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ORIGINAL RESEARCH

Protective Effects of Adeno-associated Virus Mediated Brain-derived Neurotrophic Factor Expression on Retinal Ganglion Cells in Diabetic Rats

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Abstract Adeno-associated virus vector plasmid carrying the expression cassette of brain-derived neurotrophic factor (BDNF), pAAV-BDNF, was constructed and packaged into recombinant adeno-associated virus (rAAV-BDNF). The rAAV-BDNF was intravitreally injected into streptzotocin (STZ)-induced diabetic Sprague–Dawley (SD) Rats. Data showed that over-expression of BDNF could increase alive retinal ganglion cell (RGC) number and improve its function in streptzotocin(STZ)-induced diabetic rats, which might be a new method to treat diabetic neuropathy and retinopathy.

Keywords Streptzotocin(STZ) · Diabetic retinopathy · Gene therapy · Adeno-associated virus (AAV) · Brain-derived neurotrophic factor (BDNF)

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Introduction

Diabetes mellitus (DM) is a metabolic disorder characterized by hyperglycemia and insufficiency of secretion or action of endogenous insulin (Maritim et al. 2003). Diabetes mellitus is a global problem with a devastating impact on people, both socially and economically. As of 2000 at least 171 million people worldwide suffered from diabetes, or 2.8% of the population (Wild et al. 2004). Among the U.S. residents aged 65 years and older, 10.9 million, or 26.9%, had diabetes in 2010. About 215,000 people younger than 20 years had diabetes (type 1 or type 2) in the United States in 2010 (Center for disease control and prevention 2011). The number of people with diabetes is still increasing due to population growth, aging, urbanization, and increasing prevalence of obesity and physical inactivity (Wild et al. 2004).

Diabetes is a disproportionately expensive disease. According to American diabetes association, in 2002, the per-capita cost of health care was $13,243 for people with diabetes, while it was $2560 for those without diabetes (American Diabetes Association 2007).

Most importantly, diabetes is the leading cause of kidney failure, non-traumatic lower-limb amputations, and new cases of blindness among adults in the United States Center for disease control, prevention (2011). Approximately 60% of people with diabetes will eventually develop neuropathy. Neuropathy may cause a lot of complications including retinopathy, eye problems that affect retina. Data showed that diabetic retinopathy is the leading cause of blindness in the U.S. for people between ages 20 and 64. The treatment of diabetic retinopathy is now mainly focused on aggressive intervention to control hyperglycemia (The Diabetes Control and Complications Trial Research Group 1993; UK Prospective Diabetes Study Group 1998), blood pressure,

and lipids (UK Prospective Diabetes Study Group 1998; Chaturvedi et al. 1998; Mauer et al. 2009).

Considerable evidence is emerging that indicates that retinal neurons are also altered during diabetes. Moreover, many types of neuronal deficits have been observed in animal models and patients before the onset of vascular compromise (Fletcher et al. 2007). Retinal neurons now are known to die by apoptosis fairly early in the course of diabetes. The concept of neuro-degeneration of the diabetic retina has been shown in many species, and study showed that retinal ganglion cell (RGC) numbers were decreased, particularly in the peripheral retina of diabetic retinopathy (Bloomgarden 2008).

Brain-derived neurotrophic factor, also known as BDNF, is a member of the "neurotrophin" family of growth factors which are related to the canonical nerve growth factor, NGF. BDNF acts on certain neurons of the central nervous system and the peripheral nervous system, helping to support the survival of existing neurons, and encourage the growth and differentiation of new neurons and synapses (Acheson et al. 1995; Huang and Reichardt 2001). Published data suggested that early retinal neuropathy of diabetes involves the reduced expression of BDNF in both patients with diabetic retinopathy and rat diabetic models (Seki et al. 2004; Shpak et al. 2010).

Gene therapy is the insertion, alteration, or removal of genes within an individual's cells and biological tissues to treat disease. One of the basic concepts of gene therapy is to transform viruses into genetic shuttles, which would deliver the gene of interest into the target cells. The most commonly used DNA virus vectors are based on adenoviruses and adeno-associated viruses (AAVs) (Akhtar et al. 2011).

Given the limitations and side effects of current treatments of diabetic retinopathy, gene therapy is becoming one of the choices to treat diabetic retinopathy. Here, we use adeno-associated virus vector technology to study the effect of over-expression BDNF on STZ-induced diabetic retinopathy.

Materials and Methods

Plasmid Construction and Virus Packaging

Plasmids pCAGGS and phSn1-EGFP-EPRE (phSyn1-EGFP-WPRE) were double digested by Apa I and EcoR I (New England Biolabs (Beijing) Ltd, Haidian, Beijing) following the manufacture's protocol. CBA promoter fragment derived from plasmid pCAGGS was inserted into Apa I and EcoR I double-digested phSn1-EGFP-EPRE with T4 DNA ligase (New England Biolabs (Beijing) Ltd, Haidian, Beijing) to create plasmid pAAV-EGFP (pCBA-EGFP-EPRE) (Shevtsova et al. 2004; Rancz et al. 2011).

cDNA of BDNF was amplified from rat brain total RNA using RT-PCR. Restriction enzyme sites of Hind III and EcoRI were included. cDNA of BDNF and plasmid pAAV-EGFP were double digested by Hind III and EcoRI (New England Biolabs (Beijing) Ltd, Haidian, Beijing) and DNA fragments were purified and ligated to get plasmid pAAV-BDNF (pCBA-BDNF-EPRE).

Plasmid extractions were done with QIAGEN Plasmid Mini Kit and Midi kit (Qiagen Hilden, Germany), following the manufactures' protocol.

Associated Adenovirus particles were produced by co-transfecting HEK293 cells with pAAV-GFP and pHelper or pAAV-BDNF and pHelper. Associated Adenovirus was tittered following the protocol described previously (Green and Loewenstein 2006; Grimm et al. 2003).

Cell Culture

HEK293 cells were obtained from the American Type Culture Collection (Manassas, VA) and maintained in a humidified atmosphere of 5% CO_2 at 37°C. The cells were routinely cultured in DMEM supplemented with 10% FBS (Life Technologies, Inc., Grand Island, NY), and 100 μg/ml penicillin–streptomycin.

Diabetes Induction

Adult Sprague–Dawley (SD) rats (8–10 weeks old; 250–3,005 g) were used in this study, which was approved by the Institutional Animal Care and Use Committee of the Chinese PLA General Hospital. Animals were fasted for 18–24 h and STZ (65 mg/kg) in 0.02 M citrate saline buffer was administered intraperitoneally, as described previously (Giraldi et al. 2001; Park et al. 2001; Usta et al. 2003). The control group animals received citrate buffer only. It is known that STZ induces diabetes by destroying the beta cells (Karunanayake et al. 1975). Animals in different groups were kept in cages individually and separately. The blood glucose levels were monitored using an *Accu-check* blood glucose meter (Roche Diagnostics, Basel, Switzerland). Rats with blood glucose levels $\geq$15 mM (200 mg/dl) for 2 consecutive weeks were considered diabetic. All types of surgeries and manipulations were performed under anesthesia by intraperitoneal injection of pentobarbital sodium (Nembutal; 50 mg/kg of body weight) (Abbott laboratories, Chicago, IL). Body temperature was maintained at 37°C ± 0.5°C with a heating pad and a rectal thermometer probe.

Retrograde RGC Labeling

2 weeks after inducing diabetes, deeply anesthetized rats were placed in a stereotactic apparatus (Stoelting, Kiel,

Germany), and the skin overlying the skull was cut open and retracted. The lambda and bregma sutures served as landmarks for drilling six holes. 5 μl of FluoroGold (FG) (Fluorochrome, Denver, CO, USA), dissolved in dimethylsulfoxide (DMSO) (FG 3% in NaCl with 10% DMSO), was injected into both superior colliculi as described previously (Jehle et al. 2008). To insure adequate RGC labeling, animals were given 6 days to perform the retrograde transport of FG before further experimental intervention.

Intravitreal Injection of rAAVs

6 days after retrograde RGC labeling, AAV-BDNF and AAV-EGFP (2×10^7 particles per eye) were injected intravitreally to the diabetic rats following the protocol described by Machemer et al. (1979).

Preparation of retinas

3, 6, or 9 months after intravitreal injection of AAVs, SD rats were anesthetized by intraperitoneal injection of pentobarbital sodium (Nembutal; 50 mg/kg of body weight) (Abbott Laboratories, North Chicago, Il.) and then decapitated. Both eyes were enucleated. With each eye, an incision was made at the equator of the globe with a razor blade and the anterior portion of the eye was removed by successively enlarging the incision with microscissors. The eyecup, hemisected slightly below the ora serrata, was then placed in a dish containing of 1× Ringer solution that had been bubbled with 95% O_2 and 5% CO_2. The vitreous humor and lens were removed and the retina was then detached from the retinal pigment epithelium (RPE). Then, the retina was fixed in 4% parafomaldehyde for 30 min. After fixing, the retina was flat-mounted on gelatin-coated glass slide and one drop of VECTASHIELD® Mounting Medium (Vector Lab oratories, Burlingame, CA) was dispensed to each sample and a coverslip was used to cover the sample. 18, 15, 18, and 20 retinas of AAV-BDNF group, AAV-GPF group, diabetic-no AAV group, and control group were used respectively in this study.

Quantification of RGCs

After retinas were dissected, flat-mounted on gelatin-coated glass slides, and embedded in mounting media (Vectashield; Vector Lab oratories, Burlingame, CA), tracer (FG)-positive RGCs were counted in a blind fashion under a fluorescence microscope (AxioImager; Carl Zeiss, Jena, Germany) in 12 distinct areas measuring 0.04 mm^2 each following Laquis's protocol with slight modification (Laquis et al. 1998).

Visual Evoked Potential (VEP)

VEP was recorded following Jehle's protocol (Jehle et al. 2008). In brief, rats were anesthetized, and stainless steel screws were implanted 3 mm lateral to the lambda and 5 mm behind the bregma. Reference electrodes were placed 2 mm lateral to the lambda and 2 mm in front of the bregma. The electrode assembly was encased in dental acrylic, and the wounds were sutured. At least 5 days were allowed for recovery before electrophysiological testing.

Electroretinography (ERG)

Animals were dark adapted overnight then ERG was recorded following Jehle's protocol (Jehle et al. 2008).

Data and Statistical Analyses

VEP and ERG responses were extracted by Fourier analysis with image processing and programming software (Igor Pro 5.01; WaveMetrics, Lake Oswego, OR). The amplitudes of the transient VEPs were calculated from the main negative trough to the main positive peak. For scotopic ERG, the b-wave amplitude was the difference between the trough of the a-wave and the peak of the b-wave. Oscillatory potentials were recorded with a bandpass of 75–300 Hz from the unattenuated scotopic flash ERG and were quantified as the differences between the highest peaks and the lowest troughs. All averaged data were presented as means with their corresponding SEMs. Statistical significance was assessed using ANOVA or by *t*-testing. Differences were considered significant at $P < 0.05$.

Results

Induction of Diabetes

48 h after using STZ, in diabetic group, the level of blood glucose was 32.12 ± 7.06 mmol/l ($n = 18$), which was significantly up-regulated when compared with that of control group (4.2–4.8 mmol/l ($n = 20$)), and the level of blood sugar in diabetic group was maintained at a high level during the following 2-week routine tests, indicating that all mice in diabetic group were successfully induced diabetes by intraperitoneal injection of STZ (Fig. 1).

AAV-BDNF Transduction

Transduction of AAV-GFP did not cause any inflammatory or deleterious effect on the retina. The efficiency of AAV transduction in RGC was 40%. Expression levels of

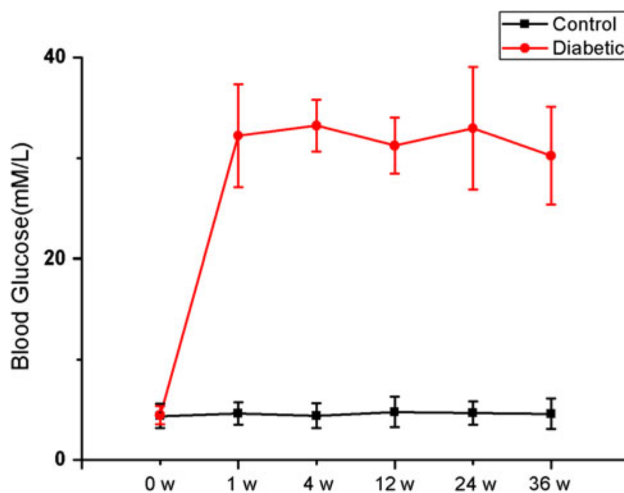

Fig. 1 The changes of average level of blood glucose in diabetic and normal rats. The level of blood glucose in diabetic rats was significantly higher than that of normal rats ($P < 0.05$), and the glucose level of diabetic rats was maintained as a high level during the study

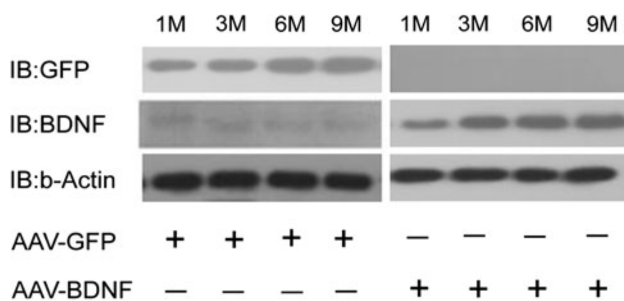

Fig. 2 Expression of BDNF after AAV-BDNF transduction in different time points. BDNF expression level in AAV-BDNF group was much higher than that of AAV-GFP group in different time points (1, 3, 6, and 9 months) after transduction

AAV-BDNF were checked at different time points (1, 3, 6, and 9 months after transduction). As shown in Fig. 2, BDNF expression level in AAV-BDNF group was much higher than that of AAV-GFP group.

RGCs

The number of RGCs was counted 3, 6, and 9 months after intravitreal injection of AAVs. As shown in Fig. 3, the number of survival RGC in diabetic group was significantly decreased ($P < 0.05$) when compared with that of control group 6 and 9 months after induction of diabetes, but no significant difference was seen between the RGC numbers of control rats and diabetic rats 3 months after induction of diabetes.

More RGCs were seen in AAV-BDNF group compared with that of AAV-GFP group 6 and 9 months after transduction (Fig. 4), and the number of RGCs stopped decreasing in

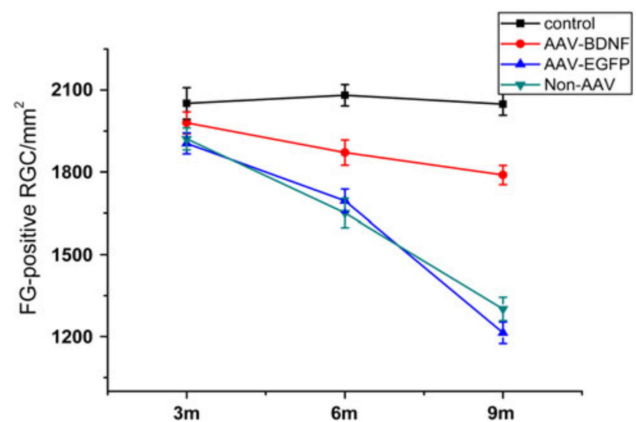

Fig. 3 RGC number in diabetic rats, and diabetic rats treated with AAV-BDNF. RGC number was significantly decreased 6 and 9 months after induction of diabetes compared with that of control rats ($P < 0.05$). RGC number in AAV-BDNF group rats was significantly increased when compared with that of AAV-EGFP group 6 and 9 months after transduction ($P < 0.05$)

AAV-BDNF group compared with that of AAV-GFP group 6 and 9 months after transduction.

VEP

P100 Amplitudes

Decreases in P100 amplitudes of diabetic group were seen 6 months after STZ induction of diabetes. P100 amplitudes of diabetic rats were significantly decreased ($P < 0.05$) when compared with those of control rats 6 and 9 months after induction of diabetes, but no significant difference was seen between the P100 amplitudes of control rats and diabetic rats 3 months after induction of diabetes. Transduction of AAV-BDNF significantly increased P100 amplitudes ($P < 0.05$) when compared with AAV-EGPF group 6 and 9 months after transduction (Fig. 5).

P100 Latency

As shown in Figs. 6 and 7, STZ induction of diabetes significantly increased P100 latency ($P < 0.05$) when compared with that of control group. And transduction of AAV-BDNF significantly inhibited the P100 latency increase ($P < 0.05$).

ERG

Oscillatory Potential (OP) Amplitudes

OP amplitudes were significantly reduced 1 month after STZ induction of diabetes when compared with control rats, and were reduced ($P < 0.05$) even more 3 and 6 months later (Fig. 8). Transduction of AAV-BDNF significantly

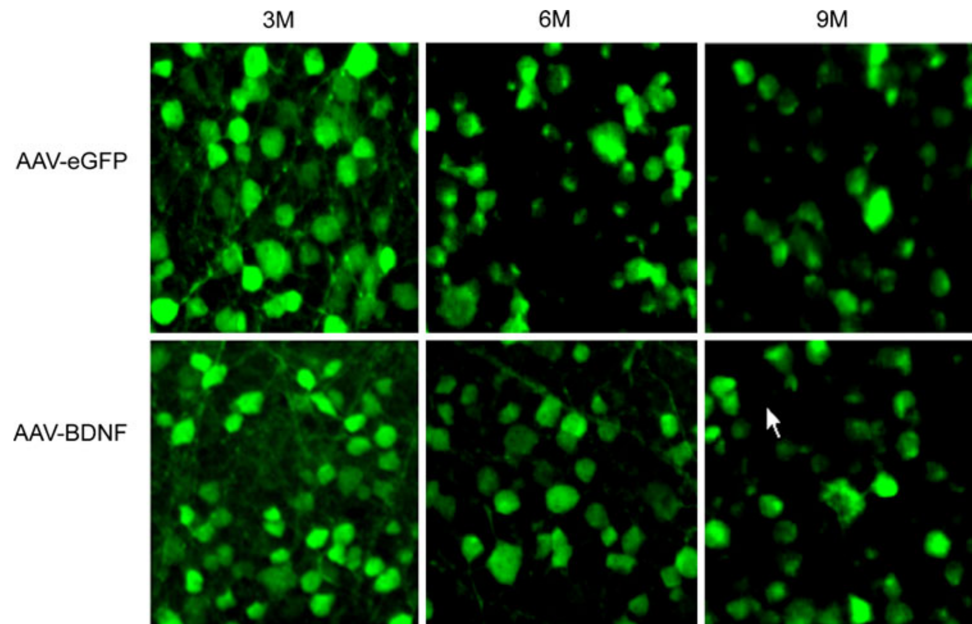

Fig. 4 Surviving RGC numbers after AAV-BDNF transduction in different time points. More RGCs were seen in AAV-BDNF group compared with that of AAV-GFP group 6 and 9 months after transduction. No significant difference was seen between the RGC numbers of control rats and diabetic rats 3 months AAV transduction

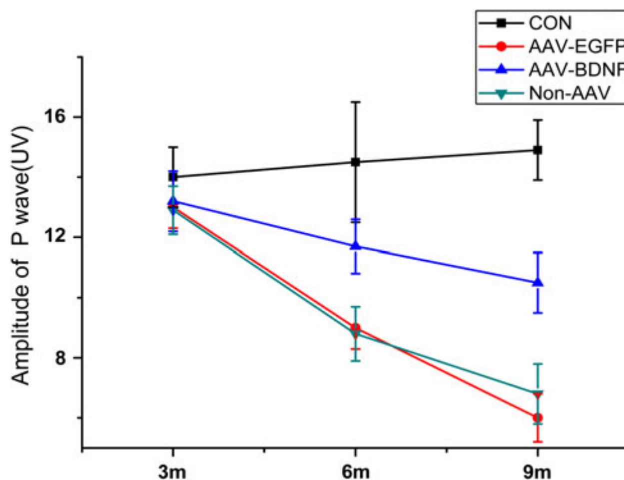

Fig. 5 Effect of BDNF on STZ-induced P100 amplitude decrease. P100 amplitudes of diabetic group were significantly decreased when compared with those of control group 6 and 9 months after induction of diabetes ($P < 0.05$), transduction of AAV-BDNF could significantly increase P100 amplitudes when compared with AAV-EGPF group 6 and 9 months after transduction ($P < 0.05$)

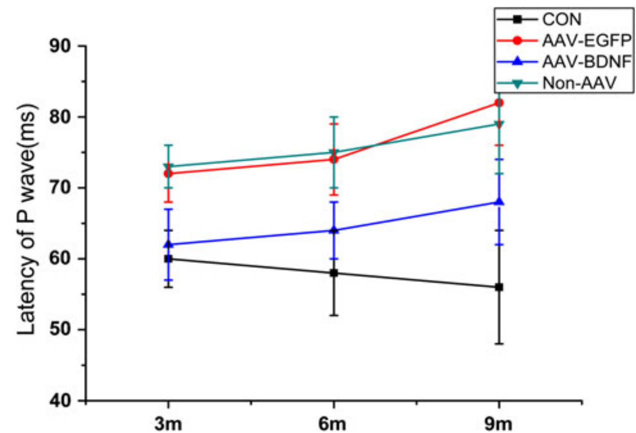

Fig. 6 Effect of BDNF on STZ-induced P100 latency increase. STZ induction of diabetes could significantly increase P100 latency when compared with control group ($P < 0.05$). And transduction of AAV-BDNF significantly inhibited the P100 latency increase when compared with AAV-EGPF group ($P < 0.05$)

blocked OP amplitudes decrease and maintained OP amplitudes at a relatively stable level ($P < 0.05$).

b-wave Amplitude

Electroretinographic analyses were performed and b-wave amplitudes were recorded. As depicted in Figs. 9 and 10, ERG b-wave amplitudes were significantly decreased ($P < 0.05$) 6 and 9 months after STZ induction of diabetes when compared with control group. And transduction of AAV-BDNF significantly inhibited these decreases ($P < 0.05$) when compared with AAV-EGFP group.

Discussion

Streptozotocin (Streptozocin, STZ, Zanosar), originally identified in late 1950s as an antibiotic, is a naturally occurring chemical that is particularly toxic to the insulin-producing beta cells of the pancreas in mammals (Vavra et al. 1959). Because of its toxicity to the insulin-producing beta cells of the pancreas, it has been used to induce diabetes to provide animal models (Mansford and Opie 1968). STZ prevents cellular reproduction, inhibits the substrate connection to the DNA or inhibits many of the enzymes involved in DNA synthesis, and finally destroys langerhans islet cells (Holemans et al. 1997). In this study, using of a 65 mg/kg dose of STZ, insured induction of diabetes in

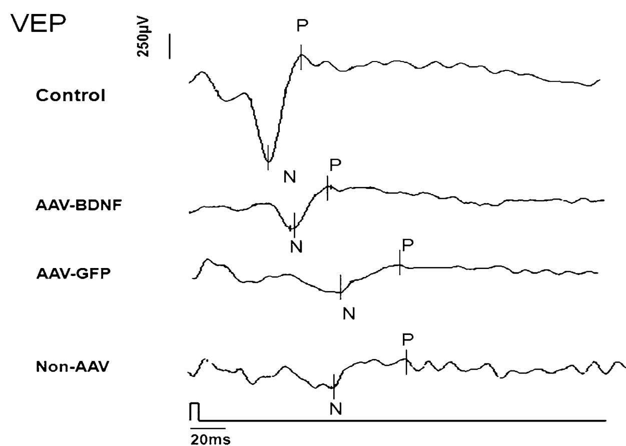

Fig. 7 VEP layout of subjects of control, non-AAV diabetic control, AAV-GFP, and AAV-BDNF. 6 months after STZ induction of diabetes, a significant increase in P100 latency was observed when compared with control group. And transduction of AAV-BDNF significantly inhibited P100 latency increase ($P < 0.05$) when compared with AAV-EGFP group

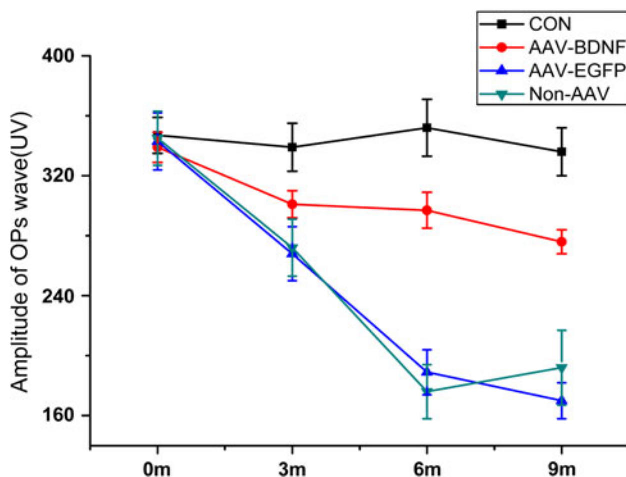

Fig. 8 Effect of BDNF on STZ-induced OP amplitude decrease. OP amplitudes were significantly reduced after STZ induction of diabetes when compared with control rats ($P < 0.05$). Transduction of AAV-BDNF significantly blocked OP amplitudes decrease compared with those of AAV-EGPF group ($P < 0.05$)

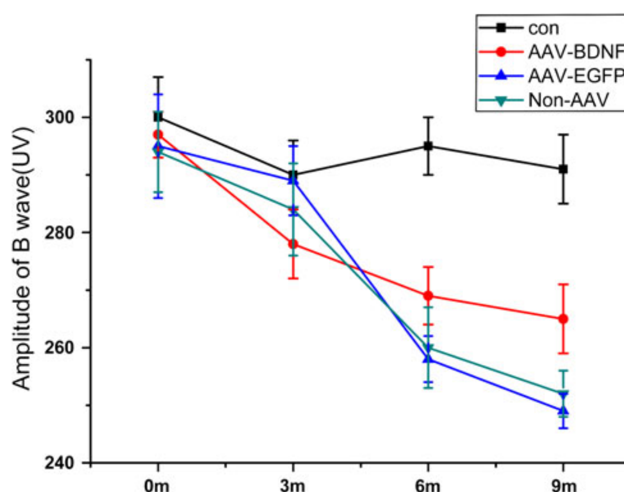

Fig. 9 Effect of BDNF on STZ-induce b-wave amplitude decrease. ERG b-wave amplitudes were significantly decreased 6 and 9 months after STZ induction of diabetes when compared with those of control group ($P < 0.05$). And transduction of AAV-BDNF significantly inhibited these decreases when compared with those of AAV-EGFP group ($P < 0.05$)

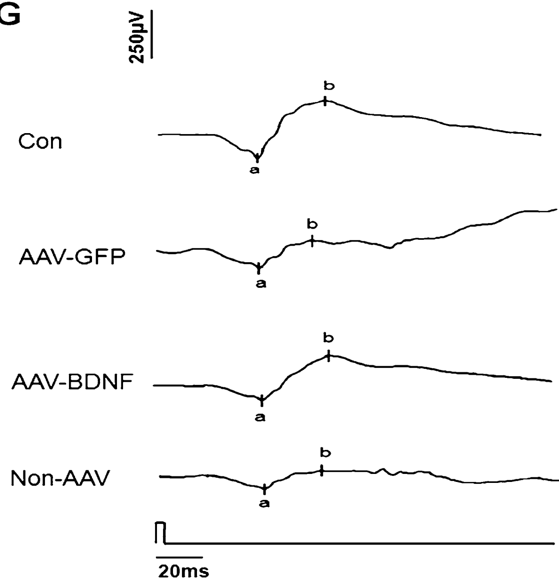

Fig. 10 ERG layout of subjects of control, non-AAV diabetic control, AAV-GFP, and AAV-BDNF. 6 months after STZ induction of diabetes, a significant decrease in b-wave amplitude was observed when compared with control group. And transduction of AAV-BDNF significantly inhibited b-wave amplitude decrease ($P < 0.05$) when compared with AAV-EGFP group

rats. High blood glucose levels were seen in adult rats within 2 days of STZ treatment and, within 2 weeks, the blood sugar levels were almost stable, which indicates irreversible destruction of langerhans islets cells, and successful induction of diabetes in SD rats.

Diabetic retinopathy remains a major cause of morbidity in diabetic patients. A variety of studies showed clinically demonstrable changes in the retinal vasculature in diabetes and this has led to the general assumption that the retinopathy is solely a microvascular disease. However, increasing evidences showed that diabetes can also damage non-vascular cells of the retina, resulting in alterations in function (Phipps et al. 2006; Kern et al. 2007). Published data showed that at least some retinal ganglion cells might die via apoptosis in diabetic patients (Kern et al. 2007). And there is general agreement that all rat strains reported to date have shown RGC loss or damage in diabetes (Kern and Barber 2008). As shown in Fig. 2, the STZ induction of diabetes in SD rats resulted in RGC loss. The RGC number of diabetic rats was significantly decreased when

compared with that of control rats 6 and 9 months after induction of diabetes.

Brain-derived neurotrophic factor (BDNF) is expressed in several retinal cells, including RGC and Müller glia in the retina, and has been previously reported to prevent RGC and amacrine cell death (Johnson et al. 1986; Parrilla-Reverter et al. 2009). rAAVs expressing BDNF or EGFP were injected intravitreally into diabetic rats, and the expression of BDNF increased the survival of RGC. As shown in Fig. 2, more RGCs were seen in rAAV-BDNF group rats compared with that of rAAV-EGFP group rats 6 and 9 months after transduction, indicating BDNF indeed prevents RGC death.

Visual evoked potentials (VEPs) are a series of signals about visual occipital cortex in response to visual stimuli and can be used as one of objective non-invasive neurophysiological parameters to reflect the visual organs and central visual pathway's functional integrity (Ozkaya et al. 2007). VEP after photo-stress, as originally suggested by Lovasik (1983) and Franchi et al. (1987), represents an electrophysiological application of the macular photostress test (MPST) proposed by Baillart and L'examen (1954). The MPST measures the period of recovery in visual acuity after dazzling of the macular region with an ophthalmoscope, it has been found to be significantly altered in several macular disorders, including diabetic retinopathy (Wu et al. 1990; Zingirian et al. 1985; Mosci et al. 1986).

The P100 response, as part of the VEP waveform, is an averaged event-related brain electrical potential. It occurs approximately 100 ms after the stimulus onset and is a highly consistent and reproducible waveform generated in the striate and parastriate visual cortex in response to a visual stimulus (Halliday 1993; Ewing et al. 1998). Several groups have found that P100 amplitude was changed in diabetes mellitus, for example, Ramen et al. has found that mean P100 amplitude in diabetic patients was significantly lower than that in controls (1997). Pan et al. also showed that P100 amplitude was reduced in diabetic patient when compared with the age-matched control group (Pan and Chen 1992). Here, in this study, we found that P100 amplitude of diabetic group was significantly decreased 6 and 9 months after induction of diabetes when compared with that of control group. And P100 amplitude decrease was inhibited by expressing of BDNF (Fig. 3), indicating that expression of BDNF could protect diabetic retinopathy.

P100 latency is the representative component of VEP with slight intra- and inter-individual variability so that the index is most commonly used (Zhao et al. 2009). There are several anecdotal reports from western countries showing alteration in VEP latencies in diabetic patients (Puvanendran et al. 1983; Algan et al. 1989; Moreo et al. 1995). Lots of studies have demonstrated significant increments in P100 latency in diabetic subjects compared with non-diabetic controls, although the percentage of patients who had abnormal results varied considerably between studies, probably because of differences in study populations and methodologies (Ewing et al. 1998; Raman et al. 1997). As shown in Fig. 4, STZ induction of diabetes significantly increased P100 latency when compared with controls. And transduction of rAAV-BDNF significantly inhibited P100 latency increase, indicating that BDNF could protect retina from diabetic damages.

ERG measures the electrical responses of various cell types in retina. It has been used to investigate functional and presumed biochemical changes at a retinal level and to uncover the mechanisms of retinal physiology and their alterations in diseases (Weymouth and Vingrys 2008). There are evidences that ERG signals are impaired in diabetes prior to the onset of clinically evident retinopathy (Lovasik and Kergoat 1993; Parisi 2001).

Oscillatory potential (OP) was first reported as a new component of the ERG in 1953 by Cobb and Morton (Cobb and Morton 1953). Soon after their description, OP was identified as a sensitive indicator of disease in diabetic retinopathy (Yonemura et al. 1962). OP wave components are believed to originate from inner retinal layers through the activity of amacrine cells (Kohzaki et al. 2008). Abnormalities in the OPs are of great interest in the assessment of diabetic retinopathy, because OP abnormalities had been shown to predict onset as well as progression of diabetic retinopathy (Vadala et al. 2002; Bresnick et al. 1984). Luu et al. has showed that all the OP components (OP1–OP4) were significantly reduced in amplitude and increased in implicit time in the diabetic groups (2010). In our study, OP amplitudes were significantly reduced 1 month after STZ induction of diabetes when compared with control rats, and were further reduced 3 and 6 months later (Fig. 5). Transduction of rAAV-BDNF significantly blocked OP amplitudes decrease and maintained OP amplitudes at a relatively stable level.

Papakostopoulos et al. (1996) reported decreased ERG b-wave amplitude in a type 1 diabetic cohort. Di Leo et al. and Caputo et al. also found reduced amplitudes for pattern electroretinograms in diabetic subjects (Di Leo et al. 1990; Caputo et al. 1990). In our study, as shown in Fig. 6, b-wave amplitudes were significantly decreased 6 and 9 months after STZ induction of diabetes when compared with controls, confirmed that ERG b-wave amplitude was decreased in diabetic patients or animals. And transduction of rAAV-BDNF significantly inhibited these decreases when compared with rAAV-EGFP group.

Taken together, STZ-induced diabetes in SD rats could induce diabetic retinopathy and BDNF could not only prevent RGC death, but also inhibit P100 amplitudes decrease and P100 latency increase, reduce OP amplitudes decrease, and block b-wave amplitudes reduction, thus help

to restore the retina function in STZ-induced diabetic retinopathy, which may offer a potential new therapeutic method for diabetic retinopathy.

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Conflicts of interest No potential conflicts of interest.

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