

Original Article

The analysis and comparison between glia maturation factor beta and gamma mRNA and protein expression in rat brain and cells

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Abstract: Glia maturation factor, a 17-kDa protein made up of 142 amino acid residues, mainly includes glia maturation factor- β (GMFB) and glia maturation factor- γ (GMFG). GMFB plays an important role in the growth and differentiation in neuron as well as glia. GMFG, a novel member of the glia maturation factor gene family, locates in the blood and immune system retaining proliferative and differentiated potential. But to date, the distribution of expression and overall function of GMFG in brain remains elusive. For the first time, we proved the existence of GMFG in the central nervous system in the level of gene, protein and cell by Real-time PCR, Western blotting and Immunofluorescence. In astrocytes, GMFB expressed in all cells and mainly located in cell nucleus, while GMFG only expressed in a part of astrocytes and located in cell nucleus and cytoplasm presenting stellated. In neuron, GMFB located in the whole cell and it is very obviously in axon and dendritic, but the expression of GMFG in neurons is weak and couldn't be detected in axon and dendritic. In addition, we found their tissue region-response distribution and age-response expression in rat brain. In conclusion, we provided a comprehensive analysis that GMFG was expressed in brain and compared the cell/tissue distribution and expression of GMFB and GMFG.

Keywords: Glia maturation factor- β , glia maturation factor- γ , astrocyte, neuron

Introduction

Glia maturation factor, a 17-kDa protein which was made up of 142 amino acid residues, mainly includes GMFB and GMFG. GMFB was predominantly detected in the vertebrate brain and initially identified as a growth and differentiation factor acting on neurons as well as glia [1-4]. The gene and amino acid sequence of GMFG is highly homologous to GMFB (71% identity with the GMFB gene and 78.9% identity with the GMFB amino acid sequence in rat), so it was named glia maturation factor- γ . In contrast to the distribution of GMFB, GMFG was predominantly detected in thymus, testis and spleen, and to a lesser extent in brain [5].

In the brain, GMFB mRNA is detectable at as early as embryonic day 10 and persists through postnatal month 14 and GMFB located in brain with a weaker expression in the heart, placen-

ta, kidney and pancreas [1-4]. GMFB is thought to play an important role in the growth and differentiation in the mammalian brain. GMFB could stimulate axon regeneration in transected rat sciatic nerve [4]. The protein kinase A (PKA)-phosphorylated GMFB is a potent inhibitor of the extracellular-regulated kinase (ERK1/ERK2), a subfamily of mitogen-activated protein (MAP) kinase [6] and it is a strong enhancer of p38 MAP kinase [7]. In addition, PKA and protein kinase C (PKC) phosphorylated GMFB could promote the activity of PKA itself [8]. Besides, GMFB of T-cell promoted its differentiation into CD4-/CD8+ cells and T-cell development in thymoma may be maintained partly by GMFB produced by the tumor cells [9].

The expression distribution pattern of the GMFG differs from GMFB. The GMFG gene is highly expressed in blood (including myeloid leukemia and lymphoid leukemia cell lines), thy-

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mus, spleen, fetal liver and lung. GMFG protein coevolved with increasing complexity of the blood/immune system retaining proliferative and/or differentiated potential [5, 10]. GMFG gene in nervous/brain tissue is not higher than that in any other tissue; in rat brain, mRNA of GMFG was localized around pyramidal cells within CA3 of hippocampus [5]. Recent study found that GMFG, which was identified GMFG as an important G-CSF response protein in myeloid cell development, plays an important in chemoattractant-stimulated directional migration in neutrophils, and regulates the polarization underpinning the neutrophil chemotaxis response via stabilizing F-actin filaments in the lamellipodia [10, 11]. GMFG observed also in the inner limiting membrane from E14 to P1 rat retina may contribute to the development and growth of glia and neurons [12]. But to date, the distribution of expression and overall function of GMFG in brain remains elusive.

Previous study showed that anti-GMFG antibody didn't detect the bands in rat brain by Western blotting [5]. But it was conflicting with subsequent reports in which GMFG was detected in the inner limiting membrane from E14 to P1 rat retinas defined central nervous system (CNS) model system [12]. That led us to examine whether GMFG protein is expressed in rat brain. Besides, we made an analysis of the cell/tissue distribution of expression between GMEB and GMFG and a preliminary study of GMFG function in the astrocyte.

Methods and materials

Ethics statement

This study was performed in strict accordance with the guidelines of caring for laboratory animals of the Ministry of Science and Technology of the People's Republic of China. All animal procedures were approved by the Committee on the Ethics of Animal Experiments of Southern Medical University. Isoflurane (Mylan, USA) was used for animal euthanasia, and every effort were made to minimize suffering.

Materials

Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from GIBCO (Carlsbad, California, USA). Mouse anti-GPADH antibody (Lot#ab37168), mouse anti-GFAP monoclonal antibody (Lot#ab10062),

rabbit polyclonal anti-GMFB antibody (Lot#-ab96694) and rabbit polyclonal anti-GMFG (Lot#ab94854) antibody were from Abcam (Cambridge, UK). Dylight 594-conjugated Affinipure Goat Anti-Rabbit IgG (Lot#94082) and Dylight 488-conjugated Affinipure goat anti-mouse IgG (Lot#82803) were obtained from Jackson Immuno Research (West Grove, Pennsylvania, USA). Goat anti-rabbit IgG IR dye 800cw (Lot#C30626-03) and goat anti-mouse IgG IR Dye 800cw (Lot#C40528-02) were from Odyssey (Licor, St Englewood, New Jersey, USA). RNAiso Plus, SYBR[®] Premix Ex Taq[™] II Kit (Perfect Real Time) and PrimeScript RT-PCR Reagent Kit (Perfect Real Time) were from TaKaRa (Da Lian, China).

Cell culture

Primary astrocytes culture: Primary culture of astrocytes were prepared from cortex of 1-day-old Sprague-Dawley rats (Laboratory Animal Center, SMU, Guangzhou, China), following a process described previously [13]. Briefly, the cerebral hemisphere was freed of the meninges and cut into small cubes (1 mm³) in DMEM. The tissue was dissociated by vortex mixing for 1.5 min, and the cell suspension was passed through 70 mm and 20 mm sterile mesh nylon filters. A volume of cell suspension containing about 4.5×10^5 cells was seeded in a 35 mm Falcon tissue culture dish. Fresh DMEM supplemented with 10% fetal calf serum was added to yield a final volume of 2 mL. Cells were cultured at 37°C in a 5% CO₂ humidified atmosphere inside a CO₂ incubator. The medium was changed twice weekly. The astrocytes which were cultured at least 4 weeks old were used for experiments.

Primary neurons culture: Cerebral cortical cultures were prepared as previously described [14] from 16-day Sprague-Dawley rats (Laboratory Animal Center, SMU, Guangzhou, China) embryos in MEM medium containing 2 mM glutamine, 10% FBS, and 1% penicillin and streptomycin. On DIV (days in vitro) 2 μM cytosine arabinoside was added for 24 h to prevent glial proliferation. The neurons were subsequently maintained in serum-free neurobasal medium (Invitrogen) containing 2% B27 supplement and 2 mM glutamine at 37°C in a humidified 5% CO₂ incubator, and were given fresh media every 3-4 days. Experiments were conducted on DIV 9-10 by exchanging the culture

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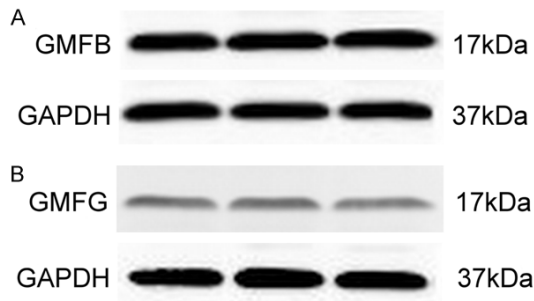


Figure 1. The expression of GMFB and GMFG in astrocytes. A: The detection of GMFB in SD rat astrocytes by Western blotting. B: The detection of GMFG in SD rat astrocytes by Western blotting.

medium with Hank's balanced salt solution (HBSS).

Western blotting

Cells were harvested and homogenized in a SDS sample buffer (25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.1% SDS, 1 mM each of PMSF, NaF, NaVO₃, and 1 µg/mL each of leupeptin, pepstatin, and aprotinin). The concentration of proteins was determined by the Bradford method with BSA as standard. The 40 µg of protein per lane were separated by 10% polyacrylamide SDS gel electrophoresis. The antibodies such as GMFB, GMFG and GAPDH were first incubated with membranes for 2 h at room temperature respectively. Goat anti-Rabbit and Mouse IgG IR Dye 800cw (1:15,000) respectively was added to incubate for 1 h at room temperature, then imaged by Odyssey analysis system. The pictures were quantified by Gel-Pro analyzer (Alpha Innotech Corp, CA), and protein levels were expressed as the relative values of the controls.

Immunofluorescence

Astrocytes and neurons were labeled by fluorescence immunostaining for GFAP and GMFB/GMFG. Cultures were fixed for 1 h with 4% paraformaldehyde in phosphate buffered saline (PBS) at room temperature. After several washes with PBS, blockade of non-specific binding was performed with goat serum for 1 h, and afterwards cells were incubated at room temperature with a mouse polyclonal anti-GFAP antibody (1/100) and rabbit polyclonal anti-GMFB/GMFG antibody (1/100) in 1% BSA-PBS for 2 h. After several washing, cells were incubated for 1h with Alexa 488 anti-mouse IgG

and Alexa 594 anti-rabbit IgG diluted 1:100 in 1%BSA-PBS. The primary antibody was omitted in controls. And then the photos were taken with confocal images. Proceed to imaging and analysis.

RNA Isolation and real-time PCR

Total RNA from fresh normal astrocyte and brain tissues sample were isolated by using RNAiso Plus reagent according to the manufacturer's instructions. The PrimeScript RT-PCR Reagent Kit (Perfect Real Time) along with 500 ng of total RNA and poly-dT primers were used for synthesis of cDNA. To determine the RNA transcript levels from cDNA, Real-time PCR was carried out using a ABI7500 instrument and SYBR® Premix Ex Taq™ II kit (Perfect Real Time) as described previously to detect the mRNA level of GMFB and GMFG. The sense primer sequence of GMFB was 5'-CCCTCTGTGCTTTA-TCTTCTCC-3', and for antisense primer was 5'-CTTCAGTTAGGTCTTCGGTGTT-3'. The sense primer sequence of GMFG was 5'-AGATGATGT-ACGCGGGAAGT-3', and for antisense primer was 5'-TCGTTGAGGTCGTCTGTGGT-3'. GAPDH gene was used as an internal control using the sense primer 5'-GGCACAGTCAAGGCTGAGAA-TG-3' and antisense primer 5'-ATGGTGGTG-AAGACGCCAGTA-3'. PCR cycling conditions were 95°C for 10 min to activate DNA polymerase, followed by 40 cycles of 95°C for 5 s, and 62°C for 34 s. Specificity of amplification products was confirmed by melting curve analysis. Independent experiments were done in triplicate.

Statistical analysis

Experiments were always reproduced a minimum of three times. Quantifiable determinations are expressed as means ± SEM of the indicated number of experiments performed in independent cultures. Significance of differences was evaluated by one-way ANOVA followed by the Student-Newman-Keuls post-hoc test when more than two conditions were evaluated. Significant changes are indicated as follows: *P<0.05, **P<0.01.

Results

Expression of GMFB and GMFG in the central nervous system of rat

Astrocyte, one of the most numerous cell types in CNS, is considered to be very important for

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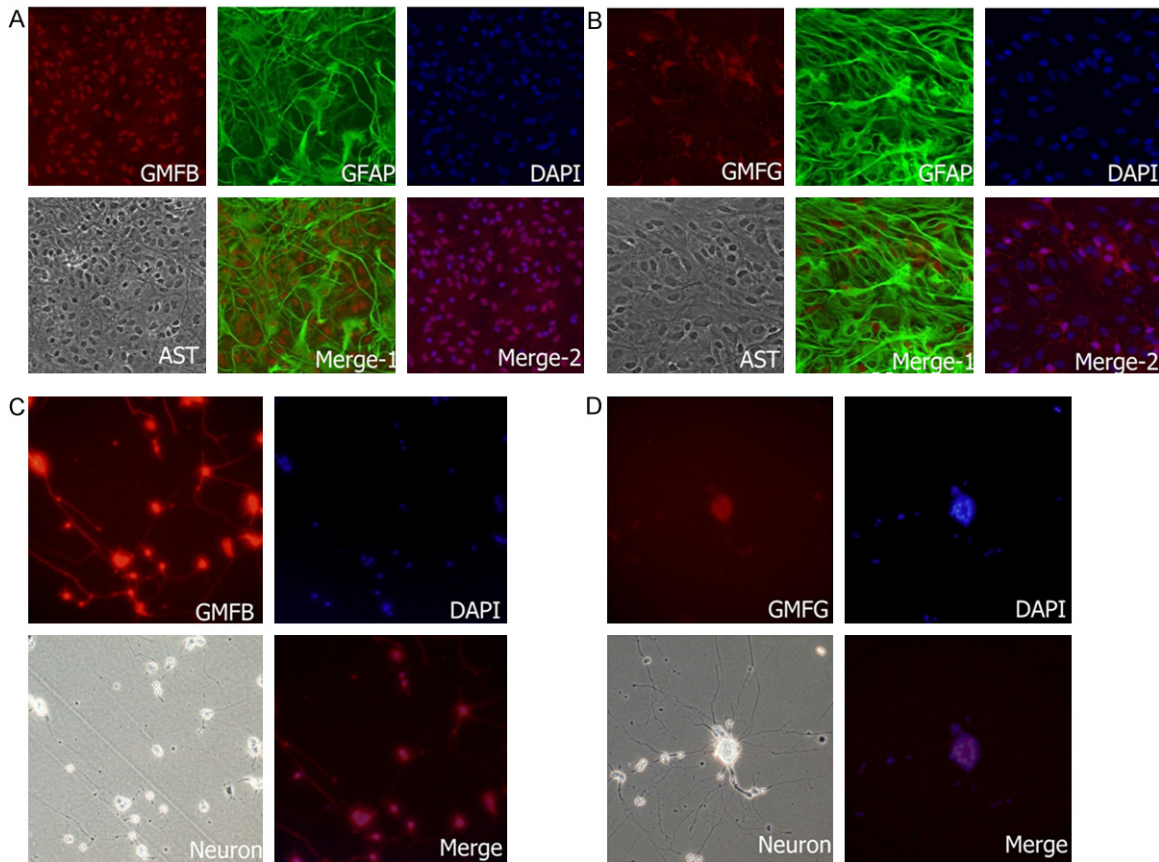


Figure 2. Characterization of expression of GMFB and GMFG in astrocytes and neurons. A: Immunofluorescence staining for GMFB (red) and GFAP (green) in SD rat astrocytes. Cell nuclei are counterstained with DAPI (blue). (Magnification: 200 ×). B: Immunofluorescence staining for GMFG (red) and GFAP in SD rat astrocytes. Cell nuclei are counterstained with DAPI (Magnification: 200 ×). C and D: Immunofluorescence staining for GMFB (red) and GMFG (red) SD rat neurons. Cell nuclei are counterstained with DAPI (Magnification: 100 ×). The results shown represent three individual experiments.

maintaining an environment in which neurons, other glial cell types and the brain endothelium function and interact properly [15], so we chose astrocyte to detect presence of GMFG. GMFB mRNA (its CT value is 19.4 when CT value of internal control GAPDH is 16.6) were expressed more strongly than those of GMFG mRNA (its CT value is 22.1 when CT value of internal control GAPDH is 16.6) in astrocytes. Western blotting analysis of the extract of astrocytes revealed the presence of GMFG with a molecular weight of 17 kDa similar to that of rat GMFB (Figure 1A and 1B). By Immunofluorescence we found that GMFB expressed in all astrocytes and mainly located in cell nucleus of astrocytes, while GMFG only expressed in a part of astrocytes and located in cell nucleus and cytoplasm where expression of GMFG present stellated (Figure 2A and 2B). In addition, we also

detected presence of GMFB in neurons where GMFB located in the whole cell and it is very obviously in axon and dendritic, but the expression of GMFG in neurons is weak and we couldn't detect it in axon and dendritic (Figure 2C and 2D).

The tissue region-response distribution of GMEB and GMFG in rat brain

To examine the expression of GMFB and GMFG proteins in brain of rat we used polyclonal anti-rat GMFB and GMFG antibodies by Immunohistochemistry Staining. Figure 3 showed that each specific antibody recognizes the corresponding protein. The specific anti-GMFB antibody visualized the bands in the whole brain and predominantly in pyramidal cells of hippocamp. In positive cases, the tumor cells, regardless of morphology, showed diffuse cytoplas-

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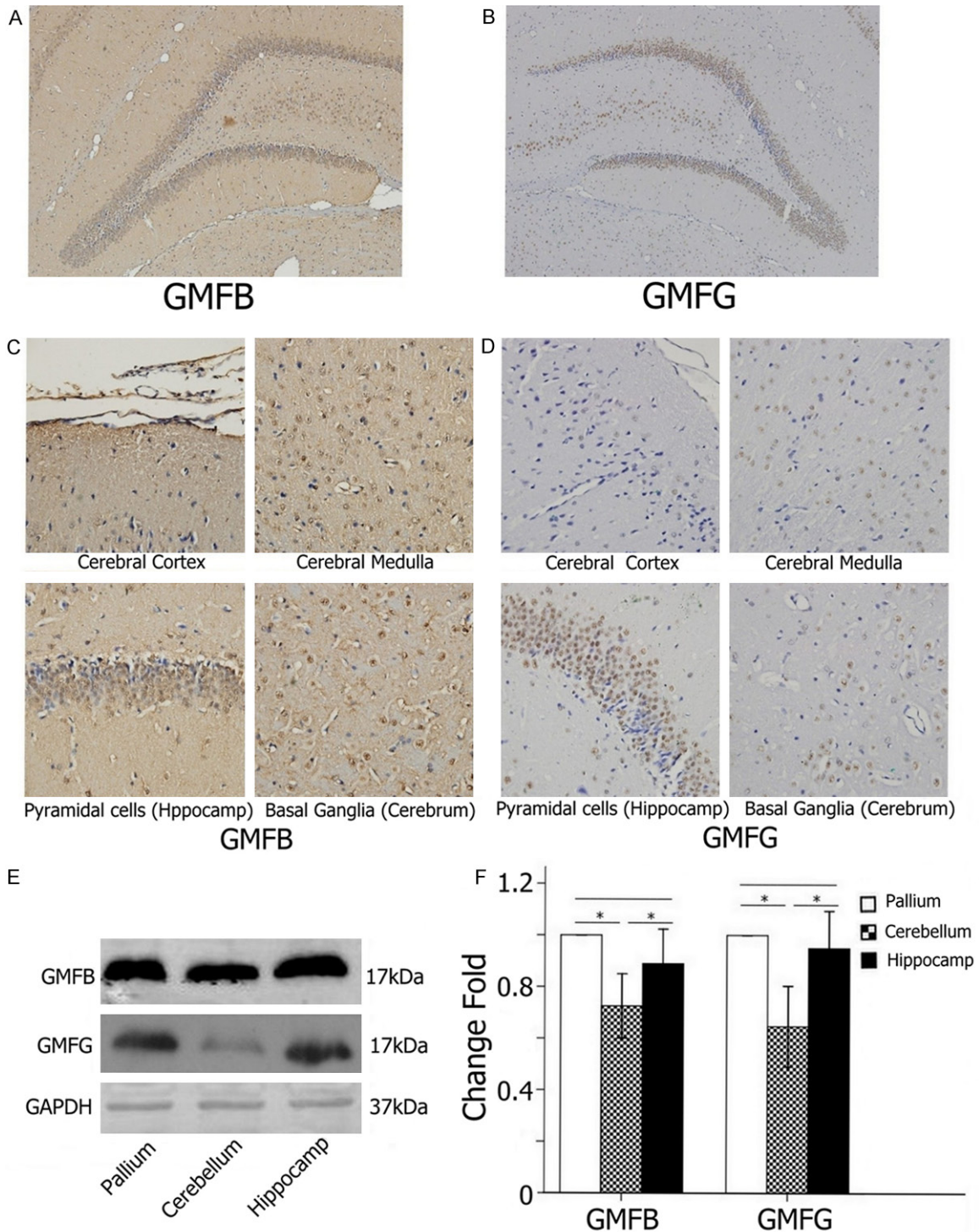


Figure 3. Characterization of the distribution of GMFB and GMFG in SD rat brain. A: Immunohistochemistry Staining for the distribution of GMFB in SD rat brain. Cell nuclei are counterstained with hematoxylin (blue) (Magnification: 40 ×). B: Immunohistochemistry Staining for the distribution of GMFG in SD rat brain. Cell nuclei are counterstained with hematoxylin (blue) (Magnification: 40 ×). C: Immunohistochemistry Staining for the distribution GMFB in SD rat brain. Cell nuclei are counterstained with hematoxylin (blue) (Magnification: 400 ×). D: Immunohistochemistry Staining for the distribution GMFG in SD rat brain. Cell nuclei are counterstained with hematoxylin (blue) (Magnification: 400 ×). E: The detection of GMFB and GMFG in SD rat brain by Western blotting. F: Quantification of protein GMFB and GMFG in the three groups including cerebral cortex, cerebellum and hippocamp. The results shown represent three individual experiments. The relative abundance of each band to its own control GAPDH band was estimated by densitometric scanning of the exposed films. The data is expressed as the means ± SEM of n=3 experiments. *P<0.05, One-way ANOVA analysis followed by Newman-Keuls post-hoc test.

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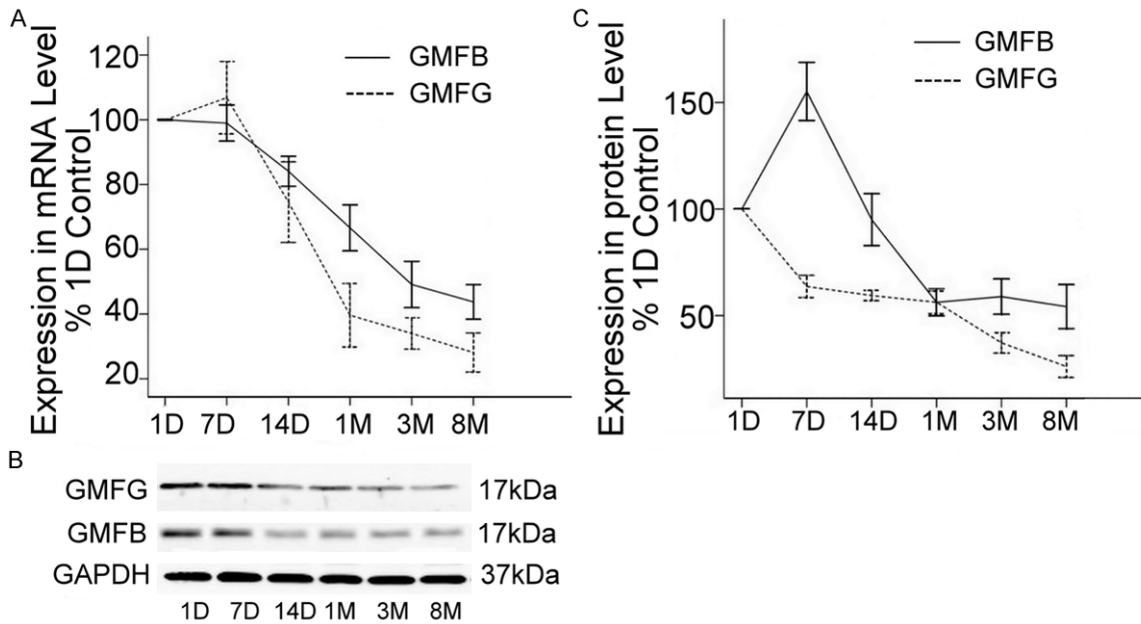


Figure 4. Characterization of age-responses expression of GMFB and GMFG. A: Quantitative PCR analysis quantifying the change in expression of GMFB and GMFG gene in different ages (postnatal day 1, day 7, day 14, month 1, month 3, month 8). Each data represents the mean \pm SD ($n = 3$). B: Western blot analysis the change in expression of GMFB and GMFG protein in different ages (postnatal day 1, day 7, day 14, month 1, month 3, month 8). C: Quantitative western blot analysis quantifying the change in expression of GMFB and GMFG protein in different ages. Each data represents the mean \pm SD ($n=3$). The data shown represent three individual experiments. The relative abundance of each band to its own control GAPDH band was estimated by densitometric scanning of the exposed films. The data is expressed as the means \pm SEM of $n=3$ experiments. $**P<0.01$, One-way ANOVA analysis followed by Newman-Keuls post-hoc test.

mic immune reactivity for GMFB; GMFB was positive in the cytoplasm and nuclei of all cells in whole brain (**Figure 3A** and **3C**). In contrast, the specific anti-GMFG antibody wasn't detected in the whole brain, GMFG was positive only in a part of astrocytes; GMFG is predominantly detected in pyramidal cells of hippocamp and to a lesser extent in cerebral medulla and basal ganglia (cerebrum), cerebral cortex is the weakest (**Figure 3B** and **3D**). Besides, by western blotting we examine the expression of GMFB and GMFG proteins in cerebellum, cerebral cortex and hippocamp. Anti-GMFB antibody and anti-GMFG antibody detected the bands very weakly in cerebellum compared to cerebral cortex and hippocamp. There was no significantly difference between cerebral cortex and hippocamp (**Figure 3E** and **3F**).

The age-responses expression of GMEB and GMFG in rat brain

We analyzed levels of GMF mRNAs in rat brain cortex from D1, D7, D14, M1, M3, and M8 after birth by real-time PCR. The expression of GMFB

mRNA was greater than GMFG mRNA in all the samples. The level of GMFB mRNA reached a peak at D7 and thereafter becomes obviously reduced; while GMFG mRNA persisted in reducing until older (**Figure 4A**). By western blotting analysis, GMFB were detected in rat brain every age phase after birth and persisted until older; GMFB protein reached the peak in the seventh days after birth with decreasing after that and plateauing from the second week to the eighth month after birth; while GMFG protein persisted in reducing from the first day to the eighth month after birth (**Figure 4B** and **4C**).

Discussion

For the first time, we proved the existence of GMFG in the central nervous system at the level of gene, protein and cell in this study. And then we compared the tissue distribution of expression between GMEB and GMFG in rat astrocyte and neuron and found their tissue region-response distribution and age-response expression in rat brain.

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Previous study showed that anti-GMFG antibody didn't detect the bands in rat brain by Western blotting, while its mRNAs were detected around pyramidal cells within CA3 of hippocampus by in situ hybridization [5]. But it is conflicting with subsequent reports in which GMFG was detected in the inner limiting membrane from E14 to P1 rat retinas defined CNS model system that comprises seven major classes of cells, ganglion cells, bipolar cells, amacrine cells, horizontal cells, a couple of photoreceptor cells, and Muller cells [12]. Firstly, we proved presence of GMFG in rat astrocyte in the level of gene, protein and cell. Meanwhile, our study also explains reason why former study didn't detect the GMFG protein in rat brain. The reasons maybe that (i) The distribution of GMFG in rat brain has the characteristics of tissue region-response (pyramidal cells of hippocampus, cerebral medulla, basal ganglia and cerebral cortex). (ii) The expression of GMFG in rat brain has the characteristics of age-responses (post-natal day 1, day 7, day 14, month 1, month 3, month 8). (iii) The expression of GMFG protein in brain was more weakly than those of GMFB or internal control GAPDH; it would take five or more minutes to visualize its band in western blotting (50 ng protein samples of astrocytes per lane).

GMFB mainly locating in brain, plays an important role in the growth, differentiation and immune response, such as stimulating axon regeneration in transected rat sciatic nerve, promoting secretion of neurotrophic factors in primary astrocytes such as BDNF and NGF, stimulating microglia to express major histocompatibility complex (MHC) II by granulocyte macrophage-colony stimulating factor (CSF) [1-4, 6, 16, 17]. By western blotting analysis, we found GMFB protein reached the peak in the seventh days after birth with decreasing after that and plateauing from the second week to the eighth month after birth; that was consistent with previous research results which show that GMFB protein exhibited more obvious developmental changes, with its level increasing slowly prenatally and plateauing at 1 week after birth [18]. Compared with GMFB locating in cell nucleus and cytoplasm where expression of GMFG presents stellated consistent with the morphology of astrocyte. In addition, we also detected presence of GMFB in neurons where GMFB located in the whole cell and it is very obviously in axon and dendritic, but the

expression of GMFG in neurons is weak and we couldn't detect it in axon and dendritic. GMFG protein has a high level of structural similarity with GMFB protein [5]. However, the two proteins showed clear differences in tissue distribution and function. The previous research showed that GMFG has a high level of structural similarity with members of the actin depolymerisation factor (ADF) domain containing family suggesting that it might play a role in there modeling of the actin cytoskeleton [19]. Several groups have shown that GMFG interact with purified Arp2/3 complex and interfere with Arp2/3 induced daughter filament growth [20, 21]. The latest studies have demonstrated that GMFG, a component of human T cell pseudopodia required for migration, influences the regulation of integrin mediated adhesion [16] and played an important role in regulating the polarization underpinning the neutrophil chemotaxis response via stabilizing F-actin filaments in the lamellipodia [10, 11].

In conclusion, we provided the first report that GMFG was expressed in brain and compared the cell/tissue distribution and expression of GMFB and GMFG. Our findings shed light on a mechanism of GMFB and GMFG in the pathogenesis of astrocytomas.

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Disclosure of conflict of interest

None.

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