Chemoarchitecture of glial fibrillary acidic protein (GFAP) and glutamine synthetase in the optic nerve of the monkey (Macaca fuscata): An immunohistochemical study

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Summary: An immunohistochemical analysis of the chemoarchitecture of glial fibrillary acidic protein (GFAP) and glutamine synthetase (GS) was conducted in the monkey optic nerve. The optic nerve has been divided into 3 regions: the prelaminar, lamina cribrosa, and retrolaminar regions. However, it currently remains unclear whether the chemoarchitecture of GFAP and GS is homogeneously organized, especially in the retrolaminar region. Strong-to-moderate GFAP immunoreactivity was observed in all 3 regions. The retrolaminar region was further divided into anterior (RLa) and posterior (RLp) retrolaminar regions. More GFAP immunoreactive punctations were observed in the RLa region than in the RLp region. Regarding GS immunoreactivity, moderately GS immunoreactive glial cells were observed in the prelaminar and retrolaminar regions. In the retrolaminar region, there were more of these cells in the RLa region than in the RLp region. GS immunoreactivity was markedly weaker in the prelaminar and retrolaminar regions than in the retina. Thus, the chemoarchitecture of GFAP and GS was heterogeneous organized in the retrolaminar region, and the RLa region was the main GS distribution site in the retrolaminar region. Since GS is a key enzyme of glutamate metabolism, these results may provide clues as to how glutamate is metabolized in the primate optic nerve.

Introduction

The optic nerve is essentially a tract in the brain, these fibers of which possess no neurolemma, and it is surrounded by meninges, which is unlike any peripheral nerve. The tract consists mainly of the axons of retinal ganglion cells. These axons are unmyelinated throughout their course in the retina of the primate and become myelinated when they leave the eye. The chemoarchitecture of the primate optic nerve has been investigated by several research groups, with a focus on the chemoarchitecture of glial fibrillary acidic protein (GFAP) in the optic nerve head of the human, especially around the lamina cribrosa region. The chemoarchitecture of neurofilament (a neuronal marker) and myelin basic protein (an oligodendrocyte marker) has also been examined in the human optic nerve. Consequently, the primate optic nerve has been divided into 3 regions: the prelaminar, lamina cribrosa, and retrolaminar regions.

Chemoarchitecture analyses in the optic nerve have focused on the marker proteins of cells. Many of these proteins are cytoskeletal filament proteins such as neurofilament proteins and/or GFAP. It remains unclear whether new evidence, provided by the chemoarchitecture of cytoskeletal filament proteins, is sufficient. Therefore, an investigation of chemoarchitecture is required, using other types of cell marker proteins such as glutamine synthetase (GS). GS is a metabolic enzyme that catalyzes the amination of glutamate to form glutamine. Immunohistochemically, GS is a marker of Müller cells in the retina and of glial cells in the optic nerve. However, information on the distribution of GS in the primate optic nerve is limited.

As described above, analyses of the chemoarchitect-
tecture of GFAP have focused on the lamina cribrosa region. It has yet to be clarified whether the chemoarchitecture of GFAP is homogeneously organized, especially in the retrolaminar region. In the present study, we conducted an immunohistochemical analysis of the chemoarchitecture of GFAP and GS in the primate optic nerve using the monkey *Macaca fuscata*.

**Materials and Methods**

**Animals and tissue preparation**

Monkey eyeballs including the optic nerve were provided by Dr. Shiro Nakagawa (Professor Emeritus, Kagoshima University Graduate School of Medical and Dental Sciences). Male monkeys (*n* = 2; adult; weighing 11.8 to 12.5 kg; *Macaca fuscata*) were initially anesthetized with ketamine hydrochloride (5 mg/kg, i.m.), followed by sodium pentobarbital (40 mg/kg, i.p.). Under deep anesthesia, the monkeys were perfused transcardially with 4% paraformaldehyde dissolved in 0.1 M PB containing 0.2% picric acid at 4°C (Nakagawa, personal communication). The eyeballs including the optic nerve were removed from the skull, stored in 4% paraformaldehyde in 0.1M PB without picric acid for 7 to 9 days, and then immersed in 30% sucrose in 0.1 M PB containing 0.2% picric acid at 4°C (Nakagawa, personal communication). The eyeballs including the optic nerve were frozen in powdered dry ice, and sectioned in the meridian plane at a thickness of 40 µm on a freezing microtome.

**Methods**

**Antibody characterization**

- **Glial fibrillary acidic protein (GFAP)**
  - The affinity purified anti-GFAP rabbit antibody (Dako, Glostrup, Denmark) recognized a single protein band of approximately 50 kDa in extracts from the mouse retina.
  - Astrocytes were immunolabeled with this antibody against GFAP. The staining obtained with the anti-GFAP antibody (Dako) in the monkey was similar to that previously reported in the marmoset.

- **Glutamine synthetase (GS)**
  - The rabbit anti-GS antibody (Sigma-Aldrich, Saint Louis, MO, USA) recognized a single protein band of 45 kDa in extracts from the rat brain. The staining of GS in immunoblotting was specifically inhibited with the GS immunizing peptide (amino acids 357–373 with N-terminally added lysine). This amino acid sequence was identical in human, bovine, rat, hamster and pig GS, and was highly conserved in chicken GS (single amino acid substitution; manufacturer’s technical information).
  - Müller cells in the retina and glial cells in the optic nerve were labeled with these antibodies against GS. The staining obtained with the anti-GS antibody (Sigma-Aldrich) in the monkey was similar to published results on the monkey.

**Immunohistochemistry**

Sections of the monkey retina and optic nerve were processed using single immunoperoxidase staining as previously described with minor modifications. Free-floating sections were bleached for 1 hour with 50% methanol and 1.5% hydrogen peroxide diluted with Milli-Q water at 4°C, and then washed three times each for 15 minutes in 0.02M phosphate buffered saline (PBS) containing 0.3% Triton X-100 (PBST). The sections were pre-incubated for 2 hours with 10% normal goat serum (NGS) in 0.1M PB containing 0.3% Triton X-100 (10% NGS blocking solution) at 4°C. The sections were incubated for 3 days with a rabbit polyclonal antibody in a 10% NGS blocking solution at 4°C (Table 1). After the primary immunoreaction, the primary antibody was washed out with PBST. The sections were then incubated for 1 day at 4°C with biotinylated anti-rabbit goat IgG (Dako, 1:1,000) in PBS containing 5% NGS. After the secondary immunoreaction, the sections were washed three times each for 10 minutes in PBS, and incubated for 1 day at 4°C with peroxidase-conjugated streptavidin (Dako, 1:1,000) in PBS. They were washed three times each for 15 minutes in 0.05 M Tris-HCl buffer (pH 7.6), and subjected to a dark-violet-black coloring reaction with 0.02% 3,3′-diaminobenzidine tetrahydrochloride (DAB; Wako Pure Chemical Industries, Osaka, Japan) and 0.6% nickel ammonium sulfate hexahydrate in 0.05 M Tris-HCl buffer (pH 7.6) containing 0.0036% hydrogen peroxide for 10-15 minutes at 4°C. After three washes in PBS, the sections were mounted onto hydrophilic silanized slides (Dako Japan, Tokyo, Japan) in an equal-parts mixture of a 0.6% gelatin solution and PBS. After being air-dried, they were dehydrated with a graded series of ethanol solutions, immersed in xylene, and embedded in Entellan new mountant (Merck KgaA, Darmstadt, Germany).

In all cases, each staining protocol was performed on a minimum of two optic nerves from two separate monkeys.

**Photomicrographs**

Tiling images of the monkey retina and optic nerve
were taken with a Virtual Slide System (VS120-L100; Olympus, Tokyo, Japan) in the extended focus imaging mode. Images were transferred to Adobe Photoshop CS5 (Adobe Systems, San Jose, CA, USA). The brightness and contrast of images were adjusted. No other adjustment was made.

Results

Division of the monkey optic nerve

We described our monkey criteria for determining the border of each region of the optic nerve below. The monkey (primate) optic nerve has been divided into three regions: the prelaminar (PL), lamina cribrosa (LC), and retrolaminar (RL) regions. The border between the LC and the RL regions was defined by conventional criteria such that it was set at the posterior limit of the scleral level of the optic nerve2–5, 15–17. Since our monkey criteria for the LC region followed Anderson15) and Balaratnasingam et al.5), the LC region was divided into two subregions: the anterior (LCa) and posterior (LCp) lamina cribrosa regions. The LCa region was located at the level of the choroid. The LCp region was the lamina cribrosa proper, and was located at the level of the sclera. Accordingly, the border between the PL and LC regions was set at the boundary between the retinal pigment epithelium and choroid (Figs. 1–3).

The RL region was divided into two subregions: the anterior (RLa) and posterior (RLp) retrolaminar regions. The boundary between RLa and RLp was set at a line 3 mm behind the border between the LC and RL regions. Accordingly, the longitudinal length of the RLa region was approximately 3 mm (Fig. 1).

Chemoarchitecture of the monkey optic nerve

Strong-to-moderate GFAP immunoreactivity was observed in the nerve fiber layer of the retina (NFL), and in all of the 3 regions: the PL, LC, and RL regions (Fig. 1A). Strongly GFAP immunoreactive filaments were observed in the NFL and PL region. These filaments were also seen in the ilm (inner limiting membrane of the optic nerve head [Elschnig]; Fig. 2E). The chemoarchitecture of GFAP in the RLa region was different from that in the RLp region. A considerable number of defects in GFAP immunoreactivity were observed in the RLa region, while a small number of these defects were noted in the RLp region (Figs. 1A, 2A,C). Moreover, there were more GFAP immunoreactive punctuations in the RLa region (Fig. 2D) than in the RLp region (Fig. 2B).

GS immunoreactivity was observed in the NFL, ilm, PL region, and in the RL region; however, it was extremely weak in the LC region (Fig. 1B). Strong GS immunoreactivity was observed in the NFL and ilm (Fig. 3E). Moderately GS immunoreactive glial cells were seen in the PL region. The chemoarchitecture of GS in the RLa region was different from that in the RLp region. Rows of GS immunoreactive glial cells were clearly observed in the RLa region, while they were detectable in the RLp region (Fig. 1B). There were more moderately GS immunoreactive glial cells in the RLa region (Fig. 3C,D) than in the RLp region (Fig. 3A,B). GS immunoreactivity was markedly weaker in the PL and RL regions than in the retina (Fig. 1B).

Discussion

Comparison with previous findings

Our monkey criteria for determining the border of each region of the optic nerve followed Anderson15) and Balaratnasingam et al.5) in order to compare between experimental data on the monkey and those on the rodent in the future. Accordingly, the border between the PL and LC regions was set at the boundary between the retinal pigment epithelium and choroid. The border between the LC and RL regions was defined by conventional criteria such that it was set at the posterior limit of the scleral level of the optic nerve2–5, 15–17.

GFAP immunoreactivity was observed in the PL, LC, and RL regions of the human optic nerve. Strong GFAP immunoreactivity was seen in the ilm (inner limiting membrane of the optic nerve head [Elschnig]) and also in the PL region2–4). Similar results were obtained in the monkey in the present study.

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Table 1. Primary antibodies used in this study.

| Antigen                              | Immunogen                                                                 | Manufacturer, cat. no., (lot no.) | Host            | Dilution   |
|--------------------------------------|---------------------------------------------------------------------------|-----------------------------------|-----------------|------------|
| Glial Fibrillary Acidic Protein (GFAP) | GFAP isolated from the cow spinal cord                                   | Dako (Glostrup, Denmark), Z0334, (00085136) | Rabbit; polyclonal | 1:2,000    |
| Glutamine synthetase (GS)            | A synthetic peptide corresponding to the C-terminus of mouse glutamine synthetase (amino acids 357–373 with N-terminally added lysine) conjugated to KLH (keyhole limpet hemocyanin), a carrier for haptens. | Sigma-Aldrich (Saint Louis, MO, USA), G2781, (115K4794) | Rabbit; polyclonal | 1:10,000  |
Fig. 1. Distribution of astrocytes and glutamine synthetase-immunoreactive glial cells in the monkey retina and optic nerve. The panels show longitudinal sections through the paramedian part, and represent single immunoperoxidase staining for an astrocyte marker (glial fibrillary acidic protein, GFAP; A) and for a glial cell marker (glutamine synthetase, GS; B). The section of B is adjacent to that of A. Arrows indicate the border between the prelaminar (PL) and lamina cribrosa (LC) regions, or the boundary between the LC and retrolaminar (RL) regions. Double-arrowheads represent the border between the anterior (RLa) and posterior (RLp) retrolaminar regions. Open arrowheads in the RLp region of A, RLa region of A, RLp region of B, and RLa region of B indicate the same structures as those in Figures 2A, 2C, 3A, and 3C, respectively. Panels A and B are tiling images that were taken with a Virtual Slide System (VS-120-L100; Olympus, Tokyo, Japan) in the extended focus imaging mode. Note that the chemoarchitecture of GFAP and GS in the RLa region differed from that in the RLp region. Regarding GFAP, a considerable number of defects in GFAP immunoreactivity were observed in the RLa region, while a small number of these defects were noted in the RLp region (A). Regarding GS, rows of GS immunoreactive glial cells were clearly observed in the RLa region, while they were detectable in the RLp region (B). Moreover, the longitudinal length of the RLa region was approximately 3 mm (A, B). NFL, nerve fiber layer of the retina. Scale bars = 1 mm and 3 mm in B for A.
Fig. 2. Distribution of astrocytes in the monkey retina and optic nerve. The panels show single immunoperoxidase staining for an astrocyte marker (glial fibrillary acidic protein, GFAP). Panels A-B and C-D represent GFAP immunoreactivity in the posterior (Rlp) and anterior (RLa) retrolaminar regions, respectively. Panels B and D are higher magnification photomicrographs in the Rlp and RLa regions, respectively. Open arrowheads in A and C indicate the same structures as those in the Rlp and RLa regions of Figure 1A, respectively. Solid arrowheads in A and C show the same astrocytes as those in B and D, respectively. Panel E represents GFAP immunoreactivity in the nerve fiber layer of the retina (NFL), prelaminar (PL) region, and lamina cribrosa (LC) region. Arrows in E indicate the border between the PL and LC regions, or the boundary between the LC and RLa regions. The double arrowhead in E shows the border between the anterior (LCa), and posterior (LCp) lamina cribrosa regions. Panels A-E are tiling images that were taken with a Virtual Slide System (VS-120-L100; Olympus, Tokyo, Japan) in the extended focus imaging mode. Note that the chemoarchitecture of GFAP in the RLa region differed from that in the RLP region. Several defects in GFAP immunoreactivity were observed in the RLa region (C), while a few of these defects were noted in the RLP region (A). Moreover, there were more GFAP immunoreactive punctuations in the RLa region (D) than in the RLP region (B). Note also that strongly GFAP immunoreactive filaments were seen in the NFL and PL region (E). Ch, choroid; ilm, inner limiting membrane of the optic nerve head [Elschnig]; Sc, sclera. Scale bars = 50 µm in D for B, 500 µm in E for A and C.
Fig. 3. Distribution of glutamine synthetase-immunoreactive glial cells in the monkey retina and optic nerve. The panels show single immunoperoxidase staining for a glial cell marker (glutamine synthetase, GS). Panels A-B and C-D represent GS immunoreactivity in the posterior (RLp) and anterior (RLa) retrolaminar regions, respectively. Panels B and D are higher magnification photomicrographs in the RLp and RLa regions, respectively. Open arrowheads in A and C indicate the same structures as those in the RLp and RLa regions of Figure 1B, respectively. Solid arrowheads in A and C show the same GS-immunoreactive glial cells as those in B and D, respectively. Panel E represents GS immunoreactivity in the nerve fiber layer of the retina (NFL), prelaminar (PL) region, and lamina cribrosa (LC) region. Arrows in E indicate the border between the PL and LC regions, or the boundary between the LC and RLa regions. The double arrowhead in E shows the border between the anterior (LCa) and posterior (LCp) lamina cribrosa regions. Panels A-E are tiling images that were taken with a Virtual Slide System (VS-120-L100; Olympus, Tokyo, Japan) in the extended focus imaging mode. Note that the chemoarchitecture of GS in the RLa region differed from that in the RLp region. There were more moderately GS immunoreactive glial cells in the RLa region (C-D) than in the RLp region (A-B). Note also that GS immunoreactivity was observed in the NFL, PL region, and RL region, but was extremely weak in the LC region (E). Ch, choroid; ilm, inner limiting membrane of the optic nerve head; Sc, sclera. Scale bars = 50 µm in D for B, 500 µm in E for A and C.
**Defects in GFAP immunoreactivity in the retrolaminar (RL) region**

In the primate, connective tissue is distributed not only in the LC region, but also in the RL region. In the RL region, axonal fibers are divided into fascicles by connective tissue sheaths that extend parallel to the direction of optic nerve bundles\(^4\,\,5\). The distribution pattern of these connective tissue sheaths was similar to that of the defects in GFAP immunoreactivity in the RL region. These defects may have been due to a GFAP negative immunoreaction against these connective tissue sheaths.

**Heterogeneous distribution of GFAP and glutamine synthetase (GS) in the RL region**

The heterogeneous distribution of axonal cytoskeleton proteins along the sagittal plane of the optic nerve was demonstrated in the RL region of the primate. These proteins were neurofilament subunits, actin, and microtubule associated protein (MAP)-1. The intensity of these proteins in the portion close to the LC/RL border was significantly different from that in the segment far from the LC/RL border\(^6\). It is probable that not only axonal cytoskeleton proteins, but also glial cell marker proteins (GFAP and GS) were heterogeneously distributed along the sagittal plane of the primate optic nerve. This notion is supported by our findings that GFAP and GS were also heterogeneously distributed along the sagittal plane of the rat optic nerve (Kawano, unpublished observations).

Moderately GS immunoreactive glial cells were distributed in the PL and RL regions, but not in the LC region. In the RL region, there were more of these glial cells in the RL\(_a\) region than in the RL\(_p\) region. Accordingly, the LC region was not a GS distribution site, whereas RL\(_a\) was the main site in the RL region.

It currently remains unknown why GS was heterogeneously distributed in the optic nerve. Since GS is a metabolic enzyme of glutamate\(^6\,\,9\), the expression of genes that regulate glutamate metabolism in the RL\(_a\) region may differ from that in the RL\(_p\) region. DNA microarray analyses may assist in clarifying these differences by using tissue specimens from the RL\(_a\) and RL\(_p\) regions. Moreover, these analyses may provide molecular biological evidence for elucidating glutamate metabolism in the RL region.

**Conclusion**

The chemoarchitecture of GFAP and GS was heterogeneously organized in the RL region, and the RL\(_a\) region was the main GS distribution site in the RL region of the monkey optic nerve. Since GS is a key enzyme of glutamate metabolism\(^6\,\,9\), the results obtained here may provide clues as to how glutamate is metabolized in the primate optic nerve.

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