Non-invasive electrical stimulation as a potential treatment for retinal degenerative diseases

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More than 38.5 million people are estimated to be affected by blindness worldwide in 2020 (Flaxman et al., 2017). Among the diseases that cause visual impairment, refractive errors are typically corrected with glasses and cataract with surgery. The leading causes of irreversible blindness nowadays are usually degenerative diseases related to the retina and its extending optic nerves, such as glaucoma, age-related macular degeneration, and retinitis pigmentosa (Quigley et al., 2006; Flaxman et al., 2017). The regenerative ability of the adult central nervous system in mammals is limited. As part of the central nervous system, impairments of the retina and optic nerve caused by trauma or diseases often lead to neurodegeneration and result in permanent blindness, which currently remains no cures. As the global population grows and ages, the number of people with vision loss will also increase. However, treatments for retinal degenerative diseases remain as unmet medical needs.

There is increasing evidence supporting that non-invasive electrical stimulation (ES) of the eye may possess therapeutic potential by preserving or restoring vision in retinal degenerative diseases (Sehic et al., 2016; Bittner and Seger, 2018). Sehic et al. (2016) highlighted the animal and clinical studies, which led to the conclusion that low current electricity can improve vision in retinal and optic nerve diseases. However, considerable issues of the human studies usually fall to the small patient groups or non-standardized ES parameters employed (Sehic et al., 2016). In part, this is also because of the general lack of insightful knowledge regarding the actions of ES. Currently, there is a pressing need for deeper understanding of the underlying mechanisms to guide and govern the designs of clinical parameters and treatment strategies.

Our group has focused on the neuroprotective effect of non-invasive ES to develop and innovate new treatment options for patients suffering from irreversible blinding eye diseases such as glaucoma, age-related macular degeneration, and retinitis pigmentosa. Understanding the underlying mechanisms and optimization of the stimulation parameters is crucial for therapeutic purposes. Optimizing ES settings also provide novel clinical implications for improving vision health in human patients.

Müller cells (MCs) are the primary type of glial cells within the retina. They are supporting cells that maintain structural and functional stabilities of retinal neurons while they are also thought to serve as a source of residential progenitor cells for retinal neuron repair and regeneration (Giannelli et al., 2011; Yu et al., 2014; Jorstad et al., 2017). Recently, we showed that low electrical current could significantly promote the proliferation of MCs and promote their neurogenic potentials (Enayati et al., 2020). Non-invasive ES stimulated proliferative MCs to migrate and transdifferentiate into a photoreceptor cell lineage. ES also improved photoreceptor survival and increased retinal functions in mice with inherited photoreceptor degeneration (Yu et al., 2020).

Our team observed an increased proliferation of primary mouse MCs, which were quantified by 5-ethynyl-2'-deoxyuridine (EdU) incorporation and immunohistochemistry (Enayati et al., 2020; Yu et al., 2020). Our data suggest that ES at a parameter of 20 Hz, 300 uA current, ramp waveform for 1 hour was most effective and induced a nearly 2-fold increase of MC proliferation compared to controls. Changes in gene expression profiles were perceived with bulk RNA sequencing analysis, and the results were verified with quantitative reverse transcription-polymerase chain reaction. We discovered that 479 genes were differentially expressed, with 188 regulatory elements significantly changed in MCs after receiving low current electricity treatment. Based on the transcriptome profiling, the data implicated that ES could drive MCs toward a progenitor and photoreceptor cell fate through downregulation of the Notch/Hes signaling pathway.

On the other hand, the Kyoto Encyclopedia of Genes and Genomes pathway analysis suggests that ES treatment led to the upregulation of calcium signaling and synaptic activities. In line with this finding, the mitogen-activated protein kinases/extracellular signal-regulated kinases pathways, which are downstream to the calcium cascade critical for cell proliferation and differentiation (Jiao et al., 2005; Guo et al., 2018), was also upregulated. A calcium channel blocker, nifedipine, abolished the effects of ES on MC proliferation and progenitor cell gene induction, supporting a critical role of calcium signaling in the process of ES-induced MC changes. Together, low current electricity positively affects the proliferative ability of MCs and upregulates the expression of progenitor cell markers. ES-treated MCs exhibited increased levels of trophic factors, such as ciliary neurotrophic factor, which in itself supports photoreceptor differentiation and survival (Xue et al., 2011).

Our *in vivo* studies were conducted to understand how low current electricity could help treat patients suffering from hitherto non-treatable eye disorders. Retinitis pigmentosa is a group of inherited retinal diseases that progressively affects the light-sensitive photoreceptors, which eventually leads to blindness. Therefore, we performed a proof-of-concept study to investigate the neuroprotective effect of non-invasive trans palpebral ES on a *Rhodopsin* knockout ($Rho^{-/-}$) mouse model. We wanted to explore whether ES could protect photoreceptors from degeneration and if ES was able to improve vision in mice with retinitis pigmentosa. $Rho^{-/-}$ mice received one or two sessions of ES or sham treatments on one random eye for seven consecutive days, and their retinal function was evaluated weekly with electroretinogram. We discovered that ES significantly improved the retinal functions as assessed by 3-Hz, 10-Hz flickers, and photopic electroretinogram, compared to sham-treated or untreated contralateral eyes. An additional ES session prolonged the effect with significantly higher b-wave amplitudes of 3-Hz and 10-Hz flickers that lasted over 4 weeks after the first ES treatment. Retinal morphologies were evaluated by immunohistochemistry to determine if ES improved the retinal function by photoreceptor preservation. Our data demonstrated that non-invasive ES prevented photoreceptor loss and significantly preserved the outer nuclear layer. Results of quantitative reverse transcription-polymerase chain reaction also showed higher expression levels of cone photoreceptor-specific genes, including

recoverin, G-opsin, and B-opsin. Based on the *in vitro* results, we were curious if ES activated the endogenous regenerative potential of MCs *in vivo*.

For this reason, daily intraperitoneal injection of EdU was performed to label proliferating cells while mice were receiving ES or sham treatments. Our data demonstrated MC proliferation as many EdU^+ cells were found to colocalize with cellular retinaldehyde-binding protein (CRALBP), a MC specific cell marker. ES not only supports the proliferation of MCs but also facilitates MC migration from the inner nuclear layer toward the outer nuclear layer. Intriguingly, we discovered a small number of EdU^+ cells that also expressed $recoverin^+$ markers, 4 weeks after the first ES was given. These findings suggest that the proliferative MCs could, in some cases, transdifferentiate into photoreceptors. To corroborate this finding, we used a Müller cell lineage-tracing mouse line Sox2ERT-ROSA26/tdTomato mice, in which the expression of the reporter gene, ROSA26/tdTomato, can be permanently induced in MCs and their progeny by tamoxifen injection. It turned out that more than 90% of tdTomato positive (tdT^+) cells were colocalized with Müller cell marker CRALBP. After ES, some of these tdT^+ cells migrated from the inner nuclear layer to the outer nuclear layer. Under conditions of photoreceptor injury, such as that induced by the retinal detachment, tdT^+/$recoverin^+$ cells could be detected within the ES-treated eyes. Similar results were found in MC cultures. ES also increased the expression of progenitor cell markers and activated photoreceptor cell fate, as shown by both quantitative reverse transcription-polymerase chain reaction and immunohistochemistry. Finally, our data support that ES function through inducing trophic factors, including basic fibroblast growth factor, to trigger cell proliferation and neurogenesis of MCs *in vivo*.

To conclude, we have investigated the neuroprotective, regenerative, and repairing potentials of ES for retinal degenerative diseases. ES changed the genetic landscape and promoted the proliferative MCs toward progenitor cell fate. Non-invasive trans palpebral ES improved photoreceptor survival and retinal function in mice with inherited photoreceptors degeneration, partly through inducing MC proliferation and partially through photoreceptor regeneration. Further studies are needed for optimizing ES conditions on different retinal degenerative disease models. By uncovering the underlying mechanisms of ES-induced intracellular changes for visual function improvements, we may establish feasible ES treatments clinically for visually impaired patients to preserve and/or restore their vision in the future.

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