Short-term Alteration of Developmental Neural Activity Enhances Neurite Outgrowth of Retinal Explants

Meng-Jung Lee[1,2] and Chuan-Chin Chiao[1,2]

[1]Institute of Systems Neuroscience, National Tsing Hua University, Hsinchu, Taiwan
[2]Department of Life Science, National Tsing Hua University, Hsinchu, Taiwan

Correspondence: Chuan-Chin Chiao, Department of Life Science, National Tsing Hua University, 101, Section 2, Kuang Fu Road, Hsinchu 30013, Taiwan;
ccchiao@life.nthu.edu.tw.

Submitted: May 3, 2016
Accepted: October 10, 2016

Citation: Lee M-J, Chiao C-C. Short-term alteration of developmental neural activity enhances neurite outgrowth of retinal explants. *Invest Ophthalmol Vis Sci*. 2016;57:6496–6506. DOI:10.1167/iovs.16-19854

Purpose. It is well known that the gradual loss of axon growth ability of retinal ganglion cells (RGCs) during development is largely determined by extrinsic signals rather than being programmed intrinsically. Spontaneous retinal waves are the major neural activity during retinal development. Thus restoring the developmental environment by providing the proper neural activity may be able to help axon regeneration of RGCs.

Methods. Retinal explants from P5 and P11 *C57BL/6* mice were treated pharmacologically or stimulated electrically, and cultured with or without brain-derived neurotrophic factor (BDNF) on coverslips or a multielectrode array for 5 days to examine the neurite outgrowth capacity of RGCs.

Results. Here we have demonstrated that neurite outgrowth of retinal explants was not affected when acetylcholine transmission was blocked pharmacologically in retinas that normally display stage II retinal waves. However, short-term induction of globally correlated neural activity at 1- to 2-minute intervals in retinas that normally display stage III retinal waves by blocking inhibitory neural transmission was found to greatly promote neurite outgrowth even in the absence of exogenous neurotrophic factors. Moreover, short-term electrical stimulation with a temporal pattern of 1- to 2-minute intervals rather than simply increasing the neural activity greatly enhanced neurite outgrowth of retinal explants of the same age.

Conclusions. These results suggest that short-term alteration of neural activity with a specific temporal pattern in retinas of later developmental stages is sufficient to enhance neurite outgrowth of retinal explants. This finding could lead to a therapeutic strategy that is able to prevent the gradual loss of the axon growth ability of RGCs in more mature retinas.

Keywords: retinal waves, electrical stimulation, retinal ganglion cells, axon regeneration, neurotrophic factors

The immature mammalian central nervous system (CNS) retains the ability to regenerate,[1] but neurons in the adult CNS are difficult to regrow after severe injury. Previous studies have shown that the gradual loss of the ability of axons to grow during the development of retinal ganglion cells (RGCs), one type of CNS neurons, is not intrinsically programmed but rather determined by extrinsic signals.[2] It follows that a restoration of the extrinsic developmental environment of the retina may be able to provide a therapeutic strategy for promoting axon regeneration of RGCs after injury or degeneration.

During retinal development, one of the most well-known phenomena is retinal waves. These are characterized as the correlated spontaneous neural activity in RGCs that is accompanied by the propagation of calcium waves, and these appear from the late embryonic stage to eye opening.[3,4] It has been shown that retinal waves are important for the refinement of binocular segregation in the dorsal lateral geniculate nucleus,[5,6] as well as the formation of the retinotopic map in the superior colliculus.[7,8] It has also been reported that a blockage of endogenous synaptic neural activity that is involved in the development of RGCs reduces axon outgrowth ability as a result of the loss of responsiveness to neurotrophic factors.[9] Furthermore, there are two major declines in the growth ability of axons in RGCs during development,[10] both close in timing to the transitions of different stages of retinal waves. Although contact-mediated inhibition by amacrine cell membranes has been shown to be a dominant factor in the loss of robust axon outgrowth in RGCs,[2] these findings suggest that endogenous neural activity, such as retinal waves, might be crucial for axon growth during development.

In addition to endogenously occurring neural activity, exogenously driven neural activity, such as electrical stimulation (ES), has also been shown to regulate regeneration in various nervous systems.[11–14] Early studies demonstrated that depolarization and ES are able to promote the neuronal survival and neurite outgrowth of isolated RGCs, mainly by increasing their trophic responsiveness in a cAMP-dependent manner.[15–19] It has also been shown that depolarization and ES exert their functionality by temporally increasing the intracellular calcium concentration of cells and by regulating downstream transcription.[20,21] As a consequence, calcium oscillations induced by patterned electrical activity have been postulated to lead to different types of transcription regulation and thus might have an effect on neural regeneration.[22–24] However, exactly how these different patterns of neural activity during retinal development promote neuronal survival and axon growth by RGCs is largely unknown.

In the present study, we have shown that abolishing stage II retinal waves did not have an effect on neurite outgrowth of retinal explants. However, the short-term induction of globally

Investigative Ophthalmology & Visual Science

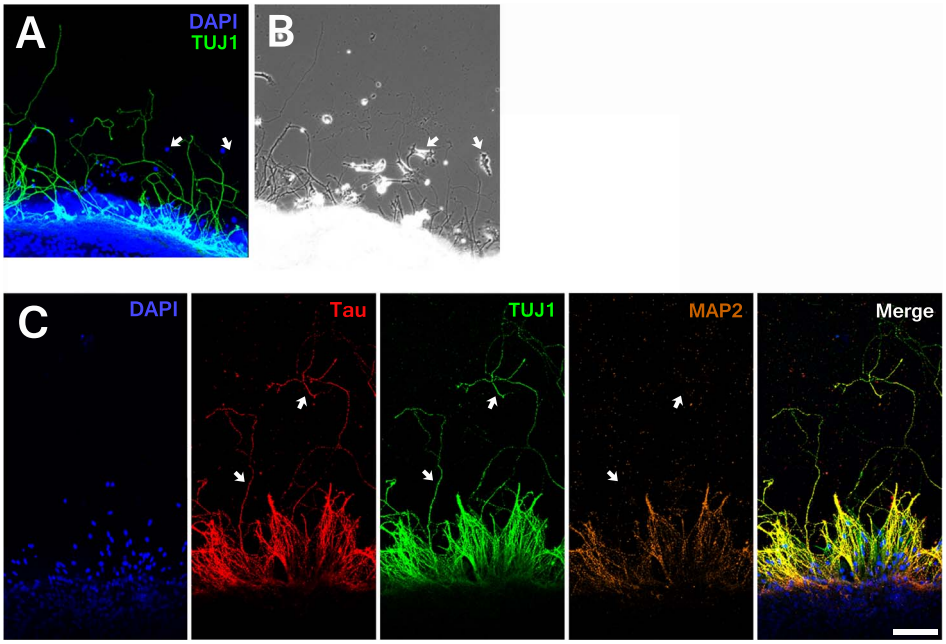

FIGURE 1. Beta III tubulin (TUJ1) is expressed in neurites grown out from RGCs but not in glial cells from the retinal explants. (**A**) A confocal image and (**B**) a phase-contrast image of whole-mount retinal explants. All morphologically recognized neurites were TUJ1 positive (*green*), and the processes of glial cells (*white arrows*) were TUJ1 negative. DAPI was used to label nuclei (*blue*). (**C**) Beta III tubulin was expressed in both Tau- and MAP2-positive neurites, but neurites longer than 200 μm were Tau positive and MAP2 negative (*white arrows*). *Scale bar*: 100 μm.

correlated spontaneous neural activities with 1- to 2-minute intervals is sufficient to enhance neurite outgrowth even without exogenous neurotrophic factors in the retinal explants that normally display stage III retinal waves. Finally, we demonstrated that ES with a similar temporal pattern promotes neurite outgrowth of retinal explants at a later stage of development.

MATERIALS AND METHODS

Retinal Explant Preparation

Retinas were isolated from postnatal *C57BL/6* mice. The animals were anesthetized and killed using an intraperitoneal injection overdose of 1:1 ketamine and xylazine, and the eyeballs were then enucleated with surgical scissors. After hemisection along the ora serrata, the lenses and vitreous humors were immediately removed. After this, the posterior eyecups were immersed in oxygenated (95% O_2 and 5% CO_2) Ames' medium (Sigma-Aldrich Corp., St. Louis, MO, USA) containing 23 mM $NaHCO_3$. At this point the retina was gently detached from the retinal pigment epithelium. The isolated retina was then cut into three pieces, and each piece of retinal explant was attached, ganglion cell side down, onto either a Cell-Tak–coated coverslip (BD Biosciences, Franklin Lakes, NJ, USA) or a multielectrode array (MEA) for ES and retinal wave recording. All procedures were approved by the Institutional Animal Care and Use Committee of the National Tsing Hua University and were in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Retinal Explant Culture

The retinal explants on coverslips/MEAs were cultured in a 5% CO_2 humidified incubator at 37°C for 5 days, and fresh growth medium containing Neurobasal-A (Gibco, Waltham, MA, USA), 0.6% glucose, 2 mM L-glutamine, 1× B-27, 10 mM HEPES, 1 mM sodium pyruvate, 2.5 μg/mL insulin, 100 μg/mL penicillin, and 6 μM forskolin, with or without brain-derived neurotrophic factor (BDNF) (50 ng/mL), was supplied daily.

Immunohistochemistry

The cultured retinal explants were fixed with 4% paraformaldehyde and 0.1% glutaraldehyde for 1 hour at room temperature and then rinsed three times in phosphate-buffered saline (PBS), 10 minutes each time. Next the fixed explants were blocked by 4% normal donkey serum and 0.1% Triton X-100 in PBS for 1 hour at room temperature to reduce nonspecific binding. The primary antibodies against class III beta-tubulin (TUJ1; 1:500; MMS-435P; Covance, Princeton, NJ, USA), microtubule-associated protein 2 (MAP2) (1:50; sc-20172; Santa Cruz Biotechnology, Dallas, TX, USA), and Tau (1:50; sc-1995; Santa Cruz Biotechnology) were then incubated with the explants overnight at 4°C. After rinsing several times in PBS, the explants were incubated with the corresponding secondary antibody (1:250; Alexa 488, Dylight 549, or Dylight 649; Jackson Lab, Franklin, TN, USA) overnight at 4 °C. Finally, the explants were mounted in mounting medium with 4′,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA, USA).

Neurite Outgrowth Identification and Quantification

Images of neurite outgrowth of the retinal explants were acquired using either an inverted microscope (Axio Observer.Z1; Zeiss, Oberkochen, Germany) or a confocal microscope (LSM510, Zeiss). Based on the confocal images, we confirmed that TUJ1 was expressed in the neurites growing out from the RGCs but not from the glial cells in retinal explants (Figs. 1A, 1B). Beta III tubulin staining in retinal whole mounts was shown to be RGC specific and was efficient in RGC identification.[25–27] Furthermore, although TUJ1 is known to be expressed in both Tau- and MAP2-positive neurites, it was found that mostly processes longer than 200 μm were Tau positive, suggesting that

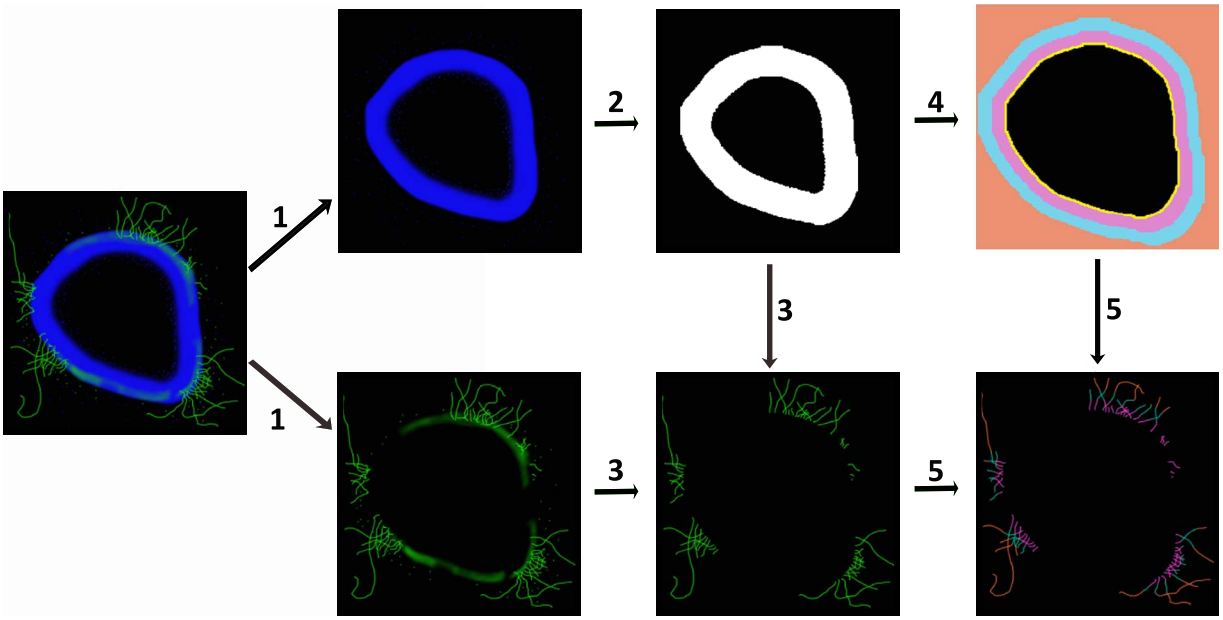

FIGURE 2. Flow chart of the approach used to quantify neurite outgrowth of the retinal explant. The image of double-immunostained retinal explant was [1] split into different color channels, *green* (TUJ1) and *blue* (DAPI). [2] The *blue* channel image was binarized, and the area of the retinal explant was defined. [3] *Green* (RGC neurites) signal inside the area of retinal explant was removed. [4] Two concentric outlines of an expanding circumference out from the retinal explant (100-μm interval) were delineated, and the area outside the explant was divided into three areas: 100 μm (*pink*), 100 to 200 μm (*light blue*), and >200 μm (*orange*). [5] Neurite areas (*green channel*) grown out from the explants were measured in these three areas separately.

they were primarily the grown axons of RGCs, not the axons from amacrine cells (Fig. 1C). To quantify the extent of neurite outgrowth, images of the retinal explants from the inverted microscope were characterized and measured directly, while those images collected using the confocal microscope were analyzed via the following specific steps (Fig. 2). The confocal images were first split into different color channels (DAPI and TUJ1), which represent the area of the retinal explants and the area of neurite outgrowth. Only neurites grown out from the explants were included in quantification, and these were defined as the total neurite area. The extent of neurite outgrowth was then characterized by dividing the total neurite area by the circumference of the explant. To further distinguish neurites of different lengths that were growing out from the explants, the neurite areas < 100, 100 to 200, and > 200 μm expanding outward from the contour of the explants were calculated separately. Note that the neurite area < 100 μm can be considered as a measure of neurite density, whereas the neurite area > 200 μm represents the amount of elongated axons. A similar approach was used by Gaublomme et al.[28] All image analyses were performed using ImageJ software (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA).

Electrical Stimulation and Recording

Retinal explants in the MEA were constantly perfused with fresh oxygenated Ames' medium (~1 mL/min) and kept at 31°C to 33°C. The explants were allowed to settle in the MEA for more than 30 minutes before recording. Electrical stimulation and recording were performed using an in vitro USB-MEA system (Multichannel Systems, Reutlingen, Germany) with a MEA chip (60MEA200/10iR-ITO-pr-T) that consisted of 60 electrodes with diameters of 10 μm that were spaced 200 μm apart to form an 8×8 array. For ES, the different temporal patterns of pulsed stimuli (1-ms and 40-mV pulses) were applied to the explants for 1 hour using 59 electrodes simultaneously, with one electrode serving as ground via a stimulus generator (STG3000 Series). For electrical recording, the neural activity of the explants was continuously measured for 20 minutes at a sampling rate of 12 kHz.

Pharmacologic Treatments

During the experiment with 1 hour of exposure to the various drugs, the retinal explants on coverslips were placed into a 6-mm culture dish with 5 mL oxygenated and prewarmed Ames' medium that had added to it various pharmacologic reagents, namely, picrotoxin (PTX) (50 μM; Sigma-Aldrich Corp.), (1,2,5,6-Tetrahydropyridin-4-yl)methylphosphinic acid (TPMPA) (50 μM; Tocris, Minneapolis, MN, USA), and strychnine (5 μM, Sigma-Aldrich Corp.).[3,29,30] After 1 hour of treatment, the explants were rinsed extensively with 30 to 50 mL Ames' medium to remove any residual drugs. Otherwise, the other various pharmacologic reagents, namely, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) (50 μM, Tocris), 2-amino-5-phosphonopentanoic acid (AP5) (50 μM, Tocris), dihydro-β-erythroidine hydrobromide (DHβE) (10 μM, Tocris), tetrodotoxin (TTX) (2 μM; Abcam, Cambridge, UK), PTX, TPMPA, and strychnine were added directly to the culture medium.[31–33] The concentrations of these pharmacologic reagents were similar to those used in previous studies.

Cell Viability Assay

The condition of various retinal explants was examined using the Vybrant apoptosis assay kit (Y3603; Thermo Fisher Scientific, Waltham, MA, USA). YO-PRO-1 in this assay is able to label both dead cells and cells undergoing apoptosis with green fluorescence.[34] The explants, after being cultured for 5 days, were incubated with 100 nM YO-PRO-1 for 1 hour in a 37 °C, 5% CO_2 humidified incubator. The explants then had images captured immediately using an inverted microscope (Axio Observer.Z1, Zeiss) and a fixed exposure time. The condition of each retinal explant was quantified by measuring the YO-PRO-1–positive area (fluorescence above the threshold)

of the explants compared to the whole area of the explant. The specificity of RGC survival was examined by using the RGC-specific marker TUJ1 to establish a correlation between RGC survival and YO-PRO-1 signal (see Supplementary Material). The results were then normalized against explants under control conditions. Image analysis was performed using ImageJ.

Wave Property Analysis

Raw data were collected from the MEA recordings and then first processed using MC_Rack software (Multichannel Systems). The processed dataset was then subjected to spike detection using the FIND_GUI toolbox[35] for MATLAB (MathWorks, Inc., Natick, MA, USA). Custom algorithms written in MATLAB were used to analyze the retinal waves. The parameters used for characterizing wave properties have been described in detail in previous studies,[36,37] which we followed closely. Briefly, the values of the interspike intervals (ISIs) from each electrode were ranked, and only the smallest 25% of the ISIs in each channel were considered as candidate bursts. To be considered as a burst, the spike count in the 1-second window following the first spike of the first interval below threshold rank had to exceed a threshold θ. The threshold θ was set to the spike count at the 95% mark of the cumulative spike count distribution. This value was calculated individually for each channel to account for the substantial variations in firing rate. The end of a burst was determined as the time when the spike count fell below $\theta/2$. Only bursts with at least 10 spikes were included in the analysis. To prevent merging of waves during long bursts, a maximum burst duration of 4 seconds was imposed. To allow multiple separate waves propagating across the retina simultaneously to be distinguished, a burst was included in a wave only if it was in spatial and temporal proximity to previously included bursts. This was implemented by first grouping temporally overlapping bursts as candidate waves and then separating spatially nonoverlapped bursts as different waves. To prevent two waves merging together when they traveled to the same location at different times, the time difference between two bursts at neighbor electrodes had to be below 2 seconds.

Results

Stage II Retinal Waves Are Not Essential for Neurite Outgrowth of Postnatal Day 5 Retinal Explants

To assess the role of developmental neural activities in facilitating neurite outgrowth of retinal explants, we first blocked neural activities by adding DHβE, a nicotinic acetylcholine receptor antagonist, together with CNQX and AP5, which are α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/kainate and N-Methyl-D-aspartate (NMDA) glutamate receptor antagonists, respectively, to the culture medium for 5 days. The specificity and efficacy of the drugs were well established in previous studies.[32,33,38,39] The extent of neurite outgrowth was reduced as expected (Figs. 3B, 3H, 3I), though it was not statistically significant due to the sample variability. To further examine whether stage II retinal waves are essential to facilitate neurite outgrowth of retinal explants, DHβE alone was added to the culture medium. Retinal waves were blocked completely by DHβE, and only residual spontaneous neural activities remained. This was confirmed by MEA recording (Fig. 3F). Surprisingly, the extent of neurite outgrowth was not affected by the blockade of stage II retinal waves (Fig. 3C), and this effect was independent of the presence of BDNF in culture medium (Figs. 3H, 3I). Similarly, preventing the generation of action potentials by adding TTX did not inhibit neurite outgrowth of retinal explants in a notable way when BDNF was present, though the TTX effect seemed to promote neurite elongation (Figs. 3D, 3H, 3I). This observation might suggest that longer growth of neurites requires synaptic transmission within the retina or that the subthreshold membrane excitation is more important than action potential in promoting longer growth of neurites. However, CNQX and AP5 alone were able to reduce neurite outgrowth of retinal explants to a similar level as when CNQX and AP5 were added together with DHβE in culture medium (Figs. 3E, 3H). Multielectrode array recording confirmed that retinal waves were not blocked by these glutamate receptor antagonists (Fig. 3G). Note that this effect was not observed in the absence of BDNF (Fig. 3I), suggesting that glutamate transmission regulates neurite outgrowth by modulating the trophic responsiveness of retinal explants.

To ensure that the reduced neurite outgrowth was not a result of cell death, retinal explants after culture for 5 days were stained with YO-PRO-1, a cell viability marker that labels cells undergoing apoptosis or those are dead. We found that the YO-PRO-1–positive areas of the retinal explants were not increased significantly in the various conditions when compared to the control explants, both in the presence and in the absence of BDNF (Figs. 3J, 3K). These findings suggest that a minimum level of neural activity is sufficient to support RGCs in postnatal day 5 (P5) retinal explants growing out neurites. More importantly, blocking neural activity mediated by cholinergic transmission (i.e., stage II retinal waves) does not affect neurite outgrowth of P5 retinal explants; instead, neural activity mediated by glutamatergic transmission seems play a more prominent role.

Short-term Induction of Globally Correlated Neural Activities Promotes Neurite Outgrowth of P11 Retinal Explants

The aforementioned results suggest that retinal waves might be independent of axon growth ability of RGCs in early development. To further examine the role of retinal waves in affecting neurite outgrowth of retinal explants in late development, we induced P11 retinal explants that normally express stage III retinal waves to display a temporal pattern of neural activity similar to stage II retinal waves by adding a cocktail of antagonists of inhibitory neurotransmitters, specifically gamma-aminobutyric acid $(GABA)_{A/C}$ and glycine receptor antagonists (picrotoxin, TPMPA, and strychnine) to the culture medium. Multielectrode array recording revealed that this removal of inhibition induced P11 retinal explants to express globally correlated spontaneous neural activities at 1- to 2-minute intervals (Fig. 4A). Surprisingly, neurite outgrowth of retinal explants was significantly enhanced at day in vitro (DIV) 5 when the cocktail of inhibitors was added at DIV 0 for 1 hour or applied repeatedly for 5 days (1 hour each day; Figs. 4B, 4D–4F), but not when the inhibitors were kept in the culture medium for 5 days continuously (Figs. 4C, 4F). Furthermore, without the presence of BDNF in the culture medium, simply inducing globally correlated spontaneous neural activities at 1- to 2-minute intervals in P11 retinal explants for 1 hour, or repeatedly for 5 days, was sufficient to completely restore neurite outgrowth ability to a state equivalent to the condition when BDNF was present (Fig. 4G). Note that the effect of neural activity was primarily on enhancing short- and intermediate-length sprouting, not the longer neurite growth (Figs. 4F, 4G), which might be an important issue in axon regeneration.

Investigative Ophthalmology & Visual Science

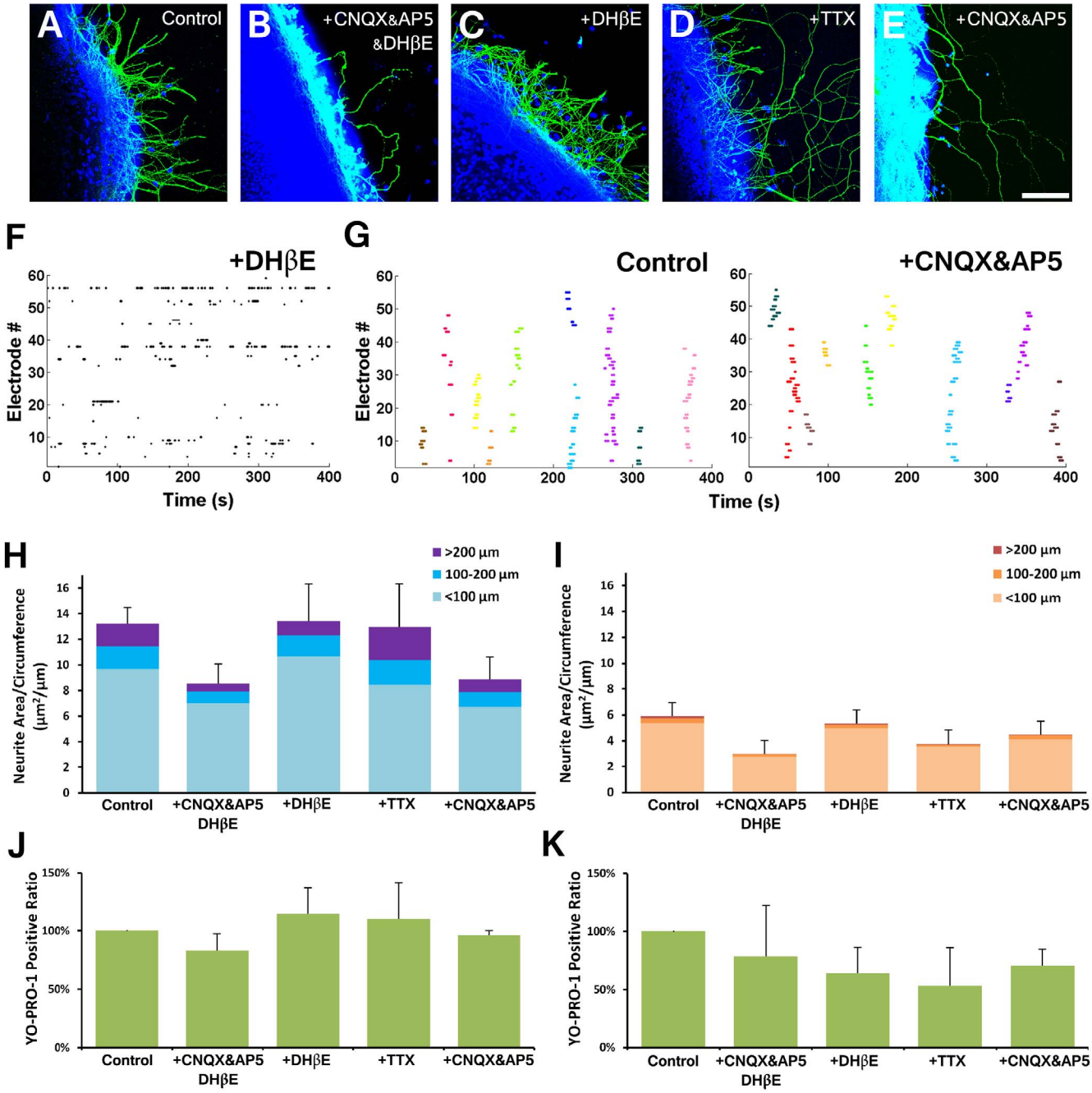

Figure 3. Neurite outgrowth of P5 retinal explants is not affected by blocking stage II retinal waves. (**A**–**E**) Confocal images of P5 retinal explants at DIV 5 when cultured in growth medium with (**A**) BDNF alone, (**B**) BDNF plus CNQX (50 μM), AP5 (50 μM), and DHβE (10 μM), (**C**) BDNF plus DHβE, (**D**) BDNF plus TTX (2 μM), and (**E**) BDNF plus CNQX and AP5. (**F**) Residual activities detected by MEA after adding DHβE. Each *black dot* represents a spike event. (**G**) Detected burst events in the retinal waves of P5 retinal explants recorded by MEA at DIV 0. Each *colored dot* represents a burst event, and the different wave events are separated by *colors*. Note that stage II retinal waves were not abolished after adding CNQX and AP5. (**H**) Growth medium with BDNF. While DHβE together with CNQX and AP5 ($n = 11$) was able to reduce neurite outgrowth, DHβE ($n = 9$) and TTX ($n = 7$) did not affect the total area of neurite outgrowth compared to the control condition ($n = 17$), though this was not statistically significant due to the sample variability. Moreover, blocking glutamate transmission by CNQX and AP5 ($n = 10$) without DHβE reduced neurite outgrowth to the same level as with DHβE in P5 retinal explants. (**I**) Neurite outgrowth was reduced when glutamate and acetylcholine transmissions were simultaneously blocked ($n = 6$), but not affected by blocking DHβE ($n = 8$), action potential ($n = 7$), or glutamate transmission ($n = 7$) in the growth medium without BDNF (control, $n = 11$), though this was not statistically significant due to the sample variability. (**J**, **K**) Cell viability was not affected after culturing the explants for 5 days in the growth medium with different reagents, whether BDNF was added to the medium or not ($n = 2$–4 for each condition in both groups). Mean ± SEM; *Scale bar*: 100 μm.

To determine if the long-term induction of globally correlated neural activities for 5 days could lead to excitotoxicity in P11 retinal explants, YO-PRO-1 was used to examine cell viability. We found that YO-PRO-1–positive areas were increased under this condition regardless of the presence or absence of BDNF in the culture medium (Figs. 4H, 4I). This observation supports that the decreased neurite outgrowth–promoting effect is a result of the long-term elevation of

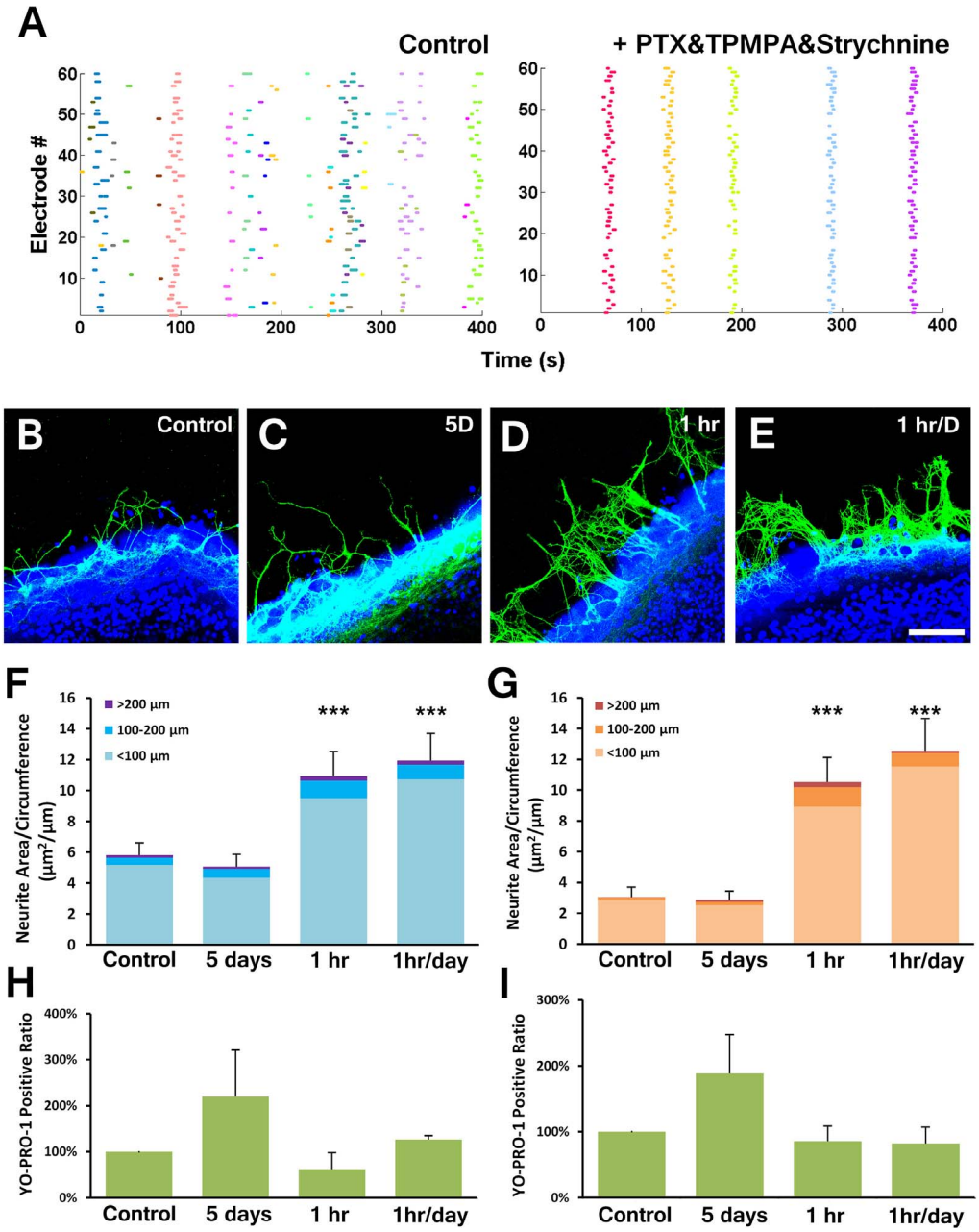

Figure 4. Short-term globally correlated spontaneous neural activities at 1- to 2-minute intervals in P11 retinal explants promote neurite outgrowth. (**A**) Detected wave events from P11 retinal explants recorded by MEA at DIV 0. Each *colored dot* represents a burst event; different wave events are separated by *colors*. Note that stage III retinal waves in the control condition were changed to globally correlated spontaneous neural activities at 1- to 2-minute intervals when a cocktail of inhibitory blockers was applied in the growth medium. (**B**–**E**) Confocal images of P11 retinal explants at DIV 5 cultured in the growth medium with (**B**) BDNF alone, and treated with a cocktail of inhibitory blockers PTX (50 µM), TPMPA (50 µM), and strychnine (5 µM) for (**C**) 5 days, (**D**) 1 hour at DIV 0, and (**E**) 1 hour per day for 5 days. (**F**, **G**) Short-term treatment with a cocktail of inhibitory blockers for 1 hour once at DIV 0 or on each day greatly enhanced neurite outgrowth of retinal explants in the growth medium with or without BDNF, while long-term treatment did not have this effect (with BDNF: control, $n = 25$; 5 days, $n = 18$; 1 hour, $n = 12$; 1 hour/day, $n = 11$; without BDNF: control, $n = 18$; 5 days, $n = 11$; 1 hour, $n = 10$; 1 hour/day, $n = 7$). (**H**, **I**) Long-term altered neural activity increased apoptosis within the retinal explants in the growth medium with or without BDNF, while short-term treatment did not have this effect ($n = 3$–6 for each condition in both groups). ***P value < 0.001, statistically different from the control condition, Kruskal-Wallis test; mean + SEM; *Scale bar*: 100 µm.

neural activity in retinal explants. Taken together, these findings indicate that short-term, but not long-term, globally correlated neural activities at 1- to 2-minute intervals are sufficient to enhance neurite outgrowth ability of retinal explants in late development even in the absence of neurotrophic factors.

Electrical Stimulation Promoting Neurite Outgrowth of Retinal Explants Depends on Postnatal Age and Stimulation Pattern

Based on the results described above, we then asked whether short-term induction of neural activities alone or some specific

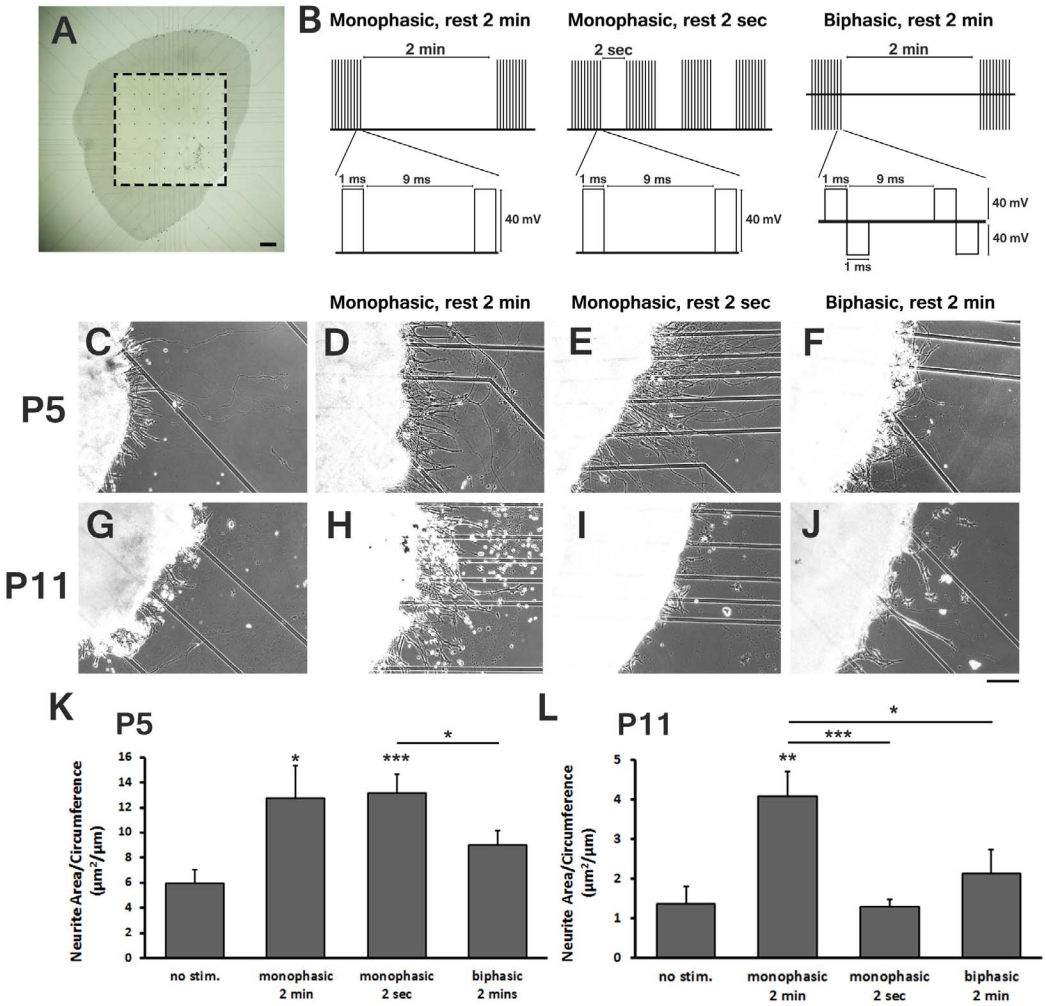

Figure 5. Different temporal patterns of electrical stimulation exert different effects on neurite outgrowth of P5 and P11 retinal explants. (**A**) Retinal explants were placed ganglion cell side down on the MEA. *Dashed lines* demarcate the area of the electrodes. *Scale bar*: 200 μm. (**B**) Temporal patterns of the three electrical stimulation protocols. (**C–F**) Phase-contrast images of P5 retinal explants cultured in growth medium with BDNF at DIV 5 when no electrical stimulation, monophasic stimulation with a rest of 2 minutes, monophasic stimulation with a rest of 2 seconds, and biphasic stimulation with a rest of 2 minutes were applied for 1 hour at DIV 0. *Scale bar*: 100 μm. (**G–J**) Similar images were obtained from P11 retinal explants at DIV 5. (**K**) All three electrical stimulation protocols promote neurite outgrowth of P5 retinal explants ($n = 7$ retinas for each protocol). (**L**) Only the monophasic electrical stimulation with a 2-minute interval had a significantly enhanced neurite outgrowth of P11 retinal explants ($n = 8$ retinas for monophasic rest 2 minutes and monophasic rest 2 seconds; $n = 7$ for biphasic rest 2 minutes). $^{*}P$ value < 0.05, $^{**}P$ value < 0.01, $^{***}P$ value < 0.001, statistically different from the control condition, unless labeled otherwise, Kruskal-Wallis test; mean + SEM.

activity pattern is required for effectively promoting neurite outgrowth. We examined if the temporal pattern with 1- to 2-minute intervals is crucial to enhance neurite outgrowth of retinal explants by applying ES directly using a MEA (Fig. 5A). Three different ES patterns (Fig. 5B) were applied for 1 hour on DIV 0 to both P5 and P11 retinal explants. The first ES pattern consisted of a set of ten 40-mV monophasic pulses at 100 Hz with a 2-minute interburst interval, which was similar to the ES patterns used in previous studies.[9,15] The second ES pattern had only a 2-second interburst interval, which was distinctly different from the first one, as a control, and thus had 60 times more stimuli than the first pattern. The third ES pattern was similar to the first one except that the pulses were biphasic instead of monophasic, which was used as an alternative ES pattern to prevent the targeted cells from accumulating too many charges over time.[23] The effects of different ES patterns on the neurite outgrowth of both P5 and P11 retinal explants were then examined at DIV 5 (Figs. 5C–5J).

All three patterns of ES promoted neurite outgrowth of P5 retinal explants (Fig. 3K), and the ES pattern with the biphasic pulses appeared to show the least prominent effect (Fig. 5K). In contrast, only the ES pattern with a 2-minute interval with monophasic pulses showed a significant effect on neurite outgrowth of P11 retinal explants, while the similar ES pattern with biphasic pulses showed only a moderate enhancement of neurite outgrowth (Fig. 5L). These findings suggest that the pattern of the ES is not a critical factor that affects neurite outgrowth in younger retinal explants, but that the temporal pattern of ES is important for the promotion of neurite outgrowth of retinal explants from a later stage of development.

Short-term Electrical Stimulation Promotes Neurite Outgrowth of P11 Retinal Explants Without Altering the Intrinsic Wave Properties

To further investigate if the enhancement of neurite outgrowth of P11 retinal explants observed at DIV 5 by applying ES for 1 hour at DIV 0 is a result of long-term alterations in their intrinsic activities (stage III retinal waves), the neural activities

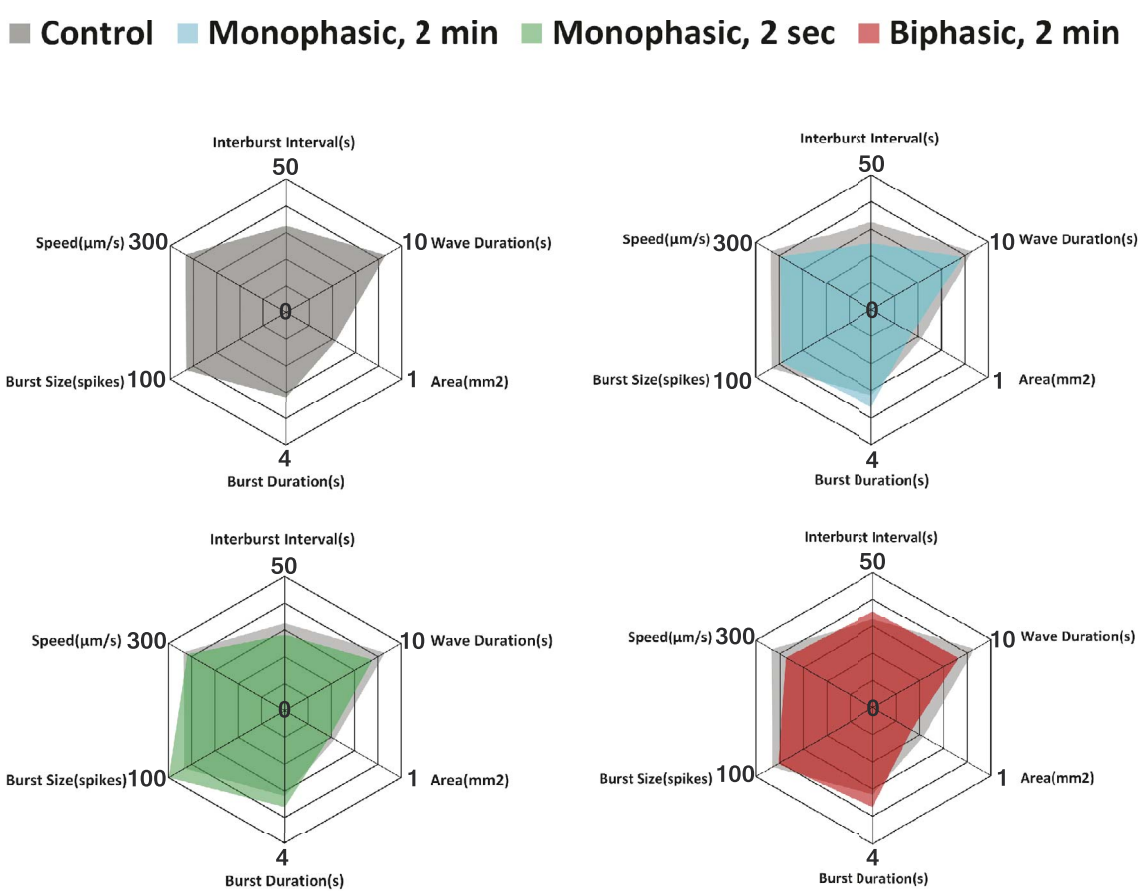

Figure 6. Electrical stimulation did not promote neurite outgrowth by causing a long-term change in the wave properties. Six wave properties from P11 retinal explants were visualized using radar plots. Three different electrical stimulation protocols did not change the retinal wave properties significantly. Control: $n = 8$ retinas; monophasic, 2 minutes: $n = 7$ retinas; monophasic, 2 seconds: $n = 6$ retinas; biphasic, 2 minutes: $n = 5$ retinas.

of retinal explants before or immediately after 1-hour ES were measured using the same MEA that provided ES to the explants. Six wave properties were extracted from 20 minutes of recorded activity patterns, including burst size, burst duration, interburst interval, wave area, wave speed, and wave duration. Although there were slight changes in some wave properties when compared with the results of the control explants and the ones after three patterns of ES, the instrinsic properties remained essentially stage III retinal waves under all four conditions (Fig. 6). These findings support that ES does not promote neurite outgrowth by altering instrinsic neural activities in the retinal explants; rather, ES for only 1 hour before the beginning of retina culture is sufficent to promote neurite outgrowth of P11 retinal explants.

Discussion

In this study, we explored the effect of neural activity on neurite outgrowth of retinal explants. The blocking of stage II retinal waves by inhibiting cholinergic transmission did not affect the neurite outgrowth of P5 retinas. However, blocking glutamatergic transmission did reduce neurite outgrowth during the same stage of retinal development. In contrast, short-term elevation of neural activity, either by ES or by pharmacologic blockade of inhibition, greatly enhanced neurite outgrowth. Furthermore, only an ES pattern with 1- to 2-minute intervals significantly enhanced neurite outgrowth of P11 retinas, while all ES patterns were able to promote neurite outgrowth of P5 retinal explants.

Glutamatergic Transmission Affects Neurite Outgrowth of Immature Retinal Explants

One of our main findings is that neural activity mediated by acetylcholine did not affect neurite outgrowth of P5 as well as P2 to P6 retinal explants (data not shown), the developmental stages in which neural activity is known to be dominated by the cholinergic waves (Fig. 3). This result did not fit our expectation because previous studies have suggested that the temporal pattern of retinal waves plays a significant role in increasing the responsiveness of neurotrophic factors in RGCs and thus their capacity for axon growth.[9] The finding that blocking stage II cholinergic waves by DHβE did not reduce neurite outgrowth in the presence or absence of BDNF suggests that retinal waves probably play only a minor role in the RGC axon growth of P5 retinas. This observation is unlikely to result from the diminished neural activity of explants in culture because retinal waves were still detectable after culturing the explants for several days under the control condition (data not shown). Rather, previously unrecognized glutamatergic transmission within P5 retinas would seem to be a major factor involved in the regulation of neurite outgrowth. It is known that mouse retinas go through a maturation process of the glutamatergic circuits between P1 and P10.[40] During this stage, bipolar cells are forming glutamate synapses with the RGCs and amacrine cells; as a result, the blocking of glutamate transmission may interfere with the maturation of the glutamatergic circuit. A previous study has shown that dark rearing results in an interruption of the maturation of the

glutamatergic circuits and also brings about the loss of the pruning process, resulting in a higher percentage of bistratified RGCs.[41] It is possible that neurite outgrowth from retinal explants was reduced due to excessive dendritic growth of the RGCs that lack this pruning process. Since the cholinergic circuit is already mature at this stage, neurite outgrowth should not be affected by blocking cholinergic transmission.[32] It is also possible that glutamatergic transmission mediated by either synaptic or extrasynaptic connections between bipolar cells and RGCs in the immature retina may control the calcium transients in the RGCs and thus regulate the extent of neurite outgrowth. Further experiments are needed to investigate the role of glutamatergic transmission at this early stage of retinal development.

Electrical Stimulation Promotes Neurite Outgrowth by Stimulating RGCs Directly

Different from previous studies showing that ES promoted neurite outgrowth in isolated RGCs,[9,15] the ES in this study was given to the retinal explants directly, and this may stimulate neurons other than RGCs in the retina. Previous studies have shown that ES targeting the whole retina indeed does lead to an increase in endogenous BDNF, possibly from glial cells.[42,43] However, the stimulating electrodes used in this study were placed on the RGC side, and, more importantly, the ES frequency (100 Hz) applied to the explants was in the range known to mainly stimulate RGCs but not other retinal neurons.[44] Even if there was an increased endogenous BDNF release caused by the ES, the concentration of exogenous BDNF added in the culture medium was presumably much higher than any increase in endogenous BDNF. Therefore, in the present study, it is likely that the ES effect with respect to promoting neurite outgrowth is a result of direct stimulation of the RGCs rather than stimulation of other cell types.

Short-term Elevation of Neural Activity Results in a Long-term Effect on the Promotion of Neurite Outgrowth

The findings showing that a short-term elevation of neural activity, either by applying ES directly (Fig. 5) or by removing inhibition pharmacologically (Fig. 4), results in a promotion of long-term neurite outgrowth of retinal explants suggests that transcriptional regulation is involved. It is well known that electrical activity can lead to calcium influx or intracellular release of calcium, and that is able to regulate downstream signaling pathways.[45,46] Recent studies have shown that calcium influx caused by ES is able to elevate cellular soluble adenylyl cyclase (sAC) activity, which in turn promotes neurite outgrowth and cell survival in isolated RGCs.[15,21,47] Notably, elevated sAC has been shown to promote neurite outgrowth by increasing cyclic adenosine monophosphate (cAMP) and protein kinase A (PKA) levels in a pathway that is distinct from the classic transmembrane adenylyl cyclase–mediated pathway, which is activated by forskolin, and this in turn also elevates cAMP and PKA levels in cells. Previous study has demonstrated that in the presence of activity blockade, the nonhydrolyzable cAMP analogue CPT-cAMP rescued axon extension from the explants, while the PKA inhibitor Rp-cAMP reduced axon extension.[9] It is likely that the effect of promoting neurite outgrowth by a short-term elevation of neural activity is also through a cAMP-dependent signaling pathway. It has been reported that both depolarization and activation of cAMP result in a rapid recruitment of TrkB, the neurotrophic BDNF receptor, to the cell soma, axons, and growth cones.[16,48] However, TrkB expression alone is not able to promote neurite outgrowth without BDNF.[9] The finding that when the intrinsic neural activity of P11 retinal explants is elevated and induced into mimicked stage II retinal waves, neurite outgrowth is promoted with or without BDNF in the culture medium, suggests that short-term induction of specific patterned neural activity might not only affect the expression and recruitment of cell surface TrkB but also increase the release of BDNF or other neurotrophic factors. Future studies to elucidate the molecular mechanism underlying the effects of short-term elevation of neural activity on promoting neurite outgrowth are likely to provide insight into the role of neurotrophic factors in neural regeneration.

The Promotion of Neurite Outgrowth Depends on the Neural Activity Pattern

We found that the temporal pattern of the ES was critical to the promotion of neurite outgrowth of retinal explants (Fig. 5). While three different patterns of ES were able to promote neurite outgrowth of P5 retinal explants and the biphasic ES was the least effective, only the pattern with a 2-minute interval had this effect on the promotion of neurite outgrowth of P11 retinal explants. Although previous studies have shown that biphasic ES causes less tissue damage due to the charge balance,[49–51] this pattern could not promote neurite outgrowth more effectively than the monophasic stimulus used in the present study. The interpulse interval between the two phases of the stimulus and the use of short-term ES may be the reasons for this discrepancy. Nevertheless, this result demonstrates that the correct temporal pattern of ES is essential and more important than simply increasing neural activities for enhancing neurite outgrowth of retinal explants. The same temporal pattern of neural activity was also found to enhance neurite outgrowth of P11 retinal explants by adding cocktails of inhibitors (Fig. 4). This temporal pattern of spontaneously correlated activity at 1- to 2-minute intervals is usually observed in an early stage of the developing retina, when the regenerative capacity of RGC axons remains relatively high. Therefore, it is likely that the pattern of neural activities at early developmental stages is an essential signal to turn on specific genes for enhancing neurite outgrowth at later developmental stages. It has been hypothesized that ES exerts its effect by regulating calcium influx, and this would seem to lead to variety of different consequences in terms of cell responses. It has also been reported that the temporal pattern parameters of ES, such as burst rate, burst duration, and burst frequency, are able to produce different intracellular calcium level oscillations and thus lead to different transcription levels.[22,24] The calcium calmodulin-activated kinase (CaMK) family of proteins is one of the main targets that are activated by calcium, and different members of the CaMK family are known to phosphorylate CREB (cAMP response element–binding protein) at different sites, which in turn regulates neural survival and transcription of genes such as *bdnf* and *ngf*.[45,52–54] These studies suggest a series of complex pathways in which calcium influx, on being modulated by diverse patterns of ES, is able to bring about very different results in terms of neurite outgrowth. Other studies also demonstrated that periodic internal waves of calcium and cAMP signaling in growth cones play a central role in the regulation of neuronal motility.[55] For example, it has been shown that cAMP oscillations in growth cones that resulted from the spontaneous activity of the developing RGCs are essential during the establishment of the retinotopic map.[56] Although the present study has demonstrated the effect of the temporal pattern of ES on neurite outgrowth, it remains unclear whether the spatial pattern of ES also plays an important role. Further investigations are needed to reveal the relationship between the patterns of neural activity and the

Investigative Ophthalmology & Visual Science

invoked downstream signaling pathways; such an approach will help to identify the optimal activity pattern for use as a therapeutic treatment.

The Limitations of Neurite Outgrowth Promoted by Neural Activity

Despite the fact that ES greatly improves the neurite outgrowth of P11 retinal explants, the overall effect is still far from that found when the neurite outgrowth of P5 retinal explants is examined (Figs. 5K, 5L). Furthermore, ES alone was not able to show any effect on RGCs from adult animals. Although the neurite outgrowth area was increased significantly when globally correlated neural activities with 1- to 2-minute intervals were provided to a level very close to that for the P5 retinal explants, the proportion of grown neurites longer than 200 μm (i.e., elongation of neurites into axons) did not increase (Figs. 4F, 4G), suggesting that only short-distance sprouting was enhanced by increased neural activity. These results indicate that the retinal explants cannot be rejuvenated by elevation of neural activity alone; they seem also to need a combination of other signals or various intrinsic factors to help the neurites extend longer or be committed to axons faster. In addition to CREB, there are many other transcription factors that regulate neurite outgrowth.[53] Among these, the Krüppel-like factor (KLF) family has been found to regulate intrinsic axon regeneration ability.[57,58] Furthermore, by deleting both Pten and Socs3 in RGCs, it has been reported that optic nerve axons after prechiasm lesion partially reinnervated and formed new synapses in the suprachiasmatic nucleus.[59] In the most recent study, it was found that enhancement of neural activity by visual stimulation, combined with elevation of the cell growth–promoting pathway involving mammalian target of rapamycin (mTOR), can significantly promote adult RGC axons to regenerate long distances and reinnervate the brain.[60] Taken together, by integrating various different approaches, it should be possible to bring about better and more useful CNS regeneration in the future.

Acknowledgments

The authors thank Chih-Tien Wang for kindly providing the recipe for culturing retinal explants. They also thank Ron Meyer and Jill Miotke for providing their laboratory protocols for the fixation and immunostaining of retinal explants. In addition, we thank Erh-Chung Chen for writing the MATLAB scripts for analyzing the properties of retinal waves, and Yung-Chieh Liu, Yueh-Chun Tsai, Ming-Kai Yang, and Pin-Yuan Chen for collecting supplementary data.

Supported by the Ministry of Science and Technology of Taiwan NSC-101-2628-B-007-001-MY3 (C-CC).

Disclosure: **M.-J. Lee**, None; **C.-C. Chiao**, None

References

1. Nicholls J, Saunders N. Regeneration of immature mammalian spinal cord after injury. *Trends Neurosci*. 1996;19:229–234.
2. Goldberg JL, Klassen MP, Hua Y, Barres BA. Amacrine-signaled loss of intrinsic axon growth ability by retinal ganglion cells. *Science*. 2002;296:1860–1864.
3. Syed MM, Lee S, Zheng J, Zhou ZJ. Stage-dependent dynamics and modulation of spontaneous waves in the developing rabbit retina. *J Physiol*. 2004;560:533–549.
4. Firth SI, Wang CT, Feller MB. Retinal waves: mechanisms and function in visual system development. *Cell Calcium*. 2005;37:425–432.
5. Stellwagen D, Shatz CJ. An instructive role for retinal waves in the development of retinogeniculate connectivity. *Neuron*. 2002;33:357–367.
6. Muir-Robinson G, Hwang BJ, Feller MB. Retinogeniculate axons undergo eye-specific segregation in the absence of eye-specific layers. *J Neurosci*. 2002;22:5259–5264.
7. McLaughlin T, Torborg CL, Feller MB, O'Leary DD. Retinotopic map refinement requires spontaneous retinal waves during a brief critical period of development. *Neuron*. 2003;40:1147–1160.
8. Mrsic-Flogel TD, Hofer SB, Creutzfeldt C, et al. Altered map of visual space in the superior colliculus of mice lacking early retinal waves. *J Neurosci*. 2005;25:6921–6928.
9. Goldberg JL, Espinosa JS, Xu Y, Davidson N, Kovacs GT, Barres BA. Retinal ganglion cells do not extend axons by default: promotion by neurotrophic signaling and electrical activity. *Neuron*. 2002;33:689–702.
10. Chen DF, Tonegawa S. Why do mature CNS neurons of mammals fail to re-establish connections following injury–functions of bcl-2. *Cell Death Differ*. 1998;5:816–822.
11. Geremia NM, Gordon T, Brushart TM, Al-Majed AA, Verge VM. Electrical stimulation promotes sensory neuron regeneration and growth-associated gene expression. *Exp Neurol*. 2007;205:347–359.
12. Brushart TM, Hoffman PN, Royall RM, Murinson BB, Witzel C, Gordon T. Electrical stimulation promotes motoneuron regeneration without increasing its speed or conditioning the neuron. *J Neurosci*. 2002;22:6631–6638.
13. Hamid S, Hayek R. Role of electrical stimulation for rehabilitation and regeneration after spinal cord injury: an overview. *Eur Spine J*. 2008;17:1256–1269.
14. Gordon T, Udina E, Verge VM, de Chaves EI. Brief electrical stimulation accelerates axon regeneration in the peripheral nervous system and promotes sensory axon regeneration in the central nervous system. *Motor Control*. 2009;13:412–441.
15. Corredor RG, Trakhtenberg EF, Pita-Thomas W, Jin X, Hu Y, Goldberg JL. Soluble adenylyl cyclase activity is necessary for retinal ganglion cell survival and axon growth. *J Neurosci*. 2012;32:7734–7744.
16. Meyer-Franke A, Wilkinson GA, Kruttgen A, et al. Depolarization and cAMP elevation rapidly recruit TrkB to the plasma membrane of CNS neurons. *Neuron*. 1998;21:681–693.
17. Schmid H, Herrmann T, Kohler K, Stett A. Neuroprotective effect of transretinal electrical stimulation on neurons in the inner nuclear layer of the degenerated retina. *Brain Res Bull*. 2009;79:15–25.
18. Meyer-Franke A, Kaplan MR, Pfrieger FW, Barres BA. Characterization of the signaling interactions that promote the survival and growth of developing retinal ganglion cells in culture. *Neuron*. 1995;15:805–819.
19. Shen S, Wiemelt AP, McMorris FA, Barres BA. Retinal ganglion cells lose trophic responsiveness after axotomy. *Neuron*. 1999;23:285–295.
20. Goldberg JL. Role of electrical activity in promoting neural repair. *Neurosci Lett*. 2012;519:134–137.
21. Stiles TL, Kapiloff MS, Goldberg JL. The role of soluble adenylyl cyclase in neurite outgrowth. *Biochim Biophys Acta*. 2014;1842:2561–2568.
22. Buonanno A, Fields RD. Gene regulation by patterned electrical activity during neural and skeletal muscle development. *Curr Opin Neurobiol*. 1999;9:110–120.
23. Corredor RG, Goldberg JL. Electrical activity enhances neuronal survival and regeneration. *J Neural Eng*. 2009;6:055001.
24. Fields RD, Neale EA, Nelson PG. Effects of patterned electrical activity on neurite outgrowth from mouse sensory neurons. *J Neurosci*. 1990;10:2950–2964.

25. Yin YQ, Cui Q, Li YM, et al. Macrophage-derived factors stimulate optic nerve regeneration. *J Neurosci*. 2003;23:2284–2293.
26. Cui Q, Yip HK, Zhao RCH, So KF, Harvey AR. Intraocular elevation of cyclic AMP potentiates ciliary neurotrophic factor-induced regeneration of adult rat retinal ganglion cell axons. *Mol Cell Neurosci*. 2003;22:49–61.
27. Snow RL, Robson JA. Ganglion-cell neurogenesis migration and early differentiation in the chick retina. *Neuroscience*. 1994;58:399–409.
28. Gaublomme D, Buyens T, Moons L. Automated analysis of neurite outgrowth in mouse retinal explants. *J Biomol Screen*. 2013;18:534–543.
29. Gavrikov KE, Dmitriev AV, Keyser KT, Mangel SC. Cation-chloride cotransporters mediate neural computation in the retina. *Proc Natl Acad Sci U S A*. 2003;100:16047–16052.
30. Farajian R, Pan F, Akopian A, Volgyi B, Bloomfield SA. Masked excitatory crosstalk between the ON and OFF visual pathways in the mammalian retina. *J Physiol*. 2011;589:4473–4489.
31. Stellwagen D, Shatz CJ, Feller MB. Dynamics of retinal waves are controlled by cyclic AMP. *Neuron*. 1999;24:673–685.
32. Blankenship AG, Ford KJ, Johnson J, et al. Synaptic and extrasynaptic factors governing glutamatergic retinal waves. *Neuron*. 2009;62:230–241.
33. Bi AD, Cui JJ, Ma YP, et al. Ectopic expression of a microbial-type rhodopsin restores visual responses in mice with photoreceptor degeneration. *Neuron*. 2006;50:23–33.
34. Moritoh S, Tanaka KF, Jouhou H, Ikenaka K, Koizumi A. Organotypic tissue culture of adult rodent retina followed by particle-mediated acute gene transfer in vitro. *PLoS One*. 2010;5:e12917.
35. Meier R, Egert U, Aertsen A, Nawrot MP. FIND—a unified framework for neural data analysis. *Neural Netw*. 2008;21:1085–1093.
36. Hennig MH, Adams C, Willshaw D, Sernagor E. Early-stage waves in the retinal network emerge close to a critical state transition between local and global functional connectivity. *J Neurosci*. 2009;29:1077–1086.
37. Maccione A, Hennig MH, Gandolfo M, et al. Following the ontogeny of retinal waves: pan-retinal recordings of population dynamics in the neonatal mouse. *J Physiol*. 2014;592:1545–1563.
38. Blankenship AG, Hamby AM, Firl A, et al. The role of neuronal connexins 36 and 45 in shaping spontaneous firing patterns in the developing retina. *J Neurosci*. 2011;31:9998–10008.
39. Dunn TA, Wang CT, Colicos MA, et al. Imaging of cAMP levels and protein kinase A activity reveals that retinal waves drive oscillations in second-messenger cascades. *J Neurosci*. 2006;26:12807–12815.
40. Blankenship AG, Feller MB. Mechanisms underlying spontaneous patterned activity in developing neural circuits. *Nat Rev Neurosci*. 2010;11:18–29.
41. Tian N, Copenhagen DR. Visual stimulation is required for refinement of ON and OFF pathways in postnatal retina. *Neuron*. 2003;39:85–96.
42. Sato T, Fujikado T, Lee TS, Tano Y. Direct effect of electrical stimulation on induction of brain-derived neurotrophic factor from cultured retinal Müller cells. *Invest Ophthalmol Vis Sci*. 2008;49:4641–4646.
43. Balkowiec A, Katz DM. Activity-dependent release of endogenous brain-derived neurotrophic factor from primary sensory neurons detected by ELISA in situ. *J Neurosci*. 2000;20:7417–7423.
44. Freeman DK, Eddington DK, Rizzo JF III, Fried SI. Selective activation of neuronal targets with sinusoidal electric stimulation. *J Neurophysiol*. 2010;104:2778–2791.
45. West AE, Chen WG, Dalva MB, et al. Calcium regulation of neuronal gene expression. *Proc Natl Acad Sci U S A*. 2001;98:11024–11031.
46. Zhang LI, Poo MM. Electrical activity and development of neural circuits. *Nat Neurosci*. 2001;4(suppl):1207–1214.
47. Martinez J, Stessin AM, Campana A, et al. Soluble adenylyl cyclase is necessary and sufficient to overcome the block of axonal growth by myelin-associated factors. *J Neurosci*. 2014;34:9281–9289.
48. Deogracias R, Espliguero G, Iglesias T, Rodriguez-Pena A. Expression of the neurotrophin receptor trkB is regulated by the cAMP/CREB pathway in neurons. *Mol Cell Neurosci*. 2004;26:470–480.
49. Miller CA, Robinson BK, Rubinstein JT, Abbas PJ, Runge-Samuelson CL. Auditory nerve responses to monophasic and biphasic electric stimuli. *Hear Res*. 2001;151:79–94.
50. Rubinstein JT, Miller CA, Mino H, Abbas PJ. Analysis of monophasic and biphasic electrical stimulation of nerve. *IEEE Trans Biomed Eng*. 2001;48:1065–1070.
51. Scheiner A, Mortimer JT, Roessmann U. Imbalanced biphasic electrical stimulation: muscle tissue damage. *Ann Biomed Eng*. 1990;18:407–425.
52. Lonze BE, Ginty DD. Function and regulation of CREB family transcription factors in the nervous system. *Neuron*. 2002;35:605–623.
53. Moore DL, Goldberg JL. Multiple transcription factor families regulate axon growth and regeneration. *Dev Neurobiol*. 2011;71:1186–1211.
54. Bleckmann SC, Blendy JA, Rudolph D, Monaghan AP, Schmid W, Schutz G. Activating transcription factor 1 and CREB are important for cell survival during early mouse development. *Mol Cell Biol*. 2002;22:1919–1925.
55. Zheng JQ, Poo MM. Calcium signaling in neuronal motility. *Annu Rev Cell Dev Biol*. 2007;23:375–404.
56. Nicol X, Voyatzis S, Muzerelle A, et al. cAMP oscillations and retinal activity are permissive for ephrin signaling during the establishment of the retinotopic map. *Nat Neurosci*. 2007;10:340–347.
57. Apara A, Goldberg JL. Molecular mechanisms of the suppression of axon regeneration by KLF transcription factors. *Neural Regen Res*. 2014;9:1418–1421.
58. Moore DL, Blackmore MG, Hu Y, et al. KLF family members regulate intrinsic axon regeneration ability. *Science*. 2009;326:298–301.
59. Li SS, He QH, Wang H, et al. Injured adult retinal axons with Pten and Socs3 co-deletion reform active synapses with suprachiasmatic neurons. *Neurobiol Dis*. 2015;73:366–376.
60. Lim JH, Stafford BK, Nguyen PL, et al. Neural activity promotes long-distance, target-specific regeneration of adult retinal axons. *Nat Neurosci*. 2016;19:1073–1084.

Investigative Ophthalmology & Visual Science