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The expression of transferrin binding protein in the turtle nervous system*

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Summary. Transferrin binding protein (TfBP) is a cytoplasmic glycoprotein that was originally isolated from the chick oviduct. As we previously demonstrated the constitutive expression of TfBP in the avian nervous system, in this study we examined whether TfBP is expressed in the reptilian nervous system. In accordance with previous findings in the chicken, oligodendrocytes were most prominently labeled by antiserum to TfBP. Great variability was observed between different regions of the central nervous system (CNS) in terms of TfBP-labeled oligodendrocyte numbers. In the retina, TfBP was localized specifically in the cells that are morphologically oligodendrocytes and present in the optic nerve and the ganglion cell layer. TfBP staining was also seen in the Schwann cells of peripheral nerves. Furthermore, choroid plexus cells and capillary endothelial cells similarly exhibited strong reactions. These results may reflect the fact that the homology of nervous system genes is conserved between close phylogenetic lines, and proove the potential of TfBP as a marker for oligodendrocytes in avian as well as reptile.

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Introduction

Oligodendrocytes (OLG), myelin-forming cells in vertebrates, are related to the iron metabolism in the mammalian CNS (Connor and Menzies, 1996). OLGs have been intensely studied in mammals but not in non-mammalian species because, unlike astrocytes that express glial fibrillary acid protein (GFAP) throughout the vertebrate phylogenetic line, molecular heterogeneity has been reported for vertebrate OLGs (Birling and Nussbaum, 1995; Birling *et al*., 1995; Anderson *et al*., 1999). Electron microscopic studies in the turtle have demonstrated that the animals possess macroglial cells that are similar to those found in mammals (Geri *et al*., 1982; Rainey and Ulinski, 1982; Davila *et al*., 1987), and recent studies have described GFAP and vimentin expression in astrocytes (Kalman *et al*., 1997; Lazzari and Franceschini, 2006). However, while a few studies have demonstrated OLGs and myelination in the developing and adult reptile brain, little is known about the immunohistochemical detection of OLGs in the reptile nervous system (Birling and Nussbaum, 1995; Monzón-Mayor *et al*., 1998; Romero-Aleman Mdel *et al*., 2003; Santos *et al*., 2006). This study was therefore designed to study OLGs in the turtle nervous system by using the antibody for transferrin binding protein (TfBP).

TfBP is a cytoplasmic glycoprotein (MW 180kDa) that was originally purified from the chick oviduct, and exhibits a transferrin-binding activity (Poola and Lucas, 1988). Unlike transferrin receptors, TfBP shares high homology with stress-regulated proteins like HSP108,

a chick heat shock protein that shows >90% amino acid sequence homology to the stress-regulated proteins of other vertebrates (Hayes *et al*., 1994). We previously showed that the TfBP antiserum selectively stains OLGs in the avian central nervous system (Cho *et al*., 1997, 1998), and TfBP has been established as an OLG marker in both embryonic and adult avian brains (Anderson *et al*., 1999; McBride *et al*., 2003; Park *et al*., 2007).

In the present study, we demonstrated TfBP expression in the turtle brain, a lower amniotic vertebrate that is phylogenetically related to birds. We also compared the localization of TfBP with those of myelin. The association of TfBP in these cell types responsible for myelination implies that TfBP may be a useful maker for oligodendrocytes in the reptilian nervous system.

Materials and Methods

Animals and antisera

The experimental procedure was approved by the Institute of Laboratory Animal Resources at Seoul National University, and all experiments were conducted in accord with NIH guidelines on the care and use of animals. Ten red-eared pond turtles, Pseudemys scripta elegans, with carapace lengths ranging from 10 to 15 cm, were used in this study. The primary antibody used was rabbit antiserum directed against chick oviduct TfBP (αOV-TfBP). The preparation and characterization of this antibody has been previously described in detail (Poola and Lucas, 1988; Hayes *et al*., 1994).

Western blot analysis

The brains were removed from turtles and chick embryos at E10 as positive controls, and homogenized in a lysis buffer (50 mM TrisCl, 150 mM NaCl, 0.02% sodium azide, 100 μg/m*l* phenylmethanesulphonyl fluoride (PMSF), 1 μg/m*l* aprotinin, and 1% Triton X-100). Samples containing 40 μg of protein were added to a 2X sample buffer (0.8 M TrisCl, 10% glycerol, 20% betamercaptoethanol, 10% SDS, and 0.02% bromophenol blue), boiled for 5 min and then loaded onto a polyacrylamide gel. The proteins were resolved by electrophoresis and then transferred from the gel onto a nitrocellulose membrane. For immunostaining, the membranes were incubated in 5% skimmed milk in phosphate-buffered saline (PBS) at room temperature for 1 h, to block nonspecific binding, and then with the TfBP antibody (1:5000) at room temperature for 2 h. Membranes were washed three times for 10 min in PBS and incubated for 1 h at room temperature with peroxidase labeled goat anti-rabbit IgG diluted 1:1000 in PBS. Immunolabeled proteins were detected by chemiluminescence using a Supersignal ECL kit (Pierce, IL, USA) and Biomax Light-1 films (Kodak, NY, USA). Two groups of experimental brain were used for Western blotting in each animal.

Immunohistochemical procedures

Turtles were cryoanesthetized at 4℃ for at least 90 min. Brains, eyeballs, optic nerves and sciatic nerves were removed under general anesthesia and then immersed in a 4% paraformaldehyde solution in phosphate-buffered saline overnight at 4℃ or into Carnoy (ethanol:acetic acid, 3:1) solution (Clark, 1984) for 4 h at room temperature. Tissues were washed three times in cold phosphate buffered saline (PBS; 0.1 M sodium PBS, pH 7.4), cryoprotected by 10–30% serial sucrose infiltrations, embedded in Optimal Cutting Temperature compound, and frozen rapidly in 2-methylbutane precooled to its freezing point in liquid nitrogen. Tissue specimens were cut into 10 μm sagittal or coronal sections using a Reichert-Jung Frigocut model 2800 cryostat (Heidelberg, Germany). Tissue sections were mounted on gelatin-coated microscopic slides, air dried, and then stored at −70℃ until required for immunohistochemistry. For free-floating tissue staining, tissues were embedded in 5% agar gel and cooled slowly at room temperature. Tissue blocks were sectioned at 100 μm on a vibratome into deionized water. Immunohistochemical staining was performed using the avidin-biotin peroxidase complex (ABC) method, as previously described (Cho and Lucas, 1995; Cho *et al*., 1997).

In order to simultaneously demonstrate pairs of antigens in the same sections, rabbit anti-TfBP was used with RCA-1 (Ricinus communis agglutinin-1) or one of the following monoclonal antibodies: anti-O4 (1:10, hybridoma), anti-NeuN (1:100, Chemicon), anti-GFAP (glial fibrillary acidic protein, BioGenex, CA, USA), or anti-vimentin (H5, Developmental Studies Hybridoma Bank, IA, USA). RCA-1 staining was observed in the cell bodies and processes of the microglia (Mannoji *et al*., 1986). Appropriate immunohistochemical staining controls included: the omission of the primary antiserum or goat anti-rabbit IgG, the substitution of normal rabbit serum for the primary antiserum, and the preincubation of primary antiserum with purified oviduct TfBP (10 mg/m*l* of diluted antiserum) for 24 h before application to tissue sections.

Histochemistry for myelin

Myelin staining was performed using the mordant-dye method (Klüver-Barrera stain) with luxol fast blue and cresyl violet (Culling *et al*., 1985).

Micrographs

Tissue sections were examined under an Olympus BX51 microscope equipped with a fluorescence attachment, and images were acquired using a ProgRes C14 CCD camera and ProgResC14 software. All digital images were adjusted for brightness, contrast, and evenness of illumination using Adobe Photoshop v. 7.0 (San Jose, CA, USA).

Results

General characteristics

Western blot analysis showed a single band of approximately 97kDa in both turtle and chicken brains (Fig. 1), suggesting that the TfBP protein is present in the turtle with properties similar to those of the chicken. Immunohistochemical staining showed intense TfBP immunostaining in central and peripheral nervous tissue sections, while control tissue sections that had been incubated in normal or preabsorbed serum were devoid of immunoreactive products. Although oligodendrocytes (OLGs) were the prominent cell type labeled by the TfBP antibody, staining was also observed in Schwann cells of the peripheral nerves, epithelial cells of the choroid plexus, and capillary endothelial cells in the turtle brain.

Brain

TfBP immunostaining patterns in brain sagittal sections are shown at low magnification in Figure 2. The numbers of TfBP-positive (TfBP$^+$) cells were highly variable in different regions of the turtle CNS. The brainstems revealed a rather uniform TfBP$^+$ cell density (Fig. 2A), but staining markedly diminished toward the diencephalon (Fig. 2B) and cerebral cortex (Fig. 2C), in which an abundance of cells were observed only in well-myelinated areas, such as the anterior commissure (Fig. 2B) and optic chiasm (Fig. 2E). At higher magnification, TfBP immunoreactive products were found to be localized in the cytoplasm of small cells—often occurring in rows in the white matter—and in a perineuronal position in the gray matter (Fig. 2D). Thus, TfBP$^+$ cells appeared to be OLGs in their morphology and distribution. As OLGs are associated with myelinated nerve tracts, we compared the localization of TfBP immunoreactivity and myelin staining in adjacent sections. As expected, a distinct parallelism was found between the two stainings in the myelin rich areas (Fig. 2E, F). In the cerebellar cortex, TfBP$^+$ cells were present primarily in the granular layer and the Purkinje cell layer but were rarely found in the molecular layer (Fig. 3A). In the Purkinje cell layer, some TfBP$^+$ cells were observed in close contact with the large somata of Purkinje cells (Fig. 3B). The choroid plexus exhibited strong positive immunostaining for TfBP (Fig. 3C). Intense cytoplasmic staining was evident in cells lining the choroid plexus while ependymal cells lining the ventricular cavity showed little immunoreactivity (Fig. 3D). TfBP immunoreactivity was also associated with blood vessels throughout the CNS (Fig. 3E, F, G) and at a lower level in capillary walls. However, blood components like red blood cells were negative.

Spinal cord

Large numbers of TfBP$^+$ cells were found in the white matter whereas the numbers of TfBP-labeled cells were markedly lower in the gray matter (Fig. 4A). At higher magnification, TfBP$^+$ cells in the white matter displayed an elongated rectangular morphology (Fig. 4F) and often occurred in rows—which is a typical feature of interfascicular OLGs whereas TfBP$^+$ cells in the gray matter often occupied a perineuronal position (arrows in Fig. 4E) and had a process-bearing appearance (Fig. 4C, E, F). To compare the localizations of TfBP and myelin, adjacent sections of the spinal cord were stained with the TfBP antiserum or by the mordant-dye method. Like TfBP immunoreactivity, myelin was found to be present mainly in the white matter (Fig. 4B, D).

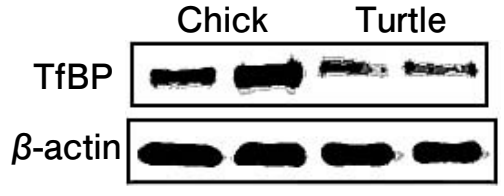

Fig. 1. Western blot analysis of the turtle brain extract (lane 3 and 4) and chicken embryo brain extract (lane 1 and 2) show a specific immunoreaction for the TfBP antibody in a single band of approximately 97kDa. β-actin was used as a loading control.

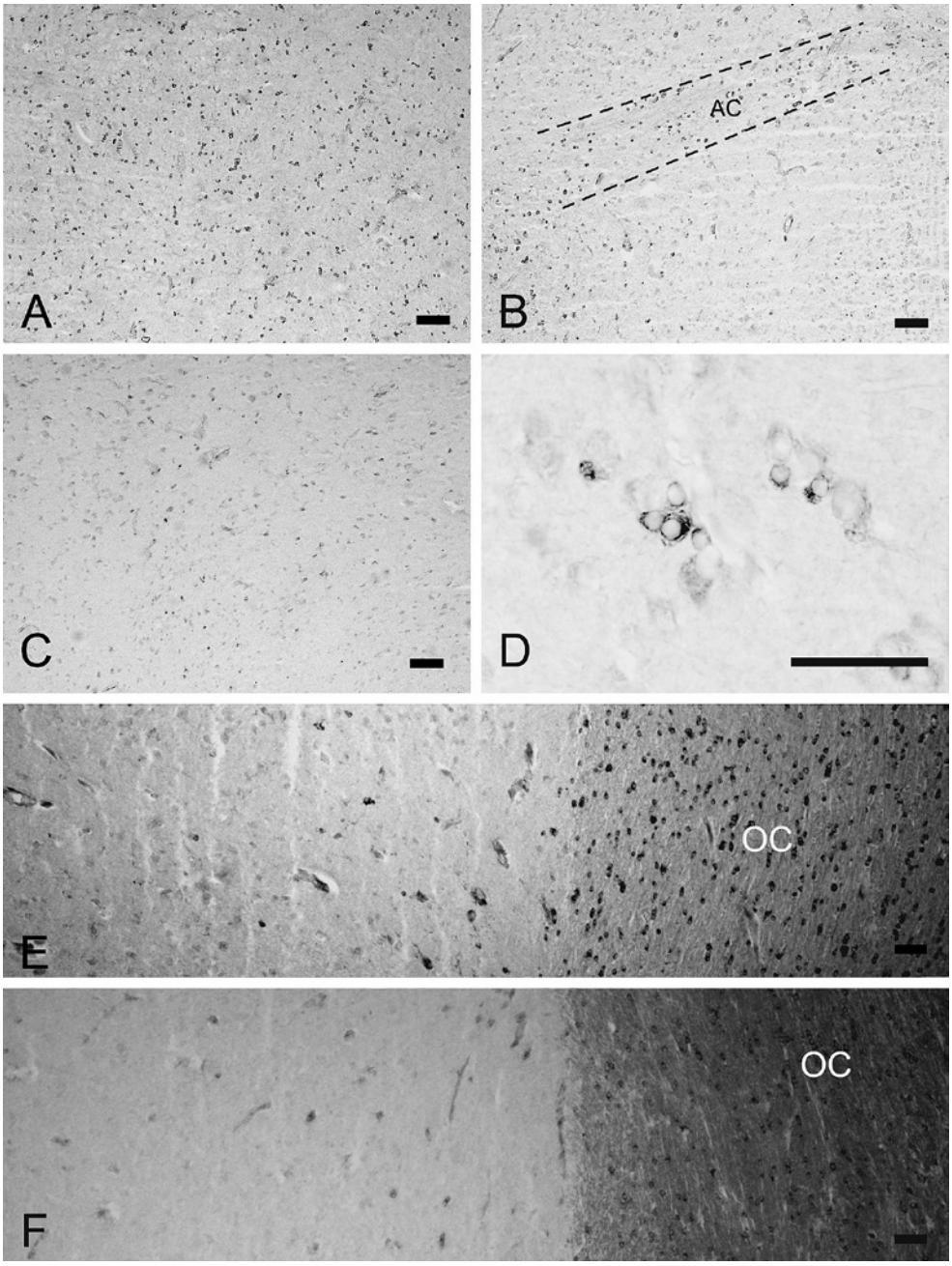

Fig. 2. Coronal sections of the turtle brain stained with TfBP antibody (**A**–**E**) and mordant-dye for myelin (**F**), showing the cellular localizations and regional distributions of the two components. Large numbers of TfBP-positive (TfBP$^+$) cells are evenly distributed throughout the brainstem (**A**), but these numbers gradually decrease toward the diencephalon (**B**) and cerebral cortex (**C**), except along myelinated tracts, such as the anterior commissure (AC in **B**), where TfBP$^+$ cells accumulate. At a higher magnification (**D**), TfBP immunoreactivity is seen localized to the cytoplasm of small cells, which resemble perineuronal oligodendrocytes. **E**, **F:** Ventral portion of the diencephalon stained for TfBP (**F**) and myelin (**G**) showing a parallelism in the localization of TfBP$^+$ cells and myelin. Note that the optic chiasm (OC) is most intensely stained for both TfBP and myelin. Scale bars = 100 μm

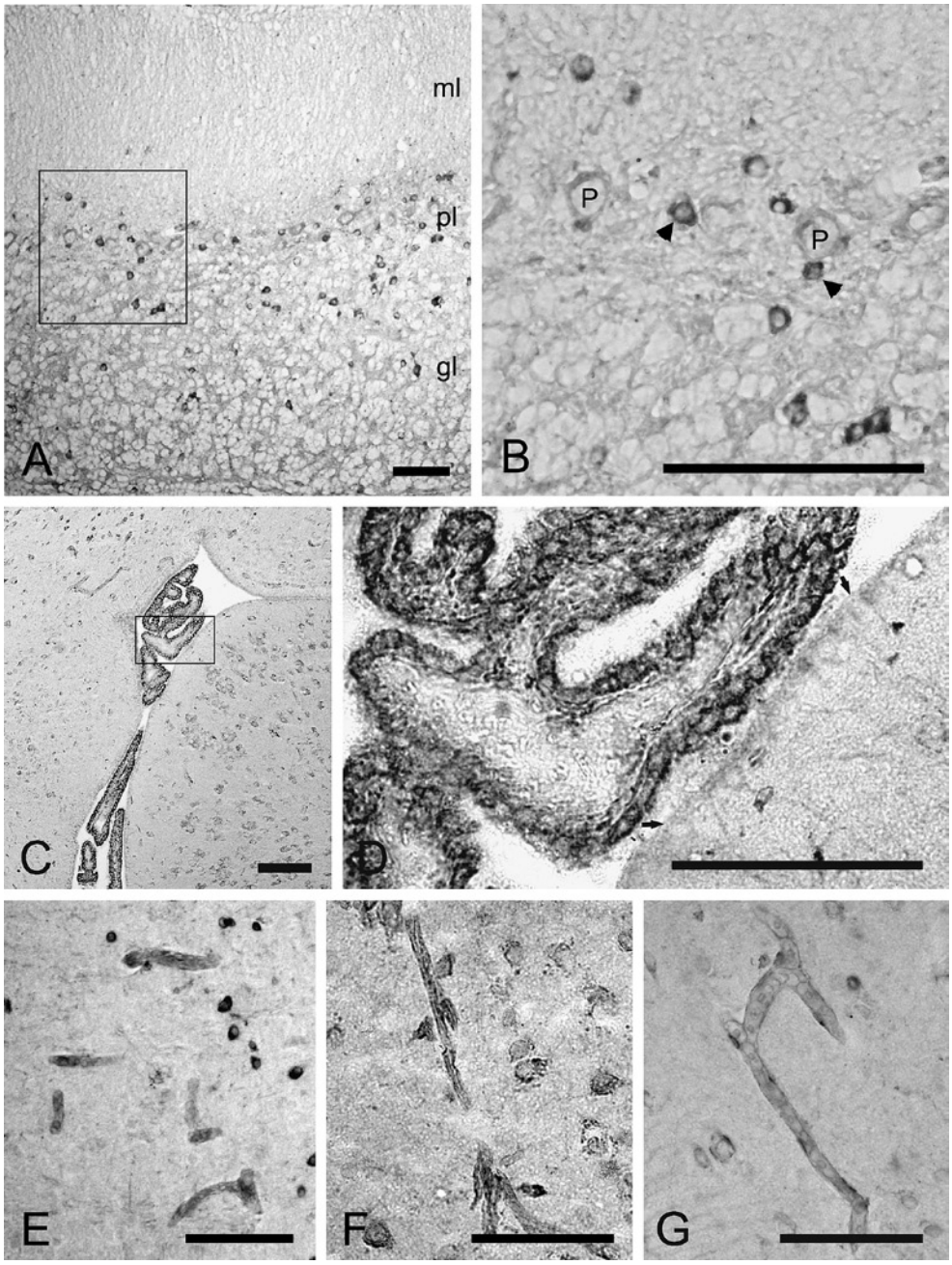

Fig. 3. Sections of turtle cerebellum (**A**, **B**) and forebrain (**C**, **D**) stained with the TfBP antibody. TfBP$^+$ cells are present primarily in the granular (gl) and Purkinje cell (pl) layers but are rarely observed in the molecular layer (ml). Strong TfBP$^+$ cells are often observed around large somata of Purkinje cells (p) in the Purkinje cell layer (arrowheads in **B**). **A:** Three layers of the cerebellar cortex. **B:** Higher magnification of the boxed area in **A**. **C:** Choroid plexus of the lateral ventricle. **D:** Higher magnification of the boxed area in **C**. Intense TfBP immunoreactivity is evident in the cytoplasm of cells lining the choroid plexus. In contrast, ependymal cells lining the ventricular cavity are not stained (arrows). **E**, **F**, **G:** Blood vessels of the optic tectum (**E**), cerebral cortex (**F**), and brainstem (**G**). A lower level of TfBP immunostaining can also be observed in the walls of blood vessels. Bs: Brain stem. Scale bars = 100 μm

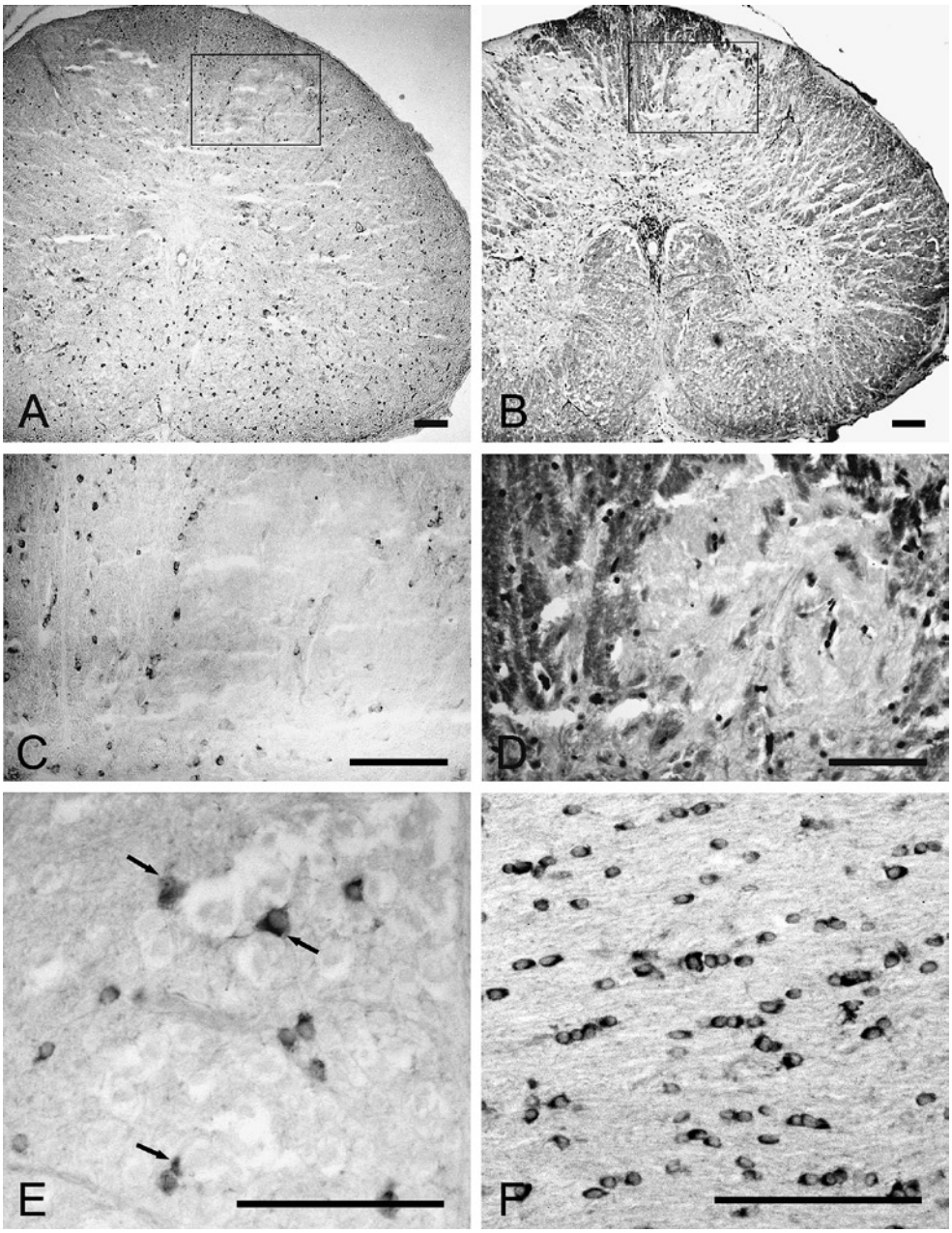

Fig. 4. Cross sections of the upper cervical spinal cord stained with the TfBP antibody (**A**, **C**, **E**, **F**) and mordant-dye for myelin (**B**, **D**). TfBP (**A**) and myelin (**B**) show a parallel distribution. Both of them can be observed primarily in the white matter but less abundantly in the gray matter. At higher magnification, $TfBP^+$ cells (**C**) and myelin-stained fibers (**D**) are found to occur in rows, although myelin is more abundant than TfBP. $TfBP^+$ cells in the gray matter often occupy a perineuronal position (arrows in **E**) and have a process-bearing appearance, while those in white matter were often arranged in rows and had an elongated rectangular morphology (**F**). **C**, **D:** The boxed area in **A** (**C**) and **B** (**D**). Scale bars = 50 μm

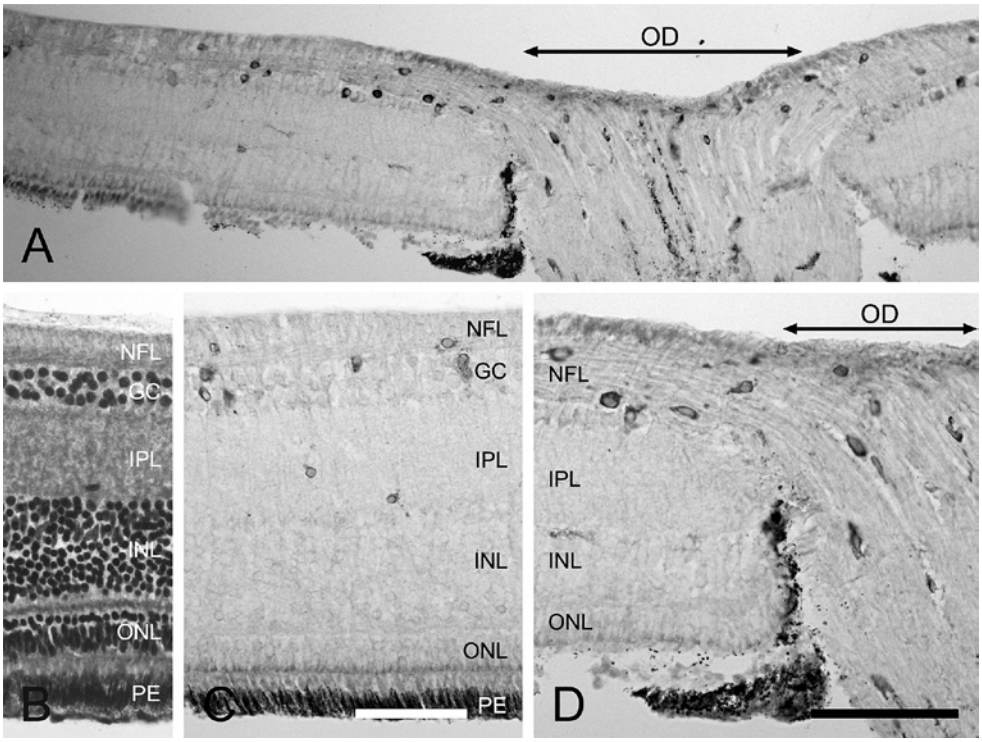

Fig. 5. Sections of the retina through the optic nerve head showing regional distribution of TfBP$^+$ cells in the central (**A**, **D**) and middle (**C**) parts of the retina. TfBP immunoreactivity is mainly found in the ganglion cell layer (GC) and the nerve fiber layer (NFL) including the optic disc (OD). A few TfBP$^+$ cells are also found in the inner plexiform layer (IPL) (**C**). **B:** Cresyl violet staining showing the layers of the turtle retina. IPL: inner plexiform layer; INL: inner nuclear layer; ONL: outer nuclear layer; PE: retinal pigment epithelium. Scale bars = 50 μm

Retina

As we have found TfBP$^+$ OLGs in the bird retina in a previous study (Cho *et al*., 1999) and OLG in the reptilian retina has been recently demonstrated (Santos *et al*., 2006), we examined whether the turtle retina has TfBP immunoreactivity. As expected, strong TfBP immunostaining was evident in the small cells of the retina, which were confined to the optic nerve fiber layer, the ganglion cell layer, and inner plexiform layer (Fig. 5A). These immunoreactive cells were interposed sporadically among ganglion cells in the eighth layer and occurred along the nerve fibers in the ninth layer of the retina but less frequently in the inner plexiform layer (Fig. 5B, C, D). The TfBP immunoreactive cells were evenly distributed in the central portion of the retina but diminished toward its periphery.

Optic and sciatic nerve

As TfBP$^+$ cells appeared to be OLGs, the myelin-forming cells in the CNS, we examined whether Schwann cells, the myelin-forming cells in the PNS, are positive to TfBP. As expected, TfBP was localized specifically to Schwann cells in the sciatic nerve. The staining intensity was as great as that of OLGs in the optic nerve, and the immunoreactivity was confined to the cytoplasm and processes of Schwann cells (Fig. 6A, B).

Double immunohistochemical staining

Because TfBP$^+$ cells in the brain parenchyma resembled OLGs in terms of morphology and distribution, double labeling of TfBP with glial or neuronal markers—such as O4 (oligodendrocyte marker), MBP (myelin marker), GFAP (astrocyte marker), RCA-1 (microglia marker), or

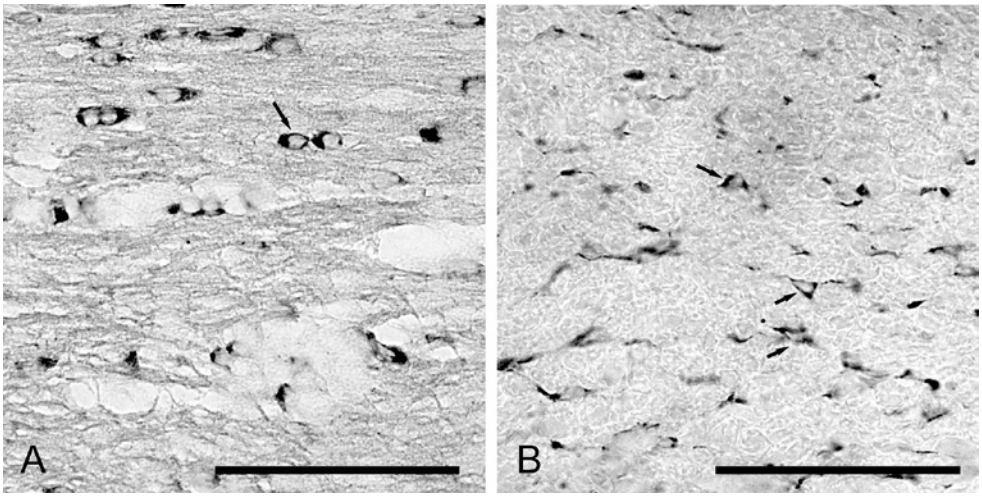

Fig. 6. Longitudinal sections of turtle optic and sciatic nerves stained with TfBP antibody. TfBP immunoreactivity can be observed in interfascicular oligodendrocytes of the optic nerve (**A**) and in Schwann cells of the sciatic nerve (**B**). Note the strong TfBP immunoreactivity of Schwann cells comparable to that of oligodendrocyes in the optic nerve (arrows in **A** and **B**). Scale bars = 100 μm

NeuN (neuron marker)—was performed in the brainstem of the turtle (Fig. 7). As expected, TfBP$^+$ cells were double labeled with O4 (Fig. 7A–C). Interestingly, some TfBP$^+$ cells were devoid of O4 immunoreactivity (Fig. 7C). We therefore, further examined if TfBP is also expressed by other cells types in the turtle central nervous system. Double labeling of TfBP with MBP, GFAP, RCA-1, or NeuN demonstrated that TfBP did not colocalize with any marker in myelin, astrocytes, microglia, or neurons (Fig. 7D–O).

Discussion

This study demonstrated TfBP expression in the turtle nervous system for the first time. At the cellular level, TfBP is specially localized in OLGs, choroid plexus cells, and capillary endothelial cells of the brain, as well as Schwann cells of the peripheral nerves. This pattern of cellular localization of TfBP in the turtle is almost the same as that described previously in the chicken (Cho *et al*., 1997, 1998), which indicates that TfBP is conserved between birds and turtles. Given the fact that birds have a close phylogenic origin with reptiles, our results may reflect the fact that the homology of nervous system genes is conserved between close phylogenetic lines. Among TfBP$^+$ cells, OLGs were the most prominent cell type, which was distributed all over the turtle central nervous system (CNS) with a high density in the white matter and very sparse population in the gray matter. Our findings are the first report of OLG distribution in the reptile brain as previous studies using antibodies for several myelin components including MBP, proteolipid protein (PLP), or myelin-associated glycoprotein (MAG), S100 protein, and glutamine synthetase were unsuccessful in demonstrating OLGs in the adult lizard brain (Birling and Nussbaum, 1995; Monzón-Mayor *et al*., 1998; Romero-Aleman Mdel *et al*., 2003; Santos *et al*., 2006). In this respect, our results showing TfBP as a marker for reptilian oligodendrocytes constitute the basis for comparative and phylogenic studies of the brain, particularly its glial components. As for the function of TfBP, its physiological significance is unclear. We previously suggested a possible role of TfBP in the iron metabolism in the brain, based on previous biochemical studies showing that TfBP demonstrates a high binding activity for transferrin and ferritin (Hayes *et al*., 1994; Poola and Kiang, 1994), in addition to our *in vivo* observation that iron and its binding proteins are colocalized in TfBP$^+$ glial cells (Cho *et al*., 1998). On the other hand, TfBP is known to be a post-transitionally modified form of HSP108, which is regulated by a number of physiological stresses (Hayes *et al*., 1994; Poola and Kiang, 1994). Therefore, TfBP may function as a stress regulating protein to cope with the potential oxidative stress that may arise from iron accumulation in oligodendrocytes. However, the clear

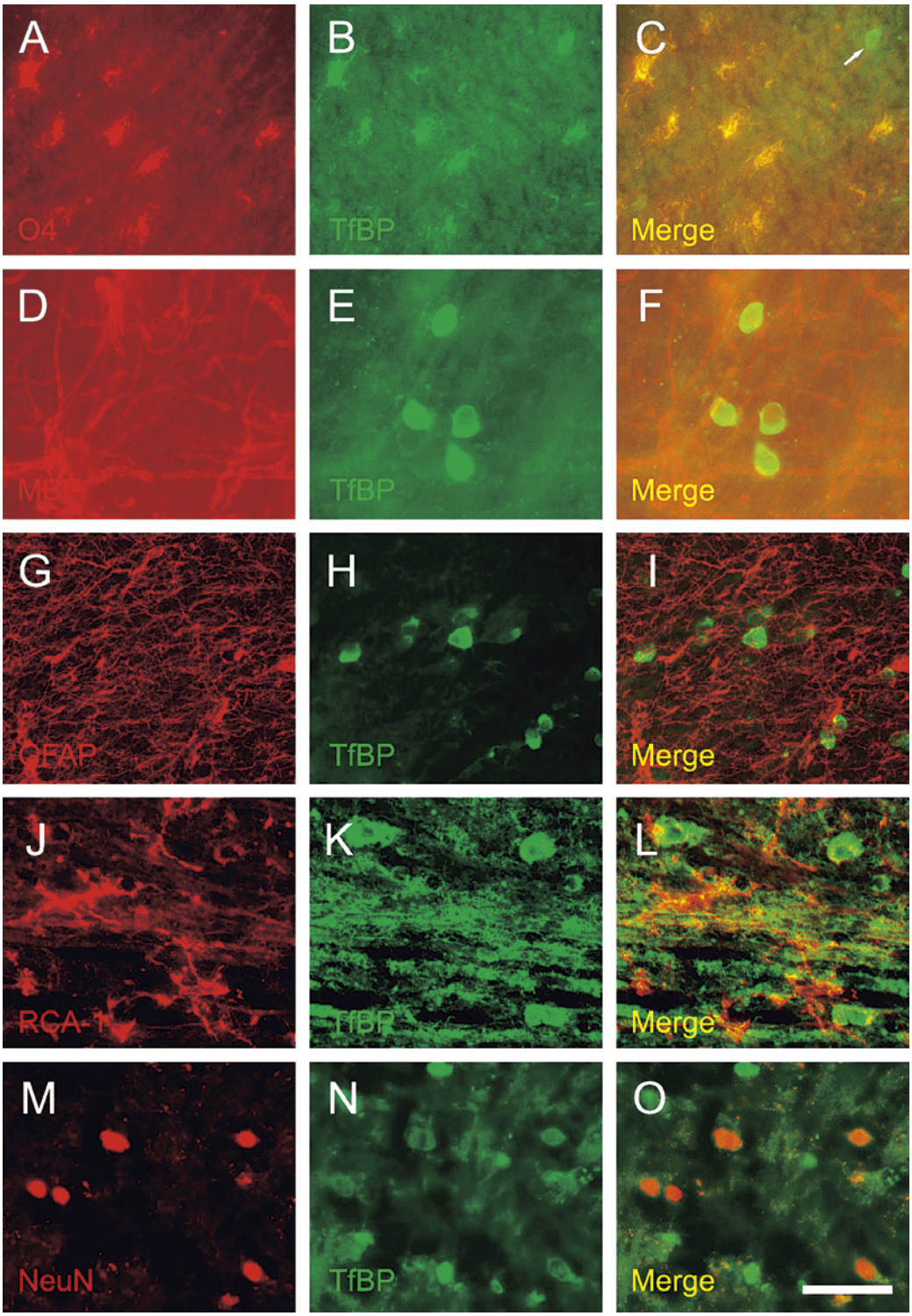

Fig. 7. Double-labeling of TfBP either with O4 (**A**, **B**, **C**), MBP (**D**, **E**, **F**), GFAP (**G**, **H**, **I**), RCA1 (**J**, **K**, **L**), and NeuN (**M**, **N**, **O**) in turtle brainstem. TfBP is co-localized with O4 but not with MBP, GFAP, RCA, or NeuN. Note that some TfBP$^+$ cells are devoid of O4 immunoreactivity (arrow in **C**). Scale bar = 50 μm

elucidation of the TfBP function requires further studies.

We have also provided morphologic and antigenic evidence of OLGs in the turtle retina. Unlike most mammals in which intraretinal myelination is absent, the fishes, amphibians, reptiles, and birds show partial intraretinal myelination (Cima and Grant, 1982; Davila *et al*., 1987; Ono *et al*., 1998; Seo *et al*., 2001; Brosamle and Halpern, 2002). A recent electron microscopic study demonstrated the presence of OLG-like cells in the lizard retina, but immunohistochemical efforts using MBP and PLP antibodies were unsuccessful in the identification of OLGs (Santos *et al*., 2006). In this respect, our results represent the first characterization of the retinal OLGs in the reptile. Our success in revealing a large pool of OLGs is due to the specific properties of TfBP. Unlike astrocytic markers, most OLG markers are regulated developmentally (Miller, 1996), so no single marker can label OLGs of a different functional status. This is particularly true when the markers are components of myelin, insofar as they appear only transiently in OLGs under myelination. They are subsequently transferred from the somata of OLGs to the myelin-containing peripheral processes: this makes it difficult to detect OLGs in the mature CNS. In contrast, TfBP is a cytoplasmic protein that is expressed abundantly in OLGs of the avian CNS (Cho *et al*., 1997). It should also be noted that myelination is completely prenatal in non-mammalian vertebrates whereas this process occurs postnatally in mammals (Hartman *et al*., 1979). For this reason, known OLG makers, particularly myelin components, are more useful for detecting a subpopulation of OLGs that are actively involved in the myelination process that occurs during ontogeny in these species (Birling and Nussbaum, 1995; Romero-Aleman Mdel *et al*., 2003). We have recently found that TfBP is also expressed during ontogeny in the immature OLGs, but is not present in the myelin sheath (Park *et al*., 2007). This property of TfBP greatly facilitates the observation of OLGs by avoiding the covisualization of OLGs and myelin. This property of TfBP was manifested by comparing a TfBP-immunostained profile with that of existing OLG markers such as O4, a marker for immature oligodendrocytes (Ono *et al*., 1997, 1998). In our previous studies, we detected $TfBP^+$ cells colocalized with $O4^+$ cells in the optic head of the adult avian retina and in the embryonic chick spinal cord (Seo *et al*., 2001, Park *et al*., 2007). As expected, TfBP and O4 were colocalized in OLGs in the turtle brainstem.

It is also notable that, in addition to $TfBP^+/O4^+$ cells, we found some $TfBP^+/O4^-$ OLGs in the double-labeled sections. In view of the fact that OLGs constitute a heterogeneous population of cells, it is suggested that TfBP may recognize the OLG population with a greater variety than any of the other OLG markers. In any case, the greater sensitivity of TfBP made it possible to demonstrate a large population of OLGs in the turtle retina. Furthermore, unlike astrocytes that express GFAP throughout the phylogenetic vertebrate line, OLG markers often show species and cellular specificities. TfBP is expressed only in the turtle and chicken; it is not expressed in other classes such as mammals, amphibians, or fishes (Cho and Hyndman, 1991; Cho and Lucas, 1995; Cho *et al*., 1997, 1998). Similarly, the Olig monoclonal antibody is a specific probe for amphibian OLGs (Steen *et al*., 1989) whereas S100 protein, which has been established as an astrocyte marker in mammals (Langley *et al*., 1982), is expressed in an OLG subpopulation in the lizard brain (Romero-Aleman Mdel *et al*., 2003). We also found TfBP immunostaing in OLGs in the snake (data not shown). Thus, it appears that TfBP is expressed in OLGs of the reptilian brain.

Taken together, the previous results and the present findings support the idea that TfBP is a reliable OLG marker in reptiles and birds. It is generally accepted that OLGs are responsible for myelination in the vertebrate CNS; therefore, it is not surprising that $TfBP^+$ OLGs were predominantly observed in the myelinated areas of the turtle CNS. Moreover, because TfBP is also expressed in Schwann cells, TfBP appears to plays a role in these myelinating cells of the turtle. Further study is required to discern the functions of TfBP in avian and reptilian brains.

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