Chemoarchitecture of glial fibrillary acidic protein (GFAP) and glutamine synthetase in the rat optic nerve: 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 rat optic nerve. The optic nerve has been divided into 3 regions: the intraretinal, unmyelinated, and myelinated regions. However, it currently remains unclear whether the chemoarchitecture of GFAP and GS is homogeneously organized, especially in the myelinated region. The intraretinal region was divided into intraretinal regions 1 (i1) and 2 (i2). GFAP immunoreactivity was very strong in the i2 and unmyelinated regions, and strong in the i1 region. GS immunoreactivity was moderate in the i1 and i2 regions, and weak in the unmyelinated region. The myelinated region was separated into myelinated regions 1 (m1) and 2 (m2). In the m1 region, GFAP immunoreactivity was strong and GS immunoreactivity was moderate; however, GFAP immunoreactivity was moderate and GS immunoreactivity was weak in the m2 region. Thus, the chemoarchitecture was heterogeneously organized in the myelinated region, with the i1, i2 and m1 regions being the main GS distribution sites. Moreover, most GS-immunoreactive glial cells were oligodendrocytes in the myelinated region. Since GS is a key enzyme in glutamate metabolism, these results may facilitate future investigations for a clearer understanding of glutamate metabolism.

Introduction

The optic nerve is in essence a tract in the brain, the fibers of which possess no neurolemma, and is surrounded by meninges, which is unlike any peripheral nerve. The tract consists mainly of the axons of retinal ganglion cells¹ (RGCs). These axons are unmyelinated throughout their course in the retina of the rodent and become myelinated when they leave the eye². The chemoarchitecture of the optic nerve has been investigated by several research groups, with a focus on the distributions of astrocytic filaments and myelinated axons³–⁷. A very high concentration of astrocytic filaments has been observed at the junction of the retina and optic nerve. The posterior limit of this concentration coincides with the beginning of axon myelination. Bundles of myelinated axons have been shown to interdigitate with astrocytes⁴. Accordingly, the rodent optic nerve has been divided into at least 3 regions: the intra-retinal (prelaminar), astrocytic filament dense (laminar), and astrocytic filament sparse (retrolaminar, retrobulbar, or myelinated) regions⁵–⁶, ⁸–⁹.

The anterior portion of the astrocytic filament dense (laminar) region was recently named the “glial lamina” in the mouse. The glial lamina has been observed in an equivalent location to that of the lamina cribrosa in humans. Furthermore, the arrangement of glial cells in the glial lamina was previously shown to be similar to that in the lamina cribrosa of humans⁵. Astrocytes do not tile in the glial lamina; each astrocyte participates in ensheathing approximately one-quarter of all axon bundles in the optic nerve, and each glial tube contains the processes of approximately nine astrocytes⁷. Thus, morphological findings have accumulated on the chemoarchitecture of the rodent optic nerve. However, the detailed chemoarchitecture of the rodent optic nerve...
remains unclear, especially in the intra-retinal (prelamellar) and astrocytic filament sparse (retrolaminar, retrolaminar or myelinated) regions. Accordingly, it is possible to hypothesize that this chemoarchitecture is not homogeneously organized in each region.

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 glial fibrillary acidic protein (GFAP). It remains unclear whether new evidence, provided by the chemoarchitecture of cytoskeletal filament proteins, is sufficient. Therefore, a chemoarchitecture analysis using other types of cell marker proteins, such as glutamine synthetase (GS), is required. 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 rodent optic nerve remains limited.

The optic nerve is composed of microglia, blood vessels, and an extracellular matrix, as well as neuronal axons, astrocytes, and oligodendrocytes. It is highly desirable to examine the chemoarchitecture of all these structures. However, we focused on the chemoarchitecture of neurons, astrocytes, and oligodendrocytes, because these cells play important roles in transmitting neuronal information. In the present study, we conducted a detailed immunohistochemical analysis of the chemoarchitecture of the normal rat optic nerve. We were specifically interested in the distributions of neurofilaments (a neuronal marker), GFAP (an astrocyte marker), RIP (an oligodendrocyte marker), and GS (a glial cell marker) in the rat optic nerve.

**Materials and methods**

**Animals and tissue preparation**

Male rats (n = 13; 12 weeks old; Slc:SD; CLEA, Japan, Tokyo, Japan) were deeply anesthetized with sodium pentobarbital (50 mg/kg, i.p.), and perfused transcardially with 4% paraformaldehyde dissolved in 0.1 M sodium phosphate buffer (PB; pH 7.4) at 4°C. The eyeballs including the optic nerve were removed from the skull, stored in the same fixative for 48 hours, and then immersed in 30% sucrose in 0.1 M PB at 4°C until they sank. The eyeballs including the optic nerve were frozen in powdered dry ice, and sectioned in the meridian plane at a thickness of 25 µm on a cryostat. Sections were collected in a cryoprotectant medium (33.3% sucrose, 1% polyvinylpyrrolidone-K-30, and 33.3% ethylene glycol in 0.067 M sodium phosphate buffer (pH 7.4) containing 0.067% sodium azide) and stored at −30°C prior to use.

All experimental protocols for this study were approved by the Committees on the Ethics of Animal Experimentation at Kagoshima University, and the protocols were conducted according to the guidelines for Animal Research of Kagoshima University and The Law (No. 50, June 2005) and Notification (No. 88, Ministry of Environment, April 2006) of the Japanese Government.

**Antibody characterization**

Please see Table 1 for a list of all antibodies used.

**Aquaporin 4 (AQP4)**

The anti-AQP4 rabbit antibody (Millipore, Temecula, CA, USA) stained a single protein band at approximately 45 kDa in the rat brain on immunoblots. This antibody was specific for AQP4, since this band was not observed when extracts from the rat brain were incubated with this antibody preabsorbed with the control protein antigen (manufacturer’s technical information).

Astrocytes in the myelinated region of the rat were immunolabeled with this antibody against AQP4. The staining obtained with the anti-AQP4 antibody in the rat was similar to published results on the rat.

**Glial fibrillary acidic protein (GFAP)**

Anti-GFAP rabbit serum (Chemicon, Temecula, CA, USA) stained a single protein band of 51 kDa in the mouse brain on immunoblots (Kawano, unpublished observations). This serum was also specific for GFAP on immunoblots in extracts from the mouse spinal cord and rat retina.

The anti-GFAP mouse monoclonal antibody (clone GA5; Chemicon) stained a single protein band at approximately 51 kDa, which corresponded to GFAP on the immunoblots of extracts from a human glioma cell line (U33CG/343MG). This antibody immunohistochemically recognized astrocytes and Bergmann glia cells. This antibody showed no cross-reactivity with vimentin (manufacturer’s technical information). This information was supported by results showing that this antibody did not recognize vimentin in Müller cells of the rodent retina (present study).

Astrocytes were immunolabeled with these antibodies against GFAP. The staining obtained with these anti-GFAP antibodies in the rat was similar to published results on the rat.

**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).

The mouse monoclonal anti-GS antibody (clone 6; BD Biotech, San Diego, CA, USA) stained a single protein band of 51 kDa in the mouse brain on immunoblots when extracts from the rat brain were incubated with this antibody preabsorbed with the control protein antigen (manufacturer’s technical information).
Biosciences Pharmingen, San Diego, CA, USA) visualized a single protein band (45 kDa) in extracts from the rat brain on immunoblots (manufacturer’s technical information).

Müller cells in the retina and glial cells in the optic nerve were labeled with these antibodies against GS\(^{11}\). The staining obtained with the two antibodies in the rat was similar to that previously reported in the mouse\(^{6, 27, 30}\) and rat\(^{11, 31}\).

**Neurofilament 200-kDa heavy chain (neurofilament 200; neurofilament H non-phosphorylated)**

The polyclonal anti-neurofilament 200 antibody (Sigma-Aldrich) recognized a single protein band of 200 kDa in extracts from the rat brain cytosolic S1 fraction (manufacturer’s technical information) and in those from the mouse brain\(^{32}\). The antibody showed wide species cross-reactivity (manufacturer’s technical information).

The mouse monoclonal antibody against neurofilament H non-phosphorylated (clone SMI-32; Covance, Princeton, NJ, USA) visualized two bands (200 and 180 kDa) on conventional immunoblots, which merged into a single neurofilament H line on two-dimensional blots\(^{33–35}\). The SMI-32 antibody reacted with a non-phosphorylated epitope from neurofilament H of most mammalian species. The reaction was masked when the epitope was phosphorylated\(^{36}\). The SMI-32 antibody immunocytochemically visualized neuronal cell bodies, dendrites, and some thick axons in the central and peripheral nervous systems, whereas thin axons were not revealed (manufacturer’s technical information).

The staining obtained with these antibodies against neurofilament 200 and/or neurofilament H was similar to published results on the mouse\(^5\) and rat\(^{22}\).

**Oligodendrocyte lineage transcription factor 2 (Olig2)**

The anti-Olig2 mouse monoclonal antibody (clone 211F11.1; Millipore) stained a single protein band of 37 kDa in the mouse brain on immunoblots. In addition to the mouse, this antibody was predicted to react with human and rat Olig2 based on 100% sequence homology (manufacturer’s technical information).

Oligodendrocyte progenitors and mature oligodendrocytes in adulthood were both immunostained with the anti-Olig2 antibody\(^{37–39}\) (manufacturer’s technical information). The staining obtained with the anti-Olig2 antibody was confined to the cell nuclei, and labeled nuclei were distributed in the myelinated region of the rat optic nerve.

**Oligodendrocytes**

Mature oligodendrocytes were immunostained with the mouse monoclonal antibody against oligodendrocytes\(^{40}\) [RIP] (clone NS-1; Chemicon). The specificity of this antibody was strictly verified in the rat CNS\(^{40}\) (central nervous system). Strong RIP staining was mainly confined to the white matter in the rat cerebellum, although moderate RIP staining was distributed in the granular layer. RIP staining was hardly detected in the molecular layer, which is composed of unmyelinated parallel fibers\(^{40}\).

The staining obtained with this antibody against oligodendrocytes [RIP] was similar to that previously reported in the rat\(^4, 28\).

**Immunohistochemistry**

Sections were processed using double immunohistochemistry as previously described\(^9\). Free-floating sections were pre-incubated for 2 hours with 10% normal goat serum (NGS) in 0.1 M PB containing 0.3% Triton X-100 (10% NGS blocking solution) at 4 °C, and were then immunoreacted for 4 days with a mixture of rabbit and mouse primary antibodies in a 10% NGS blocking solution at 4 °C (Table 1). After two rinses for 10 minutes in 0.02 M phosphate buffered saline (PBS) containing 0.3% Triton X-100 (PBST), the sections were incubated with a mixture of two secondary antibodies in PBS containing 5% NGS and 0.3% Triton X-100 for 24 hours at 4 °C. The two secondary antibodies used were Alexa Fluor 488 conjugated with the F(ab\(^\prime\))\(_2\) fragment of goat anti-rabbit IgG (H+L) (1:200; Molecular Probes, Eugene, OR) and Alexa Fluor 594 conjugated to the F(ab\(^\prime\))\(_2\) fragment of goat anti-mouse IgG (H+L) (1:200; Molecular Probes). The sections were washed once for 10 minutes in PBST, and then twice 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, the sections were subjected to nuclear staining by using a bisBenzimide (Hoechst 33258, Sigma-Aldrich; 0.1 mg/ml) solution, and coverslipped with VECTASHIELD mounting medium (Vector Laboratories, Burlingame, CA, USA).

In all cases, each staining protocol was performed on a minimum of 3 optic nerves from 3 separate rats. GFAP staining was performed on a total of 18 optic nerves from 13 separate rats. GS staining was performed on 20 optic nerves from 12 separate rats. Oligodendrocyte [RIP] staining was performed on 12 optic nerves from 7 separate rats.

In order to eliminate the possibility of any cross-reaction between the secondary and primary antibodies from the wrong species, one of the two primary antibodies was removed. No cross-reactivity was observed in these control experiments (data not shown).

**Photomicrographs**

Fluorescent photomicrographs were taken with an LSM700 confocal laser scanning microscope (Carl Zeiss Jena GmbH, Jena, Germany) at the Joint Research Laboratory, Kagoshima University Graduate School of Medical and Dental Sciences, or an FV500 confocal laser scanning microscope (Olympus, Tokyo, Japan) at the...
Table 1. Primary antibodies used in this study.

| Antigen                          | Immunogen                                                                 | Manufacturer, cat. no., (lot no.)                  | Host         | Dilution |
|----------------------------------|---------------------------------------------------------------------------|---------------------------------------------------|--------------|----------|
| Aquaporin 4                      | GST fusion protein with residues 249-323 of rat aquaporin 4 (SwissProt accession no. P47863) | Millipore (Temecula, CA, USA), AB3594, (2493949)   | Rb; polyclonal | 1:200    |
| Glial Fibrillary Acidic Protein (GFAP) | Purified bovine GFAP                                                  | Chemicon (Temecula, CA, USA), AB5804, (0604027868) | Rb; polyclonal | 1:500    |
| GFAP                             | Purified GFAP from the porcine spinal cord                            | Chemicon, MAB360, (25040157)                     | Ms; monoclonal | 1:500    |
| 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 | Sigma-Aldrich (Saint Louis, MO, USA), G2781, (115K4794) | Rb; polyclonal | 1:5,000  |
| GS                               | Sheep glutamine synthetase (1-373: Full Length)                        | BD Biosciences (San Diego, CA, USA), Pharmedgen, 610517, (01915) | Ms; monoclonal | 1:500    |
| Neurofilament 200                | Purified neurofilament 200 from the bovine spinal cord                 | Sigma-Aldrich, N4142, (059K4872)                 | Rb; polyclonal | 1:100    |
| Neurofilament H non-phosphorylated [SMI-32] | Fischer 344 rat hypothalamus                                         | Covance (Princeton, NJ, USA), SMI-32R, (E11CF00693) | Ms; monoclonal | 1:250    |
| Oligodendrocyte lineage transcription factor 2 (Olig2) | Recombinant protein corresponding to human Olig2                      | Millipore, MABN50, (2388987)                     | Ms; monoclonal | 1:500    |
| Oligodendrocytes [RIP]           | Rat olfactory bulb                                                     | Chemicon, MAB1580, (25040064)                    | Ms; monoclonal | 1:20,000 |

Abbreviations: Rb, rabbit; Ms, mouse.

Center for Chronic Viral Diseases, Kagoshima University Graduate School of Medical and Dental Sciences. 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.

Image Analysis

The quantitation of all images was performed using ImageJ (Version 1.45; developed by Wayne Rasband, National Institute of Mental Health, Bethesda, MD, USA).

Measurement of GFAP immunoreactivity and GS immunoreactivity in the optic nerve

Confocal microscopic images of GFAP immunoreactivity and GS immunoreactivity were assembled using a photomerge function in Adobe Photoshop CS5 (Layout; Reposition). Measurements were performed in the shaded area of each region depicted in Figure 1A. Before measurements, we cut the measurement areas off the images. Each measurement area was analyzed in gray scale (0-255 pixel intensity). We used a quantitative histogram function in ImageJ to calculate the mean pixel intensity in each measurement area. The mean pixel intensity in each region (each measurement area) was then used for statistical analyses.

Cell Count

GS and/or Olig2 immunoreactive cells were counted in the shaded areas of the m1 and m2 regions depicted in Figure 1A.

Statistical Analysis

All statistical analyses were performed with IBM SPSS statistics 23 for Windows (SPSS Inc., Chicago, IL, USA). Prior to statistical analyses being conducted, Shapiro-Wilk testing and Levene testing were performed on all data to determine whether data were normally and homogeneously distributed, respectively. Data were analyzed by a one-way ANOVA with Tukey’s multiple comparisons test, a one-way ANOVA with the Games-Howell multiple comparisons test, or by the Two Sample t-test. P < 0.05 was considered significant.

Results

Division of the rat optic nerve

A definition of the optic nerve division was necessary for the description and interpretation of experimental results; therefore, we described our rat criteria for determining the border of each region of the optic nerve. The rat criteria principally followed our previously reported mouse criteria.

As shown in Figure 1A, the rat optic nerve in the orbit was divided into three regions: the intraretinal (i), unmyelinated (u), and myelinated (m) regions. This classification was defined by the position of the sensory retina, the distribution of oligodendrocytes (myelinated nerve fibers), and by the strength of GFAP immunoreactivity. The border between the i and u regions was set at the boundary between the sensory retina and retinal pigment epithelium. Only a few myelinated nerve fibers were detected in the u region; however, very strong GFAP immunoreactivity was observed. The border between
Fig. 1. **A:** Diagram showing a division scheme in the rat optic nerve used in this study. In this diagram, shading demonstrated areas where quantitative analyses were performed. The optic nerve was divided into 5 regions: intraretinal regions 1 (i1) and 2 (i2), unmyelinated (u) region, and myelinated regions 1 (m1) and 2 (m2). The dashed lines made up of dashes and shorter gaps indicate the border between each region: for example, the border between unmyelinated and myelinated regions. The dashed lines made up of dashes and longer gaps show the boundary between each subregion: for example, the boundary between m1 and m2. The two up-down arrows at the right side of the u region, the lengths of which are the same, indicate that the center of the shading in this region was located on the longitudinal halfway line of this region. The three up-down arrows at the right side of the m region, the lengths of which are the same, also show the positions of shading in the m1 and m2 regions. Note that the m1 region was thicker than the m2 region. Moreover, the outline of the optic nerve was bent at the border between the m1 and m2 regions. NFL, nerve fiber layer of the retina. Scale bar = 200 µm. **B:** Distribution of astrocytes and oligodendrocytes in the normal rat optic nerve. The panel shows a longitudinal section through the paramedian part, and represents double immunostaining for an astrocyte marker (glial fibrillary acidic protein, GFAP; Alexa Fluor 488 label; green) and an oligodendrocyte marker (RIP; Alexa Fluor 594 label; magenta). Cell nuclei were labeled with bisBenzimide (Hoechst 33258; blue). The arrows indicate the border between the intraretinal and u regions or that between the u and myelinated regions. The double arrowheads represent borders between the subregions: the border between i1 and i2 regions, or the border between m1 and m2 regions. The panel is a photo montage composed of 11 square fluorescent images. These images were taken with an LSM 700 confocal microscope (Carl Zeiss, Jena, Germany). Parts of the background were filled up with a black color. Note that the outline of the optic nerve was bent at the border between the m1 and m2 regions. A small notch was observed at the bottom of the bend. Interestingly, GFAP immunoreactivity in the m1 region was stronger than that in the m2 region. Columns of astrocytes were seen to interdigitate with myelinated (RIP-immunoreactive) nerve fiber bundles in the m1 region. A cluster of GFAP-negative circles or eclipses was observed in the proximal (posterior) half of the u region. Some eclipses were longitudinally elongated. NFL, nerve fiber layer of the retina. Scale bar = 200 µm.
Chemoarchitecture of the intraretinal (i) region

Astrocytic filaments were not only longitudinally distributed along unmyelinated nerve fibers in the i1 region, but were also transversely distributed on them (data not shown). The distribution pattern of astrocytic filaments in the i2 region was similar to that in the u region, as described below. In addition, GFAP immunoreactivity was weak in the i1 region (Figs. 1B, 2D, 4P), while it was strong in the i2 region (Figs. 1B, 2D, 4P, 4T).

The inner limiting membrane of the optic nerve head [Elschnig] (ilm) was observed on the i region and on the medial-most part of the NFL. In addition, a high concentration of the intermediary tissue (hci) was seen beneath the surface of the optic nerve at the border between the i and u regions. Strong GFAP immunoreactivity was observed in the ilm and the hci. Moderate GS immunoreactivity was seen in the astrocytes (Fig. 4M–T). The intermediary tissue at the termination of the retina [Kuhnt] (imt) was detected between the i region and the retinal layers, except for the NFL and the inner limiting membrane (Fig. 4M–P).

GFAP immunoreactivity was very strong in the u region. The large proportion of astrocytic filaments in the u region extended perpendicular to the optic nerve axis (Figs. 1B, 2B, 4J). In some confocal images, these filaments were distributed alongside unmyelinated nerve fibers. Clusters of astrocytic filaments were observed between unmyelinated nerve fiber bundles (data not shown). In addition, weak GS immunoreactivity was detected in the astrocytes of the u region (Figs. 2, 4I–L).

Large GFAP-negative or GS-negative areas were observed in the proximal (posterior) half of the u region in longitudinal sections approximately through the median part. Densely packed cell nuclei were also noted in these areas, many of which were fusiform in shape and/or strongly labeled for bisBenzimide (Fig. 3). A cluster of GFAP-negative circles and eclipses was observed in the proximal (posterior) half of the u region in longitudinal sections through the paramedian or peripheral parts. Some eclipses were longitudinally elongated (Figs. 1B, 2B, D).

Division of the myelinated (m) region

The m region was further divided into two subregions: myelinated regions 1 (m1) and 2 (m2; Fig. 1A, see also Fig. 3). As shown in Figures 1–3, the m1 region was thicker than the m2 region. The outline of the optic nerve was bent around the border between the m1 and m2 regions. The border between the m1 and m2 regions was set at the bottom of the bend in the outline. A small notch was frequently observed at the bottom of the bend. Therefore, the small notch was used as a good indication of the border (Figs. 1B, 2). There were two bends in the outlines in each section of the optic nerve: one was generally close to the boundary between the u and m regions, whereas the other was far from the boundary. The bottom of the closer bend was selected as the border. This border principally coincided with the anterior limit of densely packed myelinated nerve fiber bundles (Figs. 1B, 3B, D), the anterior limit of GFAP-poor tracks in which myelinated nerve fiber bundles were located (Figs. 1B, 2B, D), and the anterior margin of an area in which the scattering of GS-immunoreactive glial cells was observed in the m2 region (Figs. 2C–D, 3C–D). The length of the m1 region was 872.2 ± 30.9 µm (Average ± SE; n = 5; Figs. 1B, 2–3).

GFAP immunoreactivity was strong (Figs. 1B, 2B, D, 4F, 5G), and columns of astrocytes (thin clusters of astrocytic filaments) were observed to interdigitate with the myelinated nerve fiber bundles (Figs. 1B, 5E–H). GS immunoreactivity was moderate (Figs. 2C–D, 3C–D, 4G, 5O), and GS-immunoreactive fibers were clearly observed (Figs. 4G, 5O). These fibers
extended longitudinally along the long axis of the optic nerve, and were RIP immunoreactive (Fig. 5M–P). Thus, the chemoarchitecture of GFAP and GS in the m1 region was different from that in the m2 region.

Moderately GS-immunoreactive glial cells were RIP immunoreactive in the m region (Fig. 5I–P). In the m1 region, 96.8 ± 0.9 % (Average ± SE; n = 3) of these cells were also Olig2 immunoreactive (Fig. 6A), while 87.9 ± 0.7 % were also Olig2 immunoreactive in the m2 region (Table 2). A significant difference was observed in the co-localization rates of Olig2/GS between the m1 and m2 regions (Two Sample t-test, P < 0.05). A quantitative study demonstrated that 70.2 ± 2.8 % (Average ± SE; n = 3) of Olig2-immunoreactive glial cells were labeled with GS in the m1 region and that 67.7 ± 5.1 % of these cells were immunostained with GS in the m2 region (Table 2). No significant difference was noted in the co-localization rates of GS/Olig2 between the m1 and m2 regions (Two Sample t-test, P < 0.05). In addition, AQP4 (aquaporin 4) immunoreactivity was hardly detected in GS-immunoreactive glial cells (Fig. 6B).

The main distribution pattern of astrocytic filaments in the m1 region was that of a thin cluster; bunches of cell nuclei (Figs. 1B, 5E), which were tightly surrounded by the clustered pattern of astrocytic filaments (Figs. 1B, 5G), were observed between myelinated nerve fiber bundles (Figs. 1B, 5E–H). The majority of bunches were longitudinally elongated (Fig. 1B). Thin clusters of the filaments were mainly composed of astrocytes. The cell nuclei of oligodendrocytes were also seen in bunches (Fig. 5E–H). In addition, bundles of densely packed and perpendicularly elongated astrocytic filaments were observed. The astrocytic filaments in these bundles constituted a part of the clusters (Fig. 4E–H). Minor forms of astrocytic filaments were aligned parallel to the optic nerve axis and distributed among myelinated nerve fiber bundles. A thick cluster was noted as a confluence of two or more thin clusters, and was mostly observed in the distal (anterior) two-thirds of the m1 region (Figs. 1B, 7). Each cluster of the filaments in the m2 region was markedly thinner than that in the m1 region. Furthermore, the number of astrocytic filaments among myelinated nerve fiber bundles in the m2 region was markedly less than that in the m1 region (Figs. 1B, 2B, 5A–H).

**Quantitative analysis**

**GFAP**

GFAP immunoreactivity was quantitatively the strongest in the i2 and u regions. It was strong in the i1 and m1 regions, moderate in the m2 region, and weak in the retina (Fig. 8A; Table 3). Statistical analyses revealed a significant difference in the strength of GFAP immunoreactivity among the different regions (a one-way ANOVA with Tukey’s multiple comparisons test, P < 0.05, n = 5; Table 4). No significant difference was observed between the i1 and m1 regions, in which GFAP immunoreactivity was strong. In addition, no significant difference was noted between the i2 and u regions, in which GFAP immunoreactivity was very strong (Tables 3–4).

**GS**

GS immunoreactivity was quantitatively the strongest in the retina. It was moderate in the i1, i2, and m1 regions, and weak in the u and m2 regions (Fig. 8B; Table 3). Statistical analyses partly revealed a significant difference in the strength of GS immunoreactivity among the different regions (a one-way ANOVA with the Games-Howell multiple comparisons test, P < 0.05, n = 5; Table 4). In every case, mean pixel intensity standardized to the u region was stronger in the retina than in the i1 region. Moreover, it was stronger in the i1, i2, and m1 regions than in the u and m2 regions in each case (Fig. 8B). Thus, the i1, i2, and m1 regions were the major sites of GS immunoreactivity in the rat optic nerve. GS immunoreactivity in these regions was markedly weaker than that in the retina.

**Discussion**

We herein described the chemoarchitecture of GFAP and GS in the rat optic nerve. In order to achieve this, the rat optic nerve was divided into 5 regions: the i1, i2, u, m1, and m2 regions (Fig. 1A; Table 3). GFAP immunoreactivity was very strong in the i2 and u regions, strong in the i1 and m1 regions, moderate in the m2 region, and weak in the retina. Significant differences were observed in GFAP immunoreactivity among these regions. The hci was seen beneath the surface of the optic nerve at the border between the i and u regions. The chemoarchitecture of the myelinated region was heterogeneously organized. Regarding GS, the i1, i2, and m1 regions were the major sites of GS immunoreactivity in the rat optic nerve; however, GS immunoreactivity in these regions was markedly weaker than that in the retina. Moreover, most moderately GS-immunoreactive glial cells were oligodendrocytes in the myelinated region.

**Statistical analysis**

Statistical analyses partly revealed a significant difference in the strength of GS immunoreactivity among the different regions (Table 3). For example, no significant difference was observed in the strength of GS immunoreactivity between the m1 and m2 regions. However, in every case, mean pixel intensity standardized to the u region was stronger in the m1 region than in the m2 region (Fig. 8B). The absence of a significant difference between these regions might be attributed to the small number of cases (n = 5).

**Comparison with previous findings**

The division scheme used in the present study was
Fig. 2. Distribution of glutamine synthetase-immunoreactive glial cells and astrocytes in the normal rat optic nerve. The panels show a longitudinal section through the paramedian part and represent double immunostaining for a glial cell marker (glutamine synthetase, GS; C; Alexa Fluor 488 label; green in D) and an astrocyte marker (glial fibrillary acidic protein, GFAP; B; Alexa Fluor 594 label; magenta in D). Cell nuclei were labeled with bisBenzimide (Hoechst 33258; A; blue in D). Panel D is an overlaid image. Arrows in A–D indicate the border between the nerve fiber layer of the retina (NFL) and the intraretinal (i) region, the boundary between the i and unmyelinated (u) regions, or the border between the u and myelinated (m) regions. The double arrowheads in A–D represent borders between the subregions: the border between intraretinal regions 1 (i1) and 2 (i2), or the border between myelinated regions 1 (m1) and 2 (m2). Panel D is a photo montage composed of 9 square fluorescent images. These images were taken with an LSM 700 confocal microscope (Carl Zeiss Jena, Germany). Parts of the background in panel D were filled up with a black color. Note that the outline of the optic nerve was bent at the border between the m1 and m2 regions. The m1 region was a major site for GS distribution in the normal rat optic nerve; however, GS immunoreactivity in this region was markedly weaker than that in the retina. A cluster of GFAP-negative circles or eclipses was observed in the proximal (posterior) half of the u part (B, D). Scale bar = 200 µm in D for A–C.
completely compatible with a previous study in the mouse\(^6\), in which the mouse optic nerve was divided into three regions: the i, astrocytic filament dense (afd), and astrocytic filament sparse (afs) regions. The i region in the rat was defined by the position of the sensory retina, similar to that in the mouse in a study by Kawano et al.\(^6\). The border between the u and m regions in the rat was set at the distal (anterior) limit of myelinated nerve fibers. This border principally coincided with the boundary delineated by the proximal (posterior) limit of very strong GFAP immunoreactivity\(^4\) (present study). Thus, the i, u, and m regions in the rat corresponded to the i, afd, and afs regions in the mouse. The division scheme used in the present study was primarily compat-
Fig. 3. Distribution of glutamine synthetase-immunoreactive glial cells and oligodendrocytes in the normal rat optic nerve. The panels show a longitudinal section through the approximately median part and represent double immunostaining for a glial cell marker (glutamine synthetase, GS; C; Alexa Fluor 488 label; green in D) and an oligodendrocyte marker (RIP; B; Alexa Fluor 594 label; magenta in D). Cell nuclei were labeled with bisBenzimide (Hoechst 33258; A; blue in D). Panel D is an overlaid image. Arrows in A–D indicate the border between the nerve fiber layer of the retina (NFL) and the intraretinal (i) region, the boundary between the i and unmyelinated (u) regions, or the border between the u and myelinated (m) regions. The double arrowheads in A–D represent borders between myelinated regions 1 (m1) and 2 (m2). Panel D is a photo montage composed of 7 square fluorescent images. These images were taken with an LSM 700 confocal microscope (Carl Zeiss Jena, Jena, Germany). Parts of the background in panel D were filled up with a black color. Note that the m1 region was thicker than the m2 region. Moreover, the outline of the optic nerve was bent at the border between the m1 and m2 regions. The bend was clearly observed on the left side of each panel. Note also that the chemoarchitecture of the m2 region was almost homogeneously organized. Strongly RIP-immunoreactive fibers were tightly packed throughout the m2 region. The length of the m2 region examined (in this study) was approximately 4 mm, and all of the m2 region examined was confined to the orbital portion of the optic nerve. A large GS-negative area was observed in the proximal (posterior) half of the u region. Densely-packed cell nuclei were seen in this area. Scale bar = 5 mm in D for A–C.
Fig. 4. Distribution of glutamine synthetase-immunoreactive glial cells and astrocytes in the nerve fiber layer of the retina (NFL) and in the optic nerve of the normal rat. The panels show a longitudinal section through the approximately median part and represent double immunostaining for a glial cell marker (glutamine synthetase, GS; C, G, K, O, S; Alexa Fluor 488 label; green in D, H, L, P, T) and an astrocyte marker (glial fibrillary acidic protein, GFAP; B, F, J, N, R; Alexa Fluor 594 label; magenta in D, H, L, P, T). Cell nuclei were labeled with bisBenzimide (Hoechst 33258; A, E, I, M, Q; blue in D, H, L, P, T). Panels D, H, L, P, and T are overlaid images. The arrows in Q–T indicate the border between the NFL and intraretinal region 1 (i1). Panels A–D, E–H, and I–L are confocal images of myelinated regions 2 (m2), 1 (m1), and the unmyelinated (u) region, respectively. The tops of these panels show the part close to the optic chiasm, while the bottoms represent the portion close to the retina. These images were taken with an LSM 700 confocal microscope (Carl Zeiss, Jena, Germany). Note that GFAP immunoreactivity was very strong in intraretinal region 2 (i2) and the u region, strong in the i1 and m1 regions, moderate in the m2 region, and weak in the NFL. Regarding GS immunoreactivity, it was very strong in the NFL, moderate in the i1, i2, and m1 regions, and weak in the u and m2 regions. GS-immunoreactive fibers were clearly observed in the m1 region (G, H). In addition, astrocytes in the i1, i2, and u regions were immunoreactive for GS. The inner limiting membrane of the optic nerve head [Elschnig] (ilm) was observed around the border between the NFL and i1 region (Q–T). Moreover, the high concentration of the intermediary tissue (hci) was seen beneath the surface of the optic nerve at the border between the i and u regions (M–P). Astrocytic filaments were densely packed and strongly immunoreactive for GFAP in the ilm and hci. Weak GS immunoreactivity was seen in the astrocytes. Int, intermediary tissue at the termination of the retina [Kuhnt]. Scale bar = 20 µm in T for A–S.
ible with another study conducted in the mouse\textsuperscript{5}). Howell et al.\textsuperscript{5)} described the i and m regions in the rat as the “prelamina”, including the medial-most part of the NFL, and the “myelinated region”, respectively. Between the “prelamina” and “myelinated region”, they clearly defined the “glial lamina” as a GFAP-rich and unmyelinated part. The u region in the rat appeared to correspond to the “glial lamina” and the rest of the unmyelinated region in the mouse\textsuperscript{5, 7}).

Previous studies showed the distributions of astrocytic filaments in the rat optic nerve\textsuperscript{3–4}). Astrocytic filaments are present along the entire length of the rat optic nerve,
and are highly concentrated in the u region. Accordingly, the rat optic nerve was divided into three regions (the i, u, and m regions) based on the distribution patterns of astrocytic filaments. According to Ffrench-Constant et al.\(^{3}\), astrocytic filaments in the u region were oriented almost entirely transversely and formed a particularly dense meshwork, while astrocytic filaments in the m region were oriented transversely and longitudinally. Astrocytic filaments in the i region were oriented longitudinally with the optic nerve and transversely with respect to the retina. The present study primarily confirmed these findings. At least three research groups have divided the rat optic nerve into three regions: the neck region, transition zone, and retrobulbar optic nerve. The neck region is located at the level of sclera. The transition zone has a conical shape with a length of approximately 250–500 µm. Scattered patches of connective tissue were observed throughout the transition zone, many of which contained vascular capillaries. Most axons were shown to be myelinated in the retrobulbar optic nerve\(^9, 17, 22\). Thus, the retrobulbar optic nerve completely corresponds to the m region. Since the proximal (posterior) half of the u region is rich in vasculature, it is reasonable to assume that the transition zone is mostly consistent with the proximal (posterior) half of the u region (see Figure 12 of Morrison et al.\(^{15}\)). In addition, the neck region roughly corresponds

Table 2. Co-localization rates of Olig2/GS, and GS/Olig2 in the m1 and m2 regions.

|          | GS | Olig2 | DL | Olig2/GS (%) | GS/Olig2 (%) | GS | Olig2 | DL | Olig2/GS (%) | GS/Olig2 (%) |
|----------|----|-------|----|-------------|-------------|----|-------|----|-------------|-------------|
| Case 17L | 63 | 85    | 61 | (96.8)      | (71.8)      | 53 | 78    | 46 | (86.8)      | (59.0)      |
| Case 20R | 107| 138   | 102| (95.3)      | (73.9)      | 97 | 111   | 85 | (87.6)      | (76.6)      |
| Case 21R | 58 | 88    | 57 | (98.3)      | (64.8)      | 65 | 86    | 58 | (89.2)      | (67.4)      |
| Average  |    |       |    | (96.8)      | (70.2)      |    |       |    | (87.9)      | (67.7)      |
| SE       |    |       |    | (0.9)       | (2.8)       |    |       |    | (0.7)       | (5.1)       |

Each numerical value shows the number of immunoreactive cells counted in areas depicted by shading in Figure 1A and represents the percentage of cells. As for the case number, case 17L shows the left optic nerve in case 17, case 20R represents the right one in case 20. GS, glutamine synthetase; DL, double-labeled; m1, myelinated region 1; m2, myelinated region 2; Olig2, oligodendrocyte lineage transcription factor 2.

Fig. 6. Cellular localization of glutamine synthetase (GS) in the myelinated region 1 (m1) of the normal rat optic nerve. A: Double immunostaining for GS (Alexa 488 label; green) and for an oligodendrocyte marker (oligodendrocyte lineage transcription factor 2; Olig2; Alexa 594 label; magenta). The arrow indicates a GS-immunoreactive glial cell co-localized with Olig2. B: Double immunostaining for an astrocyte marker (aquaporin 4; AQP 4; Alexa 488 label; green) and for GS (Alexa 594 label; magenta). Cell nuclei were labeled in blue with bisBenzimide (Hoechst 33258). The arrowhead shows a GS-immunoreactive glial cell not co-localized with AQP4. These images were taken with an LSM 700 confocal microscope (Carl Zeiss, Jena, Germany). Note that moderately GS-immunoreactive glial cells were oligodendrocytes, not astrocytes. Scale bar = 20 µm in B for A.
to the distal (anterior) half of the u region (Figs. 1B, 2A, D). Two research groups used a similar division scheme in the rat optic nerve. By referring to the adjacent layers of the eyeball, they divided the rat optic nerve into 4 regions: the retinal, choroidal, and scleral levels of the intraocular optic nerve, and the optic nerve proper\textsuperscript{16, 41}). The retinal level is mostly consistent with the i region.

The choroidal and scleral levels are mainly consistent with the distal half of the u region. Accordingly, the optic nerve proper mostly corresponds to the m region and the proximal (posterior) half of the u region.

**Chemoarchitecture of the intraretinal (i) region**

**Border between the NFL and i region**

Fig. 7. A thick cluster of astrocytic filaments in myelinated region 1 (m1) of the normal rat optic nerve. The panels show double immunostaining for an astrocyte marker (glial fibrillary acidic protein, GFAP; C; Alexa Fluor 488 label; green in D) and an oligodendrocyte marker (RIP; B; Alexa Fluor 594 label; magenta in D). Cell nuclei were labeled with bisBenzimide (Hoechst 33258; A; blue in D). Panel D is an overlaid image. The tops of the panels show the part close to the optic chiasm, while the bottoms represent the portion close to the retina. Arrows and arrowheads in A–D indicate an astrocyte and an oligodendrocyte, respectively. These images were taken with an LSM 700 confocal microscope (Carl Zeiss Jena GmbH, Jena, Germany). Note that a thick cluster of astrocytic filaments was observed between myelinated nerve fiber bundles. The thick cluster was seen as a confluence of two or more thin clusters. In addition, not only astrocytes, but also the cell bodies of oligodendrocytes were distributed in the cluster. Scale bar = 20 µm in D for A–C.
In the present study, GS was used to set the border between theNFLand i region. In the retina, GS was exclusively expressed in Müller cells to their full extent, with their cell bodies in the middle lamina of the inner nuclear layer and their radial processes terminating at the outer and inner limiting membranes. It is highly likely that the very strong GS immunoreactivity observed in the NFL can be attributable to GS expressed in Müller cells. Since Müller cells have also been labeled exclusively with antibodies against vimentin, vimentin

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Fig. 8. Quantitative analysis of protein distribution in the retina and optic nerve of the normal rat. Mean pixel intensity values standardized to the unmyelinated (u) region within each region of glial fibrillary acidic protein (GFAP; A) and glutamine synthetase (GS; B) are presented. Lines were used to connect data points. As for case numbers, case 4L shows the left retina and optic nerve in case 4, and case 13R represents right ones in case 13. Note that pixel intensity values at points in each region were expressed as a percentage of the intensity value of the u region since the distributions of GFAP and GS were homogeneously organized in this region (see Figs. 2B, C, 4J, K). i1, intraretinal region 1; i2, intraretinal region 2; m1, myelinated region 1; m2, myelinated region 2.
has been suggested as another marker for setting the border.

**Imt (intermediary tissue at the termination of the retina [Kuhnt]) and hci (high concentration of the intermediary tissue)**

The “intermediary tissue of Kuhnt” was previously shown to be a marginal glial layer separating the outer retinal layers from the i region. Interestingly, the hci was clearly observed at the level between the outer limiting membrane and retinal pigment epithelium (RPE). Not only the imt, but also the hci were the marginal glial layers, and the hci was adjacent to the imt (Fig. 4M-P).

Thus, the hci is likely to be a part of the “intermediary tissue of Kuhnt”. Moreover, the border between the i and u regions was set at the boundary between the sensory retina and RPE. Therefore, it is reasonable to assume that the hci is a clear marker for the border between the i and u regions.

The barrier functions of the hci and imt have been proposed based on the findings that the diffusion of tracers (i.e., horseradish peroxidase and lanthanum nitrate) from the choroid into the optic nerve was observed, whereas the penetration of these tracers from the optic nerve into the retina was hardly seen.

**Ilm (inner limiting membrane of the optic nerve head [Elschnig])**

On the optic disc of the human and monkey, the peri-

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1The term “intermediary tissue of Kuhnt” has been used to describe the portion comprising both the imt and hci.

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Table 3. Chemoarchitecture of glial fibrillary acidic protein (GFAP) and glutamine synthetase (GS) in the rat optic nerve.

|             | GFAP         | GFAP Statistics | GS          | GS Statistics |
|-------------|--------------|-----------------|-------------|---------------|
| m2          | ++           | 36.38 ± 5.39    | +           | 84.41 ± 12.08 |
| m1          | +++          | 60.95 ± 5.10    | ++          | 147.86 ± 22.00 |
| u unmyelinated region | ++++          | 100.00 ± 0.00  | +           | 100.00 ± 0.00 |
| i2           | ++++         | 88.28 ± 4.30    | ++          | 155.93 ± 3.28 |
| i1           | +++          | 59.00 ± 5.43    | ++          | 211.59 ± 28.28 |
| Retina       | +            | 9.56 ± 2.97     | ++++        | 673.51 ± 102.28 |

Column GFAP indicates the strength of GFAP immunoreactivity and column GS shows that of GS immunoreactivity in each subregion: ++++, very strong; ++, strong; +, moderate; +, weak. Columns GFAP Statistics and GS Statistics indicate the average ± SE of mean pixel intensity standardized to u (%) in each subregion. Abbreviations of the nomenclature are described in the far left column.

Table 4. Differences in mean pixel intensity standardized to u between each region.

| GFAP       | Retina | i1 | i2 | u | m1 | m2 |
|------------|--------|----|----|---|----|----|
| Retina     | ND     | +  | +  | + | +  | +  |
| i1         | ND     | +  | +  | - | +  | -  |
| i2         | ND     | -  | +  | + | +  | -  |
| u          | ND     | +  | +  | + | +  | -  |
| m1         | ND     | +  | +  | + | +  | -  |
| m2         | ND     | +  | +  | + | +  | -  |

| GS         | Retina | i1 | i2 | u | m1 | m2 |
|------------|--------|----|----|---|----|----|
| Retina     | ND     | -  | +  | + | +  | +  |
| i1         | ND     | -  | -  | - | -  | +  |
| i2         | ND     | +  | -  | - | -  | +  |
| u          | ND     | -  | -  | - | -  | -  |
| m1         | ND     | -  | -  | - | -  | -  |
| m2         | ND     | -  | -  | - | -  | -  |

+, Significant difference with P < 0.05*; -, No significant difference with P ≥ 0.05*; ND: Not determined; i1, intraretinal region 1; i2, intraretinal region 2; m1, myelinated region 1; m2, myelinated region 2; u, unmyelinated region. * As for glial fibrillary acidic protein (GFAP), data were normally and homogeneously distributed. Accordingly, data were analyzed by a one-way ANOVA with Tukey’s multiple comparisons test. As for glutamine synthetase (GS), data were normally, but not homogeneously distributed. Accordingly, data were analyzed by a one-way ANOVA with the Games-Howell multiple comparisons test.
vascular glia of the central retinal vessels was shown to be continuous with the glial layer, which covers the disc, the inner limiting membrane of Elschnig. At the disc margin, the inner limiting membrane of Elschnig, derived from astrocytes, was continuous with the inner limiting membrane of the retina, derived from Müller cells.

Connective tissue and glia derived from the central retinal vessels and the inner limiting membrane of Elschnig may be thickened when there is a deep central depression in the disc (physiologic cup), which partly fills the concavity and forms the central connective tissue meniscus of Kuhnt. As described here, the glial layer in the primate has been divided into two parts: the central connective tissue meniscus of Kuhnt and the inner limiting membrane of Elschnig. In the rat, these two parts were collectively termed the ilm (the present study), because it was very difficult to identify clear chemoarchitectonic differences between the glial layer covering the central part of the optic disc and that covering the peripheral part.

In contrast to the barrier functions of the int, those of the ilm have not yet been elucidated. The tracer, lanthanum nitrate, freely diffused into the optic disc mainly along the perivascular space after an intravitreal injection, and was clearly observed in the intercellular space of optic nerve fibers to the level of the pigment epithelium.

Chemochitecture of the unmyelinated (u) region

Comparison between the distal (anterior) half of the u region in the rat and glial lamina in the mouse

The glial lamina in the mouse was originally defined and named by Howell et al. It was observed in an equivalent location to that of the lamina cribrosa in humans. The arrangement of glial cells in the glial lamina is also similar to that in the lamina cribrosa of humans. Unlike the lamina cribrosa in humans, extracellular matrix plates are absent in the glial lamina. Further details on the glial lamina in the mouse have been reported as follows. The glial lamina is a clearly defined region with a robust network of GFAP-positive astrocytes in the mouse optic nerve. The astrocytes in this region form an enmeshing network of glial cells through which retinal ganglion cell axons pass, and they are intimately associated with axons. Most nuclei within this region are transversely elongated and arranged in rows running perpendicular to the long axis of the nerve. In a more posterior position, the cell nuclei are rounder in shape and aligned in longitudinal rows, parallel to the long axis of the nerve. The glial lamina begins at the level of the sclera and extends for 70–80 μm posteriorly, gradually ending before the axons become myelinated. Myelination begins approximately 120–170 μm behind the sclera. There is no extracellular matrix (collagenous) plate in this region, with abundant collagen only being observed in blood vessel walls.

The morphological characteristics of the glial lamina in the mouse were observed in the distal (anterior) half of the u region in the rat optic nerve. GFAP immunoreactivity was shown to be very strong in the distal half of the u region (the present study). As in the primate, astrocytes at the scleral level of the rat (an equivalent position with the distal half of the u part) appear to be specialized to support axons. In both species, astrocyte processes are packed with glial filaments and are oriented perpendicular to the axons. At the ultrastructural level, optic nerve head astrocytes are oriented across the optic nerve head, within the plane of the sclera, and lie perpendicular to the optic nerve axons. Astrocyte processes within axon bundles are in intimate contact with unmyelinated optic nerve axons, which is very similar to that reported in the primate optic nerve head. Thus, it is reasonable to assume that the distal half of the u region is the anterior part of the glial lamina in the rat.

Vasculature in the proximal (posterior) half of the u region

Casting studies revealed that the capillary bed in the transition zone (an equivalent position with the proximal half of the u region) is continuous posteriorly with that of the retrobulbar optic nerve (an equivalent position with the m region) and anteriorly with capillaries of the superficial optic nerve head. The capillary bed was shown to receive a blood supply from the posterior ciliary arteries by way of their branches, and the capillary bed in the transition zone then drains into the central retinal vein. In the present study, defects in GFAP immunoreactivity were mainly observed in the proximal half of the u region. It is reasonable to speculate that these defects are attributable to the capillary bed located in this region. This speculation is supported by the frequent occurrence of transversely oriented blood vessels in the proximal half of the u region.

Chemochitecture of the myelinated (m) region

Glutamine synthetase (GS) immunoreactivity in oligodendrocytes

GS immunoreactivity was frequently used as an astrocyte marker in the 1980s. However, GS immunoreactivity in oligodendrocytes was demonstrated in the rat central nervous system, including the optic tract, in the 1990s. GS immunoreactivity in oligodendrocytes was subsequently reported in the optic nerve and optic chiasm. In addition to oligodendrocytes, GS immunoreactivity was found to be localized in fibrous astrocytes. GS in fibrous astrocytes was shown to be particularly abundant in the optic chiasm. In the present study, most moderately GS-immunoreactive glial cells were oligodendrocytes in the m region, since 88–97% of these cells were Olig2 positive. Moreover, 3-12% of these cells were Olig2 negative (Table 2). These GS-positive/Olig2-negative cells might be astrocytes immunoreactive for GS.
Thus, the present study was primarily compatible with previous findings by Domercq et al.12.

We used two markers for oligodendrocytes: [RIP] and Olig2. The former ([RIP]) is a marker for mature oligodendrocytes40, while the latter (Olig2) is a marker for mature oligodendrocytes and oligodendrocyte progenitors37, 39. It is probable that GS-positive/RIP-positive cells and some GS-positive/Olig2-positive cells are mature oligodendrocytes. However, it currently remains unknown whether oligodendrocyte progenitors are GS positive. Further studies are required to clarify this issue by using a marker for oligodendrocyte progenitors, such as NG239.

**Thick clusters of astrocytic filaments in the distal (anterior) two-thirds of the m region**

Astrocytes were previously shown to interdigitate with bundles of myelinated axons in the rat optic nerve4. The present study confirmed this finding and demonstrated that the width of each thin cluster in the m1 region was wider than that in the m2 part. The present study also showed that the thick cluster of astrocytic filaments was a confluence of two or more thin clusters. The functions of the thick clusters and why the thick clusters were almost confined to the distal (anterior) two-thirds of the m1 region remain unknown. In the thick clusters, astrocytes express the following glutamate transporters on the cell membrane: GLT-1 (glutamate transporter 1), GLAST (glutamate-aspartate transporter), and EAAC1. Oligodendrocytes express a single glutamate transporter on the cell membrane: EAAC152. Oligodendrocytes also moderately express GS, a key enzyme in glutamate metabolism (the present study). Thus, thick clusters may function to control glutamate concentrations in the extracellular milieu in order to protect neuronal axons from glutamate excitotoxicity.

Another hypothesis has been proposed for these functions and reasons. Morphologically, a single oligodendrocyte has the ability to wrap its membranous processes around many axons. Each oligodendrocyte enwraps between one and 30 axonal segments (called internodes), depending on the axon diameter18). From a molecular biological standpoint, oligodendrocytes possess various neurotransmitter receptors and ion channels53–54). Electrophysiologicaly, oligodendrocytes facilitate the rapid conduction of action potentials by forming myelin and buffering extracellular K+. Moreover, the temporal coordination of action potentials, which are conducted along axons wrapped by the same oligodendrocytes, has also been suggested. The activation of oligodendrocytes by glutamate and/or extracellular K+ has been proposed to coordinate the timing of action potentials. Thus, oligodendrocytes may modulate and synchronize the conduction of action potentials along axons in the distal (anterior) two-thirds of the m1 region53–54. The conduction velocity of action potentials along the axon is markedly increased by myelination. It is reasonable to speculate that the initial segment of myelination is an appropriate place to modulate and synchronize the conduction of action potentials. Since the distal (anterior) two-thirds of the m1 region comprise the initial segment, and since oligodendrocytes were tightly surrounded by astrocytes in the thick clusters (the present study), astrocytes may maintain the activity-dependent facilitative effect of oligodendrocytes on conduction velocity in the thick clusters53–54).

**GS distribution in the normal rat optic nerve**

We demonstrated the heterogeneous distribution of GS in the normal rat optic nerve. Interestingly, neither the u nor m2 regions were the major sites of GS distribution, whereas the i1, i2, and m1 regions were. Moreover, GS immunoreactivity was markedly weaker in the i1, i2, and m1 regions than in the retina. These results were supported by the fact that GS was also heterogeneously distributed along the sagittal plane of the monkey optic nerve13). It currently remains unknown why GS is heterogeneously distributed in the optic nerve. GS is a key enzyme in glutamate metabolism10, 12). Vesicular glutamate release has been reported from axons in the white matter including the optic nerve of the rodent55–56). It is possible that the amount of vesicular glutamate released in each region of the optic nerve may be reflected in the strength of GS immunoreactivity in glial cells. Therefore, it is important to determine whether the expression of glutamate transporters in glial cells correlates with the strength of GS immunoreactivity. Since EAAC1 (excitatory amino acid carrier 1) is the major glutamate transporter in oligodendrocytes in the rodent optic nerve52, it is necessary to identify the region that is the major site of EAAC1 distribution in the rat optic nerve.

**Usefulness of the results obtained for future biochemical studies in the rat optic nerve**

The combination of laser capture microdissection and DNA microarrays has been widely used recently to obtain the gene expression profiles of small tissue specimens57–58). By using laser capture microdissection and the new division scheme, it is possible to obtain tissue specimens from each subregion. DNA microarray analyses may clarify the gene expression profiles of tissue specimens in each subregion.

The present study demonstrated that GS immunoreactivity in the m1 region was stronger than that in the m2 region. Since GS is a metabolic enzyme of glutamate, the expression of genes regulating glutamate metabolism in the m1 region may differ from that in the m2 region. DNA microarray analyses may clarify this difference by using tissue specimens of the m1 and m2 regions. Moreover, these analyses may provide molecular biological insights for discussing glutamate metabolism in the myelinated (m) region.
Conclusion

We herein described the chemoarchitecture of GFAP and GS in the normal rat optic nerve in detail. The results obtained in the present study may facilitate future molecular, histological, and functional investigations to provide a clearer understanding of the physiology of the rat optic nerve. The present study demonstrated that the i1, i2, and m1 regions were the major sites of GS distribution in the rat optic nerve. It currently remains unknown why GS was heterogeneously distributed in the optic nerve. Since GS is a key enzyme in glutamate metabolism, this issue may be clarified by investigating the expression of the gene that regulates glutamate metabolism in the optic nerve. The combination of laser capture microdissection and DNA microarrays will be useful to obtaining gene expression profiles in each region of the optic nerve. Gene expression profiles may facilitate future molecular and histological investigations to provide a clearer understanding of glutamate metabolism.

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