Neuropeptide FF Promotes Recovery of Corneal Nerve Injury Associated With Hyperglycemia

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Submitted: January 21, 2015
Accepted: October 25, 2015

PURPOSE. To investigate how the neuropeptide FF (NPFF) promotes the recovery of corneal nerve injury associated with hyperglycemia.

METHODS. Gene expression was analyzed using neurotrophin and receptor RT2 profiler polymerase chain reaction arrays in trigeminal (TG) sensory neurons. The role of NPFF in the regeneration of diabetic TG nerves was investigated in vitro by using cultured TG neurons from diabetic BKS.Cg-m+/+Leprdb/+ (db/db) mice and in vivo by following corneal injury healing responses. RP9, a selective NPFF receptor (NPFF2R) antagonist, was used to prevent the interactions between NPFF and NPFF2R.

RESULTS. Using a mRNA real-time PCR array, NPFF was found to be significantly lower in diabetic TG sensory neurons. Hyperglycemia induced the deficiency of ocular properties in db/db mice. The application of NPFF enhanced neurite elongation in diabetic TG neurons. Through subconjunctival injection, NPFF promoted corneal nerve injury recovery and epithelial wound healing in db/db mice. Furthermore, the application of NPFF rescued the activation of SIRT1 and PPAR-gamma, and downregulated the expression of PTEN and Rb in diabetic TG neurons. The promotion of NPFF on diabetic corneal epithelial healing and corneal innervations was completely abolished by RP9. Moreover, subconjunctivally injected NPFF accelerated the reinnervation of corneal nerves via the ERK1/2 pathway.

CONCLUSIONS. These results indicate that NPFF signaling through NPFF2R contributes to diabetic corneal nerve injury recovery and epithelial wound healing. Neuropeptide FF is a potential neuroregenerative factor for diabetic sensory nerve injury.

Keywords: hyperglycemia, neuropeptide, diabetic keratopathy, NPFF

目的：探讨NPFF对糖尿病角膜神经再生的作用及具体分子机制；

方法：通过PCR芯片的方法筛选糖尿病BKS.Cg-m+/+Leprdb/+ (db/db)小鼠三叉神经节（TG）中的差异基因；通过神经逆行示踪方法，结膜下注射荧光金标记三叉神经节眼支来源的神经节细胞，采用免疫组化和荧光技术显示NPFF及其受体在 db/db 小鼠及其对照鼠三叉神经节的细胞定位及表达，并通过ELISA检测NPFF蛋白在角膜和三叉神经节的表达情况。在 db/db 小鼠角膜上皮刮除模型上，结膜下注射NPFF或者空白对照药物PBS，通过角膜知觉、以及角膜神经染色评价角膜末梢神经修复情况。

结果：24w龄 db/db小鼠出现糖尿病角膜病变的典型特征：泪液分泌异常、角膜知觉减退和末梢神经异常等。通过荧光金逆行示踪，成功标记三叉神经节中眼支
Diabetes mellitus (DM) is a major cause of peripheral nerve damage. Due to this complication, DM may lead to significant morbidity and mortality.\textsuperscript{1,2} Although the peripheral nervous system has the ability to repair itself, patients with diabetes are susceptible to peripheral nerve injury. Therefore, diabetic peripheral neuropathy continues to be a significant challenge in diabetics in general. It is believed that the sensory neurons are targeted and damaged by DM before significant motor neuron involvement.\textsuperscript{3} More importantly, the decreased release of neuropeptides in neurons induced by hyperglycemia is generally associated with delayed recovery from sensory nerve injury.\textsuperscript{4,5}

Diabetes can alter the structure of corneal nerves\textsuperscript{6} and decrease the release of neuropeptides, neurotrophins, and growth factors.\textsuperscript{7} Neuropeptides that function in the axis of corneal nerves include substance P (SP),\textsuperscript{8,9} calcitonin gene-related peptide (CGRP), and vasointestinal peptide (VIP).\textsuperscript{10} The corneal nerves arise from the ophthalmic division of the trigeminal ganglion (TG). Nerve fibers are sensitive to temperature as well as to mechanical and chemical stimuli. Corneal nerves are critical for corneal epithelial wound healing.\textsuperscript{11} Evidence of a cornea-trigeminal axis and the potential role of neuropeptides has been described by Ferrari et al.\textsuperscript{12} Corneal sensitivity is decreased in diabetic patients with sterile neurotrophic corneal ulcers, and neuronal abnormalities may be the cause of diabetic keratopathy.\textsuperscript{7} Moreover, nerve function can be easily measured by assessing both corneal sensation and corneal epithelial integrity. These characteristics make the corneal TG a highly accessible injury model for studying the effect of potential modulators of peripheral nerve repair.

In the present study, using an mRNA real-time PCR array, we found that Neuropeptide FF (NPFF) significantly decreased in TG tissues of type 2 diabetic BKS.Cg-m\textsuperscript{+}/+Leprdb/J (db/db) mice compared with control mice (db/\textsuperscript{+}). Neuropeptide FF was isolated from a bovine brain in 1985 and is part of the RF-amides (Arg-Phe-NH\textsubscript{2}) family.\textsuperscript{13} One of the important functions of NPFF (FLFQPQRF\textsubscript{a}) is in pain perception.\textsuperscript{14} Additionally, NPFF displays potent effects on regulation of the core temperature,\textsuperscript{15} reduction in food intake and changes in arterial blood pressure.\textsuperscript{16} The neuropeptide FF receptor family is part of the G protein-coupled receptor superfamily. Several groups cloned two genes encoding two different receptors (NPFFR1 and NPFFR2) and two precursors in several mammalian species.\textsuperscript{17} Neuropeptide FF1 is found in its highest concentrations in the limbic system and the hypothalamus of the central nervous system. NPFFR2 is found in its highest concentrations in the diencephalon and superficial layers of the spinal cord. It was reported that most of the pharmacological effects of NPFF are due to the NPFF2R.\textsuperscript{18} At present, few studies have been conducted on the role of NPFF in the recovery of diabetic sensory nerve injury.

Therefore, in this study, using a combination of in vitro and in vivo approaches, we found that NPFF can enhance neurite elongation in diabetic TG neurons and overcome the detrimental corneal wound healing effects of hyperglycemia by stimulating corneal nerve regeneration. More importantly, the recovery effect of NPFF on corneal nerve injury was associated with functional recovery of corneal sensation under hyperglycemic conditions. Furthermore, local inhibition of the NPFF-NPFFR2 pathway slowed down diabetic corneal nerve recovery. This suggests that the NPFF-NPFFR2 pathway may be involved in the recovery of diabetic peripheral nerve injury, which is supported by the fact that the NPFFR2 exists not only in the central nervous system but also in peripheral nervous systems.\textsuperscript{17}

**MATERIALS AND METHODS**

**Animal Experiments and Tract Tracing Techniques**

This investigation was approved by the Institutional Animal Care and Use Committee, Shandong Eye Institute (Shandong Academy of Medical Sciences, Qingdao, China). All procedures and animal handling during the study conformed with the guidelines of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Diabetic BKS.Cg-m\textsuperscript{+}/+Leprdb/J (db/db), non-diabetic control db/\textsuperscript{+}, neurofluorescent thyl1-YFP, and wild-type C57BL/6 mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). The in vivo corneal injury model was prepared as previously described by Wang et al.\textsuperscript{19} The mice were randomly divided into four groups:
untreated control db/+ mice, untreated db/db mice, NPFF-treated db/db mice, and NPFF plus RP9-treated db/db mice. The left eyes of the mice in all groups remained untreated and untreated for use as normal controls. In groups 1 and 2, the right eyes were untreated after injury. Recombinant mouse NPFF (100 μM; USCN Life Science, Inc., Houston, TX, USA) and/or RP9 (10 μM), a selective NPFF2R antagonist (No. 3672, R&D Systems, Minneapolis, MN, USA), were injected into the subconjunctival site of the right eye on the same day as corneal epithelium injury in groups 3 and 4. All groups were assessed using fluorescein sodium and photographed at 0, 24, 48, and 72 hours. Fluoro-Gold (FG, 2% in saline; Fluorochrome, LLC, Denver, CO, USA) was used as a retrograde tracer to identify corneal nerves in the TG for detection of the expression of NPFF after subconjunctival injection.

**Examination of the Ocular Surface**

Examination of the ocular surface was performed as previously described by Yin et al. Rose Bengal ocular surface staining was used to assess the damage to conjunctival and corneal epithelial cells. Tear secretion was evaluated with phenol red-impregnated cotton threads (Zone-Quick, Tokyo, Japan). Anesthesiometer (Cochet-Bonnet, Luneau, France) was used to determine corneal sensation.

**Transmission Electron Microscopy (TEM)**

The cornea and TG tissues (from the ophthalmic division of the trigeminal ganglia) were dissected out quickly and divided into small pieces. All tissues were placed in 2.5% glutaraldehyde fixing solution and dehydrated with acetone. Then, the tissues were placed in a dilute solution of plastic embedding medium epoxy resin mixture. Sections 70-nm thick were embedded for orientation purposes and subsequently stained with heavy metal solutions. Changes in ultrastructure were examined using a JEM-1200EX transmission electron microscope (JEOL, Tokyo, Japan).

**PCR Array**

We collected the TG tissues from three db/db and three control db/+ mice. Expression of genes was analyzed using the neurotrophin and receptor (PAMM-031; SABiosciences, Frederick, MD, USA) RT² profiler polymerase chain reaction arrays according to the manufacturer’s protocol.

**Cell Culture, Treatment, and TG Neuronal Cell Growth and Viability Assay**

The whole TG tissue was collected from the diabetic or nondiabetic control mice. Primary TG neuronal cells were cultured as described previously. The proximal region of trigeminal ganglia containing the ophthalmic branch, which innervates the cornea, was cut into tissue explants. Trigeminal explants were put in 2-well chamber slides coated with Laminin (20 g/mL in water; cat. no. L2020; Sigma, Shanghai, China) to support neuronal adhesion and outgrowth. Then the trigeminal explants were cultured at 37°C in a humidified CO2 incubator for 72 hours in Neurobasal-A Medium supplemented with 1% B27 and 1% penicillin or streptomycin (Life Technologies, Gaithersburg, MD, USA). Then, the cultured neurons from the diabetic animals were matched according to the preharvesting blood glucose level. In addition, primary TG neuronal cells from the diabetic mice were treated with 5 μM RF9.

**Corneal Nerve Staining**

Corneal nerve staining was performed as described previously with anti-neuron-specific beta III tubulin antibody (ab7751; Abcam, Cambridge, MA, USA). The corneal nerves were counted using ImageJ software (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA).

**Immunohistochemistry and Immunoblotting**

Immunohistochemistry and immunoblotting were performed according to a previously described protocol. The primary antibody for NPFF (USCN Life Science, Inc.) was used. The blots were probed with the following primary antibodies: AKT1 (ab6076; Abcam), phospho-AKT (#4060; Cell Signaling Technology, Danvers, MA, USA), phospho-p44/42 MAPK (ERK1/2; #9101; Cell Signaling Technology), and p44/42 MAPK (ERK1/2; #9102).

**Enzyme-Linked Immunosorbsent Assay for NPFF**

Neuropeptide FF levels in TG tissues of db/db and nondiabetic control db/+ mice were determined by ELISA (USCN Life Science, Inc.) according to the manufacturer’s protocol.

**Statistical Analyses**

The results are expressed as the means ± SD. One-way ANOVA and Student’s t-test were used to determine any significant differences between the diabetic and nondiabetic control mice or cells. The least significant difference procedure was performed using GraphPad Prism software 5.0 (GraphPad Software, Inc., San Diego, CA, USA). A P value less than 0.05 was considered statistically significant.

**Results**

**Evaluation of Ocular Properties**

The data of blood sugar and body weight of db/db and nondiabetic control db/+ mice are shown in Table 1. db/db mice had significantly higher blood glucose and weight than the control mice. By ophthalmic examinations and measurements, the ocular changes were caused by hyperglycemia in db/db and nondiabetic control db/+ mice. Rose Bengal staining was much stronger in the db/db mice than in the controls (Fig. 1A; n = 4 per group, *P < 0.05), indicating a deficiency of tear film protection. The db/db mice had significantly fewer tears than the control db/+ mice (Fig. 1B; n = 4 per group, *P < 0.05). Corneal sensitivity was measured in the central, nasal, dorsal, temporal, and ventral cornea using a Cochet-Bonnetesthesiometer. The mean overall corneal sensitivities of the db/db mice and control db/+ mice were 5.4 ± 0.04 cm and 5.8 ± 0.07 cm, respectively. The central cornea of control db/+ mice was more sensitive than that of the db/db mice. Corneal sensitivity was significantly lower in the db/db mice compared with the control db/+ mice (Fig. 1C; n = 4 per group, *P < 0.05). The ultrastructural analysis of the corneas from the control group showed that corneal epithelium was tightly packed with no intercellular spaces between neighboring cells, and compact collagen fibers were observed enfolding stromal keratocytes. However, in the corneas from the diabetic mice, intracellular spaces were present between neighboring cells in the superficial corneal epithelium, and deformation was observed in lower layers of epithelial cells. Stromal keratocytes and endothelia also displayed degeneration of intracellular organelles and cytoplasmic vacuoles at various sizes. Disconnected collagen fibers resulted in structural disintegration in the corneal stroma (Fig. 1D). Electron micrographs showed a zone of dysmyelination in the TGs from the diabetic mice. Most axons were relatively intact, but there was expansion of the inner or outer mesaxon, or myelin invagination, in some of them (Fig. 1E).
NPFF Enhances Diabetic Corneal Nerve Recovery

Table 1. Average Weight and Blood Glucose Level at Time of Death

<table>
<thead>
<tr>
<th>Group</th>
<th>Numbers</th>
<th>Age, wk</th>
<th>Weight, g</th>
<th>Glucose, mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>27</td>
<td>20–24</td>
<td>25.93 ± 1.25</td>
<td>7.63 ± 0.47</td>
</tr>
<tr>
<td>Diabetic</td>
<td>51</td>
<td>20–24</td>
<td>57.59 ± 4.82</td>
<td>22.26 ± 4.21</td>
</tr>
</tbody>
</table>

BKS-db/db mice had significantly higher blood glucose than control mice, and the weight of BKS-db/db mice was also significantly higher than that of the control animals.

* P < 0.05, unpaired t-test relative to corresponding control group
† P < 0.01, unpaired t-test relative to corresponding control group.

Downregulation of NPFF in TG Tissues From Diabetic Mice

The differential expression of genes of neurotrophins and receptors in TG tissues of the db/db and control db/+ mice was detectable in the PCR array assay. A scatterplot of gene expression is shown in Figure 2A (n = 5 per group, *P < 0.05). With an arbitrary cutoff of more than 2-fold change, only one gene, NPFF, was downregulated in TG tissues of the db/db mice compared with the nondiabetic control db/+ mice. An additional seven genes displayed weaker (1.07- to 1.38-fold) but still significant changes (Table 2). Six of them were upregulated. We then investigated NPFF protein expression in TG tissues of the db/db and control db/+ mice by ELISA. As shown in Figure 2B (n = 4 per group, *P < 0.05), the expression of NPFF protein was also reduced in the TG tissues of the db/db mice compared with that of the db/+ mice. The expression of NPFF was also analyzed in TG tissue sections from the db/db and db/+ mice by immunohistochemistry (Fig. 2C).

In Vitro Effects of NPFF on Diabetic TG Neuron Growth

Because corneal sensory nerves are mainly derived from the ophthalmic branch of the TG, we investigated the effects of NPFF on diabetic TG neuron growth in vitro. The primary cultured diabetic or nondiabetic TG sensory neurons were treated with exogenous NPFF (10 μM) or RF9 (5 μM). As shown in Figure 3A, after incubation for 6 days, the neurites in the NPFF treatment groups were significantly longer than those of the non-NPFF-treated db/+ mice. Total neurite length of TG neurons treated with NPFF was greater than 5-fold (for NPFF-treated db/+ mice) and 4-fold (for NPFF-treated db/db mice) the lengths in the non-NPFF-treated db/+ or db/db mice (Fig. 3C; n = 4 per group, *P < 0.05). The neuronal growth effect of NPFF was abolished when its availability was decreased by coinoculation with RF9 (Fig. 3B). Quantitation of total neurite length in TG sensory neurons of the db/db mice treated with RF9 is shown in Figure 3D (n = 4 per group, *P < 0.05).

Effects of NPFF Treatment on the Expression of SIRT1, PPAR-γ, Pten, and Rbh Tg Tissues

The expression of NPFF in TG sensory neurons was detected after the subconjunctival injection of NPFF. Representative images of coexpression of NPFF and Fluoro-Gold in the TG are presented in Figure 4A. More Fluoro-Gold-labeled cell bodies were observed in the medial region of the ipsilateral TG, compared with the non-NPFF-treated control db/+ mice. immunofluorescence staining and ELISA analysis revealed that the protein levels of NPFF in the diabetic corneal epithelium or TG sensory neurons increased after NPFF treatment (n = 4 per group; Figs. 4B, 4C). We investigated the effects of NPFF on the expression of silent information regulator protein 1 (SIRT1), peroxisome proliferator-activated receptor gamma (PPAR-γ), phosphatase and tensin homolog (PTEN), and retinoblastoma (Rb), which was altered in the diabetic TG (Figs. 4D, 4E). The expression of SIRT1 and PPAR-γ (message/protein) was significantly upregulated in the diabetic TG at 72 hours after the subconjunctival injection of NPFF (n = 4 per group). However, the expression of PTEN and Rb (message/protein) was significantly downregulated in the diabetic TG after the subconjunctival injection of NPFF (n = 4 per group).

Effects of NPFF Treatment on Corneal Debridement Wound Healing

Fluorescence staining showed that a significant difference was exhibited with regard to the corneal epithelial healing rate at 72 hours after the corneal epithelium scrape (Fig. 5A). The defect size of the corneal epithelium in the NPFF-treated diabetic mice was significantly more improved than that in the other groups with diabetic mice (n = 8 per group; Fig. 5B).

Effects of NPFF Treatment on Corneal Nerve Density

We also found an effect of NPFF in promoting the increase of corneal nerves in diabetic mice. At 28 days after the subconjunctival injection of NPFF, the corneas were collected for immunohistochemical staining of all corneal subbasal nerves and their associated branches. As shown in Figure 5C, the subbasal nerve density increased in both the peripheral and central zones of corneas treated with NPFF from the db/db mice, compared with those of the non-NPFF-treated db/db mice. Epithelial branches penetrating into the stratified epithelium were visualized at the focal plane of the basal epithelial nuclei. In the db/db mice without NPFF treatment, few neurites were detected, whereas NPFF treatment increased epithelial branches. With NPFF treatment, the mean subbasal nerve density increased almost 1.5-fold in the peripheral cornea and almost 3-fold in the central cornea compared with the non-NPFF treatment group (n = 6 per group; Fig. 5D).

Effects of NPFF Treatment on Corneal Sensation Threshold

Because NPFF accelerated corneal nerve recovery, we then investigated whether this nerve recovery was associated with equal functional recovery. At 7 days after corneal epithelial debridement, no differences in corneal sensation were observed between all groups (Fig. 5E). However, at 28 days after epithelial debridement, the db/db mice receiving NPFF injection showed increased corneal sensation compared with those receiving PBS or no treatment. Furthermore, the corneal sensation of the db/db mice receiving NPFF injection returned to a level similar to that of the control db/+ mice (n = 6 per group).

NPFF2R Mediates the Promotion of NPFF on Recovery of Diabetic Corneal Nerve Injury and Epithelial Wound Healing via ERK1/2 Pathway in db/db Mice

The NPFP2R specific antagonist RF9 injection before topical NPFF application in the corneal epithelium scraped db/db mice was found to fully reverse the promotion of NPFF on diabetic corneal epithelial wound healing. Fluorescence staining showed that, at 48 hours after injury, the wound area in the db/db mice receiving RF9 before NPFF application was
FIGURE 1. Evaluation of ocular surface properties and ultrastructural analysis of the cornea and TG in the db/db mice. (A) Rose Bengal staining of the control db/+ mice (i) and db/db mice (ii). The scores of rose Bengal staining are shown in Figure 1A-iii. Data are the means ± SD (n = 4 per group, *P < 0.05). (B) Measurement of tear secretion with cotton threads (n = 4 per group, *P < 0.05). (C) Detection of corneal sensitivity with anesthesiometer (n = 4 per group, *P < 0.05). (D) Ultrastructural analysis of the cornea. In the diabetic group, ultrastructural alterations were observed in endothelial cells (thick arrows). Examination of the stroma in the diabetic group revealed degenerative chromatin condensation (thin arrows) and vacuoles (*) structure. Cytoplasmic vacuoles are also observed in the epithelial cells in the diabetic group (*). Formed myelin figures are

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observed in some epithelial cells in this group (#). (E) Ultrastructural analysis of the TG. The zone of the TG where the photo was taken is from the ophthalmic division of the trigeminal ganglia. Electron micrographs showed a zone of dysmyelination in the TGs from the diabetic mice. In some axons, there was inner or outer mesaxon expansion or myelin invagination (i and ii, ×50,000). Figure 1E-iii showed a higher magnification photo (×20,000). Axoplasm ("1"), axolema ("2"), and myelin ("3") were observed. Internal structures such as neurofilaments were shown in the square frame.

**Figure 2.** The downregulation of NPFF in the db/db mice. (A) Scatterplot of gene expression in the PCR array in whole-TG lysates. db/+ mice served as the control. Data are the means ± SD (n = 3 per group, *P < 0.05). (B) ELISA in whole-TG lysates. Data are the means ± SD (n = 4 per group, *P < 0.05). (C) Immunohistochemical staining of NPFF in TG of db/+ or db/db mice. (D) Neuropeptide FF was expressed in the sensory TG. (D) Transverse sections of normal adult TG with the coexpression of NF200 and NPFF in neurons. There was relatively little coexpression of NF200 and NPFF (E) The coexpression of NPFF and S100β, a marker of Schwann cells, in TG neurons. Scale bars: 50 μm.
TABLE 2. PCR Array Results

<table>
<thead>
<tr>
<th>RefSeq</th>
<th>Symbol</th>
<th>Description</th>
<th>$2^{\Delta \Delta Ct}$</th>
<th>Fold Change Diabetic/Nondiabetic</th>
<th>$P$ Value</th>
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<tbody>
<tr>
<td>NM_009827</td>
<td>Cckar</td>
<td>Cholecystokinin A receptor</td>
<td>3.4E-02</td>
<td>4.5E-01</td>
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<tr>
<td>NM_016673</td>
<td>Cntfr</td>
<td>Ciliary neurotrophic factor receptor</td>
<td>2.4E-03</td>
<td>3.0E-03</td>
<td>1.25</td>
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<tr>
<td>NM_009953</td>
<td>Crhr2</td>
<td>Corticotropin releasing hormone receptor 2</td>
<td>5.9E-02</td>
<td>7.6E-01</td>
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<tr>
<td>NM_008115</td>
<td>Gfra2</td>
<td>Glial cell line derived neurotrophic factor family receptor alpha 2</td>
<td>3.2E-01</td>
<td>3.8E-01</td>
<td>1.20</td>
</tr>
<tr>
<td>NM_198959</td>
<td>Hcrtr1</td>
<td>Hypocretin (orexin) receptor 1</td>
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<td>2.5E-02</td>
<td>1.38</td>
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<td>NM_010559</td>
<td>Il6ra</td>
<td>Interleukin 6 receptor, alpha</td>
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<td>5.2E-01</td>
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<tr>
<td>NM_018787</td>
<td>Npff</td>
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<td>NM_133192</td>
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<td>1.35</td>
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</table>

For PCR array, an additional seven genes displayed weaker (1.07- to 1.38-fold) but still significant changes. Six genes were upregulated, whereas one gene was downregulated. The six upregulated genes were cholecystokinin A receptor (Cckar), ciliary neurotrophic factor receptor (Cntfr), corticotropin-releasing hormone receptor 2 (Crhr2), glial cell line-derived neurotrophic factor family receptor alpha 2 (Gfra2), hypocretin (orexin) receptor 1 (Hcrtr1), and neuropeptide FF receptor 2 (Npffr2). The one downregulated gene was interleukin 6 receptor-alpha (Il6ra).

**Figure 3.** Neuropeptide FF promoted TG neuron growth under high-glucose conditions in vitro. (A) Neuropeptide FF effects on the neurite growth of neurons from the $\text{db/db}$ and the control $\text{db/+}$ mice. (B) The neuronal growth effect of NPFF was abolished when its availability was decreased by concomitant incubation with RF9 treatment. The data represent the results of four different experiments. Scale bar: 50 μm. (C) Quantitation of total neurite length analyzed in TG sensory neurons of the $\text{db/+}$ and $\text{db/db}$ mice ($n=4$ per group, $*P<0.05$). (D) Quantitation of total neurite length in TG sensory neurons of the $\text{db/db}$ mice treated with RF9 ($n=4$ per group, $*P<0.05$).
FIGURE 4. Effects of NPFF treatment on the expression of SIRT1, PPAR-\(\gamma\), PTEN, and Rb in TG tissues. (A) Representative images of the coexpression of NPFF and Fluoro-Gold in TG sensory neurons of the \(db/db\) mice (\(n = 8\) per group). Scale bar: 50 \(\mu\)m. More Fluoro-Gold–labeled cell bodies were observed in the medial region of the ipsilateral TG, compared with the non-NPFF–treated control \(db/+\) mice. (B) Representative images of NPFF expression in the cornea and TG sensory neurons after a subconjunctival injection of NPFF (\(n = 4\) per group). Scale bars: 50 \(\mu\)m. (C) Detection of NPFF in the cornea and TG sensory neurons after the subconjunctival injection of NPFF by ELISA (\(n = 4\) per group, *\(P < 0.05\)). Immunofluorescence staining and ELISA analysis revealed that the protein levels of NPFF in the diabetic corneal epithelium or TG sensory neurons increased after NPFF treatment. The expression levels of silent information regulator protein 1 (SIRT1), peroxisome proliferator-activated receptor gamma (PPAR-\(\gamma\)), phosphatase and tensin homolog (PTEN), and retinoblastoma (Rb) were detected by qPCR (D) and Western blot (E) in the diabetic TG at 72 hours after the subconjunctival injection of NPFF (\(n = 4\) per group, *\(P < 0.05\)). SIRT1 and PPAR-\(\gamma\) (message/protein) were significantly upregulated. However, PTEN and Rb (message/protein) were significantly downregulated in the diabetic TG after the subconjunctival injection of NPFF.
FIGURE 5. Effects of NPFF treatment on corneal debridement wound healing, corneal nerve density, and corneal sensation threshold. (A) The fluorescein-stained corneas at 72 hours after subconjunctival injection of NPFF (n = 8 per group). (B) The remaining wound area at 72 hours after subconjunctival injection of NPFF (n = 4 per group, *P < 0.05). The defect size of the corneal epithelium in the NPFF-treated diabetic mice was significantly more improved compared with that in the other groups with the diabetic mice. (C) The effect of NPFF treatment on corneal nerve density. At 28 days after the subconjunctival injection of NPFF, the corneas were collected for immunohistochemical staining of all corneal subbasal nerves and their associated branches. The subbasal nerve density increased in both the peripheral and central zones of corneas treated with NPFF from the db/db mice, compared with the non-NPFF–treated db/db mice. Epithelial branches penetrating into the stratified epithelium were visualized at the focal plane of the basal epithelial nuclei. In the db/db mice without NPFF treatment, few neurites were detected, whereas NPFF treatment increased epithelial branches. With NPFF treatment, the mean subbasal nerve density increased by almost 1.5-fold in the peripheral cornea and by almost 3-fold in the central cornea compared with the non-NPFF treatment group (n = 6 per group). (D) The quantified data of corneal subbasal nerve density (n = 4 per group, *P < 0.05). (E) Effects of NPFF treatment on corneal sensation threshold. The detection of corneal sensation in the db/db mice at 7 and 28 days after subconjunctival injection of NPFF (n = 6 per group, *P < 0.05).
significantly expanded \( (n = 8 \text{ per group}; \text{Fig. 6A}) \). A significant difference was exhibited with regard to the corneal epithelial healing rate at 48 hours after the corneal epithelium scrape in the \( db/db \) mice administered with RF9 before NPFF application \( (n = 4 \text{ per group}; \text{Fig. 5C}) \). Moreover, treatment with NPFF in corneal epithelia promoted the recovery of diabetic corneal nerves. However, this effect of NPFF was completely abolished by RF9. As shown in Figures 6B and 6D, the nerve density decreased at 28 days after administration of RF9 before NPFF application in the diabetic corneas.

In diabetes, reluctant regrowth of sensory neuron may be a major aspect of regeneration failure, depending on several
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important growth factors or their pathways. High levels of ERK signaling activity have been found in recovery of nerve injury.23,24 Additionally, the serine/threonine AKT activation is also a central pathway involved in survival, growth, proliferation, and regeneration of axons.25,26 In this study, we also detected the activation of ERK or AKT pathway in diabetic TG sensory neuron regeneration. At 48 hours after scrape, a stronger expression of p-ERK1/2 was found in the NPFF-treated diabetic corneal epithelium than that in both the untreated and antagonist-injected diabetic corneal epithelia (Fig. 6E). The levels of phosphorylated AKT and total AKT were almost identical in all samples tested. The NPFFR2 receptor mediated the reactivation of ERK1/2 by NPFF, and the promotion of NPFF on diabetic corneal nerve regeneration was also NPFFR2 mediated.

**DISCUSSION**

In this study, we found that NPFF promotes corneal nerve injury recovery and epithelial wound healing via the NPFF receptor (NPFF2R). The diabetic animal model used in this study was db/db mice, which have been used as a model for the study of diabetic neuropathy.27 Several research groups reported abnormality of the ocular surface and delayed healing of the epithelial wounds in diabetic animals. Yin et al.28 documented the corneal complications in STZ-induced DM rats, including a lack of sufficient tear secretion and neuropathy-associated denervation. Our prior studies investigated the alteration of ocular properties, especially the delayed corneal wound healing in Akita diabetic mice, which have been used as a model for the study of chronic complications of type 1 DM.22,28 In the current study, we found that compared with the normal control mice, the db/db mice had reduced tear secretion, impaired innervation, and altered ultrastructures of the cornea and TG, suggesting that the peripheral nerve and ocular properties were altered in this type 2 diabetic animal model.

Trigeminal ganglion has a neurotrophic influence on the cornea, in addition to its well-known sensory role. Therefore, the corneal nerves and TG neurons can be analyzed as a whole and can serve as an excellent model for diabetic peripheral nerve injury repair.29 To further study the molecular mechanisms of this relationship between trigeminal neurons and corneal nerve endings in diabetics, we performed a PCR array assay and found that the expression of NPFF decreased significantly in the TG tissues of the diabetic mice. Neuropeptide FF plays an important role in regulation of the central nervous system (CNS) and peripheral nervous system.16 Oehlmann et al.30 found that NPFF could be strongly expressed in the hypothalamus, brain stem, and spinal cord. In contrast with the study by Nieminen et al.,31 we found NPFF expression in TG, while Nieminen et al. found no NPFF expression in TG neurons of the rats by in situ hybridization. The reason that our results are different from those obtained by Nieminen et al.31 may be due to the different methods. Apart from the role of NPFF in pain perception and the regulation of the cardiovascular system, to our knowledge, the effect of NPFF on recovery from peripheral nerve injury in experimental diabetic neuropathy has not been reported.

Diabetes may accelerate neuronal cell death and inhibit neurite regeneration.32,33 In this study, by investigating the NPFF-induced neuronal growth in primary cultured diabetic TG sensory neurons, we found that the neurite length was significantly longer in the NPFF-treated diabetic neurons compared with the non-NPFF-treated controls. It is believed that TG innervation plays a pivotal role in the maintenance of a healthy, intact corneal epithelium. Experimentally, interruption of the ophthalmic TG pathways results in delayed healing of epithelial wounds, which can be difficult to manage clinically.7 Patients with diabetes exhibit various types of corneal pathology (diabetic keratopathy) and have decreased corneal sensitivity if they have sterile neurotrophic corneal ulcers or neuronal abnormalities.34 In the present study, using tract tracing techniques of Fluoro-Gold, NPFF was found in the TG sensory neurons after subconjunctival injection in the diabetic mice. The results also suggest that NPFF subconjunctival injection can regulate the activation of multiple signaling pathways needed for nerve regeneration. SIRT1 and PPAR-γ have been evaluated for their capacity to accelerate nerve regeneration,35,36 and downregulation of PTEN and Rb have also been found to enhance nerve regeneration.37,38 In this study, NPFF subconjunctival injection not only stimulated the reactivation of SIRT1 and PPAR-γ but also attenuated the expression of PTEN and Rb in the TG sensory neurons. Furthermore, the in vivo application of NPFF demonstrates that NPFF can serve as a potent neuron growth-promoting factor for recovery from diabetic peripheral nerve injury. When NPFF was applied to the injured corneal nerves, a remarkable nerve growth-promoting effect was noted in the corneas of the db/db mice via ERK1/2 pathway. ERK-signaling pathway was reported to play a central role in controlling Schwann cell plasticity and peripheral nerve regeneration.39 We observed the expression of NPFF in a large proportion of TG sensory neurons colabeling with S100β, a marker of Schwann cells. This suggests that NPFF may be secreted by Schwann cells in TG sensory neurons. We also observed that the ERK1/2 signaling pathway was activated in TG sensory neurons after NPFF subconjunctival injection, whereas the AKT pathway was not affected. These results indicate that the upregulation of NPFF could activate the ERK pathway specifically, and the ERK pathway may be a key downstream pathway of NPFF in diabetic corneal nerve regeneration. Our finding is consistent with the results of Sun et al.42 They studied the signal transduction downstream of NPFF using the differentiated neuroblastoma cell line dSH-SYSY, and found that NPFF rapidly and transiently stimulated the ERK pathway.

In conclusion, our study demonstrates for the first time that NPFF promotes diabetic corneal nerve regeneration and epithelial wound healing via the ERK pathway. Further studies are needed to assess the role of NPFF in Schwann cells under high-glucose conditions in vitro and in vivo.

**Acknowledgments**

The authors thank Zhen Liu (Shanghai Bio-Fcen Biotechnology Co. Ltd., Shanghai, China) for his valuable assistance.

Supported by the State Key Basic Research (973) Project of China Grant 2012CB722409 (Qingdao, Shandong, China); the National Natural Science Foundation of China Grant 81370990 and 30901657 (Qingdao, Shandong, China); and the Shandong Province Natural Science Foundation Grant BS2012YY030 (Qingdao, Shandong, China).

Disclosure: Y. Dai, None; X. Zhao, None; P. Chen, None; Y. Yu, None; Y. Wang, None; L. Xie, None

**References**


