Electric fields, contact guidance and the direction of nerve growth

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SUMMARY
Nerve orientation in response to electrical guidance cues in one direction and contact guidance cues in an orthogonal direction has been studied. Where neurites had a free choice between following contact guidance cues or electrical cues, the direction of nerve growth was determined predominantly by the vector of the applied electric field.

INTRODUCTION
The direction of nerve growth in vitro can be regulated by several extrinsic cues (e.g. Letourneau, 1982). Gradients of specific target-released chemicals may attract nerve growth (Gundersen & Barrett, 1980; Lumsden & Davies, 1983; McCaig, 1986a). A small DC electric field can direct nerve growth; nerves grow towards the cathode (Marsh & Beams, 1946; Jaffe & Poo, 1979; Hinkle, McCaig & Robinson, 1981; Patel & Poo, 1982; McCaig, 1986b). Pulsed and focal electric fields are similarly effective in directing nerve growth (Patel & Poo, 1984). The physical nature of the substrate also can determine the direction of nerve growth; nerves will follow scratches, aligned collagen fibrils or patterns of greatest adhesiveness (Ebendal, 1976; Letourneau, 1975). The extent to which any of these influences pertain in vivo is unknown; all may be involved in synergistic or opposing fashion. Interactions between two or more guidance cues therefore may be expected in vivo. Few attempts have been made to investigate interactions between known guidance cues (see Ebendal, 1976).

I have studied the behaviour of neurites faced with a choice of guidance cues. The experiments pit the nerve-orienting properties of a DC electric field of varied strength against the contact guidance properties of regular scores on the culture substratum. Given a free choice, the direction of spinal neurite growth in most cases was determined by the electric field.

METHODS
The earliest developing spinal neurites from Xenopus laevis embryos (stage 19/20 of Nieuwkoop & Faber, 1956) were used for all experiments. Cultures were prepared as described...
previously (Hinkle et al. 1981). The dorsal third of an embryo was excised and treated briefly, 10 min, with collagenase, 1 mg ml\(^{-1}\) in Steinberg's solution (composition mm\(^{-1}\): NaCl 58; KCl 0·67; Ca (NO\(_3\))\(_2\) 0·44; Mg SO\(_4\) 1·3; Tris 4·6; pH 7·9). Neural tubes were dissected out and placed in a dissociating medium for 20–30 min (divalent ion-free Steinberg's plus 0·4 mM EDTA). Cells were picked up through a fine, flame-drawn Pasteur pipette and dispersed into culture medium in the centre of a chamber formed on the base of a Falcon tissue culture dish (type 3003F). The culture medium was Steinberg's solution supplemented with 20% Liebowitz L 15 solution, 1% foetal bovine serum and 2% penicillin (5000 i.u. ml\(^{-1}\))/Streptomycin (5000 \(\mu\)g ml\(^{-1}\)) [all from Flow Laboratories, Irvine] and used at pH 7·9. The culture chamber was made with two strips of No. 1 cover glass (64 cm long) glued parallel to each other with silicone rubber, 1 cm apart. Cells were allowed 15–30 min to attach to the dish before a roof of No. 1 cover glass was applied to the chamber using silicone grease. The very shallow culture chamber thus created measured 64\(\times\)10\(\times\)0·5 mm.

Forty parallel scores had been made about 250 \(\mu\)m apart across the centre of the chamber, using a fine gauge hypodermic needle and a ruler. These ran at 90° to the long axis of the chamber. Scores were on average 50 ± 2 \(\mu\)m wide and approximately one cell diameter in depth, 30 \(\mu\)m. These formed cues for the contact guidance of neurites developing close to either side of a score.

In experimental situations an electric field also was present with a field vector along the long axis of the chamber, perpendicular to the scores. The electric field was applied through a pair of agar–salt bridges, long enough (13 cm) to eliminate any possibility of electrode products reaching the culture chamber.

Some observations were made by taking repeated photomicrographs of nerves during their first 2 to 3 h of growth as they responded to the guidance cues. Other analysis was performed on cells which had grown for 16–18 h. In some experiments the polarity of the electric field was reversed during observation. Neurones were evaluated as follows:

**Contact guidance.** This was defined as having occurred if a neurite came into contact with the edge of a score and grew along this without turning away for at least a further 50 \(\mu\)m.

**Turning or differential growth away from contact guidance.** This was defined as having occurred in neurones which had extended neurites within ‘touching distance’ of a score (the length of a filopodium, about 30 \(\mu\)m) but whose neurites either followed the score for less than 50 \(\mu\)m before turning to grow away from the score, or grew directly away from the score.

**Traversing the score.** Neurones whose neurites grew across the score into an adjacent interspace.

All measurements and growth cone filopodia counts were made from prints (\(\times\)400–500 magnification) examined under a dissecting microscope. The distribution of filopodia at growth cones in contact with a score was assessed by bissecting the growth cone with a line projecting along the direction of growth (orthogonal to the electric field) and counting filopodia on the right/left (controls) or cathodal/anodal side of the line. The distance between the edge of the phase-dark neurite tip excluding filopodia and the edge of the phase-dark score also was measured.

**RESULTS**

**Control cultures**

(1) **Contact guidance only**

In control cultures neurites may come into contact with a score to their left or to their right. Table 1 shows that in 12 culture chambers, 76% of all neurones coming into contact with a score (222/295) exhibited contact guidance of their continued growth. This was as likely to occur if the score was to the left of the cell body (76%: 99/130 neurones) as it was if the score was to the right of the cell body (75%; 123/164 neurones). Only one example of a neurite crossing a score was
seen in control cultures. There was a strong tendency for neurites that came in contact with a score to direct their growth along the edge of the score.

(2) Electric field only – no scores

Published work has shown that an electric field in the range 15–200 mV mm\(^{-1}\) caused 65–75 % of cells to grow differentially towards the cathode or to turn towards the cathode (Hinkle et al. 1981). Similar values were obtained in these experiments, with 55–73 % of neurites growing differentially or turning towards the cathode even at low field strengths, 20–30 mV mm\(^{-1}\).

Experimental cultures. Contact guidance + electric field

Table 1 shows the combined results from 18 experimental chambers in which neurones were exposed both to contact guidance and electrical guidance cues of varying strength. In total 409 neurones were analysed. Of those that came into contact with a score on the cathodal side of the cell body, 86 % (218/253) showed continued growth that followed the edge of the score, 10 % (25/253) turned or grew differentially away from cathodal-contact guidance while 4 % traversed the score to continue growing towards the cathode in the neighbouring interspace (Figs 3, 4, 5).

Of those neurones that were within one cell diameter of a score on the anodal side of the cell body, only 12 % (18/156) followed the score for more than 50 \(\mu\)m of their growth. By contrast, the vast majority 88 % (138/156) turned or grew differentially away from the score and towards the cathode (Figs 6–8). No examples of neurites crossing a score in an anodal direction were seen.

In general, neurones showed a very strong tendency to follow scores if these lay on the cathodal side of the cell body, but usually ignored scores when these were on the anodal side of the cell body, preferring instead to turn away and grow towards the cathode.

These effects were dependent on field strength. At high field strengths, all the neurones that came into contact with a score on the cathodal side of the cell body

<table>
<thead>
<tr>
<th>Mean field strength (mV mm(^{-1}))</th>
<th>Number of culture chambers</th>
<th>CG Anodal/left</th>
<th>Turn/Differential away from CG Anodal/left</th>
<th>Turn/Differential away from CG Cathodal/right</th>
<th>CG Cathodal/right</th>
<th>Traversing score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>12</td>
<td>99</td>
<td>31</td>
<td>41</td>
<td>123</td>
<td>1</td>
</tr>
<tr>
<td>20</td>
<td>4</td>
<td>12</td>
<td>35</td>
<td>16</td>
<td>48</td>
<td>4</td>
</tr>
<tr>
<td>55</td>
<td>4</td>
<td>3</td>
<td>38</td>
<td>6</td>
<td>49</td>
<td>0</td>
</tr>
<tr>
<td>130</td>
<td>5</td>
<td>1</td>
<td>33</td>
<td>3</td>
<td>67</td>
<td>3</td>
</tr>
<tr>
<td>185</td>
<td>5</td>
<td>2</td>
<td>32</td>
<td>0</td>
<td>54</td>
<td>3</td>
</tr>
</tbody>
</table>
either used this to direct their growth, or in exceptional cases (5%) managed to traverse this barrier and continued growing directly towards the cathode in the next interspace. No neurones turned or grew differentially away from contact guidance on their cathodal side (Fig. 9A). With progressively weaker field strengths down to about the threshold level for orientation (10 mV mm$^{-1}$; Hinkle et al. 1981) there was a drop off in the number of cells showing contact guidance and a corresponding increase in the numbers turning or growing differentially away from contact guidance on their cathodal side. Both parameters thus approached control values. In Fig. 9B, a similar variation with field strength can be seen in the responses of cells contacting a score on the anodal side of their cell body. At high field strengths, almost all neurites turned or grew differentially away from contact guidance with the score and grew towards the cathode. Near the threshold level for electrical guidance in vitro about one quarter of neurones showed contact guidance of their continued growth, while three quarters still preferred to follow the electrical cue and turned away from anodal contact guidance to grow towards the cathode.

**Nature of the contact guidance**

**Controls**

Some parameters describing the nature of the contact guidance were measured. During guidance the phase-dark neurite tip excluding filopodia lay about 9 μm from the edge of the score while a mean value of 2.2 filopodia were in contact with the score. The distribution of filopodia at the growth cone was asymmetric; slightly more filopodia projected towards the score than projected away from the score (Table 2).

**In an electric field**

The same parameters were measured from neurones growing alongside scores in an electric field, before and after the field was reversed (see below), but before neurites turned away to grow to the new cathode (Table 2). Neurites growing by cathodal CG were more closely applied to the edge of the score than controls and

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Figs 1, 2. Neurites in control cultures growing by contact guidance along the edge of a score. A muscle cell is present also in Fig. 2.

Fig. 3. Neurites turn through 90° to grow towards the cathode (left), then turn a further 90° to follow the score on the cathodal side. E, 136 mV mm$^{-1}$.

Fig. 4. Neurite showing contact guidance on its cathodal side (left). E, 133 mV mm$^{-1}$.

Fig. 5. Examples of neurites passing over a score while growing towards the cathode (left). Cathodal–contact guidance occurs at the neighbouring cathodal score. E, 120 mV mm$^{-1}$.

Fig. 6. Example of a neurite growing from anodal contact guidance directly towards the cathode (left). E, 21 mV mm$^{-1}$.

Figs 7, 8. Examples of neurites turning away from contact guidance on the anodal side and growing towards the cathode (left). E, 138 mV mm$^{-1}$.

Figs 1–8, scale bar, 100 μm.
had greater numbers of filopodia contacting the score. Growth cone filopodia were distributed asymmetrically, twice as many projected towards the score (cathodally) than projected away from the score (anodally).

Neurites that had grown along a score on their cathodal side represent examples of anodal-CG after reversal of the field. Over the following 2 h these neurites had fewer filopodia contacting a score, while the asymmetry of filopodia was reversed; more filopodia projected away from the score towards the new cathode than projected towards the score (the old cathode/now anode).

**Field reversal experiments**

The responses of neurites which had established contact guidance with a score on the cathodal side were studied over time following the reversal of polarity of the

<table>
<thead>
<tr>
<th></th>
<th>Number of neurites</th>
<th>Distance from score (µm)</th>
<th>Number of filopodia in contact with score</th>
<th>Filopodia distribution directed towards/away from score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls &amp; E off</td>
<td>29</td>
<td>9.3 ± 0.8*</td>
<td>1.9 ± 0.2††</td>
<td>3.4 ± 0.3§ ↔ §2.8 ± 0.3</td>
</tr>
<tr>
<td>Cathodal-CG</td>
<td>18</td>
<td>6.6 ± 0.6§</td>
<td>2.8 ± 0.2†§</td>
<td>4.1 ± 0.3§ ↔ §2.2 ± 0.2</td>
</tr>
<tr>
<td>Anodal-CG</td>
<td>15</td>
<td>11.6 ± 1.2§</td>
<td>1.0 ± 0.3‡§</td>
<td>2.4 ± 0.4§ ↔ §4.5 ± 0.6</td>
</tr>
</tbody>
</table>

** **P < 0.01; †† P < 0.005; †‡ P < 0.005; §§ P < 0.001.
electric field. Ten neurites that had established cathodal-contact guidance were observed after an immediate switch of polarity. Nine of these subsequently turned away from what had become anodal-contact guidance to grow towards the new cathode. The other neurite stopped growing. This response occurred within 52 ± 16 min. Two of these neurites sent out lateral cathode-directed branches from points along the neurite shaft (Figs 10–13).

In other experiments a control period (no field for 55 min) was allowed, after neurites had established cathodal-CG, before the polarity was switched. Twenty-two neurites (15 neurones) were observed. During the control pause in stimulation, two neurites began to turn away from CG. The others continued to grow by contact guidance along the edge of the score for 23 ± 5 μm. Reversing the polarity of the electric field making their growth anodal-CG caused 17 of the 22 neurites to turn away from anodal-CG and to grow towards the cathode (Figs 14–17). This response usually was apparent within 1 h (59 ± 9 min, n = 12: five cases took between 3 and 9 h). The three remaining neurites stopped growing and remained in contact with the score.

DISCUSSION

Contact guidance of neurite growth by an oriented substrate was recognized first by Harrison (1912) and later named by Weiss (1945). The orientation of several cell types including neurones on a variety of experimentally manipulated substrates has since been studied extensively (e.g. Dunn, 1982). Neurites in the present control cultures showed a strong tendency to direct their growth by contact guidance along the edge of a score. In general, neurites lay about 9 μm from the edge of the score but had about two filopodia maintaining contact with the score (Table 2). Filopodia were distributed asymmetrically with more projecting towards the score than projecting away from the score. It is likely to be this filopodial asymmetry and maintained contact with the score that promotes contact guidance in control cultures. It is possible that the rough, raised edge of the score offers greater adhesion to filopodia and thus causes more filopodia to project towards the score. Since filopodia did not project predominantly parallel to the score, it is unlikely that the geometry of the raised edge induced by scoring played a major part in orienting the cytoskeleton. Such a direct interaction between surface topography and cytoskeletal orientation has been suggested as the basis of some contact guidance phenomena (Dunn & Heath, 1976), although other mechanisms have been proposed (Ohara & Buck, 1979).

Further evidence that the scores ruled on the plastic surface provided cues for contact guidance comes from the observation that 72% of Xenopus myoblasts elongate parallel to a score that they contact (unpublished observation). Neurites given a free choice of following these contact guidance cues or of following orthogonal electrical cues, in general directed their growth towards the cathode where this was possible and did this even after establishing a contact guidance relationship.
Most importantly, the higher electrical fields drastically reduced the likelihood of contact guidance occurring on the anodal side while markedly increasing the numbers of neurites turning or growing differentially away from CG-anodal. Both these events showed some relation to field strength but remained markedly different from control values even at low field strengths (Fig. 9). In field reversal experiments, neurites that had grown along a score on their cathodal side and continued by contact guidance when the field was switched off, soon began to turn away from CG and grow towards the new cathode.

It seems likely that the raised edge of the scores formed a physical barrier to the passage of most neurites. Only 1/295 passed across this in controls, while only 10/409 did so in growing towards the cathode. Although these values are statistically different from each other \((P < 0.001)\), the vast majority of cathodally directed neurites nevertheless failed to traverse the score. No neurites traversed the score in an anodal direction. In support of the argument that the score forms a barrier to neurite growth, high field strengths actually increased the incidence of CG-cathodal while reducing the occurrence of turning or differential growth away from CG-cathodal. At low field strengths these parameters were no different from control values. It is likely therefore that it is only at scores on the anodal side of a cell and in the field reversal experiments that neurites have a free choice of following either contact guidance or electrical guidance cues.

The distribution of filopodia at the scores in an electric field

Twice as many filopodia contacted a score if this was on the cathodal side of the neurite than contacted a score on the anodal side of the neurite. Neurones following a score on the cathodal side also had 50% more filopodia in contact with the score than did control neurites. As in controls, filopodia were distributed asymmetrically. In neurites following cathodal scores, almost twice as many filopodia projected towards the score/cathode than projected away from the score. After field reversal, the asymmetry of filopodia distribution also reversed;

Figs 10–13. The same neurones photographed immediately before and at various times after the polarity of the electric field was reversed. E, 125 mV mm⁻¹.

Fig. 10. 8 h, cathode at right, cathodal contact guidance.
Fig. 11. +35 min, cathode at left, beginning to turn away from contact guidance.
Fig. 12. +180 min, cathode at left, two cathodal-directed branches have appeared.
Fig. 13. +13 h, cathode at left, turned away from contact guidance anodal, towards the new cathode.

Figs 14–17. The same neurones photographed in an electric field (cathode at right), after switching off the field for 1 h, and at various times after reversing the polarity of the electric field. E, 150 mV mm⁻¹.

Fig. 14. 9 h, cathode at right, cathodal contact guidance: switch off E for 57 min.
Fig. 15. +57 min, with no field, continued contact guidance of both neurites. Reverse polarity and switch on.
Fig. 16. +27 min, in E reversed, cathode at left. Both neurites are turning away from contact guidance.
Fig. 17. +55 min, in E reversed, cathode at left. Both neurites have turned away from anodal–contact guidance to grow towards the new cathode.

Scale bar, 50 μm in Fig. 14; 100 μm in all others.
twice as many filopodia now projected away from the score (to the new cathode) than projected towards the score. Filopodia asymmetry in an applied electric field has been reported previously (McCaig, 1986b). Filopodia attached to a substrate generate tension and the distribution of tension at the growth cone determines the direction of neurite growth (Bray, 1979). Presumably more tension is produced on the cathodal side by the larger number of filopodia and this can turn cells away from contact with a score on their anodal side.

In vivo, contact guidance could be involved in axon growth along other nerve axons, or along oriented glial cell processes which may form channels or tunnels to direct earliest nerve growth (Letourneau, 1982; Silver & Sidman, 1980). Contact guidance features amongst the possible cues offered to extending axons by guidepost cells in invertebrates (Bate, 1976).

Endogenous electric fields exist in some embryos. Steady currents have been measured leaving the primitive streak of the chick embryo (Jaffe & Stern, 1979), passing dorsoventrally through Xenopus skin and internal tissues (Robinson & Stump, 1984) and leaving the neural tube of the quail embryo (Erickson & Nuccitelli, 1984). The magnitude and distribution of the electric fields which these set up within the complex geometry and confined tissue spaces of an embryo have not been determined directly, but estimates show that they may approach and exceed the threshold value for electrical orientation of neurites in vitro (e.g. Erickson & Nuccitelli, 1984). It is possible that channels and tunnels, 'cross roads' and decision points for early axon growth all coexist in vivo with specifically located and oriented electric fields. Other cues, gradients of chemical attractants or gradients of substrate stickiness are likely to be present and to interact as well. A small electric field has been shown to be a more potent guiding influence on neurite growth in vitro than contact guidance. The relative influences of other cues in vitro and the relative importance of all cues in vivo has yet to be tested.

I thank the Medical Research Council, the International Spinal Research Trust and Action Research for support.

REFERENCES


(Accepted 13 January 1986)