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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 $\geq$0.0005 mV/mm, and straightforward extensions were found in fields of $\geq$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*.

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 Sisken 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 ($\geq$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 μL 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% CO_2 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 TΩ) 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 μM) 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 μm, 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 μm 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