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Electric axon guidance in embryonic retina: Galvanotropism revisited

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ABSTRACT

In addition to well-known mechanisms of chemical guidance, growing axons in the nervous system are directed by an extracellular electric field in a process known as galvanotropism. The galvanotropic behavior of nerve cells *in vitro* was first demonstrated as long ago as 1920. However, it remains unknown whether embryonic nerve tissues generate a similar electric field in order to guide growing axons. The present study reveals that an extracellular voltage gradient exists in the embryonic retina and that this gradient guides the axons of newborn retinal ganglion cells towards their targets. These findings indicate an important role for galvanotropism in the initial orientation of axons that extend over long distances, and provide insight into the mechanisms underlying the proper extension of developing axons in the embryonic brain.

1. Introduction

The prevailing belief is currently that growing axons are guided by attraction or repulsion in response to various chemical signals (chemotropism) during brain development. However, it has also been shown that cells may respond to an extracellular electric field by changing their morphology along the voltage gradient in a process termed 'galvanotropism' [1]. Galvanotropic behavior in nerve cells was demonstrated in cultured cells as early as 1920 [2]. The application of an extracellular electric field directs growing axons towards the cathode under a voltage gradient as low as 7 mV/mm [3]. However, since previous studies used culture systems to which exogenous electric fields were applied, it remains unknown whether electric gradients are normally generated in the embryonic nervous system, or whether developing axons use galvanotropism to find their targets *in vivo*. The present study reveals that an extracellular voltage gradient exists in the embryonic retina, and that axons of newborn retinal ganglion cells (RGCs) are directed towards their targets via a galvanotropic mechanism.

2. Materials and methods

2.1. Preparation of retina

The optic cup was isolated from a chick embryo incubated for three days (E3) at 38 °C. The optic cup was positioned on the bottom of a recording chamber (volume, 0.2 mL) with the inner side up. The recording chamber was mounted on the fixed stage of an upright microscope (BX51WI, Olympus, Tokyo, Japan) under a water immersion objective (×100 or ×60), and was perfused at 2 mL/min with a normal bath solution (NBS) containing (mM); 137 NaCl, 5 KCl, 2.5 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 22 glucose, buffered to pH 7.3 by adding NaOH.

2.2. Electrical recording

Extracellular potential was recorded immediately inside the inner limiting membrane of the retinal neuroepithelium with a glass microelectrode filled with 2 M NaCl (electrode resistance: 108–200 MΩ) using a conventional preamplifier for intracellular recording. Current pulses (40 pA, 5 mS in duration) were passed through the electrode at 0.5 S-interval to monitor the resistance between the electrode and the bath solution. Recordings were made at 36–38 °C.

2.3. In ovo injection of amiloride and retinal cultures

Amiloride (1 mM)-containing saline (0.1 μL) was injected into optic vesicles *in ovo* at E2 using a glass micropipette (tip diam-

Abbreviations: C, central; D, dorsal; DC, direct current; DMEM, Dulbecco's modified Eagle medium; DMSO, dimethyl sulfoxide; E, embryonic day; FBS, fetal bovine serum; ICCD, intensified charge coupled device; N, nasal; RGC, retinal ganglion cell; T, temporal; V, ventral.

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eter: 10 μm). The egg was resealed and incubated for two days. Retinas isolated from E3 and E4 chick embryos were cultured for 24 h. The culture medium (DMEM) contained 20% FBS and 10 μM amiloride. Amiloride was dissolve in DMSO at 10 mM.

2.4. Fluorescence microscopy

The retina was loaded with calcein-AM (10 μM in NBS) for 30 min at room temperature to stain live cells and axons. A Nipkow-type confocal scanner (CSU10, Yokogawa, Kanazawa, Japan) and a cooled ICCD video camera were used for fluorescence imaging.

3. Results

Optic cups (precursors to the retina) were isolated from chick embryos incubated for three days (E3). Extracellular potentials were recorded from the dorsal (D), temporal (T), nasal (N), central (C), and ventral (V) parts of the optic cup, immediately inside the inner limiting membrane through which the axons of newborn RGCs travel. Upon penetration of the inner limiting membrane from the vitreous side with a microelectrode (Supplementary Fig. S1), a positive direct current (DC) potential was recorded, with an increase in the resistance between the electrode and the bath solution (Supplementary Fig. S2). The amplitude of the DC potential was largest at the dorsal (8 mV), and almost null at the ventral (<1 mV) region of the optic cup. The amplitude of the potential was 4–5 mV at the temporal and nasal regions, and 3–4 mV at the central part of the optic cup (Fig. 1A, total number of recordings: 251 from 26 retinas). Fig. 1B illustrates the direction of the voltage gradient, indicating that the DC potential decreases towards the ventral part of the optic cup.

RGCs are first born in the central portion of the optic cup [4]. The axons of these newborn RGCs extend towards the ventral portion of the optic cup, where the future optic disc (the exit of axons from the eye) is formed (Fig. 1A, inset, modified from [4]). Thus, the observed direction of the voltage gradient appears to correspond to the known initial course of newborn RGC axons. In addition, the relative steepness of the voltage gradient (approximately 14–15 mV/mm at the central portion) exceeds the threshold for galvanotropic behavior of nerve cells described previously (7 mV/mm, [3]).

It has been shown previously that, when the positive DC potential is nullified focally at a point other than the ventral part, RGC axons extend towards the null point [5]. In fact, when a small piece of retina is removed by aspiration to make a hole, at which the DC potential would be extinguished while creating steep voltage gradients around the hole, numerous axons head to the center of the lesion sites, as have been shown in retinal organotypic cultures (Supplementary Fig. S3, reproduced from [5]). Moreover, I found that the Na^+ channel antagonist amiloride (10 μM) suppressed the positive DC potential (see Supplementary Discussion), and that the injection of amiloride into the optic vesicles (optic cup precursors) *in ovo* at E2 resulted in the disruption of RGC axon path-finding in the central portion of the retina after two days incubation (Fig. 2A and B). Organotypic cultures of retina are available from E3, and the normal traveling of RGC axons is observed in these cultures [6]. When E3 retinas were cultured in the presence of amiloride (10 μM) for 24 h, RGC axons exhibited erroneous path-finding at the peripheral regions (Fig. 2D and E), while the normal traveling was observed at the central portion (Fig. 2F). Aberrant axons were also observed in the similar cultures of E4 retinas at the peripheral part of the cultured retina after 24 h (Fig. 2G and H), while the normal axon traveling was observed at the central portion (Fig. 2I). These results may indicate that amiloride is effective only at the onset of axon extension since RGCs are born in the central-to-peripheral order as the retina expands [7]. I also found that when an exogenous electric field ($\geqslant$0.17 mV/mm) was applied to a cultured E4 retina, the cells which were stained with the live cell marker calcein-AM became rounded and dispersed, while the inner limiting membrane was lost (data not shown). Together, these data indicate that the presence of an endogenous electrical potential is necessary for the correct guidance of RGC axons.

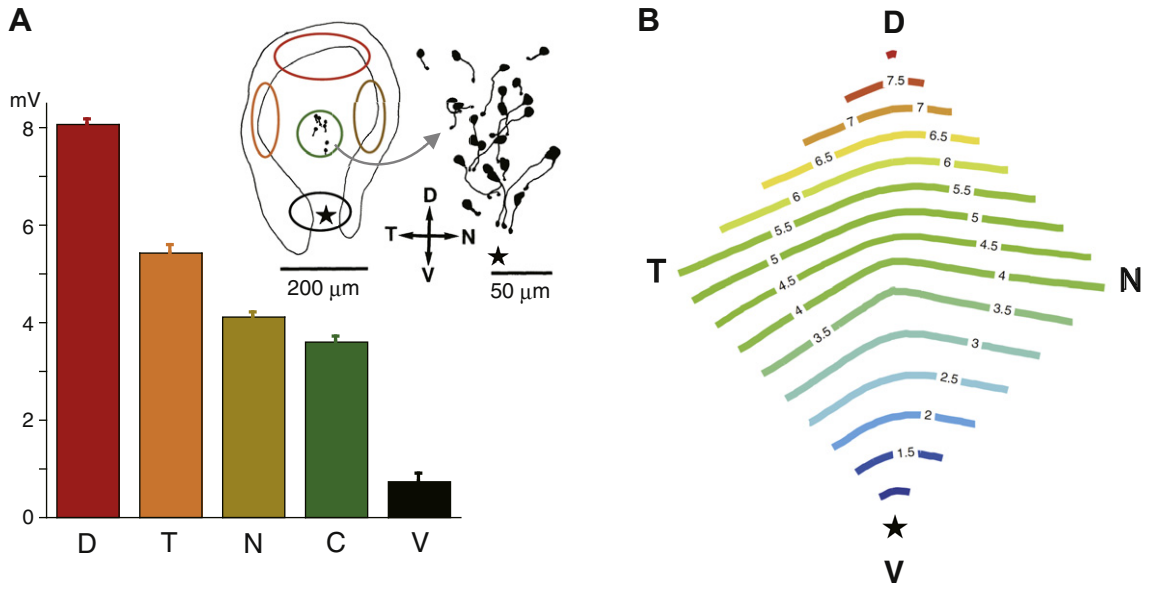

Fig. 1. (A) Amplitudes of positive DC potential recorded from dorsal (D), temporal (T), nasal (N), central (C), and ventral (V) portion of optic cup, indicated as mean ± SEM, numbers of recordings: 41 (D); 52 (T); 72 (N); 53 (C); 33 (V). Inset shows the five recording areas in optic cup, and the first axon-bearing ganglion cells, which appear in the central part. All of the axons grow ventrally towards the future optic disc (★). Modified from figure 3 in [4]. (B) The direction of the voltage gradient is indicated by rainbow scale.

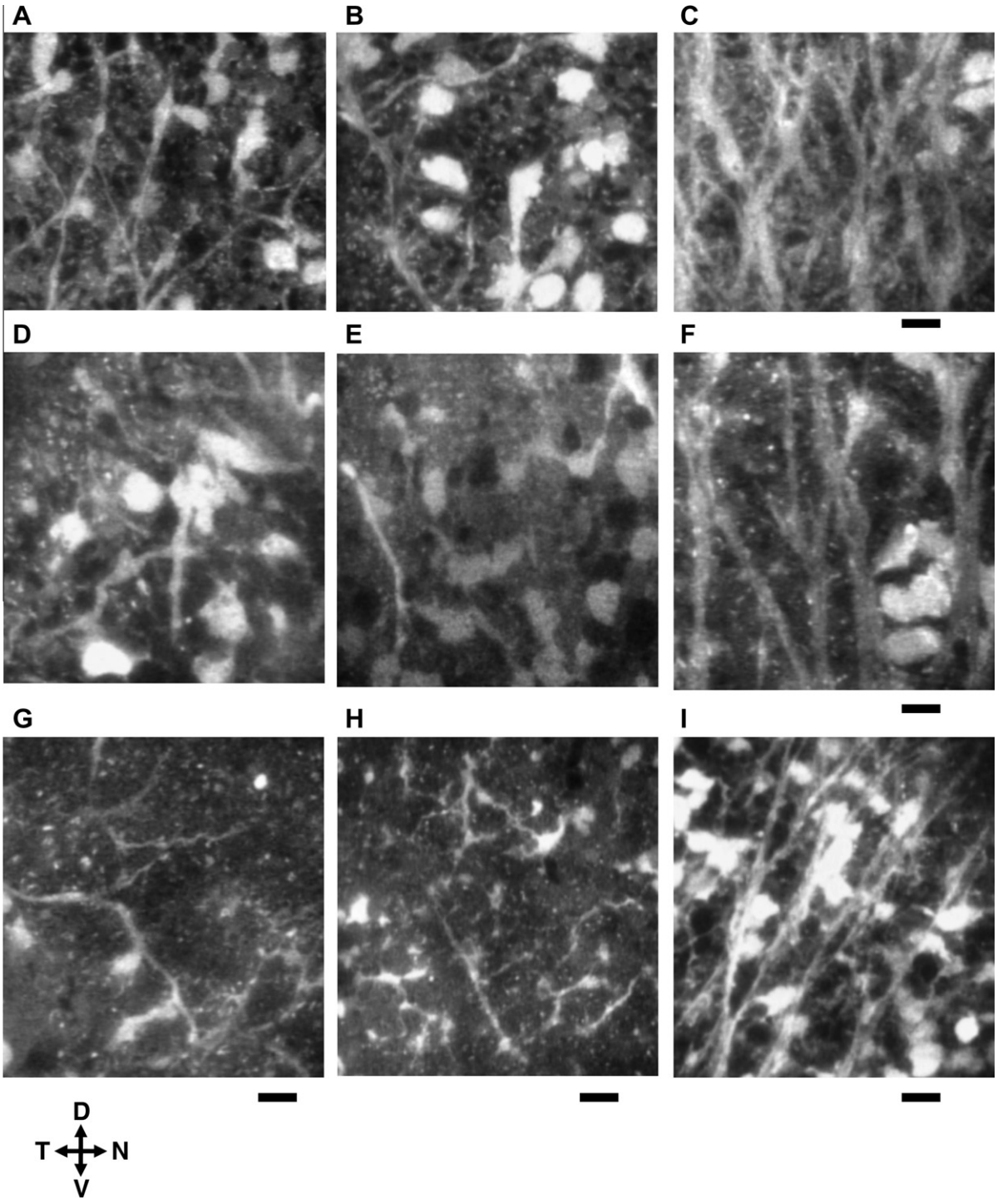

Fig. 2. Effects of amiloride on traveling of RGC axons. (A–C) Central portion of E4 retinas after injection of amiloride (A, B) or vehicle alone (C) into optic vesicles *in ovo* at E2. (D–F) E3 retinas were cultured in the presence of amiloride (10 μM) for 24 h. Dorso-temporal (D), temporal (E), and central (F) portion of the cultured retinas. (G–I) E4 retinas were cultured in the presence of amiloride (10 μM) for 24 h. Dorso-temporal (G), dorso-nasal (H), and central (I) portion of the cultured retinas. (H) and (I) were taken from the same retina. Scale bar: 10 μm.

4. Discussion

The molecular mechanisms involved in guiding RGC axons to the optic disc are not well understood [8]. Although netrin has been proposed to be an axon guidance molecule in the retina, RGC axons orient and extend towards the optic disc even in the absence of this molecule [9]. However, axons in these netrin-deficient retinas exhibit erroneous path-finding within the optic disc, and fail to exit via the optic nerve [9]. Thus, netrin-mediated signaling is essential for the local guidance of axons at certain decisive points during their travels. Together, these findings suggest that while chemotropism is crucial for the fine-tuning or local direction of developing axons, electrical gradients may serve as long-distance path-finding cues for the general orientation of the axons. In agreement, previous studies have shown that various cellular behaviors, including axonal outgrowth, are under the influence of electrical

cues [1,3]. The present study highlights the importance of galvanotropism in the initial orientation of RGC axons, and provides insight into the mechanisms underlying the extension of axons during embryonic development.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.12.115.

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