Characterization of Neural Stem Cells and Proliferating Cells in the Brain of Loach (*Misgurnus Anguillicaudatus*)

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Original Article

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ABSTRACT

Introduction: Loach (*Misgurnus anguillicaudatus*) is an essential commercial fish in China. The brain of fish is primitive and characterizes with adult neurogenesis.

Aim of Work: this study aimed to give some notes on brain histology of loach fish and characterize the sites of stem and proliferating cells in the brain.

Materials and Methods: We histologically observed the structure of the brain via hematoxylin and eosin stain. We also used fluorescent in situ hybridization to evaluate the localization of *Msi1*, *Bmi1*, and *Sox2*. They are essential genes expressed in brain stem cells of loach. Proliferating cells were evaluated by PCNA immunofluorescence. As well as GFAP immunofluorescence was used for detection of the gial property of proliferating cells.

Results: Our results indicated that the neural stem cells were located in the granular cell layer of the cerebellum, vagal lobe, facial lobe, optic tectum, and beside the third ventricle because these sites exhibited expression of *Sox2*. There were several areas of the brain that contained proliferating cells, including the cerebrum, the torus longitudinalis, and in between the optic tectum and cerebellum. In the cerebellum, the proliferating cells were present in the molecular cell layer, around the intracerebellar ventricle, in the lobus caudalis cerebella, in the eminentia granularis, and around the rhombencephalic ventricle. Proliferating cells were also observed in the three layers of the vagal lobe and throughout the facial lobe. These proliferating cells were all negative for GFAP.

Conclusion: The present study provides valuable insight into neural stem cells in loach brain, and these findings can be used to guide future studies.

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Key Words: Brain; development; loach (*misgurnus anguillicaudatus*); proliferation; stem cell.

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INTRODUCTION

Loach (*Misgurnus anguillicaudatus*) is an essential commercial fish species for aquaculture in China because of its high growth rate, tolerance to hypoxia for long periods, and production of a large number of offspring[1]. Loach brain has a long shape and comprises the telencephalon, diencephalon, mesencephalon, cerebellum, and myelencephalon[2]. The brains of vertebrates are characterized by persistent neurogenesis, even in the adult stage[3,4]. Persistent neurogenesis has been previously reported in several fish species, including the zebrafish (*Danio rerio*)[5-10], medaka (*Oryzias latipes*)[11], mozampique tilapia[12], Austrolebias charrua[13], and knifefish (*Apteronotus leptorhynchus*)[14]. To date, there have been no studies that have examined brain development and the sites of persistent neurogenesis in loach brain.

The adult neurogenesis process in the mammalian brain is restricted to the subventricular region of the lateral ventricle[15-17] and the subgranular zone of the dentate gyrus[18,19]. Neural stem cells are typically associated with the expression of the *Bmi1*, *Sox2*, and Musashi1[20-23,11,24]. The stem cells located in between the midbrain and hindbrain, have glial properties, whereas those present in the optic tectum have neuroepithelial properties[25]. Alunni *et al*.[11] identified that stem cells located in the optic tectum of the medaka, have no glial properties but are neuroepithelial with pluripotent characteristics. Some stem cells in the zebrafish brain have glial properties, and some have neuroepithelial properties[7,10]. Proliferating cell nuclear antigen (PCNA) staining has been used as a marker of cell proliferation in many types of fish. PCNA is typically used in combination with GFAP to determine the glial properties of proliferating cells[26,27,11].

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The present study aims to evaluate the developmental changes of loach brain via the general histological observations. As well as, determination of sites of persistent neurogenesis in the adult fish. We used the PCNA marker to characterize proliferating cells and the GFAP marker to distinguish glial properties. Fluorescent in situ hybridization (FISH) was also used to evaluate the localization of the *Msi1*, *Bmi1*, and *Sox2*, which are associated with stem cells, to further evaluate the locations that contained stem cells.

MATERIAL AND METHODS

Loach samples

Forty loaches were taken from the aquaculture base located in the College of the Fishery, Wuhan, China.

Ethics statement

The recommendations presented in the Guide of Huazhong Agricultural University for the Use and Care of Laboratory Animals were strictly followed throughout the present study.

Histology

The brains were collected at various stages of development and preserved in 10% neutral buffered formalin for 1 week. The samples were then dehydrated, cleared, and embedded in paraffin wax. We sectioned brain by using microtome at 5µm.Then we stained with hematoxylin and eosin, Bielschowsky's silver stain to label axons, and Holzer's stain to mark glial cells, astrocytes, oligodendroglia, microglia, and ependymal cells, as previously described by Jensen[28]. Stained sections were observed using a Zeiss light microscope.

Semiquantitative PCR

The whole brain was collected from the adult loach for the extraction of total RNA. The RNA was reverse transcribed into cDNA using the PrimescripttmRT Reagent Kit with the gDNA Eraser (PERFECT REAL-TIME). The expression of *Msi1*, *Sox2* and *Bmi1* in loach brain was evaluated using semiquantitative PCR. Primer pairs were designed using Primer Premier 6 software with using data obtained from gene cloning and sequencing of loach genome. The primers used are shown in (Table 1).

Table 1: Primers sequences used in semiquantitative PCR experiment

Gene name	forward primer (5'-3')	reverse primer (5'-3')
Sox2	CAACGGAGGCTACGGCATGATG	GTGCTCTGCTGCGAATAGGACAT
Bmi1	AGAGGTGGCAGATGAAGACAAGAGA	GTCATAGCGGCAGGACATTGTAAGT
Msi1	CAGCCATTCCTCTTACAGCGTACAA	GTCGTTCCATACAGGTCTGCCATC
β-Actin	TCCCATTGAGCACGGTATTG	ATCTTTTCTCTGTTGGCTTTGG

The following PCR conditions were used for these three genes and β-Actin: 95°C for 5 min; 35 cycles of 95°Cfor 30 s, 60°Cfor 30 s, and 72°C for 30 s; and a final extension of 72°Cfor 5 min.

Fluorescent insitu hybridization

Fish were anesthetized by being placed on ice. The head was then removed using a sharp scalpel, and the brain was collected and rapidly preserved in a 4% paraformaldehyde solution and incubated overnight at 4°C. The brains were then stored in methanol at −20°C until use. The brain samples were dehydrated using serial dilutions of ethanol (75%, 80%, 95%, and 100%), cleared with xylene, and then inserted in paraffin. Serial sections of 5 µm were obtained, mounted on positive charge slides, and stored at 4°C until use. After dewaxing, the slides were dehydrated in serial concentrations of ethanol, washed with DEPC-treated water, and then washed with DEPC-PBS.

The brain sections were acetylated with 0.25% acetic anhydride, which was added to triethanolamine (0.1 M TEA, pH 8.0) just before use, as the half-life is very short, and the sections were then incubated for 5 min at 37°C. Acetic acid was then added to reach a concentration of 0.5%, and the samples were incubated for 5 min at 37°C; 1 µg/ml proteinase K in TE buffer was added to the brain sections, and the sections were incubated for 7 min at 37°C. The slides were then washed with PBS containing glycine. For prehybridization, the brain sections were treated with a pre-hybridization buffer (2XSSC, 50% deionized formamide, 0.5 mg/ml yeast tRNA, 1× Denhardt's solution, salmon sperm DNA (0.5 mg/ml), and 0.02% sodium dodecyl sulfate pH 7.0) at 65°C for 1 h. For the preparation of a 50X Denhardt's solution, 10 g Ficoll 400, 10 g bovine serum albumin (BSA), 10 g polyvinylpyrrolidone were added to 1 L of sterile dH_2O, which was then was filtered and stored at 4°C. Denhardt's solution was warmed before use. The fluorescent-labeled oligonucleotide antisense probes were added to the brain sections in combination with the hybridization buffer (1× Denhardt's solution, 2XSSC, 50% deionized formamide, salmon sperm DNA (0.5 mg/mL), yeast tRNA (0.5 mg/mL), and 10% dextran sulfate) for the triple fluorescent hybridization process. This was conducted avoiding light to prevent loss of signal. The oligonucleotide probes were designed as described previously, with some modifications Moffitt *et al*.[29]. Negative control sense probes were used to confirm the results.

The brain sections were incubated at 60°C overnight (14–16 h). After hybridization, the sections were washed two times with 2X SSC and then with 1× SSC and DEPC-treated PBS. The slides were cover slipped and mounted with an anti-fading medium (nine parts of glycerol and one part of PBS, pH 8.5) and examined using Leica confocal microscope.

Immunofluorescence

Brains were collected and preserved in 4% paraformaldehyde solution overnight. Brains were then dehydrated in serial concentrations of ethanol, cleared with xylene, and inserted in paraffin. Then, 5 µm-thick sections were cut using a microtome. The brain sections were treated with xylene, followed by serial grades of ethanol. The tissue sections were then blocked with 5% BSA at room temperature for 1 h. The brain sections were incubated overnight at 4°C with a rabbit polyclonal anti-PCNA antibody (ab18197) and a goat polyclonal anti-GFAP antibody (ab53554) both diluted 1:400 in 5% BSA. The brain sections were then washed with PBS, incubated with the secondary antibodies, which included a goat anti-rabbit IgG (H+L) Cross-Adsorbed Ready Probes™ Secondary Antibody, Alexa Fluor 594 (in*vitro*gen) and Donkey Anti-Goat IgG H&L (Alexa Fluor® 488) (ab150129). The tissue sections incubated with secondary antibodies for 2 hours at 37°C. All slides were counterstained with DAPI to label cell nuclei. Slides were then mounted with a cover slip using anti-fading medium. Leica confocal microscope was used to image the slides.

RESULTS

General histological observations of loach brain at various stages of development

At 1 month of age, the two cerebral hemispheres were elongated in shape with a small telencephalic ventricle between them (Figure 1a). Each cerebral hemisphere was attached to the corresponding olfactory lobe. The mesencephalon formed from two small optic lobes, and each lobe comprised two layers. The outer layer of the optic lobe contained fibers, and the inner layer was rich in neurons. The optic tectum was connected medially with the torus longitudinalis and ventrally with the torus semicircularis (Figure 1b). The diencephalon was present from the thalamus and hypothalamus (Figure 1c). Additionally, the hindbrain and medulla oblongata were also observed (Figure 1d). At 2 months of age, the hindbrain comprised the corpus cerebellum and the valvula cerebellum. The corpus and valvula cerebellum gray matter were formed from the lateral molecular layer and medial granular layer, with the ganglionic or Purkinje cell layer in between (Figure1e). The myelencephalon or medulla oblongata contained two large vagal lobes located externally, each of which comprised three layers, including outer and inner layers of neurons and a middle layer of fibers. The two vagal lobes enclosed the facial lobes (Figure 1f) with an intracerebellar ventricle in the midline, where the two halves of the cerebellum fused during embryogenesis (Figure 1g).

From 3 months of age, the facial lobe was well-established and formed from the dorsal and ventral facial portions (Figure 1h). Some amount of neuromelanin was observed at the external borders of the vagal lobes, reaching its maximum at 3 months of age and then disappearing. With increasing age, the brain increased in size, but no other structural differences were observed (Figures 1 i,j).

Therefore, using these histological observations, we conclude that loach brain reaches full maturity at 3 months of age. Several neuroglia (astrocytes) were present in different locations, particularly around the ventricles, including the telencephalic (Figure 1k) and intracerebellar ventricles, and in the vagal lobe (Figure 1l). Several axons were present in the granular layer of the cerebellum, the inner layer of the optic lobe (Figures 1 m,n), and the outer layer of the vagal lobe. At 15 months of age, several nerve fibers were present in between the torus semicircularis and the inner layer of the optic tectum (Figure 1o).

Gene expression analysis

Semiquantitative PCR was used to measure the expression of *Msi1*, *Bmi1*, *Sox2*, and *β-Actin*. The expression of *Msi1*was greater than that of *β-Actin*, the expression of *Bmi1* was nearly equal to that of *β-Actin*, and the expression of *Sox2* was lower than that of *β-Actin* (Figure 2). The expression profiles of the *Msi1*, *Bmi1*, and *Sox2* in loach brain were also evaluated using FISH (Figure 3). Remarkably, both *Msi1* and *Bmi1*were not expressed in the optic lobe and cerebellum (Figure 3a). Meanwhile, the *Sox2* was expressed in the outer and inner layers of the optic lobe, torus longitudinalis, and granular cell layer of the cerebellum (Figure 3a). At the basal end of the third ventricle, there were a few neurons that exhibited high expression of both *Msi1*and *Bmi1*. Meanwhile, *Sox2* had low levels of expression in these neurons (Figure 3b). A high number of neurons were present next to the third ventricle with high expression of *Sox2* (Figure 3b). Both the vagal and facial lobes did not have the expression of *Msi1*and *Bmi1* (Figure 3c). However, *Sox2* was expressed in the outer layer of the facial lobe (Figure 3c).The negative control sense probes for the three genes were used to confirm our results. There were no signals detected for these three genes using the negative control sense probes (Figure 3d).

Immunofluorescence

GFAP is a marker for astrocytes and neural stem cells with glial properties. GFAP staining was used in combination with PCNA, a marker of proliferating cells, to detect proliferating cells with glial properties. The expression of GFAP and PCNA were evaluated at 1.5 and 3 months. PCNA staining was detected in the cerebellum, vagal lobe, facial lobe, optic tectum, and the cerebrum. At 1.5 months of age, the PCNA positive cells were GFAP negative and were observed in the dorsomedial part of the optic tectum (torus longitudinalis) and in the cerebellum (Figures 4 a,b,c,d). A few PCNA-positive cells were also observed in the ventral part of the cerebellum and were GFAP negative (Figure 4 e,f,g,h). PCNA expression was also observed in some cells at the tip of the vagal lobe (Figures 4 i,j,k,l).

At 3 months of age, cells in the torus longitudinalis and cerebellum and some cells in between the optic tectum and cerebellum had positive PCNA staining (Figures 5 a,b,c,d). In the cerebellum, many PCNA-positive cells that were GFAP negative were observed in the molecular cell layer (Figures 5 e,f,g,h), near the intracerebellar ventricle, and near the rhombencephalic ventricle (Figures 5 i,j,k,l). PCNA-positive cells were distributed randomly in the facial lobe and reacted negatively with GFAP (Figures 5 m,n,o,p). The proliferating cells in the vagal lobe were primarily concentrated in the outer and inner layers, with few in the middle layer. Many proliferating cells were distributed in the medial end of the vagal lobe, and all of these proliferating cells were GFAP negative (Figures 5 q,r,s,t).

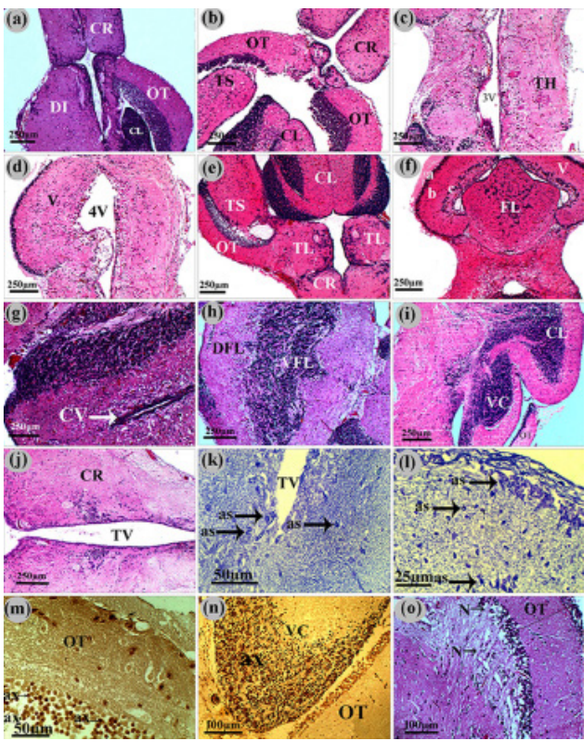

Fig. 1: The general histological observations of the loach brain at different ages. (a) at the age of 1 month, H&E. (b), (c) and (d) at the age of 1.5 months, H&E. (e) and (f) at the age of 2 months, H&E. (g) at the age of 3 months, H&E. (h) at the age of 7 months, H&E. (i) at the age of 10 months, H&E. (j) at the age of 15 months, H&E. (k) at the age of 1.5 months, Holzer's Crystal Violet Stain. (l) at the age of 3 months, Holzer's Crystal Violet Stain. (m) at the age of 10 months, Bielschowsky Silver Stain. (n) at the age of 10 months, Bielschowsky Silver Stain. (o) at the age of 15 months, H&E. CR (cerebrum), OT (optic tectum), DI (diencephalon), CL (corpus cerebellum), VC (valvula cerebellum), TL (torus longitudinalis), TS (torus semicircularis), 3V (third ventricle), TH (thalamus), V (vagal lobe) (a) outer layer, (b) middle layer, (c) inner layer of vagal lobe, 4V (rhombencephalic ventricle), FL (facial lobe), CV (intercerebellar ventricle), DFL (dorsal facial lobe), VFL (ventral facial lobe), TV (telencephalic ventricle), as (astrocyte), ax (axons), and N (nerve fibers).

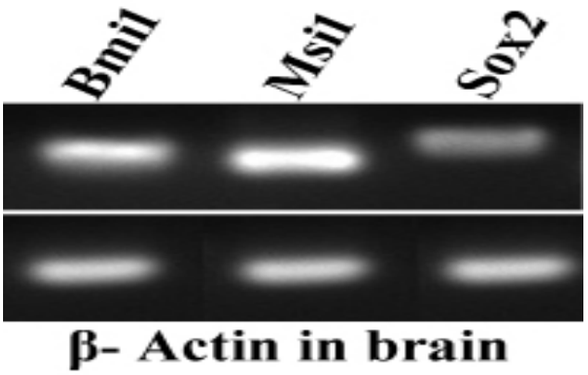

Fig. 2: Photo for gel electrophoresis result showing gene expression of *Bmi1*, *Msi1*, *Sox2* and β-Actin genes by semiquantitative PCR.

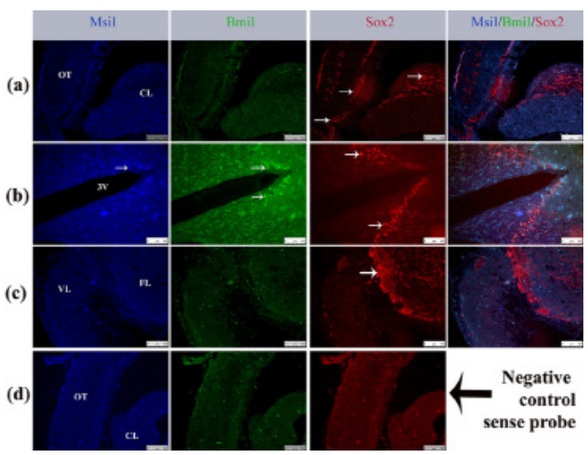

Fig. 3: The fluorescent in situ hybridization (FISH) for the detection of *Bmi1*, *Msi1*, and *Sox2* genes expressions in the loach brain. (Arrows) determine sites of gene expression. (a) the *Sox2* gene expression in optic lobe (OT), and the *Sox2* gene expression in cerebellum (CL). (b) the *Bmi1*, *Msi1*, and *Sox2* genes expression beside the third ventricle (3V). (c) the *Sox2* gene expression in the facial lobe (FL). (d) the expression of *Bmi1*, *Msi1*, and *Sox2* genes via using negative control sense probe.

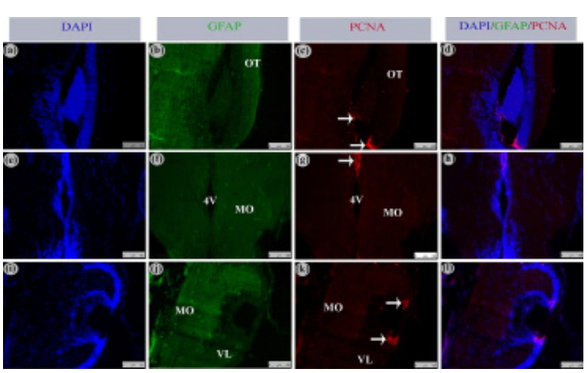

Fig. 4: The loach brain at the age of 1.5 months shows PCNA, and GFAP immunofluorescence (arrows). Optic lobe (OT), medulla oblongata (MO), and vagal lobe (VL). The DAPI stain used to counterstain nuclei.

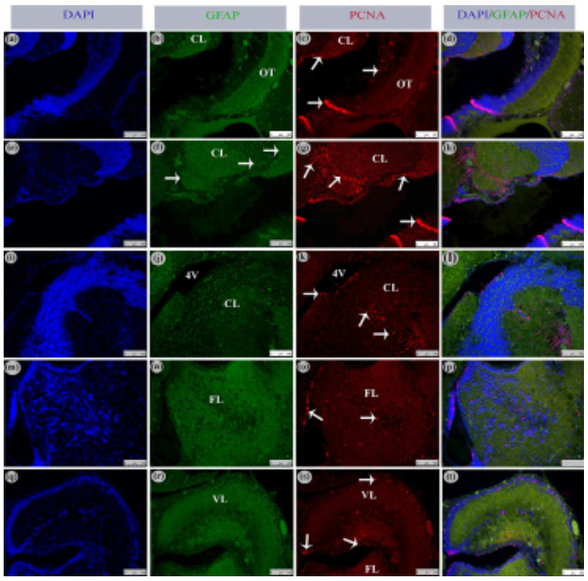

Fig. 5: The loach brain at the age of 3 months shows GFAP, and PCNA immunofluorescence (arrows). Cerebrum (CR), Optic lobe (OT), cerebellum (CL), rhombencephalic ventricle (4V), facial lobe (FL) and vagal lobe (VL). The DAPI stain used to counterstain nuclei.

DISCUSSION

In the present study, we evaluated the histological structure of loach brain. The cerebrum in loach brain has two elongated lobes separated from each other by the telencephalic ventricle. The cerebrum of loach is formed from a single layer of cells. The cerebrum of the teleost has been described as a single layer without the traditional six layers that are present in the mammalian cerebrum[30]. The mesencephalon comprised the optic tectum, torus longitudinalis, and torus semicircularis. The optic tectum consisted of two layers, with the inner containing several neurons. Our results agree with other studies that have examined the zebrafish brain, suggesting that the inner layer, the periventricular gray zone, has several neurons extending their dendrites into superficial layers[31,32]. However, the optic tectum of some other fish has six layers[33,34].

The cerebellum, facial lobe, and vagal lobe were all well-developed in loach brain. The cerebellum was large and formed from the corpus and valvula cerebella, with the intracerebellar ventricle in the midline of the corpus cerebellum. This indicates that loach has strong rapid swimming skills. Our findings for the cerebellum morphology of loach are consistent with previous findings of Abrahão and Shibatta[35].

A sizable facial lobe was present in between the two wings of the well-developed vagal lobe. These results indicate that loach has a well-developed sense of taste[2]. A well-developed sense of taste was also found in catfish[36]. The vagal lobes of the Antarctic eel cod extend throughout the caudal half of the medulla. They form the floor of the rhombencephalic ventricle caudal to the cristae but are positioned more ventrally, under and caudal to the facial lobes[33]. Goldfish (*Carassius auratus*) and some carps have well-developed gustatory centers[37]. Additionally, the vagal lobe in loach resembles what has been described previously for the Carassius carassius fish[38], with a highly organized, laminated structure consisting of an outer sensory zone, a central fiber zone, and an inner region of motor neurons. The vagal lobe is fully developed by 5–6 months in the goldfish[39], whereas in loach, it becomes morphologically developed by 3 months of age.

Neuromelanin pigment was detected in loach brain at young ages, reaching its maximum at 3 months of age and then disappearing. Therefore, these pigments may be considered a by-product of cell differentiation[40]. The expression of *Msi1*, *Bmi1*, and *Sox2* is typically associated with neural stem cells. Therefore, we used these to evaluate the localization of stem cells in loach brain. Our previous study in the retina and lens of loach demonstrated that these three genes are associated with stem cells in loach eye[41].

The expression of *Sox2* was high in the optic tectum, the granular cell layer of the cerebellum, besides the third ventricle, in the torus longitudinalis, and in the periphery of the facial lobe. These results indicate the presence of stem cells in these regions. *Sox2* plays a significant role in stem cell differentiation and self-renewal in the brain[42-44]. Fate mapping studies of the mammalian brain indicate that multipotent stem cells are controlled by the expression of *Sox2*[45]. *Sox2* also binds and regulates GFAP-positive regulatory elements[46]. The expression of *Msi1* and *Bmi1* were detected at the basal end of the third ventricle, which indicates the presence of proliferating stem cells around the third ventricle in loach brain. In zebrafish, the expression of *Msi1*was high at the early stages of development, which is similar to that of mammalian *Msi1*[24]. *Bmi1* plays a role in the repression of genes involved in senescence, which is a state in it the proliferation property of cells is terminated[47].

PCNA staining was used to observe the active proliferating areas in loach brain[48,49,27]. PCNA staining was most robust in the dorsomedial part of the inner layer of the optic tectum (at the torus longitudinalis). These cells increased in number with increased age and were GFAP negative. However, Ito *et al*.[7] and Recher *et al*.[8] described that the optic tectum of zebrafish contains cells with non-glial characteristics that can rapidly proliferate and cells with radial glial properties that proliferate slowly.

Grandel *et al*.[5] and Ito *et al*.[7] found that proliferating cells located in the dorsomedial part of the inner layer of the optic tectum express several stem cell markers, and these cells can generate both neuronal and glial cells. Meanwhile, Chapouton *et al*.[25] and Doetsch[50] identified that, in between the midbrain and hindbrain, there are stem cells with glial properties. However, our results from loach brain indicate the presence of proliferating cells in between the optic tectum and the cerebellum. These cells were GFAP negative and, thus, are proliferating neuroepithelial cells.

In the cerebellum, PCNA-positive proliferating cells were present in the molecular cell layer and around the intracerebellar ventricle and did not have glial properties, as they were GFAP negative. A high number of proliferating cells with neuroepithelial properties (non-glial) were also observed in the lobus caudalis cerebella, the eminentia granularis, and around the rhombencephalic ventricle. These indicate that neuroepithelial proliferating cells are present in the cerebellum of loach. However, Kaslin *et al*.[10] described that there are a few glial stem cells that share the neurogenesis process in the adult cerebellum. Several proliferating cells with neuroepithelial properties were detected in loach in the facial lobe and in the three layers of the vagal lobe, which is consistent with previous findings[5]. This work gives valuable information about the histological structure of brain of loach during development. As well as, gives insights about adult neurogenesis in brain of loach. Further work in the future is needed for detection of other genes involved in adult neurogenesis process in loach brain.

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CONFLICT OF INTERESTS

There are no conflicts of interest.

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