Involvement of Brain-Derived Neurotrophic Factor in Early Retinal Neuropathy of Streptozotocin-Induced Diabetes in Rats

Therapeutic Potential of Brain-Derived Neurotrophic Factor for Dopaminergic Amacrine Cells

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Although neurotrophins have been assessed as candidate therapeutic agents for neural complications of diabetes, their involvement in diabetic retinopathy has not been fully characterized. We found that the protein and mRNA levels of brain-derived neurotrophic factor (BDNF) in streptozotocin-induced diabetic rat retinas were reduced to 49% ($P < 0.005$) and 74% ($P < 0.05$), respectively, of those of normal control animals. In addition, dopaminergic amacrine cells appeared to be degenerating in the diabetic rat retinas, as revealed by tyrosine hydroxylase (TH) immunoreactivity. Overall TH protein levels in the retina were decreased to one-half that of controls ($P < 0.01$), reflecting reductions in the density of dopaminergic amacrine cells and the intensity of TH immunoreactivity within them. To confirm the neuropathological implications of BDNF reduction, we administered BDNF protein into the vitreous cavities of diabetic rats. Intraocular administration of BDNF rescued dopaminergic amacrine cells from neurodegeneration and counteracted the downregulation of TH expression, demonstrating its therapeutic potential. These findings suggest that the early retinal neuropathy of diabetes involves the reduced expression of BDNF and can be ameliorated by an exogenous supply of this neurotrophin. *Diabetes* 53:2412–2419, 2004

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BDNF, brain-derived neurotrophic factor; BSS, balanced salt solution; ELISA, enzyme-linked immunosorbent assay; GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; NT-3, neurotrophin-3; PLSD, projected least significant difference; RGC, retinal ganglion cell; STZ, streptozotocin; TH, tyrosine hydroxylase; TUNEL, transferase-mediated dUTP nick-end labeling.

Diabetic retinopathy, which is classically defined as a microvasculopathy, is being viewed as a neurodegenerative disease of the retina (1). Much evidence suggests (2–8) that changes in the functional molecules and viability of neurons in the retina occur immediately after the onset of diabetes, preceding the vascular complications in both humans and experimental animals. The most prominent changes in the electroretinogram seen in the early stages of diabetes involve oscillatory potentials that are thought to derive from dopaminergic amacrine cells (9). Therefore, alterations in the dopaminergic system are thought to be among the first significant events in the development of diabetic retinopathy (2–4).

Neurotrophins are expressed and have pleiotropic effects in the nervous system (10). Among them, brain-derived neurotrophic factor (BDNF) is expressed in retinal ganglion cells (RGCs) and Müller glia in the retina (11) and is important for the survival of RGCs (12). It has also been reported (13,14) to prevent amacrine cell death. In addition to being a survival factor, BDNF acts as a synaptic modulator and has been shown (15) to cause hypertrophy of the retinal dopaminergic system in the rat retina. Although many studies have described the important roles of BDNF in the physiology and pathophysiology of the retina, there have been no reports of the involvement of neurotrophins in diabetic retinopathy. Here, we report the degeneration of dopaminergic amacrine cells accompanied by a reduction in BDNF levels in the retina of rats with streptozotocin (STZ)-induced diabetes. Furthermore, we will demonstrate the therapeutic potential of BDNF for treating neurodegeneration of dopaminergic amacrine cells in the diabetic rat retina.

RESEARCH DESIGN AND METHODS

All experimental procedures using animals were performed in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research and our institutional guidelines for the care and use of laboratory animals. Adult male Wistar rats (Japan SLC, Hamamatsu, Japan), 9 weeks of age (250–300 g), were housed in standard lighting conditions (12-h light and 12-h dark cycle) with food and water ad libitum for at least 7 days before experimentation. STZ (70 mg/kg in

50 mmol/l sodium citrate buffer, pH 4.5; Sigma, St. Louis, MO) was administered intraperitoneally under general anesthesia with diethylether inhalation (16). Nondiabetic animals were injected with an equal volume of citrate buffer. Body weight and blood glucose concentrations were measured before the injection and weekly thereafter. Diabetes was confirmed by assaying the glucose concentration in blood obtained from the tail vein using FreeStyle (Nipro, Osaka, Japan). Rats with glucose levels >250 mg/dl were classified as diabetic. Animals were anesthetized by intraperitoneal injection of an overdose of chloral hydrate and killed by decapitation 4 weeks after the STZ injection, near the end of the light phase. The retinas were processed for further analyses.

Intraocular administration of BDNF. Multiple intraocular injections, for a total of five times, were given every 3 days, beginning 2 weeks after the intraperitoneal injection of STZ or citrate buffer, under general anesthesia with diethylether and topical anesthesia with a drop of 2% lidocaine applied to the eyes. BDNF (5 μg) in 5 μl of 0.1% BSA (Fraction V; Sigma) in balanced salt solution (BSS) (Alcon, Fort Worth, TX) was injected into the vitreous space of one eye chosen at random according to a previous report (17). An equal volume of vehicle (0.1% BSA in BSS) was injected into the other eye as a control. Retinas were dissected 2 days after the last intraocular injection. Any animal with lens damage or visible vitreous hemorrhage was excluded from the analyses.

Western blotting. To determine tyrosine hydroxylase (TH) protein levels, retinal tissues were lysed, and their protein samples were subjected to SDS-PAGE followed by Western blotting as previously reported (18). Mouse anti-TH monoclonal antibody (19) (1:10,000 dilution; a gift from Dr. Hatanaka) and peroxidase-conjugated goat anti-mouse IgG antibody (1:10,000 dilution; Jackson ImmunoResearch Laboratories, West Grove, PA) were used as primary and secondary antibodies, respectively. Protein levels of TrkB, a high-affinity receptor for BDNF, in the retina were determined by Western blotting using mouse anti-TrkB monoclonal antibody (20) (1:3,000 dilution). Anti–β-actin (1:5,000 dilution; Chemicon, Temecula, CA) was used as a loading control for Western blotting. The intensity of the bands was quantified by densitometry using NIH Image version 1.60 (National Institutes of Health, Bethesda, MD). Protein concentration was assessed by a BioRad Protein Assay Kit (BioRad Laboratories, Hercules, CA).

Enzyme-linked immunosorbent assay. Protein levels for BDNF (21), neurotrophin-3 (NT-3) (22), and Thy-1 (23) in the retina were quantified by enzyme-linked immunosorbent assay (ELISA) as previously described. ELISA samples (in duplicate) and standards (in triplicate) were applied to titer plates. The anti-BDNF antibody used in ELISA has no cross-reactivity against other neurotrophins or growth factors and did not detect BDNF protein in samples obtained from BDNF knockout mice (21). A standard curve of recombinant BDNF (1–100 pg/well), NT-3 (1–100 pg/well), or Thy-1 (300–6,000 pg/well) was plotted for each plate. The average value of the sample was normalized against the total protein concentration.

Real-time PCR. mRNA levels for BDNF, NT-3, and Thy-1 in the retinas were quantified by real-time PCR. Total RNA was extracted from retinas as previously described (11). The quality of the mRNA was evaluated by the ratio of 28S ribosomal RNA (rRNA) to 18S rRNA; samples with a ratio >1.6 were used for real-time PCR ($n = 4$ animals in each group) (24). Total RNA (50 ng) was amplified using an RT-PCR High Plus kit (Toyobo, Osaka, Japan), with the following sets of specific primers: for BDNF, 5′-TCCCTGGCTGACACTTTT GAG-3′ and 5′-ATTGGGTAGTTCGGCATTGCG-3′ (11); for NT-3, 5′-GATCC AGGCGGATATCTTGA-3′ and 5′-AGCGTCTCTGTTGCCGTAGT-3′; for Thy-1, 5′-CGCTTTATCAAGGTCCTTACTC-3′ and 5′-GCGTTTTGAGATATTTGAAGG T-3′ (25); and for β-actin, 5′-GGCATCCTGACCCTGAAGTA-3′ and 5′-GGGGT GTTGAAGGTCTCAAA-3′ (each experiment performed twice). These combinations of PCR primers gave single PCR products of 465, 182, 344, and 203 bp, respectively. The Thy-1 primers, which span an intron and thus produce different-sized products from genomic DNA (745 bp), were used to check for possible DNA contamination of the samples. Quantitative PCR amplification was carried out on a real-time PCR machine (LightCycler; Roche Diagnostics, Basel, Switzerland), using an initial incubation for 10 min at 60°C and 20 cycles of 1 s at 94°C and 15 s at 60°C, with fluorescence acquisition at 80°C in each cycle. Following the PCR cycles, a melting-curve analysis performed by a standard program showed a single melting peak for each pair of primers. Data were analyzed as previously described (24), and mRNA levels for BDNF, NT-3, and Thy-1 were standardized against β-actin mRNA levels in the same RNA sample. The mean value for the nondiabetic animals was set at 100%.

Immunohistochemistry and transferase-mediated dUTP nick-end labeling staining. Tissue preparation, cryosectioning, and immunohistochemistry were performed as previously described (11). Rabbit anti-BDNF antibody (SC-546, 1:200 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) or mouse anti-TH antibody (19) (1:1,000 dilution) was used as primary antibody. The specificities of both antibodies were confirmed by preabsorption with the antigens in the previous studies (11,19). For double immunostaining, rabbit anti-pan Trk antibody (C-14, 1:200 dilution; Santa Cruz Biotechnology) was used. The Alexa Fluor 488 goat anti-rabbit IgG antibody (1:200 dilution; Molecular Probes, Eugene, OR) and Alexa Fluor 546 goat anti-mouse IgG antibody (1:500 dilution; Molecular Probes) were used as secondary antibody. To detect individual apoptotic cells, dual-color staining for transferase-mediated dUTP nick-end labeling (TUNEL) and TH protein was carried out using a kit (In Situ Cell Death Detection Kit, Fluorescein; Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions. Cell nuclei were counterstained with Hoechst 33342 (200 nmol/l; Molecular Probes) to visualize the layer structure of the retina. Specimens were observed under an AxioPhoto 2 microscope (Carl Zeiss, Jena, Germany), and photomicrographs were captured at 1,300 × 1,030-pixel resolution using a digital camera (AxioCam; Carl Zeiss) and image processing software (AxioVision; Carl Zeiss). The strength of the excitation light and length of exposure time were kept constant for precise comparisons. Immunohistochemistry was performed using four individual animals and was repeated at least twice.

Whole-mount immunohistochemistry. Whole-mount immunohistochemistry for TH was performed on retinas of both nondiabetic and diabetic rats ($n = 4$ animals in each group), as previously reported (15), with some modifications. Briefly, fixed retinas were defattened and rehydrated in 0.1 mol/l PBS (26). Specimens were incubated with mouse anti-TH antibody (19) (1:1,000 dilution) in 5% skim milk in PBS containing 0.3% Triton X-100 for 3 days at 4°C with continuous agitation, followed by extensive washes in PBS. After a 1-day incubation with the Alexa Fluor 546 goat anti-mouse IgG antibody (1:500 dilution) at 4°C, retinas were washed in PBS and mounted flat on glass slides with the ganglion cell layer (GCL) oriented upward in 80% glycerol in PBS for observation. Image acquisition was performed as described above. Morphometric analyses were performed according to the methods described by Cellerino et al. (15): type I and type II TH-positive dopaminergic amacrine cells were identified by observing the intensity of their immunoreactivity and the size of their soma. Labeled cells in the sample consisting of five fields of 2.33 mm^2 for each retina were counted to determine the density of TH-positive cells.

Statistical analyses. All values are expressed as means ± SD. Statistical significance between groups was analyzed either by Welch's t test or one-way ANOVA followed by Fisher's projected least significant difference (PLSD) multiple comparison tests, as indicated. Values of $P < 0.05$ were taken to be significant.

RESULTS

Reduced TH protein levels in diabetic rat retinas. TH protein level, which has been used as a marker for retinal dopaminergic amacrine cells (15), and actin level, as a control, were quantified by Western blotting (Fig. 1*A*). Band intensity was determined by densitometry, and the mean value obtained for nondiabetic animals was set as 100% for each protein species. TH protein levels were standardized to actin levels in the same lane because actin levels did not differ between nondiabetic (100 ± 4%) and diabetic animals (103 ± 11%, $P = 0.73$, Welch's t test). TH levels obtained in diabetic animals were significantly lower than those of nondiabetic animals ($P < 0.01$, Welch's t test, $n = 4$ animals in each group) (Fig. 1*B*).

We also quantified Thy-1 levels as an index of the number of RGCs (27) because Thy-1 protein levels had a strong positive correlation with the RGC number (23). Neither Thy-1 protein level nor mRNA level was significantly different between nondiabetic and diabetic animals (247 ± 13 vs. 227 ± 21 ng/mg Thy-1 protein level, $P = 0.12$; and 100 ± 7 vs. 92 ± 8% relative Thy-1 mRNA level, $P = 0.30$, in nondiabetic and diabetic animals, respectively, Welch's t test, $n = 4$ animals in each group). β-Actin mRNA levels were also indistinguishable (nondiabetic, 100 ± 5%; diabetic animals, 101 ± 6%; $P = 0.78$, Welch's t test).

Reduction in TH immunoreactivity and the numbers of dopaminergic amacrine cells in the diabetic rat retina. To examine the effects of diabetes on the morphol-

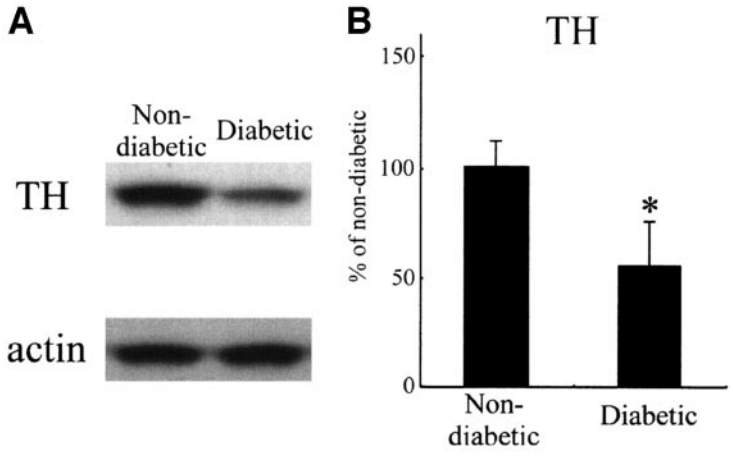

FIG. 1. Reduced TH protein levels in the diabetic rat retinas. *A*: Retinas were dissected from diabetic or nondiabetic rats 4 weeks after treatment with STZ or vehicle (citrate buffer) (n = 4 animals in each group). Protein samples (15 μg) were collected and subjected to SDS-PAGE. Western blotting for TH (*upper panel*) and actin (*lower panel*) in the retinas allowed examination of protein levels. *B*: Densitometric analyses of TH protein levels standardized against actin protein levels in the same lane were performed. TH levels obtained in diabetic animals (55 ± 20%) were lower than those of nondiabetic animals (100 ± 11%). Bars represent the means ± SD values, with the mean values for nondiabetic rat retinas set at 100%. *Welch's t tests gave $P < 0.01$ in comparison with the nondiabetic controls.

ogy of dopaminergic amacrine cells and their processes, we performed immunohistochemistry with an anti-TH antibody on radial sections of the rat retina. In both nondiabetic and diabetic rat retinas, TH-positive amacrine neurons were observed in the innermost row of the inner nuclear layer (INL) (Fig. 2*A*), as reported (15). TH-positive fibers innervating both stratum 1 (s1, arrows) and stratum 3 (s3, arrowheads) of the inner plexiform layer (IPL) could be detected in the nondiabetic rat retina (Fig. 2*A*, *left panel*). In the diabetic animals, TH-positive fibers in stratum 1 were thinning down, and those in stratum 3 were hardly visible (Fig. 2*A*, *right panel*). No positive signal could be observed in neighboring sections stained without primary antibody (data not shown). When specimens were analyzed by dual-color staining for TUNEL and TH protein, some TH-positive amacrine neurons undergoing apoptosis were identified in diabetic animals (Fig. 2*B*). In the nondiabetic animals, TUNEL-positive dopaminergic neurons could not be observed (data not shown).

Because dopaminergic amacrine neurons are very sparse, making it difficult to quantify their numbers in radial sections, we determined the density of TH-positive cells in the whole-mounted retina. Cells with a large soma that display intense TH immunoreactivity (type I) (Fig. 3*A*, arrowheads) and smaller cells with lighter TH immunoreactivity (type II, arrows) were distinguished in the rat retina, as reported (15). The distribution of TH-positive cells in the diabetic rat retinas (Fig. 3*B*, *lower panel*) was sparser than in the nondiabetic animals (Fig. 3*B*, *upper panel*). Furthermore, signal intensity of TH immunoreactivity in both the soma and cell processes of individual cells was much weaker in the diabetic rat retinas. Statistical analysis revealed a significant difference in the cell density of TH-positive dopaminergic amacrine cells in

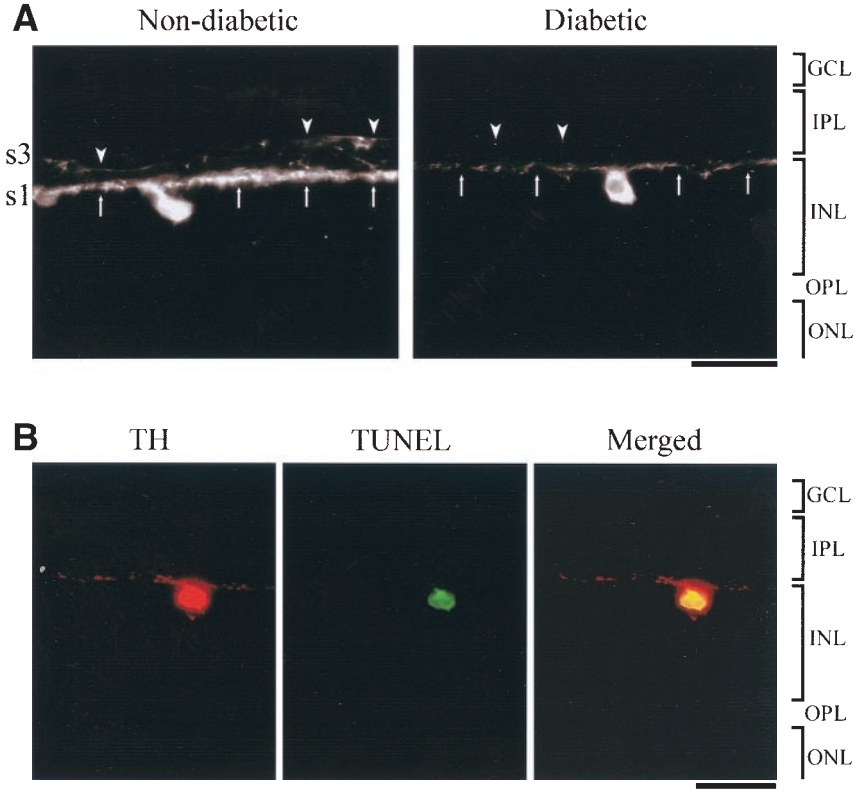

FIG. 2. Immunohistochemistry of TH and TUNEL staining on cryosections of diabetic rat retinas. *A*: Radial sections of nondiabetic and diabetic rat retinas were immunostained with anti-TH antibody to visualize dopaminergic amacrine cells and their processes (n = 4 animals in each group). In the rat retinas, TH-positive amacrine neurons were observed in the innermost row of the INL. TH-positive fibers innervating both stratum 1 (s1; arrows) and stratum 3 (s3; arrowheads) of the IPL could be detected in the nondiabetic rat retina (*left panel*). In the diabetic animals, TH-positive fibers in stratum 1 were thinner than in the nondiabetic controls, and those in stratum 3 were hardly visible (*right panel*). *B*: TUNEL staining was carried out in combination with immunostaining against TH. The image is a representative photomicrograph of a diabetic rat retina. Some of the TH-positive neurons (red) showed a TUNEL-positive signal (green), confirming the apoptosis event in dopaminergic amacrine cells of the diabetic animals. ONL, outer nuclear layer; OPL, outer plexiform layer. Scale bar = 50 μm.

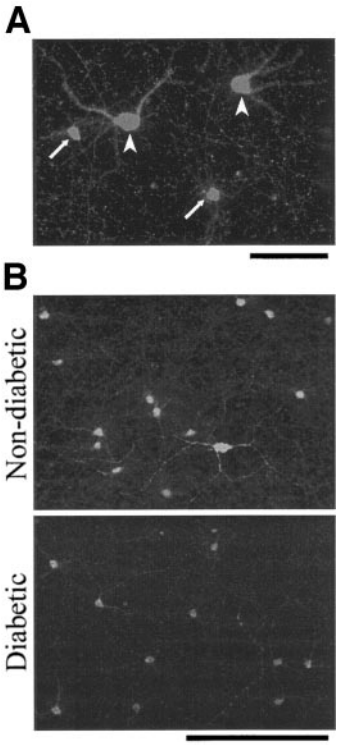

FIG. 3. Whole-mount immunohistochemistry of TH in diabetic rat retinas. Whole-mounted retinas of nondiabetic and diabetic rats were immunostained with anti-TH antibody (n = 4 animals in each group). *A*: A representative photomicrograph of a high-magnification image of a nondiabetic rat retina immunostained with anti-TH antibody. Cells with large somas that display intense TH immunoreactivity (type I, arrowheads) and smaller cells with lighter TH immunoreactivity (type II, arrows) were distinguishable. Scale bar = 50 μm. *B*: TH-positive cells in the diabetic rat retinas (*lower panel*) were more sparsely distributed than in the nondiabetic animals (*upper panel*). Furthermore, signal intensity in both the somas and cell processes of individual cells was much weaker in the diabetic rat retinas. Scale bar = 200 μm.

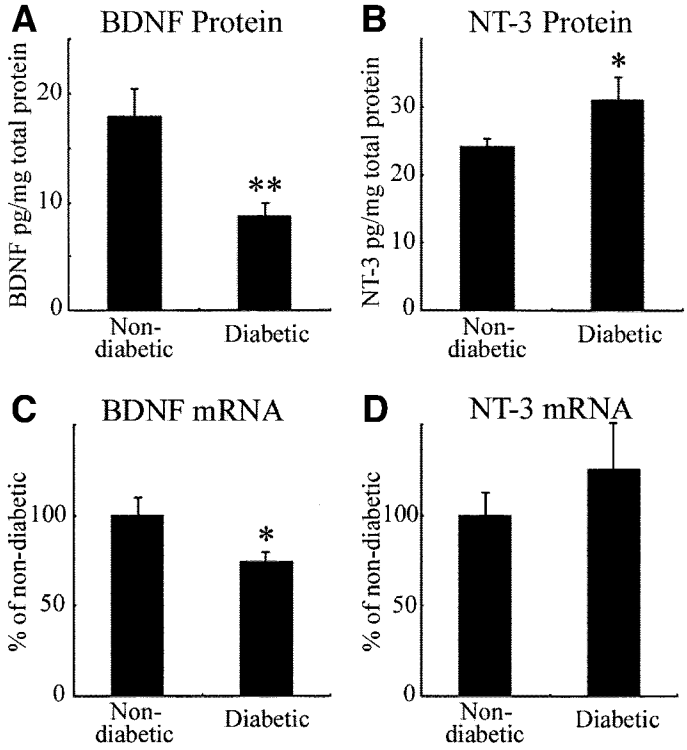

FIG. 4. Quantification of BDNF and NT-3 levels in diabetic rat retinas. Protein levels of BDNF (*A*) and NT-3 (*B*) in the retinas of nondiabetic and diabetic rats were quantified by ELISA and were represented as quantities of neurotrophin per total soluble protein (n = 4 animals in each group). Bars represent means ± SD. BDNF protein levels in the retinas of diabetic animals (8.7 ± 1.3 pg/mg total protein) were significantly decreased compared with those in nondiabetic rats (17.8 ± 2.7 pg/mg total protein). In contrast, rather higher NT-3 protein levels were observed in diabetic animals (31.1 ± 3.3 pg/mg total protein) than in nondiabetic animals (24.3 ± 1.1 pg/mg total protein). mRNA levels of BDNF (*C*) and NT-3 (*D*) were determined by real-time PCR, and their values were standardized to β-actin mRNA levels in the same RNA samples (n = 4 animals in each group). The mean value for the nondiabetic animals was set at 100%. Bars represent means ± SD. BDNF mRNA levels in diabetic animals (74 ± 6%) were significantly lower than those of nondiabetic animals (100 ± 10%), whereas NT-3 mRNA levels were not significantly different between diabetic (126 ± 26%) and nondiabetic (100 ± 12%) rats. Welch's t tests gave *$P < 0.05$ and **$P < 0.005$ in comparison with the nondiabetic animals.

whole-mounted retinas. The densities of both type I and type II TH-positive neurons were decreased in diabetic animals ($P < 0.05$, Welch's t test, $n = 4$ animals in each group) (Table 1). These results imply that degeneration of dopaminergic amacrine cells or TH downregulation in these cells occurs in the early stage of STZ-induced diabetes.

Reduction in BDNF, but not NT-3, expression levels in diabetic rat retinas. As shown in Fig. 4, BDNF protein levels in the retinas of diabetic animals were significantly decreased ($P < 0.005$, Welch's t test, $n = 4$ animals in each group). In contrast, rather higher NT-3 protein levels were observed in diabetic animals compared with those in nondiabetic animals ($P < 0.05$, Welch's t test, $n = 4$ animals in each group). BDNF mRNA levels were significantly lower in diabetic compared with nondiabetic animals ($P < 0.05$, Welch's t test), whereas NT-3 mRNA levels did not differ significantly (Fig. 4*C* and *D*).

TABLE 1
Cell density of TH-positive dopaminergic amacrine cells in diabetic rat retinas

	Type I (cells/mm^2)	Type II (cells/mm^2)
Nondiabetic	24.5 ± 1.3	11.0 ± 0.9
Diabetic	16.8 ± 1.5*	8.6 ± 0.4*

Data are means ± SD (n = 4 animals). *$P < 0.05$ compared with the nondiabetic animals. Statistical comparisons were made by Welch's t test.

Reduced BDNF immunoreactivity in both neurons and glial cells of diabetic rat retinas. BDNF immunoreactivity was seen in the cytoplasm of cells in the GCL and INL and in the process-like structures of the GCL running through the IPL (Fig. 5*A*), which correspond to RGCs, amacrine cells, and Müller glia, respectively (11). In the diabetic rat retinas, all of these cell types exhibited reduced BDNF immunoreactivity (Fig. 5*A*, *right panel*), confirming the results obtained by ELISA and RT-PCR. Protein levels of TrkB, a high-affinity receptor for BDNF, in the retina were not altered by diabetic condition, as determined by Western blotting ($P = 0.59$, Welch's t test, $n = 4$ animals in each group) (Fig. 5*B*). Trk immunoreactivity (Fig. 5*C*, green) was seen in the GCL, IPL, and INL and colocalized with TH immunoreactivity (red), even in the diabetic rat retinas (Fig. 5*C*, arrows indicate double-labeled cells), indicating that TH-positive amacrine neurons express Trk receptor.

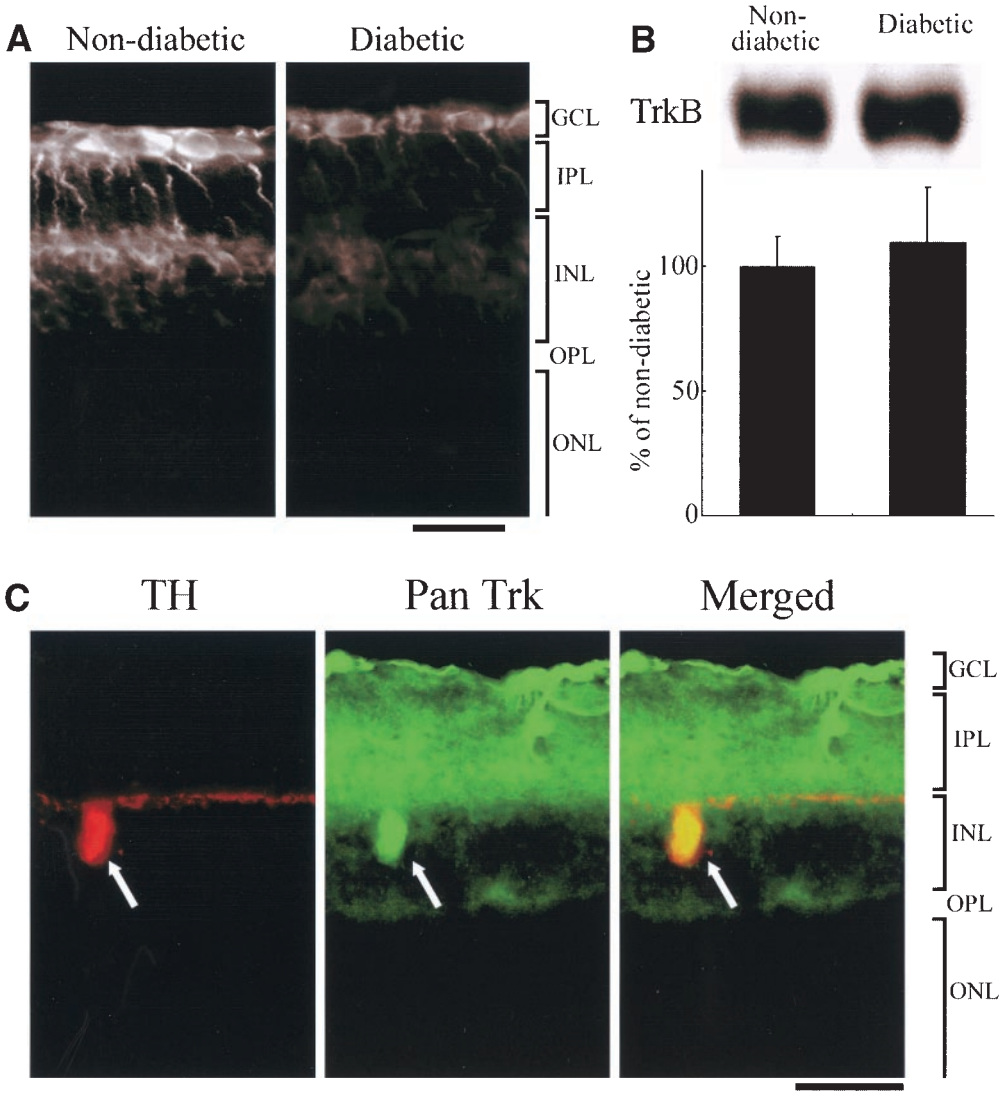

FIG. 5. Immunohistochemistry of BDNF in diabetic rat retinas. *A*: Retinal sections of nondiabetic (*left panel*) and diabetic rats (*right panel*) were immunostained with anti-BDNF antibody. Three types of BDNF immunoreactivity were observed in the retina: *1*) strong BDNF immunoreactivity associated with cytoplasm of cells in the GCL, *2*) weaker BDNF immunoreactivity in cell bodies in the INL, and *3*) process-like BDNF immunoreactivity spanning the GCL through IPL. In the diabetic rat retinas, all of these three types of BDNF immunoreactivity were weakened. ONL, outer nuclear layer; OPL, outer plexiform layer. Scale bar = 50 μm. *B*: Protein samples (40 μg) were subjected to Western blotting for TrkB. Densitometric analyses standardized TrkB protein level against actin in the same lane. TrkB protein levels in the retina were not altered by diabetic condition (100 ± 12.7 vs. 110.2 ± 22.9% relative TrkB protein level in nondiabetic and diabetic animals, respectively; n = 4 animals in each group). Bars represent the means ± SD, with the mean values for nondiabetic rat retinas set at 100%. Welch's t tests gave no significant difference in comparison with the nondiabetic animals (P = 0.59). *C*: Double immunohistochemistry with mouse anti-TH antibody (red) and with rabbit anti-pan Trk antibody (green) was performed using retinal sections of nondiabetic and diabetic rats. The image is a representative photomicrograph of the diabetic animal. Trk immunoreactivity was seen in the GCL, IPL, and INL and colocalized with TH immunoreactivity (arrow), suggesting that TH-positive amacrine neurons express Trk receptor, even in the diabetic condition. Scale bar = 50 μm.

Therapeutic effect of BDNF against degeneration of dopaminergic amacrine cells in diabetic rat retinas. To assess the therapeutic efficacy of BDNF against retinal neuropathy, multiple injections of BDNF were given to either eye of both nondiabetic and diabetic rats (n = 5 animals in each group). Overall TH protein levels in the retinas were determined by Western blotting (Fig. 6*A*) and standardized to actin protein levels. The mean TH protein level for the vehicle-treated nondiabetic rat retinas was set at 100%, and statistical analyses were performed by one-way ANOVA, followed by Fisher's PLSD multiple comparison tests (Fig. 6*B*). The intraocular injection procedure itself did not alter retinal TH protein levels in nondiabetic animals when compared with noninjected nondiabetic ones (108 ± 9%, n = 4, P = 0.49, one-way ANOVA). Retinal TH levels in the vehicle-treated diabetic eyes were markedly reduced ($P < 0.001$ in comparison with the vehicle-treated nondiabetic rat retinas). BDNF treatment efficiently maintained TH levels in the diabetic rat retinas ($P < 0.05$ in comparison with the vehicle-treated diabetic rat retinas). Furthermore, BDNF application upregulated TH protein levels even in nondiabetic animals. To assess the therapeutic potential of BDNF morphologically, we conducted immunohistochemistry on whole-mounted retinas (n = 4 animals in each group). In the diabetic animals, BDNF treatment significantly increased the cell density of both type I and type II dopaminergic amacrine neurons and TH immunoreactivity in individual cells (Table 2 and Fig. 6*C*, *lower panels*). Even in nondiabetic animals, BDNF application increased the cell density of type II dopaminergic neurons and the intensity of TH immunoreactivity in cell processes.

DISCUSSION

Dysfunction of dopaminergic amacrine cells in diabetic retinas has been suggested by studies examining retinal dopamine content (2), TH activity (3,4), and electroretinogram (9). Using TH immunoreactivity as a marker for these cells (15), we demonstrated, for the first time, the morphological or functional degeneration of dopaminergic amacrine cells during the early stage of STZ-induced diabetes, an animal model of type 1 diabetes. In the retinas of diabetic rats, overall TH protein levels were decreased (Fig. 1), reflecting reductions in the cell density of dopaminergic amacrine cells (Table 1). Alternatively, downregulation of TH expression in individual cells might occur in response to diabetes. Either condition may lead to

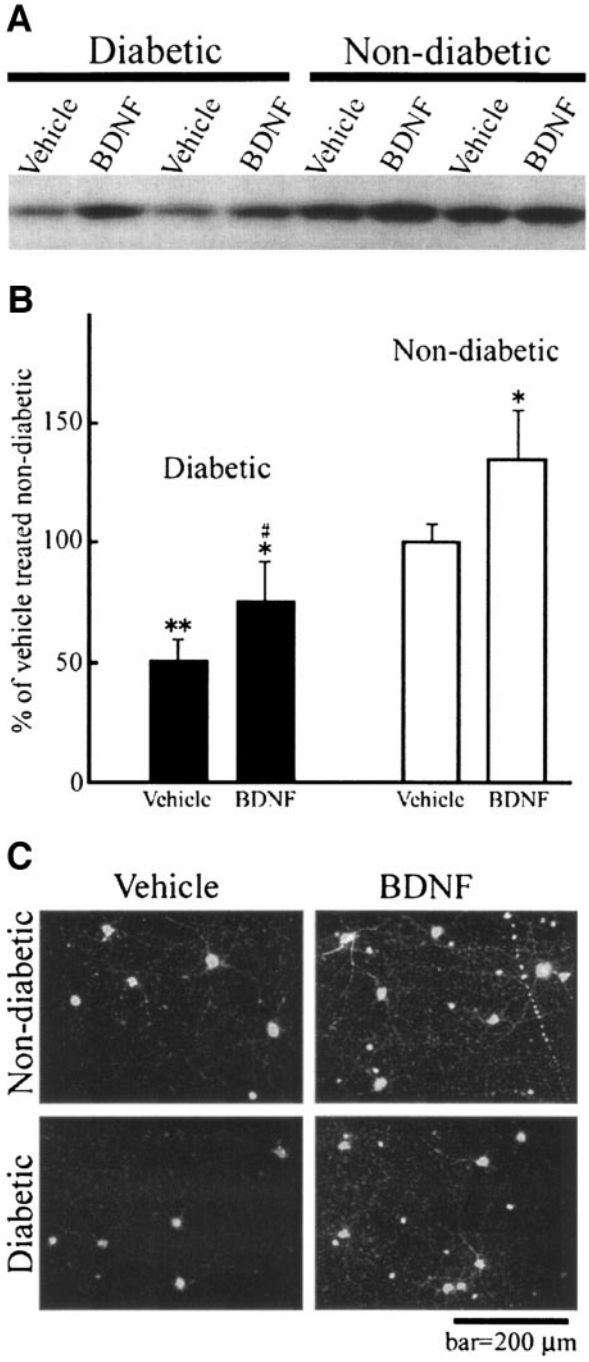

FIG. 6. Therapeutic effect of BDNF against degeneration of dopaminergic amacrine cells in diabetic rat retinas demonstrated by Western blotting and whole-mount retina immunohistochemistry. To assess the therapeutic efficacy of BDNF against retinal neuropathy, a 5-μg injection of BDNF was given to either eye of both nondiabetic and diabetic rats every 3 days, for a total of five injections, beginning 2 weeks after the intraperitoneal injection of STZ or citrate buffer. An equal volume of vehicle (0.1% BSA in BSS) was injected into the matched eye as a control. Retinas were dissected 2 days after the last intraocular injection and subjected to Western blotting (*A* and *B*) or whole-mount retina immunohistochemistry (*C*). *A*: TH protein levels in the retinas were determined by Western blotting. *B*: Densitometric analyses of TH levels standardized against actin levels in the same lane were performed, and the mean value for the vehicle-treated nondiabetic rat retinas was set at 100% (100 ± 8%). Bars represent the means ± SD for each group (n = 5 animals in each group). Retinal TH levels in the BDNF-treated diabetic eyes (76 ± 16%) were higher than those in vehicle-treated diabetic eyes (51 ± 9%) (■). BDNF application itself upregulated TH protein levels, even in the retinas of nondiabetic rats (135 ± 21%) (□). One-way ANOVA followed by Fisher's PLSD multiple comparison tests gave *P < 0.05 and **P < 0.001 in comparison with the vehicle-treated nondiabetic retinas and #P < 0.05 in comparison with the vehicle-treated diabetic rat retinas. *C*: Whole-mounted retinas of nondiabetic (*upper panels*) and diabetic (*lower panels*) animals, which received intraocular injections of either vehicle (*left panels*) or BDNF (*right panels*), were immunostained with anti-TH antibody (n = 4 each group). BDNF treatment increased the cell density of the type II dopaminergic neurons, which have smaller cell bodies, in both nondiabetic and diabetic rats. In the diabetic animals, the cell densities of both type I and type II dopaminergic amacrine neurons and TH immunoreactivity in each cell were increased with BDNF treatment (*lower panels*). Scale bar = 200 μm.

TABLE 2
Effect of BDNF on cell density of TH-positive dopaminergic amacrine cells

	Type I (cells/mm^2)	Type II (cells/mm^2)
Nondiabetic plus vehicle	25.2 ± 1.3	12.2 ± 0.2
Nondiabetic plus BDNF	25.6 ± 1.7	42.5 ± 3.5*
Diabetic plus vehicle	17.0 ± 1.2*†	8.6 ± 0.4†‡
Diabetic plus BDNF	22.0 ± 2.0‡§‖	15.9 ± 2.2†‡¶

Data are means ± SD (n = 4 animals). Statistical comparisons were made by one-way ANOVA, followed by Fisher's PLSD multiple comparison tests. *P < 0.001 vs. nondiabetic plus vehicle; †P < 0.001 vs. nondiabetic plus BDNF; ‡P < 0.05 vs. nondiabetic plus vehicle; §P < 0.01 vs. nondiabetic plus BDNF; ‖P < 0.005 vs. diabetic plus vehicle; ¶P < 0.001 vs. diabetic plus vehicle.

dopaminergic dysfunction in these cells. Because the intensity of TH immunoreactivity in individual cells was much weaker in the diabetic rat retinas, it cannot be ruled out that the numbers of TH-positive cells were underestimated due to immunoreactivity in some cells that was below the detection threshold of the immunohistochemical assay. We quantified retinal Thy-1 levels, which reflect RGC numbers (23,27), and did not find significant reduction in Thy-1 levels in diabetic rat retinas in the present study. In some studies, degeneration of RGCs in STZ-induced diabetic rats has been reported (5,6). The discrepancy between our results and these (5,6) might be due to the length of the induced diabetic condition, which was 4 weeks in our study but >15 weeks in others. As BDNF is a critical factor for RGC survival, long-term BDNF deficiency under diabetic conditions may lead to subsequent RGC death at a later time point.

There are several possible mechanisms underlying the degeneration of dopaminergic amacrine cells in the diabetic animals. First is severe insulin deprivation, which occurs in STZ-induced diabetes. Insulin is an essential factor for the survival of amacrine cells in vitro (28). In addition, systemically administered insulin accumulated in the vitreous (29) and augmented insulin receptor phosphorylation (30). A second possible underlying mechanism is hyperglycemia. Although a study (31) using the sand rat *Psammomys obesus*, which develops spontaneous hyperglycemia, did not demonstrate a statistically significant reduction in the number of dopaminergic amacrine cells, an in vitro study (32) has shown that excess glucose impairs insulin-stimulated cell survival and insulin receptor signaling in retinal neurons. A third possibility is dysfunction of Müller cells in the diabetic retina. Diabetic Müller cells showed reduced glutamate-aspartate transporter (GLAST) function (8) and impaired glutamine synthesis (7). These dysfunctions resulted in elevated glutamate levels in the diabetic retinas (7), which might induce excitotoxicity in amacrine cells.

In this study, we demonstrated reduced levels of BDNF in the diabetic retina, which may underlie the degeneration of dopaminergic amacrine cells. A null mutation of the *bdnf* gene in mice resulted in atrophy of dopaminergic amacrine cells (15). In addition, BDNF was reported to prevent the death of dopaminergic neurons in the substantia nigra (33) and retinal amacrine cells in vitro (13) and in vivo (14). Immunohistochemical analysis showed that BDNF protein levels in both RGCs and Müller cells were reduced in diabetic retinas (Fig. 5). This suggests that diabetes affects multiple cell types simultaneously. Diabetes-related changes in the expression levels of neurotrophins have been reported in peripheral nerves (34) and the brain (35). However, the endogenous neurotrophin levels in diabetic retinas had not been reported until this study, in which both protein and mRNA levels of BDNF were shown to be decreased in STZ-induced diabetic rats (Fig. 4*A* and *C*). This animal model of diabetes is associated with hypoinsulinemia. When under dietary restriction, another metabolic state associated with mild hypoinsulinemia, BDNF levels were reported to be upregulated in the brain (36); hence, it is controversial whether hypoinsulinemia itself reduces or increases BDNF levels in the central nervous system. In contradistinction to this reduction in BDNF levels, NT-3 did not show such a reduction. BDNF and NT-3 are subject to different transcriptional regulation (37), but how this process is altered under diabetic conditions is not known.

To confirm the neuropathological implications of the BDNF reduction, we administered BDNF protein into the vitreous cavities of diabetic rats. Our results demonstrate that BDNF injection efficiently rescues dopaminergic amacrine cells from neurodegeneration and counteracts the downregulation of TH level seen under diabetic conditions (Fig. 6 and Table 2). BDNF protects neurons from cell death through TrkB, activating downstream Ras/mitogen-activated protein kinase and phosphatidylinositol 3-kinase/Akt pathways (38), the latter of which is more relevant to neuronal survival (39). Neurotrophins have biological roles other than those involving the survival of dopaminergic neurons; BDNF elicited hypertrophy of dopaminergic amacrine cells in developing animals (15) and upregulated dopaminergic function of cultured ventral mesencephalic neurons (40). Although BDNF and insulin share the same intracellular signaling pathways (18), differences (18,41) and interplay (42,43) in the signaling between BDNF and insulin have been reported. Clearly, elucidation of the precise signaling mechanisms by which BDNF protects dopaminergic amacrine cells in the diabetic retina await further investigation. A previous report (44) demonstrated that TrkB localized to dopaminergic amacrine cells by means of double immunostaining using retinal specimens of various species of animals. Thus, TrkB is a strong candidate for having a role in this process. Another possibility is that BDNF, which has been shown to reduce systemic glucose levels (45), might also act in this fashion, locally in the retina.

Neurotrophins have been tested as candidate therapeutic agents for diabetic neuropathy. However, randomized, double-blind, placebo-controlled studies on the effect of subcutaneous injection of either nerve growth factor (46) or BDNF (47) in patients with diabetes failed to demonstrate a significant beneficial effect on diabetic polyneuropathy. The negative results of these clinical trials suggested that the manner and site of neurotrophin administration are critical. Furthermore, because neurotrophins do not cross the blood-brain barrier (48), it is difficult to ensure their local supply to retinal neurons. Although we injected BDNF directly to the eyeball in the present study, frequent injections of neurotrophic factors into the vitreous space of diabetic patients are not practical. An intravitreous implant that releases BDNF continuously or subretinal transplantation of BDNF-secreting cells might be more viable options. Furthermore, intraocular BDNF gene transfer using a viral vector could be attempted. However, the most practical approach is the induction of endogenous BDNF by external administration of agents with a smaller molecular weight, such as catechol compounds (49). In addition, nighttime illumination might be somewhat beneficial to the diabetic retina, by reducing oxygen consumption of the neural retina (50) and upregulating BDNF expression by light stimulation of the retina (11).

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