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Brain Development and Adult Neurogenesis in the Optic Tectum of Gray Mullet (*Mugil cephalus*)

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Abstract	Original Research Article

The Mugil Cephalus fish is widely spread all over the world, but there is little knowledge about brain development in this kind of fish. In this study, we showed the histological observations by general and histochemical staining procedures as well as, GFAP, PPARα, PPARγ and ki67 immunohistochemistry. We considered brain development at three months, eight months and fifteen months of age. The forebrain formed from two large elongated olfactory lobes; they connected by olfactory tract with the corresponding cerebrum. The diencephalon present under the telencephalon, it composed from epithalamus, thalamus, and hypothalamus. The epithalamus contained pineal gland which its lumen opened in the third ventricle. The thalamus found beside the mesencephalon around the third ventricle and contained several nuclei the most prominent one was the nucleus granulosus. The hypothalamus formed from the pituitary gland, inferior lobes and saccus vasculossus they found under the third ventricle. The mesencephalon contained two sizeable optic tectum and two torus longitudinalis. The torus longitudinalis reach complete development at the age of eight months. The most characteristic thing in the brain of Mugil cephalus was persistent neurogenesis which occurred at the level of tectal ventricle under the optic tectum; this neurogenesis became more evident at the age of fifteen months. There was a positive reaction between optic tectum and tectal ventricle to GFAP, PPARα, PPARγ, and ki67.
Keywords: brain, development, adult neurogenesis, optic tectum, Mugil cephalus, PPARs, fish.

INTRODUCTION

In all vertebrates, the neural tube gives rise to the central nervous system as its wall thickens leading to the formation of the brain and the spinal cord. The cerebrospinal fluid fills the lumen of the neural tube and the ventricular system develops laterally and is responsible for the creation of new glial cells and neurons for further formation of all brain components [1]. During embryonic development, the radial glial cells which present in the surface of lateral ventricles act as neural stem cells as they can form new neurons [2-4].

The five main brain divisions including telencephalon, diencephalon, mesencephalon, cerebellum, and rhombencephalon, were observed from the first day of hatching in sturgeon Acipenser naccarii fish [5] but take time till it reaching complete development. The olfactory bulb is a pyriform structure. It presents in front of the telencephalon and is joined to the olfactory mucosa by a long olfactory nerve; it shows four layers which from outer to inner are: the olfactory nerve fiber layer, the glomerular layer, the plexiform layer and the granule cell layer [6]. Diencephalon consisted of epithalamus, thalamus, and hypothalamus. The epithalamus was situated beneath the optic tectum and formed from the pineal gland, the thalamus located under the tegmentum, and the hypothalamus was beneath the third ventricle (diencephalic ventricle). The hypothalamus consisted of the pituitary gland, infundibulum and the inferior lobes [7]. The mesencephalon of the gilthead seabream consists of the optic tectum dorsally and the tegmentum ventrally. The optic tectum formed from two bilateral lobes each one of them connected medially with the torus longitudinalis. The torus longitudinalis (TL) present only in actinopterygian fishes; it appears lately and originates from the ventricular zone of the optic tectum [8]. The tegmentum replaces the caudal diencephalon and synencephalon and is bordered dorsally by the valvula cerebelli, laterally by the optic tectum and ventrally by the inferior lobe of the hypothalamus. The cerebellum was composed of the corpus and the valvula cerebella. The histological structure of the cerebellum gray matter was three different layers based on histological organization, including outer molecular,

Purkinje cell and inner granular layers [9]. Except in Eleginops maclovinus fish the cerebellum characterized by unordinary structure; as it formed from the central molecular layer and lateral granular regions [10]. The brain stem contained the medulla oblongata, and it continued to form the spinal cord [11].

The present study is directed to observe histological changes during brain development in Mugil cephalus fish at the age of three months, eight months and fifteen months. The brain of Mugil cephalus characterized by sizeable optic tectum; it formed from 6 layers, with noticeable signs of adult neurogenesis around tectal ventricle which found under optic tectum. The KI67, PPARα, PPARγ, and GFAP markers were used to determine their roles in the process of adult neurogenesis which occurred around tectal ventricle under the optic tectum.

MATERIALS AND METHODS

Animal and tissue processing
This research applied in Mugil cephalus fish. Fifteen Mugil Cephalus fishes were taken from mugil fish farm in Egypt at the following ages, three months, eight months and fifteen months. Their brains were rapidly collected and preserved in 4% paraformaldehyde overnight at 4℃, then dehydrated, cleared and embedded in paraffin wax for serial sectioning.

Histology and Histochemistry
The samples were cut sagittaly at 5 µm. the sections were deparaffinized and stained with the following stains:

A- Hematoxylin and eosin for general histological observation.
B- Cresyl violet to demonstrate Nissl substance.
C- Luxol fast stain for staining of Myelin, including phospholipids green and neurons red.
D- Bielschowsky silver stain for staining of axons, neuro-fibrillary tangles, and senile plaques.
E- Holzer's stain for staining of glial cells: astrocytes, oligodendroglia, microglia, and ependymal cells.

The staining procedures were according to Jensen [12]. Then slides were observed by Zeiss light microscope for taking photos.

Immunohistochemistry
For immunohistochemical examination, the tissue sections were deparaffinized with xylene and rehydrated with downgrades of ethanol. Then the tissue sections were washed by phosphate-buffer saline (PBS) PH 7.4 for 15 minutes. The antigen retrieval protocol was done by using sodium citrate buffer in preheated steamer for 30 minutes. Then the tissue sections were washed in PBS tween 20 two times for 2 minutes. Then the tissue sections were immersed in 0.3 % H_2O_2 in PBS (pH 7.4) for 30 min at room temperature to remove endogenous peroxidase activity, for the elimination of non-specific binding. This step is done in a dark, humid chamber and is done only in case of using biotinylated secondary antibodies in GFAP immunohistochemistry. The sections were rinsed with 1x PBS tween 20 three times and then blocked with blocking serum (0.3% triton X-100 plus 5% bovine serum albumin (BSA) at room temperature for 1 h. the previous step is needed for both blocking and permeabilization of tissue sections. The tissue sections were incubated with the following antibodies at 4 °C overnight in a closed humid chamber: Rabbit polyclonal anti-GFAP antibody (ab19166), Rabbit polyclonal anti-PPAR alpha antibody (ab61182), Rabbit polyclonal anti-PPAR gamma antibody (ab66343) and Rabbit polyclonal anti-Ki67 antibody (ab15580). All antibodies were diluted by 1/500 with blocking serum. For GFAP immunohistochemistry the slides were incubated with biotinylated secondary antibodies and then were incubated with streptavidin-biotin-horseradish peroxidase complex for 30 min at room temperature. The sections were washed with PBS and treated with diaminobenzidine (DAB) substrate solution. The hematoxylin was used as counter stain for nucleus observation. Then the sections were washed and dehydrated with various grades of alcohol, cleared in xylene and then mounted with DPX. Then the slides were examined under Zeiss light microscope for taking photos. For PPARα, PPARγ, and KI67 fluorescent immunohistochemistry applied, the sections were incubated at room temperature for 1 hour with secondary goat anti-rabbit IgG antibodies labeled with appropriate secondary dye at a dilution 1/400. The primary antibodies were company validated for use in fish and negative control slides were examined for validation of secondary antibodies specificity. Then they were washed by PBS then coverslip mounted with an anti-fading medium, then observed with a confocal microscope.

RESULTS
In general, the brain of gray mullet fish is formed from telencephalon or forebrain (contain 2 olfactory lobes and cerebrum), diencephalon (contain epithalamus, thalamus, and hypothalamus), mesencephalon or midbrain (contain 2 optic lobes), metencephalon or hindbrain (cerebellum) and myelencephalon or brain stem (medulla oblongata). This study is concerned with the brain development in Mugil cephalus fish at the following ages; three months, eight months and fifteen months.

At the age of 3 months
The two olfactory lobes were connected by olfactory tract with the corresponding cerebrum. The cerebrum composed of a single layer of nerve cells. The two optic lobes were present below the cerebrum and beside the third ventricle. They were separated from torus semicircularis by tectal ventricle. At this age, the torus longitudinalis didn't reach complete development (figure 1a). The optic tectum was large and consisted of

six layers, are from rostral to caudal; stratum marginal (SM), stratum opticum (OP), stratum fibrosum et griseum superficial (SFGS), stratum griseum central (SGC), stratum album central (SAC) and stratum periventriculare (SPV) (figure 1b). The diencephalon present beside the third ventricle, the most prominent part of the thalamus was the nucleus granulosus (figure 1c and figure 1d). The cerebellum still not fully differentiated like that in mature fish (figure 1e). The medulla oblongata and two vagal lobes form the most posterior part of the brain (figure 1f).

At the age of 8 months

At this age, the torus longitudinalis appeared on the medial side of each optic tectum. The cerebellum was present in between two torus semicircularis (figure 2a). The layers of optic tectum contain myelinated nerve fibers while the nerve cell bodies were concentrated only in the stratum periventriculae layer and in torus longitudinalis (figure 2b). There were some axons observed beside tectal ventricle emerging from tectal ventricle toward optic tectum (figure 2c). It noticed that the nerve cell bodies in the stratum periventriculae, torus longitudinalis, the three layers of torus semicircularis, ganglionic and Purkinje cell layer of cerebellum, nucleus isthmi and ependymal cells lining third ventricle their cytoplasm showed a high positive reaction with cresyl violet stain, Which indicates presence of nissel´s substance in the cytoplasm of these cells (figure 2d). While the molecular cell layer of cerebellum showed a moderate reactions with cresyl violet stain and there were several multipolar nerve cell bodies under the epithelium of tectal ventricle. It was found a high concentration of glial cells in the area around tectal ventricle, under the optic tectum (figure 2e). The ependymal cells lining tectal ventricle showed the presence of several nerve fibers emerging from them toward optic tectum (figure 2f). The thalamus contains several nuclei the most obvious one was the nucleus granulosus (figure 2g). The cerebellum present between two optic lobes and under it the medulla oblongata observed. It was apparent that the cerebellum gray matter formed from the lateral molecular layer and medial granular layer and ganglionic or Purkinje cell layer in between (figure 2h). It was found that the granular cell layer of the cerebellum and the peripheral cell layer of the torus semicircularis contain several axons (figure 2i).

At the age of 15 months

At this age, the brain wholly formed (figure 3). The forebrain contains two large olfactory lobes were connected with the cerebrum by a stalk of nerve fibers. These olfactory lobes include expansions of olfactory nerve which appears in the form of bundles of nerve fibers surrounding small nerve cells (figure 3a). There

were no layers in the cerebral cortex of the brain in gray mullet fish like other teleost fish (figure 3b). Under the telencephalon and beside the optic tectum the epithalamus was present (figure 3c and figure 3d). The third ventricle penetrated the lumen of the pineal gland, the most obvious kind of cells in this gland was the pinealocytes (photoreceptor cells), which were giant multipolar cells containing a central spherical nucleus, it´s cytoplasm filled with nissel's granules and has several dendrites. The thalamus was present in the third ventricle and medial to the optic tegmentum. The hypothalamus was present under the third ventricle it contains pituitary gland, inferior lobe and the saccuss vasculossus which was relatively small in Mugil cephalus fish while the inferior lobe was medium sized (these data not showed). The mesencephalon consists of two large optic lobes were connected medially with torus longitudinalis and ventrally by torus semicircularis and optic tegmentum. The nucleus isthmi were a cell mass present below the torus semicircularis and in front of the corpus cerebellum (figure 3e).

The optic tectum was very large in this kind of fish, and there was persistent neurogenesis around the tectal ventricle which was more evident in this age more than fishes at periods of three or eight months. It was apparent that neuroepithelial cells were lining the tectal ventricle some of these cells have the property of forming axons of the layers of the optic tectum others have the ability for creating nerve cell bodies of the other layers (figure 3f and figure 3g). It was noticed the presence of a network of blood vessels and capillaries have a connection with the optic tectum, it present above optic tectum and below cerebrum (figure 3h). The medulla oblongata was present at the level of the fourth ventricle (figure 3i) and ends by two small vagal lobes (figure 3j).

Adult neurogenesis in optic tectum at the age of 15 months

The GFAP antibodies showed a positive reaction in the glial cells which present around tectal ventricle as well as, between optic tectum and tectal ventricle (figure 4a), which is the most obvious site for adult neurogenesis in Mugil cephalus fish. The KI67 showed a high positive reaction in optic tectum and very high positive reaction in the newly formed somas or nerve cell bodies which move from tectal ventricle toward optic tectum (figure 4b). The PPARα at the age of fifteen months showed a high positive reaction in optic tectum, torus longitudinalis, optic tegmentum and the membrane which surround the newly formed somas during the process of adult neurogenesis (figure 4c). While, the PPARγ showed a high positive reaction in optic tectum and newly formed fibers in the area of adult neurogenesis (figure 4d).
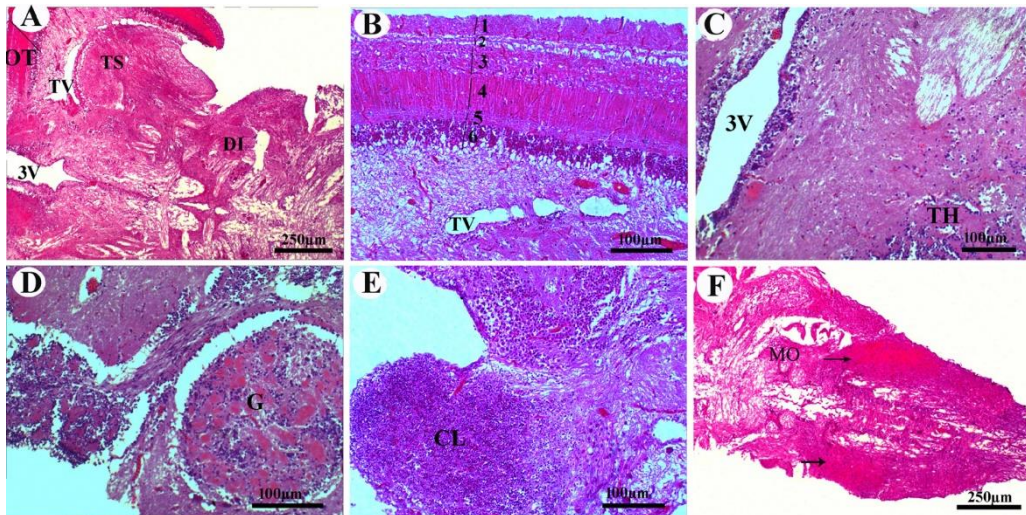
Fig-1: Brain at the age of three months (A) optic tectum (OT), tectal ventricle (TV), torus semicircularis (TS), third ventricle (3V) and diencephalon (DI). (B) layers of optic tectum; stratum marginal (1), stratum opticum (2), stratum fibrosum et griseum superficial (3), stratum griseum central (4), stratum album central (5) and stratum periventriculae (6). (C) Thalamus (TH). (D) Nucleus granulosus (G). (E) Cerebellum (CL). (F) Vagal lobes (arrows) and medulla oblongata (MO). All slides stained with hematoxylin and eosin stain
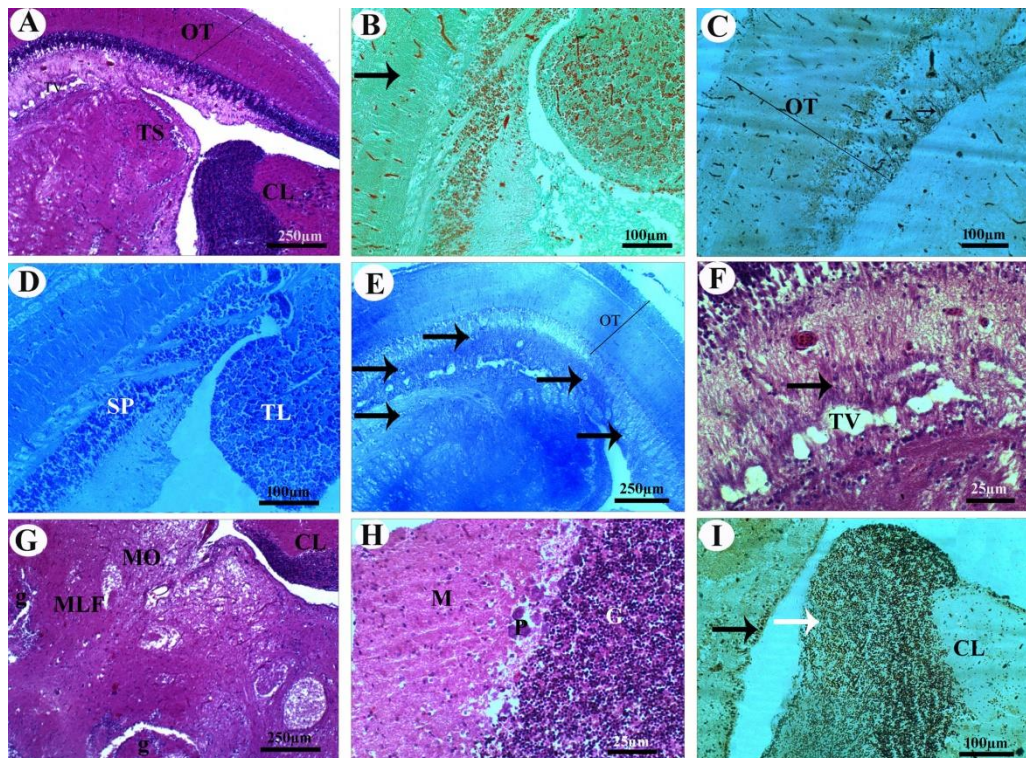

Fig-2: Photomicrographs of the brain at the age of eight months (A) optic tectum (OT), tectal ventricle (TV), torus semicircularis (TS) and cerebellum (CL) H&E. (B) myelinated nerve fibers (arrows) stained with luxol fast stain. (C) Axons around tectal ventricle (arrows) under optic tectum (OT) stained with Bielschowsky's silver stain. (D) Nerve cell bodies contain nissel granules in stratum periventriculare (SP) and torus semicircularis (TS) stained with cresyl violet stain. (E) glial cells (arrows) under the optic tectum, stained with Holzer's stain. (F) the Process of neurogenesis (arrow) around tectal ventricle H&E. (G) medulla oblongata (MO), medial longitudinal fasciculus (MLF) and nucleus granulosus (g), H&E. (H) Granular cell layer (G), ganglionic cell layer (P) and molecular cell layer (M). (I) Axons in TS (black arrow), axons in the cerebellum (white arrow) stained with Bielschowsky's silver stain
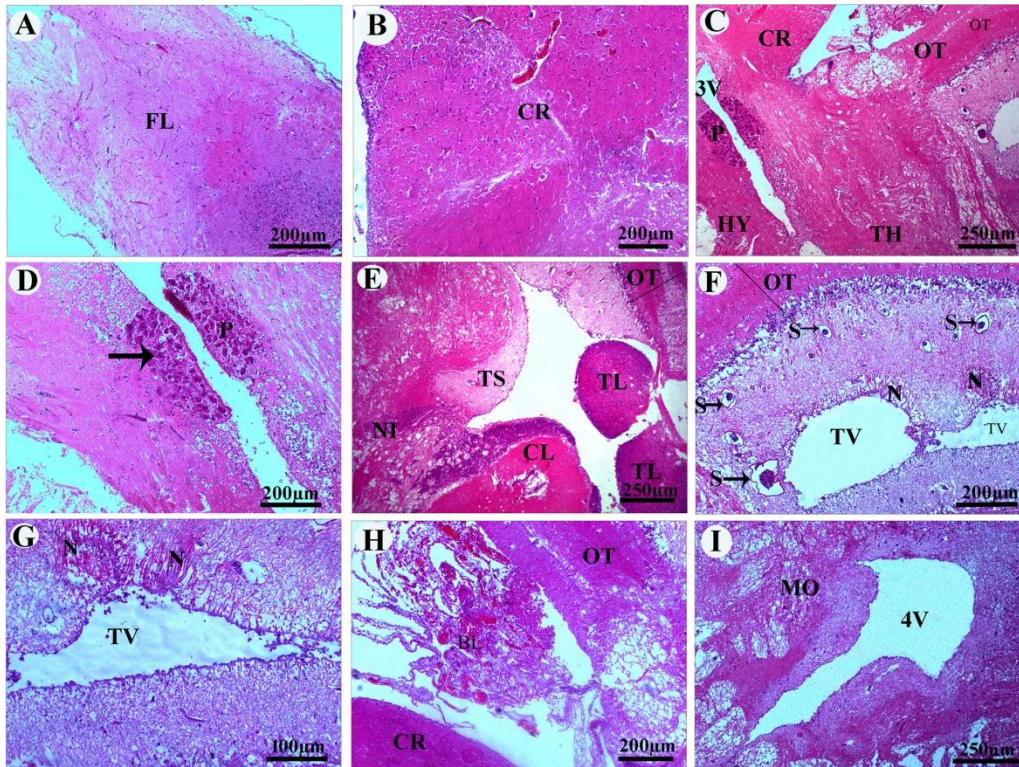
Fig-3: Photomicrographs of the brain of gray mullet at the age of 15 months. (A) Olfactory lobe (FL). (B) Cerebrum (CR). (C) Optic tectum (OT), pineal gland (P), third ventricle (3V), thalamus (TH) and hypothalamus (HY). (D) Pineal gland cells (arrow). (E) Torus longitudinalis (TL), torus semicircularis (TS), nucleus isthmi (NI) and cerebellum (CL). (F) neurogenesis process under optic tectum (OT) for the formation of new axons (N) and new nerve cell bodies enveloped by capsule (arrows) occurs around tectal ventricle (TV). (G) Newly formed axons originate from neuroepithelial cells lining tectal ventricle (TV). (H) a network of blood vessels and capillaries (BL) connected with optic tectum (OT). (I) Shows nuclei of the medulla oblongata (MO) and fourth ventricle (4V). All slides stained with hematoxylin and eosin stain
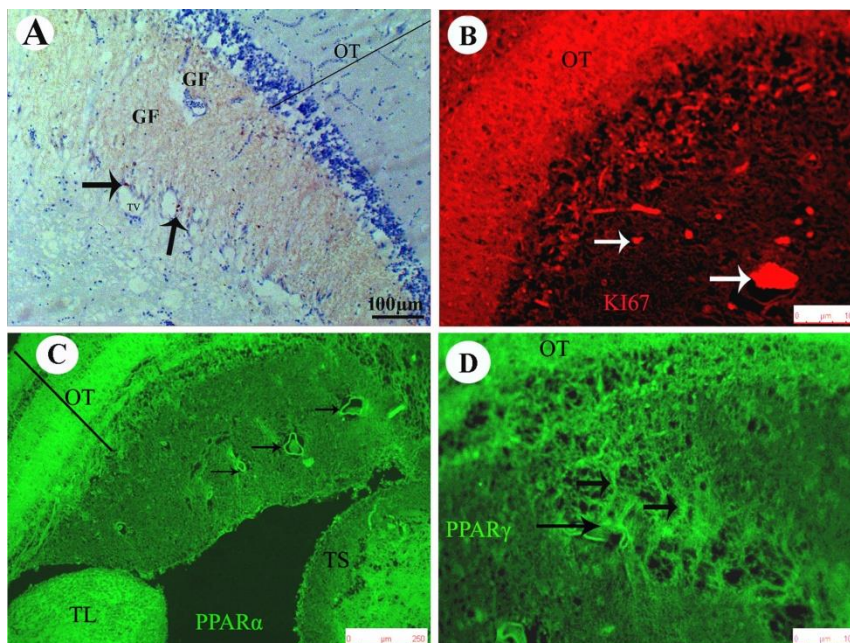

Fig-4: Photomicrographs of the brain of gray mullet at the age of 15 months. (A) the Positive reaction against GFAP antibodies in astrocytes (arrow) and glial cell fibers (GF). (B) the high positive reaction against KI67 in newly formed cells which move toward optic tectum (arrows). (C) the high positive reaction against PPARα in OT, TL and in the membrane which surrounds the newly formed cells which move toward optic tectum (arrows). (D) Positive reaction against PPARγ in the newly created axons of the optic tectum (arrows)

DISCUSSION

The olfactory lobes were medium in size, elongated in shape and connected with olfactory tract with the corresponding cerebrum, which its gray matter formed from a single layer of neurons this result was in agreement with [13, 7]. The diencephalon was formed from epithalamus, thalamus, and hypothalamus.

The epithalamus or pineal gland located below the cerebrum and its lumen open in third ventricle [14, 15]. Three types of cells were considered the main content of the pineal gland, i.e., pinealocytes (photoreceptor cells), glial (supporting) cells and second-order neurons ganglion cells [16]. The thalamus contains several nuclei the most obvious one was the nucleus granulosus this result was following [10]. The hypothalamus was found in the third ventricle and formed from inferior lobe, saccus vasculosus and pituitary gland this result previously reported by [7]. The optic tectum was large and present under the cerebrum. It consisted of six layers which were from cranial to caudal; stratum marginal (SM), stratum opticum (OP), stratum fibrosum et griseum superficial (SFGS), stratum griseum central (SGC), stratum album central (SAC) and stratum periventriculare (SPV) [17, 18].

The torus longitudinalis which appeared as semicircular structure connected with the medial end of the optic tectum [19]. Only observed at both eight and fifteen months not observed at three months age this result following [8] who described torus longitudinalis as a late-appearing structure. The optic tectum ventrally separated from torus semicircularis and optic tegmentum by tectal ventricle this result following[18,10]. The neurons in the stratum periventriculae, torus longitudinalis, the torus semicircularis, ganglionic or Purkinje cell layer of the cerebellum, nucleus isthmi and ependymal cells lining the third ventricle their cytoplasm contains several nissl´s substances which indicate high energy need for these cells.

The adult neurogenesis in optic tectum occurred in the area between optic tectum and tectal ventricle. While [20, 21] described it in the periventricular gray zone in case of zebrafish. While [22] after in vivo investigation of the neural stem cell of the zebrafish embryo revealed a unique vertebrate progenitor population in the peripheral midbrain layer (PML), the progeny of three PML clones contributed to both the optic tectum and the torus semicircularis. The glial cells were highly concentrated between the optic tectum and tectal ventricle which may indicate their role in process of adult neurogenesis. These glial cells react positively with GFAP antibodies which indicate that they were astrocytes, these results in accordance with [23, 2, 4].

As they mentioned that multiciliated ependymal cells and monociliated astrocytic neural stem cells covered the ventricular walls in the adult mammalian brain in the ventricular-subventricular zone. While, Alunni et al. [24] found that in medaka fish, the adult optic tectum progenitors were not glia, as they express neither brain lipid-binding protein (BLBP) nor glial fibrillary acidic protein (GFAP) and they showed expression of pluripotency-associated markers (Musashi1, Bmi1, and Sox2). Our result indicated the presence of some axons beside tectal ventricle which reacts positively with a silver stain which may indicate the presence of progenitor stem cells responsible for the formation of new axons and nerve cell bodies of the optic tectum.

The process of neurogenesis in between optic tectum and tectal ventricle was very few at the age of 3 months become moderate at the age of 8 months and become very obvious at the age of fifteen months. The KI67 antibodies showed a very high positive reaction in newly formed cells in the area of adult neurogenesis which indicate high proliferation activity of these cells, our result agrees with [25] who mentioned that Ki-67 expression is highly elevated in proliferating cells and can nevertheless be detected in quiescent cells. The expression of the Ki-67 protein can be used as an indicator for the growth fraction of a given cell population by indicating the proliferative activity [27]. There are three peroxisome proliferator-activated receptors; PPARα, PPARβ, and PPARγ they are nuclear receptors responsible for maintenance of lipid homeostasis [28, 29]. The PPARα expression was low at age of eight months while at age of fifteen months was high, it found to have role in adult neurogenesis as it enter in the formation of the membrane which surround the newly formed proliferating cells, while the PPARγ showed positive reaction with the newly formed axons which move toward optic tectum.

Our results were in accordance with, Braissant and Wahli [30] who reported that PPARα and PPARγ appeared in adulthood. It appeared that high PPARα expression was found in tissues with peroxisomal β-oxidation and high mitochondrial capacity [31, 31]. PPARα is considered the main mediator of peroxisome proliferation [32] and PPARγ believed to be involved in the activation of the peroxisome proliferation response [33]. The optic tectum and area of adult neurogenesis in the brain of gray mullet showed higher positive reaction agaist PPARα and PPARγ than other parts of the brain and more at fifteen months than at eight months age, this may be because this part its activity in process of adult neurogenesis increased at one year more than at eight months age so we can conclude that there is essential role of PPARα and PPARγ in the process of adult neurogenesis in adult gray mullet fish which may indicate the need of this process for peroxisome biogenesis and lipid catabolism. The cerebellum at the age of 3 months was not completely developed. The

cerebellum was large; also, Abrahão *et al.* [34] mentioned that the corpus cerebelli had the largest structure of the brain in Pseudopimelodus bufonius fish. The gray matter of cerebellum formed from three layers, were from outer to inner; molecular cell layer, ganglionic cell layer and granular cell layer, this order was in accordance with Ross and Pawlina [35, 7], while [10] described that the granular cell layer was outer and the molecular cell layer was inner. The medulla oblongata and two vagal lobes form the most posterior part of the brain [9].

This study showed for the first time the general histological observations in the brain of gray mullet at three stages of development; first was at the age of 3 months, second was at the age of 8 months, and third was at the age of 15 months. As well as immunohistochemistry for KI67, GFAP, PPARα and PPARγ to express their roles in adult neurogenesis process which observed around tectal ventricle under optic tectum.

Acknowledgments

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