INTRODUCTION

Wolfram syndrome (OMIM 222300) is an autosomal recessive neurodegenerative disorder defined by juvenile-onset non-autoimmune insulin-dependent diabetes mellitus and progressive optic atrophy (Wolfram and Wagener, 1938). The nuclear gene responsible for Wolfram syndrome was mapped to human chromosome 4 at p16.1 (Polymeropoulos et al., 1994; Collier et al., 1996), and was subsequently identified as *WFS1* (Wolfram syndrome 1) or *wolframin* (Inoue et al., 1998; Strom et al., 1998). The *WFS1* gene encodes a putative 890-amino acid protein with an apparent molecular mass of ~100 kDa (Inoue et al., 1998; Strom et al., 1998). The subcellular localization of WFS1 protein (wolframin) has been assigned primarily to the endoplasmic reticulum (ER) membrane. WFS1 protein contains nine transmembrane segments and is embedded in the ER membrane with the amino-terminus in the cytosol and the carboxy-terminus in the ER lumen (Takeda et al., 2001; Hofmann et al., 2003). Subsequent functional studies demonstrated that WFS1 protein is important in the regulation of intracellular Ca^{2+} homeostasis and its expression is induced under conditions of troubled homeostasis, including ER stress (Osman et al., 2003; Yamaguchi et al., 2004; Ueda et al., 2005; Takei et al., 2006). In addition, mutation screening analyses in Wolfram syndrome patients showed more than 50 distinct mutations of the *WFS1* gene, including stop, frameshift, deletion and missense mutations (Inoue et al., 1998; Strom et al., 1998; Hardy et al., 1999; Gómez-Zaera et al., 2001; Khanim et al., 2001; Tessa et al., 2001). Thus loss-of-function mutations in the *WFS1* gene have been linked to Wolfram syndrome. However, roles of WFS1 protein in cellular functions and the mechanism by which mutations of the *WFS1* gene cause Wolfram syndrome remain unclear.

The association of diabetes mellitus and optic atrophy has been known since at least 1858 when von Gräfe reported optic atrophy as a complication of diabetes mellitus (von Gräfe, 1858). This report is probably the first description of Wolfram syndrome (Minton et

al., 2003). The prevalence of Wolfram syndrome is one per 770,000 in the UK population, and the median age of onset of optic atrophy is 11 years (Barrett et al., 1995). The ophthalmologic signs are a progressive decrease in visual acuity, constriction of the peripheral visual field with or without central scotoma, color vision disturbance, and bilateral optic disc atrophy. Diabetic retinopathy is a rare complication (Mtanda et al., 1986; Seynaeve et al., 1994; Barrett et al., 1997). Although a profound reduction in visual function is observed in Wolfram syndrome patients, the ERG tests indicate normal or only slightly reduced responses (Niemeyer and Marquardt, 1972; Mtanda et al., 1986; Seynaeve et al., 1994; Barrett et al., 1997). These findings suggest that the site of pathology for optic atrophy lies not in the retina, but in the visual pathway proximal (posterior) to the eye including the optic nerve (Barrett et al., 1997). In addition, brains of Wolfram syndrome patients showed neuronal loss and gliosis in the lateral geniculate nucleus (LGN) and superior colliculus (SC) as well as severe degeneration of the optic nerve (Genís et al, 1997; Shannon et al., 1999). Thus, ophthalmologic and neuropathological facts concerning optic atrophy in Wolfram syndrome have been accumulated. However, the site of pathology, i.e. causative cell types, for the optic atrophy remains unclear. To obtain insights into causative cell types for the optic atrophy, it is necessary to examine *WFS1* expression not only in the retina, but also in the optic nerve, the optic chiasm, the optic tract, and in the retino-recipient nuclei, since there is a possibility that the optic atrophy is due to neuronal loss in the retino-recipient nuclei caused by loss-of-function mutations in the *WFS1* gene (retrograde transsynaptic degeneration). The insights into the causative cell types may provide hypotheses about the pathogenesis of the optic atrophy in Wolfram syndrome.

In the rodent central nervous system (CNS), *Wfs1* expression has previously been described in neurons of the cerebral cortex, the basal ganglia, the hypothalamus, the brainstem motor and sensory nuclei, the reticular formation, and of the cerebellar cortex, as

well as in hippocampal CA1 pyramidal neurons (Takeda et al., 2001, Ishihara et al., 2004). In the visual system, recent studies suggest that WFS1 protein is expressed in retinal ganglion cells (RGCs) and optic nerve glia of the cynomolgus monkey (Yamamoto et al., 2006). To know species differences in the localization of WFS1 protein in the visual system, to obtain insights into causative cell types for optic atrophy in Wolfram syndrome, and to establish a basis for functional studies of WFS1 in the visual system, we performed a detailed histochemical analysis of the distribution of *Wfs1* mRNA and protein in the normal mouse visual system including the retina, the optic nerve, the optic chiasm, the optic tract, the retino-recipient nuclei, and the visual cortex.

MATERIALS AND METHODS

Animals and tissue preparation

Male mice (n=24; 7–8 weeks old; C57BL/6NCrlCrlj; Charles River Laboratories Japan, Inc., Yokohama, Kanagawa, Japan) were used in this study. Seven-week old mice were used for the retrograde labeling of RGCs, and 8-week mice, for immunoblot and histochemistry.

For immunoblot analysis, the mice were deeply anesthetized with sodium pentobarbital (50 mg/kg, i.p.). The brain, retina, and optic nerve were dissected from the skull on ice, immediately frozen in liquid nitrogen, and stored at –70 °C prior to use. For histochemistry by using frozen sections, the mice 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 and the brain were removed from the skull, stored in the same fixative for 48 hours, and then immersed in 30 % saccharose 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. The brains were frozen and coronally cut at a thickness of 40 μm. The sections were collected in a cryoprotectant medium (33.3% saccharose, 1% polyvinylpyrrolidone (K-30), and 33.3% ethylene glycol in 0.067M sodium phosphate buffer (pH 7.4) containing 0.067% sodium azide; Warr et al., 1981) and stored at –30 °C prior to use. For immunohistochemistry by using whole-mount retinae, the mice were anesthetized, and perfused as described above. The eyeballs were removed from the skull, and stored in the same fixative for 48 hours. The retina was gently dissected, and immersed in 30 % saccharose in 0.1 M PB at 4°C until they sank. The retina was shock-frozen in acetone at –70 °C, and stored in a freezer at the same temperature prior to use.

All experimental protocols for this study were approved by the committees on the Ethics of Animal Experimentation at Kagoshima University or at Yamaguchi University School of Medicine. And the protocols were conducted according to the guidelines for Animal Research of Kagoshima University or Yamaguchi University School of Medicine and The Law (No. 105) and Notification (No. 6) of the Japanese Government.

Retrograde labeling of retinal ganglion cells

Seven-week old mice were anesthetized with a mixture containing ketamine hydrochloride (60 mg/kg, i.p.) and xylazine (6 mg/kg, i.p.). A full strength solution of Fluorescent Latex Microspheres (Red fluorescent RetroBeads; rhodamine label, Lumafluor, Naples, FL) was stereotaxically injected through a glass micropipette (20–30 μm in tip diameter) by air pressure. The injection was made into the bilateral SC (6 mice). After a period of 4 days, the mice were re-anesthetized and perfused as described above. In addition, Fluoro-Ruby (dextran-tetramethylrhodamine, Molecular probes, Eugene, OR) was used as another retrograde tracer. A 10% (w/v) solution dissolved in saline was injected. The injection was made into the bilateral LGN (2 mice), the bilateral SC (2 mice), or both the bilateral LGN and the bilateral SC (2 mice). The survival period was set at 3-5 days. Other procedures were the same as those in Fluorescent Latex Microspheres injection cases.

Antibodies

Preparation of the affinity purified rabbit anti-Wfs1 N-terminus (anti-Wfs1) antibody recognizing the N-terminus 179 amino acids of mouse Wfs1 protein was described previously (Cryns et al., 2003). In brief, a cDNA fragment encoding the N-terminal 290 amino acids of mouse Wfs1 was cloned into pGEX-6P-1 plasmid (Amersham Biosciences, Tokyo, Japan) to produce chimeric proteins consisting of the N-terminal sequence of Wfs1 (1-290) and the C-terminal glutathione *S*-transferase (GST) protein, termed GST-Wfs1N. The cDNA encoding the N-terminal 179 amino acids of mouse Wfs1 was also cloned into the

pMAL-c2 plasmid (New England Biolabs, Ipswich, MA) to produce a fusion protein consisting of the N-terminal sequence of mouse Wfs1 and maltose-binding protein (MBP), termed Wfs1N-MBP. The GST-Wfs1N and Wfs1N-MBP chimeric proteins were expressed in *Escherichia coli* (JM109 strain) and induced by 1 mM isopropyl-thiogalactopyranoside (IPTG). Bacterial lysates were mixed with glutathione Sepharose 4B (Amersham Biosciences), and amylose resin (New England Biolabs) for purification of the GST-Wfs1N, and Wfs1N-MBP. The bound GST-Wfs1N chimeric protein was eluted from glutathione Sepharose 4B by addition of 10 mM reduced glutathione solution. The eluted GST-Wfs1N chimeric protein was used for immunizing Japanese White rabbits. Rabbit antisera were collected and affinity purified by using Wfs1N-MBP bound to the amylose resin.

The following antibodies were obtained from commercial suppliers and applied in order to label specific cell types of the retina and optic nerve. Horizontal cells and non-displaced amacrine cells were immunolabeled with a mouse monoclonal antibody against calbindin-D-28K (Haverkamp and Wässle, 2000). Rod and (putative) ON-cone bipolar cells were immunostained with a mouse monoclonal antibody against G-protein Goα (Haverkamp and Wässle, 2000). Cholinergic amacrine cells were labeled with a goat polyclonal antibody against choline acetyltransferase (ChAT) (Jeon et al., 1998; Haverkamp and Wässle, 2000; Kang et al., 2004). RGCs including their dendrites and axons were immunolabeled with a mouse monoclonal antibody against tubulin, βIII isoform (Sharma and Netland, 2007). According to criteria by Sharma and Netland (2007), large cells intensely labeled with this antibody in the GCL were recognized as RGCs. Nuclei of RGCs were immunostained with a mouse monoclonal antibody against Brn-3a POU-domain transcription factor (Xiang et al, 1995). Müller cells and glial cells in the optic nerve were labeled with a mouse monoclonal antibody against glutamine synthetase (Haverkamp and Wässle, 2000). Astrocytes were immunolabeled with a rabbit serum or a mouse monoclonal antibody against glial fibrillary

acidic protein (GFAP) (Morcos and Chan-Ling, 2000). As for the anti-GFAP rabbit serum, this serum stains a single protein band of 51 kDa in the mouse brain on immunoblot (Jalil et al., 2005). Oligodendrocytes were immunostained with a mouse monoclonal antibody against oligodendrocytes [RIP] (Friedman et al., 1989). Specificity of this antibody was strictly verified in the rat CNS (Friedman et al., 1989). In the rat cerebellum, strong RIP staining was mainly confined to the white matter, although moderate RIP staining was distributed in the granular layer. In the molecular layer, composed of the unmyelinated parallel fibers, RIP staining was hardly detected (Friedman et al., 1989). In the mouse cerebellum, staining pattern of this antibody (data not shown) was identical to that in the rat cerebellum. In the mouse optic nerve, staining pattern was also identical to that in the rat one (Saari et al., 1997; Morcos and Chan-Ling, 2000). Microglia were labeled with a rat monoclonal antibody against mouse CD11b (Mac-1 α chain) (Dräger, 1983; Reichert and Rotshenker, 1996). The staining patterns of these antibodies were identical to those previously published, and their source, host species, antigen, and specificity are listed in Table 1.

Immunoblot analysis

The tissue of the brain, retina, and optic nerve was homogenized in phosphate buffered saline (PBS) containing 1 mM dithiothreitol (DTT) by repeated passages through a 25-gauge needle and then centrifugated at 500*g* for 10 minutes at 4 °C to remove debris. Protein concentration of the supernatant was determined by the Bio-Rad DC Protein Assay (Bio-Rad Laboratories, Hercules, CA). The supernatant was mixed with SDS-PAGE sample buffer and boiled for 5 minutes. Forty (40) μg amounts of protein for brain and retina samples, or 20 μg amount of protein for optic nerve sample were loaded onto 5-20% precast polyacrylamide gradient gels (Atto Corporation, Tokyo, Japan). The proteins were electro-transferred to a polyvinylidene difluoride (PVDF) membrane in Tris-glycine-methanol transfer buffer. The membrane was blocked for 1 hour at room temperature in buffer 1 (150

mM NaCl, and 100 mM Tris-HCl, pH 7.5) containing 2% blocking reagent (Roche Diagnostics GmbH, Penzberg, Germany) (buffer 2) and then incubated for 16 hours at 4 °C with the anti-Wfs1 antibody (1:1000) in blocking solution (buffer 2). The membrane was then rinsed with PBS three times and incubated for 1 hour at room temperature with peroxidase-conjugated affinipure $F(ab')_2$ fragment goat anti-rabbit IgG (H+L) (1:2000) (Jackson ImmunoResearch Laboratories, West Grove, PA). Antigen-antibody complexes were visualized by enhanced chemiluminescence (ECL) system (GE Healthcare UK, Buckinghamshire, UK), and exposed onto Fuji medial X-ray films (RX-U) (Fujifilm, Tokyo, Japan).

To determine the specificity of anti-Wfs1 antibody, we preabsorbed the antibody with GST-Wfs1N chimeric protein (antigen). The antigen (50 μg/ml) was incubated with the anti-Wfs1 antibody (1:1000) for 3 hours at 4 °C before incubation with the PVDF membrane, and then the immunoblot was performed as described above. The GST-Wfs1N chimeric protein was newly generated. The service offered by Hokudo (Sapporo, Japan) was used to produce the protein. The protocols were the same as described above except for the strain of *Escherichia coli*. For expression of the protein, the B21(DE3)RIL strain was used instead of the JM109 strain.

In situ hybridization histochemistry

To synthesize a cRNA probe for in situ hybridization, a 870-base fragment of the mouse *Wfs1* cDNA was amplified by RT-PCR, and subcloned into the vector pCR-Blunt (Invitrogen, Carlsbad, CA). The primers used were No. 00276, 5'-CGG GAT CCA TGA ACT CAG GCA CCC CAC CT-3', and No. 00277, 5'-GGA ATT CCA CCT TCT GGC GTA GTG GCA G -3'. The fragment encoded the 5'-end of the protein-coding region, including all of exon 2 and the first triplet of exon 3. Two independent clones containing the insert with a different orientation (pCR-clone 3 for sense, pCR-clone 9 for antisense) were

used. A sense or an antisense cRNA probe was obtained by in vitro transcription with a DIG RNA labeling kit (SP6/T7; Roche Diagnostics).

In situ hybridization histochemistry was carried out as described previously (Fujinaga et al., 2004). Free-floating sections washed for 5 minutes in diethylpyrocarbonate-treated phosphate-buffered saline (DEPC-PBS) were pretreated with 0.2 N HCl for 20 minutes, washed twice for 5 minutes in DEPC-PBS, and then acetylated in 0.1 M triethanolamine-HCl (pH 8.0) containing 0.25% acetic anhydride for 10 minutes. Before the hybridization step, sections were washed again twice for 5 minutes with DEPC-PBS. All pretreatments were performed at 4 °C. Following the pretreatment, sections were preincubated in hybridization buffer (50% deionized-formamide; 10 mM Tris-HCl, pH 7.5; 1 mM EDTA, pH 8.0; 600 mM NaCl; 1 X Denhardt's solution; 10% dextran sulfate; 0.25% sodium dodecyl sulfate; and 200 μg/ml yeast tRNA) at 55 °C for 1 hour and then hybridized with DIG-labeled anti-sense cRNA probes (0.5 μg/ml; denatured at 95 °C for 5 minutes and cooled at 4 °C for 5 minutes shortly before use) in the same buffer at 55 °C for 16 hours. After hybridization, the sections were washed with 2 X SSC (300 mM NaCl, and 30 mM sodium citrate, pH 7.0) containing 50% formamide at 55 °C for 1 hour, rinsed in wash buffer (500 mM NaCl, 10 mM Tris-HCl, pH 8.0, and 1 mM EDTA, pH 8.0) for 10 minutes and then incubated with RNase A (20 μg/ml; Sigma-Aldrich, St. Louis, MO) in wash buffer at 37 °C for 30 minutes. After being rinsed in wash buffer again for 10 minutes, they were soaked in 2 X SSC containing 50% formamide and 0.2 X SSC containing 50% formamide at 55 °C for 30 minutes each. To perform the immunoreaction, the sections were blocked in buffer 2 (buffer 1 (150 mM NaCl, and 100 mM Tris-HCl, pH 7.5) containing 2% blocking reagent) at 20 °C for 1 hour and then incubated in buffer 2 containing alkaline phosphatase-conjugated sheep anti-DIG antibody (Roche Diagnostics) diluted 1:3000 at 20 °C for 16 hours. After two washes in buffer 1 for 10 minutes, they were rinsed in buffer 3 (100 mM NaCl, 50 mM $MgCl_2$, 100 mM Tris-HCl, pH

9.5) for 5 minutes and incubated with NBT/BCIP substrate (1:50; Roche Diagnostics) in buffer 3 at 37 °C for 2–4 hours to visualize the immunocomplex. The coloring reaction was stopped with buffer 4 (1 mM EDTA, and 10 mM Tris-HCl, pH 8.0), and the sections were washed in PBS, mounted on glass slides using a 0.6% gelatin solution, and air-dried. The slides were coverslipped with VectaMount mounting medium (Vector Laboratories, Burlingame, CA). As a control, a sense cRNA probe was used instead of the anti-sense cRNA probe. Signals detected by the anti-sense *Wfs1* cRNA probe were categorized as positive if they were stronger than those detected by the sense probe in an adjacent section (control section).

Immunohistochemistry

Single immunoperoxidase staining Sections of the retina, the optic nerve and the brain were processed by the immunoperoxidase method as previously described (Sheng et al., 2004). 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 they were washed three times each for 15 minutes in 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 the anti-Wfs1 antibody diluted 1:10000 in a 10% NGS blocking solution at 4 °C. After the primary immunoreaction, primary antibody was washed out with PBST. Then the sections were incubated for 3 hours at 4 °C with biotinylated anti-rabbit goat IgG (DAKO Cytomation, Glostrup, Denmark; 1:500) in PBS containing 5 % NGS. After the secondary immunoreaction, the sections were washed three times each for 10 minutes in PBS, and incubated for 3 hours at 4 °C with a mixture of rabbit peroxidase anti-peroxidase (PAP; DAKO Cytomation, 1:500) and peroxidase-conjugated streptavidin (DAKO Cytomation, 1:500) in PBS containing 1% NGS (enhanced HRP-labeling reaction). Then, 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 0.0006% hydrogen peroxide for 10-15 minutes at 4 °C. After three washes in PBS, the sections were mounted onto glass slides in a 0.6% gelatin solution. After being air-dried, they were dehydrated with a graded series of ethanol solutions, immersed in xylene, and embedded in NEW M•X mountant (Matsunami, Osaka, Japan).

To determine the specificity of the anti-Wfs1 antibody in sections, the GST-Wfs1N chimeric protein (5 μg/ml) was incubated with the anti-Wfs1 antibody (1:10000) for 6 hours at 4 °C before incubation with sections of the retina, the optic nerve, and of the brain, and then immunohistochemistry was performed as described above. To verify the non-specific immunoreaction by the secondary antibody (biotinylated anti-rabbit goat IgG), normal rabbit immunoglobulin (DAKO Cytomation) was used instead of the primary antibody (anti-Wfs1). For the cytoarchitectonic analysis, adjacent series of the brain sections were subjected to Nissl staining by using cresyl violet (acetate) (Merck KgaA, Darmstadt, Germany).

Single and double immunofluorescent staining To identify cell types expressing Wfs1, we performed double immunohistochemistry in retinal and optic nerve tissue. The monoclonal and goat polyclonal antibodies listed in Table 1 were used for additional primary antibodies. In experiments using the mouse monoclonal antibodies, free-floating sections were pre-incubated for 2 hours in a 10% NGS blocking solution at 4 °C, then immunoreacted for 4 days with a mixture of the anti-Wfs1 antibody (1:5000) and each of the monoclonal antibodies in a 10% NGS blocking solution at 4 °C. After three rinses for 10 minutes in 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')$_2$ fragment of goat anti-rabbit IgG (H+L) (1:200; Molecular Probes, Eugene, OR) and Alexa Fluor 594 conjugated to the F(ab')$_2$ fragment of goat anti-mouse IgG (H+L) (1:200; Molecular Probes). The sections were washed three times for 10 minutes in PBS, and mounted onto glass slides in a 0.6% gelatin solution. 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). For staining using the rat monoclonal antibody, Alexa Fluor 488 conjugated to goat anti-rabbit IgG (H+L), highly cross-absorbed (1:200; Molecular Probes) and Alexa Fluor 568 conjugated to goat anti-rat IgG (H+L) (1:200; Molecular Probes) were used as secondary antibodies. For staining using the goat polyclonal antibody, normal donkey serum was used instead of NGS. As for secondary antibodies, Alexa Fluor 488 conjugated to donkey anti-rabbit IgG (H+L) (1:200; Molecular Probes) and Alexa Fluor 594 conjugated to donkey anti-goat IgG (H+L) (1:200; Molecular Probes) were used.

In double immunohistochemistry for detecting GFAP and GS simultaneously in the optic nerve, the rabbit polyclonal anti-GFAP antibody (1:1000) and the mouse monoclonal anti-GS antibody (1:500) were used as primary antibody. Other procedures were the same as those for double immunohistochemistry by using the anti-Wfs1 and each of the mouse monoclonal antibodies. To eliminate the possibility of any crossreaction between secondary antibodies and primary antibodies from the wrong species, one of the two primary antibodies was removed. In these control experiments, no cross-reactivity was observed (data not shown).

Single immunofluorescent staining was applied to Wfs1/tracer double labeling. The anti-Wfs1 antibody (1:5000) alone was used for primary antibody, and Alexa Fluor 488 conjugated with the F(ab')$_2$ fragment of goat anti-rabbit IgG (H+L) (1:200; Molecular

Probes) alone, for secondary antibody. Other procedures were the same as those for double immunohistochemistry by using the anti-Wfs1 and each of the mouse monoclonal antibodies. In Fluorescent Latex Microspheres injection cases, coversplip was made by using Fluoromount-G (SouthernBiothech, Birmingham, AL) instead of the VECTASHIELD mounting medium (Vector Laboratories) in order to prevent Fluorescent Latex Microspheres from fading (Manufacturer's technical information by Lumafluor).

To determine Wfs1 specific immunoreactivity in Wfs1/Goα and Wfs1/FLM double-labeling studies, the anti-Wfs1 antibody was preabsorbed with the antigen as described in the "single immunoperoxidase staining" section.

Photomicrographs and terminology

Brightfield photomicrographs were taken using a DS-5Mc color digital camera (Nikon, Tokyo Japan) equipped with an Eclipse 80i photomicroscope (Nikon). Fluorescence photomicrographs were taken with a FV500 confocal laser scanning microscope (Olympus, Tokyo, Japan) at the Center for Chronic Viral Diseases, Kagoshima University Graduate School of Medical and Dental Sciences, or a LSM510 (Carl Zeiss Jena GmbH, Jena, Germany) at the Institute for Biomedical Research and Education in Yamaguchi University Science Research Center. Images were transferred to Adobe Photoshop 6 (Adobe Systems, San Jose, CA), and brightness, contrast, and picture sharpness were adjusted. No other adjustment was made.

The nomenclature used for the different regions of the brain followed that of Paxinos and Franklin (2001).

RESULTS

Immunoblot analysis of Wfs1 protein expression

To determine organ-specific Wfs1 protein expression in the brain, retina, and optic nerve, and to characterize the specificity of the anti-Wfs1 antibody, we performed immunoblot analysis. The anti-Wfs1 antibody detected Wfs1 protein bands of ~100 kDa in extracts from the brain, retina, and optic nerve. In addition, a Wfs1-immunoreactive band of ~70 kDa in extracts from the retina was observed (Fig. 1, anti-Wfs1). These protein bands were not seen when extracts from the brain, retina, and optic nerve were incubated with the anti-Wfs1 antibody preabsorbed by incubation with the GST-Wfs1N chimeric protein (antigen) (Fig. 1, Absorbed).

Wfs1 mRNA expression in the normal mouse retina

To determine cell-specific *Wfs1* expression in retinal cells, we performed RNA in situ hybridization on normal mouse retinal sections by using a mouse *Wfs1* anti-sense-riboprobe. The most intense *Wfs1* mRNA signals were observed in cell bodies of the ganglion cell layer (GCL; Fig. 2A). Moderate signals were distributed in the inner and outer rows of the inner nuclear layer (INL), and in the inner segment of photoreceptors (PR). The moderate signals in the outer row showed a dash-shaped appearance and were located along the boundary between the INL and outer plexiform layer (OPL). Weak signals were seen in the intermediate row of the INL and outer nuclear layer (ONL; Fig. 2A). Few detectable signals were found in control experiments with a mouse *Wfs1* cRNA sense probe. In these experiments, there was a tendency for reaction deposits to be randomly scattered over the entire retina, including the inner plexiform layer (IPL; Fig. 2B).

Wfs1 protein expression in the normal mouse retina

To examine cell-specific protein localization, retinal sections were immunostained by using the anti-Wfs1 antibody. The most intense immunoreactivity was observed in the OPL,

the inner row of the INL, the GCL, and in the optic nerve fiber layer (NFL). Moderate immunoreactivity was found in the inner segment of photoreceptors (PR), the outer nuclear layer (ONL), and in the outer row of the INL. In the outer row of the INL, Wfs1-immunoreactive cell bodies were seen with their processes extending along the intense immunoreactivity in the OPL (pointed by arrowheads in Fig. 2C). Weakly Wfs1-immunoreactive cell bodies were seen in the middle row of the INL. In the IPL, three labeled strata were observed, and the immunoreactivity in the intermediate stratum was stronger than that in the other two strata. Radially extending Wfs1-immunoreactive fibers were clearly seen from the INL to the NFL with these fibers terminating at the inner limiting membrane (Fig. 2C). When a normal rabbit immunoglobulin was used instead of the anti-Wfs1 antibody, as a control for Wfs1 immunohistochemistry, no clear immunoreactivity by the secondary antibody was seen in cytoplasms of the normal mouse retina (data not shown). When the antibody preabsorbed by incubation with the GST-Wfs1N chimeric protein (antigen), no clearly immunoreactive cytoplasm was observed (Fig. 2D).

To confirm which cell types express Wfs1, retinal sections or whole-mount retinae were double-immunostained with the anti-Wfs1 antibody and with each of the antibodies against several retinal marker proteins. In the outer row of the INL, Wfs1 immunoreactivity was observed in horizontal cells, which were immunolabeled with antibodies against calbindin-D-28K (a horizontal cell marker). In these cells, intensity of Wfs1 imunoreactivity was weak-to-moderate, and the immunoreactivity was seen in both cell bodies and processes (Fig. 3A-C). In the outer and intermediate rows of the INL, faint Wfs1 immunoreactivity was detected in putative ON-cone, and rod bipolar cells, which were immunolabeled with antibodies against G-protein Goα (a bipolar cell marker; Fig. 3D-F). In control (preabsorption) experiments, Wfs1 immunoreactivity was hardly seen in Goα-immunoreactive bipolar cells (Fig. 3G-I; compare Fig. 3D with 3G). In the inner row of the

INL, strong Wfs1 immunoreactivity was observed in non-displaced amacrine cells, which were immunolabeled with antibodies against ChAT (a cholinergic amacrine cell marker; Fig. 3J-L) or against calbindin-D-28K (an amacrine cell marker; Fig. 3M-O). In the IPL, the three Wfs1-immunoreactive strata were overlapped with three calbindin-D-28K-immunoreactive strata (Fig. 3M-O). Out of these strata, the outer and inner ones were overlaid with two cholinergic strata (Fig. 3J-L).

In the GCL, strong Wfs1 immunoreactivity was observed in cholinergic displaced amacrine cells, which were immunolabeled with antibodies against ChAT (a cholinergic amacrine cell marker; Fig. 4A-F). In addition, Wfs1-immunoreactive but non-cholinergic cells were distributed in this layer (Fig. 4A-C). To examine whether these cells are RGCs, we performed Wfs1/tracer double labeling and Wfs1/retinal ganglion cell-specific-marker double immunofluorescent staining. Wfs1 immunoreactivity was detected in RGCs that were retrogradely labeled in vivo with Fluorescent Latex Microspheres (Fig. 4G-I) or with Fluoro-Ruby (Fig. 5A-C). Additionally, Wfs1 immunoreactivity was also detected in RGCs that were immunolabeled with antibodies against tubulin, βIII isoform (Fig. 4M-O) or Brn-3a POU-domain transcription factor (Fig. 4P-R). To verify whether Wfs1 immunoreactivity is positive in RGCs, we performed control (preabsorption) experiments. In these experiments, Wfs1 immunoreactivity was hardly seen in Fluorescent Latex Microspheres-labeled RGCs (Fig. 4J-L; compare Fig. 4G with 4J). There is a tendency that Wfs1 immunoreactivity in cholinergic displaced amacrine cells is stronger than that in RGCs. Thus immunoreactivity for Wfs1 was observed in all neuron types: photoreceptors, horizontal cells, bipolar cells, non-displaced and displaced amacrine cells, and RGCs (Figs. 3,4,5A-C).

In glial cells, immunoreactivity for Wfs1 was observed solely in Müller cells, which were exclusively immunolabeled with antibodies against GS. In Müller cells, Wfs1 immunoreactivity was strong in the endfeet adjacent to the inner limiting membrane,

moderate in internal radial processes, but weak in their cell bodies localized to the intermediate row of the INL (Fig. 5D-F). Wfs1 immunoreactivity was not seen in GFAP-immunoreactive astrocytes, or mouse CD11b-immunoreactive microglia (Table 2).

Optic nerve

Because a definition of the optic nerve subdivision is necessary for the description and interpretation of the experimental results, we will briefly describe our criteria for determining the border of each part of the optic nerve. In the orbit, the mouse optic nerve was divided into three parts: intraretinal (i), astrocytic filament dense (afd), and astrocytic filament sparse (afs). This classification was defined by the position of the sensory retina and by the distribution of astrocytic filaments. The border between the i and afd parts was set at the boundary between the sensory retina and the retinal pigment epithelium. Astrocytic filaments were seen to be elongated transversely at the border (Fig. 6A,C), while those in the i part were observed to be extended longitudinally. In the afd part, a very high concentration of astrocytic filaments was seen. A majority of astrocytic filaments in the afs part were long and extended perpendicular to the optic nerve axis, while a minority were seen to be short and expanded parallel to the axis. The border between the afd and afs parts was defined by the distal (anterior) limit of longer transverse filaments in the afs part (Fig. 6A,C). The length of the afd part was around 200 μm. The area containing GS-immunoreactive cells in the mouse optic nerve corresponded to the afs part (Fig. 6).

***Wfs1* mRNA expression in the normal mouse optic nerve**

Next, to determine whether *Wfs1* mRNA is expressed in the optic nerve, we performed RNA in situ hybridization on longitudinal sections of the normal mouse optic nerve by using a mouse *Wfs1* anti-sense-riboprobe. Weak-to-moderate *Wfs1* mRNA signals were observed in the afd, and afs parts. In the afd part, *Wfs1* mRNA-hybridized cells were densely concentrated, while in the afs part, they were sparsely distributed. Weak *Wfs1* mRNA

signals were detected in the i part (Fig. 7A). In control experiments by using a mouse *Wfs1* cRNA sense probe, few mRNA signals were observed (Fig. 7B).

Wfs1 protein expression in the normal mouse optic nerve

We next examined cell-specific Wfs1 protein localization in the optic nerve. Weak-to-moderate Wfs1 immunoreactivity was clearly seen in the afd, and afs parts (Fig. 7C,E,F). Wfs1-immunoreactive cells were aligned along optic nerve fiber bundles, and more tightly distributed in the afd than afs part. Background Wfs1 immunoreactivity on optic nerve fiber bundles was greater in the afd than afs part (Fig. 7C,E,F). Weak Wfs1 immunoreactivity was detected in the i part (Fig. 7C). When a normal rabbit immunoglobulin was used instead of the anti-Wfs1 antibody, no immunoreactivity was seen in the optic nerve (data not shown). When the antibody preabsorbed by incubation with the GST-Wfs1N chimeric protein (antigen), immunoreactivity was not observed (Fig. 7D).

To confirm which glial cell types express Wfs1, other longitudinal sections were double-immunostained with the anti-Wfs1 antibody, and with antibodies against GFAP (an astrocyte marker), oligodendrocytes [RIP] (an oligodendrocyte marker), GS (a glial cell marker), or mouse CD11b (a marker for microglia). Wfs1 immunoreactivity was observed solely in astrocytes (Fig. 8A-I). No Wfs1 immunoreactivity was found in oligodendrocytes (Fig. 8J-O), GS-immunoreactive glial cells (Fig. 8P-R), or in microglia (data not shown; Table 3).

***Wfs1* mRNA expression in the normal mouse vision-related brain structures**

We examined whether *Wfs1* mRNA was expressed in the optic chiasm, the optic tract, the retino-recipient nuclei, and in the visual cortex (vision-related brain structures). Moderate *Wfs1* mRNA signals were observed in layer II of the primary and secondary visual cortical areas. In layer II, moderate signals were distributed along the boundary between layers I and II, and weak signals were seen deeper down (Fig. 9A). In the retino-recipient nuclei, *Wfs1*

mRNA signals were weak in the suprachiasmatic nucleus (SCN) and in the SC. In the SC, *Wfs1* mRNA-hybridized cells were dispersed in the zonal, superficial gray, and intermediate gray layers (Fig. 9D,F). Extremely weak or no *Wfs1* mRNA signals were seen in the optic chiasm, the optic tract, the LGN, the pretectum, and in the medial terminal nucleus (data not shown). In control experiments by using a mouse *Wfs1* cRNA sense probe, few mRNA signals were observed (data not shown).

Wfs1 protein expression in the normal mouse vision-related brain structures

We next examined whether Wfs1 protein was expressed in the vision-related brain structures. Wfs1 immunoreactivity was found in the visual cortex, the SCN, and in the SC (Figs. 9B,E, 10A). In the visual cortex, moderately Wfs1-immunoreactive neurons were distributed in layer II of the primary and secondary visual cortical areas. The more strongly immunoreactive neurons were distributed in the more superficial part of the layer. In addition, Wfs1-immunoreactive punctations were observed in layer V(Fig. 9B,C). In the SCN, moderately Wfs1-immunoreactive neurons were seen in the dorsomedial part (Fig. 10A). In the SC, moderately immunoreactive cells were distributed in the zonal, superficial gray, and intermediate gray layers (Fig. 9E,F). Extremely weak or no immunoreactivity was seen in the optic chiasm, the optic tract, the LGN, the pretectum, and in the medial terminal nucleus (Fig. 10C,D; Table 4). When a normal rabbit immunoglobulin was used instead of the anti-Wfs1 antibody, no immunoreactivity was seen in the visual cortex, the SCN, and in the SC (data not shown). When the antibody preabsorbed by incubation with the GST-Wfs1N chimeric protein (antigen), immunoreactivity was not observed in these structures (Fig. 10B, data not shown).

DISCUSSION

We determined the expression pattern of the *Wfs1* gene in the normal mouse retina, optic nerve, SC, SCN, and in the visual cortex, at both the mRNA and protein levels. In these structures, not only neurons but also glial cells expressed Wfs1. In the retina, Wfs1 was expressed in all neuron types: photoreceptors, horizontal cells, bipolar cells, displaced (ON) and non-displaced (OFF) amacrine cells, and RGCs. Müller glial cells also expressed Wfs1. In the optic nerve, astrocytes were the site of Wfs1 expression. In the brain, Wfs1 was expressed in the zonal, superficial gray, and intermediate layers of the SC, in the dorsomedial part of the SCN, and in layer II of the primary and secondary visual cortical areas.

Specificity of anti-Wfs1 antibody

In the immunoblot analysis, the anti-Wfs1 antibody clearly recognized a single ~100 kDa protein band of extracts from the brain and optic nerve (Fig.1). The band size was consistent with an apparent molecular mass of ~100 kDa that was examined in extracts from rodent brains (Takeda et al., 2001; Ishihara et al., 2004). This protein band disappeared when the antibody was preincubated with the antigen (GST-Wfs1N chimeric protein) (antibody-absorption experiment) (Fig. 1). Thus, the specificity of the antibody has been characterized in the mouse optic nerve as well as in the brain.

In the immunoblot analysis of retinal extracts, the antibody detected both ~100 kDa and ~70 kDa bands. As for the ~70 kDa Wfs1-immunoreactive band, a few reports have appeared. It is not known which kind of protein around ~70 kDa the antibody detected. If the protein of this band is a part of the Wfs1 protein, the appearance of the band likely resulted from degradation of the Wfs1 protein or from an alternative RNA splicing of the *Wfs1* gene in retinal cells. Since not only the ~100 kDa band but also the ~70 kDa band disappeared in the antibody-absorption experiment, the protein of ~70 kDa is most probably a part of the Wfs1 protein containing a portion of the N-terminus antigen site (amino acids 1-179), that is,