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Electric Field Depends on the Substratum: Contributions of Adhesivity and Net Surface Charge

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We investigated the influence of the growth surface on the direction of *Xenopus* spinal neurite growth in the presence of a dc electric field of physiological magnitude. The direction of galvanotropism was determined by the substratum; neurites grew toward the negative electrode (cathode) on untreated Falcon tissue culture plastic or on laminin substrata, which are negatively charged, but neurites growing on polylysine, which is positively charged, turned toward the positive electrode (anode). Growth was oriented randomly on all substrata without an electric field. We tested the hypothesis that the charge of the growth surface was responsible for reversed galvanotropism on polylysine by growing neurons on tissue culture dishes with different net surface charges. Although neurites grew cathodally on both Plastek substrata, the frequency of anodal turning was greater on dishes with a net positive charge (Plastek C) than on those with a net negative charge (Plastek M). The charge of the growth surface therefore influenced the frequency of anodal galvanotropism but a reversal in surface charge was insufficient to reverse galvanotropism completely, possibly because of differences in the relative magnitude of the substratum charge densities. The influence of substratum adhesion on galvanotropism was considered by growing neurites on a range of polylysine concentrations. Growth cone to substratum adhesivity was measured using a blasting assay. Adhesivity and the frequency of anodal turning were graded over the range of polylysine concentrations (0 = 0.1 < 1 < 10 = 100 μg/ml). The direction of neurite growth in an electric field is therefore influenced by both substratum charge and growth cone-to-substratum adhesivity. These data are consistent with the idea that spatial or temporal variation in the expression of adhesion molecules in embryos may interact with naturally occurring electric fields to enhance growth cone pathfinding.

Key Words: **electric field; laminin; poly-L-lysine; substratum; neurite; laminin.**

INTRODUCTION

Naturally occurring voltage gradients driven by the ion transport properties of embryonic epithelia are important for neuronal development (Hotary and Robinson, 1990, 1992). The developing amphibian spinal cord (neural tube) maintains a substantial potential difference across itself, with the resultant electric field across the neural tube cells ranging from about 400 to 1000 mV/mm (Hotary and Robinson, 1991; Shi and Borgens, 1994). Collapse of the transneural tube potential in those embryos causes profound developmental defects of the brain and spinal cord (Hotary and Robinson, 1994; Shi and Borgens, 1994; Borgens and Bohnert, 1995).

In addition to their role in neurogenesis, electric fields applied to the damaged mammalian CNS stimulate anatomical and functional recovery (Borgens and Bohnert, 1997; Borgens *et al.*, 1990, 1993). A corollary to studies in whole animals is the observation that neurons respond directionally to electric fields *in vitro* by specific changes in cell shape, a process called galvanotropism (reviewed by McCaig *et al.*, 1994). *Xenopus* spinal neurons growing on tissue culture plastic exhibit faster growth rates toward the cathode (slower anodally), increased cathodal branching, turning of neurites toward the cathode, and asymmetric changes in growth cone morphology at field strengths substantially below those present in embryos. The impli-

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cations for neurite guidance during development and potential regenerative therapies following CNS injury make it important to determine the factors that affect the direction of neurite growth in combination with weak, steady dc electric fields.

Substratum discontinuities of extracellular matrix molecules (Snow *et al.*, 1996) and receptor ligands (Orioli and Klein, 1997) within developing embryos are thought to provide important guidance cues to migrating growth cones. Despite the individual significance of substratum cues and electric fields as neuronal guidance factors and the fact that substratum variations and voltage gradients occur simultaneously within embryos, their interaction in determining neuronal architecture remains largely unexplored.

We studied the combined effects of various uniform growth substrata and small dc electric fields. Tissue culture plastic substrates were used for which there is abundant information about "typical" growth cone behavior and because electric field responses have been examined almost exclusively on tissue culture plastic. The extracellular matrix molecule laminin was tested because it is a potent neurite-promoting factor that is likely to be encountered by developing neurites (McKerracher *et al.*, 1996). The rationale for using polylysine was threefold: (i) It is used frequently to promote neuronal adhesion and differentiation. (ii) It has been proposed that on highly adhesive substrates, such as polylysine, growth cones would not be able to respond to directional cues (Bray, 1992). (iii) In the context of growth cone steering, the extracellular matrix has been viewed largely as an adhesive substratum that stabilizes growth cone filopodia, thus providing the traction required for growth cone advance. The strong positive charge and adhesive characteristics of polylysine allowed us to explore the influences of adhesivity and surface charge on galvanotropism.

Our data indicate that the growth surface has a profound influence on the direction of neurite growth in a weak dc electric field. The surface charge density and adhesivity of the substratum affect the direction of growth cone turning. Substratum discontinuities within embryos in combination with naturally occurring electric fields may therefore act in a cooperative way to direct neurite growth during development.

MATERIALS AND METHODS

Cell Culture

Primary cultures of neural tube cells were prepared from stage 20 *Xenopus* neural tubes (Nieuwkoop and Faber, 1956) according to the method of Hinkle *et al.* (1981), with minor alterations. Specifically, we aimed to minimize chamber-to-chamber variability by pooling all neural tubes for one experiment in the Ca^{2+}–Mg^{2+}-free Steinberg's solution (disaggregating medium). Disaggregating medium was then replaced with an appropriate volume of culture medium (200 μl per chamber) and the resulting cell suspension was triturated with a Rainin RC 20 pipet tip. Culture medium consisted of (v/v) 20% Leibowitz L15 medium, 2% penicillin (5000 IU/ml)/streptomycin (5000 μg/ml), 1% fetal bovine serum (all from ICN Biomedicals, Ltd, Irvine, Scotland) made up in Steinberg's solution (58 mM NaCl, 0.67 mM KCl, 0.44 mM $Ca(NO_3)_2$, 1.3 mM $MgSO_4$, 4.6 mM Tris–HCl, pH 7.9).

Electric Field Application

The basic requirements for the application of electrical fields to cells *in vitro* have been described (Robinson, 1989; McCaig *et al.*, 1994). The electric field chamber design has been published previously (see Fig. 1 in McCaig *et al.*, 1994). The base of the chamber was a round 100-mm-diameter, 20-mm-high tissue culture plastic dish to which two 12 × 64-mm strips of No. 1 coverglass were secured parallel to each other, 1 cm apart with silicone sealant (Dow Corning 3140RTV). Chambers were allowed to cure for at least 24 h and were then used immediately for experiments or were treated with an appropriate substrate molecule (see below).

Cells suspended in culture medium were plated into the trough defined by the parallel coverglass strips and then a third coverslip (24 × 60 mm) was secured on top using a noncuring silicone compound (Dow Corning MS4). Electrical contact to the culture chambers was made using two 20-cm-long U-shaped tubes filled with Steinberg's solution gelled with 1% agar. One end of each bridge rested in a saline-filled beaker which, in turn, contained an Ag/AgCl coil electrode connected to a dc constant current source. The other end of each bridge terminated in a pool of culture medium continuous with that in the central trough containing the cells.

The field was initiated about 1 h after plating; therefore, most cells had adhered to the dish surface but had not yet sprouted neurites at the time of field initiation. Electrical field strength was determined directly at the beginning and end of each experiment by measuring the voltage drop across the 60-mm-long coverslips. Typically, fields were applied to six chambers and six other chambers remained disconnected from the power supply (controls) for the duration of the 8- to 12-h experiment.

Substrate Preparation

All substrate treatments were performed immediately prior to plating the cell suspensions. Poly-L-lysine substrates were prepared by spreading 1 ml of 100 μg/ml poly-L-lysine (Sigma, molecular weight approx 51,000) over the central region of the dish. After about 30 min the dishes were rinsed twice with a large volume of distilled water and allowed to air-dry prior to use.

The procedure for preparation of laminin substrates was similar except that 1 ml of a freshly prepared solution of laminin isolated from the basement membrane of the Engelbreth–Holm–Swarm mouse sarcoma (Sigma; 20 μg/ml) was applied to the central trough of the field chamber for 30 to 45 min prior to washing. The dishes were air-dried before plating the cells. In an attempt to maximize the amount of laminin adsorbed to the surface, laminin was sometimes applied to dishes already treated with poly-L-lysine as described above (this is the "polylysine + laminin" substratum).

Two different substrates were sometimes applied to a single chamber by dividing the central trough into three 2 × 1-cm regions that were each treated separately. For example, the entire trough was treated with poly-L-lysine as described above and then a 2 × 2-cm square coverglass was placed over the central third of the chamber. Laminin solution was drawn under the coverglass by capillary action and allowed to remain there for 45 min. The border of the laminin-treated section was marked by drawing a needle gently along the edge of the coverglass to make a fine scratch in the dish surface. After 45 min the coverglass was removed and the

laminin-treated section was rinsed, being careful not to allow any of the wash volume contact the polylysine-treated regions of the dish. A dish treated in this way could have two polylysine-treated sections (ends) and one polylysine + laminin-treated section (center) or vice versa. This method was also used to produce dishes with untreated regions and polylysine-treated regions.

The influence of the surface charge on the direction of galvanotropism was tested using Plastek C and Plastek M polystyrene tissue culture dishes. These dishes were treated by the manufacturer (Mat Tek Corp., Ashland, MA) with a gas plasma using a proprietary process that added either primarily nitrogen-containing (Plastek C) or oxygen-containing (Plastek M) functional groups to the dish surface. As a result, Plastek C dishes provide a positively charged growth surface and Plastek M dishes provide a negatively charged growth surface.

Data Collection and Analysis

Data were collected as photographs of live or 4% Formalin-fixed cells. All cells in each chamber whose neurite paths could be identified unambiguously were photographed for analysis. Photographs were taken on Kodak Technical Pan film using a Nikon Diaphot TMD inverted phase-contrast microscope.

A thorough description of the data analysis system has been published (Cork and Rajnicek, 1990). Photographic negatives are projected onto a digitizing pad (Summagraphics, SummaSketch II) interfaced with an IBM-compatible computer. The position of the center of the cell body is marked with the digitizer cursor as well as the points at which neurites sprout from the somas. The positions of any growth cones are then marked and the length of each neurite is traced. The computer stores simple coordinate data for relevant points and these are then used to calculate several asymmetry indices and measures of neurite growth for large populations of cells.

For all determinations of asymmetry the anode is at 0° (or 360°) and the cathode is at 180°. Asymmetric distribution of initiation sites on the cell bodies was detected by dividing the cell body into anode-facing ($0 \pm 180°$) and cathode-facing ($180 \pm 180°$) halves with respect to the electric field direction (for control cells these represent the right and left sides of the cells, respectively). The method used to detect field-induced asymmetry of neurite elongation, branching, and turning is summarized in Fig. 1. The direction of net neurite growth was calculated using the projection of each neurite onto the field line (Fig. 1A). A neurite is classified as projecting cathodally if the X value of the digitizer coordinate for the growth cone is less than the X value of the digitizer coordinate for the initiation site of the same neurite (i.e., $X_{\text{growth cone}} - X_{\text{initiation site}} < 0$). Similarly, the direction of neurite branching relative to the field direction was determined by comparing the X coordinates of the center of the cell body and the base of the branch.

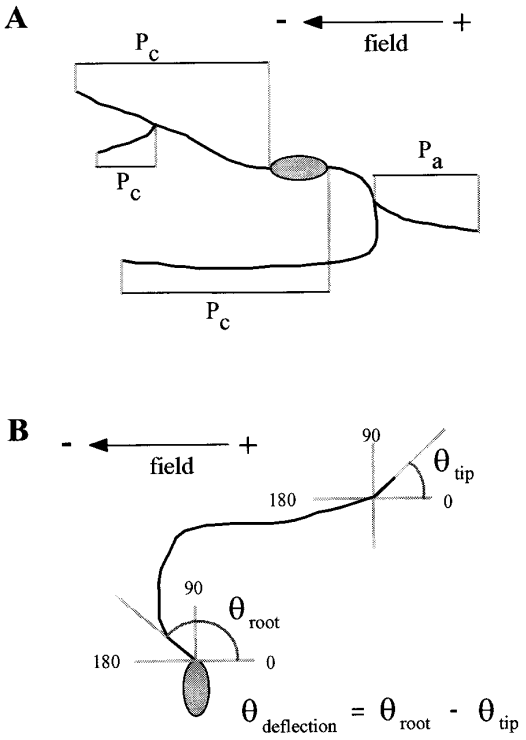

FIG. 1. Method for quantitating asymmetric neurite growth. (A) Overall direction of neurite growth. The net projection of each neurite onto the field line was calculated from the X coordinates of the initiation site relative to the center of the growth cone: P_c, projection oriented toward the cathode; P_a, projection toward the anode. This cell has three cathode-facing neurites and one anode-facing neurite. Each branch was treated as a separate neurite. The mean projection length in each direction was calculated as the sum of cathodal or anodal projections divided by the number of cathodal or anodal projections, respectively (B) Direction of growth cone deflection (turning). The angle of the distal tip of the neurite (θ_{tip}) was subtracted from the angle of the proximal region (θ_{root}) of the same neurite to yield the angle of turning relative to the field direction ($\theta_{deflection}$).

Growth Cone Adhesivity Assay

Neurons were grown in 60-mm diameter Falcon tissue culture dishes that were untreated or treated with polylysine (molecular weight 26,000, 56,000, or > 300,000 at 0.1, 1, 10, or 100 μg/ml) as described above. Neurons were plated into a pool of 100 μl of medium spread over the center of the dish and were left undisturbed for at least 2 h before examining for neurite growth. The adhesivity of growth cones to various substrata was tested using the method of Lemmon *et al.* (1992). Culture medium was blown at the growth cone directly from a glass pipet (4 μm inner diameter) positioned 20 μm directly in front of the growth cone. The pipet was connected to a WPI pneumatic picopump using compressed nitrogen gas as the pressure source. The output pressure on the tank was 60 psi and the pulse pressure was maintained at 50 psi. Culture medium was then blown repeatedly at the growth cone in discrete pulses until the growth cone dislodged completely from the substratum. The blast duration was increased in 10-ms steps from 10 to 100 ms and then was increased in 20-ms steps to a maximum of 200 ms. The same pipet tip was used for all growth cones because the assay is influenced by small changes in tip diameter. The tip diameter was monitored visually throughout each experiment and the experiment was terminated and not included in analysis if the pipet tip became blocked by debris (such as yolk platelets) or was broken. The performance of the pipet was also checked by performing the blasting assay on growth cones on untreated Falcon tissue culture dishes at the start and end of each experiment. If the mean blast duration (see below) was unchanged for these data sets, the tip was considered to have performed uniformly throughout the experiment. Data were pooled from 2

TABLE 1
The Influence of the Growth Surface on Neuronal Morphology

Substratum	No. of neurites	No. of growth cones	Neurites/cell	Branches/ neurite[b]	Neurite length (μm)
Falcon	326	371	1.9 ± 0.06	0.2 ± 0.04	178 ± 7
Polylysine	458	581	1.7 ± 0.07[a]	0.5 ± 0.03	78 ± 3
Laminin	225	327	1.9 ± 0.05	0.9 ± 0.06	112 ± 6
Polylysine + laminin	1000	1336	1.9 ± 0.02	0.6 ± 0.03	155 ± 4

Note. Values represent means ± SEM. Cells were grown for 10 to 12 h without an electric field. These data are from the control cells in Tables 2–4.
[a] $P < 0.001$, comparing mean neurites/cell on polylysine to each other substratum, two-tailed t test
[b] $P < 0.001$, each mean is different from each other mean, two-tailed t test.

experiments in which 10 growth cones were assayed successfully on each of the 13 substrates without damage to the pipet tip. The mean of the maximum blast duration required to strip each growth cone from the surface was taken as a measure of the growth cone adhesivity to the substratum and is reported as the "mean blast duration."

RESULTS

The Influence of the Substratum on Neuronal Morphology

Control cells grown in the absence of an electric field showed substrate-dependent differences in neuronal morphology (Table 1), confirming that our substrate treatment method altered the growth surface effectively. The most striking morphological effects were observed for cells plated onto polylysine, on which cells sprouted fewest neurites, neurites were wider, and shorter and growth cones were usually flat with large, broad lamellipodia and numerous filopodia. The substratum also affected the extent of branching, with laminin inducing most branches (Table 1). Neuronal morphologies on polylysine and laminin were similar to those for embryonic rat sympathetic neurons on the same substrates (Lein and Higgins, 1989).

Field-Induced Asymmetry of Neurite Initiation Sites

Neurite initiation sites are distributed asymmetrically on the somas of field-treated *Xenopus* neurons growing on Falcon tissue culture plastic (Patel and Poo, 1982). Our data confirm this observation for Falcon substrata and extend it to include neurons on dishes coated with laminin but not polylysine or polylysine + laminin (Table 2). This observation is different from that of Hinkle *et al.* (1981), who concluded that initiation sites were uniformly distributed around somas on Falcon plastic substrates. This discrep-

TABLE 2
Substratum-Dependent Asymmetry of Neurite Initiation Sites

Substratum	No. of dishes	No. of neurons	No. of initiation sites	% Cathode-facing	% anode-facing
Control Cells					
Falcon	2	171	326	43	55
Polylysine	8	267	458	46	53
Laminin	5	117	225	45	54
Polylysine + laminin	5	526	1000	48	52
Field-treated cells					
Falcon	4	482	802	67[b]	32
Polylysine	8	321	520	52	47
Laminin	3	112	184	59[a]	40
Polylysine + laminin	4	462	820	51	49

Note. Neurons growing on various substrates were exposed to fields of 82 to 128 mV/mm for 10 or 12 h. These data represent the same cells as in Tables 3 and 4. Somas were divided visually into equal halves and the total number of cathode-facing and anode-facing initiation sites in each half was compared to 50% using a Z score (Downie and Heath, 1974). Superscripts indicate significant bias in initiation sites.
[a] $0.05 > P \geq 0.001$.
[b] $P < 0.0001$.

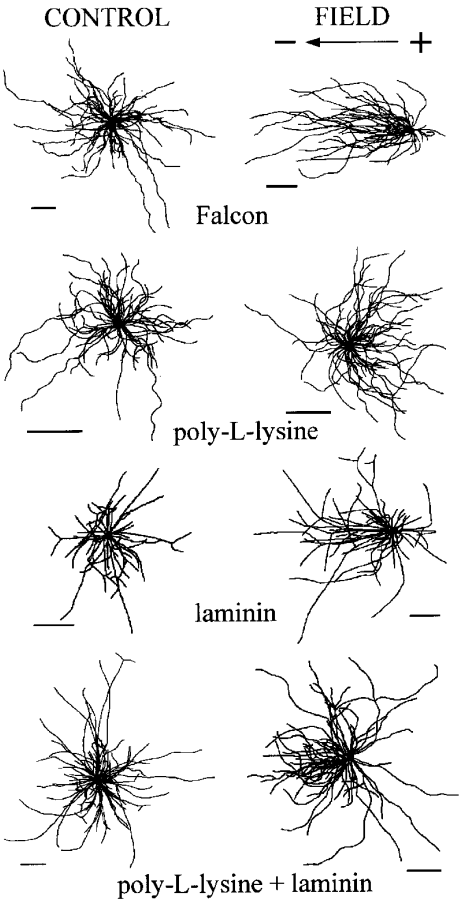

FIG. 2. Populations of neurons in which the cell bodies have been superimposed and every neurite has been traced. Control neurite growth was random but field-treated neurites grow toward the cathode on Falcon tissue culture plastic, laminin, and polylysine + laminin but they grow toward the anode on polylysine. Number of neurons shown on Falcon is 28 control and 21 field (119 mV/mm), on poly-L-lysine is 25 control and 36 field (115 mV/mm), on laminin is 16 control and 24 field (100 mV/mm), and on polylysine + laminin is 26 for control and 26 for field (120 mV/mm). Scale bars are 100 μm.

ancy is most likely due to reabsorption of anode-facing neurites in the present study, in which fields were applied for much longer periods than those used by Hinkle *et al.* (1981).

Field-Induced Asymmetry of the Direction of Neurite Growth

The growth surface had a profound influence on the direction of neurite growth in the presence of a weak electric field (Fig. 2). Most neurites grew toward the cathode on Falcon, laminin, or polylysine + laminin but most grew toward the anode on polylysine (Fig. 2, Tables 3 and 4). Although the number of cathode-facing neurites was increased on Falcon, laminin, and polylysine + laminin, the mean cathodal projection length was increased only on Falcon and laminin, suggesting that neurites elongated more quickly toward the cathode than the anode on those substrate but not on polylysine + laminin (Table 3). This is probably due to preferential stimulation of growth rates for cathode-facing neurites compared to anode-facing ones as observed previously for *Xenopus* neurites on Falcon dishes (McCaig, 1987).

The substratum influenced the direction of growth cone turning in an electric field. More neurites turned toward the cathode than the anode on Falcon and laminin substrates and the mean angle turned toward the cathode was greater than that toward the anode. On polylysine substrates, however, more neurites turned anodally and the mean angle turned toward the anode was greater than that toward the cathode. On polylysine + laminin, the mean angle turned toward the cathode was increased but equal numbers of growth cones turned toward each electrode.

The electric field enhanced cathodal branching on Falcon, laminin, and polylysine + laminin substrata (Table 4), further contributing to the overall cathodal stimulation of growth on those substrata. On polylysine, however, equal numbers of branches were found in cathode or anode-facing directions (Table 4).

Variation in the direction of growth in an electric field was related to the substratum directly rather than variation between cells cultured on different days or solubilized substratum proteins in the culture medium. Neurons were plated onto untreated Falcon dishes in which only the central third of the chamber (or the third at each end of the chamber) was coated with polylysine. Alternatively, the entire chamber was coated with polylysine and only the central third (or the third at each end of the chamber) was coated with laminin. In this way, cells from the same suspension were plated in a single chamber on two different substrata and could be exposed to the same electric field with all other growth conditions being identical. Under these circumstances, the direction of neurite growth in an electric field was the same as that found using separate chambers (compare Table 5 to Tables 2–4). The direction of galvanotropism was appropriate to the substratum in these chambers and was not influenced by whether the data were collected from the central region of the chamber or the end regions. This validates our chamber design and indicates that there was no influence of oxygen gradients or electric field-induced gradients of tropic substances in the culture medium.

Influence of Surface Charge on the Direction of Galvanotropism

Of all the substrata tested only polylysine reversed the direction of galvanotropism from cathodal to anodal. Since polylysine is strongly cationic we investigated the hypothesis that the net positive charge on polylysine (relative to the net negative charge on Falcon and laminin) was responsible for the reversal of the electric field response. Neurons

TABLE 3

The Influence of the Growth Surface on the Overall Direction of Galvanotropic Neurite Growth

Substrate	% Projections (net growth) that are		Length of neurite projection onto the field line (mean + SEM)	
	Cathode-facing	Anode-facing	Cathodal projection (μm)	Anodal projection (μm)
Control cells				
Falcon	49	51	104 ± 10	102 ± 10
Polylysine	49	51	45 ± 5	45 ± 5
Laminin	48	52	70 ± 12	64 ± 10
Polylysine + laminin	51	49	90 ± 6	86 ± 6
Field-treated cells				
Falcon	81[c]	19	173 ± 10[d]	44 ± 3
Polylysine	43	57[b]	46 ± 5	49 ± 5
Laminin	63[c]	37	69 ± 11[e]	43 ± 6
Polylysine + laminin	54[a]	46	77 ± 6	70 ± 6

Note. These data represent the same cells in Tables 2 and 4. Percentages were compared to 50% using a Z score. Neurite projection lengths were compared (anode vs cathode) using a two-tailed Student t test.

[a] $0.05 > P \geq 0.001$.

[b] $0.001 > P \geq 0.0001$.

[c] $P < 0.0001$.

[d] $P < 0.001$.

[e] $P = 0.039$.

were grown on polystyrene tissue culture dishes treated by the manufacturer with a gas plasma to produce either a net negative (Plastek M) or a net positive (Plastek C) surface charge. Neurites grew randomly on both types of dishes without a field but responded strongly toward the cathode on Plastek M dishes (Fig. 3, Table 6), which bear a net

TABLE 4

The Influence of the Growth Surface on Galvanotropic Neurite Deflection and Branching

Substratum	No. of growth cones	Direction of neurite deflection		Degree of turning (mean ± SEM)		No. of branches	Direction of branching	
		% that turn cathodally	% that turn anodally	Cathodal deflection (degrees)	Anodal deflection (degrees)		% Cathode-facing	% Anode-facing
Control cells								
Falcon	371	51	48	39 ± 2.3	34 ± 2.2	45	44	55
Polylysine	581	48	49	30 ± 1.7	28 ± 1.4	123	47	53
Laminin	327	51	47	30 ± 2.4	27 ± 2.0	102	51	49
Polylysine + laminin	1336	52	46	34 ± 1.4	33 ± 1.2	235	51	49
Field-treated cells								
Falcon	910	70[c]	28	41 ± 1.3[c]	23 ± 1.4	110	83[c]	17
Polylysine	637	37	61[c]	28 ± 1.7	40 ± 1.7[c]	117	50	50
Laminin	257	56[a]	42	35 ± 2.9[c]	22 ± 2.0	73	68[a]	32
Polylysine + laminin	1162	51	47	35 ± 1.4[b]	30 ± 1.3	337	58[a]	42

Note. These represent the same cells as in Tables 2 and 3. Significant differences are indicated by superscripts. The % of neurites turning anodally or cathodally and the % of anode- and cathode-facing branches were compared to an expected frequency of 50% (for control cells) by calculating a Z score (Downie and Heath, 1974). 1 to 3% of neurites did not turn. The mean angles of deflection were compared (anodal versus cathodal) by a two-tailed Student t test.

[a] $0.05 > P \geq 0.001$

[b] $0.05 > P \geq 0.02$

[c] $P < 0.0001$

TABLE 5

Influence of the Growth Surface on the Galvanotropic Responses of Neurites Growing in Single Culture Chambers

Substratum	No. of neurons	No. of initiation sites	No. of growth cones	No. of branches	Initiation sites (%)		Direction of net growth (% neurites)		Deflection (% neurites)		Branching (%)	
					Cathode-facing	Anode-facing	Project cathodally	Project anodally	Turn cathodally	Turn anodally	Cathode-facing	Anode-facing
Polylysine[a]	202	346	395	49	45	54	37	63[e]	33	64[e]	55	45
Polylysine + laminin[a]	280	444	549	105	56	43	59[e]	41	48	51	69[e]	30
Falcon[b]	307	598	817	219	56[d]	43	79[c]	21	69[c]	29	78[e]	22
Polylysine[b]	129	252	271	19	43	56	31	69[e]	28	71[e]	37	63

Note. Statistical analysis: Percentages were compared to an expected frequency of 50% using a Z score (Downie and Heath, 1974). Significant differences are indicated by superscripts. 1 to 2% of neurites did not turn. Control cells showed no orientation responses (data not shown, but see Fig. 3).

[a] The entire central trough of the electrical field chamber was covered with poly-L-lysine (~56,000 MW; 100 μg/ml) and then either the two end regions (1 × 2 cm each) or only the central third (1 × 2 cm) of each trough was treated with laminin (20 μg/ml). Cells were exposed to fields of 100 to 140 mV/mm for 7 to 12 h. Data for 11 field chambers were pooled.

[b] Poly-L-lysine (~56,000 MW; 100 μg/ml) was applied either to the central third (1 × 2 cm) of the trough (leaving the end regions untreated) or to both of the ends (1 × 2 cm each) of the trough, leaving the central region untreated. Cells were exposed to fields of 97 to 117 mV/mm for 8 to 10 h. Data for 8 field chambers were pooled.

[c] $0.05 > P \geq 0.001$.

[d] $0.001 > P \geq 0.0001$.

[e] $P < 0.0001$.

negative surface charge similar to that on Falcon dishes (surface charge data provided by Mat Tek Corp., the Plastek manufacturer). Neurites also responded toward the cathode on Plastek C but the magnitude of the response was significantly decreased compared to that on Plastek M (Fig. 3, Table 6). These data suggest that surface charge contributes to galvanotropism but charge alone is insufficient for reversal of galvanotropism on polylysine. For comparison a qualitative summary of galvanotropic responses on all substrates is provided in Table 7.

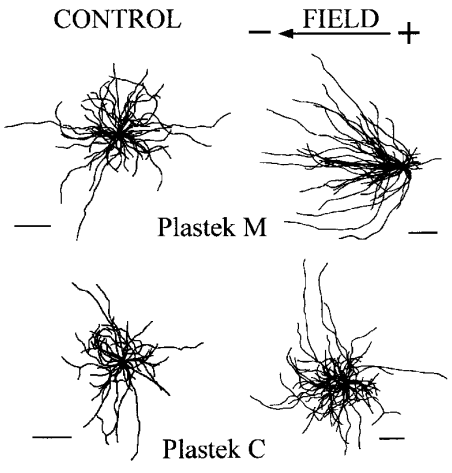

FIG. 3. Neurons in which cell bodies have been superimposed. Neurons were grown on Plastek M (negatively charged) or Plastek C (positively charged) culture dishes. The number of neurons shown on Plastek M is 25 for controls and 33 for field (104 mV/mm) and on Plastek C is 17 for control and 26 for field (105 mV/mm). Scale bars are 100 μm.

Influence of Substratum Adhesivity on Galvanotropism

In addition to being positively charged, polylysine-treated surfaces are strongly adhesive to cells. We investigated the hypothesis that anodal turning of neurites was related to substratum adhesivity by correlating the extent of anodal turning on polylysines of various molecular weights (26,000, 56,000, or > 300,000) over a range of range of concentrations (0.1, 1, 10, or 100 μg/ml). Measurements of relative growth cone adhesivity (Fig. 4A) on each substratum were compared to anodal turning data (Fig. 4B) on the same substrates. Growth cone to substratum adhesivity was quantitated by determining the mean pulse duration required to blast a growth cone completely off the substratum with repeated, controlled bursts of culture medium. Substratum to growth cone adhesivity and the extent of anodal growth cone turning were affected similarly by the presence of substratum polylysine (untreated Falcon dishes = $0.1 < 1 < 10 = 100$ μg/ml) but were not affected by the molecular weight of the polylysine (Fig. 4). The amount of polylysine bound to the substratum (therefore positive charge density) and not polymer length appears to be the important factor in anodal growth cone turning and adhesivity.

TABLE 6
The Influence of Net Surface Charge on Neurite Galvanotropism

Substratum	Net surface charge[a]	No. of neurons	No. of neurites	Initiation sites: % Cathode-facing	Initiation sites: % Anode-facing	No. of growth cones	Net neurite growth: % Project cathodally	Net neurite growth: % Project anodally	Direction of neurite deflection: % Turn cathodally	Direction of neurite deflection: % Turn anodally	No. of branches	Branching: % Cathode-facing	Branching: % Anode-facing
Plastek M	1.2 mequiv/cm^2 negative	366	706	60[b]	39	912	75[b]	25	60[b]	38	206	80[b]	20
					NS			**		**			*
Plastek C	0.7 mequiv/cm^2 positive	410	792	59[b]	40	1036	66[b]	34	51	48	245	71[b]	29

Note. Cells were exposed to fields ranging from 80 to 108 mV/mm for 8 h (total of 6 chambers for each substratum). Percentages were compared to an expected frequency of 50% for control cells using a Z score (Downie and Heath, 1974). Significant differences are indicated by superscripts. Control cells showed no asymmetry in any growth parameter. 2% of neurites did not turn. Asterisks indicate a significant increase (**$P < 0.001$; or *$0.05 > P > 0.02$) in the % of neurites displaying enhanced anodal growth on Plastek C compared to Plastek M calculated using a d test (Bailey, 1981). NS indicates no significant difference between the response on the two substrates.

[a] Estimates provided by Mat Tek Corporation, manufacturer of Plastek tissue culture dishes. Charges represent meqiv/cm^2 of univalent positive or negative charges.

[b] $P < 0.0001$.

DISCUSSION

During development neurites traverse a variety of surfaces on route to their targets. Substratum cues, which are regulated spatially and temporally in embryos, are presented within a complex milieu of coexisting guidance factors such as physical and chemical topography (e.g., Snow *et al.*, 1996; Orioli and Klein, 1997; Rajnicek *et al.*, 1997), gradients of diffusible chemoattractant or -repellent molecules (e.g., Culotti and Kolodkin, 1996; McFarlane and Holt, 1997) and naturally occurring dc electric fields (e.g., Robinson and Messerli, 1996). Although the properties of these neurite guidance cues have been determined individually, their effects in combination are relatively unexplored, especially for electric fields. The only reports to combine substratum effects with weak electric fields indicate that *Xenopus* spinal cord neurites grow toward the cathode of an electric field rather than responding to scratches in the substratum (McCaig, 1986) and that they integrate cues provided simultaneously by stripes of substratum-bound laminin and an orthogonally oriented electric field (Britland and McCaig, 1996). In light of the variety of molecules encountered by embryonic neurites *in vivo*, and the self-driven electrical potential of 400 mV/mm present across the cells of the developing *Xenopus* neural tube (Hotary and Robinson, 1991), we determined the directional responses of *Xenopus* spinal neurites on various uniform substrata in dc electric fields in the physiological range (80 to 146 mV/mm).

The Substratum Determines the Direction of Neurite Growth in an Electric Field

In a steady, dc electric field, most neurites grew cathodally on Falcon tissue culture plastic or laminin substrates. On these substrates, most branches were directed cathodally, most growth cones turned cathodally, growth cones turned through larger angles cathodally than anodally, and

TABLE 7
Qualitative Summary of Galvanotropic Responses

Substratum	Net surface charge	Initiation site asymmetry	Direction of net neurite growth	Direction of turning	Direction of branching
Falcon	Negative	−	−	−	−
Polylysine	Positive	○	+	+	○
Laminin	Negative	−	−	−	−
Polylysine + laminin	Probably negative	○	−	−	−
Plastek M	Negative	−	−	−	−
Plastek C	Positive	−	−	○	−

Note. The symbols indicate the direction of the galvanotropic response, where + indicates net anodal growth, − indicates net cathodal growth, and ○ indicates uniform growth in all directions. These data summarize qualitatively the quantitative results in Tables 2–6.

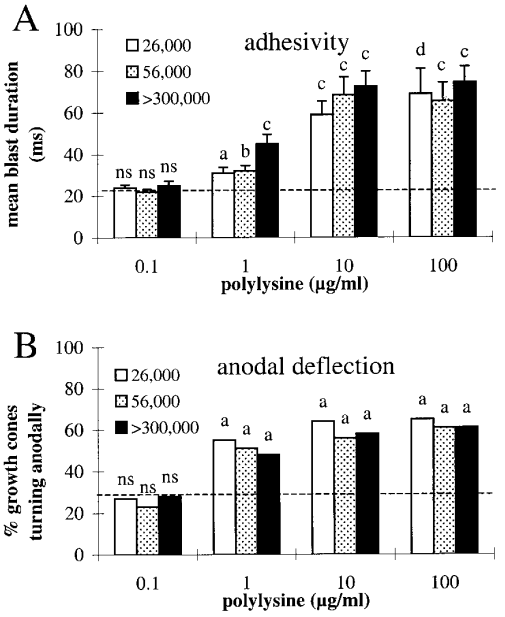

FIG. 4. The relationship of polylysine to substratum adhesivity and anodal growth cone turning. Bars represent polylysines with different molecular weights. (A) Relative substratum adhesivity for growth cones was assessed by measuring the mean blast duration (± SEM) required to dislodge a growth cone from the substratum. The dotted line represents untreated Falcon tissue culture plastic substrata (22 ± 2.3 ms, $n = 20$). Data were pooled from two experiments in which all growth cones ($n = 20$ for each substratum) were blasted using the same pipet. The letter above each bar is the P value compared to untreated Falcon (two-tailed Student's t test): ns, not significant; a, 0.0175; b, 0.0065; c, < 0.0001; d, 0.0004. (B) The percentage of growth cones that turn anodally depends on polylysine concentration, not molecular weight. The dotted line indicates the control percentage (see Table 4) of growth cones that turn anodally on Falcon dishes in a field. Letters above each bar are the P values compared to untreated Falcon by a d test (Bailey, 1981): ns, not significant; a, < 0.001. Number of growth cones measured on 26,000, 56,000, and > 300,000 molecular weights, respectively: at 0.1 μg/ml polylysine $n = 763$, 547, and 795; at 1 μg/ml $n = 611$, 308, and 510; at 10 μg/ml $n = 512$, 271, and 676; and at 100 μg/ml $n = 501$, 637, and 288. The values for 1 and 10 μg/ml were different ($P < 0.002$) at 26,000 and > 300,000, but not 56,000 molecular weight by a d test (Bailey, 1981).

neurites grew faster cathodally than anodally (inferred from neurite projection lengths). These data support previous reports of cathodal galvanotropism for *Xenopus* spinal neurites on Falcon tissue culture plastic (reviewed by McCaig *et al.*, 1994) and suggest that these responses may be an accurate reflection of those on more natural substrates, of which laminin is a major component.

The morphology and galvanotropic responses of neurites on polylysine were different from those on plastic or laminin. Most growth cones on polylysine turned toward the anode and they turned through larger angles anodally than cathodally. When culture dishes were treated first with polylysine and then with laminin (polylysine + laminin), the galvanotropic response contained elements of the responses observed on polylysine and laminin separately, suggesting that laminin did not mask the effects of polylysine completely. This may reflect relative differences in the concentrations of molecules adsorbed to the dishes (polylysine was used at 100 μg/ml and laminin was used at 20 μg/ml), which would affect the substratum surface charge density and adhesivity.

Substratum Effects on Galvanotropism Are Not Due to Soluble Laminin or Polylysine

To interpret the influence of the substratum on galvanotropism it is important to determine whether the responses are due to substratum-bound molecules directly or to molecules released from the treated growth surface. A potential contribution of soluble polylysine must be identified because soluble cations, such as polylysine, block calcium channels (Brown *et al.*, 1995) and calcium is required for cathodal galvanotropism of *Xenopus* growth cones (Stewart *et al.*, 1995), although possibly not under all circumstances (Palmer *et al.*, 1997). Additionally, soluble cations in combination with raised extracellular calcium induce anodal turning of *Xenopus* growth cones in an electric field (Erskine *et al.*, 1995). Similarly, any influence of soluble laminin must be identified because soluble and substratum-bound laminin stimulate distinct changes in growth cone morphology and behavior (Rivas *et al.*, 1992; Chamak and Prochiantz, 1989). The electric field responses of neurons in chambers with alternating areas of polylysine/polylysine+laminin or Falcon/polylysine were identical to those on the relevant uniform substratum. Therefore, if solubilized substratum molecules were present in the bulk medium, their concentrations were not sufficient or their actions were too short ranged to affect substratum-dependent galvanotropism in these low-density cultures.

Do Substratum Effects on Differentiation Affect Galvanotropism?

The substrate's influence on galvanotropism may be indirect, with different substrata inducing distinct types of neurons to differentiate, each with different inherent responses to electric fields. This remains a possibility because the embryonic neural tube contains a heterogeneous population of neurons and we did not attempt to identify neuronal subpopulations in our cultures and relate them to the direction of galvanotropism. Alternatively, the substratum might determine the axonal or dendritic identity of neurites that differentiate, each with different inherent galvanotropic responses. We have not tested this idea directly for *Xenopus* neurites but a focally applied electric field has distinct directional effects on the growth of rat hippocampal axons compared to dendrites emerging from the same soma (Davenport and McCaig, 1993) and extracellular matrix molecules affect the type of processes that

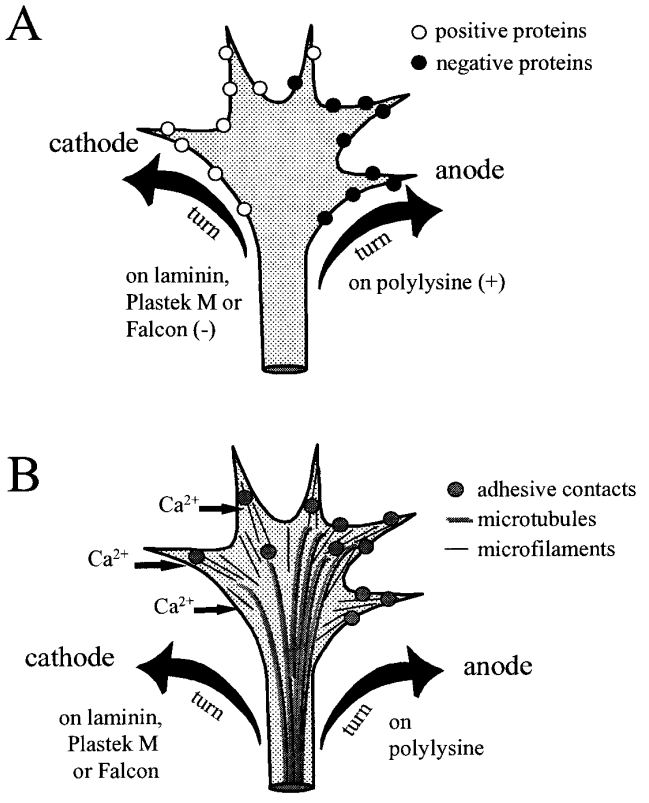

FIG. 5. Hypothetical mechanisms for substrate-dependent galvanotropism. (A) Increased electrostatic attraction of the anodal or cathodal side of the growth cone leads to turning. Positive membrane proteins accumulate cathodally and negative membrane proteins accumulate anodally. On negatively charged substrata, such as laminin, Falcon, or Plastek M, growth cones may adhere more strongly cathodally and on positively charged substrata, such as polylysine, growth cones may adhere more strongly anodally via electrostatic attraction. This mechanism is consistent with our data that a critical positive substratum charge density enhances growth cone binding to polylysine and that a critical positive surface charge density is required for anodal turning. (B) Interaction of several electric field effects may produce substrate-dependent galvanotropism. Regardless of substrate type, the cathode-facing regions of the growth cone experience increased calcium influx via field-induced membrane depolarization or accumulation and activation of membrane proteins such as calcium channels or acetylcholine receptors. While the role of calcium in galvanotropism is controversial (Stewart *et al.,* 1995; Palmer *et al.,* 1997), increased calcium in the cathode-facing side of the growth cone could stimulate cytoskeletal dynamics and membrane addition cathodally. Substratum-bound polylysine stimulates substratum adhesion, localized actin polymerization (Forscher *et al.,* 1992), and anodal turning (Fig. 4B). Therefore lamellipodial stability, filopodial stability, and substratum adhesion may be enhanced anodally on polylysine. Anodal turning would result if these effects predominated over cathode-directing events.

differentiate in other neuronal systems (Chamak and Prochiantz, 1989; Lein and Higgins, 1989).

Another possible explanation for an influence of the growth surface on galvanotropism is that the substratum affects the physical properties of neurites which alters their ability to respond to electric fields. For example, the growth surface affects the number and distribution of functional calcium channels so that calcium entry occurs selectively in neurites cultured on laminin but not on concanavalin A (Ross *et al.,* 1988). Concanavalin A (Patel and Poo, 1982; McCaig *et al.,* 1994) and calcium channel blockers (Stewart *et al.,* 1995) inhibit cathodal orientation of *Xenopus* neurites in electric fields but calcium's role is still controversial (Palmer *et al.,* 1997). It would be useful, therefore, to determine whether the substrates used in the present study induce similar differences in calcium channel activity in *Xenopus* neurites.

Substratum Surface Charge and Adhesivity Influence Galvanotropism

The location and size of cell-to-substratum contacts on neurons are influenced strongly by the growth surface (Gundersen, 1987) and the extent of substrate adhesion is related closely to surface charge (Sugimoto, 1981). Since about 90% of cell surface charges are negative at physiological pH, a strong electrostatic attraction exists between cells and positively charged substrates, such as polylysine. Anodal galvanotropism was induced only by polylysine (positively charged) and not by laminin or Falcon plastic (both negatively charged) so we explored the possibility that substratum adhesivity and surface charge influenced the direction of growth cone turning. Neurons were grown either on tissue culture plastics that differed in their net surface charge or on varying concentrations of polylysine. Adhesivity and anodal growth cone turning on polylysine were concentration dependent ($1 < 10 = 100$ μg/ml). The extent of anodal turning therefore appears to be related directly to the amount of substratum-bound polylysine. We did not attempt to determine the surface charge of our polylysine-treated surfaces but we assume that the shift in galvanotropism from cathodal to anodal with increasing polylysine concentration reflects, at least in part, an effect of increasing positive growth surface charge density.

The influence of surface charge was tested using tissue culture plastic substrates for which surface charge information was available from the manufacturer. Plastek M dishes bear a net negative charge (1.2 meqiv/cm^2) and Plastek C dishes bear a net positive charge (0.7 meqiv/cm^2). Although the relative frequency of anodal turning was increased on Plastek C (positively charged) relative to Plastek M (negatively charged, Table 6), the reversal of charge was insufficient to reverse the polarity of galvanotropism. This implies that the positive surface charge affects anodal turning but the inability of Plastek C dishes to reverse the response completely may reflect the relatively low net positive charge density on Plastek C compared to the higher net negative charge density on Plastek M.

What Is the Mechanism for Substrate-Induced Reversal of Galvanotropism?

Proteins are redistributed within the membranes of cells exposed to electric fields (Stollberg and Fraser, 1988) and asymmetry of proteins such as acetylcholine receptors (Erskine and McCaig, 1995), calcium channels (Stewart *et al.*, 1995), and growth factor receptors (McCaig *et al.*, 1995; McCaig and Erskine, 1996) is the most likely cause of *Xenopus* growth cone galvanotropism (see McCaig *et al.*, 1994; McCaig and Erskine, 1996 for reviews). Assuming an asymmetry exists in growth cone membranes, an interaction of substratum charge and adhesivity could determine the direction of galvanotropism via localized electrostatic attraction (Fig. 5A). It is likely however that several coincident guidance cues act, presumably via the cytoskeleton, to yield substrate-dependent galvanotropism (Fig. 5B). This seems reasonable because for growth cones on polylysine localized actin assembly (Forscher *et al.*, 1992) and anodal turning (Fig. 4B) are both linked to the substratum's positive charge density. The substratum also affects *Xenopus* microtubule transport and dynamics (Chang *et al.*, 1998).

Developmental Relevance

Our data indicate that substratum variations encountered by migrating growth cones affect pathfinding. The influence of multiple substratum cues present in growth cone "decision-making" regions of embryos is receiving increasing attention (e.g., Tang *et al.*, 1994; Rajan and Denburg, 1997) but this is the first demonstration that variation in the growth surface reverses the direction of growth cone steering in an electric field of physiological magnitude. We acknowledge that growth cones do not normally encounter tissue culture plastic or polylysine substrata *in vitro* but our data linking substratum adhesivity and surface charge with the direction of galvanotropism indicate that regional (and/or temporal) differences in the expression of charged cell surface molecules may affect the direction of galvanotropism in embryos and contribute to axon guidance. This seems particularly likely since the charge separation (hence charge density) of soluble chondroitin sulfate proteoglycans influences galvanotropism of *Xenopus* growth cones (Erskine and McCaig, 1997).

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