A Novel Neuroprotective Small Molecule for Glial Cell Derived Neurotrophic Factor Induction and Photoreceptor Rescue

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Abstract

Purpose: Degenerative diseases of the retina, such as retinitis pigmentosa and age-related macular degeneration, are characterized by the irreversible loss of photoreceptors. Several growth factors, including glial cell derived neurotrophic factor (GDNF), have been shown to rescue retinal neurons. An alternative strategy to direct GDNF administration is its induction in host retina by small molecules. Here we studied the ability of a novel small molecule GSK812 to induce GDNF *in vitro/in vivo* and rescue photoreceptors.

Methods: GDNF induction *in vitro* was assessed in human ARPE-19, human retinal progenitor cells (RPCs) and mouse pluripotent cell-derived eyecups. For time course pharmacokinetic and GDNF induction studies in C57Bl/6 mice, GSK812 sustained release formulation was injected intravitreally. The same delivery approach was used in the rhodopsin knockout mice and Royal College of Surgeon (RCS) rats to assess long-term GDNF induction and photoreceptor rescue.

Results: The suspension provided sustained GSK812 delivery with 28 µg of drug remaining in the eye 2 weeks after a single injection. GSK812 suspension injection in C57Bl/6 mice resulted in significant upregulation of GDNF mRNA (>1.8-fold) and protein levels (>2.8-fold). Importantly, GSK812 treatment resulted in outer nuclear layer preservation in $\rho^{-/-}$ mice with a 2-fold difference in photoreceptor number. In the RCS rat, the GSK812 injection provided long-term rescue of photoreceptors and outer segments, accompanied by function preservation as well.

Conclusions: GSK812 is a potent neuroprotectant that can induce GDNF in normal and diseased retina. This induction results in photoreceptor rescue in 2 models of retinal degeneration.

Keywords: GDNF, neuroprotection, photoreceptors

Introduction

RETINAL DEGENERATIVE DISORDERS, such as retinitis pigmentosa and age-related macular degeneration, are characterized by irreversible loss of retinal neurons. Several therapeutic strategies are being explored to delay or substitute for such loss: neuroprotection,¹ gene correction,² cell replacement,³ optogenetics,⁴ and photosensitive chips.⁵ One benefit of neuroprotection is that it can be applied to many neurodegenerative diseases at early stages, while cell and tissue structure is still preserved.

One approach to neuroprotection is the use of growth factors and small molecules. Several of these compounds, such as ciliary neurotrophic factor (CNTF),⁶ brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF),⁷

pigment epithelium-derived factor (PEDF),⁸ glial cell derived neurotrophic factor (GDNF), vitamin A,⁹ docosahexaenoic acid,¹⁰ lutein,¹¹ valproic acid¹² and rasagiline,¹³ have been shown to rescue retinal structure in various animal models of retinal disease, although none of them has yet emerged as a viable therapeutic option. This is partially due to limited delivery options of growth factors: they can be administered as recombinant proteins, overexpressed in host cells by genetic vectors or delivered by transplantation of over-expressing cells. In addition to the high cost of these approaches, the ability to control the level of growth factor bioavailability and its delivery to targeted cells is limited.

Indirect neuroprotection is an emerging alternative. With a growing knowledge of growth factor induction and release, one can manipulate the production of growth factors

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in host cells using small-molecule triggers. This approach has been extensively studied for GDNF production due to its importance in central nervous system development and repair. For example, it has been shown that GDNF may be induced by various classes of molecules¹⁴ such as amitriptyline¹⁵ (tricyclic antidepressant) and valproic acid¹² (VPA, histone deacetylase inhibitor). Systemic treatment with VPA resulted in partial photoreceptor rescue in the rd10 mouse model of photoreceptor degeneration.¹² However, previous clinical studies showed no significant improvement in vision, following administration.¹⁶ Both of these small molecules are of particular interest due to their known pharmacokinetic properties after systemic application, however, the effective concentrations identified for these compounds *in vitro* are far above 10 μ M, which complicates potential clinical application.

In this study, we describe a novel small molecule GSK812, which was previously identified in a GDNF induction phenotypic screen in embryonic stem cell (ES)-derived astrocytes and rat C6 glioma cell line by GlaxoSmithKline. GSK812 has polypharmacology antagonizing multiple receptors, including dopamine 2 and dopamine 3 receptors and serotonin 2A, 2C, and 5HT6 receptors. The formula is shown in Supplementary Fig. S1 (Supplementary Data is available online at www.liebertpub.com/jop): 7-[4-(4-chloro-benzyloxy)-benzenesulfonyl]-8-methoxy-3-methyl-2,3,4,5-tetrahydro-1H-3-benzazepine. We hypothesized that this compound may induce GDNF in retinal tissue *in vitro* and *in vivo*. Also, this induction is sufficient to prevent/delay retinal degeneration in rhodopsin knockout mice and Royal College of Surgeon (RCS) rats. In our study, we tested GDNF induction in several cell lines and mouse iPS-derived eyecups and showed that this molecule induces GDNF at concentrations below 50 nM. We observed that a single intravitreal injection of GSK812 leads to GDNF upregulation at the mRNA and protein level in both healthy and diseased mouse retinas. A sustained released formulation (suspension) was developed to achieve sustained delivery after a single injection. With this approach, micrograms of the compound remained in the eye for at least 2 weeks after intravitreal delivery as shown by pharmacokinetic analysis. Importantly, a single intravitreal injection of this sustained release formulation of GSK812 led to sufficient GDNF induction to rescue photoreceptors in a severe retinal degeneration model, rhodopsin knockout mice ($\rho^{-/-}$).

Our results suggest that GSK812 is a potent neuroprotectant, with the effect at least partially mediated by GDNF induction. We believe that this is a viable therapeutic strategy for retinal degenerative disorders, and growth factor induction in the retina may pave the way for use of small molecules to treat diseases such as retinitis pigmentosa.

Methods

The experiments outlined below were designed to investigate the following: (1) GDNF induction by GSK812 in retinal-specific *in vitro* assays; (2) GDNF induction by GSK812 in normal and diseased mouse retina; and (3) long-term effect of GSK812 on the retina in a mouse model of retinitis pigmentosa.

The work with human fetal cells was reviewed by the MEEI/SERI Institutional Review Board and found to be an exemption. All studies were conducted in accordance with the GSK Policy on the Care, Welfare, and Treatment of

Laboratory Animals and were reviewed by the Institutional Animal Care and Use Committee either at GSK or by the ethical review process at the institution where the work was performed.

Data are presented as mean $\pm$ SEM. Statistical analysis was performed using a *t*-test ($P < 0.05$) with Bonferroni *post hoc* test or ANOVA ($P < 0.05$).

Formulation of GSK812 solutions and suspensions

For GSK812 solutions for *in vivo* experiments, the drug was dissolved in a vehicle consisting of 10 mM sodium phosphate buffer (pH 6.0), 50 mg/mL mannitol, 50 mg/mL Captisol at a final stock concentration of 1 mM. The stock solution was then filtered through 0.45- μ m filters and subsequently diluted with the vehicle into desired concentrations of 100, 30, and 10 μ M. For GSK812 suspensions (sustained release), the drug was suspended in a vehicle containing 4 mg/mL poloxamer 188, 20 mg/mL PEG 3350, and 45 mg/mL mannitol at 60 mg/mL by vortexing the drug/vehicle blend to form a whitish uniform suspension. The particle size of the suspension was measured to be D_{10} = 6.8 μ m; D_{50} = 14.2 μ m; and D_{90} = 26.5 μ m. The pH was also adjusted to 6.0.

For GSK812 solutions for *in vitro* experiments, the drug was dissolved in DMSO to make 10 μ M concentrations, followed by serial dilutions.

GDNF induction assays in vitro

GDNF induction *in vitro* was assessed using 3 retinal-specific cell systems: ARPE-19, as a substitute for human retinal pigment epithelium¹⁷; human retinal progenitor cells (hRPC),¹⁸ as a substitute for human retinal neurons/glia; and mouse eyecups, differentiated from induced pluripotent stem cells.¹⁹ To study GDNF induction, we plated thawed ARPE19 cells (passage 33; ATCC) or hRPC on fibronectin (Akron)-coated plates (Nunc) in a stimulation medium at a density of 10k cells/sq.cm. The flasks were incubated at standard conditions: 37°C, 5% CO₂, 100% humidity. Cells were plated 24 h before GSK812 treatment and were grown in UltraCULTURE medium (Lonza), supplemented with 1 $\times$ MEM NEAA (Gibco), 1 $\times$ L-glutamine (Gibco), 1 $\times$ antibiotic/antimycotic (Gibco), 2 ng/mL basic fibroblast growth factor (bFGF) (Peprotech), and 1 ng/mL epidermal growth factor (Peprotech). For quantitative polymerase chain reaction (qPCR) analysis, the experiments were performed in 35-mm Petri dishes in 2 mL of medium with GSK812 concentration of 100 nM (DMSO-based solution). Total mRNA was isolated at 0, 15, 30, 60, 120, 240 min poststimulation. For protein expression determination by ELISA, the experiments were performed in 96-well plates in 100 μ L of medium with each condition (concentrations ranging from 0 to 10 μ M) reproduced in 3 wells. For ELISA, plates were removed from the incubator 24 h poststimulation and freeze-thawed followed by CHAPS lysis buffer treatment for total mRNA extraction. The ELISA for GDNF was performed using the GDNF DuoSet kit (R&D systems) as per the manufacturer's instructions.

To study GDNF induction in retina-like tissue structures, mouse eyecups were differentiated from wild-type mouse-induced pluripotent stem cells according to the protocol described previously.²⁰ At day 26 of differentiation, differentiation was confirmed by Crx-expression, and eyecups were cut to

about 0.5 mm² in size and replated as a suspension in a 96-well format in Neurobasal media (Gibco), supplemented with 1×L-glutamine (Gibco) and 1×N₂ supplement (Gibco). Samples were treated 24 h later with GSK812 in concentrations ranging from 0 to 10 μM. The sample collection and ELISA were performed in the same way as for ARPE-19 and hRPC. All ELISA experiments were repeated twice.

Intravitreal injections

All animal procedures were performed under general (intraperitoneal ketamine/xylazine) and topical anesthesia with proparacaine drops (Alcon). Tropicamide (Alcon) was applied for pupil dilation. After injection, the eye was treated with an antibiotic ointment (bacitracin/neomycin/polymyxin). The same procedures were applied to control eyes. The test compound or vehicle was administered intravitreally using beveled borosilicate glass needle (~100 μm in diameter), connected to a Hamilton syringe through polyethylene tubing. During the injection, a corneal paracentesis was performed to reduce intraocular pressure and minimize the immediate drug reflux.

GDNF induction by GSK812 solution in wild-type and rhodopsin knockout mice

To study GDNF induction by GSK812 solution, 1 μL of 10 μM (*n*=5) or 100 μM (*n*=5) water-based GSK812 or 1 μL of vehicle (*n*=5) GSK812 was injected into 4-week-old wild-type (C57/Bl6; Jackson labs) and rhodopsin knockout²¹ mice (a gift from P. Humphries, colony at Schepens Eye Research Institute). Three hours after the injection, animals were euthanized using CO₂ and cervical dislocation. The treated and untreated eyes were enucleated and placed in 250 μL of chilled RNAlater buffer (Qiagen) and stored at 4°C. Later eyes were dissected and total neural retinas with retinal pigment epithelium were collected into 400 μL of lysis buffer (Qiagen RNeasy kit) with 5 mM beta-mercaptoethanol (Sigma). Total RNA was isolated using the RNeasy kit (Qiagen) and eluted with 100 μL of ultrapure water (Life technologies). All flow through from RNA isolation (~2.4 mL) was collected and mixed with 10 mL of ice-cold (-20°C) acetone for protein precipitation. The protein was precipitated at -20°C for 30 min and then pelleted at 3500 rpm for 20 min. Pellets were washed once with 100% ethanol and then reconstituted with 300 μL of RIPA buffer with 0.2 mM 4-benzenesulfonyl fluoride hydrochloride (Tocris).

mRNA content was estimated by qPCR. To prepare cDNA, we have used the Superscript III kit (Thermo Fisher) with oligo(dT) random primers, according to the manufacturer's instructions. We have loaded equal amount of total RNA for cDNA preparation. The qPCRs were run in 25 μL volume with Power SYBR Green PCR Master Mix (Thermo Fisher). The results were read by StepOne Real-Time PCR System. The results were normalized to GAPDH.

The protein content was assessed by western blotting in reducing conditions. Protein solution in lysis buffer was mixed with the loading buffer, boiled for 10 min, and then loaded on the acrylamide gel. After electrophoresis, the proteins were transferred to the polyvinylidene fluoride membrane. The membranes were blocked with 3% Bovine serum albumin for 1 h at room temperature and stained with anti-GDNF (Santa Cruz) or anti-βActin (Abcam) antibodies

overnight, at +4°C followed by staining with secondary horseradish peroxidase-conjugated antibodies (RD Systems). The intensity of the staining was determined using luminescent substrate (Thermo Fisher) and X-ray film (Denville).

Pharmacokinetic studies of GSK812 sustained release formulation (suspension)

To profile pharmacokinetics of GSK812 after a single intravitreal injection, 2 μL of GSK812 suspension (60 mg/mL) was administered intravitreally to 3-week-old wild-type (C57/Bl6) mice. For enucleation, animals were euthanized at several time points after injection: 1 h (*n*=5), 1 day (*n*=3), 2 days (*n*=3), 7 days (*n*=3), and 14 days (*n*=3). The eyes were enucleated and placed in chilled phosphate-buffered saline and kept on ice. The eyes were dissected within 30 min after enucleation: total retina and vitreous were collected into preweighed tubes, weighted, and snap-frozen. For GSK812 content in the eye analysis, retina and vitreous humor samples were diluted in 0.1% formic acid in 90:10 (v:v) acetonitrile:water before being analyzed for GSK812 using an analytical method based on protein precipitation, followed by high-performance liquid chromatography - mass spectrometry (HPLC/MS/MS) analysis. Using a 25 μL aliquot of sample, the lower limit of quantification for GSK812 was 1.00 ng/mL. The computer systems that were used on this study to acquire and quantify data included Analyst Version 1.6.1 and SMS2000 version 2.3.

GDNF induction by GSK812 sustained release formulation (suspension) in wild-type mice

To study GDNF induction by GSK812 suspension (60 mg/mL), 2 μL of suspension (*n*=35) or vehicle (*n*=5) was administered intravitreally to 3-week-old wild-type (C57/Bl6) mice. Animals were euthanized at several time points after injection for enucleation and RNA/protein analysis (*n*=5 for each time point, *n*=4 for 7 and 14 days): vehicle at 1 h, GSK812 at 1 h, 1 day, 2 days, 7 days, and 14 days. The treated and untreated eyes were enucleated and were collected for mRNA and protein expression as previously described.

Photoreceptor rescue by GSK812 suspension in rhodopsin knockout mice

To study photoreceptor rescue by GSK812 suspension (60 mg/mL), 2 μL of suspension (*n*=5) or vehicle (*n*=5) was administered intravitreally to 4-week-old rhodopsin knockout mice. Ten weeks after the injection, eyes were enucleated and fixed in Davidson solution for histological processing: paraffin embedding and sectioning. Six-micrometer-thick sections were cut in the area close to the optic nerve head (ONH) and stained with hematoxylin/eosin (H&E) or processed for immunohistochemical analysis (IHC).

H&E-stained sections were imaged (Olympus imaging system DP72) and the total photoreceptor number was estimated based on the nuclei count in the outer nuclear layer (ONL). Cells were counted in each 100 μm retina span starting from ONH on 3 sections for each eye (5 eyes per group). Total photoreceptor number per section was also calculated.

FIG. 1. GDNF induction *in vitro* in mouse eyecups differentiated from miPSc, ARPE-19, and hRPC by GSK812 solution. GSK812 induced GDNF protein in mouse retinal cells, differentiated from iPSc (a), hRPC (b), and ARPE-19 (c) in dose-dependent manner as measured by ELISA. Time course studies in ARPE19 (d) showed that GDNF mRNA peaks at 30 min postinduction. Data are shown as $M \pm SEM$. GDNF, glial cell derived neurotrophic factor; hRPC, human retinal progenitor cells; M, mean; miPSc, mouse induced pluripotent stem cells.

For immunohistochemistry, sections were deparaffinized, heated (95°C) in citrate buffer (pH 6.0) for 30 min, then blocked for 1 h, and stained with the antibodies for the following antigens: photoreceptors: opsin blue (Millipore), cone arrestin (Millipore), opsin red/green (Millipore), recoverin (Millipore); Muller glia and astrocytes: Lhx2 (Millipore), glutamate synthase (Abcam), glial fibrillar acidic protein (Millipore); bipolar cells: protein kinase C alpha (Santa Cruz); and GDNF (Santa Cruz). All primary antibodies were applied at 1:200 dilution overnight at +4°C, followed by staining with Cy3-conjugated secondary antibodies for 3 h at room temperature (Jackson ImmunoResearch).

Photoreceptor rescue by GSK812 suspension in RCS rats

To study photoreceptor rescue by GSK812 suspension (60 mg/mL), 2 μ L of suspension ($n=7$) or vehicle ($n=7$) was administered intravitreally to 3-week-old pigmented RCS rats. Fourteen weeks after the injection, optokinetic assessment of visual function and full-field photopic electroretinography (25 cd·s/m² flash; Espion, Diagnosys LLC) were performed. The eyes were enucleated and fixed in Davidson solution for histological processing: paraffin embedding and sectioning. Six-micrometer-thick sections were cut in the area close to the ONH and stained with hematoxylin/eosin (H&E) or processed for IHC. For IHC, sec-

tions were deparaffinized, heated (95°C) in citrate buffer (pH 6.0) for 30 min, and then blocked and stained with the antibodies for recoverin (Millipore).

Results

GDNF induction in vitro

Our goal was to determine if GDNF induction could be achieved by means of a small molecule, GSK812, in diseased retina. The first step was to determine if GSK812 could induce GDNF in several *in vitro* models of the retina: mouse eye cups, human retinal progenitor cells, and the ARPE-19 cell line. We observed significant dose-dependent GDNF induction by GSK812 in all cell culture systems tested. Mouse eyecups, differentiated from induced pluripotent cells, showed the highest sensitivity: the upregulation of GDNF protein was observed at 30 nM concentration of GSK812 (Fig. 1a). This system also showed the highest concentration of GDNF detected—up to 55 pg/mL. However, it was also characterized by a higher baseline level—around 20 pg/mL, compared to 5–10 pg/mL in adherent human cell lines tested. The hRPC (Fig. 1b) and ARPE-19 (Fig. 1c) upregulated GDNF after GSK 812 treatment at 1 and 3 μ M, respectively. The time course of GDNF induction (normalized by GAPDH) by GSK812 in ARPE-19 showed that GDNF mRNA peaked at 30 min after stimulation (1.8-fold increase)

FIG. 2. GDNF induction by GSK812 solution in wt and $\rho\text{-}^{-/-}$ mice. GDNF expression is increased in healthy wild-type retina on both transcript (a) and protein (b) levels 3 h after intravitreal injection of 10 and 100 μM GSK812 solution. In the diseased retina ($\rho\text{-}^{-/-}$), the induction of GDNF mRNA (c) is not statistically significant ($P=0.11$ and $P=0.15$ for t -test). However, the protein (d) is induced >2 fold by both concentrations ($P=0.01$ and $P=0.11$, t -test, respectively). Data is shown as $M \pm \text{SEM}$. wt, wild-type.

and then declined (Fig. 1d). These results indicate that GSK812 was able to induce GDNF in multiple *in vitro* models of the retina.

GDNF induction by GSK812 aqueous solution in wild-type and rhodopsin knockout mice

Since GSK812 was an inducer of GDNF expression in ARPE19 cells, hRPC, and mouse eye cups, we next tested to

see if GSK812 would induce GDNF mRNA and protein in wild-type and rhodopsin knockout ($\rho\text{-}^{-/-}$) mouse eyes using an aqueous solution of GSK812. We observed significant induction of GDNF by 10 and 100 μM GSK812 solution on mRNA in wild-type mice (Fig. 2a)— 1.3 ± 0.1 - and 1.9 ± 0.4 -fold compared to vehicle-treated control, respectively, and in rhodopsin knockout mice we have also observed a trend toward induction (Fig. 2c) at 10 μM concentration— 1.8 ± 0.4 -fold. Protein was upregulated in vehicle-treated

FIG. 3. Pharmacokinetic and efficacy time course studies in wt mice. Significant amount of the test compound remained in the eye for at least 2 weeks following single intravitreal injection of GSK812 suspension (a). The drug concentration in the vitreoretinal sample decreased from 21 to 5 μg per mg of tissue ($P=0.05$, ANOVA). This amount was sufficient to sustainably upregulate GDNF in the retina at mRNA ($P=0.01$, ANOVA) (b) and protein ($P<0.0001$, ANOVA) (c) levels. While mRNA induction remained at 1.2- to 1.8-fold increase versus vehicle, protein expression increased with time and reached 3.2-fold induction. Data are shown as $M \pm \text{SEM}$.

(1.5 ± 0.3 -fold) and GSK812-treated eyes (2.3 ± 0.4 -fold, both), compared to untreated control (Fig. 2b) in wild-type animals. In rhodopsin knockout mice, protein was upregulated in vehicle-treated (1.2 ± 0.2 -fold) and GSK812-treated eyes (2.8 ± 0.2 and 2.6 ± 0.5 for 10 and 100 μ M, respectively) (Fig. 2d). Therefore, we concluded that GSK812 was able to induce GDNF mRNA and protein in wild-type and rhodopsin knockout mice.

GSK812 concentration after suspension injection in wild-type mice

Our next aim was to determine if GSK812, when given as suspension formulation, would result in sustained release of GSK812 and therefore sustained induction of GDNF. To test if GSK812 drug substance would last over a period of time via a single intravitreal injection as a suspension, wild-type mice were injected and samples were collected at different time points over 14 days. We observed a decline in GSK812 depot size during the course of the study, but the drug was still present after 2 weeks. The GSK812 content in the collected vitreoretinal samples decreased from 21 ± 2 μ g (1 h) to 5 ± 1 μ g (2 weeks) per milligram of tissue. It corresponds to 80 ± 8 μ g at 1 h postinjection to 28 ± 3 μ g (14 days postinjection, Fig. 3a) of total GSK812 amount. The presence of GSK812 after 14 days led us to conclude that it may be possible to utilize this formulation to produce long-term induction of GDNF.

GDNF induction by GSK812 suspension in wild-type mice

The rationale for developing a suspension formulation of GSK812 was to determine if GDNF could be induced in the retina at a sustained, but modest level, by a small molecule. To answer this question, further characterization of the pharmacodynamics of the GSK812 slow release suspension was performed with 10 and 100 μ M GSK812 solutions. GSK812 suspension treatment showed GDNF induction (Fig. 3b, c), similar to 10 μ M GSK812 aqueous solution (Fig. 2a, b). The GDNF mRNA remained upregulated at all time points (1.4 ± 0.1 , 1.3 ± 0.1 , 1.2 ± 0.2 , 1.8 ± 0.4 , 1.8 ± 0.2

at 1 h, 1 day, 2 days, 7 days, and 14 days, respectively) (Fig. 3b). The protein level increased over time (0.9 ± 0.2 , 1.6 ± 0.3 , 2.4 ± 0.4 , 2.7 ± 0.3 , and 3.2 ± 0.3 at 1 h, 1 day, 2 days, 7 days, and 14 days, respectively) (Fig. 3c). The achieved level of GDNF induction by the sustained release formulation exceeded levels observed in previous studies with a single GSK812 injection.

Photoreceptor rescue by GSK812 suspension in rhodopsin knockout mice

We tested whether the GDNF induction achieved by GSK812 suspension could prevent the near complete loss of the photoreceptors in the rhodopsin knockout mouse. The rhodopsin knockout mouse has photoreceptor degeneration that models the human disease of retinitis pigmentosa. This animal model is characterized by moderate progression of photoreceptor loss, starting at 4 weeks of age with only a single layer of photoreceptors (predominantly cones) remaining by 15 weeks of age.²² It was hypothesized that if GSK812 suspension formulation was injected before the massive degeneration of the cones at week 3, photoreceptors may be maintained by the induction of GDNF through GSK812.

The treatment resulted in 2.1-fold increase in photoreceptor cell number pan-retinally compared to uninjected and vehicle (1210 vs. 516 vs. 508 total photoreceptor cells per section, respectively) with significant retinal rescue of the photoreceptor layer throughout all retinal regions (Fig. 4a, b).

We did not observe a significant difference in photoreceptor structure (outer segments), however, the signal was more intense in the treated group, perhaps due to higher cell number in total (Fig. 5). Muller glia (Lhx2, glial fibrillary acidic protein, glutamine synthetase) and bipolar cell protein kinase C alpha [PKC α] marker expression was similar between groups (Fig. 6). GDNF expression was confirmed in the ONL (Fig. 6).

Photoreceptor rescue by GSK812 suspension in RCS rats

Finally, we tested if we can achieve the photoreceptor rescue in another species model of retinal degeneration—pigmented RCS rats. These animals have mutation in

FIG. 4. Photoreceptor rescue in $\rho^{-/-}$ mice by GSK812 suspension. Single intravitreal injection of GSK812 suspension in 4-week-old $\rho^{-/-}$ mice was sufficient to provide long-term rescue of photoreceptors (10 weeks after treatment). The spider plot of photoreceptor count (a) showed pan-retinal preservation of photoreceptors in the treated group. Data are shown as $M \pm SEM$. We have observed a significant increase in ONL thickness, and no changes in the retinal pigment epithelium, inner nuclear or ganglion cell layers (b) at 10 weeks after the treatment. Scale bar 100 μ m. ONL, outer nuclear layer.

FIG. 5. Photoreceptor marker expression following GSK812 treatment. We have observed slightly increased intensity of recoverin (red) staining in the $\rho^{-/-}$ retina 10 weeks after GSK812 treatment compared to age-matched controls and vehicle-treated retina. It may be attributed to a higher cell number in ONL. We have not observed any difference in the expression of cone visual pigments (opsin blue and opsin red/green) or length of outer segments (white arrows). DAPI (blue) was used as a nuclear counterstain. Scale bar 100 μm .

MerTK gene, which affects photoreceptor outer segment phagocytosis by retinal pigment epithelium. As a result, the rods and cones in these animals undergo progressive degeneration starting at week 3. RCS rats have been widely used in studying the effect of neurotrophic factors on photoreceptor rescue.

In our study, we have observed a profound effect of a single GSK812 injection on retinal structure. Fourteen weeks after the intravitreal suspension injection, the outer nuclear layer of these animals contained up to 5 layers of photoreceptors (Fig. 7a) compared to 1 intermittent layer in nontreated and vehicle-treated control. These photoreceptors had outer segments and expressed recoverin (Fig. 7d) as shown by immunocytochemistry. We also observed single recoverin-positive cells in the outer nuclear layer of the untreated and sham-treated degenerated retinas.

This rescue of structure was sufficient to mediate partial preservation of visual function as detected by electroretinography (Fig. 7b): electroretinography b-wave amplitude was 23.1 ± 1.0 and $28.9 \pm 2.5 \mu\text{V}$ in vehicle-treated versus GSK812 suspension-treated eyes. The optokinetic recordings

demonstrated differences in highest setting (1 cycle per degree)— 6.0 ± 0.27 s vs. 8.2 ± 0.4 s in vehicle-treated versus GSK812 suspension-treated eyes, respectively (Fig. 7c).

Discussion

A neuroprotective strategy for treating retinal degenerative disorders is an attractive approach due to its potential to treat variants of retinitis pigmentosa as well as other neurodegenerative diseases of the eye. Although in reality this approach may only delay disease progression, a significant delay of photoreceptor degeneration in these diseases may in fact positively impact quality of life for many decades, and it is therefore an attractive strategy to further investigate. Moreover, its applications are extensive and in most of the cases do not require a full understanding of the genetic background of the disease.

Several approaches of neuroprotection have been tested with at least 6 classes of growth factors showing promise in various animal models of retinal degeneration and photoreceptor loss: CNTF,²³ NGF,²⁴ BDNF,²⁵ bFGF,²⁶ pigment

FIG. 6. The expression of glial and bipolar cell markers following GSK812 treatment. Immunostaining for GDNF (red) showed the predominant localization of the protein in the outer nuclear layer in all groups: untreated $\rho^{0/-}$ (14 weeks of age), vehicle-treated (10 weeks after the injection), and GSK812-treated retina (10 weeks after the injection). Glial activation marker Lhx2 and other Muller glia markers—GFAP and GS, as well as bipolar cell marker PKCα were expressed at similar levels in all groups. DAPI (blue) was used as a nuclear counterstain. Scale bar 100 μm . GFAP, glial fibrillary acidic protein; GS, glutamine synthetase; PKCα, protein kinase C alpha.

FIG. 7. Photoreceptor rescue in RCS rats by GSK812 suspension. Single intravitreal injection of GSK812 suspension in 3-week-old RCS rat was sufficient to provide long-term rescue of photoreceptors (14 weeks after treatment). We have observed significant increase in ONL thickness, and no changes in the retinal pigment epithelium, inner nuclear or ganglion cell layers (a) at 14 weeks after the treatment. Scale bar 100 μ m. This correlated with the improvement in function as observed by electroretinography (b) and optokinetic response (c). ERG data are presented as a b-wave amplitude ($n=7$). Optokinetic recordings are presented as time (s) spent in tracking the rotating drum with the respective eye during the 60-s recording interval ($n=7$). Data are shown as $M \pm SEM$. ERG, electroretinography; RCS, Royal College of Surgeon.

epithelium-derived growth factor,²⁶ and glial cell derived neurotrophic factor^{27–29} (GDNF). Among these, GDNF is of a particular interest due to its known broad spectrum of targets, including dopaminergic neurons, motor neurons of spinal cord, as well as retinal ganglion cells and photoreceptors of the eye. GDNF was first described in 1993 as a survival factor for midbrain dopaminergic neurons³⁰ and since then has been shown to promote development,³¹ synaptogenesis,³² and survival of a wide variety of neurons,¹⁴ including ganglion cells³³ and photoreceptors.³⁴ Also, its overexpression is not known to cause deleterious significant side effects, such as electroretinogram depression described for CNTF.³⁵

The mechanisms of GDNF delivery into the retina vary: it can be administered as recombinant protein in solution or in slow delivery systems, it can be constitutively overexpressed in host cells by adeno-associated viruses and plasmids, or overexpressed in transplanted cells, such as neural progenitors or Schwann cells (add refs to these studies). These approaches come with 2 major limitations: lack of control over the expression levels and the targeted delivery of GDNF to the specific location within the retina. The subretinal injection of adeno-associated virus (AAV) or cells seems to solve the latter issue, resulting in significant, although only local, rescue of photoreceptors. The indirect neuroprotective approach, when a growth factor is induced in host cells, may potentially abrogate these problems. Indeed, GDNF induction *in vitro* has been extensively studied with multiple triggers identified: bFGF, dopamine, amitriptyline, valproic acid, and rasagiline, among others.¹⁴ However, GDNF induction was observed at μM to mM concentrations of the stimulating compounds, which complicates the translation of these findings to clinical treatments.

In this study, we show that the novel dopamine and serotonin receptor antagonist GSK812 induces GDNF in retinal cells at concentrations as low as 30 nM. However, the actual mechanism is not known. The role of dopamine in GDNF induction has been previously studied in astrocytes and central nervous system neurons.¹⁴ We observe more than 2-fold induction in all 3 retinal systems tested: ARPE-19, hRPC, and mouse eyecups. Interestingly, the latter assay shows both the highest sensitivity (30 nM) and levels of GDNF (50 pg/mL) produced, which may be related to the natural tissue-like structure of the assay target. The induction of GDNF mRNA *in vitro* was significant, but lower compared to GDNF protein levels—which were only increased 1.8-fold. A similar ratio (low mRNA induction and more significant protein induction) was observed in our *in vivo* experiments in both wild-type and retinal degenerative (rhodopsin knockout) mice.

We also have not seen dose response in our *in vivo* studies with similar GDNF induction effect observed following the injection of 10 and 100 μM compound. This may be due to the saturation effect achieved at low concentration.

We developed a sustained release formulation via suspension of GSK812 particles that could be deposited in the vitreous and offer prolonged bioavailability of the compound. Pharmacokinetic studies showed that a single 2 μL injection provides a sufficient amount of the compound locally for at least 2 weeks—more than 28 μg remained in the eye after single injection. The modeling shows that drug may be present for up to 4 weeks, resulting in prolonged GDNF induction.

This 2-fold GDNF upregulation is enough to rescue photoreceptors in rhodopsin knockout mice (2.1-fold increase in photoreceptor number compared to vehicle treated) and RCS

rats to a level that was previously achieved with the transplantation of GDNF-overexpressing Schwann cells.³⁶ It is also comparable to the results of AAV-mediated GDNF overexpression in rat models, such as S334ter²⁸ and RCS.²⁹ Immunohistochemistry showed that GDNF is mostly localized in the outer retina (outer nuclear/outer segment layers). The rescue of photoreceptors was more significant in the rat model, which may be related to the rate of degeneration progression.

We achieved pan-retinal neuroprotection by a single intravitreal injection, which is a great advantage for clinical translation. We believe that this endogenous neuroprotection approach will be beneficial while applied during the onset of retinal degeneration.

Conclusions

We demonstrate GDNF induction *in vitro* and *in vivo* by a novel small molecule GSK812. We also have shown that a single intravitreal injection of sustained release GSK812 formulation is sufficient to rescue photoreceptors in 2 inherited models of retinal degeneration.

Implications

The indirect neuroprotection approach through GDNF induction is a promising therapeutic strategy, highly relevant for clinical translation.

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Author Disclosure Statement

No competing financial interests exist.

References

1. Pilli, S., Zawadzki, R.J., and Telander, D.G. The dose-dependent macular thickness changes assessed by fd-oct in patients with retinitis pigmentosa treated with ciliary neurotrophic factor. *Retina*. 34:1384–1390, 2014.
2. Maguire, A.M., High, K.A., Auricchio, A., et al. Age-dependent effects of RPE65 gene therapy for Leber's congenital amaurosis: a phase 1 dose-escalation trial. *Lancet*. 374:1597–1605, 2009.
3. Schwartz, S.D., Hubschman, J.-P., Heilwell, G., et al. Embryonic stem cell trials for macular degeneration: a preliminary report. *Lancet*. 379:713–720, 2012.
4. Lagali, P.S., Balya, D., Awatramani, G.B., et al. Light-activated channels targeted to ON bipolar cells restore visual function in retinal degeneration. *Nat. Neurosci.* 11:667–675, 2008.
5. da Cruz, L., Coley, B.F., Dorn, J., et al. The Argus II epiretinal prosthesis system allows letter and word reading and long-term function in patients with profound vision loss. *Br. J. Ophthalmol.* 97:632–636, 2013.

6. Chew, E.Y., Clemons, T.E., Peto, T., et al. Ciliary neurotrophic factor for macular telangiectasia type 2: results from a phase 1 safety trial. *Am. J. Ophthalmol.* 159:659–666.e1, 2015.
7. Huo, S.J., Li, Y.C., Xie, J., et al. Transplanted olfactory ensheathing cells reduce retinal degeneration in Royal College of Surgeons rats. *Curr. Eye Res.* 37:749–758, 2012.
8. Wang, Y., Subramanian, P., Shen, D., Tuo, J., Becerra, S.P., and Chan, C.-C. Pigment epithelium-derived factor reduces apoptosis and pro-inflammatory cytokine gene expression in a murine model of focal retinal degeneration. *ASN Neuro.* 5:e00126, 2013.
9. Radu, R.A., Yuan, Q., Hu, J., et al. Accelerated accumulation of lipofuscin pigments in the RPE of a mouse model for ABCA4-mediated retinal dystrophies following Vitamin A supplementation. *Invest. Ophthalmol. Vis. Sci.* 49:3821–3829, 2008.
10. Mirza, M., Volz, C., Karlstetter, M., et al. Progressive retinal degeneration and glial activation in the CLN6 (ncl) mouse model of neuronal ceroid lipofuscinosis: a beneficial effect of DHA and curcumin supplementation. *PLoS One.* 8:e75963, 2013.
11. Woo, T.T.Y., Li, S.-Y., Lai, W.W.K., Wong, D., and Lo, A.C.Y. Neuroprotective effects of lutein in a rat model of retinal detachment. *Graefes Arch. Clin. Exp. Ophthalmol.* 251:41–51, 2013.
12. Mitton, K.P., Guzman, A.E., Deshpande, M., et al. Different effects of valproic acid on photoreceptor loss in Rd1 and Rd10 retinal degeneration mice. *Mol. Vis.* 20:1527–1544, 2014.
13. Eigeldinger-Berthou, S., Meier, C., Zulliger, R., Lecaude, S., Enzmann, V., and Sarra, G.-M. Rasagiline interferes with neurodegeneration in the Prph2/rds mouse. *Retina.* 32:617–628, 2012.
14. Saavedra, A., Baltazar, G., and Duarte, E.P. Driving GDNF expression: the green and the red traffic lights. *Prog. Neurobiol.* 86:186–215, 2008.
15. Hisaoka, K., Nishida, A., Koda, T., et al. Antidepressant drug treatments induce glial cell line-derived neurotrophic factor (GDNF) synthesis and release in rat C6 glioblastoma cells. *J. Neurochem.* 79:25–34, 2001.
16. Bhalla, S., Joshi, D., Bhullar, S., Kasuga, D., Park, Y., and Kay, C.N. Long-term follow-up for efficacy and safety of treatment of retinitis pigmentosa with valproic acid. *Br. J. Ophthalmol.* 97:895–899, 2013.
17. Tong, J.-P., Lam, D.S.C., Chan, W.M., Choy, K.W., Chan, K.P., and Pang, C.P. Effects of triamcinolone on the expression of VEGF and PEDF in human retinal pigment epithelial and human umbilical vein endothelial cells. *Mol. Vis.* 12:1490–1495, 2006.
18. Baranov, P.Y., Tucker, B.A., and Young, M.J. Low-oxygen culture conditions extend the multipotent properties of human retinal progenitor cells. *Tissue Eng Part A.* 20:1465–1475, 2014.
19. Decembrini, S., Koch, U., Radtke, F., Moulin, A., and Arsenijevic, Y. Derivation of traceable and transplantable photoreceptors from mouse embryonic stem cells. *Stem Cell Reports.* 2:853–865, 2014.
20. Eiraku, M., and Sasai, Y. Mouse embryonic stem cell culture for generation of three-dimensional retinal and cortical tissues. *Nat. Protoc.* 7:69–79, 2012.
21. Humphries, M.M., Rancourt, D., Farrar, G.J., et al. Retinopathy induced in mice by targeted disruption of the rhodopsin gene. *Nat. Genet.* 15:216–219, 1997.
22. Wang, R., Jiang, C., Ma, J., and Young, M.J. Monitoring morphological changes in the retina of rhodopsin-/- mice with spectral domain optical coherence tomography. *Invest. Ophthalmol. Vis. Sci.* 53:3967–3972, 2012.
23. Wen, R., Tao, W., Li, Y., and Sieving, P.A. CNTF and retina. *Prog. Retin. Eye Res.* 31:136–151, 2012.
24. Sun, X., Xu, X., Wang, F., et al. Effects of nerve growth factor for retinal cell survival in experimental retinal detachment. *Curr. Eye Res.* 32:765–772, 2007.
25. Caffé, A.R., Söderpalm, A.K., Holmqvist, I., and van Veen, T. A combination of CNTF and BDNF rescues rd photoreceptors but changes rod differentiation in the presence of RPE in retinal explants. *Invest. Ophthalmol. Vis. Sci.* 42:275–282, 2001.
26. LaVail, M.M., Unoki, K., Yasumura, D., Matthes, M.T., Yancopoulos, G.D., and Steinberg, R.H. Multiple growth factors, cytokines, and neurotrophins rescue photoreceptors from the damaging effects of constant light. *Proc. Natl. Acad. Sci. U. S. A.* 89:11249–11253, 1992.
27. Hauck, S.M., Kinkl, N., Deeg, C.A., Swiatek-de Lange, M., Schöffmann, S., and Ueffing, M. GDNF family ligands trigger indirect neuroprotective signaling in retinal glial cells. *Mol. Cell Biol.* 26:2746–2757, 2006.
28. Dalkara, D., Kolstad, K.D., Guerin, K.I., et al. AAV mediated GDNF secretion from retinal glia slows down retinal degeneration in a rat model of retinitis pigmentosa. *Mol. Ther.* 19:1602–1608, 2011.
29. Touchard, E., Heiduschka, P., Berdugo, M., et al. Non-viral gene therapy for GDNF production in RCS rat: the crucial role of the plasmid dose. *Gene Ther.* 19:886–898, 2012.
30. Lin, L.F., Doherty, D.H., Lile, J.D., Bektesh, S., and Collins, F. GDNF: a glial cell line-derived neurotrophic factor for mid-brain dopaminergic neurons. *Science.* 260:1130–1132, 1993.
31. Rothermel, A., and Layer, P.G. GDNF regulates chicken rod photoreceptor development and survival in reaggregated histotypic retinal spheres. *Invest. Ophthalmol. Vis. Sci.* 44:2221–2228, 2003.
32. Ledda, F., Paratcha, G., Sandoval-Guzmán, T., and Ibáñez, C.F. GDNF and GFRalpha1 promote formation of neuronal synapses by ligand-induced cell adhesion. *Nat. Neurosci.* 10:293–300, 2007.
33. Kyhn, M.V., Klassen, H., Johansson, U.E., et al. Delayed administration of glial cell line-derived neurotrophic factor (GDNF) protects retinal ganglion cells in a pig model of acute retinal ischemia. *Exp. Eye Res.* 89:1012–1020, 2009.
34. Politi, L.E., Rotstein, N.P., and Carri, N.G. Effect of GDNF on neuroblast proliferation and photoreceptor survival: additive protection with docosahexaenoic acid. *Invest. Ophthalmol. Vis. Sci.* 42:3008–3015, 2001.
35. McGill, T.J., Prusky, G.T., Douglas, R.M., et al. Intraocular CNTF reduces vision in normal rats in a dose-dependent manner. *Invest. Ophthalmol. Vis. Sci.* 48:5756–5766, 2007.
36. Keegan, D.J., Kenna, P., Humphries, M.M., et al. Transplantation of syngeneic Schwann cells to the retina of the rhodopsin knockout (rho-/-) mouse. *Invest. Ophthalmol. Vis. Sci.* 44:3526–3532, 2003.

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