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Integrated interactions between chondroitin sulphate proteoglycans and weak dc electric fields regulate nerve growth cone guidance in vitro

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SUMMARY

During development and regenerative growth, neuronal pathways are defined in part by several endogenous cues that collectively determine directed growth. The interactions between such cues largely are unknown. To address potential interactions, we have examined in vitro the combined effect on nerve growth of two endogenous growth cone guidance cues: chondroitin sulphate proteoglycans and weak dc electric fields. Addition to the culture medium of a chondroitin 6-sulphate/keratan sulphate containing PG (BNC-PG) markedly enhanced the cathodal re-orientation of embryonic *Xenopus* neurites in an electric field, whereas a proteoglycan containing chondroitin 4-sulphate (RC-PG) was inhibitory. These effects of BNC-PG and RC-PG were reproduced by their chondroitin sulphate glycosaminoglycan side chains alone. Chondroitin 6-sulphate or chondroitin 4-sulphate, respectively, enhanced and inhibited cathodally-directed nerve re-orientation. This was dependent on the integrity of the glycosaminoglycan chain structure; when digested into their disaccharide subunits both molecules became inactive. Keratan sulphate, a minor component of BNC-PG, was found to be inhibitory, whereas dermatan sulphate, an epimer of chondroitin 4-sulphate, had no effect. We conclude that in vitro specific interactions between these two nerve guidance cues do occur and that the specificity of the response is critically dependent on the charge pattern of the proteoglycans chondroitin sulphate side chains. The expression of a host of proteoglycans with differing glycosaminoglycan side chains varies in both time and place in the developing nervous system, thus the scope is vast for spatial and temporal modulation of nerve guidance by interacting cues.

Key words: Development, Glycosaminoglycan, Axon guidance

INTRODUCTION

Nerve guidance is critical in development and regeneration. To achieve this attractive and repulsive cues are transduced by growth cone filopodia and lamellipodia into directed cell motility (reviewed by Letourneau et al., 1991). Individual filopodia sense the local microenvironment and can amplify signals from the periphery (Davenport et al., 1993) with such sensitivity that the information from a single filopodium can re-orient nerve growth (O'Connor et al., 1990). Various orienting influences are known: selective adhesion or repulsion (Letourneau, 1975; Burmeister and Goldberg, 1988), gradients of tropic molecules (Gundersen and Barrett, 1980; Kennedy et al., 1994; Zheng et al., 1994) and weak extracellular electric fields (reviewed by McCaig et al., 1994; McCaig and Erskine, 1996). Such cues are regulated spatially and developmentally, thus migrating growth cones encounter a changing array of co-existent guidance signals. Nevertheless, few in vitro studies have combined guidance cues and yet interactions seem inevitable. Chondroitin sulphate proteoglycans (PGs)/glycosaminoglycans (GAGs) are one of the components of the embryonic extracellular matrix (Aquino et al., 1984; Flaccus et al., 1991; Oakley and Tosney, 1991; Bicknese et al., 1994; Landolt et al., 1995; Miller et al., 1995; Fernaud-Espinosa et al., 1996). Alone they can promote (Iijima et al., 1991; LaFont et al., 1992; Fernaud-Espinosa et al., 1994; Dou and Levine, 1995) or inhibit (Carbonetto et al., 1983; Verna et al., 1989; Snow et al., 1990, 1991; Oohira et al., 1991; Friedlander et al., 1994; Dou and Levine, 1995) and consequently direct nerve growth (Snow et al., 1991; Brittis et al., 1992; Snow and Letourneau, 1992). We have studied how these PGs interact with the strong guidance signal provided by a small applied electric field (Hinkle et al., 1981; Patel and Poo, 1982).

Endogenous electric fields exist in avian, amphibian and mammalian embryos, whilst disrupting these results in serious developmental abnormalities, including malformation of the central nervous system (see Discussion). In tissue culture, electric fields profoundly influence many aspects of nerve growth and provide a good model for examining potential interactions between guidance signals. In vitro, weak dc electric fields, similar in magnitude to those detected in embryonic systems (Robinson and Stump, 1984; Hotary and Robinson, 1990, 1994; Shi and Borgens, 1994, 1995a), stimulate neurite sprouting (Hinkle et al., 1981), induce growth cone turning (towards the cathode (negative pole); Hinkle et al., 1981; Patel and Poo, 1982; Erskine et al., 1995), enhance nerve growth rates (McCaig, 1990b; Erskine et al., 1995), enhance and direct neurite branching (towards the cathode;

McCaig, 1990a; Erskine et al., 1995) and alter the distribution of growth cone filopodia (McCaig, 1986, 1987). Control experiments have demonstrated that these responses occur as a direct result of the electric field and are not due to secondary effects such as the production of chemical gradients within the culture medium or the physical dragging of the neurites by the electrophoretic movement of fluid (Hinkle et al., 1981; Patel and Poo, 1982). Thus, it is the electric field, acting on the neurite itself, which modulates nerve growth.

To investigate whether the efficacy of electric field-induced guidance can be modulated by other components present in a neurites extracellular environment, we examined the effect of two extracellular matrix molecules, a chondroitin 6-sulphate/keratan sulphate PG (BNC-PG) and a chondroitin 4-sulphate containing PG (RC-PG) on galvanotropism. BNC-PG enhanced the cathodal turning of neurites in an electric field, whereas RC-PG was inhibitory. The chondroitin sulphate GAG moieties of these molecules alone mimicked the effects of the intact PGs, indicating that the GAG chains are responsible for their regulatory activities and that the core protein is not required. Potential mechanisms underlying these specific interactions are discussed. Such interactions in vivo between endogenous PGs/GAGs and bioelectric fields, or indeed similar interactions between other guidance cues, would add an extra level of subtlety to the control of growth cone pathfinding. A preliminary account has appeared in abstract form (Erskine and McCaig, 1993).

MATERIALS AND METHODS

Cell culture

All experiments were performed in accordance with national guidelines using tissue from embryos of *Xenopus laevis*. Developmental stages were assessed using the criteria of Nieuwkoop and Faber (1956). The culture methods were adapted from Jones and Elsdale (1963) and have been described previously (Hinkle et al., 1981; McCaig et al., 1994). Briefly, dissociated neurones from the neural tube of stage 20 *Xenopus* embryos were cultured in small chambers formed from two strips of No. 1 coverglass glued parallel to each other in the base of an untreated plastic tissue culture dish (Falcon 3003), 1 cm apart. A third coverglass formed the roof of the chamber. The final dimensions of the chamber, through which current was passed, were 64 mm × 10 mm × 0.5 mm. The culture medium consisted of (v/v) 20% Liebowitz L15 culture medium, 2% penicillin (5,000 i.u. ml^{-1})/streptomycin (5,000 μg ml^{-1}), 1% fetal bovine serum (all from ICN Biomedicals Inc., 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 7.8).

Global electric field application

Electric fields were applied 2-4 hours after the cells were plated, to neurites which had already sprouted, and were maintained for 5 hours. Agar-salt bridges, 15 cm long, were used to connect Ag/AgCl electrodes in beakers of Steinberg's solution to pools of excess culture medium at either side of the chamber. This prevents diffusion of electrode products into the cultures. Field strengths were measured directly at the beginning and end of the 5 hour observation period by bridging the cultures with a volt meter. No fluctuations in field strengths were observed. All data were pooled from a minimum of 2 separate experiments and, where the magnitude of the applied field differed between cultures, the range of field strengths used is given.

Experimental treatments

The PGs (BNC-PG/RC-PG) were purchased from ICN Pharmaceuticals Inc. (Costa Mesa, CA, USA) and the various GAGs (porcine rib cartilage Type A chondroitin sulphate (chondroitin 4-sulphate), shark cartilage Type C chondroitin sulphate (chondroitin 6-sulphate), porcine skin Type B chondroitin sulphate (dermatan sulphate); bovine cornea keratan sulphate) and their disaccharide subunits from Sigma Chemical Co. (St Louis, MO, USA). These molecules have all been demonstrated to alter growth cone behaviour in a variety of neuronal types (e.g. Snow et al., 1990, 1994; LaFont et al., 1992; Fernaud-Espinosa et al., 1994; Dou and Levine, 1995).

PGs and GAGs were dissolved in culture medium to the required concentration (0.1 μg ml^{-1} PGs; 10 μg ml^{-1} GAGs) and, using a push-pull technique and hand-held Pasteur pipettes were exchanged for normal medium 30 minutes after the cells were plated.

Analysis of neurite orientation

Neurones were photographed hourly for 5 hours and all measurements made from enlarged tracings. Neurites were selected for analysis on the basis of the following criteria: (1) actively growing throughout the 5 hour observation period (see Fig. 5; mean neurite growth rate in all cultures greater than 20 μm $hour^{-1}$), (2) initial length longer than 10 μm and (3) initial angle of growth outwith 15° of cathode or anode. The direction of nerve growth was defined as the angle between a line projecting through the centre of the growth cone and a line drawn at right angles to the long axis of the culture chamber. The direction of growth was taken to be 0° for a neurite perpendicular to the field, –90° for a neurite growing directly towards the cathode and +90° for a neurite growing directly towards the anode. In control cultures (no electric field), –90° was taken as growth directly towards the right of the chamber and +90° as growth directly towards the left. A neurite was categorised as having turned cathodally or anodally if, throughout the 5 hour observation period, it showed a sustained deviation of greater than 15° from its original direction of growth. This would eliminate neurites which turned first one way, then the other. These would be categorised as not turning.

Statistics

Statistical analysis of mean angles turned were made using unpaired, two-tailed Student's *t*-test and Mann-Whitney U-test. A *d*-test, which is a modification of the basic *t*-test principle and is suitable for comparing percentages based on two large samples (Bailey, 1981), also was used.

RESULTS

Neurite orientation in control cultures (no electric field)

In control cultures (no electric field) neurites grew randomly. During the 5 hour observation period, the majority of neurites, 65% (116/179; 10 cultures) showed no sustained change in their direction of orientation and, overall, neurites turned only –1±3° (n=179) from their original direction of growth (Table 1; Fig. 1). Randomly directed growth persisted in cultures containing either of the PGs (Table 1; Fig. 1). Thus, neither the culture conditions nor the PGs alone imposed any direction on nerve growth.

Neurite orientation in electric field stimulated cultures

In weak extracellular electric fields *Xenopus* neurites cultured on tissue culture plastic re-orient cathodally in a predictable, quantifiable and field strength dependent manner (Hinkle et al., 1981; Patel and Poo, 1982; Erskine et al., 1995). In 5 separate cultures exposed to applied fields in the range of 50-133 mV mm^{-1} for

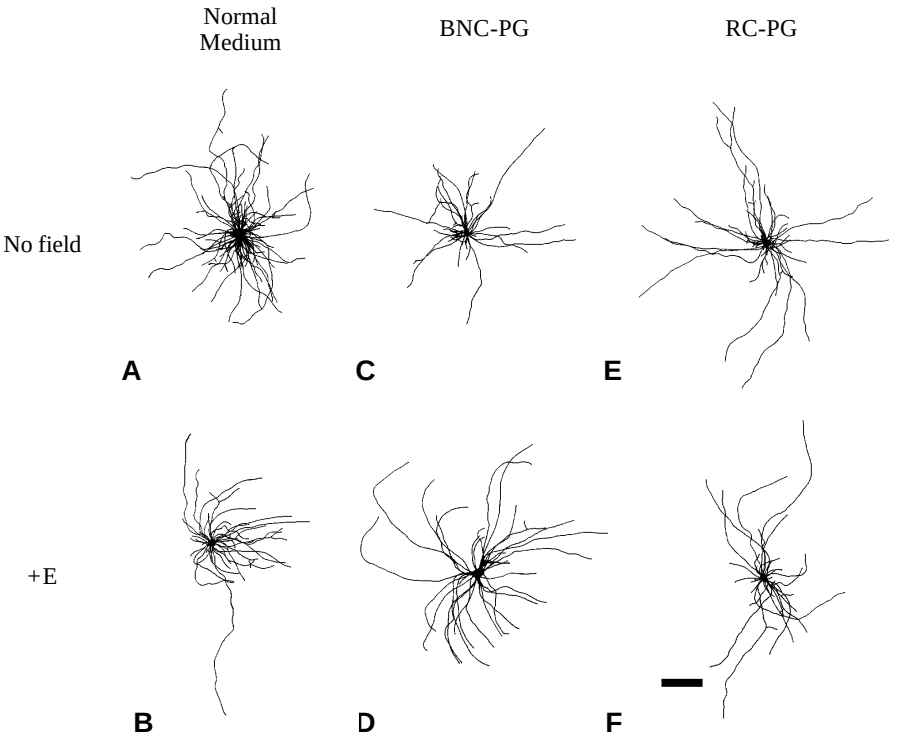

Fig. 1. Composite line drawings of neurite outgrowth in cultures of dissociated *Xenopus* neural tube cells. Enlarged tracings were made of the outgrowth from individual neurones and the composite pictures produced by superimposing their cell bodies. (A,B) Normal medium; (C,D) plus BNC-PG; (E,F) plus RC-PG. (A,C,E) Neurite outgrowth in the absence of an electric field. In each picture nerve growth is directed randomly, the majority of neurites extending in more or less straight lines. (B,D,F) Neurite outgrowth after 5 hours field exposure. In the field plus BNC-PG (D) significantly more neurites have turned to grow cathodally than in normal medium (B), whereas in the field plus RC-PG (F) very few neurites exhibit directed growth. For each picture the long axis of the culture chamber, and thus the field vector, runs horizontally with the cathode at the right. Bar, 100 μm.

5 hours 62% (64/103; range, 60%-68%) of neurites turned cathodally, increasing to 85% (82/97; 5 cultures; range, 79%-95%) at 143-200 mV mm^{-1} (P<0.001; Erskine et al., 1995). Within each of these range of field strengths, no quantitative difference in the numbers of neurites re-orienting was observed (see Erskine et al., 1995) allowing the data to be combined and compared with that from neurites exposed to test substances.

Effect of chondroitin sulphate PGs on electric field-induced neurite orientation

Addition of BNC-PG (0.1 μg ml^{-1}) to the culture medium enhanced significantly the extent of field-induced neurite orientation (Table 2; Fig. 1). In applied fields of 50-133 mV mm^{-1} 82% (27/33) of neurites in the field plus BNC-PG turned cathodally compared to 62% in the field alone (P<0.001; Table 2; Fig. 1). The mean angle turned towards the cathode during the 5 hours of field exposure also increased from −28±4° (n=103) in normal medium to −49±8° (n=33) in the presence of BNC-PG (P<0.05; Table 2). By contrast, in cultures containing RC-PG (0.1 μg ml^{-1}) galvanotropism was inhibited (Table 2; Fig. 1). Both the number of neurites turning cathodally (33%; 10/30) and the overall angle turned towards the cathode (−9±6°; n=30) were decreased significantly (P<0.01 compared to field matched control).

Effect of GAGs on electric field-induced neurite orientation

PGs are composed of a core protein to which different types of GAGs are attached. BNC-PG contains approximately 100 chondroitin sulphate chains and about 40 keratan sulphate chains. RC-PG is very similar, except it is lacking keratan sulphate. Additionally, the chondroitin sulphate chains of RC-PG are sulphated almost entirely in the 4-position, whereas those of BNC-PG are sulphated mainly in the 6-position (80%; Fig. 2). To test whether the observed effects of these two PGs were mediated by their GAG side chains, various isolated GAGs were added to the culture medium. The effects of chondroitin 4-sulphate, chondroitin 6-sulphate, dermatan sulphate (an epimer of chondroitin 4-sulphate) and keratan sulphate were determined.

Table 1. Neurite orientation in control cultures (no electric field)

	Number (%) of neurites			
Proteoglycan	Turned to right*	Did not turn	Turned to left	Mean angle turned†
None	33 (18%)	116 (65%)	30 (17%)	−1±3° (179)
BNC-PG	5 (11%)	33 (75%)	6 (14%)	2±4° (44)
RC-PG	9 (26%)	18 (53%)	7 (21%)	−4±4° (34)

*For definition of turning see Materials and Methods.
†Negative angles indicate turning towards the right of the chamber, positive angles towards the left. Numbers in parenthesis = number of neurites.
All data were pooled from a minimum of 3 separate experiments.

Table 2. Modulation of field-induced neurite orientation by chondroitin sulphate proteoglycans

	Number (%) of neurites			
Proteoglycan	Turned to cathode	Did not turn	Turned to anode	Mean angle turned
None	64 (62%)	30 (29%)	9 (9%)	−28±4 (103)
BNC-PG	27 (82%)*	4 (12%)†	2 (6%)	−49±8 (33)†
RC-PG	10 (33%)*	15 (50%)†	5 (17%)	−9±6 (30)†

Range of field strengths (applied for 5 hours) = 50-133 mV mm^{-1}. All data were pooled from a minimum of 2 separate experiments.
*P<0.001, †P<0.05, compared to field alone.

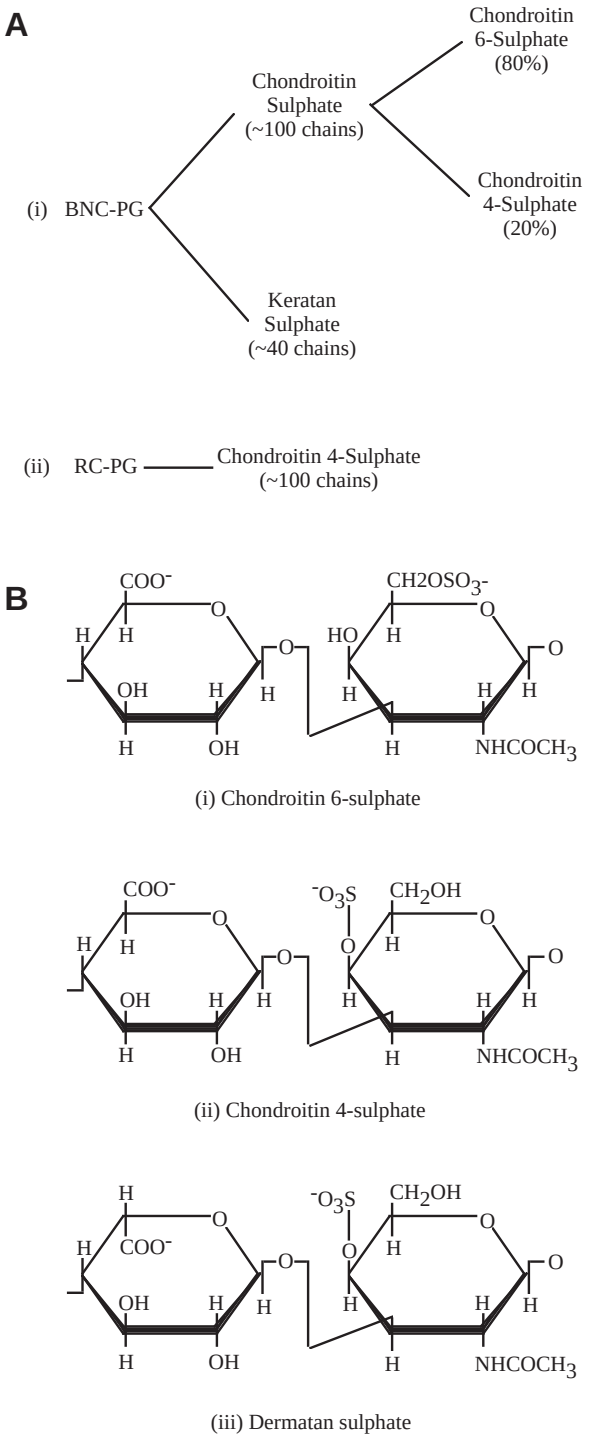

Fig. 2. (A) Glycosaminoglycan composition of (i) BNC-PG and (ii) RC-PG. (B) Structure of disaccharide subunits of (i) chondroitin 6-sulphate, (ii) chondroitin 4-sulphate, and (iii) dermatan sulphate.

At low field strengths (50-133 mV mm^{-1}) chondroitin 4-sulphate and dermatan sulphate (10 μg ml^{-1}) did not alter the extent of field-induced neurite orientation significantly (Fig. 3A). However, in the field plus chondroitin 6-sulphate (10 μg ml^{-1}) 85% (56/66) of neurites re-oriented cathodally, a significant increase over the field alone (62%; $P<0.001$), field plus chondroitin 4-sulphate ($P<0.01$) or field plus dermatan sulphate ($P<0.01$). Not only did more neurites respond, they did so to a much greater extent. There was a two fold increase in the mean angle turned by neurites during the 5 hour observation period (Fig. 4A). Neurites turned −57±5° ($n=66$) in an electric field plus chondroitin 6-sulphate compared to −28±4° ($n=103$; $P<0.001$) in the field alone, −31±7° ($n=32$; $P<0.01$) in the field plus chondroitin 4-sulphate and −25±6° ($n=39$; $P<0.001$) in the field plus dermatan sulphate. By contrast, addition of 10 μg ml^{-1} keratan sulphate to the culture medium decreased the extent of field-induced cathodal turning (Figs 3A, 4A). In an electric field (50-133 mV mm^{-1}) plus keratan sulphate only 34% (13/38) of neurites turned cathodally, a significant decrease ($P<0.01$ compared to the field alone). The total angle turned towards the cathode also was inhibited, neurites turning only −14±4° ($n=38$; $P<0.05$).

The effect of the GAGs on neurite orientation at higher field strength also was determined. In larger extracellular electric fields (143-200 mV mm^{-1}) 85% (82/97) of neurites cultured in normal medium turned cathodally (Fig. 3B). Dermatan sulphate had no effect on any of the parameters examined whereas chondroitin 4-sulphate and keratan sulphate decreased significantly the number of neurites turning cathodally in higher fields to 66% (39/59; $P<0.01$), and 56% (23/41; $P<0.001$), respectively (Fig. 3B). The rate of neurite orientation and the total angle turned towards the cathode also were decreased significantly (Fig. 4B). By contrast, 85% (33/39) of neurites grown in chondroitin 6-sulphate re-oriented cathodally, an insignificant change compared to the field alone (Fig. 3B). However, these neurites oriented significantly faster than those in the other treatments, turning 58% of their observed maximum orientation in the first hour of field exposure compared to 22% in the field alone and 10% in the field plus chondroitin 4-sulphate (Fig. 4B). They continued to turn faster over the next 2 hours such that by 3 hours field exposure they had achieved 95% of their maximum orientation compared to 66% in the field alone and 39% in the field plus chondroitin 4-sulphate (Figs 4B, 5). By 5 hours 'catching-up' had occurred, neurites in the field alone now having turned as far as those in the field plus chondroitin 6-sulphate (Figs 4B, 5). Thus, since BNC-PG and its predominant GAG chondroitin 6-sulphate (Fig. 2) both enhanced field-induced neurite re-orientation, whereas RC-PG and chondroitin 4-sulphate both inhibited galvanotropism (Table 2; Figs 3, 4), it is likely that the observed effects of these PGs are mediated by their chondroitin sulphate side chains.

Effect of disaccharide subunits of chondroitin 4-sulphate and chondroitin 6-sulphate on field-induced neurite orientation

GAGs are long chains formed from repeating disaccharide subunits. The component sugars of chondroitin 6-sulphate and chondroitin 4-sulphate, which, respectively, enhanced and inhibited field-induced neurite re-orientation, differ only in the position of their sulphate group. Dermatan sulphate is also 4-sulphated but contains L-iduronic acid as opposed to D-glucuronic acid (Fig. 2). The effect of the disaccharide subunits of chondroitin 4-sulphate and chondroitin 6-sulphate on galvanotropism was tested. At a concentration of 10 μg $ml^{-1,}$ neither of these disaccharides had any significant effect on field-induced neurite orientation (Table 3). In applied fields of

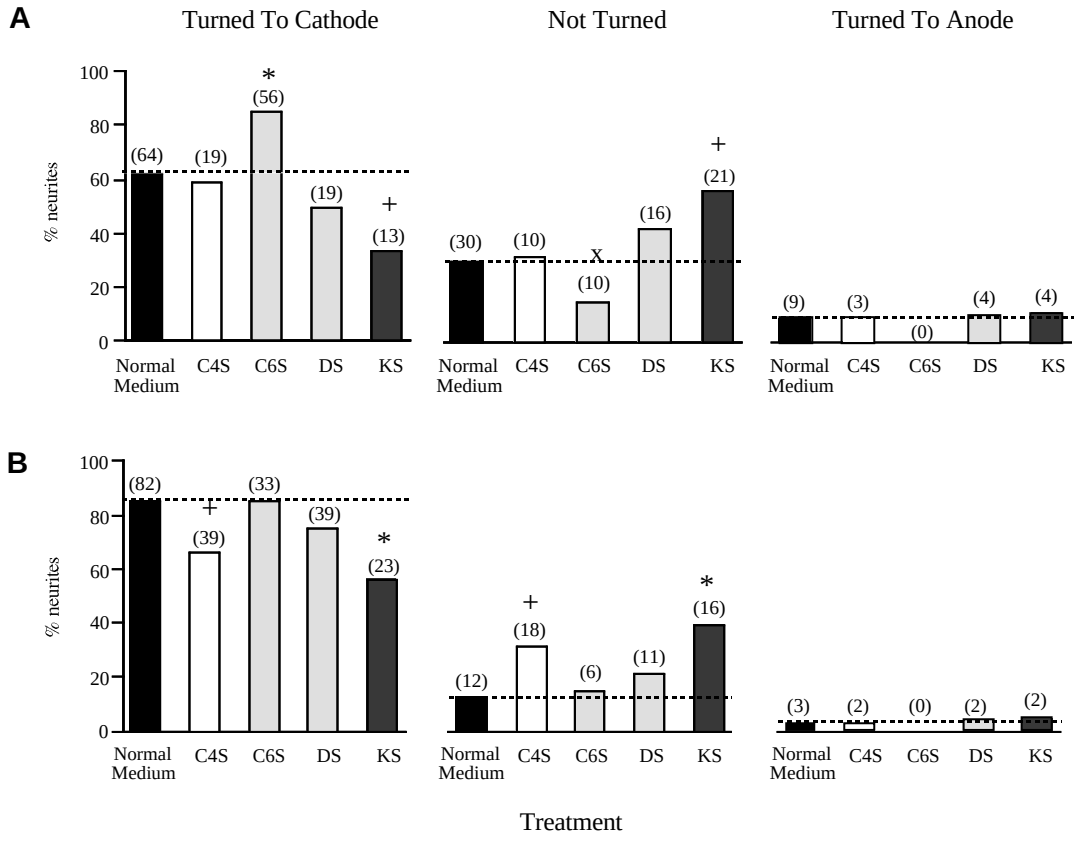

Fig. 3. Neurite orientation in applied electric fields of (A) 50-133 mV mm^{-1} or (B) 143-200 mV mm^{-1} in the presence and absence of various GAGs. C4S, chondroitin 4-sulphate; C6S, chondroitin 6-sulphate; DS, dermatan sulphate; KS, keratan sulphate. *P<0.001, ^{+}P<0.01, ^{x}P< 0.05 compared to normal medium. Numbers above bars = number of neurites. Data were pooled from a minimum of 2 independent experiments.

50-133 mV mm^{-1}, 58% (22/38) and 64% (14/22) of neurites cultured in the disaccharides of chondroitin 6-sulphate and chondroitin 4-sulphate, respectively, turned cathodally, an insignificant change compared to the field alone (62%; Table 3). The rate of neurite orientation and the overall angle turned towards the cathode also were not altered significantly (Table 3). The activity of these GAGs therefore must require their chain structure to be intact.

Neurite growth rates

In weak dc electric fields, neurites grow significantly faster than in control cultures (no electric field; Erskine et al., 1995; Fig. 6A). Addition of either BNC-PG or RC-PG to the culture medium induced a significant decrease in both control and field-stimulated nerve growth rates although, in both cases neurite extension was still significantly faster in the presence than in the absence of an electric field (Fig. 6A). These changes in growth rates are unlikely to underlie the PG-induced alterations in neurite re-orientation (Table 2; Fig. 1). Both PG had similar effects on nerve growth rates but opposing effects on field-induced neurite re-orientation. Moreover, there is no relationship between the angle an individual neurite re-orients in an electric field and its rate of extension (Fig. 6B); slow growing neurites are just as likely to turn cathodally as those growing at faster rates.

Table 3. Electric field-induced neurite orientation in the presence and absence of chondroitin sulphate disaccharides

	Number (%) of neurites			
Disaccharide	Turned to cathode	Did not turn	Turned to anode	Mean angle turned
None	64 (62%)	30 (29%)	9 (9%)	−28±4 (103)
C4S-DS	14 (64%)	6 (27%)	2 (9%)	−31±5 (22)
C6S-DS	22 (58%)	12 (32%)	4 (11%)	−23±6 (38)

Range of field strengths = 50-133 mV mm^{-1}. C4S-DS = disaccharide subunit of chondroitin 4-sulphate; C6S-DS = disaccharide subunit of chondroitin 6-sulphate. All data were pooled from a minimum of 2 separate cultures.

DISCUSSION

The aim of these experiments was to examine in vitro the potential interactions between electric fields and another nerve growth cone guidance cue: chondroitin sulphate PGs. The guidance properties of the electric field were enhanced significantly by addition of BNC-PG to the culture medium. Both the number of neurites orienting and the angle through which they turned were increased significantly. By contrast RC-PG inhibited galvanotropism. Thus, in vitro, specific interactions between cues mediating the direction of nerve growth do occur and can up- or down-regulate the extent of neurite re-orientation.

Proteoglycans and nerve growth

Proteoglycans are extracellular matrix molecules composed of a protein core to which various types of carbohydrate side chains (GAGs) are attached. In vitro both soluble and substrate bound chondroitin sulphate PGs can, depending on the conditions, either stimulate (Iijima et al., 1991; LaFont et al., 1992;

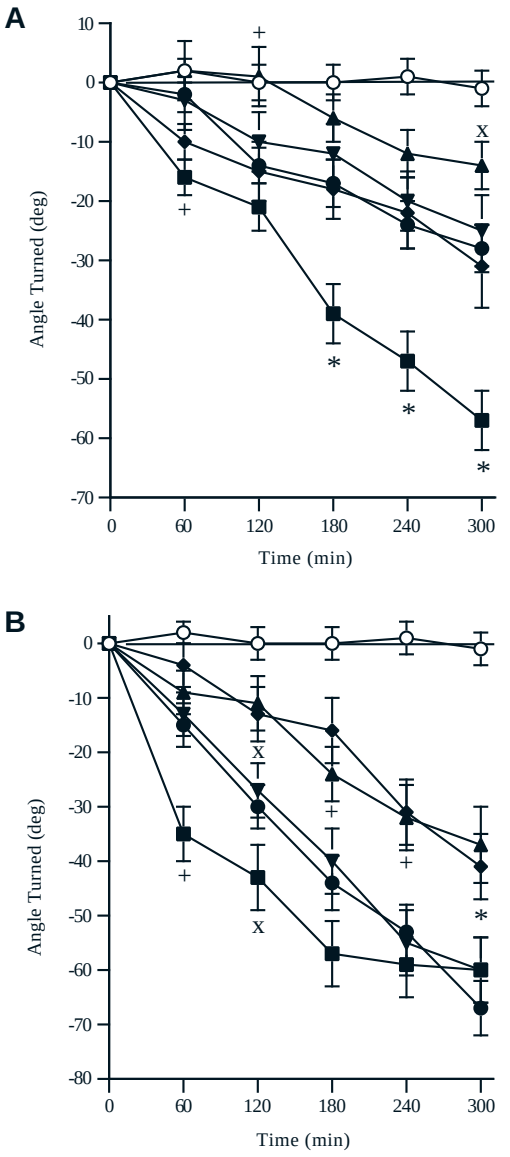

Fig. 4. Mean (± s.e.m.) angle turned by neurites over a 5 hour observation period in the presence and absence of various GAGs. In control cultures (normal medium, no electric field; ○) neurites grew in a random manner, turning −1±3° (n=179) in 5 hours. In applied fields of 50-133 mV mm^{-1} (A) or 143-200 mV mm^{-1} (B) neurites turned cathodally. ●, field alone; ■, field-plus chondroitin 6-sulphate; ◆, field plus chondroitin 4-sulphate; ▼, field plus dermatan sulphate; ▲, field plus keratan sulphate. (A) At low field strengths, chondroitin 6-sulphate (■) increased significantly the rate of neurite orientation and the overall angle turned. Keratan sulphate (▲) decreased these parameters. (B) At higher field strengths stronger orientation occurred under all conditions. Chondroitin 4-sulphate (◆) and keratan sulphate (▲) decreased significantly the rate of neurite re-orientation and the total angle turned, whereas chondroitin 6-sulphate (■) enhanced the rate of neurite re-orientation. Positive angles indicate turning towards the anode, negative angles towards the cathode. *P<0.001, ^{+}P< 0.01, ^{x}P<0.05 compared to field alone. Number of neurites as in Fig. 3.

Fernaud-Espinosa et al., 1994; Dou and Levine, 1995) or inhibit (Carbonetto et al., 1983; Verna et al., 1989; Oohira et al., 1991; Snow et al., 1990, 1991; Friedlander et al., 1994; Dou and Levine, 1995) nerve growth. In all but a few cases (e.g. Iijima et al., 1991; Oohira et al., 1991) these effects of the PGs have been found to be mediated by their GAG side chains.

The effects on nerve galvanotropism of the two PGs tested also are likely determined by the composition of their GAG side chains. Chondroitin 6-sulphate, the predominant GAG of BNC-PG (Fig. 2) also stimulated field-induced neurite re-orientation whereas chondroitin 4-sulphate, the only GAG present in RC-PG, was partially inhibitory. Keratan sulphate, a minor component of BNC-PG was also inhibitory. Presence of the core protein was not necessary for any of the observed activities of these PGs.

Cellular mechanisms underlying the interactive affects of electric fields and proteoglycans on nerve growth

Several mechanisms have been proposed to explain the biological effects of chondroitin sulphate PGs, which when in solution, act by binding directly to growth cones (Snow et al., 1996). Firstly PGs could, as a consequence of the large negative charge carried by their GAG side chains, alter cell surface charge and thereby neurite growth. Secondly, the GAGs could be cleaved from their core protein, endocytosed and transferred to the nucleus where they participate in the regulation of cell activity (LaFont et al., 1992; Fernaud-Espinosa et al., 1994). Lastly, PGs/GAGs could bind to specific membrane proteins and, by altering their function or activating second messenger systems, influence nerve growth. Contact with chondroitin sulphate PG bound to a bead or the culture substrate raises growth cone intracellular calcium (Snow et al., 1994).

Decreasing the negative surface potential of cultured embryonic *Xenopus* neurites by increasing the cation concentration of the culture medium inhibits electric field-induced cathodal re-orientation (Erskine et al., 1995). However, surface charge considerations alone cannot account for the observed effects of the PGs tested; chondroitin 6-sulphate, chondroitin 4-sulphate and dermatan sulphate are similarly charged (Fig. 2) yet had strikingly different effects on nerve galvanotropism (Figs 3, 4, 5). Instead our results strongly suggest that the GAGs act by interacting with specific membrane proteins on the growth cone and that the spacing pattern of their charged groups is the critical factor in determining their relative activities. Chondroitin 4-sulphate and chondroitin 6-sulphate differ only in the position of their sulphate group (Fig. 2) and become inactive when broken down into their disaccharide subunits (Table 3). Dermatan sulphate, which had no effect on galvanotropism, has the same sulphation pattern as chondroitin 4-sulphate but contains L-iduronic acid as opposed to D-glucuronic acid (Fig. 2). This implies that it is the pattern of the spacing between the charged sulphate/carboxyl groups which determines the markedly different activities of these GAGs. Cations with charged groups in specific configurations inhibit the binding of herpes simplex virus to its cellular receptor (Langeland et al., 1988). Thus, as a consequence of their distinct charge patterns, chondroitin 4-sulphate and chondroitin 6-sulphate may interact with and alter the functioning of specific membrane proteins in the growth cone leading either to enhancement or inhibition of electric field-induced neurite re-orientation. Such growth cone proteins could include voltage gated calcium channels, activation of which is essential

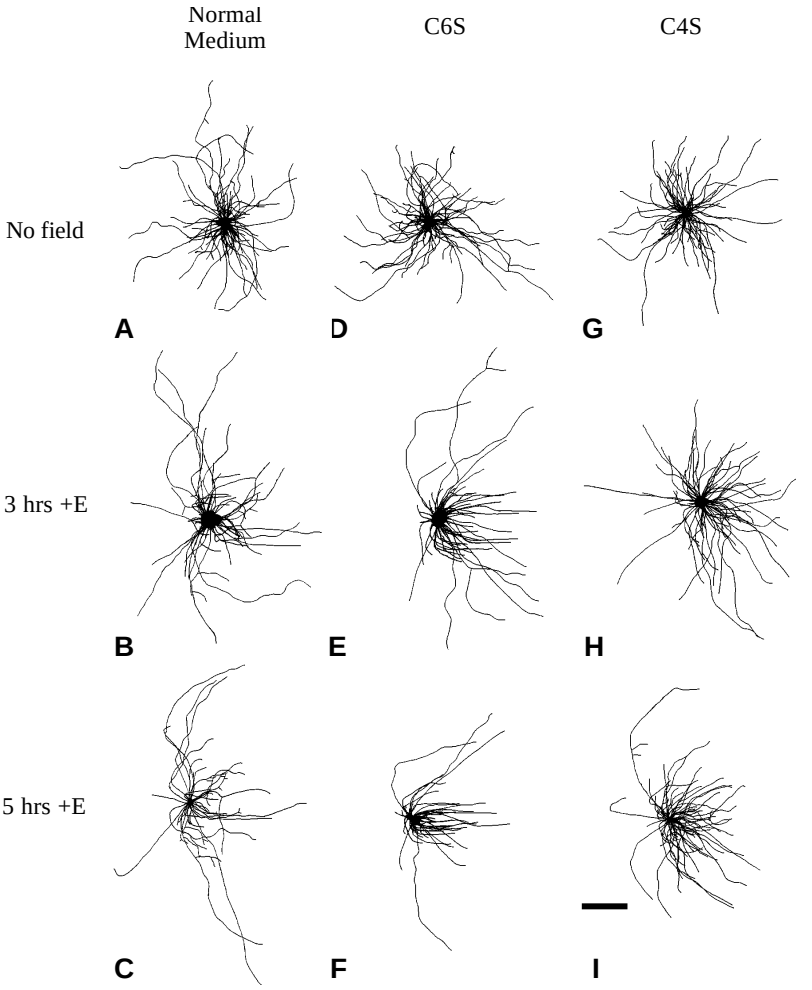

Fig. 5. Composite pictures of neurite outgrowth in normal medium (A,B,C), 10 μg ml^{-1} chondroitin 6-sulphate (D,E,F) or 10 μg ml^{-1} chondroitin 4-sulphate (G,H,I). (A,D,G) Control neurites grown for 5 hours in the absence of an electric field. (B,E,H) Neurite orientation after 3 hours exposure to applied electric fields of 166-200 mV mm^{-1}. In the electric field alone (B) some cathodal orientation has occurred, neurites turning −44±5° (*n*=97), whereas neurites in the field plus chondroitin 6-sulphate (E) show striking cathodal re-orientation. Already the majority of neurites have re-oriented to grow directly towards the cathode, turning −57±6° (*n*=39). By contrast, in the field plus chondroitin 4-sulphate (H) very little orientation has occurred, neurites having only turned −16±6° (*n*=56) by this time. (C,F,I) Neurite orientation after 5 hours field exposure. Strong cathodal orientation is now evident in the field alone (C) or field plus chondroitin 6-sulphate (F). In the field plus chondroitin 4-sulphate (I) significantly less orientation has occurred. The long axis of the culture chamber runs from left to right with the cathode at the right. Bars: 50 μm (B,E,H); 100 μm (A,C,D,F,G,I).

for galvanotropism (Stewart et al., 1995), neurotransmitter receptors, several of which modulate field-induced neurite orientation (Erskine and McCaig, 1995), or perhaps yet unidentified specific receptors for the individual GAGs.

Different responsiveness of neurites to chondroitin 4-sulphate/chondroitin 6-sulphate/dermatan sulphate has been reported previously. Outgrowth of chick dorsal root ganglion neurites is strongly inhibited by a substrate composed of a chondroitin 6-sulphate containing PG(BNC-PG), whereas a PG containing chondroitin 4-sulphate (RC-PG) exerted a much weaker effect (Snow et al., 1990). Addition of chondroitin 6-sulphate but not of dermatan sulphate to the culture medium promotes axon-like outgrowth from embryonic rat thalamic neurones (Fernaud-Espinosa et al., 1994). On a L1 substrate, chondroitin 6-sulphate weakly inhibits neurite outgrowth whereas chondroitin 4-sulphate enhances this parameter (Dou and Levine, 1995). Charge pattern therefore may be a common factor in determining many of the biological effects of PGs.

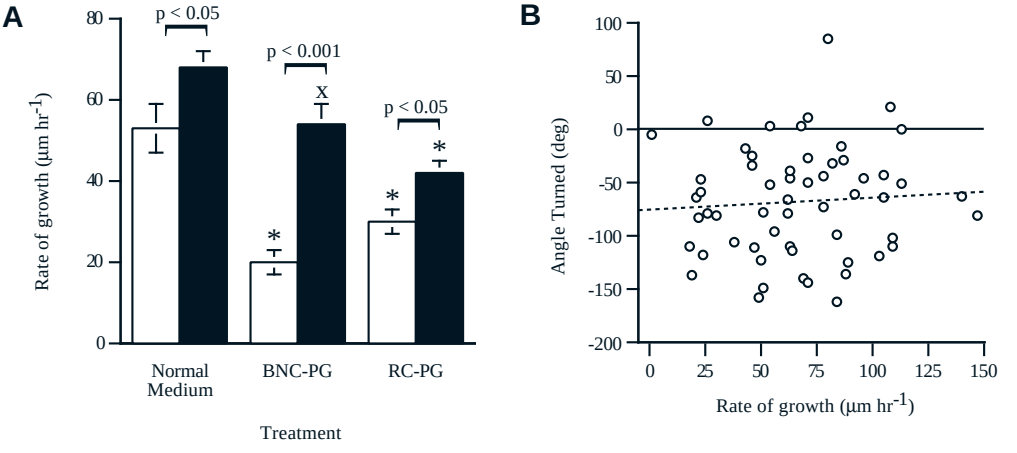

Fig. 6. (A) Effect of BNC-PG and RC-PG on mean (± s.e.m.) nerve growth rates. Open bars, no electric field; solid bars, plus electric field (110-133 mV mm^{-1}). **P*<0.001, ^{x}P<0.05 compared to normal medium. Number of neurites as in Tables 1, 2. (B) Relationship between overall angle turned in an electric field (120-133 mV mm^{-1}) and rate of nerve growth. Broken line = regression line (*P*>0.2).

Physiological relevance

Endogenous electrical currents have been measured in both avian and amphibian embryos, preceeding and during the period of earliest axon outgrowth. In *Xenopus* and axolotl a potential difference is maintained across the cells of the neural tube, driven by active transport by the cells lining the inner aspect of the neurocel. This gives rise to a voltage gradient greater than 400 mV mm^{-1} across the neuroepithelium (Hotary and Robinson, 1991; Shi and Borgens, 1994). Blocking this current with pharmacological agents induces a range of developmental abnormalities (Shi and Borgens, 1994, 1995b). In chick embryos large currents leave the posterior intestinal portal during the period of tail gut reduction, producing an intraembryonic voltage gradient of up to 33 mV mm^{-1} (Hotary and Robinson, 1990). Re-routing the direction of this current by implanting conductive shunts severely disrupts normal development, particularly of the tail region (Hotary and Robinson, 1992). Finally, in *Xenopus* and axolotl embryos, three further endogenous voltage gradients have been detected and quantified (Robinson and Stump, 1984; Hotary and Robinson, 1994; Metcalf et al., 1994; Shi and Borgens, 1995a). Two simultaneous currents have been measured in the subectodermal space underlying the neural plate which polarise the embryo in its rostro-caudal and medio-lateral axes. Both these currents appear coincident with the start of neurulation and disappear upon closure of the neural folds (Shi and Borgens, 1995a). A third, less transient current exits the blastopore and gives rise to sustained voltage gradients of 27-40 mV mm^{-1} (Robinson and Stump, 1984; Hotary and Robinson, 1994). Localised and specific pharmacological or physical collapse of any of these currents prevents normal development.

Chondroitin sulphate PGs are also expressed in the embryonic nervous system (Aquino et al., 1984; Flaccus et al., 1991; Snow et al., 1991; Brittis et al., 1992; Bicknese et al., 1994; Fernaud-Espinosa et al., 1996). However, at present, there is little information on exactly what PGs are expressed where and when during development. Nonetheless, our results indicate that, in areas where endogenous electric fields and PGs are co-expressed, interactions are likely to occur and play an important role in modulating nerve guidance.

Other guidance cues interact and modulate nerve guidance

The existence in vivo of multiple co-existent guidance cues strongly suggests that growth cone guidance occurs under the integrated influence of these disparate signals. This study is part of a series which indicates that interactions between differing types of potential guidance cues is widespread, at least in vitro. A gradient of the neurotransmitter acetylcholine alone can guide *Xenopus* growth cones (Zheng et al., 1994). Growth cones exposed simultaneously to an applied electric field and the muscarinic antagonist atropine show markedly enhanced cathodal re-orientation; those exposed to the field plus the nicotinic antagonist d-tubocurarine are inhibited from re-orienting cathodally (Erskine and McCaig, 1995). Galvanotropism also is enhanced by two other chemotropic factors, the neurotrophins NT-3 and BDNF (McCaig et al., 1995). These studies have a twofold significance. Firstly, they establish that interactive effects between co-existent guidance cues do occur. Secondly, they enhance the likelihood that endogenous electric fields have a physiological relevance by demonstrating that electric fields interact with three recognised guidance cues: PGs, neurotransmitters and neurotrophins.

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