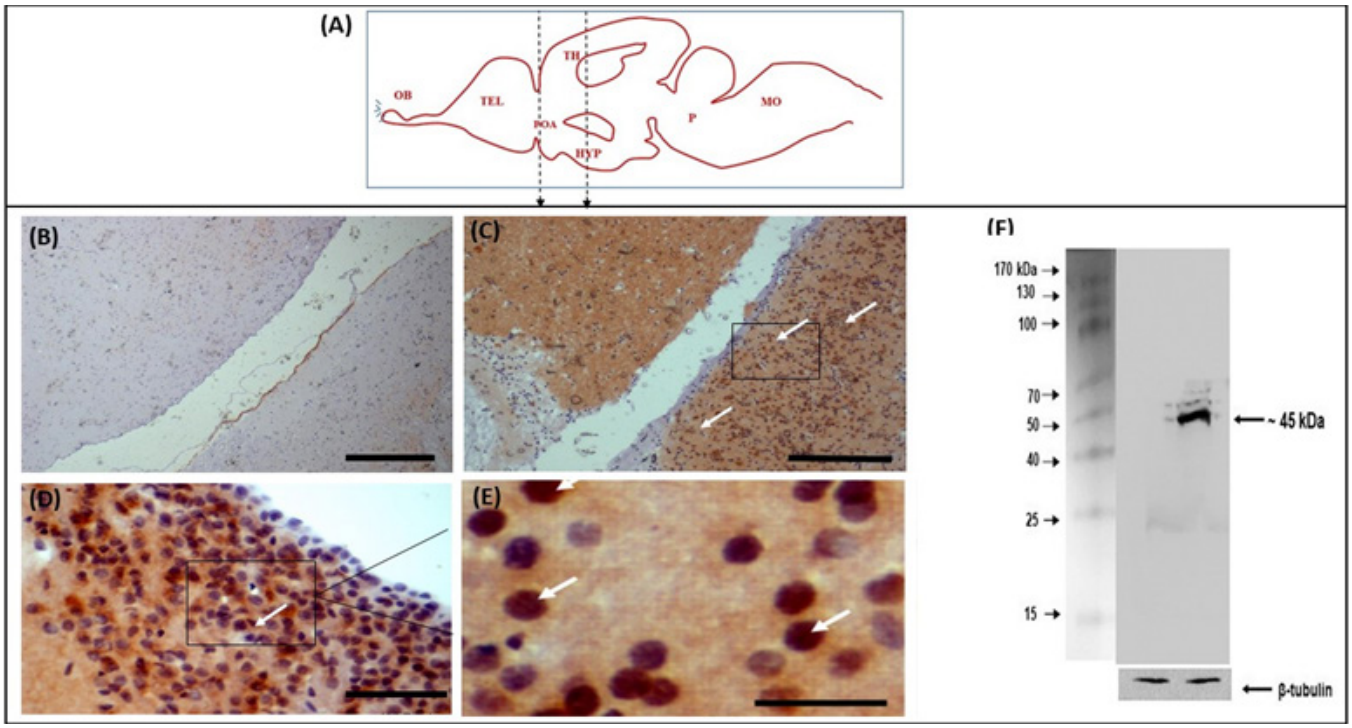

Figure 6 **(A)** Immunohistochemistry of *Tgf-β* protein in the brain. **(B)** Schematic sagittal image of catfish brain depicting various regions. The immunoreactivity of *Tgf-β* was seen in POA-HYP. **(C-D),** though negative control by preabsorbed antibody treatment showed no immunoreactivity. **(E)** Arrow indicates immune-reactive brain cells in POA-HYP region. Scale bar specifies, B: 50 µm; C: 50 µm; D: 25 µm; E: 10 µm. **(F)** Shows corroboration of western blot with a prominent band of ~45 kDa size.

emerging male and female brains as well as in gonads, and lower level in other tissues implying its role in these regions. In a previous study, the expression of *tgf-β* was found in the brain, gills[23], spleen, and kidney[24] of teleosts. Observation of *tgf-β* expression in brain, spleen, kidney, macrophages, and leukocytes of *O. mykiss* and in sea bream, *Sparus aurata* revealed its multiple functions in biological processes[23,25]. In the present study, higher expression of *tgf-β* was shown in both male and female at 75, 100, and 125 dph during brain ontogeny. However, in *O. mykiss*, the most prominent difference was found in the intestine and gonad where *Tgf-β*1a expression was 88.6 times higher than *Tgf-β*1b[26]. During ontogeny, *Tgf-β*1a was expressed significantly high in all progressive stages than *Tgf-β*1b, while minor differences in expression were seen amongst all developmental stages for both the isoforms[26]. It is reported that inadequacy of *Tgf-β*1 triggers perturbed functioning of the hypothalamic-hypophyseal-gonadal axis, which inhibits synthesis of luteinizing hormone in mammals, leading to low testosterone levels in males and abnormalities of estrous cycle in females[20]. In catfish, higher expression of *tgf-β* in prespawning phases signifies that the gene might be regulated by gonadotropin-releasing hormone (GnRH) and gonadotropin (GTH) axis via GnRH periodicity[27]. Further, GTHs showed cyclic differences ensuring their control on gonads in catfish[28–30]. Immunolocalization of *Tgf-β* protein predominantly expressed in POA-HYP supports a key role for this correlate in catfish brain. Some lines of evidence support that *Tgf-β* is a crucial molecule that regulates neuronal survival synergistically with GDNF, BDNF, NT, and other neurotrophic factors. Consecutively, these factors regulate the release and synthesis of *Tgf-β* from neurons[6,10]. Two different members of *Tgf-β* act as modulators of ovarian function in teleost[2]. In grass carp, the expression of *tgf-β1* mRNA was seen in the pituitary, brain, head kidney, spleen, and thymus[31]. Incidentally, no functional studies have been carried out to implicate the expression analysis of *tgf-β*. Nevertheless, the present study in terms of tissue distribution, ontogeny, reproductive cycle, and immunohistochemistry indicates an important role for *Tgf-β* in catfish brain.

5. Conclusion

To conclude, prominent expression of *tgf-β* mRNA and protein in the brain appears to propose a substantial impact for this correlate via growth factor signalling. Further, tissue distribution and ontogeny study revealed higher expression in the developing brain. Immunolocalization of *Tgf-β* protein expression in the POA-HYP by IHC indicates its conceivable role in relation to neuronal development through GnRH and GTH axis which might further support gonadal function.

6. Conflict of Interest

The authors declare no conflict of interest. The authors are accountable for the content and writing of the manuscript.

7. Acknowledgments

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