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Retinal Expression of Fgf2 in RCS Rats with Subretinal Microphotodiode Array

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PURPOSE. To test the hypothesis that subretinal electrical stimulation from a microphotodiode array (MPA) exerts a neuroprotective effect in Royal College of Surgeons (RCS) rats through the induction of growth factors.

METHODS. At postnatal day 21, RCS rats were divided into four groups 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 1 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 Igf1) 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 greater for the A group (4.67 ± 0.72) than for the M group (2.80 ± 0.45; \(P = 0.0501\)), S group (2.05 ± 0.45; \(P < 0.01\)), and C group (1.30 ± 0.22; \(P < 0.001\)). No significant change was detected for Bdnf, Cntf, Fgf1, Gdnf, and Igf1. Four weeks after surgery, the A group had significantly higher dark- and light-adapted ERG b-waves than for the M and C groups (\(P < 0.01\)). Simultaneously, mean relative Fgf2 expression was again greater for the A group (3.28 ± 0.61) than for the M (1.28 ± 0.32; \(P < 0.05\)) and C (1.05 ± 0.04; \(P < 0.05\)) groups.

CONCLUSIONS. 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. (Invest Ophthal Vis Sci. 2009;50:4523–4530) DOI:10.1167/iovs.08-2072

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more, 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 IGF-1 receptor antagonist.5,2 Thus, it seems plausible that the induction of a neuroprotective growth factor is responsible, at least in part, for the preservation of retinal function and photoreceptors in RCS rats that received subretinal electrical stimulation.20

We hypothesized that neuroprotection of the RCS rat retina in response to subretinal electrical stimulation3 results from 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 the expression of several growth factors (brain-derived neurotrophic factor [Bdnf], ciliary neurotrophic factor [Gntf], acidic fibroblast growth factor [Fgf1], basic fibroblast growth factor [Fgf2], glia-derived neurotrophic factor [Gdnf], and insulin-like growth factor [Igf1]) in RCS rats that received subretinal electrical stimulation from an active MPA (A), minimally active MPA (M), sham surgery (S), or no treatment control (C). One week after implantation surgery, Fgf2 expression was selectively induced compared with 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 4 weeks after surgery, ERG b-wave amplitudes were significantly larger in the A group than in 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 Matthew LaVail, PhD, of the University of California at San Francisco, was used in this study. The mutation was bred onto the Long Evans background; thus, pigmented animals were used throughout this study. In the dystrophic RCS rat, both copies of the receptor tyrosine kinase gene Merk are mutated, resulting in a premature stop codon and improper protein synthesis.34 The lack of the MERTK protein is responsible, at least in part, for the preservation of retinal function and photoreceptors in RCS rats that received subretinal electrical stimulation.33 Results from subretinal electrical stimulation20 for the RCS rat retina in response to subretinal electrical stimulation3 results from 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 the expression of several growth factors (brain-derived neurotrophic factor [Bdnf], ciliary neurotrophic factor [Gntf], acidic fibroblast growth factor [Fgf1], basic fibroblast growth factor [Fgf2], glia-derived neurotrophic factor [Gdnf], and insulin-like growth factor [Igf1]) in RCS rats that received subretinal electrical stimulation from an active MPA (A), minimally active MPA (M), sham surgery (S), or no treatment control (C). One week after implantation surgery, Fgf2 expression was selectively induced compared with 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 4 weeks after surgery, ERG b-wave amplitudes were significantly larger in the A group than in the other treatment groups. These results suggest that Fgf2 induction may mediate neuroprotective effects from subretinal electrical stimulation.

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**Implant Description and Characterization**

Implants were fabricated essentially as described previously.5,3 In this study, 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 Ω 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 resultant dielectric layer was 1200 ± 50 Å thick and covered both sides of each MPA. 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. No metal electrodes were applied to the minimally active MPAs, so each face consisted of a smooth, continuous layer of silicon dioxide. The photovoltaic charge-injection characteristics (μA/cm²/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 mW/cm²) was shown on the photoactive side of the active MPAs or either side of the minimally active MPAs as current was measured in the PBS electrolyte at one measurement every 10⁻⁴ seconds.

**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 eye cup. 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 reagent (TRIZol; Invitrogen, Carlsbad, CA) at 30,000 rpm for 2 × 15 seconds 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. Resultant RNA was dissolved in 100 μL RNase-free dH2O and further purified and concentrated to a final volume of 12 μL with a spin column (MinElute RNasey; Qiagen, Valencia, CA). RNA yields, as determined by measuring A260 nm, varied between approximately 0.5 and 2.0 μg per sample, and the ratios of A260 nm to A280 nm varied between 1.7 and 2.0.

Optimal RNA and cDNA dilutions were determined by RT and subsequent PCRs on a seven-step, twofold 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 RNA per RT decreased 18S Ct differences, implying incomplete RT at high RNA amounts, and using less than 50 ng RNA per RT increased Ct for genes of interest. For each RNA sample, 100 ng total RNA was reverse transcribed (Quantitect Reverse Transcriptase; Qiagen) in a final 20-μL volume according to the manufacturer’s instructions. The resultant cDNA was diluted 20-fold, and 5 μL 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, Ifg1) each of a primer pair (18S forward, 5’-TTGTTGTGTCCCAGCTGAAGGC-3’; 18S reverse, 5’-GTCGGCACATTATGGTGTCG-3’; Bdnf forward, 5’-AACCCAGAAAAAGCACCACAAA-3’; Bdnf reverse, 5’-CTTGTGGCTGAATGGGACAGAA-3’; Cntf forward, 5’-GGACCTCTGTGACCGTTCTA-3’; Cntf reverse, 5’-TCATCTCCTCAGAAAGATCA-3’; Fgf1 forward, 5’-AACCAAGTCTCTCAAGGCTG-3’; Fgf1 reverse, 5’-GAGCGGGTATAAACGCCCTTC-3’; Fgf2 forward, 5’-GCCGGCTCTACTGCAAGA-3’; Fgf2 reverse, 5’-CTTCGACATTCTCCTGATAGC-3’; Gdnf forward, 5’-CACTGTCTCTGGCACCACTCTG-3’; Gdnf reverse, 5’-AGGCTGAATGTGGTTCTCTF-3’; Ifg1 forward, 5’-TGAGACGCCTCTCATTGGTGTCG-3’; Ifg1 reverse, 5’-GGTTTCGTGCCTCCTCCTAGC-3’; 12.5 μL of 2X reaction cocktail (iQ SYBR Green Master Mix; Bio-Rad, Hercules, CA) and nuclease-free dH2O to 25 μL. Primers were designed with Primer3 (http://fokker.wi.mit.edu/primer3/). cDNA was amplified in 96-well plates on a thermal cycler end of each 72°C extension. Melt analysis conditions were heating 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 more than 100 times more current than retinas in the M group.

**Electrophotographic Assessment of Retinal Function**

Dark-Adapted Responses. Representative dark-adapted ERGs from animals at 1 and 4 weeks after 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 prominent a- and b-waves with oscillatory potentials present on the leading edge of the b-wave. The negative spike at 0.05 ms was attributed 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 after 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 than in 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 with the C and M groups, as shown in Figure 2B. The representative waveforms in Figure 2B show smaller waveforms compared with the 1-week recordings (Fig. 2A), reflecting the progressing photoreceptor degeneration in the RCS rats. Figure 2D shows the average a- and b-wave

**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-ms 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 compared with ~30 nA, respectively).
amplitudes across intensity. The A group had a significantly larger b-wave amplitudes in response to intensities greater than $-0.6 \log cd \cdot s/m^2$ (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). Figures 3A and 3C illustrate no significant differences in the light-adapted waveform or average b-wave amplitude, respectively, between treatment groups at 1 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 cd $\cdot$ s/m$^2$ (two-way ANOVA, $P = 0.003$; Fig. 3D).

**Growth Factor Expression Analyses by RT-PCR**

Nine days after surgery, expression analyses by RT-PCR of Gsf1, Fgf1, Fgf2, Gdnf, Igf1, and Bdnf (not shown) showed a consistent, large, and significant elevation of Fgf2 expression in the A group compared with all other treatment groups, whereas little to no change across treatment groups was observed for the other genes (Fig. 4). Additional surgeries and 9-day analyses of the Fgf2 expression ratio again showed significantly elevated Fgf2 expression in the A group (Fig. 5A) but also revealed an increase as treatment progressed from control to sham to minimally active implantation to active implantation (C, $1.30 \pm 0.22$; S, $2.03 \pm 0.45$; M, $2.80 \pm 0.45$; and A, $4.67 \pm 0.72$; $F_{3,36} = 6.67$; $P = 0.001$). 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 ($3.24 \pm 0.61$) compared with the other treatment groups: M, $1.28 \pm 0.32$; C, $1.05 \pm 0.04$ ($P < 0.05$, post hoc Holm-Sidak) (Fig. 5B). Over time, 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, the induction of growth factors in applications of neuronal electrical stimulation, and previous results that indicated some degree of neuroprotection in the retina was provided by retinal electrical stimulation, we hypothesized that electrical stimulation affects neuroprotective pathways by the induction of known neuroprotective growth factors. The present results indeed show an increased expression of Fgf2 with MPA implantation. As shown previously, 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 approximately 100 times more electric current than the minimally active implant in response to light, it is inferred that the 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 after implantation for the A group compared with all other groups. This was based on the expectation that the effects on gene expression would be detectable soon after implantation, in advance of any detectable neuroprotection. For gene expression analysis, 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 nonimplanted 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 $\text{Fgf2}$ 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; Fig. 4), whereas little change in mean expression was noted for $\text{Fgf1}$, $\text{Cntf}$, $\text{Gdnf}$, $\text{Igf1}$, and $\text{Bdnf}$. In addition, $\text{Fgf2}$ expression at 4 weeks remained elevated in the A group. These data suggest that subretinal electrical stimulation specifically initiated and sustained increased $\text{Fgf2}$ gene expression.

**Fgf2 Induction from Acute Injury and Chronic Electrical Stimulation**

As a response to injury alone, $\text{Fgf2}$ induction was expected and observed. The present results show approximately a twofold increase in mean $\text{Fgf2}$ expression in the S group compared with the C group. According to published work in which the effect of a retina-piercing injury on growth factor expression was analyzed, detectable $\text{Fgf2}$ induction might be expected from a retina-piercing injury in a 21-day-old rat with sampling performed 9 days after injury, as shown here (Figs. 4, 5A).
Similarly, it is not surprising that Cntf and IGF1 induction 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.46,47

Fgf2 expression was induced in electrically stimulated retinas beyond injury-induced levels 1 week after implantation. The A group showed a significant increase in mean Fgf2 expression (4.7 ± 0.72) compared with the S and C groups (2.03 ± 0.45 and 1.3 ± 0.22) and a noticeable increase in Fgf2 levels compared with 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 that in the S and A groups because the M group experienced some degree of electrical stimulation, albeit much less than did 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, suggest that electrical stimulation is more important for Fgf2 induction than chronic implantation of the MPA because 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 on implantation that could be related to 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 unknown. However, rat Müller cells are known to be a source of FGF2 in response to various stimuli,47,48 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.28 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 to delay photoreceptor apoptosis26 in RCS rats. However, it is not clear whether or how electrical stimulation from the MPA blocks L-type VDCCs. In fact, in vitro induction of Fgf227 and Bdnf26 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 cells26 could not have been caused by 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, given that trauma to the retina is known to induce Fgf2, another possible mechanism is recurring trauma from the MPA sufficient to prolong Fgf2 induction. Future experiments will be directed toward 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,20 whereas 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 were relevant to neuroprotection because this had not been observed before. By 4 weeks of implantation, the A group showed significantly larger dark-adapted b-wave amplitudes than the other treatment groups, as previously reported.19 Additionally, the 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 time point 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 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.35 Additional studies, including quantitative analysis of FGF2 protein in response to subretinal electrical stimulation and inhibition of FGF2 signaling, will be required to determine whether 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 the neuroprotection of photoreceptors. Specifically, CNTF has been shown to prolong the life of photoreceptors in several different retinal degeneration (RD) animal 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.49,50 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 RD animal models.

Electrical stimulation as a treatment for retinal degeneration could be applied externally or internally. Morimoto et al.31 also

**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 the 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 with the M and C groups. Post hoc Holm-Sidak analysis significance scores were as follows: * \( P = 0.0501 \); * \( P < 0.05 \); ** \( P < 0.01 \); *** \( P < 0.001 \). Error bars are ± SEM.**
showed that transcorneal electrical stimulation of RCS rats exerted a neuroprotective effect on photoreceptors. This transcorneal approach requires current to be periodically applied through 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 Fgf1 with transcorneal electrical stimulation 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 whether retinal neuroprotection provided by electrical stimulation can be extended to other RD animal models and determining whether 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.

References

RETRACTION

“Effect of Superposed Electromagnetic Noise on DNA Damage of Lens Epithelial Cells Induced by Microwave Radiation,”

DOI: 10.1167/iovs.07-1333. PMID: 18436834.

An investigation by the Editor-in-Chief, Paul L Kaufman, IOVS, found that this paper was previously published. It is against the policy of the Association for Research in Vision and Ophthalmology (ARVO) to consider any paper or component of a paper that has been published or is under consideration for publication elsewhere.

The paper is therefore being retracted by ARVO from the IOVS journal.