Nerve regeneration and wound healing are stimulated and directed by an endogenous electrical field in vivo

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Summary

Biological roles for naturally occurring, extracellular physiological electric fields have been proposed over the past century. However, in the molecular era, many biologists presume that electric fields have little physiological relevance because there has been no unequivocal demonstration of their importance at the single-cell level in vivo. We have used an in vivo rat corneal model, which generates its own endogenous electric field and show that nerve sprouting, the direction of nerve growth and the rate of epithelial wound healing are controlled coordinately by the wound-induced electric field.

Key words: Nerve regeneration, Wound healing, Cornea, Electric field

Introduction

Cell migration and neuronal growth cone guidance are controlled by growth factors, the extracellular matrix and other soluble and substrate bound molecules (Gipson and Inatomi, 1995; Sutherland et al., 1996; Zheng et al., 1996; Mueller, 1999; Song and Poo, 1999; Imanishi et al., 2000; Song and Poo, 2001; Duchek and Rorth, 2001; Martin et al., 2002; Xiang et al., 2002). Steady DC electric fields (EFs) also control these cell behaviours in culture (McCaig and Zhao, 1997; Zhao et al., 1999a; Zhao et al., 1999b) have been measured over many hours in vivo and are required for normal development and regeneration (Hotary and Robinson, 1991; Hotary and Robinson, 1992; Shi and Borgens, 1995; Robinson and Meserli, 1996; Sta Iglesia and Vanable, 1998). A physiological role for EFs in nerve guidance is not well established however, because to date there have been no unequivocal demonstrations of EF effects at the cellular level in vivo. This work provides this evidence using the wounded mammalian cornea as a model system. We chose this preparation because: (1) it generates its own endogenous electrical field instantaneously and this penetrates up to 1 mm from the wound edge; (2) sensory nerves close to the wound sprout new processes that grow towards the wound edge; (3) epithelial cells proliferate and migrate coordinately to close the wound.

The endogenous EF generated at an epithelial wound is an intrinsic property of all transporting epithelia that separate ions and sustain a transepithelial potential difference (Fig. 1). Unwounded cornea establishes an internally positive transcorneal potential difference (TCPD) of around +40 mV, by actively pumping Na^+ and K^+ inwards and Cl^- outwards across the epithelial layers (Candia, 1973; Klyce, 1975; Candia and Cook, 1986). Wounding the epithelial sheet creates a hole that breaches the high electrical resistance established and maintained by epithelial tight junctions and this short-circuits the epithelium, locally. The TCPD therefore drops to zero at the wound. However, because normal ion transport continues in the unwounded epithelium, the TCPD remains at normal values around 500 μm to 1 mm from the wound edge. It is this gradient of electrical potential difference, 0 mV at the short-circuited lesion, + 40 mV 500 μm from the wound in unwounded tissue, that establishes a steady, laterally oriented EF with the cathode at the wound (Fig. 1). So in contrast to the TCPD generated across the intact epithelium, which has an apical to basal orientation, the wound-induced EF has a vector orthogonal to this. It runs laterally under the basal surfaces of the epithelial cells and returns laterally within the tear film across the apical surface of the epithelium (Fig. 1). Importantly, wound-induced EFs will persist until the migrating epithelial leading edges re-seal the wound and re-establish a uniformly high electrical resistance across the tissue.

Two studies using extracellular microelectrode recordings have confirmed the existence of such steady wound-induced EFs in bovine cornea and in guinea pig and human skin. In skin, the peak voltage gradient at the wound edge was 140 mV/mm and in cornea 42 mV/mm, although the latter is an underestimate (Barker et al., 1982; Chiang et al., 1992).

The TCPD and therefore the wound-induced EF that inevitably ensues can be manipulated pharmacologically and this allows a test of the hypothesis that the wound-induced EF regulates and directs both nerve sprouting and wound closure. Four drugs that increase the TCPD by different mechanisms and two drugs that collapse the TCPD were used. We show that enhancing or reducing these electrical signals, respectively enhanced or reduced the extent of nerve sprouting, the direction of nerve sprouting and the rate of epithelial wound healing. This is the first definitive evidence in vivo, that a naturally occurring, endogenous EF controls two interdependent cell behaviours: nerve regeneration and wound healing.

Materials and Methods

Transcorneal PD measurements in vitro

Excised rat corneas were clamped in Ussing chambers with a 3 mm

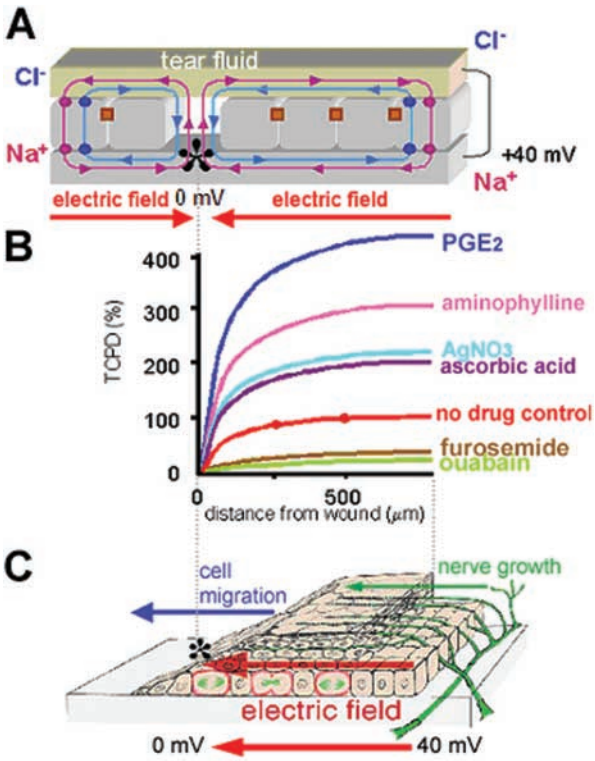

Fig. 1. The origin and effects of the wound-induced EF in rat cornea. (A) Simplified schematic representing the stratified rat corneal epithelium as a single layer of cells resting on a basement membrane and covered with a tear film. Cells are connected by tight junctions (brown squares), which form the major electrical resistive barrier of the epithelium. Intact mammalian corneal epithelium transports Na^+ inwards (pink) and Cl^- outwards (blue) and this separation of charge establishes a transcorneal potential difference (TCPD) of around +40 mV (internally positive). At a wound (asterisk), ionic currents flow underneath the cell layers, pass through the lesion and have return paths in the tear fluid layer. This short-circuits the TCPD, which drops to 0 mV at the wound, but the TCPD remains at normal values 500 μm from the wound. This establishes a steady, laterally oriented EF (red arrows) directed towards the wound (Chiang et al., 1992). (B) The TCPD varies as a function of distance from the wound edge and we manipulated this pharmacologically. Control plot (red) represents directly measured data, with 100% of the normal TCPD present 500 μm from the wound edge (Chiang et al., 1992). The effects of various drugs are shown. For example, prostaglandin E2 (PGE2) increased the TCPD more than fourfold (425%). The drop off with distance to the wound is inferred by comparison to the no drug control graph. The *x* axis maps onto the diagrams in (A) and (C). PGE2 enhances chloride efflux, aminophylline and ascorbic acid inhibit phosphodiesterase breakdown of cAMP, which also enhances Cl^- efflux, $AgNO_3$ increases both early Na^+ uptake and later Cl^- efflux. Ouabain inhibits the Na^+/K^+ ATPase and furosemide inhibits the active Cl^- efflux. (C) Detail of the wound in (A). The EF vector is defined as the flow of positive charge (red arrow). Current flows out the wound (asterisk), which acts as a cathode. Several cell behaviours close to the wound are directed by the wound-induced EF. Cell migration and nerve sprouting (green) are directed cathodally, towards the wound edge and mitotic spindles (green) align parallel to the EF. Cell division therefore also is oriented by the naturally occurring EF (Song et al., 2002).

diameter hole immediately after dissection, perfused continuously at 10 ml/minute with Kreb's Ringers (pH 7.4), and equilibrated with 95% O_2 and 5% CO_2. We recorded the TCPD by routine methods using a DVC-1000 amplifier (World Precision Instruments). Aminophylline (10 mM), PGE2 (0.1 mM), ascorbic acid (1 mM), $AgNO_3$ (1 mM), ouabain (10 mM), furosemide (1 mM), neomycin (10 mM) and d-tubocurare (1 mM) were added individually to both sides of the cornea to manipulate the TCPD.

Equatorial and circular epithelial wounds in vivo

Sprague-Dawley rats (27-30 days old, male or female) were anaesthetised with intramuscular Hypnom (0.3 ml/kg) and intraperitoneal Diazepam (0.5 ml/kg). To study wound-induced nerve sprouting, two nasal to temporal parallel incisions through the whole corneal epithelium were made and the epithelial layer was removed with the basement membrane intact, leaving a horizontal wound, 1-1.5 mm wide. To assess wound healing rates, a circular wound was made through the whole epithelium with a trephine and a 3.5 mm diameter disc of epithelium was removed with the basement membrane intact under an ophthalmic microscope. Sterile conditions were maintained for all experiments. Post-surgical recovery was uneventful and corneal wound healing proceeded without infection.

Application of drugs

For wound healing and nerve sprouting assessment in vivo, the following drugs were used: 10 mM aminophylline, 0.1 mM prostaglandin E2 (PGE2), 1 mM $AgNO_3$, 1 mM ascorbic acid, 0.1 mM ouabain, 1 mM furosemide, 10 mM neomycin or 1 mM d-tubocurare. Each drug was applied topically to lesioned corneas every 2 hours after wounding, for up to 30 hours. All agents were diluted in a balanced salt solution: 140 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5 mM glucose and 10 mM HEPES, with pH adjusted to 7.4. Balanced salt solution alone was used for control corneas.

Wound healing

Wound healing was assessed at 0, 10, 20 and 30 hours. Animals were lightly anaesthetised and the circular lesion area labeled with fluorescein and photographed. Lesion radius was measured from a minimum of four experiments with each treatment.

Scanning confocal microscopy to assess nerve sprouting

Control animals were killed using CO_2 at 0, 6, 16 and 24 hours after wounding and drug-treated animals were killed after 16 or 24 hours. Lesioned corneas were removed, fixed, permeabilized and the nerves stained with 1:200 anti-βIII tubulin (Promega), or 1:500 anti-GAP-43 (Sigma) monoclonal antibody at 4°C for 12 hours followed by a FITC-conjugated secondary antibody (Sigma). Epithelial cell nuclei were labeled with propidium iodide (Vector). Optical sectioning of whole-mounted cornea in the Z-axis was performed on an MRC 1024 confocal microscope (Bio-Rad Laboratories). The thickness of the whole series was from 20 to 30 μm with Z steps at 2 μm. 3D reconstruction was performed using Bio-Rad Lasersharp 2000 Version 3.1 software and all 3D images were projected to 2D. All nerves sprouting within 1 mm of the wound edge were counted. The angle (0-180°) between the sprouting nerves and the wound edge was measured to quantify the orientation of nerve sprouting. The proportion of nerves lying between 80° and 100° (classed as 'perpendicular') was compared statistically between drug treatments. Additionally, the 0-180° angles (θ) were transformed to 0-90° by |180–θ|, and the absolute orientation of nerve sprouts was analysed by Rayleigh's distribution, to give a mean polarization index (PI) of $\{\Sigma n\cos[2(\theta–90)]/n\}$ (Zhao et al., 1999b). If the entire population of sprouting nerves met the wound edge perpendicularly, at an angle of 90º, this equation becomes cos 2(90–90)=cos 0 and this gives a polarization index of +1. If all nerves ran parallel to the wound edge, the equation becomes cos 2(0–90)=cos–180 and this gives a

polarization index of –1. Nerves sprouting in random directions would have a mean PI of 0.

To determine whether nerves changed their direction of growth and turned to grow perpendicularly towards a wound edge, the number of new sprouts that turned through more than a 10° angle towards the perpendicular was counted within the healing tissues at different time points. Absolute nerve numbers were converted to percentages to assess differences between different groups.

To test the influence of the wound-induced EF on the direction of neurite sprouting, neurite angles were analysed at different distances from the edge (0-250 μm, 250-500 μm and 500 μm-1 mm), because the strength of the EF measured at a wound drops off sharply within the first 0.5 to 1 mm from the edge (Fig. 1B).

Statistical analysis

Two-sided Pearson Chi-Square test and Student's *t* test were used.

Results

The cornea generates wound-induced electrical fields that can be manipulated pharmacologically

Wounding the cornea was previously shown to generate an endogenous, laterally oriented EF under the epithelium, by abolishing the TCPD locally at the wound site (Chiang et al., 1992) (Fig. 1). We measured the TCPD in normal and drug-treated rat cornea using Ussing chambers (Fig. 1B). PGE2, aminophylline, $AgNO_3$ and ascorbic acid all increased the TCPD and hence the EF, two to fourfold (425±25%; 288±13%; 200±14% and 192±8% respectively; minimum n=4, P<0.01). Ouabain and furosemide reduced the TCPD fivefold (to 19±3% and 28±3% respectively n=6, P<0.01; Fig. 1B). We chose this particular array of drugs because they modulate the TCPD by different cellular mechanisms (see Discussion). Neomycin was also used because it did not alter the TCPD (data not shown), but it did enhance the epithelial wound closure rate (see below) and also because it inhibits some EF-induced cell behaviours (McCaig and Dover, 1991; Erskine et al., 1995).

The wound-generated EF regulates corneal wound healing

Circular wounds in control corneas healed at 23±3 μm/hour in the first 10 hours (Fig. 2, Table 1). Enhancing the wound-generated EF with PGE2, aminophylline, $AgNO_3$, or ascorbic acid approximately doubled early healing rates whereas collapsing the EF with ouabain or furosemide, by contrast, significantly reduced healing rates within 10 hours (Fig. 2, Table 1).

The above experiments were designed to test the effects of pharmacological agents that modulated the wound-induced EF on the rate of wound healing and also on the extent and direction of nerve sprouting (see below). In addition to testing the effects of manipulating the EF, we sought to test the role of drugs that we had shown previously interfered with the ability of nerve cells to transduce an EF. Neomycin and d-tubocurare both prevent cathode-directed neuronal growth cone turning in culture (Erskine et al., 1995; Erskine and McCaig, 1995) (see Discussion). In contrast to ouabain, neomycin increased wound closure rates to 56±2 μm/hour by 30 hours (Fig. 2, n=9, P<0.05) but d-tubocurare had little effect (data not shown).

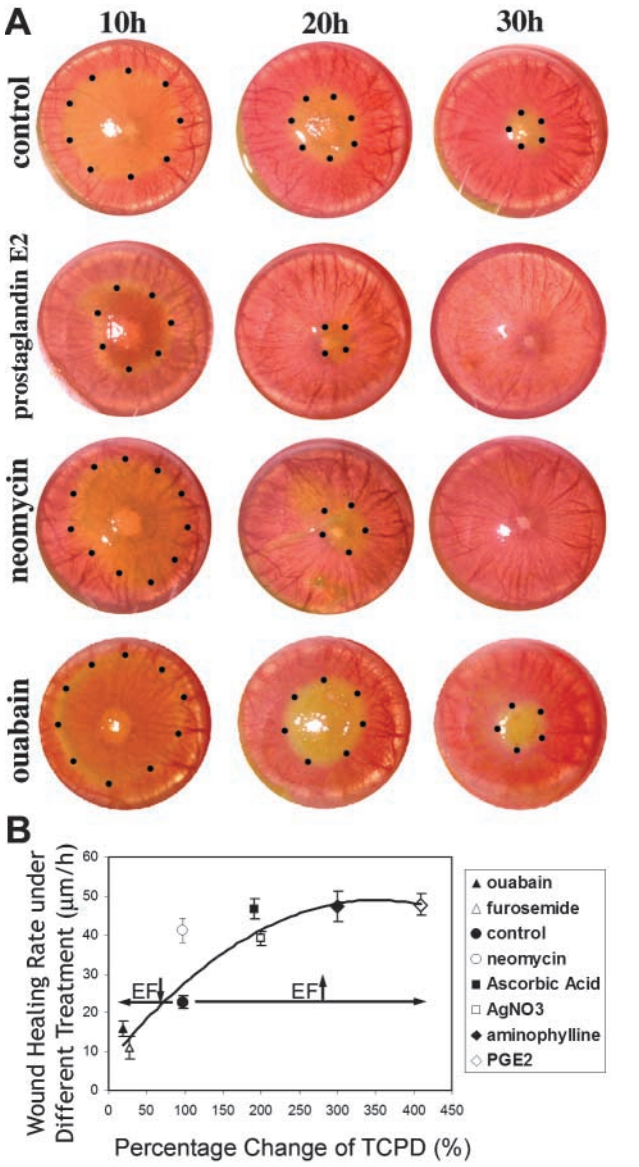

Fig. 2. The wound-induced EF controlled wound-healing rates. (A) Circular lesions in rat cornea (3.5 mm in diameter at 0 hour) are labeled yellow with fluorescein and outlined with dots and shown at 10, 20 and 30 hours after wounding. Control (top row) healing is incomplete by 30 hours. Enhancing the wound-generated EF with PGE2 (second row), caused wound closure before 30 hours. Aminophylline, $AgNO_3$ and ascorbic acid also enhanced wound-healing rates (not illustrated). Collapsing the EF with ouabain (bottom row) or furosemide (not shown) slowed wound healing which was incomplete by 30 hours. Neomycin also enhanced the wound-healing rate up to 30 hours after a wound (third row). (B) Corneal epithelial wound-healing rates varied with the wound-generated EF. Enhancing (with ascorbic acid, $AgNO_3$, aminophylline and PGE2) or reducing (with ouabain and furosemide) the TCPD and therefore the steady wound-induced EF pharmacologically (x-axis), respectively enhanced and reduced the wound healing rate (μm/hour, mean±s.e.m. of 0-10 hours post wound). Regression formula for the correlation between TCPD and wound healing rate is $y=34+0.12x-0.0002x^2$, Pearson correlation=0.91, correlation is significant at 0.01 level. A minimum of four experiments was performed in each group. Neomycin did not affect the TCPD, but enhanced the wound-healing rate significantly.

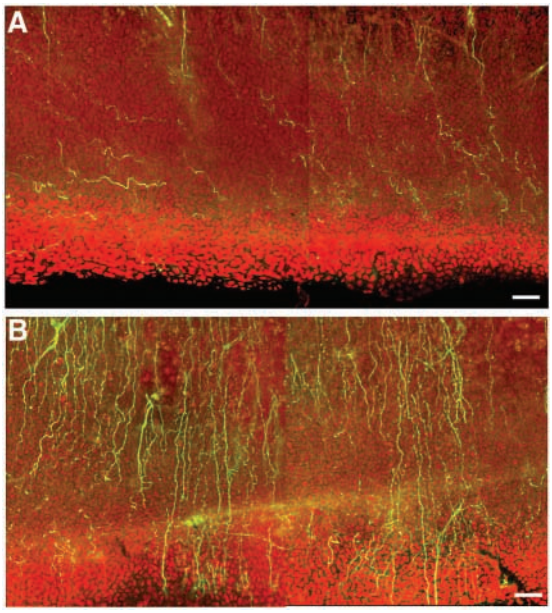

Fig. 3. Early response of corneal nerves during wound healing. Anti-βIII tubulin staining for corneal nerves at 0 hour (A) and 24 hours (B) after wounding. There were fewer nerves detectable at 0 hour and most were randomly distributed. At 24 hours after wounding, nerve numbers were increased dramatically. Nerve sprouts run parallel to each other and are oriented perpendicularly toward the wound edge (bottom). Bars, 50 μm.

Wounding the cornea induces directed nerve sprouting

Wounding the cornea stimulates wound-directed nerve sprouting (Rozsa et al., 1983). In equatorial epithelial wounds in adult rat this was a striking response, with bundles of nerve sprouts oriented parallel to each other and largely perpendicular to the straight wound edge. The response became obvious around 16 to 24 hours after wounding (Fig. 3). Initially, we used anti-βIII-tubulin to stain both existing nerves and new nerve sprouts within the cornea. However, because the wound-directed nerve response revealed with anti-βIII-tubulin was so robust, we chose to stain subsequently with anti-GAP-43, which labels a subpopulation of stout, regenerating sensory nerve sprouts and this made quantification easier (Fig. 4).

We measured the angles at which nerves projected towards the wound margin and used this to calculate a polarization index (PI). Nerves approaching the wound edge at angles of 90±10° were classed as 'perpendicular'. Randomly distributed nerve orientation gives a mean angle of 45° (and therefore a mean PI of 0) and a population of sprout angles closer to 'perpendicular' gives a mean closer to 90° (and therefore a mean PI nearer 1). At 16 hours, the PI of all nerve angles from control (untreated) wounds was 0.17±0.02, but by 24 hours it was 0.69±0.05 (P<0.01). Nerves therefore became markedly 'perpendicular' between 16 and 24 hours (compare Fig. 4C with 4D).

The wound-generated EF is causal in directing corneal nerve sprouting

Enhancing the wound-generated EF with PGE2 (Fig. 4C), aminophylline (Fig. 4F), ascorbic acid or $AgNO_3$ (not shown) tripled the proportion of early perpendicular nerves at 16 hours and massively increased the PI (Fig. 4J, P<0.01; PI=0.17, 0.73, 0.77, 0.54 and 0.57 for 16 hours control, 16 hours aminophylline, PGE2, ascorbic acid and $AgNO_3$ respectively). Collapsing the wound-generated EF with ouabain (Fig. 4G) and furosemide (not shown) caused a twofold drop in perpendicular nerves by 24 hours and the PI dropped towards zero (Fig. 4G and J, P<0.05; PI=0.69±0.06, 0.20±0.05 and 0.27±0.07 for 24 hours control, ouabain and furosemide respectively).

Interestingly, following pharmacological manipulation of the TCPD, the PI of nerve sprouts and the TCPD changed proportionately (Fig. 4J). The regression formula for the correlation between TCPD and PI of nerve angles was y=–3.7+1.3x–0.0017x^2, giving a Pearson correlation of 0.93, which is significant at the 0.05 level (two-sided, P=0.02). This indicates that enhancing or reducing the EF caused a directly proportionate increase or decrease in directed nerve sprouting and therefore implicates the endogenous EF as one of the key factors controlling the orientation of nerve sprouts towards a corneal wound.

The neuronal nicotinic acetylcholine receptor antagonist d-tubocurare and the phospholipase C inhibitor neomycin both completely inhibited EF-directed turning of nerve growth cones in culture. They therefore identify key receptor and second messenger elements of the signaling pathway used to induce EF-directed nerve guidance (Erskine et al., 1995; Erskine and McCaig, 1995). We tested whether they also inhibited wound-directed corneal nerve sprouting. Neomycin (Fig. 4I) and d-tubocurare (Fig. 4H) more than halved the incidence of perpendicular nerves at 24 hours, the mean angle of neurites sprouting towards the wound edge (and therefore the PIs) also decreased greatly. In control (untreated) corneas, the PI of nerve sprouts at 24 hours was 0.69±0.06. In corneas treated with neomycin or d-tubocurare, the PI values had dropped to 0.21 and 0.38 respectively (P<0.05), indicating randomly directed nerve sprouting. Importantly, neomycin enhanced the wound-healing rate whereas d-tubocurare did not affect wound healing (Table 1 and Discussion).

Morphology of the neuronal response

Four morphological sub-categories of nerve sprouts were present within 1 mm of the wound edge. These were: (1) new nerve outgrowth from intact nerves (66-86%, Fig. 5A); (2) new sprouts rising from basal layers (4-20%, Fig. 5B); (3) new sprouts branching off a cut nerve (3-11%, Fig. 5C); (4) complex sprouts, where the nerve origin and orientation could not be determined (5-10%, Fig. 5D). Subsequent analyses were made on new sprouts from intact nerves (1 above), because these represent the majority of the whole population (66-86%) and contribute most of the changes in nerve angles and PI.

Table 1. Wound healing rate under different treatments for 0-10 hours

Treatment	Control	PGE2	Aminophylline	AgNO3	Ascorbic acid	Ouabain	Furosemide	Neomycin
Rate of wound healing±s.d. (μm/hour)	23±3	48±3	47±4	39±2	47±3	16±2	11±3	43±3

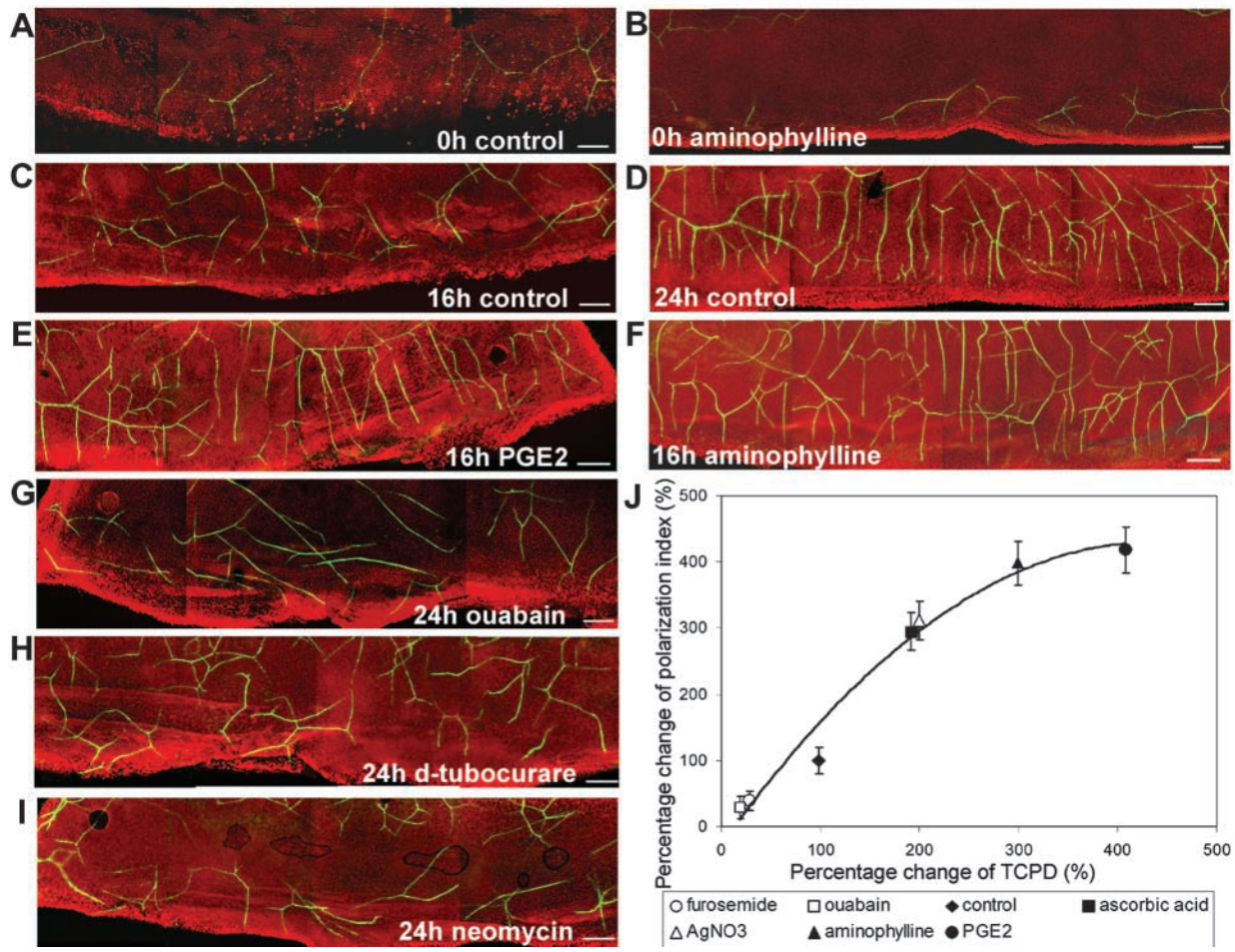

Fig. 4. Nerves grow perpendicularly towards a wound edge. (A-I) Enhancing or reducing the wound-induced EF increased or decreased perpendicular sprouting. FITC anti-GAP-43 and propidium iodide staining for whole-mounted, wounded cornea. By 24 hours, the frequency of perpendicular nerve sprouts doubled in untreated corneas (compare A and D). Enhancing the wound-generated EF with PGE2 (E) and aminophylline (F), more than doubled the frequency of perpendicular nerve sprouts at 16 hours compared to 16 hour control (C). At 24 hours, perpendicular nerve sprouting was abolished in corneas treated with ouabain, d-tubocurare and neomycin (G, H and I, respectively). All images are representative projections from three separate experiments. The corneal wound edge is at the bottom margin. (J) Perpendicular nerve sprouting towards a wound edge is proportionate to the wound-induced EF. Enhancing or reducing the TCPD and therefore the steady wound-induced EF pharmacologically (*x* axis), respectively enhanced and reduced the polarization index of nerve sprouting angles (*y* axis). On the *x* axis, control TCPD is shown as 100%, and pharmacologically modified TCPDs are compared with this. On the *y* axis, the PI for controls is shown as 100%, and the PIs for other treatment groups are scaled accordingly. Regression formula for the correlation between TCPD and proportion of perpendicular nerves is $y=-3.7+1.3x-0.0017x^2$, Pearson correlation=0.93, correlation is significant at 0.05 level (two-sided, $P=0.02$). The number of wounded, whole-mounted corneas was between four and eight for each treatment and the total number of nerve sprouts was between 46 and 186. Bars, 100 μm.

EF-directed nerve growth depends on time and distance from the wound edge

We showed above that nerve sprouts were directed towards the wound edge by the wound-induced EF. In addition, indirect information on the time dependency and EF strength dependency of directed nerve sprouting can be obtained by analysing directed nerve growth as a function of distance from the wound edge. This is because tissues 600 μm from the wound will have been at the cut edge 20 hours previously if healing occurs at 30 μm/hour, and because the EF intensity is strongest at the wound edge and drops off exponentiallyinto the intact cornea (Fig. 1). Analyses were made in three corneal zones: 0-250 μm, 250-500 μm and 500 μm-1 mm from the wound edge. Fig. 6 shows that new nerve sprouts were oriented perpendicularly towards the wound edge up to 1 mm from the wound edge. Nearest the edge in untreated corneas (0-250 μm), the PI was 0.39±0.04 at 16 hours and had doubled to 0.81±0.06 by 24 hours (Fig. 6). This indicates that the EF continues to have a profound effect in orienting new sprouts within the leading edge of the healing epithelial sheets for at least 24 hours. Because the PI values at 16 and 24 hours in the 250 μm-1 mm sector were smaller than at the very edge (0.20±0.05 and 0.65±0.04 respectively) this further indicates that the orienting effect of the EF on nerve growth was strongest at the wound edge, but persisted albeit with a weaker influence into tissues 250 μm-1 mm behind the edge. All four drugs that enhanced the EF (PGE2, aminophylline, $AgNO_3$ and ascorbic acid) roughly doubled the orientation of nerve growth towards the wound edge within the first 16 hours. Interestingly, this enhancement was evident up to 1 mm from the wound and showed no drop off with distance from the wound edge, as was

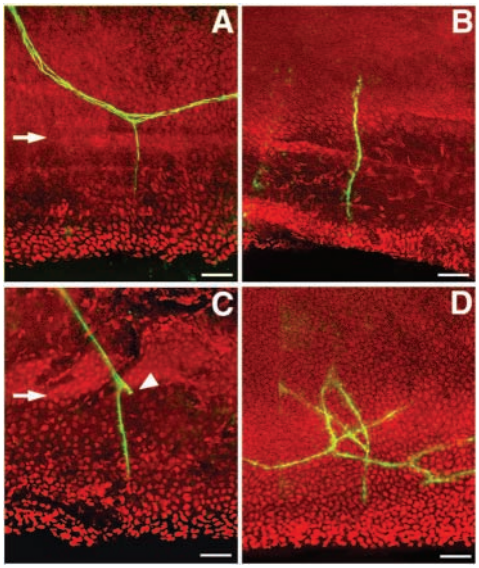

Fig. 5. Classification of four types of new nerve sprouts. (A-D) Nerves in whole-mounted, wounded corneas are green (FITC anti-GAP-43) against a red propidium iodide-stained background. Four types of new nerve sprouts were seen: (A) New growing sprouts from intact nerves; (B) New growth from basal layers; (C) New growth from a cut stump (arrowhead shows the cut end of the nerve); (D) complex nerves with no clear orientation. New sprouts from intact existing nerves formed the majority of the total new nerve population and were the only type of nerve changing dramatically with time and different drug treatment (A). Arrows in (A,C), cut edge at 0 hours. Bars, 50 μm.

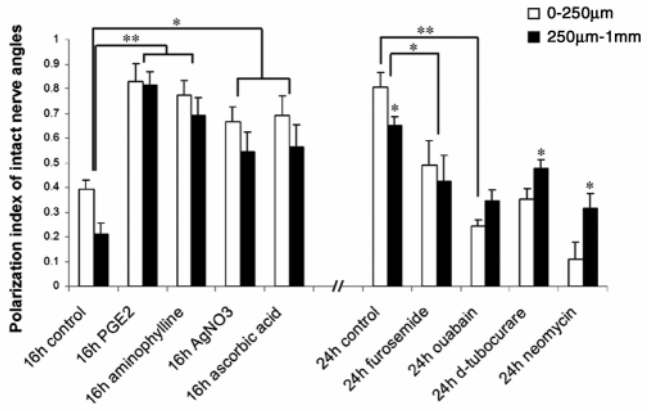

Fig. 6. Directed nerve sprouting as a function of time and distance from the wound (EF strength). The polarization index of nerve sprouting angles was calculated for new sprouts from intact nerves. From 16 to 24 hours, new sprouts oriented significantly with time (compare 16 and 24 hour control PI values, $P<0.01$). In control corneas, new sprouts within 0-250 μm of wound edge were oriented more perpendicularly than those at 250 μm-1 mm, where the EF strength drops off ($P<0.01$ for 16 hours; $P<0.05$ for 24 hours). At 16 hours, all four drugs that increased the EF (PGE2, aminophylline, $AgNO_3$ and ascorbic acid) enhanced perpendicular orientation up to twofold (compare 16 hour control PI with 16 hour PGE2 and aminophylline PI values, both $P<0.01$). At 24 hours, reducing EF with ouabain or furosemide significantly reduced nerve orientation (compare 24 hour control PI with 24 hour ouabain or furosemide PI values, $P<0.01$ or $P<0.05$ respectively), and this was more obvious at 0-250 μm (compare 24 hour ouabain 0-250 μm PI with 250 μm-1 mm PI, $P<0.05$). Tubocurare and neomycin also significantly reduced nerve orientation (compare 24 hour control PI with 24 hour tubocurare and neomycin PI values, $P<0.05$ and 0.01, respectively).

seen in control corneas (Fig. 6). This has two implications: (1) that the drug-enhanced EF induced faster nerve orientation and (2) that it penetrated further into the tissues to induce this. By contrast, both when the EF was suppressed by ouabain or by furosemide and when transduction of the EF was inhibited by d-tubocurare or by neomycin (Fig. 6), the normally striking nerve orientation seen in control corneas at 24 hours was profoundly inhibited. Importantly, the effects of ouabain, d-tubocurare and neomycin in inhibiting directed nerve sprouting were most marked in the leading edge zone, where the EF would be strongest. The endogenous EF therefore, controlled the orientation of new, regenerating sprouts in a time- and field strength-dependent manner.

Nerve sprouts turn to respond to the wound-induced EF

Many studies have shown that a physiological EF induced robust turning of cultured neuronal growth cones (McCaig et al., 2002). To determine whether new nerve sprouts changed direction to align with the vector of the endogenous EF in vivo, the proportion of sprouts with angles that changed by more than 10° towards the perpendicular were counted at different times. Fig. 7A shows that 16 hours after wounding around 20% of nerves had turned to project more perpendicularly towards the wound. After 16 hours of treatment with all four of the drugs that enhanced the EF there was a 59-80% increase in the proportion of nerves turning to align with the endogenous EF (Fig. 7A, left panels). In addition, between 16 and 24 hours the proportion of nerve sprouts that had turned to lie more perpendicularly more than doubled (Fig. 7A, compare 16 hours control with 24 hours control; 21% to 48%). This sustained ability of the EF to induce continual turning of sprout growth over a 24 hour period was inhibited severely both by treatment with ouabain and furosemide that suppressed the EF and by treatment with neomycin and d-tubocurare that inhibit transduction of the EF (Fig. 7A, right panels; 48% down to 17-25%). In short, new nerve sprouts turned to align with the EF vector, which lies perpendicular to the wound edge. The proportion that did so increased when the EF was increased and decreased when the EF was suppressed. Importantly, sprouts made both left- and right-handed turns to come to lie perpendicular to the wound-induced EF (Fig. 7B).

The wound induced EF enhanced nerve sprouting

To determine whether the wound-induced EF stimulated nerves to sprout, sprout numbers were counted per unit length of wound edge. Fig. 8 shows that within the tissue 1 mm from the wound at 16 hours, there were two sprouts per mm of wound edge. New sprouts continued to appear with time, as at 24 hours there were 3.5 sprouts/mm of wound edge ($P<0.01$). Sprout numbers increased more rapidly at wounds treated with all four drugs that increased the endogenous wound-induced EF. Treatment with aminophylline for example more than doubled sprout numbers at 16 hours (Fig. 8), whereas all four drugs that suppressed the EF effects slowed and/or suppressed the appearance of new sprouts by 24 hours.

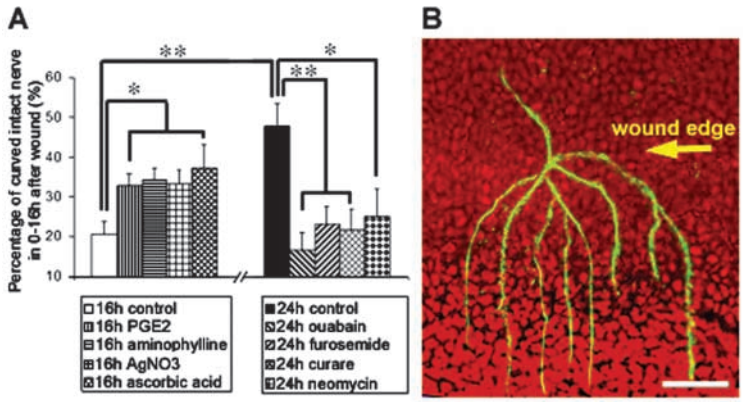

Fig. 7. Percentage of curved nerves from intact branches 0-16 hours after wounding. (A) All drugs that increased the EF (PGE2, aminophylline, $AgNO_3$ and ascorbic acid) enhanced the nerve turning response in the period 0-16 hours after a wound (compare 16 hours control value with percentages in the four treatments at 16 hours, $P<0.05$). This nerve turning response roughly doubled during the 16-24 hour period after wounding in controls (compare 16 hour control value with 24 hour control value, $P<0.01$). Reducing the EF with ouabain or furosemide, significantly suppressed the nerve turning response at 24 hours (compare 24 hour control value with 24 hour ouabain or furosemide value, $P<0.01$). Preventing transduction of the EF with tubocurare or neomycin also significantly suppressed this turning response at 24 hours ($P<0.01$, and $P<0.05$ respectively). (B) Representative photomicrograph of nerve turning. New sprouts that turned through more than 10° to project toward the wound edge were counted at either 16 hours or 24 hours after wounding. The image is a projection from three separate experiments and shows that nerves turned in either direction to come to project perpendicular to the wound edge along the EF vector. Arrow shows the wound edge at 0 hour. Bar, 50 μm.

Sprout numbers were also assessed as a function of distance from the wound edge to determine how far this stimulatory effect of the EF penetrated into the tissue. Fig. 9A shows that at the wound edge (0-250 μm), sprout numbers increased dramatically; threefold between 16 and 24 hours (compare Fig. 9D and 9E). By contrast Fig. 9B shows that more distant from the wound edge in the 500 μm-1 mm zone there was no increase in sprout numbers between 16-24 hours (compare Fig. 9F and 9G). Taken together these observations indicate that the effect of the EF in stimulating nerve sprouts was limited to the wound edge, where the EF was most intense (Fig. 1) and that this wound edge effect persisted for at least 24 hours.

A different picture emerges in examining the effects of the four drugs that enhanced the EF. Fig. 9A,B shows that sprout numbers increased two- to threefold, both at the wound edge (0-250 μm) and further back from this (500 μm-1 mm, compare Fig. 9F,G with 9H,I). Increasing the intensity of the EF therefore increased the extent to which its effects were felt further from the wound edge.

Finally, Fig. 9C shows that the effects of the EF in stimulating sprout numbers penetrated at least 500 μm from the wound. In addition, the four drugs that inhibited either the EF magnitude or the transduction of the EF, each led to a suppression of sprout numbers 500 μm from the wound edge (compare Fig. 9J with 9K).

Discussion

We have confirmed that a steady DC electric field arises instantaneously at rat corneal wounds and that this regulates three interdependent cell behaviours. The wound-induced EF controlled epithelial healing rates, induced nerves to turn in order to grow perpendicularly towards the wound edge and also stimulated directed nerve sprouting. Reducing or increasing the electrical signal with drugs that work by different cellular mechanisms, predictably inhibited or enhanced all three responses.

Wound healing is regulated by the endogenous EF at the wound

Electrical control of wound healing has been described previously although the effective polarity is debated (Chiang et al., 1992; Sta Iglesia and Vanable, 1998). Here we have shown that increasing or reducing the naturally occurring, wound-induced EF, which has the cathode at the wound, respectively speeded up and slowed down wound healing rates. Cathodal migration of corneal epithelial cells (CECs) in culture is transduced through the EGF signaling pathway (Zhao et al., 1999a). It depends on serum growth factors, is restored by adding EGF to serum-free medium, is inhibited by function-blocking antibodies to EGF and involves EF-induced upregulation and cathodal asymmetry of the EGF receptor, of elements of the MAP kinase signaling pathway and of filamentous actin (Zhao et al., 1996; Zhao et al., 1999a; Zhao et al., 2002). Similar events may drive the EF-induced wound healing reported here. Irrespective of the cellular mechanisms involved, there has been little clinical exploitation of the profound effects of an EF on wound healing. Our

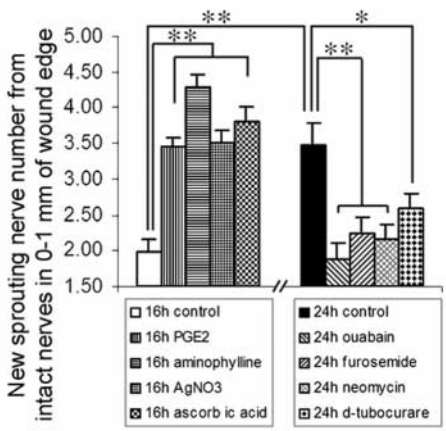

Fig. 8. The number of new nerve sprouts was increased by the wound-induced EF. From 16 to 24 hours after wounding, new sprouts grew from the intact nerves (compare 16 and 24 hour control values, $P<0.01$). Pharmacologically enhancing the endogenous EFs also significantly increased new sprouts as early as 16 hours (compare 16 hour control values with values for all other treatments at 16 hours, $P<0.01$). Ouabain, furosemide, neomycin and d-tubocurare all reduced nerve sprout numbers 24 hours after wounding (when compared with 24 hour control value).

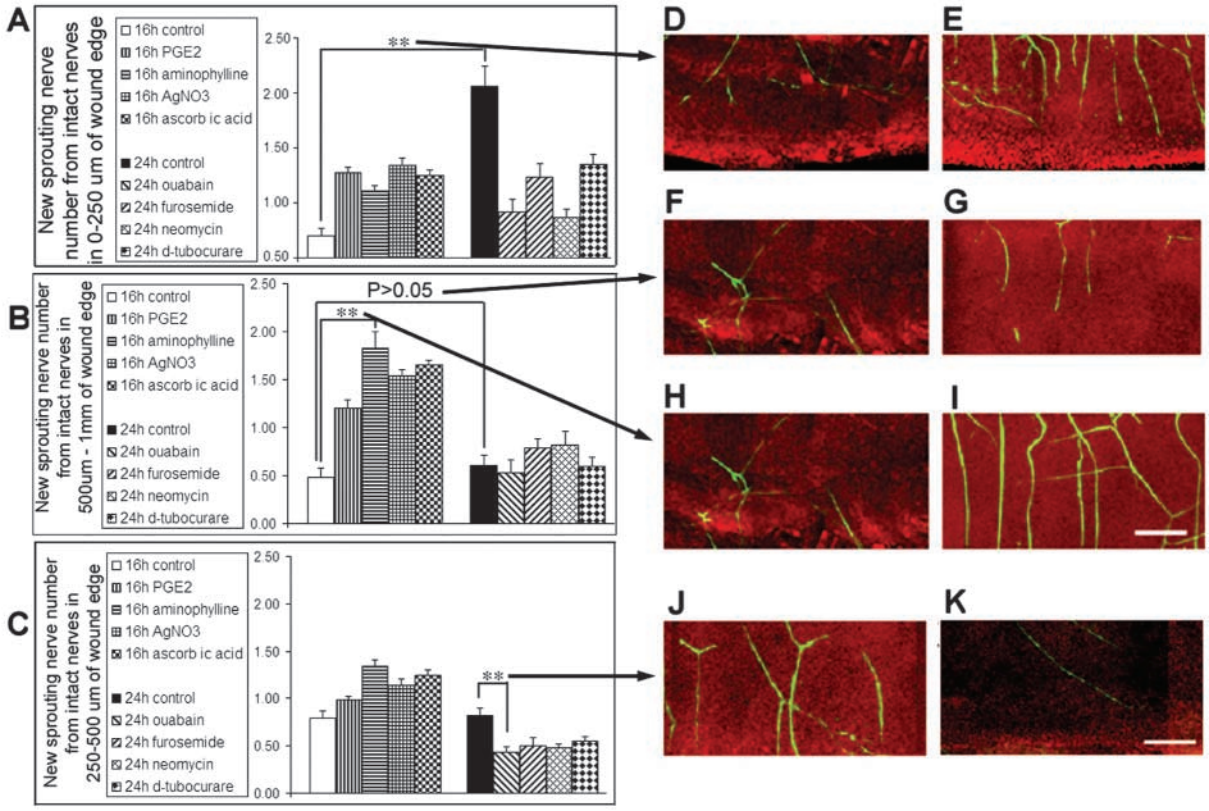

Fig. 9. The number of new sprouts is a function of time and distance from the wound (EF strength). (A) At the wound edge (0-250 μm), new sprouts more than doubled in control corneas over the period from 16 hours (D) to 24 hours (E) (P<0.01), indicating that the EF effect was most potent at the leading edge between 16 and 24 hours. (B) At 500 μm-1 mm from the wound edge, there was no change in sprout number between 16 hour (F) and 24 hour (G) control values (P>0.05). Pharmacologically enhancing the endogenous EF, significantly increased nerve sprouts at 16 hours, for instance, compare the 16 hour control cornea (H) with the 16 hour aminophylline-treated cornea (I), (P<0.01), indicating that for all four drug-enhanced EFs the effects had penetrated to a depth of 1 mm between 0 and 16 hours. (C) At 250-500 μm from the wound edge, suppressing the endogenous EF with ouabain (K) or furosemide and preventing EF transduction with neomycin or tubocurare all significantly reduced nerve sprout numbers at 24 hours compared to 24 hour control values (J) (P<0.01), indicating that the EF effect had penetrated to a depth of at least 500 μm. Bars, 50 μm.

demonstration that a doubling of healing rates can be achieved through enhancing the natural, wound-generated EF pharmacologically may promote this and indeed stimulate efforts to use EFs in conjunction with current growth factor-based therapies.

The direction of nerve growth is regulated by the endogenous EF at a wound

With respect to the directed neuronal growth and turning responses, at least two well-recognised guidance cues are likely to be present at corneal wounds and may induce or contribute to these effects. First, a host of growth factors and cytokines are released upon wounding and these could give rise to chemical gradient effects. Second, a healing epithelial sheet may induce tension within trailing tissues that could orient nerve sprouts. When the EF was modulated pharmacologically, faster epithelial closure rates were frequently correlated with the extent of perpendicular nerve sprouting, suggesting a causal link. In this scenario, wound-directed nerve sprouting could be secondary to epithelial movement-induced tensions and not a primary response to the EF.

Three observations indicate that the EF induced perpendicular nerve growth. Firstly, with regard to potential tension effects, both ouabain and neomycin prevented perpendicular nerve growth (Fig. 4G,I). However, ouabain slowed and neomycin increased wound healing, probably reducing and increasing epithelial closure-induced tension respectively. Because wound closure rates could increase (in neomycin), whereas nerve sprouts failed to orient perpendicular to the edge, closure-induced tension is unlikely to play a major role in orienting nerve sprouts.

Secondly, many growth factors and cytokines are thought to regulate epithelial cell migration and nerve sprouting at wound sites (Gipson and Inatomi, 1995; Sutherland et al., 1996; Zheng et al., 1996; Mueller, 1999; Song and Poo, 1999; Imanishi et al., 2000; Song and Poo, 2001; Duchek and Rorth, 2001; Martin et al., 2002; Xiang et al., 2002). A standing chemical gradient, though not demonstrated, might orient nerve sprouts at untreated wounds, where a standing electrical gradient has been demonstrated. To our knowledge the drugs we used to enhance the wound-generated EF altered only the TCPD and did so by different mechanisms. PGE2 enhances chloride efflux, aminophylline and ascorbic acid inhibit phospodiesterase breakdown of cAMP, which also enhances Cl^- efflux (Chalfie et al., 1972; Klyce et al., 1973; Beitch et

al., 1974; Buck and Zadunaisky, 1975) and $AgNO_3$ increases both early Na^+ uptake and later Cl^- efflux (Klyce and Marshall, 1982). Similarly, we chose drugs that suppressed the wound-generated EF, by reducing the TCPD in different ways. Ouabain inhibits the Na^+/K^+ ATPase and furosemide inhibits the active Cl^- efflux (Pataraca et al., 1983; Scharschmidt et al., 1988). Importantly therefore we have used six disparate drugs with differing mechanisms of action, but which converge on one common denominator, an altered TCPD and wound-induced EF. None of these drugs is known to modulate growth factor or cytokine release, or chemical gradient formation, therefore a primary EF-induced effect is the most likely explanation of modulated wound healing and directed nerve sprouting.

Thirdly, both d-tubocurare and neomycin have been shown previously to prevent cathodal turning of amphibian growth cones cultured in a physiological EF, indicating that activation of neuronal nicotinic acetylcholine receptors and of phospholipase C are required for this response (Erskine et al., 1995; Erskine and McCaig, 1995). Both drugs also prevented nerves from turning and growing along the EF vector, perpendicular to the wound edge. The simplest interpretation is that nerves were prevented from transducing the existing wound-related electrical signal, because these receptor and second messenger signaling elements were blocked.

The magnitude of the nerve orientation and nerve sprouting responses as a function of distance from the wound edge was also consistent with an EF effect. The profile of the EF measured directly in the wounded cornea predicts that the strongest EF will exist closest to the wound edge and that this will drop off around 500 μm-1 mm from the wound edge (Chiang et al., 1992) (Fig. 1B).

(1) At 16 hours, sprouts were perpendicular to the wound edge only in the leading 250 μm and not between 250 μm and 1 mm; (2) Increasing the EF with four different drugs caused oriented sprouting earlier (by 16 hours) and this penetrated further into the wound, up to 1 mm from the wound edge (Fig. 6); (3) ouabain and furosemide, which collapsed the EF, suppressed wound-directed nerve sprouting most effectively at 250 μm from the leading edge. Although none of these observations formally excludes chemical gradient or tension-based effects, collectively, because they are consistent with the profile of the EF, they strengthen the case for electrical control of nerve orientation and nerve sprouting.

Intriguingly, the proportion of nerves sprouting was also regulated by the wound-induced EF. Sprout numbers increased closest to the wound edge, were enhanced by enhancing the EF (with four differently acting drugs) and inhibited by reducing the EF with ouabain and furosemide. It is also significant that ouabain and furosemide did not prevent nerves from sprouting (16 hour control and 24 hour ouabain sprout numbers were similar, Fig. 8), but did inhibit new sprouts from growing directly towards the wound edge. This argues against any nonspecific poisoning role for the cardiac glycoside in preventing perpendicular nerve orientation.

In conclusion, this work demonstrates for the first time that naturally occurring EFs regulate the extent and the direction of nerve growth in vivo, by inducing nerves to turn and that they also regulate the rate of wound healing. Current thinking on nerve guidance is dominated by the discovery of many molecules expressed in gradient form that direct nerve growth (Mueller, 1999). Our data indicate that in some situations, such chemical gradients must coexist with electrical gradients and that the physiological electrical signals direct nerve growth. Because these endogenous electrical signals can establish molecular gradients of charged protein molecules within embryonic tissues (Messerli and Robinson, 1997), it is appropriate that the interactions between chemotropic and electrotropic guidance of nerves be addressed more widely.

In addition to the clinical importance of EF-regulated wound healing, there is also clinical significance in the observations that nerve sprouts increase in number and that their growth is directed by the naturally occurring or pharmacologically enhanced EF. Applied EFs have been shown to enhance spinal nerve regeneration in the severed dorsal columns of adult guinea pig (Borgens, 1999) and to restore some function in guinea pigs and dogs with spinal cord damage (Borgens et al., 1987; Borgens et al., 1999). In addition, the efficacy of EF therapy in treating human spinal cord injuries is currently being tested in clinical trials (see www.vet.purdue.edu/cpr/).

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