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Identification and characterization of a MBP isoform specific to hypothalamus in orange-spotted grouper (*Epinephelus coioides*)

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Abstract

Myelin basic protein (MBP), as a major component of the myelin sheath, has been revealed to play an important role in forming and maintaining myelin structure in vertebrate nervous system. In teleost, hypothalamus is an instinctive brain center and plays significant roles in many physiological functions, such as energy metabolism, growth, reproduction, and stress response. In comparison with other MBP identified in vertebrates, a smallest MBP is cloned and identified from the orange-spotted grouper hypothalamic cDNA plasmid library in this study. RT-PCR analysis and Western blot detection indicate that the *Ec*MBP is specific to hypothalamus, and expresses mainly in the tuberal hypothalamus in adult grouper. Immunofluorescence localization suggests that *Ec*MBP should be expressed by oligodendrocytes, and the expressing cells should be concentrated in hypothalamus and the area surrounding hypothalamus, such as NPOpc, VC, DP, NLTm, and NDLI. The studies on *Ec*MBP expression pattern and developmental behaviour in the brains of grouper embryos and larvae reveal that the *Ec*MBP-expressing cells are only limited in a defined set of cells on the border of hypothalamus, and suggest that the *Ec*MBP-expressing cells might be a subpopulation of oligodendrocyte progenitor cells. This study not only identifies a smallest MBP isoform specific to hypothalamus that can be used as a molecular marker of oligodendrocytes in fish, but also provides new insights for MBP evolution and cellular distribution.

Keywords: Myelin basic protein; Orange-spotted grouper; Hypothalamus; Oligodendrocyte

1. Introduction

Myelination is a complex biological process in the vertebrate central nervous system (CNS) where oligodendrocytes (OL) extend their plasma membranes that enfold the axons (Asipu et al., 2001). It is involved in migration, proliferation, and differentiation of oligodendrocytes progenitor cells and complex interactions between the processes of mature oligodendrocytes and the axonal tracts of neurons (Baumann and Pham-Dinh, 2001). The myelin sheaths wrap around the axon of selective neurons in central and peripheral nervous systems and provide an electrical insulating pathway for rapidly transmitting information (Huang et al., 2002). Myelin basic protein (MBP), as a major component of the myelin sheath, has been revealed to play an important role in forming and maintaining the myelin structure by attracting oligodendrocytic membranes via protein-lipid interactions (Asipu and Blair, 1997), but knowledge of how myelination is regulated or how MBP-expressing cells interact with neurons is still incomplete (BrÖsamle and Halpern, 2002).

MBP exists as several different isoforms that result from alternative splicing of the primary mRNA transcripts (de Ferra et al., 1985; Kamholz et al., 1986, 1987, 1988; Streicher and Stoffel, 1989; Asipu and Blair, 1997; Givogri et al., 2000), and multiple isoforms have been cloned and identified from human and mouse (Harauz et al., 2004). In fish, several MBP cDNAs were reported (Spivack et al., 1993; BrÖsamle and Halpern,

Abbreviations: DP, dorsal posterior thalamic nucleus; CNS, central nervous system; *Ec*MBP, *Epinephelus coioides* myelin basic protein; IL, inferior lobe; MBP, myelin basic protein; NDLI, diffuse nucleus of inferior lobe; NLTv, medial part of the lateral tuberal nucleus; NPOpc, parvocellular part of the parvocellular preoptic nucleus; RT-PCR, reverse transcription-polymerase chain reaction; nt, nucleotide; OL, oligodendrocytes; OPCs, oligodendrocyte progenitor cells; ORF, open reading frame; P, pituitary; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate saline buffer; PI, propidium iodide; PLP, proteolipid; SDS, sodium dodecyl sulfate; SMART, switch mechanism at the 5′ end of RNA templates; SSC, standard saline citrate; SV, saccus vasculosus; TH, tuberal (medline) hypothalamus; TBS, Tris-buffered saline; UTR, untranslated region; VC, central part of the ventral telencephalon

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2002). In elasmobranches, two MBP cDNAs, encoding 155 amino acids and 141 amino acids, were cloned from dogfish (*Squalus acanthias*) and skate (*Raja erinacia*) (Spivack et al., 1993). In teleost, a MBP cDNA, encoding 88 amino acids, was cloned from zebrafish (BrÖsamle and Halpern, 2002). In comparison with higher conservation among mammalian MBPs, fish MBPs were found to have higher sequence divergence (BrÖsamle and Halpern, 2002). The lower conservation might imply that the myelination in fish and other anamniotes might be different from that in mammals, and related to the evolutionary issues. Thus, further molecular identification and studies on cellular localization and developmental behaviour remain to be performed in fish and lower vertebrates. It will contribute significant insights into evolutionary and functional aspects of myelination.

Groupers of the genus *Epinephelus* are widely distributed throughout the tropical and subtropical waters of the world and regarded as favourite marine food fish (Yeh et al., 2003). As being protogynous hermaphrodites (Chen et al., 1977; Sadovy and Colin, 1995), the orange-spotted grouper (*Epinephelus coioides*) has been considered as good model for development and reproduction (Yao et al., 2003; Wang et al., 2004; Li et al., 2005). Recently, we have constructed a SMART cDNA plasmid library from the orange-spotted grouper pituitary and gonads, and initiated a series of molecular studies (Yao et al., 2003, 2007; Jia et al., 2004a,b; Wang et al., 2004; Li et al., 2005; Zhou et al., 2005; Ji et al., 2006; Xia et al., 2007).

Hypothalamus–pituitary-gonad is an important endocrine axis to regulate reproduction and sex differentiation. Also, teleost hypothalamus is an instinctive brain center and plays significant roles in many physiological functions, such as energy metabolism (Yu et al., 2000), growth, reproduction (Foran and Bass, 1999), and stress response (Wingfield and Sapolsky, 2003). Therefore, the identification of genes specific to hypothalamus might provide some useful sights for hypothalamic physiological functions. Recently, we performed the studies on identification of genes expressed in the hypothalamus of male orange-spotted grouper using EST and RT-PCR strategy. A total of 1006 ESTs were sequenced and analyzed. Moreover, the expression patterns of 26 unknown genes were analyzed in various tissues (Zhou et al., 2006). Interestingly, a smallest MBP full-length cDNA (named *Ec*MBP) was identified. RT-PCR analysis showed *Ec*MBP is expressed predominantly in hypothalamus, which suggested *Ec*MBP might play especial physiological role in grouper. So, in this study, we analyzed the specific MBPs molecular characterization, described its expression pattern in brains of adult and developing embryos and larvae, and revealed its developmental behaviour.

2. Materials and methods

2.1. Sequencing analysis

By screening 1006 clones of the male orange-spotted grouper hypothalamic cDNA plasmid library, four clones showed a high similarity with the MBP identified from zebrafish (BrÖsamle and Halpern, 2002). The glycosylation site and phosphorylation site were predicted by softwares at the ExPASy Molecular Biology Server (http://expasy.pku.edu.cn). All sequences were searched for similarity using BLAST at web servers of the National Center of Biotechnology Information (Altschul et al., 1997). Multiple sequence alignment was performed using the CLUSTALW 1.8 program and phylogenetic tree was constructed (Thompson et al., 1994). Antigenic peptides were determined using the method of Kolaskar and Tongaonkar (1990).

2.2. Tissue distribution by RT-PCR

The total RNAs of liver, kidney, spleen, fat, heart, muscle, pituitary, hypothalamus, telencephalon, cerebellum, midbrain, medulla oblongata, ovary and testis were isolated from orange-spotted grouper using SV Total RNA Isolation System (Promega), respectively, and then were reverse-transcribed with M-MLV Reverse Transcriptase (Promega) and oligo(dT)8–12 (Promega, Madison, USA) as described by the manufacturer. Total volume of each reaction is 25 μl containing 2 μl of the isolated RNAs, 0.5 mM dNTP, 0.5 μM each primers, 200 units M-MLV RT, and 25 units of rRNasin® Ribonuclease Inhibitor with 1× M-MLV buffer (10 mM Tris–HCl, 25 mM KCl, pH 8.3, 0.6 mM $MgCl_2$ and 2 nM DTT). The reaction mixtures were incubated at 37 °C for 1 h.

All of the resultant cDNAs were respectively diluted 1:10 to use as templates for PCR with Taq DNA polymerase (MBI, Fermentas). One pair of primers (*Ec*MBP-F: GAATTCATATGGCTACGGCGAGCACCTCAG and *Ec*MBP-R: GCTCGAGTTATGAAGCAAAGCTCCTG) were synthesized (Sangon, Shanghai) and used to identify tissue distribution. Amplification reactions were performed in volume of 25 μl containing 1 μl cDNA as template DNA, 0.2 μM each primer, 0.5 units Taq polymerase (MBI, Fermentas), 0.1 μM of each dNTP, 1× buffer for Taq polymerase (MBI, Fermentas). Each PCR cycle included denaturation at 95 °C for 40 s, annealing at 60 °C for 50 s, and extension at 72 °C for 50 s. Thirty cycles were performed, followed by a final extension at 72 °C for 5 min. As a positive control for the RT-PCR analysis, α-tubulin (tubulin-F: GTGCACTGGTCTTCAGGGGTT and tubulin-R: GGGAAGTGGATGCGTGGGTAT) was amplified to determine the template concentration and to provide a semi-quantitative external control for PCR reaction efficiency under the same reaction conditions as *Ec*MBP.

2.3. Production of fusion proteins and the polyclonal antibody

The *Ec*MBP cDNAs coding mature protein were amplified used primers (*Ec*MBP-F: CGAATTCATATGGCTACGGCGAGCACCTCAG and *Ec*MBP-R: GGCTCGAGTTATGAAGCAAAGCTCCTG) and double-digested with *Eco*RI and *Xho*I. The digested cDNAs were inserted in frame to the *Eco*RI and *Xho*II double-digested expression vector pET32a (+)(Novagen), and transformed into the expression cell, *E. coli* BL21 (DE3). Protein expression was induced with IPTG (final concentration 1 mM), and the bacterial cells were harvested respectively by centrifugation after culturing for 4 h in a medium containing 50 μg/ml of ampicillin at 37 °C. The fusion protein Trx-*Ec*MBP was purified by His-binding Kit (Novagen) and used to immunize rabbit as normal method.

2.4. Western blot analysis

The specificity of polyclonal antibody was evaluated by Western blot analysis. The protein extracts prepared from hypothalamus and tuberal hypothalamus were subjected to Western blot analysis. SDS-PAGE was carried out using a 15.5% gel following the procedure described by Schagger and von Jagow (1987) to separate low molecular weight proteins. After they separated on SDS-PAGE gel, the proteins were electrophoretically blotted to PVDF membranes. After blocking with 5% dry milk in TS buffer [100 mM NaCl, 100 mM Tris–HCl, pH7.5], the membranes were incubated with rabbit antisera against *Ec*MBP at a dilution of 1:500 in TS buffer containing 1.0% dry milk and 0.05% Tween 20 at room temperature for 1 h. The membranes were washed three times for 15 min each in TS buffer and then incubated with 1:2000 diluted alkaline phosphatase conjugated goat anti-rabbit IgG. After washing three times for 15 min each in TS buffer, detection was performed using BCIP/NBT.

Two controls were designed to determine polyclonal antibody specificity. In one control the *Ec*MBP antisera were replaced with pre-immune serum respectively. In another control the *Ec*MBP antisera was replaced by antisera

pre-adsorbed with pure antigen for 16 h at 4 °C. The preadsorption was carried as described by Xia et al. (2007). In brief, the optimal ration of purified *E. coli* expressed antigen to anti-*Ec*MBP antiserum was determined by serial array of dilution. Incubate 0.1% diluted anti-*Ec*MBP antiserum with 20 μg/ml purified fusion protein at 4 °C for 16 h in 0.5% nonfat milk powder/TBST solution with mild agitation. Then the antigen-antibody mixture was centrifuged at 16,000 × *g* for 20 min, and the supernatant was carefully pipetted into a clean vial and used in place of the normal antiserum in the following western blot and immunofluorescence analysis as controls.

The protein extracts prepared from pituitary, inferior lobe of hypothalamus, tuberal hypothalamus, telencephalon, midbrain, cerebellum, medulla oblongata, spinal cord, eyes, liver, kidney, spleen, fat, heart, muscle, and ovary were subjected to Western blot analysis.

2.5. Immunofluorescence and microscopy

The brains with the pituitary from 1-year-old orange-spotted grouper, larvae at 1, 2, and 3 dpf were embedded in O.C.T. Compound (Sakure, Tokyo, Japan), and then fast cooled in liquid nitrogen. Serial transversal sections (10 μm thick) were cut with frozen microtomy (Leica CM1850), and collected on TESPA-coated slides. The sections were fixed by 4% paraformaldehyde for 15 min at RT, and then the tissue was washed several times in PBS. The sections were incubated for 1 h with 5% dry milk in PBS at room temperature to prevent non-specific binding of antibodies. The sections were incubated for 12 h at 4 °C with *Ec*MBP antisera diluted 1:100. After five rinses in PBST, they were exposed to 10% goat serum for 1 h and finally to fluorescein-conjugated goat anti-rabbit IgG (H + L) for 1 h diluted 1:100. After three time washes in PBS (5 min each), the sections were stained by PI (final concentration 1 mg/ml) for 5 min and washed for four times (5 min each). Finally, the sections were observed with a Leica cofocal fluorescence microscope. Two controls were also designed to determine polyclonal antibody specificity as same as Western blot analysis described previously.

3. Results

3.1. Molecular cloning and characterization of a smallest MBP

A total of 1006 ESTs were sequenced from the male orange-spotted grouper hypothalamic cDNA plasmid library (Zhou et al., 2006), and four ESTs were revealed to have a high similarity with zebrafish MBP (BrÖsamle and Halpern, 2002). According to the EST sequence, we designed primers and cloned the full-length cDNA using RACE strategy. As shown in Fig. 1, the full-length cDNA is 844 bp, and has an ORF of 222 bp for encoding a polypeptide of 74 aa. It has a 85 bp of 5′-untranslated region (UTR) and a 534 bp of 3′UTR including the poly (A) tail. A consensus polyadenylation signal AATAAA is located at 21 bp upstream from the poly (A) tail. N-linked glycosylation site analysis by NetNGlyc 1.0 did not find any potential N-glycosylation site, but YinOYang 1.2 showed eight potential O-glycosylation sites (Thr 3, Thr 6, Ser 7, Ser 54, Ser 55, Thr 60, Ser 71 and Ser 74). NetPhos 2.0 analysis revealed six phosphorylation sites (Ser 7, Ser 39, Ser 42, Ser 49, Ser 55 and Thr 60).

Homology searches suggested that the cloned molecule should be a MBP homologue in *E. coioides*. As shown in Fig. 2A and B, the deduced polypeptide has the highest (63.5%) amino acid identities with the matched regions of zebrafish MBP, and has 35.7, 40.7, 37.0, 34.1, 34.6, and 37.4% amino

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GACTGTGGGCTGTGGACTGGCTTGGAAGAGGACTACTGAGTAGATAGACAGAGGTCAACC 60

CCACCCAACCTCAGCAACTACTAGCATGGCTACGGCGAGCACCTCAGGGCAGAGCACCTT 120
                        M  A  T  A  S  T  S  G  Q  S  T  F

CGGACTCGGGAGGAAAAAGAAGACCCCTGGTCTCATGGACCAGATTACCAAGTTCTTTGG 180
  G  L  G  R  K  K  K  T  P  G  L  M  D  Q  I  T  K  F  F  G

GGGAGACAAAAAGAAAAGGAGCAAGGGGTCGTTCCGAGGTCACCTGGCCTCCCCACCCCA 240
  G  D  K  K  K  R  S  K  G  S  F  R  G  H  L  A  S  P  P  Q

GCAATCCTCCGCTCGCCGCCGGACCAATGAAAATGCTGTGGTGCATTTCTTCAGGAGCTT 300
  Q  S  S  A  R  R  R  T  N  E  N  A  V  V  H  F  F  R  S  F

TGCTTCATAAACACTGTTTCCTGTCTGTTTGCCCAAATGCAACAGTGATGCTCTCACCCA 360
  A  S  *

GGATCAGGAGATACCAAGTCCCGTCCACCCCCTAAACGCTGGAGCACCATCTTCTAAGCT 420

CTCCGCCGAGGACCGAACCACAGATCCAACACCAATGGGACAAGACAAAAGTGGGGAACA 480

AGATCTGCAAGCGGCGGCGTCACAACCTCAAGAAATGTTAATCCACTAACACAAGGCTTC 540

CTGTATGTTTTTGTCAGAGTATGAAATCCACTCTCCAGGGATGTAGGTGGTCACTGGGCC 600

TTTTTTGTTTAATCCTAAATATCTTGTCTACTTGTTTATAAAAGGTTTGGTCGTGCTTTT 660

ATAAAATATCTTACTGTAAAAATCACCCATTTTGAGTTTTTTGGTCTGATTGTGGCTATT 720

GTCTTGCTGGTCTTCAAGGTTTTGTTCATGGCAAAATGGTTTTGGATTTGGCTCCCGGCA 780

CTTGCAATTTTGGGTACCTTAATAAACTGGGGGTTTCGCCGAAAAAAAAAAAAAAAAAAA 840

AAAA                                                          900
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Fig. 1. Nucleotide and deduced amino acid sequences of full-length cDNA of *Ec*MBP cloned from orange-spotted grouper.

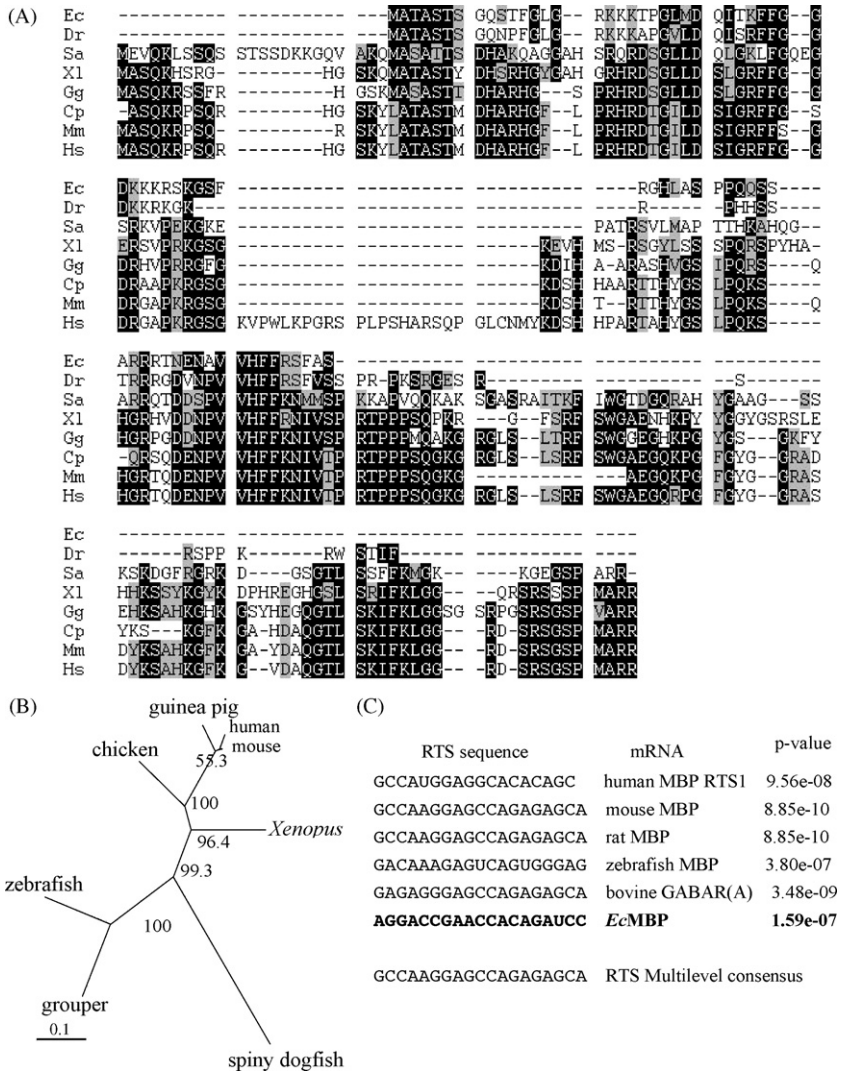

Fig. 2. Sequence analysis of *Ec*MBP. (A) Amino acid sequence alignment of *Ec*MBP (Ec), zebrafish MBP (Dr, GenBank No. NP_001018319), spiny dogfish MBP (Sa, GenBank No. CV652320), *Xenopus* MBP (Xl, GenBank No. Ab000736), chicken MBP (Gg, GenBank No. NP_990611), guinea pig MBP (Cp, GenBank No. P25188), mouse 14 kDa MBP (Mm, GenBank No. AAA39499), and human 18.5 kDa MBP isoform (Hs, GenBank No. NP_001020261). (B) Phylogenetic relationships of *Ec*MBP with other vertebrate MBPs. (C) Comparison of mRNA transportation signals (RTS) detected in various transported transcripts to a common motif (RTS multilevel consensus) extracted from 13 mRNA species previously found to be transported intracellularly. *Ec*MBP RTS (bold) shows significant similarity to the known RTSs in MBP and other transported RNAs.

acid identities with the matched regions of spiny dogfish MBP, *Xenopus* MBP, chicken MBP, guinea pig MBP, mouse major MBP isoform (14 kDa) and human major MBP isoform (18.5 kDa), respectively, although it has only 48.5, 16.9, 19.9, 17.1, 16.6, 23.4 and 18.0% identities with their complete MBP sequences respectively. In comparison with other MBPs, it is the smallest among all of the known MBPs, and the most similar to zebrafish MBP. The significant difference is shorter 23 aa in C-terminal of the grouper MBP than that in zebrafish MBP. Therefore, the newly cloned molecule was designated *E. coioides* MBP, *Ec*MBP.

*Ec*MBP also possesses common MBP properties similar to zebrafish MBP and other major MBPs. For example, a conserved motif of RNA transportation signal (RTS) was also found from the 3′UTR of *Ec*MBP cDNA in a similar range (*P*-value = 1.59e−07) (Fig. 2C) as identified previously in mammalian and zebrafish MBP mRNAs (Ainger et al., 1997; BrÖsamle and Halpern, 2002). *Ec*MBP is a highly basic protein too, because the calculated isoelectric point is 11.90.

3.2. Specificity of anti-EcSOX3 antibody

The coding region of *Ec*MBP cDNA was cloned into vector pET32a (+), and the expressed *Ec*MBP recombinant protein *in vitro* was used to raise polyclonal antibody in rabbit. To ensure the antibody specificity, the epitopes in the deduced polypeptide were firstly analyzed by semi-empirical method for prediction of antigenic determinants on protein antigens (Kolaskar and Tongaonkar, 1990), and two epitopes (“FRGHLASPPQQ” and “ENAVVHFF”) were revealed. Their average antigenic propensity is 0.9869, and has the

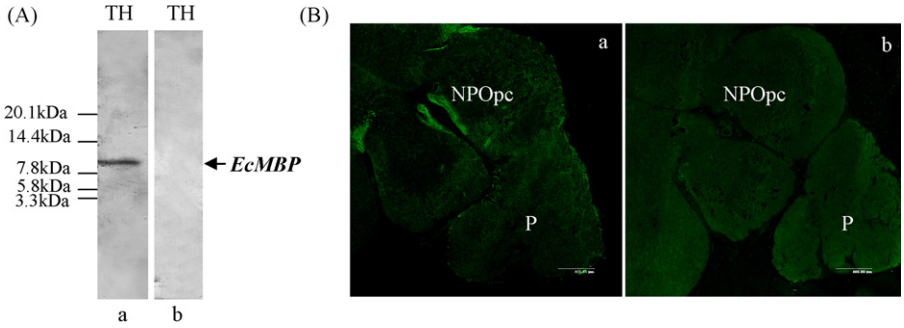

Fig. 3. Specificity detection of anti-*Ec*BMP antibody. (A) Western blot detection of anti-*Ec*MBP serum specicity. The grouper tuberal hypothalamus extracts were immblotted by anti-*Ec*MBP serum (a) and the pre-adsorbed anti-*Ec*MBP serum with extra *Ec*MBP protein (b). (B) Immunofluorescence detection of anti-*Ec*MBP serum specicity. Grouper brain sections were immunostained by anti-*Ec*MBP serum (a) and the pre-adsorbed anti-*Ec*MBP serum with extra *Ec*MBP protein (b).

highest identities with the matched regions of zebrafish MBP and other MBP isoforms.

Subsequently, the antibody specificity was confirmed by Western blot and immunofluorescence analysis. As shown in Fig. 3, the anti-*Ec*MBP antibody specifically recognizes an about 10 kDa positive protein band in tuberal hypothalamus extract, while the pre-adsorbed antiserum can not detect the specific 10 kDa polypeptide in the same tuberal hypothalamus extract, because the anti-*Ec*MBP antibody is abolished by the recombinant *Ec*MBP protein (Fig. 3A). In the same way, strongly immunoreactive signals of *Ec*MBP are revealed in medial part of the hypothalamus lateral tuberal nucleus (NLTm) by the anti-*Ec*MBP antibody (Fig. 3Ba), whereas no positive signals are observed when the pre-adsorbed antiserum is applied (Fig. 3Bb). As several MBP isoforms with different molecular weight (21.5–14.0 kDa) have been purified from mouse, human and chicken, and the anti-*Ec*MBP antibody recognizes only a single protein band in the tuberal hypothalamus extract, the anti-*Ec*MBP antibody is therefore specific to the grouper shorter MBP.

3.3. Hypothalamus-specific expression of EcMBP in the grouper adults

Tissue distribution of *Ec*MBP transcripts was firstly examined by RT-PCR analysis. RT-PCR analysis was carried out using total RNAs from the grouper adult tissues, such as liver, kidney, spleen, fat, heart, muscle, pituitary, hypothalamus, telencephalon, cerebellum, midbrain, medulla oblongata, ovary and testis. As shown in Fig. 4A, *Ec*MBP is expressed predominantly in hypothalamus, whereas lower level of *Ec*MBP transcripts can be detected only in brain tissues, such as midbrain, pituitary, telencephalon, and cerebellum. No signals were detected from other analyzed tissues including spleen, fat, heart, muscle, medulla oblongata, ovary and testis.

Preliminarily, the same 14 tissues, including liver, kidney, spleen, fat, heart, muscle, pituitary, hypothalamus, telencephalon, cerebellum, midbrain, medulla oblongata, ovary and testis, were subjected to Western blot detection, but no positive *Ec*MBP protein band was observed in all of the tissue extracts (data not shown). To solve the contradiction between RT-PCR analysis and Western blot detection, we further anatomized the hypothalamus. As shown in Fig. 4B, the hypothalamus was divided into three distinct portions, such as inferior lobe (IL), tuberal (medline) hypothalamus (TH), and saccus vasculosus (SV) (Rink and Wullimann, 2004). When the three portion extracts and the whole hypothalamus extract were subjected to Western blot, an about 10 kDa positive protein band was detected only in the tuberal hypothalamus extract, whereas no *Ec*MBP protein was found in the whole hypothalamus extract and other two extracts (Fig. 4C). Furthermore, the tuberal hypothalamus extract and other tissue extracts were simultaneously subjected to Western blot detection, the positive *Ec*MBP protein band was again observed only in the tuberal hypothalamus extract (Fig. 4D). The above data suggested that the *Ec*MBP might express specifically in hypothalamus, and mainly in the tuberal hypothalamus.

3.4. Distribution and cellular localization of EcMBP in adult brain

The anti-*Ec*MBP antibody was used to trace distribution and localization of *Ec*MBP on serially transversal brain sections from adult orange-spotted grouper. Fig. 5 is schematic lateral view of the brain and represents levels of the sections shown in Fig. 6. The left of Fig. 6 shows the schematic drawings and the observed regions of the successive rostrocaudal transverse sections. Consistent with the results of Western blot detection, no positive signals were detected in sections A, B and G. In the sections C, D, E and F, strongly immunoreactive signals of *Ec*MBP were observed in hypothalamus, the connection area between hypothalamus and telencephalon and the area surrounding hypothalamus, mainly in parvocellular part of the parvocellular preoptic nucleus (NPOpc) (Fig. 6C and D), central part of the ventral telencephalon (VC), dorsal posterior thalamic nucleus (DP), medial part of the lateral tuberal nucleus (NLTm) (Fig. 6E), and diffuse nucleus of inferior lobe (NDLI) (Fig. 6F). No positive signals were found in cerebellum, midbrain, medulla oblongata, pituitary, and spinal cord (Fig. 6A, B and G).

Double staining of PI for cellular nucleus and immunofluorescence for *Ec*MBP and higher magnification further revealed detail distribution characterization of the *Ec*MBP immunoreactive signals. As shown in Fig. 7, the *Ec*MBP

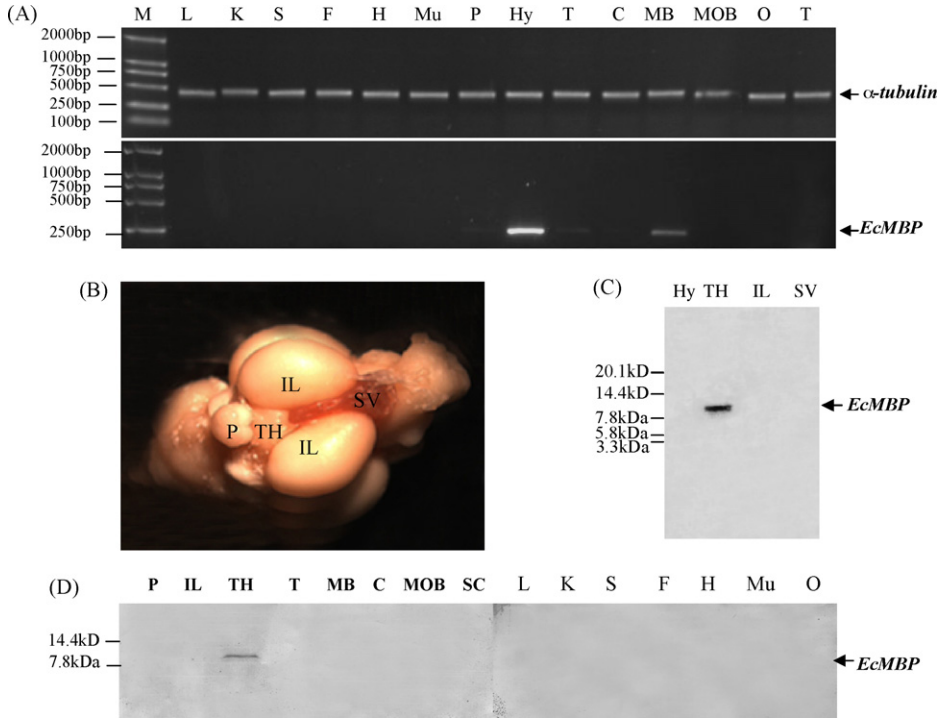

Fig. 4. Hypothalamus-specific expression of *Ec*MBP in the grouper adults. (A) *Ec*MBP tissue distribution detected by RT-PCR. α-Tubulin was used as RT-PCR control. M is the 2 kb DNA Ladder marker. (B) Ventral view of an adult orange-spotted grouper brain. The hypothalamus of orange-spotted grouper is divided into three distinct portions, including inferior lobe (IL), tuberal (medline) hypothalamus (TH), and saccus vasculosus (SV). Pituitary (P) is attached closely to tuberal hypothalamus. In order to show the shape and location of TH, pituitary is moved to side. (C) Western blot analysis of proteins isolated from the whole hypothalamus (Hy), tuberal hypothalamus (TH), inferior lobe (IL), and saccus vasculosus (SV). (D) *Ec*MBP tissue distribution detected by Western blot. L, liver; K, kidney; S, spleen; F, fat; H, heart; Mu, muscle; P, pituitary; Hy, hypothalamus; IL, inferior lobe; TH, tuberal hypothalamus; Te, telencephalon; C, cerebellum; MB, midbrain; MOB, medulla oblongata; O, ovary; T, testis.

immunoreactive signals are mainly localized on nerve fiber bundles, and intensive immunofluorescence is concentrated around the fiber bundles, implying that the immuno-stained fibers are the myelinated fibers. Fig. 7A shows the transverse fiber bundles on which the myelin sheaths are intensively stained by the *Ec*MBP immunoreactive signals. In longitudinal fiber bundles, a large number of more exiguous fibers were immunoreactively visualized to be linearly arrayed in the myelinated axons (Fig. 7B). Under higher magnification, the oligodendrocytes that express *Ec*MBP could be distinguished in the *Ec*MBP immunoreactive areas (Fig. 7C). The data suggest that *Ec*MBP should be expressed by oligodendrocytes, and the expressing cells should be concentrated in hypothalamus and the area surrounding hypothalamus.

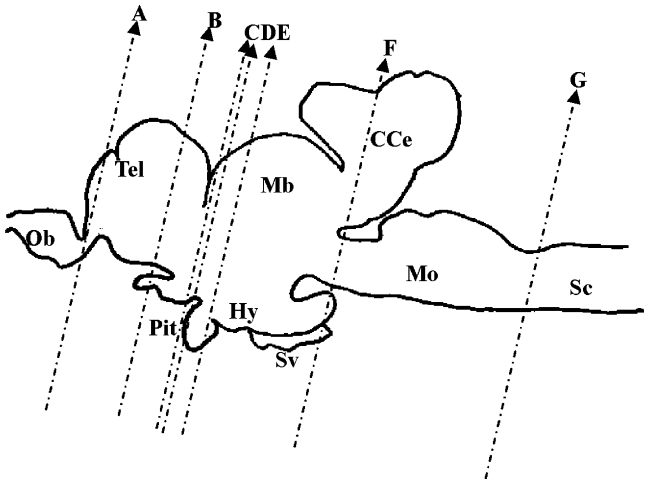

Fig. 5. Lateral view of the orange-spotted grouper brain showing the levels of sections in the figure. CCe, corpus of the cerebellum; Hy, hypothalamus; Mb, middle brain; Mo, medulla oblongata; Ob, olfactory bulb; Pit, pituitary; Sc, spinal cord; Sv, saccus vasculosus; Tel, telencephalon.

3.5. Expression pattern and developmental behaviour of EcMBP in the developing embryos and larvae in grouper

Expression pattern and developmental behaviour of *Ec*MBP were further investigated by immunofluorescence localization in the grouper embryos and larvae. No immunoreacted signals were observed in embryos at 1 day postfertilization (dpf) (Fig. 8A). At 2 dpf, a small number of *Ec*MBP-positive cells were first detected on the border of hypothalamus (Fig. 8B). Within the *Ec*MBP-expressing cells in hypothalamus, the *Ec*MBP proteins were obviously found to localize only one side of cytoplasm under higher magnification (Fig. 9). By 3 dpf, the processes with intensive *Ec*MBP signals had been extended from one side of the *Ec*MBP expressing cells, and one long myelinated fiber tract was found to form a connecting bridge between hypothalamus and mesencephalic tegmentum along third ventricle (Fig. 8C and D). Interestingly, the *Ec*MBP-expressing cells are only limited in a defined set of cells on the border of hypothalamus, and constitute a tube-like structure

(Fig. 8C). According to cellular morphology and localization characterization, we suggested that the *Ec*MBP-expressing cells might be a subpopulation of oligodendrocyte progenitor cells.

4. Discussion

In this study, we have made three interesting findings. First, we have cloned and identified a smallest MBP in vertebrates

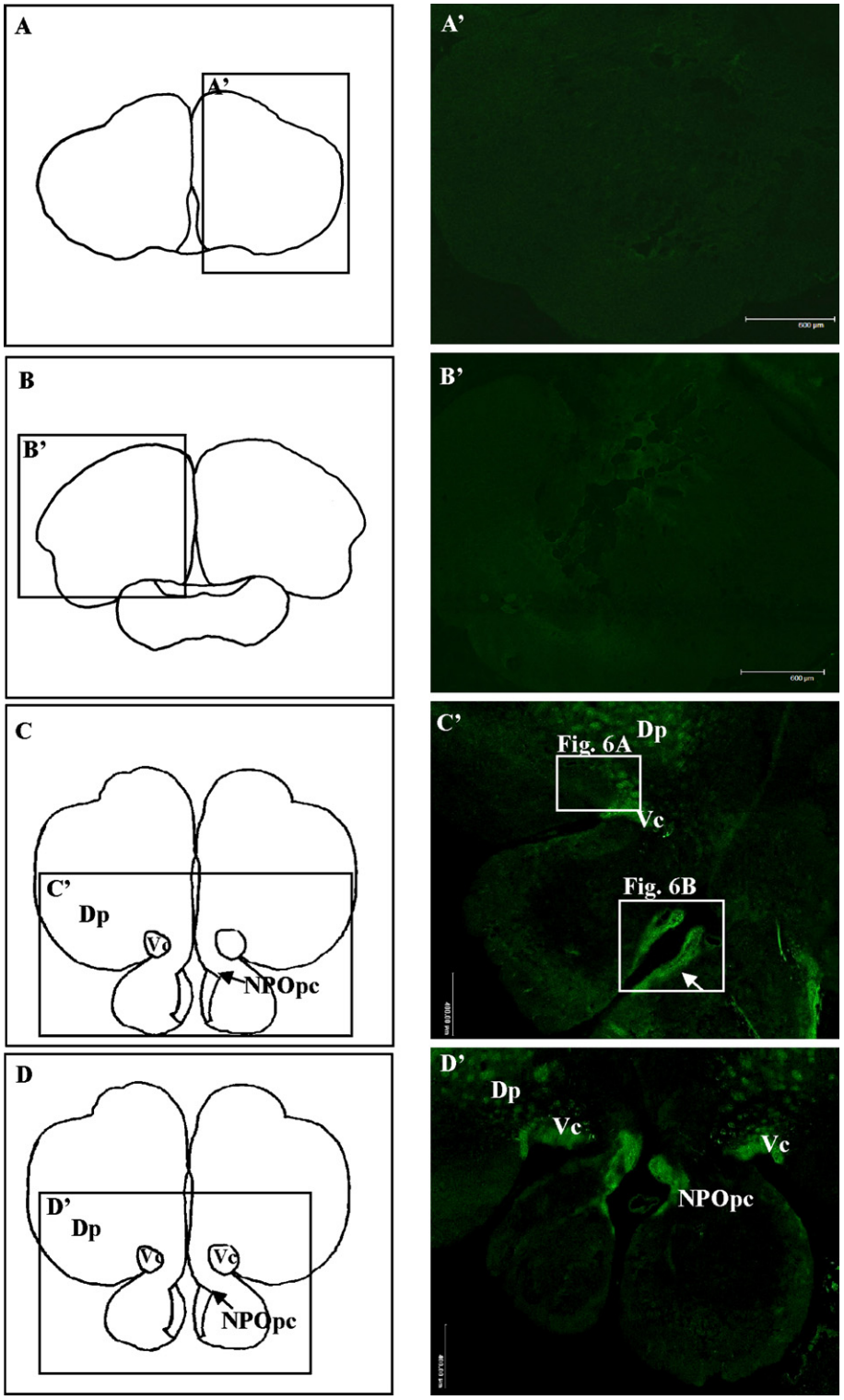

Fig. 6. Distribution of *Ec*MBP in adult brains. (A–G) Schematic drawings of successive transverse sections of orange-spotted grouper brain. (A′–G′) Photomicrographs of the squared areas in transversal sections through the brains of adult orange-spotted grouper. Bar scale in (A′), (B′), and (G′) represents 600 μm, bar scale in (C′), (D′), and (F′) represents 400 μm, and bar scale in (E′) represents 200 μm. The strong *Ec*MBP immunoreactive signals were observed in parvocellular part of the parvocellular preoptic nucleus (NPOpc), central part of the ventral telencephalon (VC), dorsal posterior thalamic nucleus (DP), medial part of the lateral tuberal nucleus (NLTv), and diffuse nucleus of inferior lobe (NDLI).

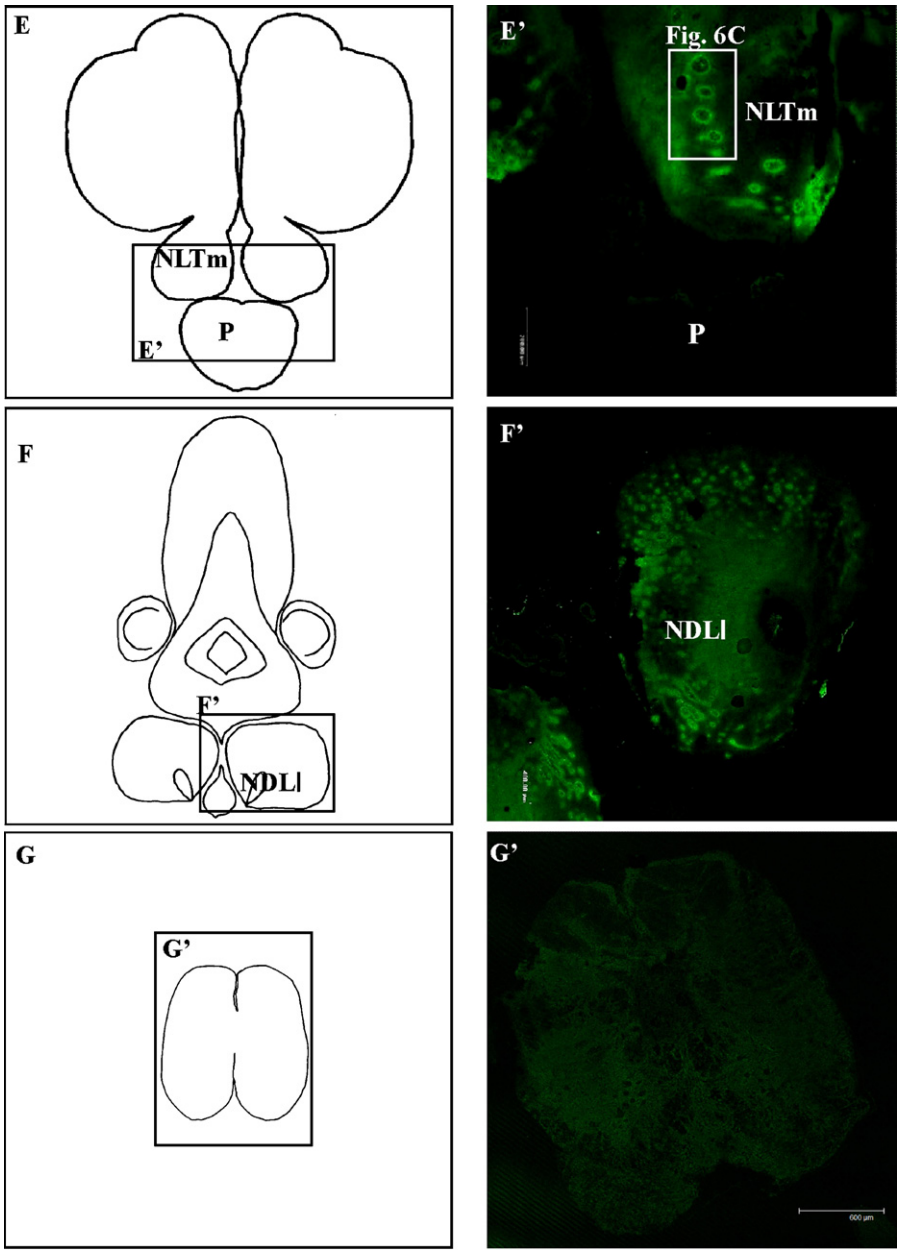

Fig. 6. (*Continued*).

from the male orange-spotted grouper hypothalamic cDNA plasmid library. Second, we have demonstrated in the grouper adult that the *Ec*MBP is specific to hypothalamus, and expresses mainly in the tuberal hypothalamus. Third, investigations on the *Ec*MBP expression pattern and developmental behaviour in the grouper embryos and larvae have revealed that the *Ec*MBP-expressing cells are only limited in a defined set of cells on the border of hypothalamus, and suggested that the *Ec*MBP-expressing cells might be a subpopulation of oligodendrocyte progenitor cells in hypothalamus.

MBPs constitute a large family of proteins and have been demonstrated to be the products of differential splicing of a single gene transcript (Harauz et al., 2004). In mouse, five different isoforms of 21.5, 18.5, 17.24, 17.22, and 14.0 kDa, formed by this differential splicing, have been identified. In human, there are four isoforms with molecular weight 21.5, 20.2, 18.5, and 17.2 kDa. In addition, five different isoforms with molecular weight 21.5, 18.5, 17.2, 16, and 14.2 kDa were also purified from chicken (Määttä et al., 1997). A lot of studies have indicated that the major MBP isoform in the adult human and bovine CNS is 18.5 kDa, and is 14 kDa in mouse and rat (Harauz et al., 2004). In the sequenced 1006 ESTs, no other MBP or MBP-like paralogs were obtained in the grouper hypothalamic cDNA plasmid library (Zhou et al., 2006), suggesting that *Ec*MBP might major isoform in the grouper hypothalamus. The newly cloned *Ec*MBP cDNA encodes a polypeptide of 74 aa, and its calculated molecular weight is only 8.1 kDa, although it has an about 10 kDa protein band in SDS-PAGE gel (Fig. 4C–D). Homology searches revealed that the *Ec*MBP is most similar to zebrafish MBP (88 aa) (BrÖsamle

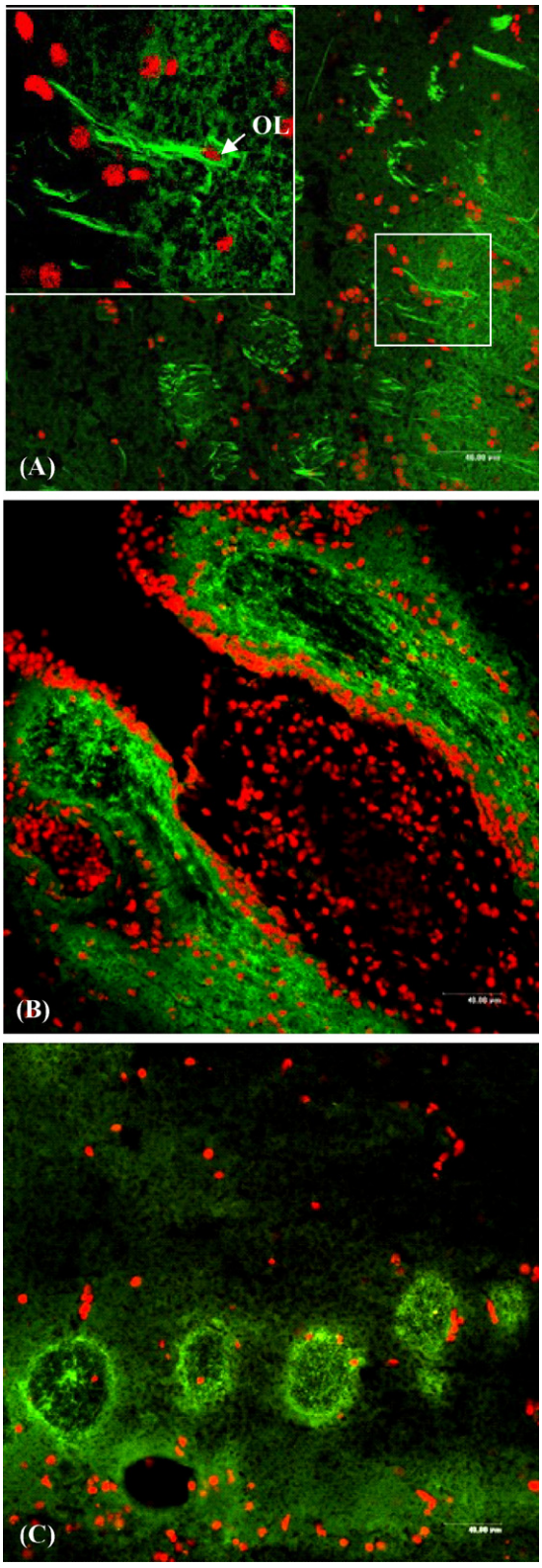

Fig. 7. Amplificatory photomicrographs of the squared areas in Fig. 5C′ and E′ showing cellular detail of *Ec*MBP-expressing cells and nerve axons. Double staining of PI for cellular nucleus and immunofluorescence for *Ec*MBP is shown. (A) *Ec*MBP-expressing oligodendrocyte (indicated by box and arrow) in the squared areas in Vc in Fig. 5C′. (B) *Ec*MBP-immunoreactive longitudinal fiber bundles in the squared areas in NPOpc in Fig. 5C′. (C) *Ec*MBP-immunoreactive transverse fiber bundles in the squared areas in NLT in Fig. 5E′. Bar scale represents 40 μm.

and Halpern, 2002). The zebrafish MBP contains only 88 amino acids and has low similarity to mammalian and other vertebrate MBPs, but its overall properties, such as domain composition and isoelectric point, are consistent with the functional role for myelination. *Ec*MBP was also found to be low conserved compared with other MBPs in vertebrates, but it possesses common MBP properties similar to zebrafish MBP and other major MBPs. For example, *Ec*MBP is a highly basic protein, and contains a conserved motif of RNA transportation signal (RTS) in its cDNA 3′UTR. In comparison with other MBPs, therefore, *Ec*MBP is the smallest among all of the known MBPs.

MBP family comprises a large number of isoforms, and therefore has complex structure-function relationships. The 18.5 kDa isoform in human and mammals, long thought as a major MBP, has thoroughly been studied because of its ubiquity and importance in myelination and in stability maintenance of the myelin sheath (Baumann and Pham-Dinh, 2001). Moreover, certain MBP isoforms, such as embryonic MBPs and Golli MBPs, are expressed prior to myelin formation in the brain and in several non-neural tissues and cells, suggesting that MBPs might have multiple roles (Campagnoni, 2003). For example, MBP can induce insulin and glucagon release from pancreatic islets (Kolehmainen et al., 1990; Kolehmainen, 1995a,b; Kolehmainen and Sormunen, 1998), and is also known to interact with numerous signaling proteins, such as calmodulin (CaM), suggesting that some isoforms might be involved in intracellular signaling processes (Barylko and Dobrowolski, 1984; Dobrowolski et al., 1986; Libich et al., 2003a,b; Polverini et al., 2004). In comparison with the known MBPs in vertebrates, the *Ec*MBP identified in the current study is of higher specificity of tissue and cell expression. Generally, most of MBPs are synthesized in myelin producing cells: oligodendrocytes in the CNS and Schwann cells in the PNS. For example, zebrafish MBP mRNA is expressed in the Schwann cells along the two branches of the anterior lateral line nerve and the posterior lateral line nerve. It also is expressed in the oligodendrocytes along the ventral hindbrain bundle and in the spinal cord (BrÖsamle and Halpern, 2002). In this study, we have not only demonstrated that the *Ec*MBP is expressed specifically in the tuberal hypothalamus in the grouper adult, but also revealed that the *Ec*MBP-expressing cells are only limited in a defined set of cells on the border of hypothalamus in the developing grouper larvae. The data implicate that the *Ec*MBP is expressed only in oligodendrocytes or a subpopulation of oligodendrocyte progenitor cells, not in Schwann cells of peripheral nervous system.

Oligodendrocytes arise from migratory and poliferative OPCs. In mammals, oligodendrocyte maturation involves a series of defined developmental stages including OPCs, immature OLs, differentiated premyelinating OLs, and mature myelinating OLs (Xin et al., 2005). OPCs arise from proliferative zones at multiple sites in brain. Forebrain, embryonic anterior entopeduncular area and brainstem are major sites of oligodendroliogenesis (Thomas et al., 2000; Ivanova et al., 2003; Liu et al., 2003). Several transcription factors, such as *Olig1* and *Olig2*, were identified and shown to

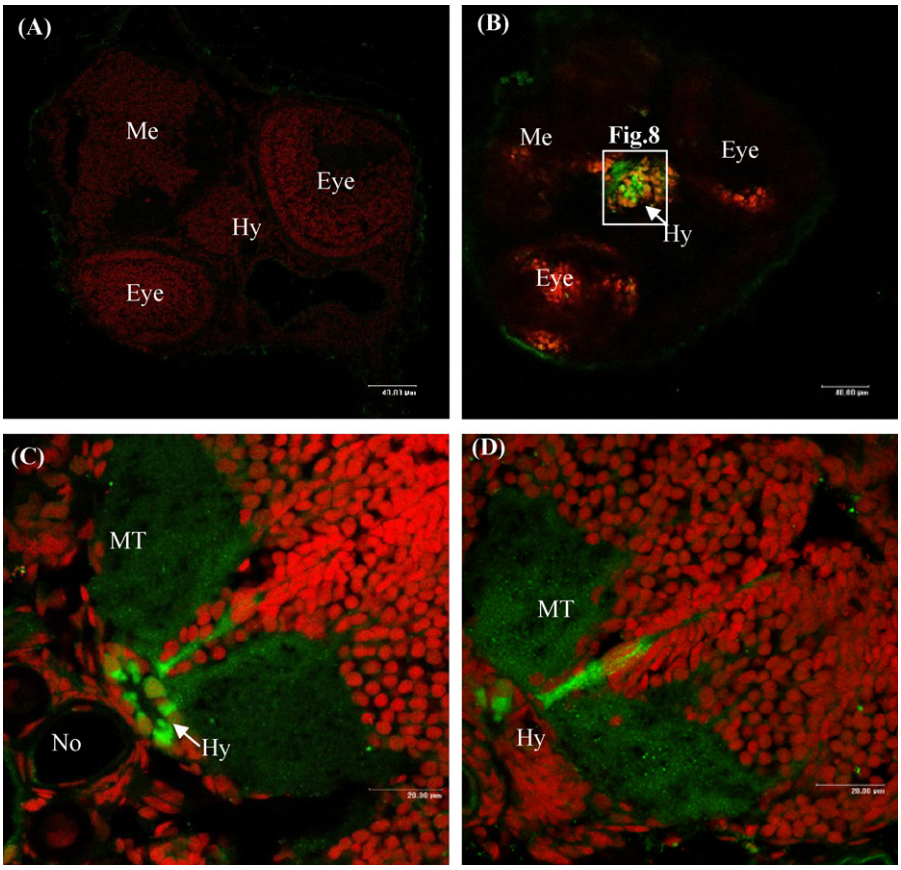

Fig. 8. Expression pattern and developmental behaviour of *Ec*MBP in the brains of developing grouper embryos and larvae. Double staining of PI for cellular nucleus and immunofluorescence for *Ec*MBP is shown. (A–D) Photomicrographs of transversal sections through the brains of orange-spotted grouper embryos and larvae at 1, 2, and 3 dpf, respectively. Bar scale represents 40 or 20 μm. (A) None *Ec*MBP-positive cells was detected in the larvae hypothalamus at 1 dpf; (B) *Ec*MBP-positive cells in the larvae hypothalamus at 2 dpf; (C) *Ec*MBP-positive cells in the larvae hypothalamus at 3 dpf; (D) section adjacent to C, showing one long myelinating fiber tract forming a connecting bridge between hypothalamus and diencephalon along third ventricle. Hy, hypothalamus; Me, mesencephalon; MT, midbrain tegmentum; No, notochord; Te, telencephalon.

be essential for generating oligodendrocyte lineage cells during embryogenesis (Lu et al., 2000; Zhou et al., 2000). *Olig2* is essential for initiation of oligodendroliogenesis from neural progenitor cells and the formation of immature OLs, whereas *Olig1* is a central regulator of oligodendrocyte myelinogenesis in brain and that axonal recognition and myelination by oligodendrocytes are separable processes (Lu et al., 2001, 2002; Takebayashi et al., 2002; Zhou and Anderson, 2002; Xin et al., 2005). Despite many similarities in oligodendrocyte morphology and function throughout the CNS, the data from Xin et al. (2005) indicated oligodendroliogenesis during brain development is more complex than for spinal cord in several respects, demonstrated the region-specific regulation of myelin gene expression and provided direct evidence for at least two populations of OPCs in the CNS that regulate myelinogenesis through distinct mechanisms. Previous studies have been revealed fundamental similarities in glial cell development and myelination between teleosts and mammals, suggesting that genetic studies in zabrafish might contribute to the understanding of human diseases of myelin (BrÖsamle and Halpern, 2002; Park et al., 2002). Recently, a serial of studies on oligodendroliogenesis and myelination were performed in zebrafish (Park et al., 2002, 2005; Yoshida and Macklin, 2005; Cunliffe and Casaccia-Bonnefil, 2006; Kazakova et al., 2006; Kirby et al., 2006; Pogoda et al., 2006; Schweitzer et al., in press). The special expression pattern that *Ec*MBP is expressed specifically in the grouper tuberal hypothalamus and only in a subpopulation of oligodendrocyte progenitor cells suggested that the oligodendroliogenesis during fish brain development might be much complicated. Further investigation whether other oligodendrocyte markers, such as *Olig1*, *Olig2*, and *sox10*, also express in the especial subpopulation of oligodendrocyte progenitor cells in grouper hypothalamus will be very useful for understanding the specific oligodendroliogenesis. Of course, *Ec*MBP might be used as a molecular marker for studying origin, development, proliferation, differentiation and migration of oligodendrocytes in fish.

The hypothalamus, known as an instinctive brain center, is implicated in a multitude of vital bodily processes including cardiovascular regulation, sleep, metabolism, stress, thermoregulation, water and electrolyte balance, appetite regulation, sexual behavior and immune responses (Koutcherov et al., 2003). In this study, the strong *Ec*MBP immunoreacted signals were observed in NPOpc, VC, DP, NLTm, and NDLI. It has been well demonstrated that the pituitary of teleosts is innervated by axons arising from the NPO and NLT (Peter

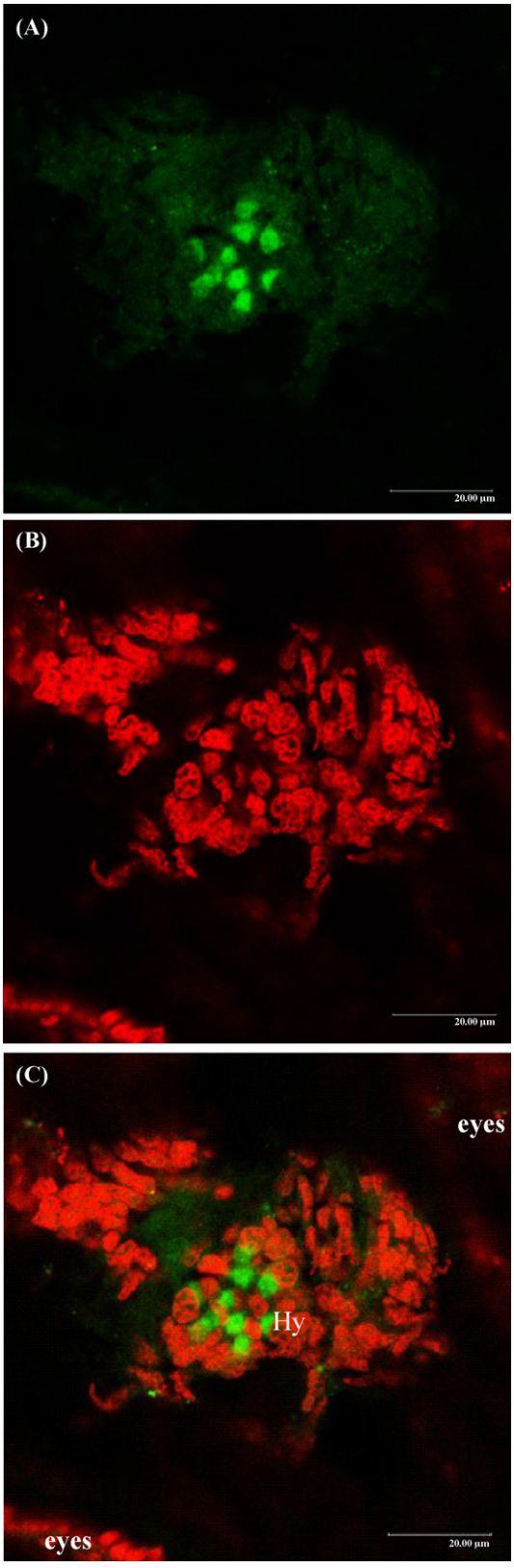

Fig. 9. Intracellular localization of *Ec*MBP in expressing cells under higher magnification. (A) *Ec*MBP immunofluorescence; (B) PI staining; (C) merged image showing the *Ec*MBP located only in one side of the cytoplasm. Hy, hypothalamus.

and Fryer, 1983). The preoptic region and lateral tuberal nucleus influence release of reproductive hormones from the anterior pituitary. In teleost fish, several hormones, receptors or enzymes were detected in NPO and NLT by using in situ hybridization and immunological methods, such as GnRH (Baby et al., 2000; Montero et al., 1994; Mousa and Mousa, 2003), Neuropeptide Y (Cerdá-Reverter et al., 2000; Chiba et al., 2002; Peng et al., 1994), FSH and LH (Parhar et al., 2003), melanocyte-stimulating hormone (Chiba, 2001), somatostatin (Cardenas et al., 2000; Mousa and Mousa, 1999), Galanin (Rao et al., 1996; Unniappan et al., 2004), estrogen receptor (Andreassen et al., 2003; Menuet et al., 2002; Pellegrini et al., 2005) and so on. In the present study, the special function of *Ec*MBP is unknown. However, the features, predominantly expressed in NPO and NLT, suggested it might be involved in the hypothalamic control of reproductive functions and further studies on its functions will be very useful for understanding of molecular mechanism of hypothalamic physiological functions.

Acknowledgements

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