BDNF is Upregulated by Postnatal Development and Visual Experience: Quantitative and Immunohistochemical Analyses of BDNF in the Rat Retina

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PURPOSE. This study sought to elucidate changes in the levels and distribution of brain-derived neurotrophic factor (BDNF) in the retina throughout aging and depending on visual experience.

METHODS. Protein and mRNA levels of BDNF were quantified by enzyme-linked immunosorbent assay (ELISA) and semiquantitative reverse transcription–polymerase chain reaction (RT-PCR), respectively. Levels were assayed in the retinas of rats on postnatal day (P)2, P7, and P14 (approximate time of eye opening) and at 1 month (M), 3M, 8M, and 18M of age. Changes in BDNF expression and localization in the retina were assessed by immunohistochemistry. The effect of monocular deprivation during infancy on retinal BDNF expression was also examined, by ELISA and immunohistochemistry.

RESULTS. Both protein and mRNA levels of BDNF in the rat retina increased after P14. Immunohistochemical analyses revealed that the increase in BDNF protein levels occurred in retinal ganglion cells (RGCs) between P14 and 1M. BDNF immunoreactivity in Müller cell processes was observed in the inner nuclear layer at 1M, but not at P14. The levels of BDNF protein in the retinas of visually deprived eyes were lower than those of control eyes, as quantified by ELISA. Immunohistochemistry showed that BDNF immunoreactivity in RGCs was diminished by visual deprivation, whereas Müller cells were unaffected.

CONCLUSIONS. These observations indicate that BDNF expression in RGCs is upregulated in an activity-dependent manner, whereas that in Müller cells is regulated only by development. (*Invest Ophthalmol Vis Sci.* 2003;44:3211–3218) DOI: 10.1167/iovs.02-1089

The neurotrophin family of ligands contains nerve growth factor, brain-derived neurotrophic factor (BDNF), neurotrophin-3, and neurotrophin-4/5. Neurotrophins and their cognate receptors, TrkA, TrkB, and TrkC, are expressed primarily in neurons to mediate pleiotropic effects, promoting the differentiation, maturation, and survival of neurons in both the peripheral and central nervous systems.[1] Accumulating evidence also suggests that neurotrophins affect synaptic functions.[2,3] In the retina, BDNF plays a critical role in the promotion of survival. BDNF is particularly important in the survival of retinal ganglion cells (RGCs) in vitro[4] and in vivo.[5,6] Neurotrophins act as synaptic modulators both in the retina and the brain. In the *Xenopus* retina, BDNF increased the complexity of optic axon terminal arbors[7]; BDNF increased the number of retinal dopaminergic synapses in the rat retina.[8]

The presence of BDNF in the retina was first described in *Xenopus* by use of an RNase protection assay.[9] Immunocytochemistry of retinal cell culture revealed that BDNF protein was found primarily in RGCs.[9] In the rat retina, BDNF mRNA[10] and protein[11] localize to the ganglion cell layer (GCL) and inner nuclear layer (INL), as demonstrated by in situ hybridization and immunohistochemistry, respectively. The colocalization of BDNF mRNA and protein at these sites strongly suggests that BDNF is synthesized locally within the retina. In addition to local synthesis, BDNF undergoes retrograde transport from the tectum to the retina in amphibians,[12] birds,[13] and rodents,[14] possibly exerting an effect as a target-derived neurotrophic factor. Furthermore, anterograde transport of BDNF from the chick retina to the tectum[15] also suggests a role for this molecule as an anterophin.[16]

The high-affinity BDNF receptor TrkB is also present within the retina.[17,18] TrkB mRNA[10] and protein[11] are detectable in the GCL, inner plexiform layer (IPL), and INL. As neurotrophins and their receptors colocalize in the retina, neurotrophins likely act on retinal neurons in an autocrine and/or paracrine manner.[12]

It is thought that the expression of BDNF, but not other neurotrophins, is regulated by neural activity.[19] The mechanisms regulating BDNF expression in the retina, however, remain elusive. In this study, we examined the age-associated changes in the levels of BDNF within the rat retina. We quantified the levels of BDNF protein and mRNA using a two-site enzyme-linked immunosorbent assay (ELISA) and a semiquantitative reverse transcription–polymerase chain reaction (RT-PCR), respectively. We also assessed retinal BDNF expression and localization by immunohistochemistry. To examine the dependence of changes in BDNF expression on visual experience, we subjected rats to monocular deprivation during infancy, followed by quantification and cellular localization of BDNF protein in the retina by ELISA and immunohistochemistry.

MATERIALS AND METHODS

Animals

All experimental procedures using animals were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the institutional guidelines for care and use of laboratory animals. Male Wistar rats (Japan SLC, Hamamatsu, Japan) were housed in standard lighting conditions (12-hour light–dark cycle) for at least 7 days before experimentation. To examine age-related changes in BDNF protein and mRNA levels in the retina, animals were anesthetized by chloral hydrate and killed by decapitation on postnatal

day (P)2, P7, P14, and at 1 month (M), 3M, 8M, and 18M of age near the end of the light phase. Retinas were separated under a dissecting microscope. In younger animals, four (P2) or two (P7) retinas were pooled for one sample. P14 and 1M rats were used for immunohistochemical analyses ($n = 3$ animals each). To analyze the effects of monocular deprivation, one eyelid margin of Brown Norway rats (Charles River, Yokohama, Japan) was trimmed after anesthesia with chloral hydrate (0.42 mg/g body weight) on P10. A soft contact lens–like shield dyed black was inserted beneath the eyelid,[20] and the lid was tightly sutured with 5-0 silk.[21] The animals were killed on P25, and their retinas were processed for ELISA, Western blot analysis, or immunohistochemistry ($n = 4$ animals for each of the experiments). Untreated eyes of these animals served as the control.

Enzyme-Linked Immunosorbent Assay

To examine age-related changes in BDNF protein levels in the retina, P2 ($n = 5$ samples of 10 rats), P7 ($n = 5$ samples of 5 rats), P14 ($n = 15$ retinas of 15 rats), 1M ($n = 11$ retinas of 11 rats), 3M ($n = 11$ retinas of 11 rats), 8M ($n = 8$ retinas of 8 rats), and 18M ($n = 5$ retinas of 5 rats) animals were used. To analyze the effect of monocular deprivation on retinal BDNF protein levels, four individual animals were used. Retinas were homogenized by sonication with homogenization buffer (50 mM Tris [pH 7.5], 300 mM NaCl, 0.1% Triton X-100, 10 μg/mL aprotinin, 0.1 mM benzethonium chloride, 1 mM benzamidine, and 0.1 mM phenylmethylsulfonyl fluoride), followed by centrifugation for 15 minutes at $12,000g$ at 4°C.[22,23] The quantity of total protein in the supernatants was assessed by protein assay (Bio-Rad Laboratories, Hercules, CA), according to the manufacturer's instruction.

BDNF protein levels were determined by ELISA, as previously described.[22,23] Briefly, titer plates were coated with primary antibody and blocked with bovine serum albumin. ELISA samples (in duplicate) or standards of BDNF (in triplicate) were incubated. After the wells were washed, biotinylated secondary antibody was added. Bound biotinylated secondary antibody was detected by incubation with streptavidin-β-galactosidase. β-Galactosidase activity in each well was measured by incubation with fluorogenic substrate, 4-methylumbelliferyl-β-D-galactoside. The quantity of fluorescent products was measured by a fluorometer (Fluorolite-1000; Dynatech Laboratories, Chantilly, VA) with excitation at 364 nm and emission at 448 nm. A standard curve in the range of 1 to 100 pg of human recombinant BDNF was plotted for each titer plate. The average value of the sample was normalized to the total soluble protein concentration.

Reverse Transcription–Polymerase Chain Reaction

Total RNA was extracted from retinas of P2 ($n = 4$ samples of 8 rats), P7 ($n = 3$ samples of 3 rats), P14 ($n = 5$ retinas of 5 rats), 1M ($n = 5$ retinas of 5 rats), 3M ($n = 4$ retinas of 4 rats), 8M ($n = 3$ retinas of 3 rats), and 18M ($n = 3$ retinas of 3 rats) rats, with RNA extraction reagent (Isogen; Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. The quality of mRNA was evaluated for each sample by the ratio of 28S ribosomal RNA (rRNA) to 18S rRNA. Samples retaining a 28S-to-18S rRNA ratio greater than 1.6 were used for RT-PCR.[24] Total RNA (50 ng) was amplified by RT-PCR (RT-PCR High Plus Kit; Toyobo, Osaka, Japan) with the following sets of specific primers: for BDNF, 5′-TCCCTGGCTGACACTTTTGAG-3′ and 5′-ATTGGGTAGTTCGGCATTGCG-3′; and for β-actin, 5′-CCCTAAGGCCAAC-CGTGAAAAGATG-3′ and 5′-GAACCGCTCATTGCCGATAGTGATG-3′ (in duplicate).[25] The predicted sizes of the amplified BDNF and β-actin products were 465 and 433 bp, respectively. RT-PCR amplification was performed on a commercial system (Gene Amp PCR System 9700; Applied Biosystems, Foster City, CA), using initial incubations of 30 minutes at 60°C and then 2 minutes at 94°C followed by cycles of 1 minute at 94°C and 1.5 minutes at 62°C. Thirty cycles for BDNF and 22 cycles for β-actin were determined by control experiments to be the appropriate number of cycles for unsaturated amplification. A final 7-minute incubation at 62°C facilitated terminal extension. PCR prod-

ucts were separated by 1.5% agarose gel electrophoresis and stained with ethidium bromide. The intensity of fluorescence for each PCR product was quantified with a fluorescent imaging system (FLA-2000; Fuji Photo Film, Tokyo, Japan). RT-PCR experiment was repeated twice for each RNA species, and the average value obtained from the experiments was analyzed.

Western Blot Analysis

Retinal TrkB protein levels in the monocularly deprived animals were determined by Western blot analysis. SDS-PAGE and Western blot analysis were performed as described.[26] Retinal tissues derived from the control and deprived eyes of four experimental animals were lysed in lysis buffer (2% sodium dodecyl sulfate and 62.5 mM Tris [pH 6.8]) by sonication. After determination of protein concentration, samples (40 μg/lane) were subjected to SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were incubated with either mouse anti-TrkB monoclonal antibody (the kind gift of Shinichi Koizumi, Research Institute of Novartis Pharmaceuticals, Tsukuba, Japan; 1:3000)[27] or mouse anti-actin monoclonal antibody (Chemicon, Temecula, CA; 1:5000). After extensive washing, membranes were exposed to peroxidase-conjugated anti-mouse IgG antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) at a 1:10,000 dilution. Peroxidase activity was visualized on x-ray film by chemiluminescence (Western Lightning Chemiluminescence Reagent Plus; PerkinElmer Life Sciences, Boston, MA). The intensity of the bands corresponding to TrkB and actin was quantified by densitometry, with NIH Image, ver.1.60 (available by ftp from zippy.nimh.nih.gov/ or from http://rsb.info.nih.gov/nih-image; developed by Wayne Rasband, National Institutes of Health, Bethesda, MD). TrkB protein levels were normalized to the levels of actin within each sample. The mean value for the control eyes was set at 100%.

Immunohistochemistry

Before performing BDNF immunohistochemistry, we determined the specificity of rabbit anti-BDNF antibody (SC-546; Santa Cruz Biotechnology, Santa Cruz, CA), an antibody previously used for immunostaining.[11] The antibody was preabsorbed with BDNF-linked affinity beads (AffiGel-10; Bio-Rad Laboratories) overnight at 4°C. The immunoreactivity of preabsorbed and nonabsorbed antibody was assayed by both dot blot assay and immunohistochemistry. In the dot blot assay, human recombinant BDNF (50 ng) was blotted onto a nitrocellulose membrane (Protran; Schleicher & Schuell, Keene, NH). Membranes were incubated with either preabsorbed or nonabsorbed antibody at a 1:1000 dilution followed by exposure to peroxidase-conjugated anti-rabbit IgG antibody (Dako, Glostrup, Denmark, 1:10,000 dilution). Peroxidase activity was detected as above. To determine the immunohistochemical specificity of the anti-BDNF antibody, preabsorbed or nonabsorbed antibody (1:200 dilution) was applied to retinal specimens of 1M rats as primary antibody.

For immunohistochemistry, rats were anesthetized with chloral hydrate and killed by decapitation near the end of the light phase. Eyes were enucleated, and the anterior segments, including the lens, were removed. Posterior eyecups were fixed for 1 hour with 4% paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.4), followed by several washes in PB. Samples were then immersed in 30% sucrose in PB overnight at 4°C for cryoprotection. Eyecups of P14 and 1M animals, or those of control and deprived eyes were embedded in the same molds with optimal cutting temperature (OCT) compound (Tissue Tek; Sakura, Tokyo, Japan) and frozen to achieve similar staining conditions for both samples. Radial sections (10 μm) were cut with a cryostat, mounted on glass slides (APS-coated Superfrost Micro glass slide; Matsunami Glass, Tokyo, Japan), and air dried. A portion of the specimens was stained with Mayer's Hematoxylin solution (Wako, Osaka, Japan) and Eosin alcohol solution (Wako). Immunohistochemistry was performed as described.[28] Rabbit anti-BDNF antibody (SC-546, 1:200 dilution) and rabbit anti-phospho-CREB (Ser133; Cell Signaling Technology, Beverly, MA; 1:100) were used as primary antibodies. For double

immunostaining, either mouse anti-microtubule associated protein-2 (MAP2) antibody (Chemicon, 1:500 dilution) or mouse anti-vimentin antibody (Boehringer Ingelheim, Ingelheim, Germany; 1:500 dilution) was combined. Fluorescence-conjugated goat anti-rabbit IgG secondary antibody (1:200 dilution, Alexa Fluor 488; Molecular Probes, Eugene, OR) was used either alone or in combination with fluorescence-conjugated goat anti-mouse IgG antibody (Alexa Fluor 546, 1:500 dilution; Molecular Probes). Cell nuclei were counterstained with 150 nM propidium iodide (Sigma, St. Louis, MO) in the absence of double immunostaining. Specimens were observed under photomicroscope (AxioPhoto2; Carl Zeiss Meditech, Jena, Germany), and photomicrographs were captured at a 1300- × 1030-pixel resolution using a digital camera (AxioCam; Carl Zeiss Meditech). Images were processed using image-processing software (AxioVision; Carl Zeiss Meditech). The strength of excitation light and length of exposure time were kept constant for precise comparisons. Immunohistochemistry was performed using three or four individual animals and was repeated at least twice to confirm the reproducibility.

Statistical Analysis

Statistical analyses were performed with one-way ANOVA followed by the Duncan multiple-comparison tests. All data are expressed as the mean ± SD.

RESULTS

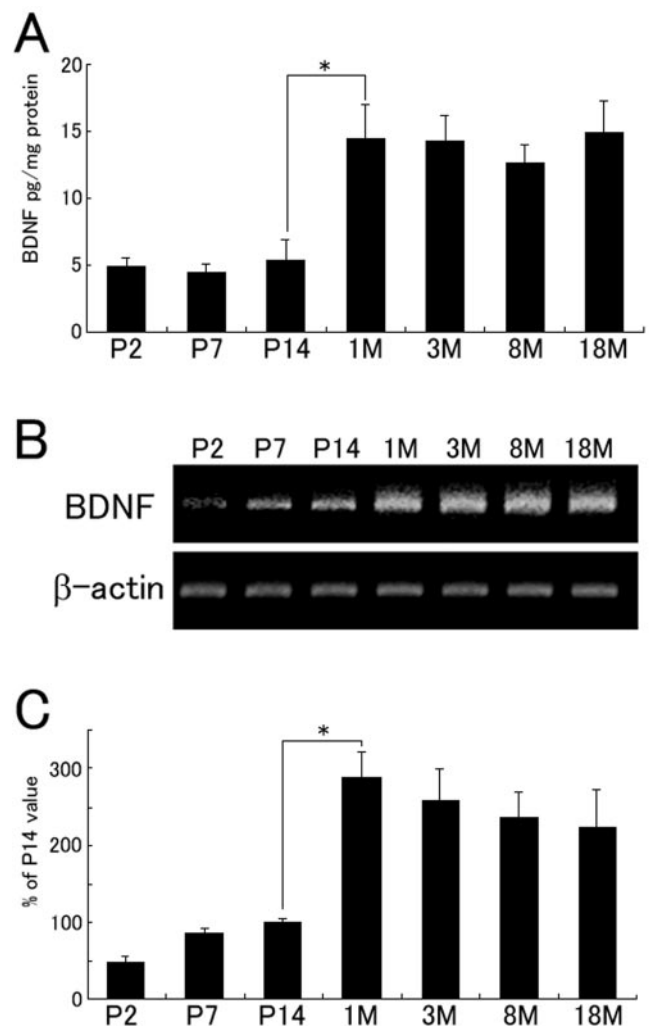### Quantification of BDNF Protein Levels in the Rat Retina during Postnatal Development

BDNF protein levels in the rat retina were quantified by ELISA. BDNF protein levels in the retina, represented as quantity of neurotrophin per total soluble protein, were 4.9 ± 0.6 pg/mg total protein and 4.4 ± 0.6 pg/mg total protein on P2 and P7, respectively (Fig. 1A), and remained constant until P14 (5.3 ± 1.5 pg/mg total protein). A large increase in retinal BDNF protein levels occurred in rat development between P14 and 1M (14.5 ± 2.5 pg/mg total protein; $P < 0.001$). These levels, however, did not change significantly between the ages of 1M, 3M (14.2 ± 1.9 pg/mg total protein), 8M (12.6 ± 1.4 pg/mg total protein), and 18M (14.9 ± 2.3 pg/mg total protein; $P >$ 0.05 in all pair-wise comparisons). BDNF protein levels in the adult (3M) retina were similar to those in the cerebral cortex (16.6 ± 1.1 pg/mg total protein), but lower than in the hippocampus (94.3 ± 10.6 pg/mg total protein) and superior colliculus (31.4 ± 5.1 pg/mg total protein).

Quantification of BDNF mRNA Levels in the Rat Retina during Postnatal Development

mRNA levels of BDNF were determined by semiquantitative RT-PCR. Fluorescence intensity of RT-PCR products from BDNF and β-actin mRNA templates (Fig. 1B) was quantified using a fluorescence imaging system. Values were standardized to β-actin mRNA, the levels of which were consistent throughout the ages examined. The mean level obtained in P14 animals was set as 100% for each mRNA species. BDNF mRNA levels in the retina on P2 were 48% ± 7% and those on P7 were 87% ± 6% (Fig. 1C). Consistent with the protein levels obtained by ELISA, a large increase in BDNF mRNA levels also occurred between P14 (100% ± 4.9%) and 1M of age (288% ± 34%; $P <$ 0.001). BDNF mRNA levels measured at 1M, 3M (259% ± 39%), 8M (235% ± 35%), and 18M (224% ± 49%) of age did not differ significantly ($P > 0.05$, in all pair-wise comparisons).
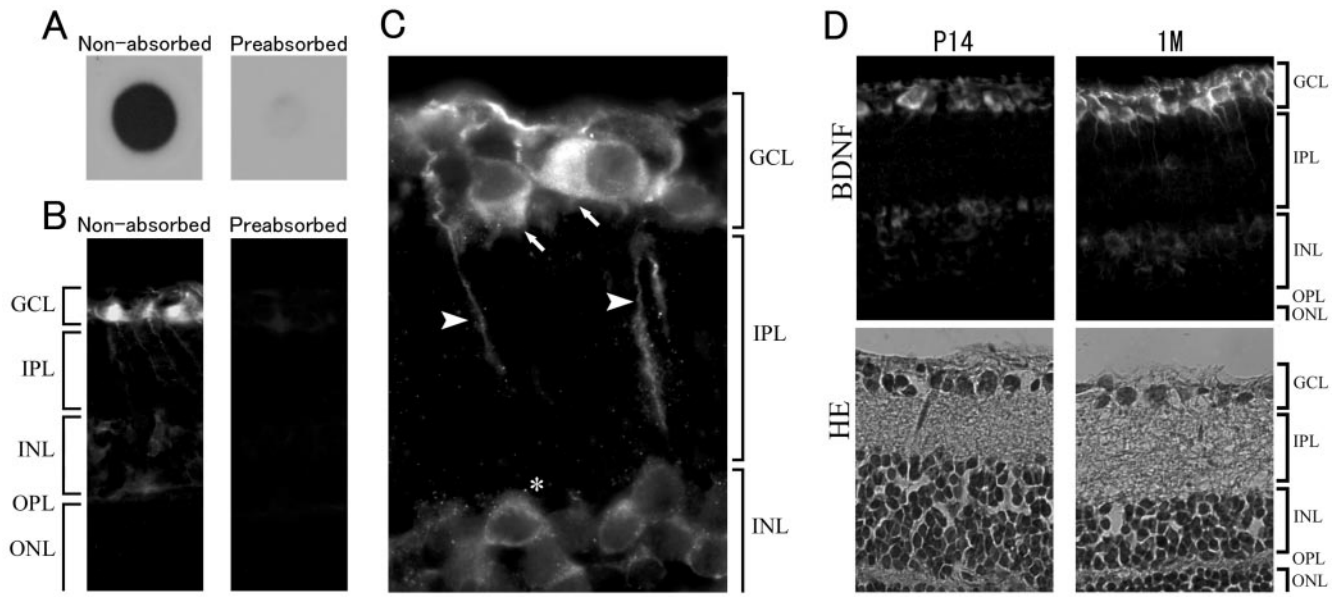
Immunohistochemistry of BDNF in the Retina: Its Localization and Developmental Changes

We used a rabbit anti-BDNF polyclonal antibody (SC-546; Santa Cruz Biotechnology) for immunohistochemical analyses.[11] Im-

FIGURE 1. Quantification of protein and mRNA levels of BDNF in the rat retina during postnatal development. Retinas of P2, P7, P14, 1M, 3M, 8M, and 18M rats were used. (**A**) BDNF protein levels were determined by ELISA and are shown as the quantity of neurotrophin per total soluble protein (in picograms/milligram). Data are expressed as the mean ± SD for P2 ($n = 5$ samples of 10 rats), P7 ($n = 5$ samples of 5 rats), P14 ($n = 15$ retinas of 15 rats), 1M ($n = 11$ retinas of 11 rats), 3M ($n = 11$ retinas of 11 rats), 8M ($n = 8$ retinas of 8 rats), and 18M ($n = 5$ retinas of 5 rats). (**B**) Total RNA was extracted from retinas of P2 ($n = 4$ samples of 8 rats), P7 ($n = 3$ samples of 3 rats), P14 ($n = 5$ retinas of 5 rats), 1M ($n = 5$ retinas of 5 rats), 3M ($n = 4$ retinas of 4 rats), 8M ($n = 3$ retinas of 3 rats), and 18M ($n = 3$ retinas of 3 rats) old rats. BDNF and β-actin mRNA levels were quantified by semiquantitative RT-PCR. Each mRNA fragment was amplified from 50 ng of total RNA with specific primer pairs (in duplicate). The image displays an example of RT-PCR products from BDNF and β-actin mRNA after separation by 1.5% agarose gel electrophoresis and visualization with ethidium bromide. The fluorescence intensity of PCR products was quantified with a fluorescence imaging system. (**C**) Levels of mRNA for BDNF were standardized to β-actin mRNA levels amplified from the same RNA sample. Data are expressed as the mean ± SD for P2, P7, 1M, 3M, 8M, and 18M animals, with the mean for P14 animals set as 100%. (**A, C**) *$P < 0.001$ in comparison with P14; by one-way ANOVA followed by the Duncan multiple comparison test.

munoreactivity of this antibody was almost completely abolished after preabsorption with affinity-gel–conjugated BDNF, as revealed by dot blot analysis and immunohistochemistry (Figs. 2A, 2B). When primary antibody was omitted, only very low signals were observed (data not shown). These results confirmed the specificity of the antibody. On immunostaining

FIGURE 2.   Immunohistochemistry of BDNF in the rat retina. (**A**) The specificity of anti-BDNF antibody (SC-546; Santa Cruz Biotechnology) was determined by preabsorption before immunohistochemistry. Examination of specificity by dot blot assay was achieved by blotting human recombinant BDNF onto membranes and then incubating them with nonabsorbed or preabsorbed anti-BDNF antibodies. Only nonabsorbed antibody exhibited strong immunoreactivity against BDNF. (**B**) The specificity of anti-BDNF antibody was also confirmed by immunohistochemistry. Retinal sections of 1M rats were incubated with either nonabsorbed or preabsorbed antibody. When immunostained with nonabsorbed anti-BDNF antibody, strong immunoreactivity was detected in the GCL, and weak staining was detected in the INL. The ONL did not react with the antibody. Specimens demonstrated only faint reactivity in the GCL and INL when stained with preabsorbed antibody. (**C**) A representative photomicrograph of inner part of neural retina of 1M rats immunostained with anti-BDNF antibody. Three types of BDNF immunoreactivity are observed in the neural retina: strong BDNF immunoreactivity associated with cytoplasm of cells in the GCL (*arrows*); weak BDNF immunoreactivity in some cell bodies in the INL (✱), and processlike BDNF immunoreactivity spanning the GCL through IPL (*arrowheads*). (**D**) Retinal sections obtained of P14 and at 1M rats were immunostained with anti-BDNF antibody, or stained with hematoxylin-eosin. Scale bar, 50 μm.

of retinal specimens with nonabsorbed antibody, strong BDNF immunoreactivity was detected in the GCL, and weak activity was detected in the INL (Fig. 2B, left). A representative photomicrograph of the inner part of the neural retina of 1M rat immunostained with anti-BDNF antibody (Fig. 2C) demonstrates three types of BDNF immunoreactivity observed in the neural retina. The first is strong BDNF immunoreactivity associated with cytoplasm of cells in the GCL (Fig. 2C, arrows). BDNF immunoreactivity is also observed in a population of cell bodies in the INL adjacent to the IPL (✱), although this staining is of a weaker intensity than that in the GCL. In addition, a processlike pattern of BDNF immunoreactivity spans the GCL running through the IPL (arrowheads).

We compared BDNF levels and distribution in the retina at P14 and 1M of age by immunohistochemistry. BDNF immunoreactivity in cell bodies in the GCL became stronger with increasing development, whereas BDNF immunoreactivity in the INL remained constant (Fig. 2D, upper panels). The processlike BDNF immunoreactivity in the GCL/IPL was not detectable in P14 rat retinas. No morphologic differences in hematoxylin-eosin (HE) staining could be observed between retinal sections of P14 and 1M (Fig. 2D, lower panels).
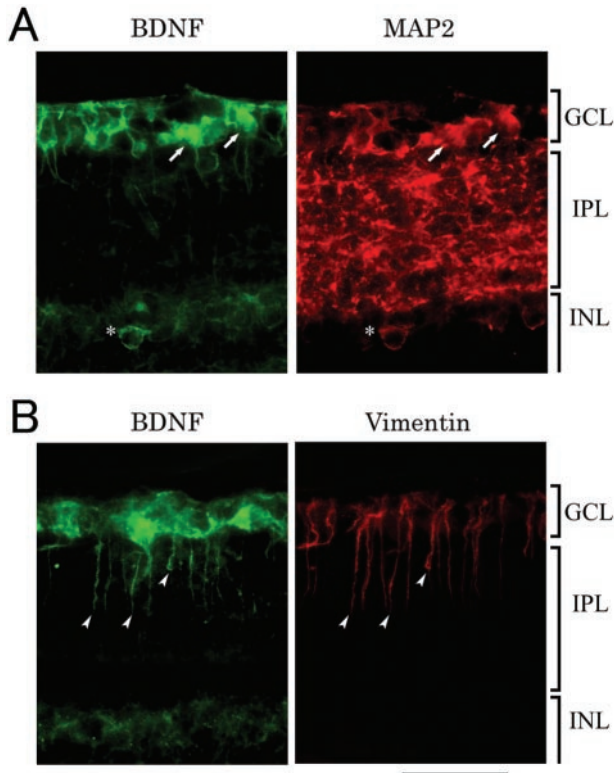
Colocalization of BDNF Immunoreactivity with MAP2- and Vimentin Immunoreactivity in the Retina

To identify the types of cells expressing BDNF, we performed double-stain immunohistochemistry. Retinal sections of 1M rats were stained with anti-BDNF antibody and antibodies against either MAP2, a marker of RGCs and amacrine cells, or vimentin, a marker of Müller glia. Strong MAP2 immunoreactivity (Fig. 3A, red) was seen in the GCL and IPL and in the INL

adjacent to the IPL. In the GCL, BDNF immunoreactivity was colocalized with MAP2 immunoreactivity (Fig. 3A, arrows indicate double-labeled cells in the GCL), indicating that RGCs contain BDNF. We also tested antibodies against Thy-1.1, a RGC-specific cell surface protein in the rat retina,[29] in double immunostaining. BDNF immunoreactivity was also colocalized with Thy-1.1 immunoreactivity, confirming that RGCs contain BDNF (data not shown). Some cell bodies in the INL adjacent to the IPL also stained positively with both antibodies (Fig. 3A, asterisk). Similar staining patterns colocalizing BDNF and MAP2 were observed in P14 rat retinas (data not shown). Vimentin immunoreactivity was detected spanning from the GCL to the IPL in a processlike pattern, colocalizing with processlike BDNF immunoreactivity (Fig. 3B, arrowheads). These data indicate that Müller cells also express BDNF. Although weaker, vimentin immunoreactivity in P14 rat retinas could also be observed in a similar processlike shape (data not shown).

Reduction in BDNF Protein Levels in the Retinas of Visually Deprived Eyes

To deprive an eye of activity, we performed unilateral suturing of a rat eyelid combined with the insertion of a contact lens-like shield. This procedure did not damage the anterior segment of the eye. We did not detect any significant changes in the light microscopic anatomy of retina (data not shown). The levels of BDNF protein in the retinas of deprived eyes at P25, as measured by BDNF ELISA, were 9.7 ± 0.9 (pg/mg total protein), significantly lower than those of control eyes (14.9 ± 0.7 pg/mg total protein; $P < 0.01$; Fig. 4A). Neither TrkB nor actin protein in the retina was altered by monocular deprivation, as determined by Western blot analysis (Fig. 4B, 4C).

FIGURE 3. Colocalization of BDNF immunoreactivity with MAP2 and vimentin immunoreactivity in the rat retina. Retinal sections of 1M rat retinas were immunostained with rabbit anti-BDNF antibody in combination with either mouse MAP2 (**A**) or mouse anti-vimentin (**B**) antibody. Anti-MAP2 antibody labeled the cell bodies and processes of retinal ganglion cells (RGCs), and amacrine cells and anti-vimentin antibody identified Müller glial cell processes. (**A**) Strong MAP2 immunoreactivity (**A**, *red*) was seen in the GCL and IPL and in the layer of the INL adjacent to the IPL. Strong BDNF immunoreactivity (*green*) in the GCL colocalized with MAP2 immunoreactivity (*arrows*). A population of cell bodies in the INL adjacent to the IPL were also double-labeled (✳). (**B**) Vimentin immunoreactivity (*red*) was detected stretching from the GCL to IPL in a branched pattern, which was associated with processlike BDNF immunoreactivity (*green*; *arrowheads*). Scale bar, 50 μm.

Downregulation of BDNF Expression in RGCs but Not in Müller Cells by Visual Deprivation

To confirm that visual deprivation was achieved by the method of unilateral eyelid suturing, we compared the immunoreactivity of phosphorylated cAMP-response element binding protein (CREB), which had been known to be increased by light stimuli in the GCL,[30] between control and deprived eyes. Phosphorylated CREB, the activated form of this protein, was decreased in light-deprived RGCs (Fig. 5A). The effect of visual input deprivation on retinal BDNF expression was assessed by immunohistochemistry. BDNF immunoreactivity in cytoplasm of cells in the GCL was largely reduced in the retinas of deprived eyes, in comparison with untreated eyes (Fig. 5B). In contrast, monocular deprivation did not affect the processlike BDNF immunoreactivity or BDNF immunoreactivity in cell bodies of the INL. The results indicate that BDNF expression was decreased by visual deprivation in RGCs alone.

DISCUSSION

Using a sensitive and specific two-site ELISA,[22,23] we quantified the levels of BDNF proteins within the retina, a primary sen-

sory organ that receives visual inputs (Fig. 1A). Levels of BDNF dramatically increased between P14 and 1M of development. The increase in BDNF protein paralleled those in mRNA (Fig. 1C). Both the retina itself[15] and the optic tectum[12,13] have been proposed as possible sources of neurotrophins in the retina. Concerted changes in protein and mRNA levels of BDNF within the retina suggest that locally synthesized BDNF protein is the primary contributor to the increase observed between P14 and 1M. The increase of BDNF mRNA in the retina between P14 and P60 was also reported by in situ hybridization.[10] The tendency was similar to the previous report that BDNF mRNA level in the feline visual cortex rose by the end of the critical period.[31]

To assess the effect of aging on BDNF expression, we quantified BDNF levels in the retinas of older rats up to at 18M of age. In the central nervous system, neurotrophin levels generally remain constant after adulthood. Stable levels of BDNF expression have been reported in the hippocampus,[23] frontal cortex,[23] and pituitary gland.[32] In agreement with these observations in the brain, the levels of BDNF in the retinas of aged animals remained relatively stable (Figs. 1A, 1C).

We examined changes in the cellular distribution of BDNF protein during postnatal development by immunohistochemistry. BDNF immunoreactivity in the retina was detected in the GCL and INL (Fig. 2B, left panel), as previously reported.[11] Colocalization of BDNF and MAP2 immunoreactivity in the GCL and INL by immunofluorescence indicated that BDNF was localized in RGCs or displaced amacrine cells in the GCL (Fig. 3A, arrows) and in amacrine cells in the INL (Fig. 3A, asterisk), as MAP2 exists in cell bodies and processes of these types of cells.[33] Vimentin is a marker for Müller cells, the predominant glia in the vertebrate retina. This molecule is restricted to the inner portion of Müller cell processes, reaching to the level of the inner limiting membrane.[34] Processlike BDNF-positive profiles overlapped with vimentin immunoreactivity (Fig. 3B, arrowheads); thus, BDNF protein also localizes to Müller cells.
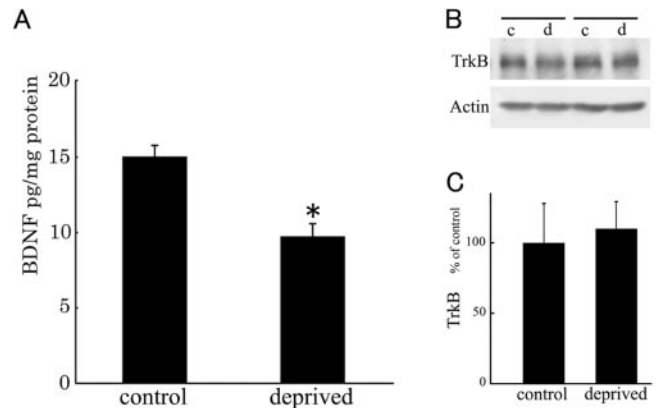
FIGURE 4. BDNF and TrkB levels in the retina of monocularly deprived rats. Unilateral eyelid suture was performed on P10, followed by retinal dissection on P25. Contralateral untreated eyes were served as the control. (**A**) Retinal BDNF protein levels of monocularly deprived animals were determined by ELISA. Data are expressed as the mean ± SD of results in four animals. *$P < 0.01$ in comparison with control animals; one-way ANOVA followed by the Duncan multiple comparison test. (**B**) TrkB and actin protein levels were determined by Western blot analysis. Western blot analysis for TrkB and actin in the retinas of control (c) and deprived (d) eyes allowed examination of protein levels. (**C**) Densitometric analysis standardized TrkB protein levels to actin protein levels obtained for the same sample. Data are expressed as the mean ± SD of results in four animals, with the mean in control eyes normalized to 100%. One-way ANOVA followed by the Duncan multiple comparison tests did not show any significant differences between control and deprived eyes.
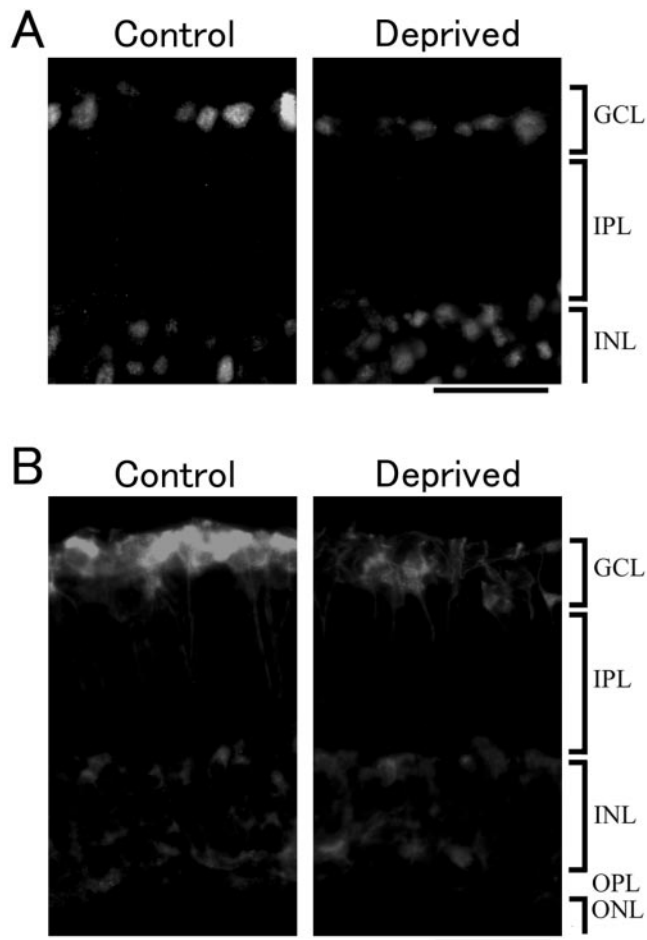
FIGURE 5. BDNF immunohistochemistry in the retina of monocularly deprived rats. Retinal sections of control and deprived eyes were immunostained with an antibody against phospho-CREB (**A**) or BDNF (**B**). (**A**) Phospho-CREB immunoreactivity in the nuclei of retinal ganglion cells (RGCs) in deprived eyes decreased in comparison with fellow control eyes. (**B**) BDNF immunoreactivity in the cytoplasm of RGCs in deprived eyes decreased in comparison with fellow control eyes. In contrast, BDNF immunoreactivity in Müller cell processes was not affected by monocular deprivation. Scale bar, 50 μm.

Whereas Di Polo et al.[35] used an adenoviral vector to transduce the BDNF gene into Müller cells as a pathway to deliver BDNF to RGCs, no previous studies have demonstrated that Müller cells contain endogenous BDNF, with the exception of results obtained in the tench retina.[36] Our results indicate that the Müller cells of mammals normally contain BDNF.

We demonstrated by ELISA and immunohistochemical analyses that BDNF protein levels in RGCs and Müller cells increased after eye opening, which occurs approximately on P14 in rodents. These results suggest that visual experiences upregulate the levels of BDNF protein. As previously reported,[37] the structure and morphology of the retina in P14 and 1M rats were very similar at the level of light microscopy (Fig. 2D, lower panels). In addition, the gross functional and morphologic features of the maturing eye just before eye opening appear very similar to the eye of an adult rodent. Electron micrographs detailing synaptic formation,[38] electroretinogram as a measure of eye function,[39] the activity of γ-aminobutyric acid (GABA) uptake system,[40] and GABA(A) receptor[41] and glutamate transporter[42] levels are almost comparable by the time of eye opening with those seen in adults. Several electrophysiological studies, however, have demonstrated that re-

sponsiveness of RGCs to visual stimuli increased after eye opening.[43–45] In the present study, we deprived rodents of visual experience which may affect neuronal functions to test the effect of visual stimuli on BDNF expression in the retina, because the expression of BDNF is regulated by neural activity in brain neurons.[19,46] Many studies examining the effects of visual experience on the development of the visual system in the brain have identified a period of high plasticity, known as the critical period.[47] The critical period for rats is between P14 and P45,[48] which begins at eye opening. Indeed, dark rearing[49] and monocular deprivation[50] decreased BDNF expression in the visual cortex. Visual input–dependent alteration of functional molecules within the retina, however, has not been examined. We performed unilateral eyelid suture to induce monocular deprivation. The procedure is not invasive, thus enabling a direct comparison between the deprived and control eye in an individual rat. BDNF protein levels in the retina of deprived eyes were reduced to 65% of the levels in control fellow eyes (Fig. 4A). BDNF levels, however, remained higher than those in control animals before eye opening, suggesting that the inhibition of BDNF expression in the retina by monocular deprivation was partial. Immunohistochemical analyses showed a marked reduction of BDNF immunoreactivity in RGCs of deprived eyes, whereas almost no changes were observed in Müller cell processes (Fig. 5). These results are in good agreement with previous reports demonstrating that expression of BDNF in neurons is activity-dependent, whereas that in glial cells is not affected by activity.[46,51] We propose two possibilities explaining why the inhibitory effect of sensory deprivation on BDNF expression in the retina was partial. Müller cells, that mature later than RGCs,[52] may contribute a source of BDNF that is developmentally upregulated but independent of neural activity. Although we used Brown Norway rats to minimize light reaching the retina, the partial inhibition may be attributable to low levels of light passing through the eyelid and shield. Although elucidation of the molecular mechanisms underlying the upregulation of BDNF by sensory inputs awaits further study, the transcriptional regulation of BDNF mRNA may be activated by changes in the activity of transcription factors such as CREB. Because the activation of CREB is known to play an important role in BDNF expression,[53] reduced levels of phospho-CREB, the activated form, in light-deprived RGCs (Fig. 5A) may cause the downregulation of BDNF in the retina. Furthermore, light-induced CREB phosphorylation in the GCL of the rat retina[30] suggests that sensory input evokes neural activities and subsequent CREB phosphorylation, activating BDNF transcription. Thus, BDNF protein levels in RGCs increase in response to visual input. In contrast to BDNF, the protein levels of TrkB, the high-affinity receptor for BDNF, were not affected by monocular deprivation (Figs. 4B, 4C).

We have demonstrated age-associated changes in the levels of BDNF in the retina. The expression levels of BDNF in the retina increased after eye opening. Elevation of BDNF protein levels was observed in both RGCs and Müller cells after eye opening. In animals subjected to monocular deprivation, the BDNF protein level in RGCs of the deprived eyes of visual experience was downregulated. Thus, BDNF production in RGCs increased in an activity-dependent manner, whereas that in Müller cells remained independent of sensory input. Although activity-dependent induction of BDNF is well established in neurons of the central nervous system, we demonstrate that this upregulation also occurs in RGCs under physiologic conditions. These results will help clarify the physiological roles of neurotrophins in the retina and mechanisms regulating neurotrophin expression. The discovery that BDNF is present in Müller cells suggests their supporting role as a source of BDNF for retinal neurons both under physiological

and pathologic conditions. In addition, the induction of endogenous BDNF in Müller cells by external administration of agents, such as catechol compounds,[54] may serve as a therapeutic approach for ischemic or degenerative disorders of RGCs and the optic nerve, as occurs in central retinal artery occlusion, diabetic retinopathy, and glaucoma.

Acknowledgments

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