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Retinal Expression of *Fgf2* in RCS Rats with Subretinal Microphotodiode Array

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Abstract

Purpose—Test the hypothesis that subretinal electrical stimulation from a microphotodiode array (MPA) exerts a neuroprotective effect in RCS rats through the induction of growth factors.

Methods—RCS rats were divided into four groups at P21 in which one eye per rat received treatment: (**A**) active MPA, (**M**) minimally-active MPA, (**S**) sham surgery, or (**C**) no surgery and the opposite eye was unoperated. Dark- and light-adapted ERGs were recorded one week after surgery. A second set of **A**-, **M**-, and **C**-treated RCS rats had weekly ERG recordings for 4 weeks. Real-time RT-PCR was used to measure relative expression of mRNAs (*Bdnf*, *Fgf2*, *Fgf1*, *Cntf*, *Gdnf*, and *Igfl*) in retina samples collected 2 days after the final ERG.

Results—One week after surgery, there was a slight difference in dark-adapted ERG b-wave at the brightest flash intensity. Mean retinal *Fgf2* expression in the treated eye relative to the opposite eye was greatest for the **A** group (4.67 $\pm$ 0.72) compared to the **M** group (2.80 $\pm$ 0.45, $p=0.0501$), **S** group (2.03 $\pm$ 0.45, $p<0.01$), and **C** group (1.30 $\pm$ 0.22, $p<0.001$). No significant change was detected for *Bdnf*, *Cntf*, *Fgf1*, *Gdnf*, and *Igfl*. Four weeks after surgery, the **A** group had significantly larger dark- and light-adapted ERG b-waves compared to the **M** and **C** groups ($p<0.01$). Simultaneously, mean relative *Fgf2* expression was again greatest for the **A** group (3.28 $\pm$ 0.61) compared to the **M** (1.28 $\pm$ 0.32, $p<0.05$) and **C** groups (1.05 $\pm$ 0.04, $p<0.05$).

Conclusion—The results show subretinal implantation of an MPA induces selective expression of *Fgf2* above that expected from a retina-piercing injury. Preservation of ERG b-wave amplitude 4 weeks after implantation is accompanied by elevated *Fgf2* expression. These results suggest that *Fgf2* may play a role in the neuroprotection provided by subretinal electrical stimulation.

Introduction

Retinal degenerations such as macular degeneration and retinitis pigmentosa are a significant health concern. Retinitis pigmentosa (RP) is found approximately once in 3500 individuals.¹ As of 2003, age-related macular degeneration (AMD) affected an estimated 20-25 million people worldwide, and the incidence of AMD is projected to triple in 30 to 40 years.² A multitude of mutations and risk factors underlie the mechanisms of RP³ and AMD,⁴⁻⁷ however all forms of these retinal degenerations result in irreversible photoreceptor cell death and thus visual impairment or blindness.⁸

For RP and AMD patients, neuroprotective strategies aim to prevent or delay irreversible photoreceptor death and thereby prolong useful vision. A variety of neuroprotective approaches including growth factor administration,⁹ antioxidant combination therapy,^{10, 11} steroids,¹² bile acids,¹³ and calcium channel blockers¹⁴ have been shown to extend neuroprotection to photoreceptors in retinal degeneration animal models. Administering growth factors to eyes of such animal models through direct injection,¹⁵ encapsulated, engineered ciliary neurotrophic growth factor (CNTF)-secreting cells,¹⁶ or transfection with a growth factor encoding vector¹⁷ is well-documented to protect the retina from some diseases or injury. Likewise, inducing expression of endogenous growth factor genes through trauma to the eye¹⁸ also provides neuroprotection in the retina of rodent retinal degeneration models.

Electrical stimulation may also be capable of exerting a neuroprotective effect on retinal neurons. RP patients who, as part of a clinical trial, received a microphotodiode array implanted into the subretinal space of one eye displayed measurable increases in visual acuity and color vision (Chow A, et al. *IOVS* 2005;46:ARVO E-Abstract 1140). Eyes of Royal College of Surgeons rats (RCS) implanted with subretinal microphotodiode arrays (MPAs) similar to those used in human trials showed a delay in the loss of photoreceptor function as determined by electroretinogram (ERG) b-wave amplitude and higher photoreceptor counts near the implantation site versus control eyes.¹⁹ RCS rats receiving electrical stimulation transcorneally also showed a delay in photoreceptor death, preservation of retinal thickness, and preservation of retinal function.²⁰ Lastly, in rats with a transected optic nerve, transcorneal electrical stimulation spared axotomized ganglion cells.²¹

In non-retinal neurons, electrical stimulation has been associated with neuroprotection. For example, electrical stimulation of spiral ganglion cells following a deafening stress²² and the cerebellar fastigial nucleus prior to mid cerebral artery occlusion²³ resulted in prolonged neuron survival. Electrical stimulation has been associated with neuroprotection of dopaminergic neurons in a rat model of Parkinson's disease.²⁴

Electrical stimulation has been shown to induce neuronal expression of neuroprotective growth factors in culture²⁵⁻²⁷ and in vivo.²⁸⁻³⁰ Some of these studies also showed that growth factor induction in response to electrical stimulation was associated with neuroprotective effects.^{20, 26-29} Furthermore, a causal relationship between electrical stimulation, *Igf1* induction, and neuroprotection of axotomized ganglion cells was established when ganglion cell rescue by transcorneal electrical stimulation was abrogated by an IGF1 receptor antagonist.³¹ Thus, it seems plausible that induction of a neuroprotective growth factor(s) is responsible, at least in part, for preservation of retinal function and photoreceptors in RCS rats that received subretinal electrical stimulation.¹⁹

We hypothesized that neuroprotection of the RCS rat retina in response to subretinal electrical stimulation³² is due to heightened activity of neuronal survival pathways that are activated by growth factors. We therefore predicted that transcript levels of neuroprotective growth factors would be elevated in RCS rats that received subretinal electrical stimulation from MPAs. Here we report expression of several growth factors (brain-derived neurotrophic factor (*Bdnf*), ciliary neurotrophic factor (*Cntf*), acidic fibroblast growth factor (*Fgf1*), basic fibroblast growth factor (*Fgf2*), glial-derived neurotrophic factor (*Gdnf*), and insulin-like growth factor (*Igf1*) in RCS rats that received either subretinal electrical stimulation from an active MPA (**A**), a minimally-active MPA (**M**), a sham surgery (**S**), or no treatment control (**C**). One week after implantation surgery, *Fgf2* expression was selectively induced compared to the other growth factors, with an increasing gradient of expression from the **C** group, to the **S** group, to the **M** group, to the highest expression in the **A** group. Four weeks after surgery, *Fgf2* expression was still greatest in the **A** group. Additionally, at four weeks after surgery, ERG b-wave amplitudes were significantly larger in the **A** group compared to the other treatment groups. These results

suggest that *Fgf2* induction may mediate neuroprotective effects from subretinal electrical stimulation.

Methods

Animals, Surgical Procedures, and Experimental Design

The dystrophic RCS rat retinal degeneration model, originally obtained from Dr. Matthew LaVail, Ph.D. of the University of California at San Francisco, was used in this study. The mutation was bred onto the Long Evans background, and thus pigmented animals were used throughout this study. In the dystrophic RCS rat, both copies of the receptor tyrosine kinase gene, *Mertk*, are mutated, resulting in a premature stop codon and improper protein synthesis.³³ The lack of the MERTK enzyme prohibits phagocytosis of photoreceptor outer segments³⁴ and is believed to be the cause of the photoreceptor degeneration, which begins at about postnatal day 12 (P12) and is complete by about P95.³⁵ Animals were reared and maintained throughout the study on a 12 hours light (~100 lux), 12 hours dark schedule, and provided food and water *ad libitum*.

Electrical stimulation was provided by a subretinally-implanted MPA that has been described previously.^{36, 37} To control for potential neuroprotective effects from injury¹⁸ or light exposure³⁸, animals were divided into 4 treatment groups: non-surgical control (C, n = 11), sham surgery (S, n = 9), minimally-active MPA implantation (M, n = 10), and active MPA implantation (A, n = 19). At P21, surgeries were performed as described previously.³⁹ For A and M treatments, implants remained in the subretinal space for the duration of the study, while the S treatment involved inserting the MPA into the subretinal space and immediately removing it. One week (7 days) after implantation, 8, 9, 6, and 14 animals in the C, S, M and A groups, respectively, were subjected to ERG and then sacrificed 2 days later (9 days post-implantation). All other animals were followed with weekly ERGs to 4 weeks (28 days) after implantation surgery and then sacrificed 2 days after the final ERG (30 days post-implantation). Animal sacrifice was performed with an overdose of pentobarbital. All experiments involving animals were reviewed and approved by the Institutional Animal Care and Use Committee of the Atlanta VA and were in full compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Visual Research.

Electroretinographic Assessment of Retinal Function

ERGs were recorded under both dark- and light-adapted conditions, as previously described.¹⁹ Briefly, rats were dark-adapted overnight and prepared under dim red light. After sedation with ketamine (60 mg/kg) and xylazine (7.5 mg/kg), the cornea was anesthetized (1.0% tetracaine) and dilating drops (1.0 % tropicamide, 1% cyclopentolate) instilled in both eyes. With body temperature maintained at 37°C on a homeothermic heating pad, the electrical response of the retina was recorded with a nylon fiber embedded with silver particles placed across the surface of the cornea⁴⁰ and wetted with 1% methylcellulose. Responses were referenced and grounded by needle electrodes placed in the cheek and tail, respectively. A series of full-field flash stimuli was presented by a Ganzfeld dome under dark-adapted conditions (−3.4 to 2.1 log cd-s/m²). Interflash interval increased with flash intensity from 10 to 60 seconds. After 10 minutes of light-adaptation (30 cd/m²) a second series of flash stimuli (−0.82 to 2.1 log cd-s/m²) were presented in the presence of the adapting light at 3 Hz to isolate cone responses. Acquired responses were filtered (1 to 1500 Hz) and stored on a commercial ERG system (UTAS 3000, LKC Technologies, Gaithersburg, MD). ERG recordings performed 1 week consisted of five dark-adapted and 4 light-adapted steps. Weekly ERG recordings of rats followed to 4 weeks after surgery served to measure retinal function as well as provide increased electrical output from the MPA,³² and thus consisted of 10 dark-adapted and 7 light-adapted steps.

Implant Description and Characterization

Implants were fabricated essentially as described previously.³⁷ Briefly, active MPAs were made from p-type starting material, and contained an array of pixels consisting of n-type doped regions on the front (pixelated) surface. In contrast, the minimally-active MPAs were made without the intentional introduction of any additional dopants, so the minimally-active MPAs were not expected to have any semiconductor junctions or to exhibit photovoltaic behavior. The minimally-active MPAs were manufactured from p-type monocrystalline silicon wafers with a resistivity of 50 ohm cm. The starting material was polished on both sides and selectively thinned (by wet etching in KOH) to form membranes 23 μm thick. A dielectric layer of silicon dioxide was formed by dry thermal oxidation of the silicon. The resulting dielectric layer is 1200 ± 50 Å thick, and covers both sides of each MPA. Finally, implantable discs were cut from the thin portions of the wafer by etching annuli completely through the 23- μm membranes using a reactive ion etch process. Finally, no metal electrodes were applied to the minimally-active MPAs, so each face consists of a smooth, continuous layer of silicon dioxide.

The photovoltaic charge-injection characteristics ($\mu\text{A}/\text{cm}^2/\text{W}$) of active and minimally-active MPAs were measured in a PBS electrolyte under a wide range of illumination conditions using an infrared source (870 nm). A pulse (1 ms) of light ($10.5 \text{ mW}/\text{cm}^2$) was shown on the photoactive side of the active MPAs or either side of the minimally-active MPAs as current was monitored in the PBS electrolyte at one measurement every 10^{-4} second.

Growth Factor Expression Analysis

Before enucleation, the superior portion of each eye was marked with a pen to maintain orientation. Eyes were enucleated, cornea and lens were removed, and four radial cuts were made to flatten the eyecup. A 2.3 mm diameter trephine was used to dissect the portion of retina overlying the implant (**A** and **M** groups), the incision site (**S** group) or an equivalent position (**C** group).

For each tissue sample, reverse transcription (RT) followed by real-time polymerase chain reaction (PCR) was performed. Samples were homogenized in 100 μL of TRIzol® (Invitrogen, Carlsbad, CA, USA) at 30,000 rpm for 2×15 s using a *TH* homogenizer equipped with a 5 mm stainless steel probe (Omni International, Marietta, GA). Total RNA isolation was performed according to the manufacturer's instructions. Resulting RNA was dissolved in 100 μL of RNase-free dH_2O and further purified and concentrated to a final volume of 12 μL with an RNeasy minelute column (Qiagen Inc., Valencia CA). The RNA yields, as determined by measuring $A_{260 \text{ nm}}$, varied between ~0.5 and 2.0 μg per sample, and the ratios of $A_{260 \text{ nm}}$ to $A_{280 \text{ nm}}$ varied between 1.7 and 2.0.

Optimal RNA and cDNA dilutions were determined by reverse transcription and subsequent PCRs on a 7 step, two fold dilution series of RNA (12.5, 25, 50, 100, 200, 400, and 800 ng RNA/RT) and inspecting for single cycle shifts in cycle threshold (Ct) between dilutions. Using more than 200 ng of RNA per RT decreased 18S Ct differences, implying incomplete reverse transcription of 18S at high RNA amounts, and using less than 50 ng of RNA per RT increased Ct for genes of interest. For each RNA sample, 100 ng of total RNA was reverse transcribed with Quantitect Reverse Transcriptase in a final 20 μL volume according to manufacturer's instructions (Qiagen Inc., Valencia CA). The resulting cDNA was diluted 20 fold, and 5 μL of diluted cDNA (equivalent of 1.25 ng RNA/PCR) was used in a real-time PCR reaction with 100 nM (*Fgf1* and *Gdnf*) or 200 nM (*18S*, *Bdnf*, *Cntf*, *Fgf2*, *Igf1*) each of a primer pair (*18S* F = 5'-GTTGGTTTTTCGGAAGTGAAGGC-3', *18S* R = 5'-GTCCGCATCGTTTATGGTCG-3', *Bdnf* F = 5'-AACCAGAAAAAGCACCAAAA-3', *Bdnf* R = 5'-CTTGTGCTGAATGGACAGAA-3', *Cntf* F = 5'-GGACCTCTGTAGCCGTTCTA-3', *Cntf* R = 5'-TCATCTCACTCCAACGATCA-3', *Fgf1* F = 5'-AACCCAACTGCTCTACTGC-3',

Fgf1 R = 5'- GAGCCGTATAAAAGCCCTTC-3', *Fgf2* F = 5'- GCGGCTCTACTGCAAGA-3', *Fgf2* R = 5'-CGTCCATCTTCCTTCATAGC-3', *Gdnf* F = 5'- CCATGTTCTAGCCACTCTG-3', *Gdnf* R = 5'- AGGCTGAAGTTGGTTTCCTT-3', *Igf1* F = 5'- TGGACGCTCTTCAGTTCGTG-3', *Igf1* R = 5'-GTTTCCTGCACTTCCTCTAC-3') 12.5 µL of 2X iQ SYBR Green Supermix (BioRad, Hercules, CA, USA), and nuclease-free dH₂O to 25 µL. Primers were designed using Primer3.⁴¹ The cDNA was amplified in 96-well plates on the MyIQ thermalcycler (BioRad, Hercules, CA, USA). Conditions were: 95°C, 3 min; 40 cycles of (94°C, 30 s denaturation; 55°C, 30 s annealing; 72°C, 30 s extension); 72°C, 7 min, with fluorescence recorded at the end of each 72°C extension. Melt analysis conditions were: heating from 55°C to 95°C in 0.5°C increments, 15 s per increment, with fluorescence recorded at each increment. The Ct of each reaction was determined from the BioRad iCycler's PCR baseline subtracted and curve fit option. Three real-time PCRs were done for each cDNA sample - primer pair combination, and the average Ct was calculated. Taking 18S as the endogenous control, growth factor transcript abundance (expression) relative to control eyes or the opposite eye was calculated from the average PCR cycle thresholds using the $2^{-\Delta\Delta C_t}$ method after Livak and Schmittgen (2001).⁴² The expression ratio was computed to minimize animal-to-animal variability in gene expression.

Statistical analyses

For replicate analyses of treatment groups, the mean and standard error of the mean was calculated. Preliminary growth factor expression data was analyzed for significance with Student's t-test. One-way (*Fgf2* expression) and two-way (ERG a- and b-wave amplitudes) analysis of variance tests were performed to determine if there were significant differences within all treatment groups, and the Holm-Sidak post-hoc test was applied to examine significant differences between two treatment groups at $\alpha = 0.05$ using SigmaStat® 3.5 statistical analysis software (Systat Software, Inc. Point Richmond, CA).

Results

Implant Characterization

The minimally-active MPAs exhibit a photovoltaic effect, suggesting that a photoactive junction was inadvertently formed during their manufacture. At all illumination levels, however, the active MPAs deliver more charge than the minimally-active MPAs. Figure 1 shows that the active MPA delivers greater than 100 times more stimulus current than the minimally-active MPA in response to illumination of ~10 mW/cm² of near IR (870 nm) light. At this wavelength, the MPA has a responsivity of 0.345 A/W (see Figure 1 in ⁴³) and will respond in a linear fashion to increasing light intensity. Thus, the difference is less pronounced at lower illumination levels, and more pronounced at greater illumination levels, since the charge capacity of the iridium oxide electrodes on the active MPAs far exceeds the small capacitive coupling available to the minimally-active MPAs. Because lighting conditions used during the study ranged from darkness to the brightest ERG flash (2.1 log cd s/m²) with the large fluctuations between these extremes, it is estimated that retinas in the **A** group experienced >100 times more current than retinas in the **M** group.

Electroretinographic assessment of retinal function

Dark-adapted responses—Representative dark-adapted ERGs from animals at 1 and 4 weeks post-surgery are shown in Figure 2. Figure 2A shows the typical ERG waveform to a bright flash (2.1 log cd s/m²) from a representative RCS rat in each treatment group at 28 days of age. To this flash intensity, all treatment groups had a prominent a- and b-wave with oscillatory potentials present on the leading edge of the b-wave. The negative spike at 0.05 msec was due to the electrical activity of the MPA or M-device at light onset and can be seen in each waveform from the **A** or **M** group. Figure 2C shows the average intensity response

functions for the a- and b-wave at 1 week post-implantation from each treatment group. There were no significant differences in the a-wave amplitude. However, the b-wave amplitude was significantly larger in the **A** and **S** groups versus the **M** and **C** groups at the brightest flash intensity (Two-way repeated ANOVA, $p = 0.02$).

After 4 weeks of treatment by the MPA device, there was a significant difference in the overall waveform amplitude of the **A** group compared to **C** and **M** groups, as shown in Figure 2B. The representative waveforms in Figure 2B show smaller waveforms compared to the 1-week recordings (Figure 2A), reflecting the progressing photoreceptor degeneration in the RCS rats. Figure 2D shows the average a- and b-wave amplitudes across intensity. The **A** group had a significantly larger b-wave in response to intensities greater than $-0.6 \log \text{ cd s/m}^2$ (two-way ANOVA, $p = 0.003$). No significant differences in a-wave amplitude were found between treatment groups.

Light-adapted responses—Figure 3 shows cone-isolating, light-adapted responses recorded 1 and 4 weeks after implantation. Note that the loss of cone function in the progressive photoreceptor degeneration can be clearly seen by the decreasing amplitude responses between 1 and 4 weeks (28 and 49 days of age, respectively). Figure 3A and 3C illustrate no significant differences in the light-adapted waveform or average b-wave amplitude, respectively, between treatment groups at one week after treatment. However, by 4 weeks after treatment, the **A** group had significantly larger responses as shown by the representative waveforms in Figure 3B. The b-wave amplitude in the **A** group was significantly larger than the **C** and **M** groups at intensities greater than $0.39 \log \text{ cd s/m}^2$ (two-way ANOVA, $p = 0.003$; Figure 3D).

Growth Factor Expression Analyses by RT-PCR

Nine days after surgery, expression analyses by RT-PCR of *Cntf*, *Fgf1*, *Fgf2*, *Gdnf*, *Igf1* and *Bdnf* (not shown) showed a consistent, large, and significant elevation of *Fgf2* expression in the **A** group compared to all other treatment groups, whereas little-to- no change across treatment groups was observed for the other genes (Figure 4). Additional surgeries and nine-day analyses of the *Fgf2* expression ratio again showed significantly elevated *Fgf2* expression in the **A** group (Figure 5A), but also revealed an increase as treatment progressed from control to sham to minimally-active implantation to active implantation (**C**: 1.30 ± 0.22 , **S**: 2.03 ± 0.45 , **M**: 2.80 ± 0.45 , and **A**: 4.67 ± 0.72 , $F_{3,36} = 6.67$, $P = 0.001$). Post-hoc Holm-Sidak analysis revealed significant differences between the **A** and **S** groups ($p < 0.01$) and the **A** and **C** groups ($p < 0.001$), whereas $p = 0.0501$ for the **A** to **M** group comparison.

Thirty days after surgery, *Fgf2* expression was still elevated in the **A** group (3.24 ± 0.61) compared to the other treatment groups: **M**, 1.28 ± 0.32 ; **C**, 1.05 ± 0.04 ($p < 0.05$, post-hoc Holm-Sidak) (Figure 5B). Over time, the *Fgf2* expression in the **A** group trended lower by 30 days after surgery, but was not significantly different from the 9-day level ($p = 0.076$).

Discussion

Based on known neuroprotective effects of growth factors in various rodent models of retinal degenerative diseases,⁹ induction of growth factors in applications of neuronal electrical stimulation,²⁶⁻²⁹ and previous results that indicated some degree of neuroprotection in the retina was provided by retinal electrical stimulation, (Chow A, et al. *IOVS* 2005;46:ARVO E-Abstract 1140)^{19, 20, 28, 44} we hypothesized that electrical stimulation effects neuroprotective pathways via induction of known neuroprotective growth factors. The present results indeed show an increased expression of *Fgf2* with MPA implantation. As shown previously,¹⁹ MPA implantation in the RCS rat had a positive effect on retinal function 4 weeks after implantation (ERG). Because the active MPA is known to produce about one hundred times more electric current than the minimally active implant in response to light, it is inferred that amount of

subretinal current was the only difference between rats implanted with the active MPA and rats implanted with the minimally active device. Consequently, the present data imply that subretinal electrical stimulation from the MPA provides neuroprotection to the RCS rat retina concomitant to *Fgf2* induction.

In this study, we began growth factor expression analyses 9 days after MPA implantation even though significant neuroprotection (preservation of maximum ERG b-wave responses and photoreceptor nuclei) was first noted 4 and 6 weeks post-implantation for the **A** group compared to all other groups.¹⁹ This was based on the expectation that effects on gene expression would be detectable soon after implantation, in advance of any detectable neuroprotection. For analyzing gene expression, we chose to use RT-PCR for its sensitivity and simplicity. We sampled a 2.3 mm diameter piece of retinal tissue centered on the 1 mm diameter implant (or an equivalent location in the non-implanted eyes) as opposed to sampling the entire retina to optimize detecting any changes in gene expression in retina overlying the implant. Expression analyses at 1 week showed that mean *Fgf2* transcript abundance increased as treatments progressed from no injury (**C**), to injury (**S**), to injury plus chronic foreign body plus low level electrical stimulation (**M**), to injury plus chronic foreign body plus higher level electrical stimulation (**A**) (Figure 4), whereas little change in mean expression was noted for *Fgf1*, *Cntf*, *Gdnf*, *Igf1*, and *Bdnf*. In addition, *Fgf2* expression at 4 weeks remained elevated in the **A** group. These data suggest that subretinal electrical stimulation specifically initiated and sustained increased *Fgf2* gene expression.

Fgf2 induction from acute injury and chronic electrical stimulation

As a response to injury alone, *Fgf2* induction was expected and observed. The present results show about a two-fold increase in mean *Fgf2* expression in the **S** group compared to the **C** group. According to published work,⁴⁵ in which the effect of a retina-piercing injury on growth factor expression was analyzed, detectable *Fgf2* induction might be expected from a retina-piercing injury in a 21 day-old rat with sampling done 9 days after injury, as shown here (Figures 4-5A). Similarly, it is not surprising that induction of *Cntf* and *Igf1* were not observed with injury at this early age given the small amount of induction (*Cntf*) and decreased expression (*Igf1*) of these genes seen in the injury response studies.^{45, 46}

Fgf2 expression was induced in electrically-stimulated retinas beyond injury-induced levels 1 week post-implantation. The **A** group showed a significant increase in mean *Fgf2* expression (4.7 ± 0.72) compared to the **S** and **C** groups (2.03 ± 0.45 and 1.3 ± 0.22) and a noticeable increase in *Fgf2* levels when compared to the **M** group (2.80 ± 0.45 , $p = 0.0501$). These data may reflect that chronic implantation of a foreign body into the subretinal space, electrical stimulation, or both, induced *Fgf2* beyond sham surgery. If electrical stimulation contributes to *Fgf2* induction in a dose dependent manner, it might be expected that *Fgf2* expression in the **M** group would be intermediate between the **S** and **A** groups, since the **M** group experienced some degree of electrical stimulation, albeit much less than the **A** group. Indeed, the 9-day data could be interpreted as showing a dose-response relationship between the charge delivered to the retina and *Fgf2* induction. Data from 30 days after surgery, however, suggests that electrical stimulation is more important for *Fgf2* induction than chronic implantation of the MPA since *Fgf2* remains elevated in the **A** group, whereas the **M** group is nearly indistinguishable from the **C** group. Thus it appears there is an initial burst of *Fgf2* induction immediately upon implantation that could be related to both implantation and electrical stimulation, and continued electrical stimulation from the active MPA maintains the induction to at least 30 days after implantation.

The source and mechanism of *Fgf2* induction in response to subretinal electrical stimulation are not known. However, rat Müller cells are known to be a source of FGF2 in response to various stimuli^{46, 47} including an alternating current (1 ms pulses, 20 Hz, 1, 5, and 10 mA, 20

mm between electrodes) that is applied to cultured rat Müller cells.²⁷ Thus, the Müller cell seems a likely site of *Fgf2* induction in response to electrical stimulation.

The mechanism of induction is less clear. Pharmacologically blocking L-type voltage-dependent calcium channels (VDCCs) has been shown to induce *Fgf2* expression¹⁴ and delay photoreceptor apoptosis⁴⁷ in RCS rats. However, it is not clear if or how electrical stimulation from the MPA blocks L-type VDCCs. In fact, in vitro induction of *Igf1*²⁶ and *Bdnf*²⁵ by electrical stimulation of cultured rat Müller cells was dependent on functional L-type VDCCs. Thus, induction of *Fgf2* by identical treatment of cultured rat Müller cells²⁷ could not have been due to a block of L-type VDCCs. Nevertheless, this does not rule out the possibility that treatment with the MPA does inactivate L-type VDCCs. Alternatively, since trauma to the retina is known to induce *Fgf2*, another possible mechanism is recurring trauma from the MPA that is sufficient to prolong *Fgf2* induction. Future experiments will be directed towards investigating these possibilities.

Relationship between *Fgf2* and retinal function

As expected from our previous studies, the ERG response did not show a difference 1 week after implantation,¹⁹ while *Fgf2* expression was increased. It is not apparent whether the increases in b-wave amplitude at the highest flash intensity for the **A** and **S** groups are relevant to neuroprotection since this had not been observed before. By 4 weeks of implantation, the **A** group showed significantly larger dark-adapted b-wave amplitudes compared to the other treatment groups, as previously reported.¹⁹ Additionally, preservation of light-adapted response was also found in the **A** group at 4 weeks after implantation, suggesting preservation of cone function. Preservation of retinal function at this timepoint, may be a direct result of the selective increase in *Fgf2* expression that promotes a neuroprotective environment. Thus, the current working hypothesis is that electrical stimulation in the **A** group was capable of achieving a threshold of *Fgf2* induction that is necessary to elicit neuroprotective effects such as preserved retinal function in degenerating retina. This neuroprotective effect is capable of anatomically preserving photoreceptors, as evidenced by a previous study of RCS rats after 8 weeks of implantation with MPAs.³² Additional studies including quantitative analysis of FGF2 protein in response to subretinal electrical stimulation and inhibition of FGF2 signaling will be required to determine if the *Fgf2* induction reported here plays a primary or ancillary role in neuroprotection in this model system.

Electrical stimulation as a neuroprotective treatment for retinal disease

Neuroprotection in general is a phenomenon with potential clinical applications to treat neurodegenerative diseases. In the eye, growth factors appear to be the most potent of well-tolerated molecules for neuroprotection of photoreceptors. Specifically, CNTF has been shown to prolong the life of photoreceptors in several different animal retinal degeneration (RD) models, and clinical trials have begun to assess its safety and efficacy to delay the progression of photoreceptor and vision loss in patients with AMD and RP.^{48, 49} Here we began to explore the mechanism behind neuroprotection provided from a subretinally-implanted microphotodiode array. Results suggest that the electrical stimulation induced endogenous *Fgf2*, another growth factor known to exert neuroprotective effects in animal RD models.

Electrical stimulation as a treatment for retinal degeneration could be applied externally or internally. Morimoto et al., 2007²⁰ also showed that transcorneal electrical stimulation of RCS rats exerted a neuroprotective effect on photoreceptors. This transcorneal approach requires current to be periodically applied via a contact lens electrode, and it presumably must traverse portions of the cornea and anterior chamber to elicit effects in the retina. Alternatively, a subretinal MPA, though more invasive, has the potential benefits of continuous and precise current delivery to the targeted neurons. The induction of *Igf1* with transcorneal electrical

stimulation²⁰ and *Fgf2* with subretinal electrical stimulation, as shown here, may indicate that growth factor expression may be induced by different stimulation parameters. Irrespective of the mode of delivery, determining if retinal neuroprotection provided by electrical stimulation can be extended to other RD animal models, and determining if the effects are responsive to stimulation parameters are questions that bear further examination if this approach is to be used as a therapy for human retinal degenerative diseases.

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Figure 1.

In vitro testing of the active and minimally-active microphotodiode arrays. MPAs were placed in PBS and exposed to an 870 nm flash of 10.5 mW/cm² intensity and 1 msec duration. The graphs show representative current output of active (A) and minimally-active (B) MPAs from this testing. Note the change in current scale from microamps to nanoamps between A and B, respectively. The active MPA produces current that is more than two orders of magnitude above that from the minimally-active MPA (~8 μA versus ~30 nA, respectively).

Figure 2.

Retinal function as assessed by dark-adapted ERG b-wave at 1 and 4 weeks post-implantation. **A** – Representative waveforms from each treatment group in response to a bright flash stimulus (2.1 log cd-s/m²) at 1 week post-implantation. The eyes from the **A** and **S** groups have slightly larger b-wave amplitudes than the eyes from the **C** and **M** groups. The large negative spike at 0.05 msec reflects the electrical activity of the **A** and **M** devices. **B** – Representative waveforms from **A**, **C**, and **M** groups at 4 weeks post-implantation. The response from the **A** group is twice as large as the response from the **C** and **M** groups. **C** – Average ($\pm$ sem) amplitude of a- and b-waves at 1 week after surgery from **A** (n=12), **C** (n=9), **M** (n=7), and **S** (n=7) groups. The b-wave amplitudes of the **A** and **S** groups are significantly larger than the **M** and **S** groups at the brightest flash intensity. No differences were found in a-wave amplitudes at this time. **D** – Average amplitude of a- and b-waves at 4 weeks after surgery from **A** (n=5), **C** (n=3), and **M** (n=4) groups. The b-wave amplitude from the **A** group is significantly larger than the **M** and **C** groups at most intensities. There were no differences in a-wave amplitudes between the groups.

Figure 3.

Cone function assessed by light-adapted ERGs at 1 and 4 weeks post-implantation. **A and B**- Representative light-adapted waveforms from each treatment group at 1 and 4 weeks post-implantation, respectively. While no differences are observed in the 1 week waveforms, the response from the **A** group is ~45% larger than the **C** and **M** waveforms at 4 weeks after implantation. **C**- Average light-adapted b-wave amplitude at one week after implantation from **A** (n=12), **C** (n=9), **M** (n=7), and **S** (n=7) groups showed no differences. **D**- Average light-adapted b-wave amplitude from **A** (n=5), **C** (n=3), and **M** (n=4) groups at 4 weeks after implantation showed significantly increased amplitudes in the **A** group at mid to bright intensities.

Figure 4.

Summary of growth factor expression analyses by RT-PCR done 9 days after surgery. Results are reported as growth factor expression in the treated eye relative to control eye. *Fgf2* expression in the A group was greater than in all other groups, $p < 0.05$. Number of replicates for A, M, S, and C treatment groups were: *Fgf2*, *Cntf*, and *Fgf1*: 4, 3, 4, 6; *Gdnf*: 4, 3, 4, 5; *Igf1*: 8, 0, 3, 10, respectively. Error bars are $\pm$ sem.

Figure 5.

Fgf2 expression analyses by RT-PCR at 9 and 30 days after surgery. Results are reported as *Fgf2* expression in the treated eye relative to opposite eye at 9 days (**A**) and 30 days (**B**) after surgery. **A** – Nine days after surgery, *Fgf2* expression tended to increase as treatment progressed from **C** to **S** to **M** to **A**. **B** – Thirty days after surgery, *Fgf2* expression remained elevated in the **A** group compared to the **M** and **C** groups. Post hoc Holm-Sidak analysis significance scores were: #, $p = 0.0501$; *, $p < 0.05$; **, $p < 0.01$, ***, $p < 0.001$. Error bars are $\pm$ sem.