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# Expression of Glial Cell Line-Derived Neurotrophic Factor and Its Receptors in Cultured Retinal Müller Cells Under High Glucose Circumstance

XINPING ZHU,[1,2] YAN SUN,[1] ZHONGPING WANG,[1,3] WEIGANG CUI,[1] YUWEN PENG,[1]* AND RUIXI LI[1]*

[1]Department of Anatomy, Histology and Embryology, Shanghai Medical College, Fudan University, Shanghai, China
[2]Department of Ultrasound, East Hospital, Tongji University, Shanghai, China
[3]Department of Immunology, Jiujiang University, Jiujiang, China

ABSTRACT

This study aimed to explore the effect of high glucose concentration on the expression of glial cell line-derived neurotrophic factor (GDNF) and its family ligand receptors (GFRs) GFRα1 and GFRα2 in Müller cells and the protective role of GDNF in cultured Müller cells under high glucose circumstance. Cultured Müller cells (untreated or treated with 200 ng/mL of GDNF) were exposed to high glucose conditions (20 mmol/L glucose). We found that the expression levels of GDNF and GFRα1 mRNA and protein increased gradually over time under high glucose and exogenous GDNF-treated conditions, whereas the upregulation in GFRα2 expression was observed only in the early stage of high glucose conditions. Exogenous GDNF not only decreased apoptosis in cultured Müller cells under high glucose circumstance, but also accelerated the levels and speed of synthesis of GDNF and GFRα1 proteins in Müller cells. These results suggest that Müller cells can synthesize GDNF and GFRs under high glucose conditions, and GDNF may play important role in protecting Müller cells during the early stage of diabetic retinopathy. The difference in GFRs expression indicated that GDNF and neurturin may exert different effects on Müller cells under high glucose circumstance. Anat Rec, 295:532–539, 2012. © 2012 Wiley Periodicals, Inc.

**Key words: GDNF; GFR; Müller cells; diabetic retinopathy**

Diabetic retinopathy (DR) is one of the most common complications of diabetes and has features of chronic inflammatory disease. Many studies suggest that Müller cells, the principal retinal glia, play critical roles in this disorder. Recent evidence suggests that DR is not only a microangiopathy but also a disease involving neurons and glial cells. The changes of Müller cells in DR include changes in the expression of glial fibrillary acidic protein (Asnaghi et al., 2003) and glyceraldehyde phosphate dehydrogenase, impaired glutamate metabolism (Du et al., 2004; Ward et al., 2005), and accelerated neuronal apoptosis (Akkina et al., 2001).

Glial cell line-derived neurotrophic factor (GDNF) is a growth factor of many neuronal populations in the central, peripheral, and autonomous nervous system. The GDNF family ligands such as GDNF, neurturin (NTN), artemin, and persephin interact with GDNF family receptors and activate intracellular signaling pathway through the Ret receptor tyrosine kinase. GDNF can bind to GFRα1 and a small amount of GFRα2 (Lindqvist et al., 2004).

*Correspondence to: Ruixi Li, Department of Anatomy, Histology and Embryology, Shanghai Medical School, Fudan University, 138 Yixueyuan Road, Shanghai 200032, China. Fax: +86-21-54237445. E-mail: reixilee@163.com or Yuwen Peng, Department of Anatomy, Histology and Embryology, Shanghai Medical School, Fudan University, 138 Yixueyuan Road, Shanghai 200032, China. Fax: +86-21-54237445. E-mail: ywpeng@fudan.edu.cn

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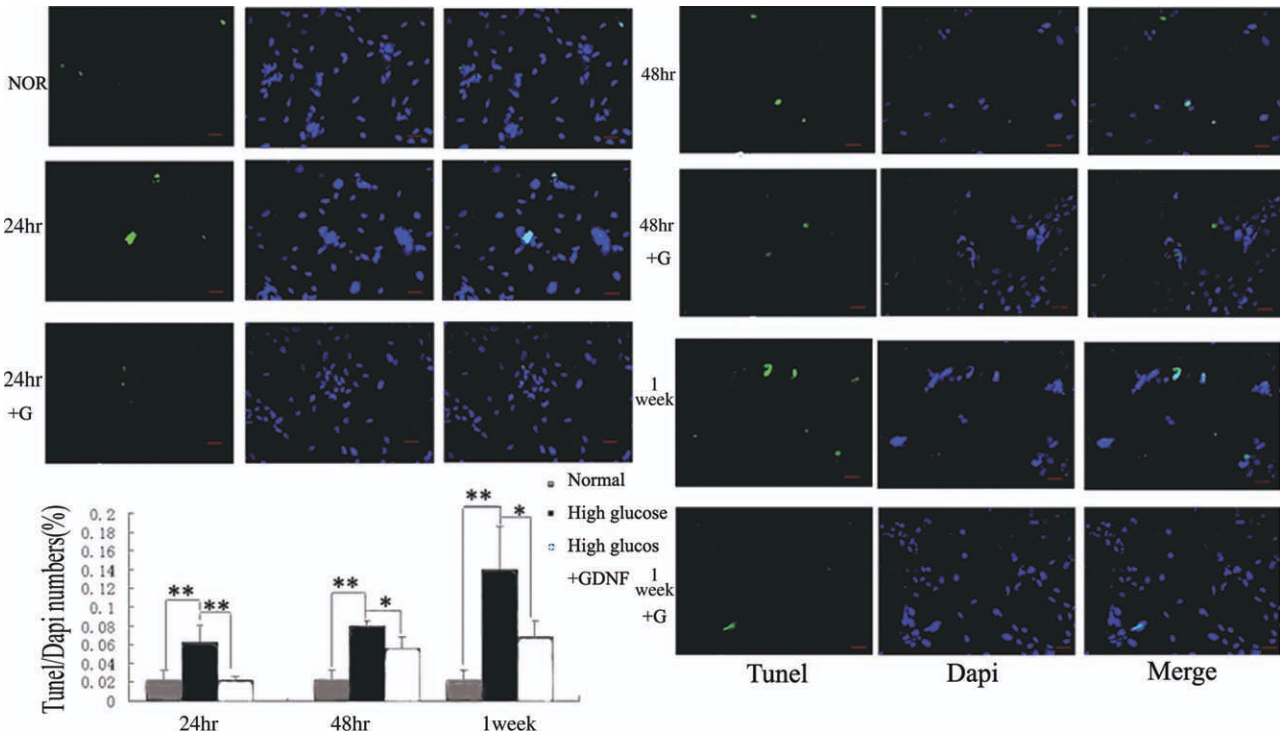


Fig. 1. Apoptotic signals (green) in high glucose groups untreated or treated with GDNF (100 ng/mL) at different time points. TUNEL staining showed that the number of apoptotic-positive cells increased under high glucose circumstance. The number of positive cells decreased significantly in the GDNF-treated group. Data are expressed as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$. Bar = 150 μm.

In the eye, GDNF is primarily expressed in the retina. Some studies have found that the expression levels of GNDF and GFRα1, or GFRα2 in the mice retina are likely to occur via horizontal, amacrine, or ganglion cells and not in Müller cells or photoreceptor cells. But some other studies have demonstrated GDNF and its receptors in Müller cells (Igarashi et al., 2000), in the model of light-induced photoreceptor-damaged or axotomized retinal ganglion cells. These data showed that Müller cells could express GDNF and GFRs in retinopathy. However, the changes in GDNF and GFRs in seriously damaged Müller cells during DR are still not clear.

Although cytokine production, oxidative stress, and glucose-induced vascular toxicity have all been implicated as key causes of DR, high blood glucose level is a major causative factor in the early stage of DR. Several intracellular biochemical pathways have been associated with high glucose conditions (Busik et al., 2008). We examined the expressions of GDNF and its receptors in cultured Müller cells under high glucose conditions to reveal the potential effects of GDNF on Müller cells during the early stage of DR.

## MATERIALS AND METHODS

### Cell Culture

All experimental procedures were approved by the Institutional Animal Care and Use Committee of Shanghai Fudan University, China. Sprague–Dawley rats, 3–5 days old, were sacrificed, and Müller cells were isolated and cultured by 10% fetal bovine serum D/F-12 solution (Seitz et al., 2010). Cells were transferred three to four times, and immunofluorescence method was used to detect polyclonal antibody against vimentin for cell purity.

Cultured cells were divided into three groups: control group, high glucose group, and high glucose with GDNF group. Prior to in vitro experiments, Müller cells were pretreated with 10 mmol/L glucose for 24 hr and then Müller cells (untreated or treated with 100 ng/mL of GDNF) (Harada et al., 2002) were exposed to 20 mmol/L glucose circumstance (Busik et al., 2008). Müller cells were divided as 24 hr, 48 hr, 72 hr, or 1 week time groups for different culture conditions.

### Terminal Transferase dUTP Nick-End Labeling

The cells were tested with the terminal transferase 2′-Deoxyuridine, 5′-Triphosphate (dUTP) nick-end labeling (TUNEL) reaction to detect apoptosis using the commercial kit (Dead End™ Fluorometric TUNEL System; Roche, Progema, Madison, WI). The number of TUNEL and nuclei was counted in the whole slides, and the data were expressed as the percentage of TUNEL-positive cells relative to total cell population in each group.

### Immunohistochemistry

Müller cells attached to chamber slides were washed with 0.1 M phosphate buffered saline (PBS) and fixed in 4% paraformaldehyde solution for 20 min. Cells were washed with 0.1 M PBS and the fixed cultures were treated for 1 hr with blocking solution (2% donkey serum, 0.02% Triton X-100 in PBS). The cells were then

incubated for 1 day at 4°C with a rabbit anti-GFRα1 (1:2,000, RD) antibodies, goat anti-GFRα2 (1:2,000, RD) antibody, anti-GDNF (1:500, Santa Cruz) antibody, mouse anti-vimentin (1:2000 Abcam) antibody, and 4′,6-diamidino-2-phenylindole (DAPI; 0.5 μg/mL, Sigma). The cells were then rinsed three times in PBS, followed by Alexa Fluor 488 goat anti-mouse and Alexa Fluor 568 goat anti-rabbit or rabbit anti-goat for 2 hr at room temperature, and subjected to DAPI nucleus staining.

## Analysis of GDNF and Its Receptors in Müller Cells by Real-Time Reverse Transcriptase PCR and Western Blot

### *Real-time Reverse Transcriptase-PCR*

The expression levels of GDNF, GFRα1, and GFRα2 mRNA in Müller cells were detected under high glucose conditions in the presence or absence of GDNF, with β-actin as a normalizing control. The specific polymerase chain reaction (PCR) primer sequences of these genes designed by Beacon designer 2 software were as follows: GDNF forward: 5′-AGTTATGGGATGTCGTGGCTGTCT-3′; GDNF reverse: 5′-TTCGGGCATATTGGAGTCACTGGT-3′; GFRα1 forward: 5′-AGGCCTTGAAGCAGAAGTCTCTGT-3′; GFRα1 reverse: 5′-ATATGAACGGGACTGCCCGGAATA-3′; GFRα2 forward: 5′-AGTGTCATCACCACCTGCACATCT-3′; GFRα2 reverse: 5′-ACTCCCTGGACTGATGTTTGTCGT-3′; actin forward: 5′-TTGCTGACAGGATGCAGAAGGAGA-3′; actin reverse: 5′-ACTCCTGCTTGCTGATCCACATCT-3'. Müller cells were trypsinized and harvested at different time points. Total RNA was isolated using Trizol reagent (Invitrogen), reverse transcription kit (MBI Fermentas), Quant qRT-PCR (SYBR Green I) Kit (Tiangen), and cDNA was acquired according to the M-MLV procedures (Promega) with 2 μg of total RNA. Two-step real-time RT-PCR reactions were performed using the ABI PRISM 7000 Detection System, which included cycle 1 (1×): 94°C 30 sec; and cycle 2 (40×): 94°C, 30 sec; 72°C, 30 sec. Absorbance data were collected at the end of every extension (60°C) and graphed using ABI Prism 7300 SDS Software. The real-time PCR data were analyzed by $2^{-\Delta\Delta Ct}$.

### *Western blot*

At different time points, the cells were rinsed with D-hanks for Western blotting test (Dun et al., 2006). After transfer, membranes were blocked for 1 hr at room temperature with 3% nonfat dry milk with agitation in tris-buffered saline and tween 20 (TBST). GDNF, GFRα1, GFRα2 were detected with the following antibodies diluted in blocking solution: rabbit polyclonal anti-GFRα1 (1:1,000, RD), goat polyclonal anti-GFRα2 (1:1000, RD) and anti-GDNF (1:250, Santa Cruz), and mouse polyclonal anti-β-actin (1:10,000, RD). After 24-hr incubation in primary antibodies, blots were incubated for 1 hr with horseradish peroxidase-conjugated goat anti-rabbit IgG, rabbit anti-goat or goat anti-mouse IgG (1:2,000, Jackson Immuno-Research) diluted in 5% dry milk containing TBST. After washing with TBST (three times, 10 min each), immunoreactivities were visualized using the enhanced chemiluminescence detection system (Pierce).

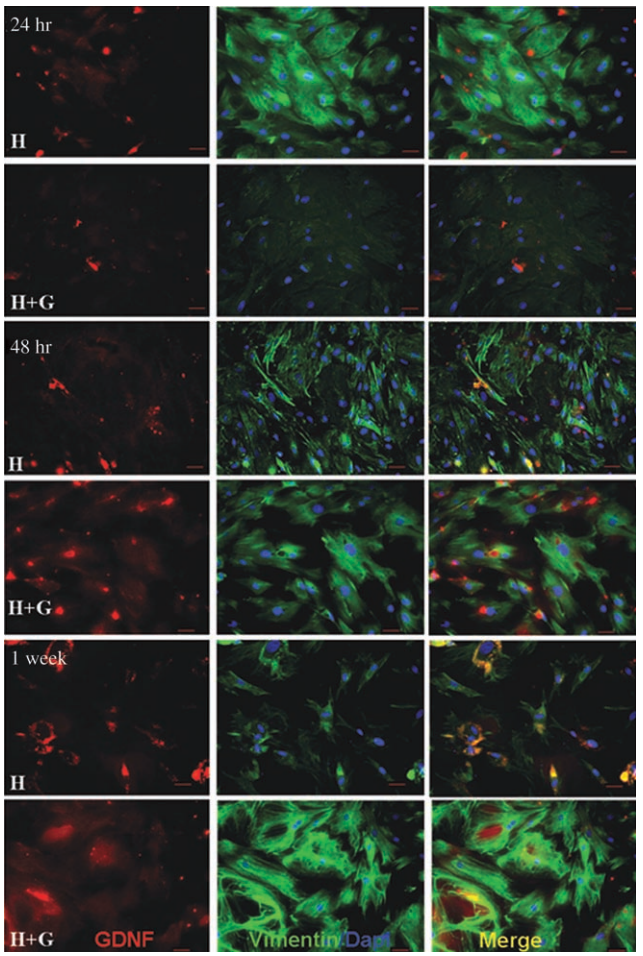


Fig. 2. Immunofluorescence of GDNF (red) and the Müller cells marker vimentin (green) in high glucose groups untreated or treated with GDNF (100 ng/mL) at different time points. Under high glucose conditions, the expression of GDNF is upregulated with increase in time and exogenous GDNF treatment enhanced this process. Bar = 100 μm.

## Statistical Analysis

Data were expressed as the mean ± SEM. $P$ values of $<0.05$ were regarded as statistically significant and $<0.01$ as highly significant.

# RESULTS

## Apoptotic Changes in Müller Cell and Protective Effects of GDNF Under High Glucose Circumstance

TUNEL-positive Müller cells were observed under high glucose for 24 hr (Fig. 1). With the prolongation of high glucose for cultured Müller cells, the number of positive signals increased. In GDNF-treated group, the number of TUNEL-positive cells decreased at the same time. We detected the percentage of TUNEL-positive cell numbers/DAPI numbers in different time groups, and the data showed a statistically significant difference ($P < 0.05$, Fig. 1). There was a highly significant difference ($P < 0.01$) between the high glucose groups untreated or treated with exogenous GDNF and the

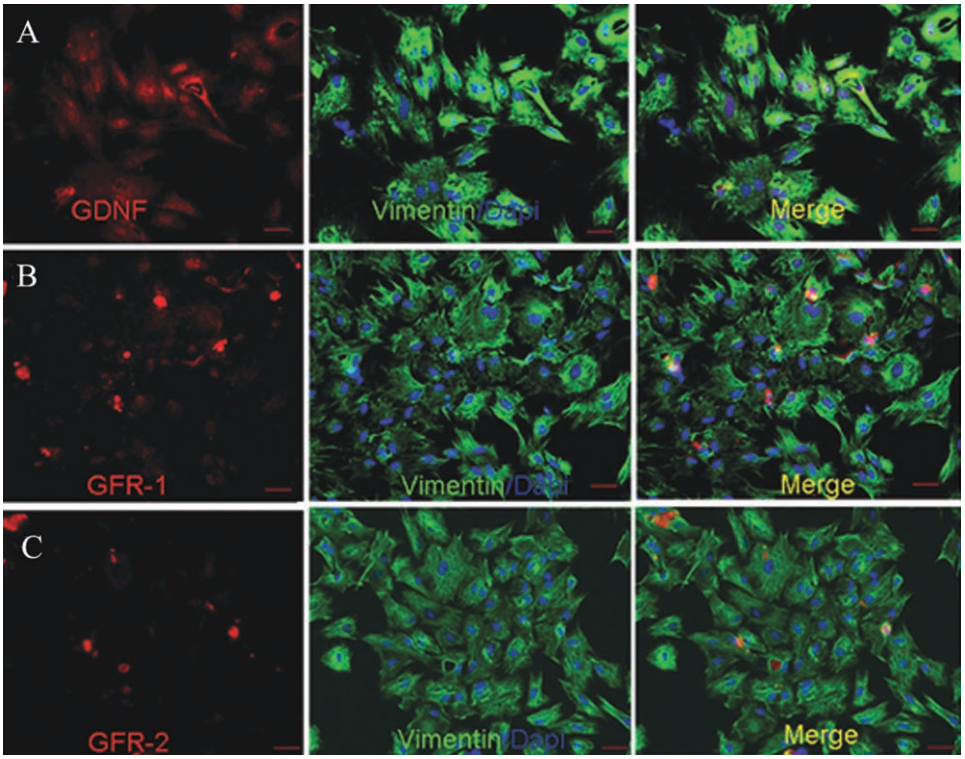


Fig. 3. Müller cells expressing GDNF- (**A**), GFRα1- (**B**), and GFRα2 (**C**)-positive signals in the control group. The GDNF, GFRα1, and GFRα2 expressed very faint positive signals, and GFRα1 and GFRα2 are not expressed in cell membrane. Bar =100 μm.

control group, except for 24-hr GDNF-treated group. This data showed that high concentration of exogenous GDNF could effectively reduce apoptosis in the cultured Müller under high glucose concentration.

## Detection of GDNF in Müller Cells

Immunoreactive-positive signals for GDNF were detected in all the samples. The characteristics of GDNF signaling in Müller cells are represented in Fig. 2. In the control group, only few faint GDNF immunoreactive-positive cells were observed (Fig. 3A). In the high glucose groups (untreated or treated with exogenous GDNF), the number of positive cells increased with increase in time. There was an obviously positive difference between the high glucose group untreated with GDNF and the high glucose group treated with the exogenous GDNF at 24 and 48 hr, but no significant difference was observed at 1 week.

The expression of GDNF mRNA was identified in all samples by real-time RT-PCR. The level of GDNF mRNA expression increased gradually both under high glucose- and GDNF-supplemented conditions. The results of GDNF mRNA expression are shown in Fig. 4A. There was a significant difference in the expression level of GDNF mRNA between the high glucose groups untreated or treated with exogenous GDNF and the control group ($P < 0.05$) at 1 week. No significant differences were observed at 24 and 48 hr.

GDNF protein was identified in all samples by Western blotting. The characteristics of GDNF protein expression and results in Müller cells are represented in Fig. 5A. There was a highly significant difference in the expression of GDNF protein between the high glucose groups untreated or treated with exogenous GDNF and the control group ($P < 0.01$). In response to exogenous GDNF stimulation, Müller cells enhanced the secretion of GDNF. There was a highly significant difference ($P < 0.01$) in protein synthesis levels between the two experimental groups at all time points.

## Detection of GFRα1 in Müller Cells

Immunoreactive-positive signals for GFRα1 were detected in all the samples. The characteristics of GFRα1 expression in Müller cells are represented in Fig. 6. In the control group, only few faint GFRα1 immunoreactive-positive cells were observed (Fig. 3B). After high glucose stimulation, the number of positive cells increased with increase in time and this process was accelerated with exogenous GDNF.

The expression of GFRα1 mRNA was identified in all samples by real-time RT-PCR. The level of GFRα1 mRNA expression increased gradually both under high glucose- and GDNF-supplemented conditions with time. The results are shown in Fig. 4B. There was a highly significant difference in the expression of GFRα1 mRNA between the high glucose groups untreated or treated with exogenous GDNF and the control group ($P < 0.01$).

GFRα1 protein was identified in all the samples, except the control group by Western blotting. The characteristics of GFRα1 protein expression and results in

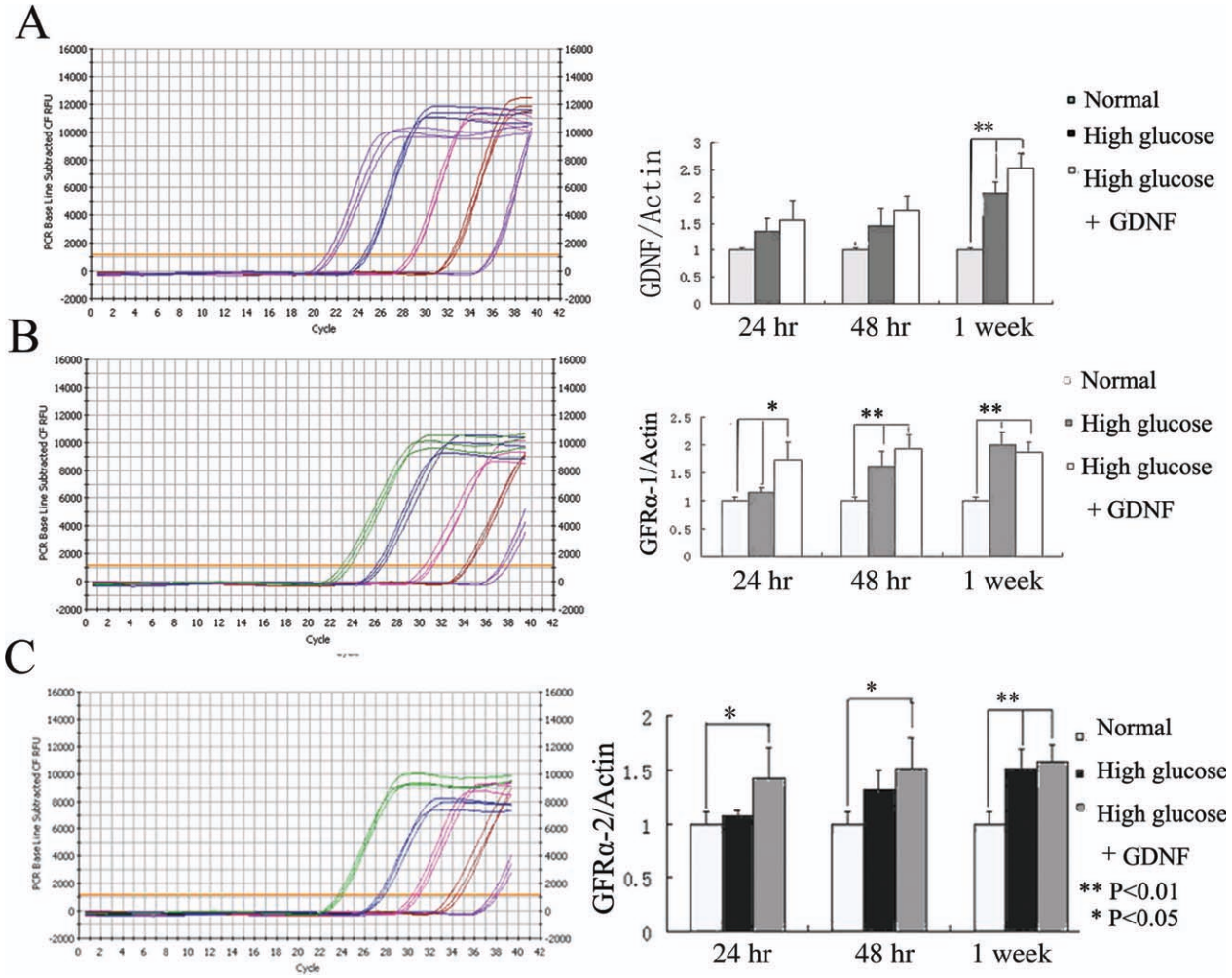


Fig. 4. Exogenous GDNF treatment affects the (**A**) GDNF, (**B**) GFRα1, (**C**) GFRα2 mRNA gene expression in Müller cells compared to the simple high glucose and normal conditions. β-Actin was used as a loading control and the data were quantified. Data are expressed as mean ± SEM. *$P < 0.05$, **$P < 0.01$.

Müller cells are shown in Fig. 5B. In response to exogenous GDNF stimulation, Müller cells enhanced the synthesis of GFRα1. There was a highly significant difference in protein synthesis level ($P < 0.01$) between the two experimental groups.

### Detection of GFRα2 in Müller Cells

Immunoreactive-positive signals for GFRα2 were detected in all the samples. The characteristics of GFRα2 expression in Müller cells are represented in Fig. 7. In the control group, few faint GFRα2 immunoreactive-positive cells were observed (Fig. 3C). After high glucose circumstance, the number of the GFRα2-positive cells increased at 24 hr and then decreased. Exogenous GDNF enhanced the positive cells at 24 hr, but no obvious difference was observed at 48 hr and 1 week.

The expression of GFRα2 mRNA was identified in all the samples by real-time RT-PCR. The level of GFRα2 mRNA expression increased gradually both under high glucose- and GDNF-supplemented conditions. The results are shown in Fig. 4C. There was a significant difference in the expression of GFRα2 mRNA between the high glucose groups untreated or treated with exogenous GDNF and the control group ($P < 0.01$), and a statistically significant difference was observed between the two experimental groups at 24 hr ($P < 0.05$).

GFRα2 protein was identified in all the samples, except the control group by Western blotting. The characteristics of GFRα2 protein expression and results in Müller cells are represented in Fig. 5C. The synthesis of GFRα2 protein was enhanced in Müller cells at 24 hr in the two experimental groups, and then the level of GFRα2 protein was downregulated. There was no statistically significant difference in protein synthesis level between the two experimental groups at any time point.

## DISCUSSION

Here, we studied whether Müller cells can synthesize GDNF and GFRs during the early stage of DR and whether the exogenous GDNF can affect the Müller cell survival. We cultured Müller cells in vitro and applied high glucose circumstance to mimic the early stage of

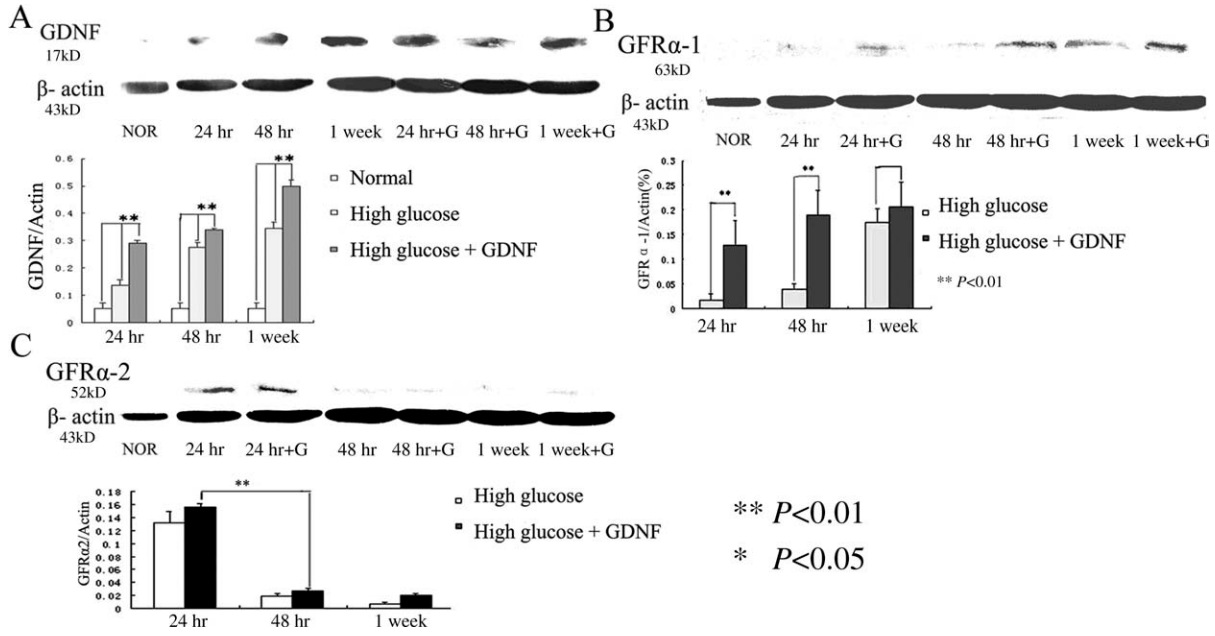


Fig. 5. Western blot analysis of GDNF protein. Representative protein expression of (**A**) GDNF, (**B**) GFRα1, (**C**) GFRα2 was evaluated at 24 hr, 48 hr, and 1 week time points. Furthermore, expression profiles were evaluated in the high glucose groups untreated or treated with GDNF. Product sizes specific for each product are indicated. Expression of GDNF, GFRα1, and GFRα2 was densiometrically analyzed from Western blots. Each sample was measured against β-actin and compared to normal Müller cells. Data are expressed as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$.

DR. Our study also revealed the relationship between GDNF and Müller cells in response to high glucose stimulation.

Studies have demonstrated that GDNF is a critical factor in regulating the vascular permeability of the blood–retinal barrier (BRB) in retina (Nishikiori et al., 2005). The GDNF receptor expression in light-damaged retina showed that GFRα2 was upregulated in Müller cells during photoreceptor degeneration. These results suggest that GDNF (Jomary et al., 2004), NTN and their receptors are involved in the regulation of trophic factor production in retinal glial cells, and that functional glia-neuron network may use GDNF family for the protection of neural cells during retinal degeneration (Harada et al., 2003). Some studies have also demonstrated that GDNF secreted from glial cells is a critical factor in regulating the vascular permeability of the BRB which comprised capillary endothelial cells and glial cells (Nishikiori et al., 2007). But other studies showed the GFRα1 expression in horizontal, amacrine, and ganglion cells, whereas GFRα2 expression was only detected in amacrine and ganglion cells, and no expression of GFRα1 or GFRα2 was detected in Müller cells (Brantley et al., 2008). These contradictory results of GDNF and its receptors synthesis by Müller cells have prompted us to investigate the relationship between GDNF and Müller cells.

Quantitative analysis of apoptotic cells revealed that the treatment of GDNF effectively reduced the damage of Müller cells under high glucose circumstance. This result was similar to that of reduced neuron cells or photoreceptor damage induced by GDNF in the diabetic rat model, demonstrating that GDNF can protect Müller cells. This also suggested that Müller cells may respond to the extracellular signals of GNDF under high glucose condition, but the specific receptor is unknown.

By comparing the expression levels of GDNF and GFRs (GFRα1 and GFRα2) between high glucose condition groups and the control group, we found that the expression level was weak in the control group. Importantly, our study revealed that exogenous GDNF in the early stage of DR could not only reduce Müller cells impairment but also promote the synthesis of GDNF and its receptors in Müller cells. However, Müller cells expressing GDNF and GFRα1 need a longer period of time under high glucose circumstance. Some studies showed that Müller cells did not express the GFRα2 receptor and, therefore, may not be involved in NTN signaling (Wolf et al., 2008). Our study showed similar results in the control group, but in the early stage (24 hr) of high glucose circumstance, we found a temporary upregulation of GFRα2, and this upregulation was enhanced by exogenous GDNF. This difference may explain the conflict in opinions whether Müller cells express GDNF and its receptors. This phenomenon also suggested that exogenous GDNF may have a protective role in Müller cells and could accelerate the synthesis of GDNF and GFRα1 in Müller cells under high glucose circumstance. This modulability in Müller cells may serve as a protective mechanism against the development of DR by regulating endothelial integrity (Igarashi et al., 2000). GDNF could exert its neuroprotective effect through Müller cells (Hauck et al., 2006) and by promoting the expression of the glial L-glutamate transporter, an endogenous neuroprotective mechanism against glutamate-mediated excitotoxicity (Delyfer et al., 2005).

Quantitative analysis of GDNF and its receptors revealed that the mRNAs levels of GDNF and GFRα1,

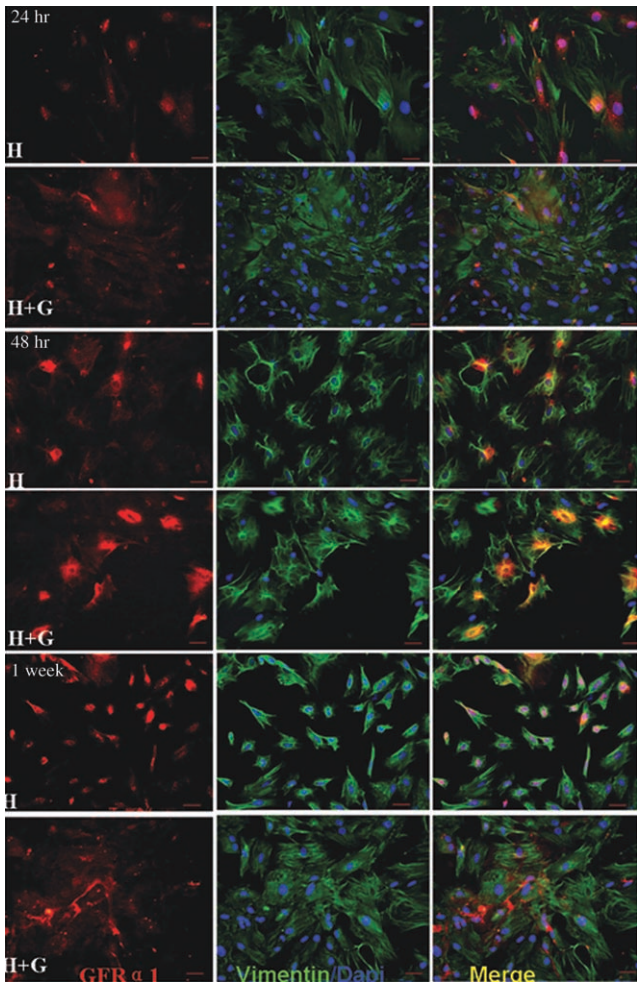


Fig. 6. Immunofluorescence of GFRα1 (red) and the Müller cells marker vimentin (green) in the high glucose groups untreated or treated with GDNF (100 ng/mL) at different time points. Under high glucose conditions, expression of GFRα1 is upregulated with increase in time period. GDNF treatment accelerated this process. Bar = 100 μm.

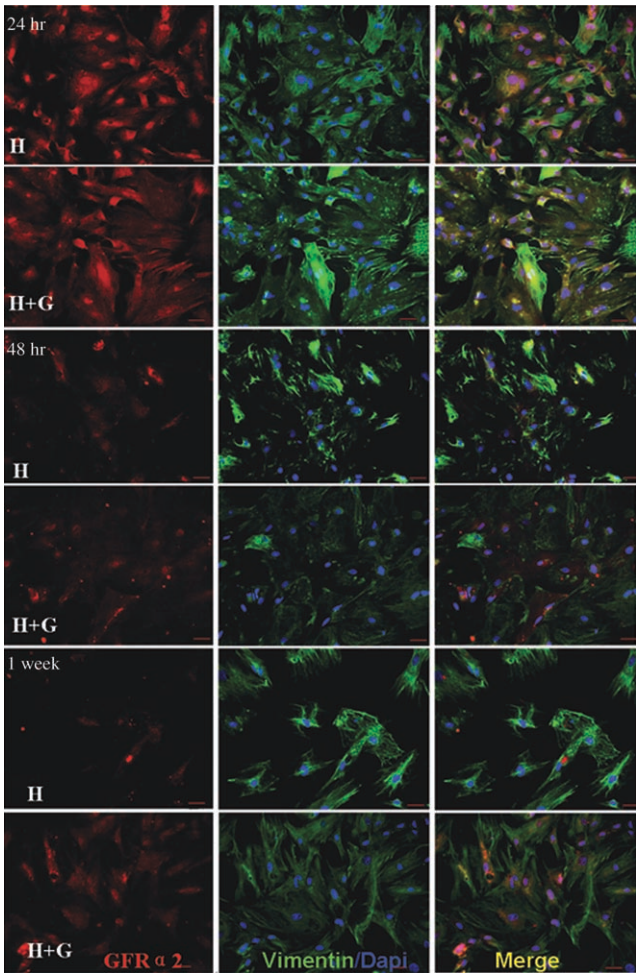


Fig. 7. Immunofluorescence of GFRα2 (red) and the Müller cells marker vimentin (green) in the high glucose groups untreated or treated with GDNF (100 ng/mL) at different time points. Under high glucose conditions, the expression of GFRα2 is upregulated at 24 hr, and GDNF treatment enhanced this process. Expression of GFRα2 did not upregulate with increase in time. Bar = 100 μm.

GFRα2 in Müller cells were upregulated under high glucose circumstance, and there was no statistically significant difference between the two high glucose condition groups untreated or treated with the exogenous GDNF. However, the protein expression detected by immunohistochemistry and Western blotting showed that the synthesis of GDNF and GFRα1 was effectively accelerated by exogenous GDNF. The difference in mRNA and protein results suggested that the mechanisms of GDNF and its receptors synthesis in Müller cells are not only due to the high glucose circumstance but also involved the extracellular concentration of GDNF.

Taken together our findings showed that the GDNF functions not only to protect neurons and photoreceptors (Creedon et al., 1997; Politi et al., 2001) but also has a role in protecting Müller cells. Müller cells have the ability to express GFRα1, GFRα2 and to secrete GDNF under high glucose conditions. The mRNA levels of intracellular GDNF and GFRα1 and GFRα2 are expressed in Müller cells, but the protein levels may be affected by extracellular GDNF concentration. Our results revealed the difference between mRNA and protein expressions in Müller cells. The results also suggested that exogenous GDNF can upregulate the level and speed of GDNF synthesis in Müller cells and may have a protective role in Müller cells survival during the early stage of DR.

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