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Immunohistochemical Distributions of Fibroblast Growth Factor 1 and 2 in the Mouse Central Nervous System in Different Time Course

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Abstract

Fibroblast Growth Factor 1 (FGF1) and FGF2 are involved in the development of the central nervous system. We evaluated the spatiotemporal distributions of FGF1 and FGF2 in the ICR male mouse cerebrum from Postnatal day 0 (P0) to P360. Western blot analysis of whole cerebrum extracts confirmed that FGF2 gradually increased during P10 to P90 and thereafter reached a plateau. FGF2 was detected as early as P0 and invariable throughout the study period. Cryosections of the cerebrums were subjected to immunohistochemistry, to demonstrate the localization of FGF1 and FGF2. The study has detected the time-course of the appearance of FGF1 and FGF2 immunoreactivity in the mouse brain. The occurrence of active neurite outgrowth, synapse formation, and sprouting was identified including eyelids opening, Corti's organ, terminal differentiation, and initiation of myelination.

Keywords: Central nervous system; Fibroblast growth factors; Immunohistochemistry

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Introduction

The development of the nervous system in most species entails an essential diversification of cells, including the generation of glia and neurons. For predefined species, the timing of development of neurons and their appearance is highly reproducible in the developing nervous system and an accurate schedule is important to organize the normal cytoarchitecture [1]. Neurons are produced primarily during the embryonic period in the mammalian Central Nervous System (CNS), whereas majority of glia were produced after birth [2].

The Fibroblast Growth Factor (FGF)s, also known as heparin binding growth factor due to their high affinity for heparin and heparin like substance, are polypeptides which have potent mitogenic or neurotrophic actions on variety of mesodermal, ectodermal and neuroectodermal cell types [3,4]. In human, 23 members of the FGF family has been identified till now. FGF 1 is also known as acidic Fibroblast Growth Factor (aFGF), and FGF 2 is also known as basic Fibroblast Growth Factor (bFGF).

Recent studies of both aFGF and bFGF and their receptors have emphasized a physiological role of these molecules in the nervous system [5,6]. Again, exogenously applied aFGF and/or bFGF mediates rescue effects on injured sensory neurons and supports neurite outgrowth of transitioned nerves also emphasizes that these molecules seem to play a physiological role during nerve regeneration.

Thus, while many of the molecular features of the FGF system have become evident over last few years, the normal physiological role of FGFs is still not well understood [7-9]. In contrast to many studies on the expression of FGF receptors as well as aFGF and/or bFGF producing or containing cells in the central nervous system, a very few studies have been conducted on their developmental appearance and cellular distribution to date, which is very crucial for gaining insight about the normal physiological role of these factors in this tissue [10,11]. Present study has been initiated to identify the time course of the developmental appearance and to localize the positive

cellular population for both aFGF and bFGF in order to investigate the possible physiological role of these factors in the development and maintenance of CNS. Investigation for the cellular distribution of aFGF and bFGF has been mainly done by immunohistochemistry and western blot technique was used for confirmation.

This study aims to identify the time course of the appearance of development of neurons and investigates the possible physiological role of positive cellular population in the development and maintenance of CNS. This study has developed an analytical and experimental framework with which the complications of the mouse brain can be observed and analyzed at cellular level. By using this approach, this study has been able to determine all major cell types of brain and to classify subtypes of neuronal cells.

Materials and Methods

The experiments were performed on ICR male mice. Separate cages were allotted to pregnant females in which they were kept under alternate 12-h periods of darkness and light. Newborn mice were kept with their mothers. Food and water were freely provided to all mice. Ethical approval was taken from the authority of Hiroshima University facilities for Laboratory Animal and Research and all experiments were carried out according to their guidelines regarding the care and use of animals for experimental procedure.

Immunohistochemistry and western blot analysis were performed in mice of different age group following birth: Day-0, day-10, day-20, day-30, day-90, day-180 and day-360. Each group consisted of 6 mice. The brains of mice were collected from anesthetized mice on the specific days and were frozen with dry ice and stored at -80°C or in liquid nitrogen.

For immunohistochemical staining, 12 µm thick evenly spaced coronal sections were cut with a cryostat at -20°C to cover all anatomical areas. Sections were rapidly dried on slides and fixed with periodate-lysin-paraformaldehyde (PLP: 10 mM $NaIO_4$, 75 mM lysine, 37.5 mM phosphate buffer, pH 7.4 containing 2% paraformaldehyde) for 10 min at Room Temperature (RT).

Sections were incubated with 5% normal goat serum (Vector Labs, Burlingame, CA) diluted in 0.01 M Phosphate Buffered Saline (PBS) for 1 hour at RT and subsequently either with rabbit polyclonal anti-aFGF antibody (0.5 µg/ml, Santa Cruz Biotechnology, Santacruz, CA) or with rabbit polyclonal anti-bFGF antibody (2 µg/ml, Santa Cruz Biotechnology, Santacruz, CA) for 40 and 16 h respectively at 4°C. The sections were then incubated with biotinylated goat anti-rabbit IgG (1:400, Vector Labs) for 90 min at RT followed by incubation with avidin-biotin peroxidase complex (1:400, Vectastain Elite ABC kit, Vector Labs) for 1 h at RT. Each incubation step was followed by three washes of 10 min each with 10 mM PBS (pH: 7.4).

Chromatographically purified rabbit IgG was used for negative control and the sections were incubated with respect to the time and primary antibody concentration. All re agents were diluted with 10 mM PBS containing 5% normal goat serum (Vector Labs). 3-3' Di-Amino-Benzidine (DAB) chromogen concentrate (Shandon, Pittsburg, PA) and H_2O_2 in 0.05 M Tris HCl buffer (0.01% v/v) were used for 5 to 8 min to visualize immunoreactivity. The sections were washed in distilled water to stop the DAB reaction. For Western blot analysis, whole brains were obtained in the time frame parallel to those of the immuno-histochemical studies.

Lysis buffer comprising of 0.01 M Tris-HCL buffer containing 0.001 M phenylmethylsulphonyl fluoride, 0.15 M NaCl, 1% NP-40 and 0.005 M EDTA was used to homogenize the whole brain. The homogenate was rotated at 4°C for 1 h and centrifuged at 14,000 Xg for 5 min at 4°C. Bio-Rad Protein assay was used to collect aliquoted supernatant and determine protein concentration. The crude tissue extracts of different age points were classified to sodium dodecyl sulfate-15% polyacrylamide gel electrophoresis for western blots. A semi dry blotting machine was used to transfer a polyvinylidene difluoride membrane. The membrane was washed in TBS-T (0.1 M Tris buffered saline with 0.001% Tween 20) containing 0.2% casein for 1 h at RT for 3 times, 15 min each. The membranes were then incubated with either anti-aFGF or anti-bFGF (1:1000, Santa Cruz) overnight at 4°C. The membranes were then incubated in Horse Radish Peroxidase (HRP) conjugated donkey anti-rabbit IgG (1:8000, Santa Cruz) for 1 h at RT. Each incubation step was followed by three washes (15 min each) with TBS-T. Chemiluminescence detection was carried out with ECL (Lumi-Light plus, Roche, IN) for 10 min and developed by using imaging films (Kodak Biomax ML, NY).

Equal loading of proteins was confirmed by immunoblotting with anti-actin antibody (1:1000, Santa Cruz) and HRP-conjugated goat anti-rabbit IgG as secondary antibody (1:5000, Santa Cruz). Specificity of anti-FGF antibodies were determined by immune-blotting with anti-FGFs antibodies pre-absorbed by its' corresponding recombinant proteins (aFGF, Pepro Tech. Inc., London, UK; bFGF, R&D Systems, MN). No band was produced in the lane incubated with pre-absorbed antibody.

Results

Immunohistochemistry

Tiny glial cells with small perikaryal and stretched-out procedures were stained with the antibody to acidic FGF. The detection of basic FGF immunoreactivity was observed in the small to large-sized neurons throughout the CNS. It was represented in the form of cytoplasmic covering of the cell body dendrites and axons of the marked cells. In specific populations, aFGF immunoreactivity became detectable at significantly varied times of development. It was not detectable between the days 0 and 5 in mouse brain. In the diencephalon and brainstem, cellular populations became positive on day 15 and a constant adult level was accomplished between day 30 and 60 in positive sub-populations and though positive emerged first in the cerebral cortex on day 180 following the birth. However, bFGF immunoreactivity was constantly examined within the predefined time period. Transient pattern between aFGF or bFGF immunoreactivity was not portrayed by any of the cell populations. Variations were mainly detected in the cerebral cortex, substantia nigra, mammillary body, thalamus, hippocampus, locus coeruleus, and ventral tegmental area in the distribution pattern. The population in the subventricular zone were positive to aFGF, while the ependymal cells were positive to bFGF after the formation of ventricles walls. The postnatal appearance of FGFs in all the various regions in the central nervous system is presented in Figure 1.

Telencephalon

Intense bFGF staining occurred in numerous cells, while faint aFGF staining occurred only on 180th and 360th day in mouse brain. Similarly, the appearance of aFGF was first identified in the telencephalon on day 10, which included some subcortical regions such as SCh, MPA, and globus pallidus. The appearance of aFGF was first noted in a 120-days old mouse, but the appearance of bFGF occurred within the predefined time frame. On day 10, Rad, L Mol,

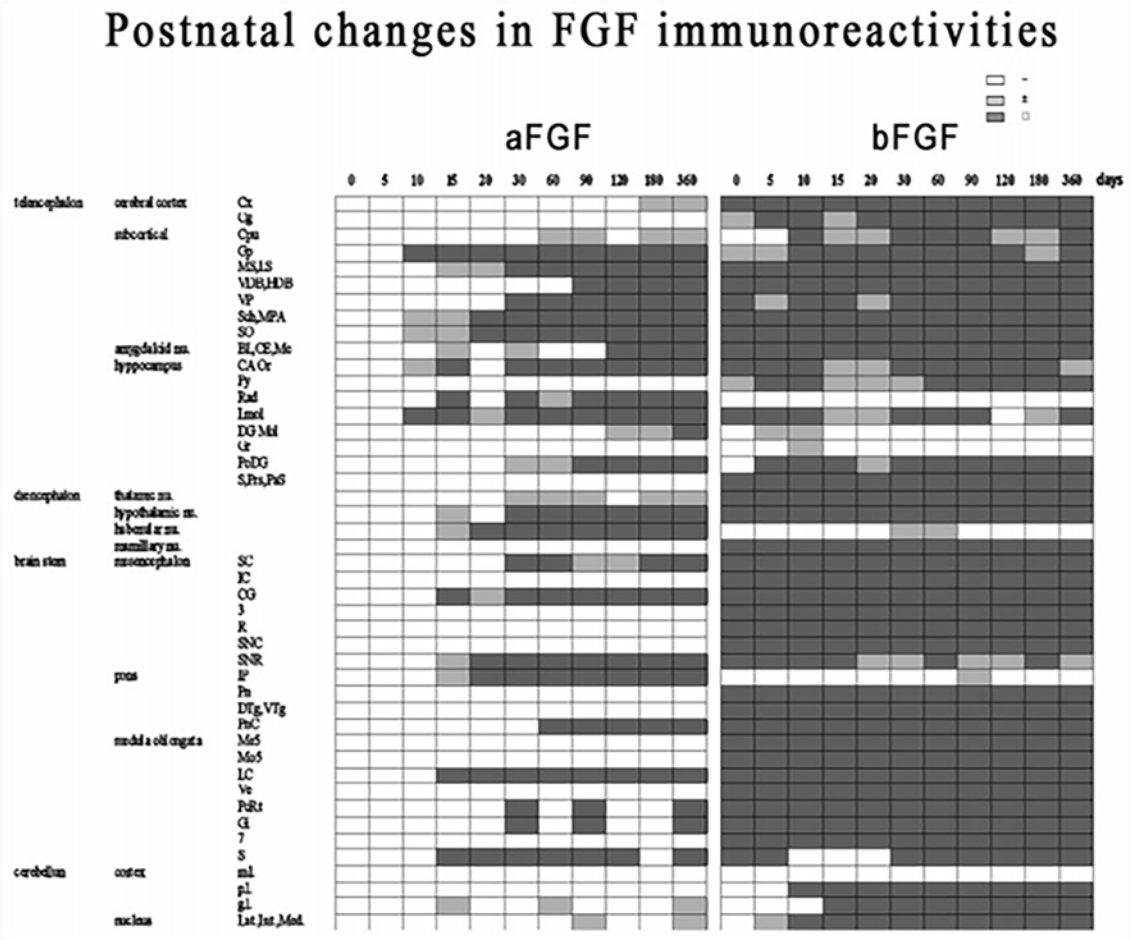

Figure 1: Schematic illustration of the postnatal appearance of FGFs in all the various regions in the central nervous system.

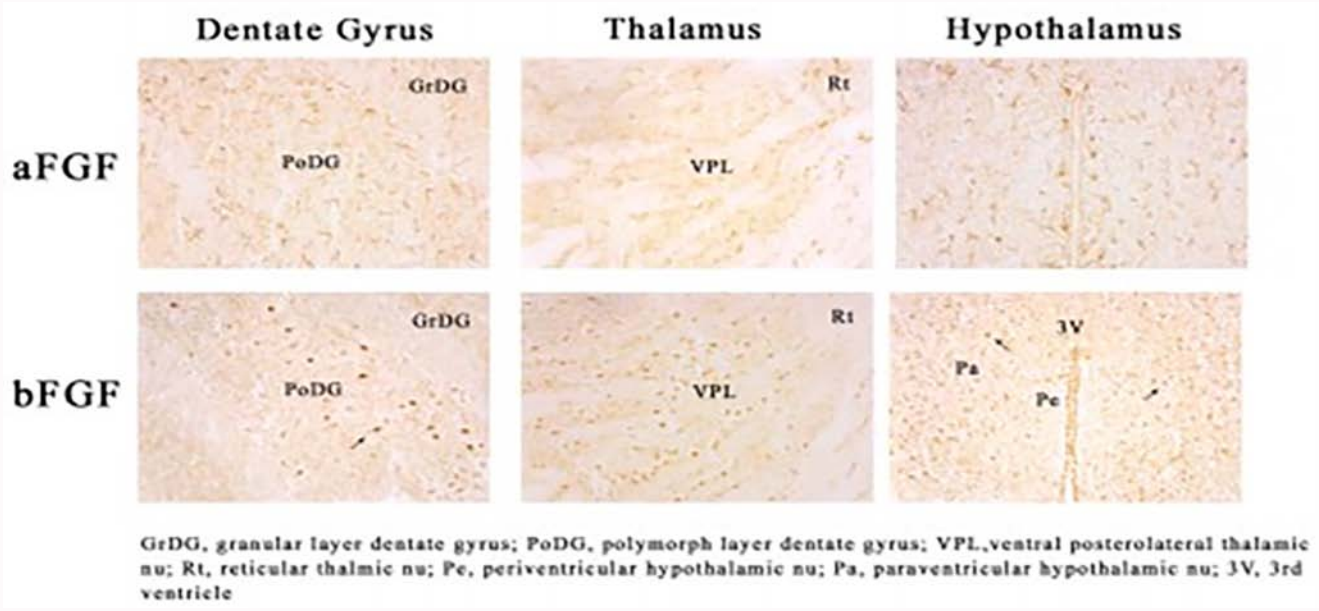

Figure 2: Different cellular distribution of aFGF and bFGF in the central nervous system in 180-day old mouse.

and CA Or were the initial groups of populations with positive aFGF staining. Afterwards, Gr and PoDG were detected positive to aFGF on day 30 and 120 respectively. aFGF immunoreactivity did not show any positivity with pyramidal cell layer, PrS, PaS and Granular layer. All these populations showed positivity from day 0 regardless of Rad, Granular layer, and DF Mol for bFGF immunostaining.

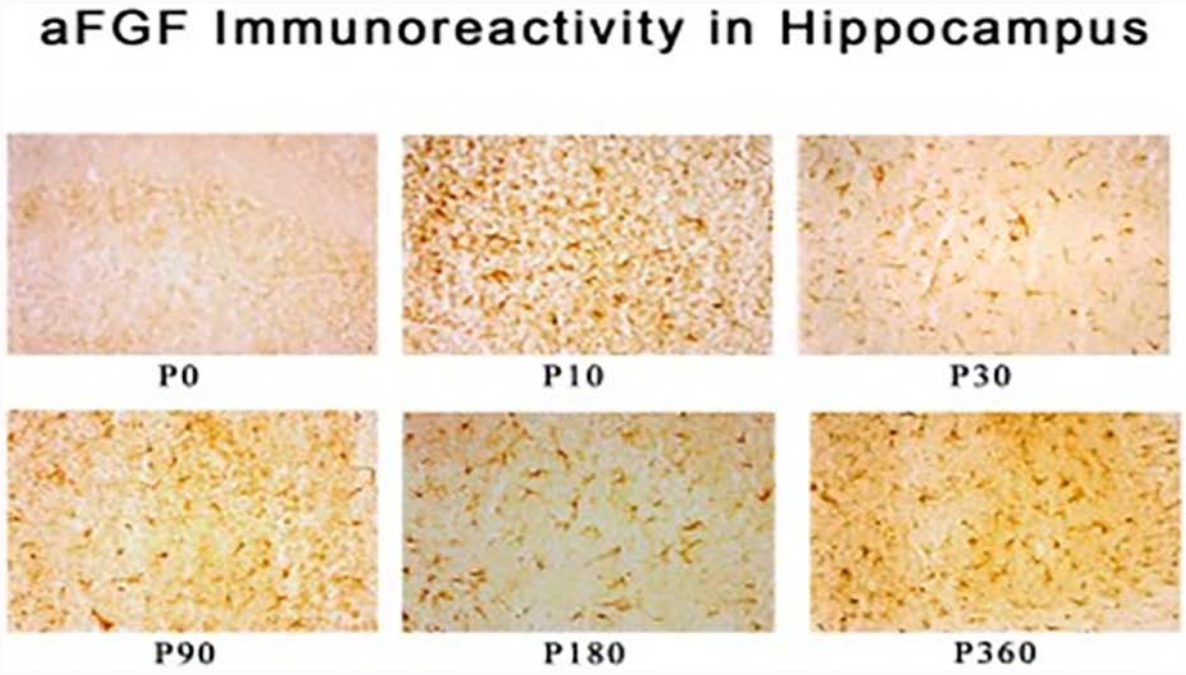

Figure 3: aFGF immunoreactivity in the hippocampus of mouse brain.

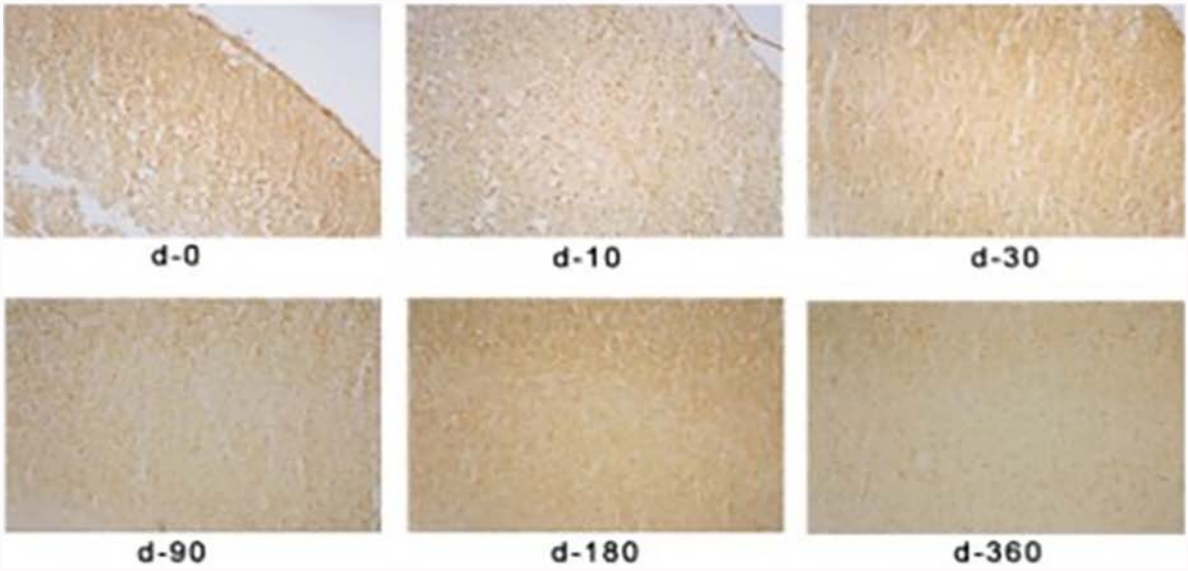

Figure 4: bFGF immunoreactivity in the cerebral cortex of mouse brain.

Subventricular zone

The ependymal cells showed positivity to bFGF, while astrocytes showed positivity to aFGF in the subventricular zone with the formation of ventricles walls. In the hypothalamic and habenular nucleon, aFGF immunoreactivity initially occurred on day 15 and then emerged in the thalamic nucleon on day 30. aFGF immunostaining was not detected in mamillary nucleus. However, the presence of bFGF immunoreactivity was detected in the hypothalamic, mamillary, and thalamic nuclei but it was not detected in the habenular nucleus (Figure 2).

Brain-stem

The presence of aFGF immunoreactivity was detected in the SNR and CG on day 15 and then it was detected in the SC on day 30 after birth. No aFGF immunoreactivity occurred in SNR cells (Figure 3). However, all the subpopulations in the mesencephalon confirmed the presence of bFGF immunoreactivity which included SNR and SNC (Figure 4). The presence of aFGF immunoreactivity was detected in the IP on day 15 and aFGF was positive in PN and PNC on day 60. VTg and DTg were not positively linked with aFGF. However, all these subpopulations irrespective of IP showed the confirmed presence of bFGF immunoreactivity. aFGF immunoreactivity was positive in S and LC from day 15 onwards. However, bFGF immunoreactivity was detected in all the subpopulations from day 0 onwards.

Cerebellum

All the subpopulations confirmed the presence of bFGF

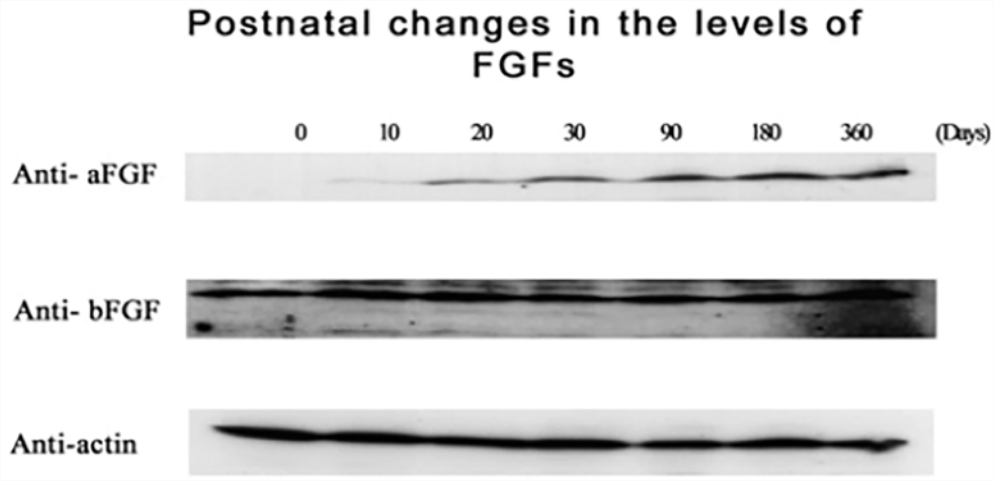

Figure 5: Western blot analysis showing different postnatal changes in the protein levels for the two FGFs in the mouse brain.

immunoreactivity but did not show the presence of aFGF immunoreactivity.

Western blot analysis

The immunohistochemical outcomes were supported by the findings of western blot analysis (Figure 5). By day 10, a band of almost 16.5 kD linking with aFGF could be detected and its extent could be maximum. However, a band of almost 22.5 kD linking with bFGF was constantly revealed within the predefined time period.

Discussion

bFGF immunoreactivity was detected in the neurons, while aFGF immunoreactivity was detected in the astrocytes. Neurons developed in the initial place throughout the development of CNS, while majority of the glial cells formed in a later stage. Astrocytes and oligodendrocytes generated and became mature in this later stage. Majority of the cells developed post-partum in the rodents throughout the first 10 days with maximum mitotic activity. Astrocytes controlled their mitotic activity and emerged as comparatively stable populations by the end of the second postnatal week. It has been observed that the time-course of the appearance of aFGF immunoreactivity was in accordance with the maturation and appearance time table of astrocytes [12,13].

A previous study revealed similar findings related to the time course of appearance of the FGFs. No detection of aFGF immunoreactivity was reported in day 0 tissues by western blotting and it was identified for the first time on day 14. The time points of their observation varied from the current study and were confined to 120 days old mouse only. A closer time interval reported was up to 1 year after birth. This might be the reason that aFGF immunoreactivity can be detected in the brain of a 10 days old mouse in the initial stage [14]. A previous study has reported the confirmation of bFGF in all dopaminergic neurons, while aFGF was confined to a subset of DA neurons. This discrete localization of aFGF was compared with bFGF and it was claimed that these two molecules might have different tropic roles [15].

Since the level of aFGF is very low, it does not respond throughout embryogenesis of the whole brain and its mRNA was unidentifiable throughout this stage. The most interesting phase is the increase in brain aFGF after birth, while taking into account the fact that some of the maturation steps occur in the brain development. The mature brain shows a plateau of aFGF. In recent times, the conventional view has been modified by identifying adult brain regions that harbor neural stem and their continual production of new neurons within life [16]. Thereby, this protein is assumed to have a regenerative role in maintaining neuronal survival and tissue homeostasis [17].

The primary neuronal precursors show astrocytes-like properties in adult mammals and are broadly disseminated in the adult CNS. The occurrence of adult neurogenesis is observed but in two particular brain regions: the hippocampal sub-granular zone and subventricular zone [18]. Astrocytes are also reported to be in conjunction with vasculature. Hence, they might offer an essential component of the cellular aspects that control adult neurogenesis in the dental gyrus. Neurogenesis can be actively regulated by astrocytes from postnatal hippocampus towards adult neural stem cells.

Conclusion

No general agreement has been identified regarding the distribution pattern of FGFs in the CNS; nevertheless, the findings reported here are in-line with only a few studies. In addition, a significant sequence homology has been exhibited by FGF family that comprises approximately 23 members. Therefore, different antibodies have been evolved in this experiment from previous experiments. Western blot analysis has been performed for confirming the immunohistochemical findings independently. Both techniques have shown better correlation with respect to the study outcomes.

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