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Weak electric fields serve as guidance cues that direct retinal ganglion cell axons *in vitro*



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ABSTRACT

Growing axons are directed by an extracellular electric field in a process known as galvanotropism. The electric field is a predominant guidance cue directing retinal ganglion cell (RGC) axons to the future optic disc during embryonic development. Specifically, the axons of newborn RGCs grow along the extracellular voltage gradient that exists endogenously in the embryonic retina (Yamashita, 2013 [8]). To investigate the molecular mechanisms underlying galvanotropic behaviour, the quantification of the electric effect on axon orientation must be examined. In the present study, a culture system was built to apply a constant, uniform direct current (DC) electric field by supplying an electrical current to the culture medium, and this system also continuously recorded the voltage difference between the two points in the medium. A negative feedback circuit was designed to regulate the supplied current to maintain the voltage difference at the desired value. A chick embryo retinal strip was placed between the two points and cultured for 24 h in an electric field in the opposite direction to the endogenous field, and growing axons were fluorescently labelled for live cell imaging (calcein-AM). The strength of the exogenous field varied from 0.0005 mV/mm to 10.0 mV/mm. The results showed that RGC axons grew in the reverse direction towards the cathode at voltage gradients of ≥ 0.0005 mV/mm, and straightforward extensions were found in fields of ≥ 0.2 –0.5 mV/mm, which were far weaker than the endogenous voltage gradient (15 mV/mm). These findings suggest that the endogenous electric field is sufficient to guide RGC axons *in vivo*.

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1. Introduction

Growing axons are directed by an extracellular electric field in a process known as galvanotropism [1]. The galvanotropic behaviour of nerve cells has been demonstrated in cultures by applying electrical currents [2–7]. Recently, an endogenous voltage gradient has been shown to exist in embryonic retinas and the electric field has been suggested to direct the axons of newborn retinal ganglion cells (RGCs) to the future optic disc [8]. To investigate the molecular mechanisms mediating galvanotropic behaviour, the electric effect on axon orientation must be examined quantitatively. The field strength for directing axons was determined in previous studies; however, discrepancies in the electric field strength for orienting neurites were found. Ingvar [2], Karssen and Sager [3], and Siskin and Smith [4] used weak electric fields (less than 0.01 mV/mm) to demonstrate galvanotropic effects, and in contrast, Marsh and

Beams [5], Jaffe and Poo [6], and Hinkle et al. [7] have shown that the threshold field strengths for the cathodal orientation of neurites are 50–60 mV/mm, 70 mV/mm, and 7 mV/mm, respectively. In the latter studies, uniform electric fields were used, and the experimental procedures were described in detail; therefore, these threshold values appear to be more reliable. However, the weak electric fields applied in the former studies should be re-examined under more precisely controlled conditions.

In the present study, a culture system was designed to create a constant, uniform DC electric field by continuously recording a voltage difference between the two points in the culture medium, and also by using a negative feedback circuit to regulate supplied currents. The results showed that weak electric fields (≥ 0.0005 mV/mm) were effective for the cathodal orientation of RGC axons.

2. Materials and methods

2.1. Preparation of retinal strips

A neural retina was isolated from an embryonic day 6 (E6) chick embryo in a Ca^{2+} - Mg^{2+} -free Hank's solution. The retina

Abbreviations: DC, direct current; DIC, differential interference contrast; DMEM, Dulbecco's modified Eagle medium; E, embryonic day; FBS, foetal bovine serum; HEPES, 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid; RGC, retinal ganglion cell; sCMOS, scientific complementary metal oxide semiconductor

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was spread on a black membrane filter (Sartrius) with the inner side up. The retina-membrane assembly was cut into a strip in the nasotemporal direction (*i.e.*, perpendicular to the optic fissure) along the lines 1 mm and 2 mm dorsal to the optic nerve head, so that the width of the retinal strip was 1 mm. The temporal and nasal portions of the strip were trimmed off the central part, of which length was less than 3 mm. To identify the directions of the retinal strip, the temporal end was cut perpendicularly to the dorsal and ventral edges, and the nasal end was cut obliquely to them to make the ventral edge longer. Other details are described in Halfter et al. [9].

2.2. A culture chamber and solutions

A round culture chamber was made from an acrylic disc (25 mm in diameter, 2 mm in thickness). A trough was made at the middle of the disc. The size and shape of the trough are shown in [Supplementary Fig. S1](#). A cover glass (25 mm in diameter) was secured to either side of the acrylic disc with silicone grease to make the bottom of the trough. The chamber was put on an ice-cooled aluminium block. A retina-membrane strip was put on the bottom of the trough with the retinal side up. The dorso-ventral axis of the retina was adjusted to be parallel to the long axis of the trough by using a fine tungsten needle. The temporal edge of the retina-membrane strip was contacted with the wall of the trough. Then, the retinal strip was embedded in Matrigel[®] (Corning) containing 20% FBS and 4% chicken serum (Gibco). Another cover glass (15 mm in diameter) was secured over the top of the trough ([Supplementary Fig. S1B](#)). After incubation for 5 min in a humidified air at 37 °C for gelling, the trough (100 μ L in volume) was filled with DMEM containing 10% FBS and 2% chicken serum. The culture chamber was put on an acrylic disc (30 mm in diameter, 10 mm in thickness), which was placed in a well of a 6-well culture plate. Distilled water was injected into this well around the acrylic disc. Then, a pair of glass tubes (3 mm in diameter) containing the culture medium was inserted in the both ends of the trough. The other end of each tube was placed in the neighbouring well filled with excess culture medium (14 mL in volume). This well was electrically connected to another well containing 25 mM-HEPES-buffered DMEM by using an agar-salt bridge (saline gelled with 1% agar). A schematic diagram of the whole circuit is shown in [Supplementary Fig. S2A](#). The pH of the solution in each well was checked with phenol red. The remaining one well of the 6-well culture plate was filled with distilled water. The 6-well filled culture plate was put in an airtight jar (2.5 L in volume) with a bag of CULTUREPAL[®] (Mitsubishi Gas Chemical Company, Inc., Tokyo), which produced 5% CO₂ in the jar. The jar was put in a dry incubator for 24 h at 37.5–37.9 °C.

2.3. Electrodes

A pair of Ag/AgCl disc electrodes was placed in the wells containing HEPES-buffered DMEM. Two monitor electrodes were inserted in the culture medium at the edges of the top cover glass, so that the distance between them was 15 mm (a and b in [Supplementary Fig. S1B](#) and [Fig. S2A](#)). The monitor electrode was fabricated by pulling a glass tube (3 mm in diameter). The tip diameter of the glass pipette was about 0.3 mm. It was filled with saline gelled with 1% agar. An Ag/AgCl pellet connected to an Ag/AgCl wire was inserted into the agar-salt gel. It was connected to a voltage follower (LMC662CN) of a high input resistance (> 1 T Ω) and an ultra low bias current (2 fA). LMC662CN was placed in the airtight jar. Connectors to the Ag/AgCl disc electrodes and LMC662CN were assembled in the wall of the jar in an airtight manner using silicone grease.

2.4. A negative feedback circuit

[Supplementary Fig. S2B](#) shows a diagram of the negative feedback circuit to regulate the voltage difference between the two points in the culture medium (a and b). R2 represents the resistance between them. R1 and R3 include all the other resistances. The voltage difference was continuously recorded by a differential amplifier, of which output was connected to the inverting input (–) of a high-voltage operational amplifier (BB3582J). A reference voltage (V_{ref}) was supplied to the non-inverting input (+) of the operational amplifier. A feedback current (I) passed through R1, R2, R3, and a feedback resistor (R_f). In this way, the output voltage of the differential amplifier was kept to be equal with V_{ref} . The differential amplifier had an offset output voltage, which was due to differences in offset voltages of the monitor electrodes and the voltage followers. Such an offset output voltage was automatically canceled by subtracting the output voltage that was sampled every 100 s during the period (0.5 s) when the feedback current loop was opened ($I=0$) by using a pulse-driven relay switch. Thus, the duty cycle of the current supplied to the culture medium was 99.5%. [Supplementary Fig. S3](#) shows the details of the whole circuit. The pulse-driven relay opens SW 2 and closes SW 1 during the sampling period. Hum Bug (Quest Scientific, North Vancouver, Canada) was used to eliminate line noises. DC amplifier included a low-pass filter (500 Hz).

2.5. Live cell staining and fluorescence imaging

After incubation for 24 h, the culture chamber was placed on the stage of an upright microscope (BX51WI, Olympus). The trough of the chamber was perfused with HEPES-buffered DMEM without phenol red for 5 min at 2 mL/min, and then with the same solution containing calcein-AM (10 μ M) for 5 min at room temperature. After waiting for 30 min, the calcein-AM-containing solution was washed out for 5 min with HEPES-buffered DMEM. A Nipkow-type confocal scanner (CSU10, Yokogawa, Kanazawa, Japan) and an sCMOS camera (ORCA[®]-Flash4.0 V2, Hamamatsu photonics, Hamamatsu, Japan) were used for fluorescence imaging.

3. Results

3.1. Control cultures without supplying currents

The orientation of RGC axons growing *in vitro* mirrors the pattern of axon growth during normal development *in vivo* [9]. For example, RGC axons emerge from the side of a retinal strip originally closest to the optic nerve head. In the present study, retinal strips were explanted from the segment that was originally dorsal to the optic nerve head; therefore, numerous axons emerged from the ventral side of the retinal strip ([Fig. 1A](#)). For live cell imaging, the growing axons and cells in the retinal strip were labelled with a fluorescent dye (calcein-AM, [Fig. 1B](#)). The axons continued to grow ventrally, as long as 500 μ m, as revealed by fluorescence imaging ([Fig. 1C](#)). On the dorsal side of the retinal strip, growing axons were rarely observed ([Fig. 1D](#)). In eight control cultures, the retinal strip did not produce more than ten axons that extended over 100 μ m on the dorsal side; however, multiple axons extended upwards into the Matrigel[®] above the retinal strip ([Fig. 1E](#)).

3.2. Test cultures with supplying currents

To identify the effects of an exogenous electric field on axon orientation the dorsal side of the retinal strip was placed facing the cathode. This direction of the electric field was opposite to the direction of the endogenous field, which is ventrally directed

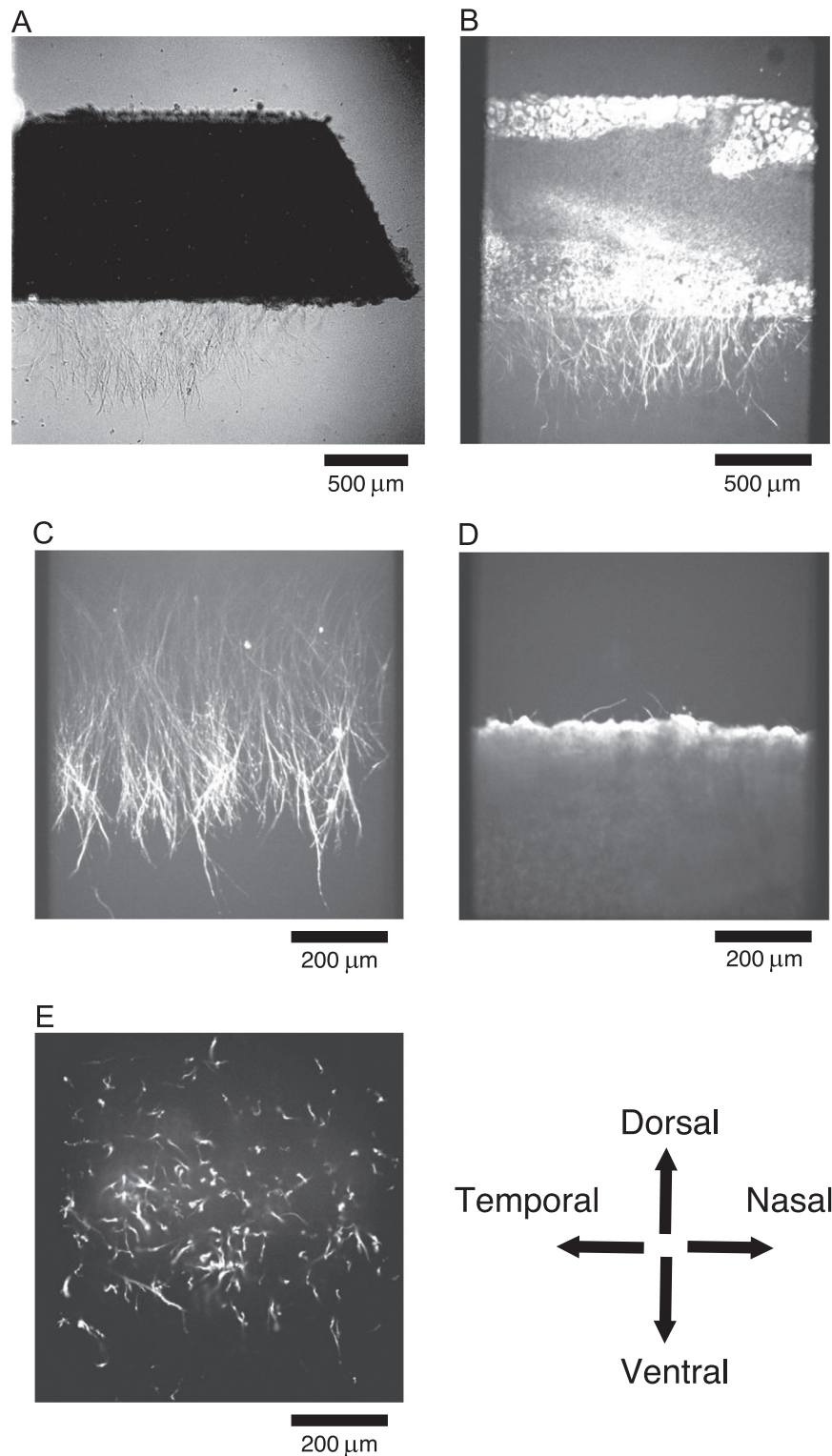


Fig. 1. A retinal strip cultured for 24 h without supplying electrical currents: (A) DIC image, (B) fluorescence image stained with calcein-AM, (C) the ventral side, (D) the dorsal side, and (E) axons extending upwards into Matrigel[®] above the retinal strip. (A)–(E) were taken from the same retinal strip.

in vivo [8]. The strength of the reverse electric field varied from 0.0005 mV/mm to 10.0 mV/mm, and its effects on axon orientation were examined by fluorescence imaging. In the reverse electric field, more than ten axons, some of which appeared to be fasciculated, extended over 100 μm on the dorsal side (Fig. 2A), and these reverse outgrowths were observed with a 0.0005 mV/mm electric field (Fig. 2B). Above the retinal strip, dorsally growing axons and/or an axon plexus was observed (Fig. 2C).

Straightforward extensions were found in fields of ≥ 0.2 –0.5 mV/mm (Fig. 2D). Table 1 summarizes the number of retinal strips cultured in the reverse electric fields and shows the very wide range of strengths of effective electric fields applied in this study. Despite the reverse electric field, numerous axons emerged from the ventral side of the retinal strips as in the control culture; however, a few axons had wavy trajectories or displayed turns towards the ventral side (Supplementary Fig. S4).

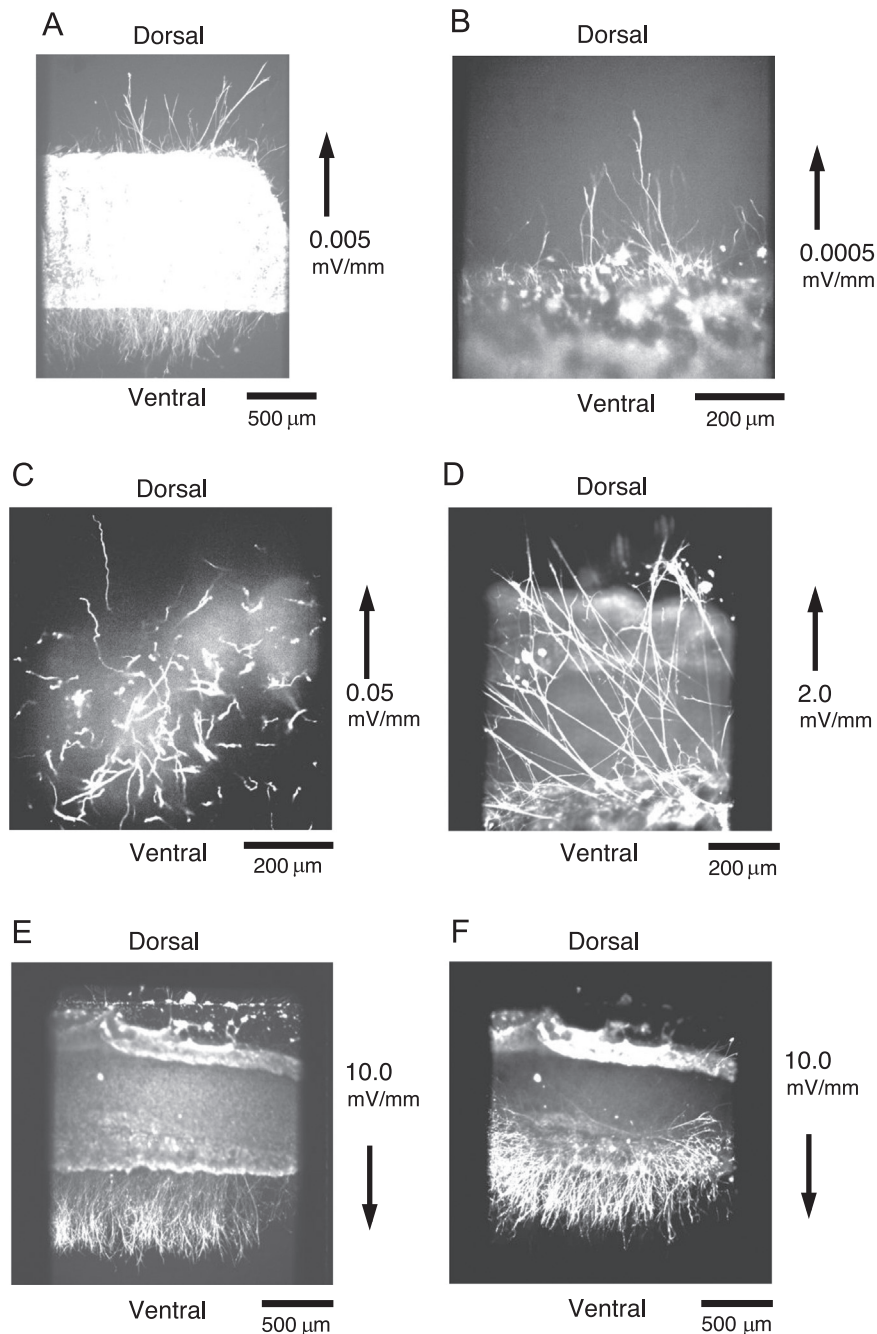


Fig. 2. Retinal strips cultured for 24 h with supplying electrical currents. Directions and strengths of electric fields are indicated on the right side of each panel: (A) outgrowing axons on the dorsal and ventral sides of a retinal strip. (B) Axons extending from the dorsal side of a retinal strip. (C) Dorsally growing axons and a plexus of axons in Matrigel[®] above a retinal strip. (D) Axons extending straightforwardly in Matrigel[®] above a retinal strip. (E) Outgrowing axons on the ventral side of a retinal strip. (F) Axons growing ventrally in Matrigel[®] above the retinal strip shown in (E).

In cultures with an electrical current applied in the forward direction from the dorsal side to the ventral side, no difference was found in axon orientation on either side of the retinal strips as compared with the control culture (Fig. 2E). However, one marked difference included axons extending upwards in the Matrigel[®] and shifting ventrally above the retinal strip. These ventral shifts were observed in electric fields of 0.0005 mV/mm. With increases in the field strength, the axons in the Matrigel[®] extended ventrally above the retinal strip (Fig. 2F).

4. Discussion

A major advance in the present study is not only about revealing the effects of electric fields on directionality of the growing axons but also about establishing a reliable method to investigate the electric field *in vitro*, which enables us to investigate possible relationship between biochemical guidance cues and physicochemical effects of the electric fields. The method developed in the present study may also be useful to

Table 1
Effects of reverse electric fields on axon orientation.

Field strength (mV/mm)	Numbers of retinal strips		
	Total cultured	With axonal plexus ^a	With dorsal axons ^b
0	8	0/8	0/8
0.0005	3	3/3	3/3
0.001	2	2/2	2/2
0.002	2	2/2	2/2
0.005	2	2/2	2/2
0.01	2	2/2	2/2
0.02	2	2/2	1/2
0.05	2	2/2	2/2
0.1	2	2/2	2/2
0.2	3	3/3	3/3
0.5	2	2/2	2/2
1.0	2	2/2	2/2
2.0	2	2/2	2/2
5.0	2	2/2	2/2
10.0	2	2/2	2/2

^a Retinal strips with a plexus in Matrigel[®] above the retinal strips.

^b Retinal strips with more than ten outgrowing axons extending over 100 μ m on the dorsal side of the retinal strips.

investigate the molecular mechanisms mediating galvanotropic behaviour.

In the control culture, RGC axons emerged from the ventral side of the retinal strips following the original orientation. Halfter et al. [9] have suggested that the axons were already oriented in this direction before the explant, and they continued to grow in this direction, explaining the maintenance of the polarity of outgrowing axons. RGC axons most likely do not change their course as they lose sensitivity to an electric field after being oriented by the initial cue. Axons of RGCs that were born just before or after the explant do not typically grow ventrally as these axons were not oriented by the endogenous cue. Alternatively, these RGC axons extend upwards into Matrigel[®] above the retinal strip (Supplementary Fig. S5A), which may be caused by growth along an intrinsic electric field generated by the retinal strip itself. Ionic currents due to Na⁺ transport by retinal neuroepithelial cells flow out from the inner side of the retina [8], and these currents may continue to flow upwards into the Matrigel[®] since the inner side of the retinal strip was up.

In the test culture, voltage gradients were created against the endogenous electric field to distinguish between the effects of the exogenous and endogenous fields. The reverse electric field caused the reverse outgrowths of RGC axons, which emerged from the dorsal side of the retinal strips. Above the retinal strip, a plexus was formed by the axons that had extended upwards (Supplementary Fig. S5B). These axons may be perturbed by the exogenous electric field to different degrees, which may result in plexus formation. With increases in the field strength, the upper axons directly extended towards the cathode (Supplementary Fig. S5C). In the culture with an electrical current in the forward direction, the upper axons shifted ventrally above the retinal strip (Supplementary Fig. S5D). With increases in the field strength, these axons extended ventrally together with the axons that had emerged from the ventral side of the retinal strip following the original orientation (Supplementary Fig. S5E).

The present results showed that voltage gradients ranging from 0.0005 mV/mm to 10.0 mV/mm (~4 orders of magnitude) cause cathodal orientations of RGC axons (Table 1). No clear difference was noticed among the strengths in Table 1. This finding sheds light on the previous studies that showed significant effects of

weak electric fields on neurite outgrowths. Ingvar [2], Karssen and Sager [3], and Sisken and Smith [4] used 2–4 nA, 10 nA and 11.5 nA/mm², respectively. Marsh and Beams [5] presumed that the current density used by Ingvar was 1 nA/mm² and that the current density used by Karssen and Sager was less than 10 nA/mm². Assuming that the specific resistance of the culture medium is 54 Ω cm [5], the voltage gradient was 0.0005 mV/mm in the Ingvar work, was applied at less than 0.005 mV/mm by Karssen and Sager, and was 0.006 mV/mm in Sisken and Smith study. In contrast, Marsh and Beams [5], Jaffe and Poo [6], and Hinkle et al. [7] showed that the threshold field strengths for neurite cathodal orientation were 50–60 mV/mm, 70 mV/mm, and 7 mV/mm, respectively. The apparent discrepancy in the field strengths for galvanotropic effects between these two groups of studies may be due to various factors such as non-uniformity of the current density in the culture medium, the species and developmental stages of animals used in the studies, and differences in nerve tissue preparation, culture periods, types of neurites (axons or dendrites), and substrates for neurite outgrowth. In the present study, retinas of embryonic chicks at E6 were used, while Marsh and Beams [5] used fragments of medulla of chick embryos at later stages (E7–10). Jaffe and Poo [6] also used fragments of embryonic chick dorsal root ganglia at the same stage (E7–9). It is likely that the sensitivity to electric fields declines with developmental stages. Hinkle et al. [7] used dissociated cells of amphibian embryos, which were cultured on non-polarizable tissue culture plastic, without the support of culture substrates such as collagen or laminin. These molecules not only support axon growth as a scaffold but also play an important role in axon orientation, because RGC axons grow randomly when the retina is treated with collagenase or trypsin [9], and also because Matrigel[®], which also contains collagen and laminin, is necessary for axons to extend along electric fields. The present results showed that voltage gradients ranging from 0.0005 mV/mm to 10.0 mV/mm had significant effects on axon orientation under the same experimental conditions. These results may support the differences in the various studies and also suggest that such a wide dynamic range is a remarkable characteristic of galvanotropism. The present study ultimately shows that the endogenous electric field (15 mV/mm [8]) is sufficient for the correct guidance of RGC axons *in vivo*.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at <http://doi:10.1016/j.bbrep.2015.08.022>.

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