

# Essential role of heparan sulfates in axon navigation and targeting in the developing visual system

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## SUMMARY

Heparan sulfate (HS) is abundant in the developing brain and is a required co-factor for many types of fibroblast growth factor (FGF) signaling *in vitro*. We report that some HSs, when added exogenously to the developing *Xenopus* optic pathway, severely disrupt target recognition causing axons from the retina to bypass their primary target, the optic tectum. Significantly, HS sidechains from a neuroepithelial perlecan variant that preferentially bind FGF-2, HS(FGF-2), cause aberrant targeting, whereas those that preferentially bind FGF-1 do not. Charge-matched fragments of HS(FGF-2) show that the mistargeting activity associates with the FGF-binding fragments. Heparitinase removal of native HSs at the beginning of optic tract formation retards retinal axon elongation; addition of

FGF-2 restores axon extension but axons lose directionality. Late HS removal, after axons have extended through the tract, elicits a tectal bypass phenotype indicating a growth promoting and guidance function for native HSs. Our results demonstrate that different HS sidechains from the same core protein differentially affect axon growth *in vivo*, possibly due to their distinct FGF-binding preferences, and suggest that growth factors and HSs are important partners in regulating axon growth and guidance in the developing visual system.

Key words: FGF-2, FGF receptor, retinal axons, tectum, axon guidance, *Xenopus*

## INTRODUCTION

When axons from the retina reach the diencephalic/midbrain border, they depart from other axon tracts and selectively enter their target, the optic tectum. This process of target recognition is an essential early step in establishing functional visual connections and relies on the ability of growth cones at the tips of advancing axons to detect and respond to cues patterning the neuroepithelial substrate. Although the molecular events underlying this behavior are not well characterized, recent evidence implicates the fibroblast growth factor receptor (FGFR) and FGF-2 in the *Xenopus* visual system. Disruption of FGF signaling in growing retinal ganglion cell (RGC) axons by dominant negative FGFR inhibition and by exogenous FGF-2 causes target recognition defects in which affected axons bypass the tectum (McFarlane et al., 1995, 1996). A third and crucial co-factor for many FGF-FGFR interactions is heparan sulfate (Yayon et al., 1991). Here we have asked whether HS also plays a role in retinal axon guidance by interfering with HS function in a living brain preparation.

HS proteoglycans (HSPGs) are widely expressed in the embryonic brain and their expression is developmentally regulated (Halfter, 1993; Herndon and Lander, 1990; Nurcombe et al., 1993; Oohira et al., 1994; Teel and Yost,

1996). Free HS sidechains potentially affect neuronal differentiation in a variety of assays, implicating a functional role for endogenous HSPGs. For example, HS alters the characteristics of neurite outgrowth from neurons *in vitro* (Dow et al., 1991; Giuseppetti et al., 1994; Haugen et al., 1992a; Isahara and Yamamoto, 1995; Verna et al., 1989), while exogenous HS or enzymatic removal of endogenous HS disrupts axon navigation in the developing cockroach limb (Wang and Denburg, 1992). In addition, HS sidechains from rat brain N-syndecan, an HSPG, inhibit the neurite outgrowth promoting activity of the heparin-binding growth-associated molecule (HB-GAM; Kinnunen et al., 1996).

HS sidechains can bind to a variety of cell surface and extracellular matrix (ECM) proteins, including those known to stimulate neurite outgrowth (Cole et al., 1986; Haugen et al., 1990; Kinnunen et al., 1996; Lander, 1990), and can potentiate the activities of various secreted factors (Bradley and Brown, 1990; Burgess and Maciag, 1989; Serafini et al., 1994; Wanaka et al., 1993). The interactions between HS and the FGF family of heparin-binding growth factors are particularly well documented. The biological activities of FGF-1 and FGF-2 depend on their ability to bind cell surface or extracellular matrix HS (Klagsbrun and Baird, 1991; Schlessinger et al., 1995). Cells that express the high affinity FGFRs, but lack HS,

neither bind nor respond to FGF-2 (Rapraeger et al., 1991; Yayon et al., 1991). By binding and delivering FGFs to their high affinity tyrosine kinase FGFR, HSPGs act as co-receptors and are often referred to as low affinity FGFRs (Kiefer et al., 1991; Schlessinger et al., 1995; Spivak-Kroizman et al., 1994). In addition, HSPGs are thought to increase local concentrations of FGF by sequestering and protecting it from degradation (Gospodarowicz and Cheng, 1986).

Recent evidence shows that some types of HS chains contain defined carbohydrate sequences that bind preferentially to FGF-2 (Gallagher and Turnbull, 1992; Ornitz et al., 1995; Rusnati et al., 1994). By adding modified HS sidechains to the same core protein, a perlecan-like HSPG (HSPG-PRM) in the mouse brain neuroepithelium switches its binding affinity from FGF-2 to FGF-1 within a 2-day period of embryonic development (E9-E11; Ford et al., 1994; Nurcombe et al., 1993; Joseph et al., 1996). This switch coincides with the transition that the neuroepithelium undergoes from mitogenesis to the beginning of neuronal differentiation and suggests that developmentally regulated glycosylations of HS side chains can intricately regulate FGF signaling in the developing CNS (Nurcombe et al., 1993). The differential growth factor binding characteristics of HS might account for the finding that some brain-derived HSs potentiate the activity of FGF-2, while others prevent FGF-2 binding to its receptor (Guimond et al., 1993; Hondermarck et al., 1992). Thus, by regulating the availability and biological activity of FGFs, and possibly other heparin-binding factors, HSPGs might help to localize signals important for axon growth and guidance.

In the present study we have disrupted native HS function *in vivo* by adding different types of HS exogenously and by removing endogenous HS. Our results indicate that HS is important for two aspects of retinal axon growth, elongation in the optic tract and tectal targeting. This dual functional role is similar to that previously identified for the FGFR (McFarlane et al., 1996) and is consistent with a model in which the activation of FGFRs on retinal growth cones is regulated by specific interactions with native HS and FGF-2 that pattern the neuroepithelial substrate. Additionally, our results suggest that guidance in the optic tract involves an HS-dependent mechanism.

## MATERIALS AND METHODS

### Embryos

*Xenopus laevis* embryos were obtained from hormone-induced matings of adult frogs and *in vitro* fertilization according to standard procedures (Gimlich and Gerhart, 1984; Gurdon, 1977). Embryos were raised in 10% Holtfreter's solution; the temperature was varied between 14°C and 27°C to control their speed of development. Staging was according to the normal tables of Nieuwkoop and Faber (1967). Motile embryos were anesthetized with tricaine (ethyl-3-aminobenzoate methanesulfonic acid; Aldrich) for experimental manipulations.

### Exposed brain preparation and labeling of retinal projections

Exposed brain experiments were performed as described previously (Chien et al., 1993). Surgical procedures were carried out in 100% modified Ringers (MR; Gimlich and Gerhart, 1984). In a typical experiment, stage 32 embryos were immobilized by pinning in a

Sylgard Petri dish and the epidermis and eye were removed from the left side of the head; the embryos were then transferred to experimental or control solutions and allowed to develop to stage 40 (approximately 26 hours). The times of initial brain exposure (stage 32-35/56) and fixation (stage 37/38 to 40) were varied depending on the particular experiment. Experimental and control solutions were 80% modified Ringers with HSs or HS-degrading enzymes added to the experimental solution.

Retinal axons from the remaining right eye were anterogradely labeled with horseradish peroxidase (HRP; type VI; Sigma) as described previously (Cornel and Holt, 1992). For whole-mount visualization of the optic projection, brains were dissected out of the embryos after fixation (1% glutaraldehyde), reacted with diaminobenzidine (DAB; Sigma), and mounted projection side up in Coverbond (BSP) with a coverslip supported by two reinforcement rings (Avery). For fluorescent immunohistochemistry, embryos were fixed in 4% paraformaldehyde at 4°C overnight and sectioned at 12 µm on a Leitz cryostat. Drawings of HRP-filled projections were made with a camera lucida attached to a Zeiss photomicroscope and scanned using a ScanJet IIc (Hewlett-Packard) to obtain digital images. Quantitation of projection length and width was done using NIH Image and macros to normalize brain size (see Chien et al., 1993; McFarlane et al., 1995).

### Reagents

The following reagents were used: heparin, HS from bovine mucosa (Sigma); heparitinase (Seikagaku) at 0.25-0.5 U/ml with no protease inhibitors added. HS(FGF-2) and HS(FGF-1) were isolated from mouse E10 neuroepithelial-derived 2.3D tissue culture medium as described previously (Brickman et al., 1995; Ford et al., 1994; Joseph et al., 1996; Nurcombe et al., 1993) broadly following the techniques of Turnbull and Gallagher (1990). HS(FGF-2) fragments were prepared by spiking the sample with previously isolated tritiated HS(FGF-2) and subsequently partially digesting with heparitinase (0.25 U enzyme for 2 hours at 37°C). The reaction was stopped by boiling and the sample subjected to size exclusion chromatography on a Bio-Gel P10 column (1 cm × 120 cm; Bio Rad Laboratories) in 0.2 M ammonium bicarbonate buffer. The pools of different sized fragments were detected by scintillation counting. Each pool of similarly sized fragments was isolated and the sizes of interest rechromatographed to ensure size homogeneity. Equivalent charge density was ensured by running all of the fragments over anionic exchange columns as previously described (Nurcombe et al., 1993) and step-eluting at 0.4 M NaCl. The pools containing the 18-mers and the 16-mers were desalted by freeze drying and exposed to an FGF-2 affinity column (Affi-Gel 10; Bio Rad). The non-binding fractions were those released below 0.5 M NaCl and the fraction released above 1.0 M NaCl was considered to be the binding fraction.

Suramin (a gift from Dr D. Donaghue) was added to exposed brains at 10 µM. For mitotic labeling, stage 40 experimental embryos were injected abdominally with 5 mg/ml BrdU (Sigma), fixed 3 hours later, and processed according to standard procedures. To assess cell viability in the brain, 1% Trypan blue (Sigma) in MR was added to heparin-treated embryos for 1 hour. Embryos were fixed in 4% paraformaldehyde overnight at 4°C, and dead cells were identified in wholemount brains. ApopTag Plus *in situ* apoptosis detection kit (Oncor Inc.) was used for analysis of apoptosis in the retina following heparitinase treatment (0.25-0.5 U/ml between stages 33/34 and 40).

### Immunohistochemistry

Fixed embryos were embedded in OCT (Miles) and cryostat sectioned at 12 µm. For single antibody labeling, sections were washed with PBT (phosphate-buffered saline, 0.2% BSA fraction V (Fisher Scientific), 0.5% Triton X-100 (Sigma) pH 7.4), blocked in PBT containing 5% normal goat serum, and incubated overnight in primary antibody at 4°C followed by fluorescein-conjugated secondary antibody. To visualize RGC axons and HS together, sections from

HRP-filled embryos were double-labeled, first with an anti-HRP antibody and rhodamine-conjugated secondary antibody and second with an anti-HS primary and fluorescein-conjugated secondary antibody. Sections were mounted in glycerol with an anti-bleaching agent (0.1% paraphenylenediamine) and viewed with a Nikon Diaphot 200 inverted microscope attached to a Noran Odyssey confocal scanning laser microscope. The color confocal images were combined into double-labeled images using the Photoshop (Adobe Inc.) program for Macintosh.

Primary antibodies used in this study include: HepSS-1 and 4H10 (Seikagaku; Kure and Yoshie, 1986; David et al., 1992) which recognize a subset of HS chains at 1:200 dilution and an anti-HRP polyclonal antibody (Sigma) from rabbit at 1:500 dilution; R5 which labels radial glia cells (a gift from Dr U. Dräger; Dräger et al., 1984) at 1:1; 6F11 which recognizes the extracellular domain of NCAM at 1:10 (Sakaguchi et al., 1989); 6-11B-1 which labels acetylated tubulin (Sigma) at 1:200, an anti-BrdU monoclonal IgG antibody (Sigma) from mouse at 1:1000. Secondary antibodies were from Jackson ImmunoResearch Laboratories.

### Retinal cultures

Eye primordia were dissected from stage 25 embryos and cultured as described previously (Harris and Messersmith, 1992; McFarlane et al., 1995). Dissociated cells were plated onto polyornithine/laminin-coated coverslips in 35 mm Petri dishes containing 4 ml of L15 culture medium (CORE cell culture facility, UCSD) supplemented with 0.1% BSA fraction V in either the presence or absence of 20 ng/ml human recombinant FGF-2 (GIBCO) and/or HS(FGF-2) at 1-1000 ng/ml. Cultures were fixed after 24 hours in 1% glutaraldehyde for 45 minutes on ice. Mounted cultures were viewed with phase optics on an inverted Nikon Diaphot 300 microscope and digital images were captured using a CCD camera (Cohu). Neurite length was measured using the NIH Image program for Macintosh. Only the longest neurites of isolated cells were included.

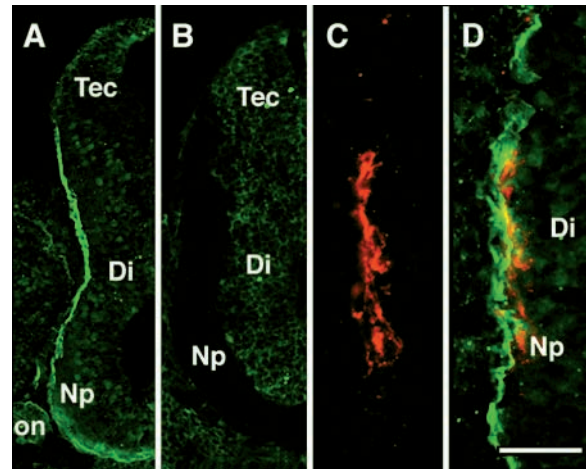
### <sup>125</sup>I-FGF-2 binding assays

Embryonic stage 40 brains were dissected and transferred into 50  $\mu$ l of 100% MR with 0.2% BSA containing either HSs or control carbohydrates. HSs included: 0.1 mg/ml heparin, 0.1 mg/ml FGF-2-binding HS (HS(FGF-2)), 0.1 mg/ml FGF-1-binding HS (HS(FGF-1)), and 0.1 mg/ml HS isolated from bovine mucosa (Sigma); control carbohydrates included: 1 mg/ml chondroitin sulfate C and 1 mg/ml sucrose. The brains were incubated for 3 hours at room temperature with 15 ng/ml <sup>125</sup>I-labelled FGF-2 (NEN DuPont, specific activity 144  $\mu$ Ci/ $\mu$ g), washed 4 times with PBS containing 0.2% BSA fraction V, transferred into new tubes, and counted in a scintillation counter.

## RESULTS

### HSs are present in the embryonic visual pathway

To determine whether HSs are present in the developing optic tract, sections of *Xenopus* embryonic brains were immunolabeled with two different anti-HS antibodies (HepSS-1 and 10E4) during the period when retinal axons first navigate through the contralateral optic tract (stages 33/34 and 35/36) and enter their target, the optic tectum (stages 37/38-40). Both antibodies revealed similar expression patterns in the brain. In the diencephalon, strong HS immunostaining was localized to the ventral two thirds of the optic tract close to the pial surface (Fig. 1A). This positive staining diminished in the dorsal brain and was absent in the rostral optic tectum. The specificity of the immunolabeling was tested by removing endogenous HS with heparitinase (0.25-0.5 U/ml), a broad-spectrum mixture of heparin lyase II and III which catalyzes the eliminative



**Fig. 1.** Pattern of native HS staining in the embryonic optic pathway. Confocal micrographs of transverse sections of the embryonic brain (left side) immunostained for HS using HepSS-1 antibody. (A) HS immunostaining is localized to a narrow band close to the pial surface of the diencephalon (Di) at stage 35/36 and is not present in the tectum (Tec). (B) Absence of HS staining following exposure of optic tract to heparitinase from stage 32 to 40. (C,D) Double immunolabeled section showing the earliest population of retinal ganglion cell axons (red) growing in the HS-rich region (green) close to the pial surface of the diencephalon. The rhodamine filter only was used in C. Np, neuropil; on, optic nerve. Dorsal is up, lateral is left. Scale bar, 100  $\mu$ m (A,B) and 50  $\mu$ m (C,D).

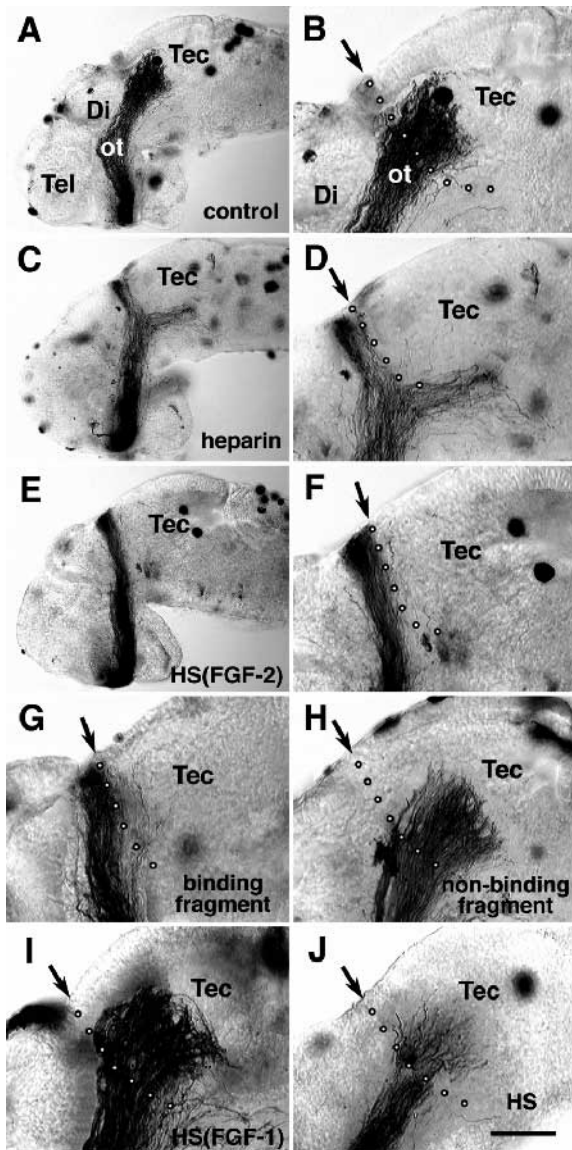
cleavage of N-acetyl-D-glucosaminide linkages. No HS immunostaining was detectable following the enzyme treatment (Fig. 1B). The HepSS-1 and 10E4 antibodies, however, may only detect a subset of HSs, leaving open the possibility that other types of HSs are expressed in immunonegative areas. Nonetheless, the observed expression pattern demonstrates that certain HSs are present in the developing optic pathway and that their expression diminishes near the tectum.

To visualize HS expression in relation to the nascent retinal projection, sections were double-labeled with antibodies against HS and horseradish peroxidase (HRP) following anterograde transport of HRP from the retina. Staining was found to overlap within the superficial 10-20  $\mu$ m of the ventral-to-mid optic tract (Fig. 1C,D) where most retinal ganglion cell (RGC) growth cones are positioned as they navigate through this region. These axons are subsequently displaced interiorly by later arriving growth cones (Holt, 1989). Thus, RGC axons extend through an HS-rich region in the optic tract but enter an HS-poor region when they reach the tectum. This distribution of HS correlates with FGF-2 staining in the developing pathway (McFarlane et al., 1995) suggesting the possibility that these two molecules interact to regulate axon growth.

### Exogenous HSs disrupt target recognition

To determine whether HSs are involved in RGC axon growth and guidance, different types of HSs were applied to retinal axons in exposed brains during the period when they first navigate to and innervate the tectum. In these experiments, the skin epidermis was removed from the left side of the embryos' heads to expose the underlying developing optic tract at stage





**Fig. 2.** Exogenously applied FGF-2-binding HS disrupts target recognition. Lateral views of wholemount brains at stage 40 showing the trajectories of HRP-filled optic projections. Brains were exposed to different types of HSs at 0.1 mg/ml for a duration of 18–24 hours, from stage 33/34 to 40. B, D, F are higher power views of A, C and E respectively. (A, B) Control projection forms a defined optic tract (ot) in the diencephalon (Di) and crosses smoothly into the tectum (Tec). Dotted line and arrow demarcate the anterior tectal border. (C, D) Projection exposed to heparin extends correctly through the diencephalon but fails to enter the tectum. The projection bifurcates at the entrance to the tectum and axons course dorsally and posteriorly around the target. (E, F) Retinal axons growing in the presence of the full length HS(FGF-2) veer dorsally at the tectal border and bypass the tectum. (G) FGF-2-binding fragments of the full length HS(FGF-2) also cause axons to grow past the target without entering it, whereas (H) non-FGF-2-binding fragments of HS(FGF-2) have no effect. Brains exposed to HS(FGF-1) (I) and to a non-FGF-2-binding full length HS (J) have normal optic projections that enter the tectum. Tel, telencephalon; dorsal is up, anterior to the left. Scale bar, 200  $\mu$ m in A, C and E and 100  $\mu$ m in B, D and F–J.

33/34. HSs were chronically present in the medium bathing the exposed part of brain through to stage 40, when the optic projection was visualized with HRP histochemistry.

#### Heparin and full length FGF-2-binding HS sidechains

RGC axons normally follow a stereotyped pathway through the diencephalon (the optic tract) and exhibit a smooth trajectory as they cross the diencephalic-midbrain border to enter the optic tectum (Fig. 2A,B; also see Chien et al., 1993; Harris et al., 1987). Heparin treatment had little effect on axonal trajectories within the optic tract, but disrupted axon behavior at the tectal border causing them to veer sharply in dorsal or ventral directions instead of continuing caudally to enter the tectum (Fig. 2C,D). In the majority of cases, the entire retinal projection bypassed the tectum growing around either its anterior margin (dorsal phenotype, for example see Fig. 2E,F,G), or around both anterior and ventral margins (bifurcation phenotype, see Fig. 2C,D; Table 1). To examine the involvement of HS/FGF interactions in axonal target recognition we used HS(FGF-2), an HS isolated from a murine neuroepi-

thelial cell line that binds specifically to FGF-2 (Ford et al., 1994). This HS(FGF-2) was recently identified as the glycosaminoglycan sidechain of an embryonic form of perlecan (Joseph et al., 1996), a well known FGF-2-binding HSPG (Aviezer et al., 1994). The full length HS(FGF-2) impaired axon targeting in a manner similar to heparin, causing over 90% of the projections to bypass the tectum (Fig. 2E,F and Table 1). In dose-response experiments, concentrations of heparin and HS(FGF-2) as low as 10  $\mu$ g/ml caused aberrant targeting (Table 1).

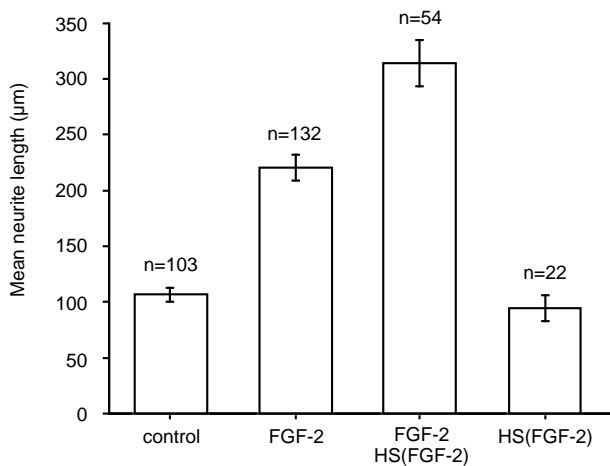
HS-treated axons did not arrest at the tectal border but extended significantly further than control axons, often crossing the dorsal midline via the posterior commissure to invade the other side of the brain (40/53 heparin-treated and 26/31 HS(FGF-2)-treated embryos) suggesting that HS(FGF-2) can enhance axon elongation. To examine this more directly, HS(FGF-2) was tested for its ability to promote retinal neurite extension in vitro. Previous results have shown that FGF-2 promotes neurite extension in dissociated retinal cultures (McFarlane et al., 1995). When retinal neurons were cultured in the presence of both FGF-2 and HS(FGF-2), an additional increase in neurite length compared to FGF-2 treatment alone was observed (Fig. 3). This indicates that HS(FGF-2) can augment FGF-2-enhanced neurite outgrowth and suggests that

**Table 1. Percentage of optic projections showing bypass phenotype**

Type of HS	Concentration			
	1.0 mg/ml	0.1 mg/ml	0.01 mg/ml	0.001 mg/ml
heparin	83% (6)*	87% (61)	54% (11)	0% (8)
HS(FGF-2)	79% (14)	91% (33)	75% (8)	0% (8)
binding		79% (14)	47% (15)	
non-bind.	0% (6)	7% (15)		
HS(FGF-1)	25% (4)	10% (21)		
HS	0% (8)	0% (8)		

Percent of total embryos showing either a dorsal (see Fig. 2F) or bifurcation (see Fig. 2D) bypass phenotype. Numbers in parenthesis indicate total number of embryos. HS(FGF-2) is the full length GAG sidechain, 'binding, non-bind.' indicate the FGF-2 binding and non-FGF-2-binding subfragments of the full length side chain.

\*Concentration producing general toxic effect on embryo development.



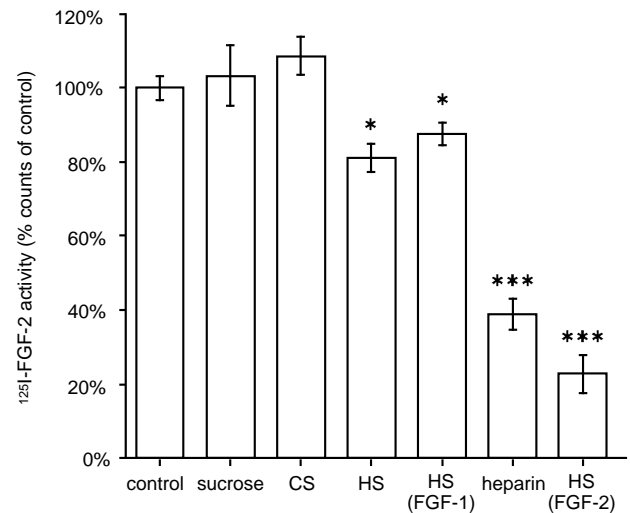
**Fig. 3.** FGF-2-binding HS augments FGF-2 induced neurite outgrowth in retinal cultures. Graph shows the mean length of the longest neurite of retinal cells growing in 24 hour dissociated cultures. Neurites in cultures treated with 20 ng/ml FGF-2 are significantly longer compared to control ( $P < 0.001$ ) whereas addition of 100 ng/ml FGF-2-binding HS (HS(FGF-2)) alone did not alter neurite length compared to control ( $P > 0.4$ ). A combination of FGF-2-binding HS with FGF-2 significantly increased neurite length compared to FGF-2 treatment alone or control ( $P < 0.001$  for both comparisons).  $n$  is the total number of neurites from 2-3 different cultures, and error bars are s.e.m.

HS(FGF-2) activates, rather than inhibits, FGFR signaling in this system.

#### FGF-2-binding HS fragments and FGF-2 non-binding HSs

The full length HS(FGF-2) sidechains used in the above experiments contain discrete domains that bind FGF-2 interspersed with areas that do not (Nurcombe et al., 1993; Turnbull et al., 1992). To test whether the biological activity associates with the FGF-2-binding domains, the sidechains were fractionated into 9 disaccharide, charge-matched units of FGF-2-binding and non-binding subfragments. The FGF-2-binding fragments potently induced targeting errors, similar in phenotype to that found with full length HS(FGF-2) sidechains (Fig. 2G). In contrast, the non-binding fragments had no effect on axon growth (Fig. 2H and Table 1). To investigate whether the bypass phenotype was specific to HSs that bind FGF-2, two further full length HSs were tested: HS(FGF-1) which preferentially binds FGF-1 and is derived from the same perlecan-like HSPG as the HS(FGF-2) (Brickman et al., 1995; Ford et al., 1994; Nurcombe et al., 1993), and a commercially available HS with little FGF-2 binding capability (see below). Neither of these HSs affected axonal pathfinding or targeting at concentrations where heparin and HS(FGF-2) showed dramatic effects (Fig. 2I,J and Table 1).

To further investigate possible HS/FGF-2 interactions underlying the biological effects of HS, the ability of different HSs to competitively interfere with exogenous FGF-2 binding to *Xenopus* brains was evaluated.  $^{125}\text{I}$ -labeled FGF-2 ( $^{125}\text{I}$ -FGF-2) was added to live brains isolated from stage 40 embryos in the presence or absence of different HSs and control carbohydrates. Only heparin and the FGF-2-binding HSs significantly reduced  $^{125}\text{I}$ -FGF-2 binding ( $P < 0.001$ , Fig. 4). Chondroitin



**Fig. 4.** FGF-2-specific HSs interfere with  $^{125}\text{I}$ -FGF-2 binding to intact brains. The ability of different carbohydrates to competitively interfere with exogenous FGF-2 binding to brains dissected from stage 40 embryos is displayed as % of counts per minute compared to control experiment with no carbohydrates added. Whereas the FGF-2-specific HSs (heparin and HS(FGF-2)) strongly inhibit  $^{125}\text{I}$ -FGF-2 binding to dissected brains ( $P < 0.001$ , \*\*\*), HS(FGF-1) and a commercially available HS (HS) only slightly reduce binding ( $P < 0.02$ , \*). Neither chondroitin sulfate (CS) nor sucrose had any effect. All carbohydrates were added at 0.1 mg/ml except for CS and sucrose which were added at 1 mg/ml.  $n = 6$  brains for sucrose, CS, and HS(FGF-2); and  $n = 9$  brains for heparin, HS(FGF-1), HS, and controls pooled from 3 independent experiments. Error bars are s.e.m.

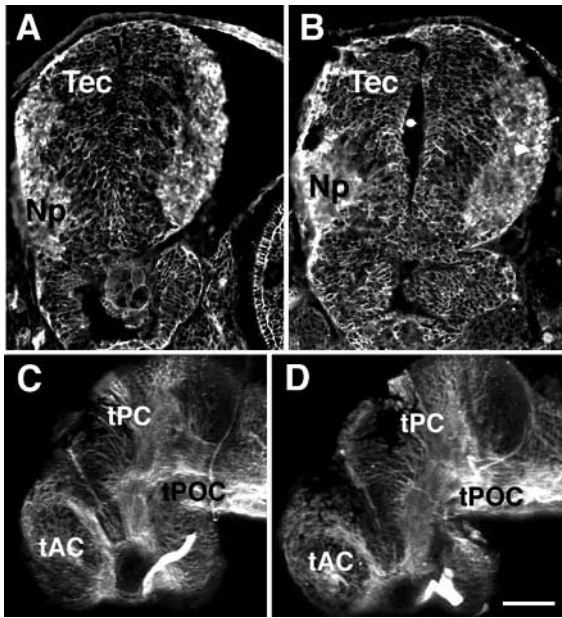
sulfate and sucrose had no effect, while non-FGF-2-binding and FGF-1-binding HSs slightly inhibited binding, possibly reflecting the ability of all HSs to weakly bind FGFs. These results confirm that the FGF-2-binding HSs used in this study are able to out compete exogenous FGF-2 binding to the endogenous low affinity HS sites in embryonic *Xenopus* brains.

#### Organization of neuroepithelium unaltered by heparin treatment

Exogenously applied HSs could indirectly impair RGC axon targeting by perturbing the neuroepithelial substrate through which they grow. To examine effects of heparin treatment on the neuroepithelium, cell viability (trypan blue; Hoechst nuclear stain) and the expression of differentiated morphological markers (anti-NCAM (Fig. 5A,B); anti-radial glia, data not shown) were investigated. In all cases, similar staining patterns were seen for heparin-treated and control brains. Furthermore, cell counts of BrdU incorporation of dividing cells in the forebrain showed no effect of heparin on cell proliferation ( $33.9 \pm 2.8$  per 10  $\mu\text{m}$  cross-section in heparin-treated embryos compared to  $35.5 \pm 2.8$  in controls,  $P > 0.1$ , 36 sections each). Nor did heparin treatment alter the organization, or number, of newly formed axon tracts as visualized with anti-acetylated tubulin (Fig. 5C,D). Thus, exogenously applied free heparin does not appear to affect the gross organization of the neuroepithelium or the main axon tracts.

#### 'Pulse' experiments define time of HS action

To determine the time course of HS action, HSs were added to



**Fig. 5.** Gross organization of neuroepithelium is unchanged by heparin treatment. Embryos were exposed to either control or heparin solutions (0.1 mg/ml) at stage 33/34 and examined at stage 40. (A,B) Confocal images of control (A) and heparin (B) treated brain sections stained with anti-NCAM. NCAM is abundant in the neuropil (Np) as well as in the neuroepithelium and is unaffected by heparin treatment. Tec, tectum, exposed side is to the left, dorsal is up. Scale bar, 50  $\mu$ m. (C,D) Major axonal tracts are not changed by heparin treatment as seen with anti-acetylated tubulin staining in wholemount control (C) and heparin-treated (D) brains. tAC, tract of the anterior commissure; tPC, tract of the posterior commissure; tPOC, tract of the post-optic commissure. Dorsal is up, anterior to the left. Scale bar, 100  $\mu$ m.

exposed brains at different developmental stages for varying durations. When heparin (0.1 mg/ml) was applied at early stages (33/34) and washed out before stage 37/38, prior to the arrival of the first RGC axons at the tectum, no influence on target innervation was observed (0% mistargeting,  $n=6$  optic projections). However, if heparin was applied later, when axons first begin to reach the tectum (stage 37/38), a strong tectal bypass phenotype was observed (88% mistargeting,  $n=8$  optic projections). Thus, targeting errors occur only when heparin is present at the time axons first encounter the tectum. These results indicate a rapid mode of action for exogenously applied heparin, arguing that HSs do not cause long-term changes in the neuroepithelial substrate, but rather act on RGC axons directly, or interfere with RGC axon-substrate interactions.

### HS removal retards axon extension and impairs pathfinding and targeting

To test for an endogenous role for HSPGs in the formation of the optic projection, native HSs were removed from the optic tract by treatment of exposed brains with heparitinase. The elimination of HS from the exposed neuroepithelium was confirmed by HepSS-1 antibody staining (Fig. 1B). When brains were treated with heparitinase (0.25–0.5 U/ml) during the period of initial retinal axon growth through the diencephalon (stages 32–37/38, approx. 13 hours), axons failed to extend far along the optic tract giving rise to abnormally short

**Table 2.** Effects of heparitinase treatment on axonal growth, pathfinding and target recognition in the optic projection

Experiment	Phenotype*		
	Normal trajectory	Shortened tract	Aberrant growth
Early heparitinase stage 32–37/38	0/15	12/15	3/15
Heparitinase+FGF-2 stage 32–37/38	0/4	0/4	4/4
Prolonged heparitinase stage 32–40	1/10	3/10	6/10
Late heparitinase stage 36/37–40	1/12	2/12	9/12
Prolonged suramin stage 32–40	0/17	17/17	0/17

\*Number of total embryos showing either a normal trajectory (see Fig. 6A), a short projection (see Fig. 6B) or aberrant growth with pathfinding and/or target recognition errors (see Fig. 6C,D).

projections (Fig. 6B; Table 2). To control for possible non-specific effects of heparitinase on the neuroepithelial substrate, the gross organization of the neuroepithelium was examined using Hoechst nuclear stain and anti-NCAM staining and found to be normal (data not shown). In addition, no evidence was found for increased apoptosis in the retina following heparitinase treatment (data not shown). When brains were treated with heparitinase for a longer duration (stages 32–40, approx. 26 hours) axons continued to extend but, interestingly, made gross errors in pathfinding and targeting (Table 2; phenotype similar to that shown Fig. 6C). When heparitinase was applied later in development during the period when retinal axons first invade the tectum (stages 36/37–40, approx. 16 hours; Table 2), axons showed a strong tectal bypass phenotype (Fig. 6D). These results indicate that endogenous HSs play a role in promoting axon elongation through the optic tract and suggest that they also help in relaying or patterning guidance information in the neuroepithelial substrate.

### Added FGF-2 overcomes heparitinase-induced growth inhibition

FGF-2 is present in the developing optic pathway and can stimulate the extension of retinal neurites in vitro, suggesting that in vivo FGF-2 promotes the extension of retinal axons through the optic tract (McFarlane et al., 1995). Suramin (10  $\mu$ M), a drug that prevents growth factors from binding to their receptors (Voogd et al., 1993), produced significantly shorter projections compared to control (Table 2), indicating that normal RGC axonal growth through the diencephalon requires the activity of one or more growth factors. Since HS is a crucial co-factor in FGF-2 signaling, we asked whether the retarded axonal extension observed with the enzymatic removal of endogenous HSs might result from disabling extracellular FGF-2 activity. FGF-2 (100 ng/ml) and heparitinase (0.25–0.5 U/ml) were added simultaneously to exposed optic tracts at stage 33/34 and assayed at stage 37/38. Under these conditions, the length of the optic projections was similar to controls (Fig. 6C; Table 2), demonstrating that activation of FGF signaling can restore normal rates of axon extension. The directional growth of these FGF-2-restored axons, however, was extremely aberrant in the diencephalon similar to those caused by prolonged heparitinase treatment alone, and they continued to

**Fig. 6.** Effects of enzymatic removal of native HS on retinal axon growth. (A) Control brain showing a normal optic projection at stage 37/38. (B) 'Early' heparitinase treatment during the period of axon extension through the optic tract, stage 32 to 37/38, causes abnormally short projections that fail to reach the tectum. (C) When FGF-2 (100 ng/ml) is added concomitant with heparitinase, the growth inhibition is alleviated and axons extend to normal lengths. However, many pathfinding errors occur with axons veering off the optic tract into the telencephalon and diencephalon (arrowheads) and axons bypass the tectum (arrow). (D) 'Late' heparitinase treatment during the period of initial tectal invasion, stage 36/37 to 40, causes the bypass phenotype typical of defective target recognition. Tel, telencephalon; Di, diencephalon; Tec, tectum; ot, optic tract; arrows depict anterior border of the tectum. Scale bar, 200  $\mu$ m.

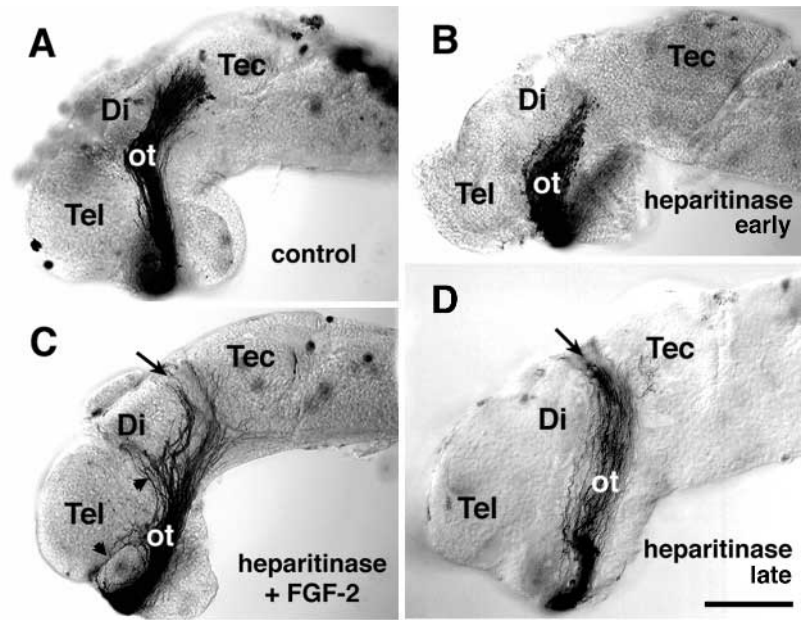


exhibit targeting errors, as seen with exogenous FGF-2 application alone (Fig. 6C; McFarlane et al., 1995).

## DISCUSSION

The results presented here strongly indicate that HSs are important in axon growth and guidance. First, FGF-2-binding HSs are potent inhibitors of target recognition and can competitively inhibit the binding of FGF-2 to embryonic *Xenopus* brains. Second, HSs are present in the embryonic diencephalon where retinal axons grow, and absent in the rostral tectum, a pattern that parallels that of FGF-2 (McFarlane et al., 1995). Third, digestion of these endogenous HSs produces dramatic defects in axon growth, navigation and targeting. Fourth, exogenous FGF-2 can overcome the growth inhibitory effect of HS removal.

Exogenous HS causes marked, often contrasting, effects on neurite outgrowth in vitro (Dow et al., 1991; Giuseppetti et al., 1994; Haugen et al., 1992a; Isahara and Yamamoto, 1995; Verna et al., 1989). This diversity of action could be due to the ability of HS to competitively interfere with the activity of a variety of native HS-binding molecules. In showing that exogenously applied HSs act rapidly and reversibly, without causing gross defects in neuroepithelial organization, our results argue against a slow mode of action such as changes in gene expression, and are consistent with the idea that HS exerts its action in the developing *Xenopus* visual system by perturbing the interaction of HS-binding molecules with their receptors in the growth cones. In a similar fashion, a different class of glycosaminoglycan, chondroitin sulfate (CS), is thought to cause its diverse effects through competitively interfering with CS-binding molecules (Emerling and Lander, 1996). Several lines of evidence support FGF-2 as a candidate HS-binding molecule whose function is disrupted by perturbing HS activity. (1) The pattern of native HS immunostaining is similar to FGF-2 (McFarlane et al., 1995) in that it is strong in the optic tract and absent in the tectum. (2) The tectal bypass

phenotype is similar to that caused by direct perturbations of FGF signaling. (3) Only FGF-2-binding HS and fragments cause the bypass phenotype. (4) Axon growth is retarded in the optic tract by removal of native HS and FGF-2 can relieve this growth inhibition; blocking FGFR function in RGCs similarly retards axon growth (McFarlane et al., 1996). (5) HS augments FGF-2-stimulated neurite outgrowth. Finally, de-N-sulfated heparin also caused mistargeting (data not shown) which agrees with studies showing sulfation to be necessary for HS to bind FGF-2 (Rusnati et al., 1994) but that N-sulfation specifically is not involved (Ornitz et al., 1995).

Our previous studies have indicated that axon mistargeting can result from either a sustained increase or decrease in FGFR activity in retinal growth cones (McFarlane et al., 1995, 1996). This led to a model based on the idea that retinal growth cones respond to changes in FGFR signaling that occur at the tectal border (McFarlane et al., 1996). Since levels of FGF-2 diminish sharply at the tectal border, we suggested that this might provide the trigger for such a signaling change, which, in turn, could activate a molecular program responsible for target entry. The observation that HS immunoreactivity is localized in a like fashion suggests that native HS and FGF-2 together could participate in demarcating the optic tract where axons grow, from the target where axons arborize, and is in line with the idea that regulation of FGFR signaling at the tectal border is a key factor in target recognition. The mistargeting of axons after HS and heparitinase treatment is also consistent with this model. HS(FGF-2) could potentially sustain high levels of FGFR activation, like exogenous FGF-2, rendering retinal growth cones insensitive to changes in external ligand concentration. Indeed, HS(FGF-2) seems to be stimulatory in its action in this system since it augments FGF-enhanced neurite outgrowth in vitro and does not inhibit axon extension in vivo. In contrast, removal of endogenous HS by heparitinase treatment, which presumably decreases FGFR function, similar to the dominant negative FGFR, would likewise render growth cones unable to register changes in ligand concentration.

The exact mechanism of HS requirement for FGF activity has

not been elucidated. HSs can stimulate as well as inhibit FGF-2 function, so the mechanism is not straight forward (Guimond et al., 1993; Hondermarck et al., 1992). Recent models attribute the role of the HS cofactor to its ability to present multiple FGFs and thus bring about receptor dimerization (Schlessinger et al., 1995; Spivak-Kroizman et al., 1994). In addition, several studies have indicated that HSs can bind directly to specific FGFRs (Kan et al., 1993; Seddon et al., 1995; Wang et al., 1995) and even activate FGFRs without FGF ligand (Gao and Goldfarb, 1995). Furthermore, HSPGs can dynamically regulate their HS sidechains and, in so doing, can change their peptide-binding preferences (Nurcombe et al., 1993). Comparison of the disaccharide sequences of the FGF-2- and FGF-1-binding HS sidechains has demonstrated the existence of distinct motifs that confer differential binding specificity (V. Nurcombe and colleagues, unpublished data), a finding that might help to explain the diverse action of HSs from different sources. Thus, HSPGs in vivo probably function to regulate precisely the availability and activity of specific FGF ligands with specific high affinity receptors. Interestingly, the specific FGF-2-binding HS used in the current study has recently been shown to specifically interact with the murine FGFR-1 (Brickman et al., 1995). Thus, the bypass phenotype seen with the exogenous application of this specific HS may result from it uniquely interfering with the endogenous interaction of FGF-2 with only one of the four families of FGFRs. FGFR-1 is present in the developing *Xenopus* eye primordium (Friesel and Dawid, 1991; M. Zuber and C. E. Holt, unpublished data), raising the possibility that the interaction between FGF-2 and FGFR-1 is specifically required for retinal axon target recognition.

One of the striking features of the bypass phenotype induced by HS(FGF-2) is its robust penetrance. Almost the entire population of retinal axons veer aberrantly around the tectum and 91% of brains treated at 0.1 mg/ml exhibit the phenotype. This degree of penetrance is similar to that obtained with exogenous FGF-2 but is markedly higher than that seen with expression of the dominant negative FGFR where approximately 50% of the axons show targeting errors. This might reflect the fact that 'overexpression' phenotypes tend to be more severe than single knockouts of the same protein (Chiba et al., 1995; Nose et al., 1994). Alternatively, exogenous HSs and heparitinase might disrupt not just FGFR signaling but also other molecules important for target recognition.

The short projections observed after early heparitinase treatment indicate that native HSs play a role in promoting RGC axon elongation through the optic tract. A possible explanation for this phenotype is that in the absence of HSs, endogenous FGF-2 may be unable to adequately activate the high affinity FGFR to stimulate normal axonal elongation rates. Indeed, when FGFR function is blocked, RGC axons grow at a markedly slower rate (McFarlane et al., 1996). In addition, the HS staining pattern co-localizes with the previously described expression of FGF-2 in the optic pathway indicating that the elements of functional FGF-2 signaling are present for RGC axons growing through the ventral and mid-diencephalon. Furthermore, applying an excess of exogenous FGF-2 to HS-free brains results in optic projections similar in length to control projections. This last finding may reflect the ability of excess FGF-2 to cause receptor dimerization sidestepping the function of the low affinity HS receptor (Roghani et al., 1994).

In addition to promoting FGF-2-dependent axon growth in the optic tract, endogenous HSs appear to impart directional information to growing axons. Our results show that when RGC axons are induced to grow in a HS-depleted diencephalon the majority of axons exhibit striking errors in pathfinding. This misrouting phenotype is not seen with exogenous HS where the optic tract trajectories appear normal, nor with any treatments that interfere specifically with FGF/R signaling. This suggests the possibility that native HSs in the diencephalon bind and localize the activity of (non-FGF) guidance molecule(s) that specifically identify the optic tract neuro-epithelium. Indeed, heparin is known to bind and thus potentially regulate the activities of molecules that guide axons such as netrin and collapsin/semaphorin (Raper and Kapfhammer, 1990; Serafini et al., 1994). In addition, HSs are required for NCAM-mediated adhesion of retinal cells and may act as an alternative receptor for laminin and other ECM molecules in the CNS (Cole et al., 1986; Haugen et al., 1992a,b).

Two of the HSs that had distinct effects in this study, HS(FGF-1) and HS(FGF-2), were isolated from the same perlecan variant. The developmentally regulated glycosylation changes of this HSPG in the developing mouse brain are thought to switch the responsiveness of cells to external growth factors (Nurcombe et al., 1993). A similar type of mechanism may also exist during axon navigation such that an axon's responsiveness to certain guidance cues depends on the type of cofactor present. It is not known which HSPGs might be playing a role in *Xenopus* visual system development. Perlecan is a large ECM proteoglycan that is present in the basal lamina of the mouse CNS (Joseph et al., 1996). Our data suggest that HS is not just restricted to the basal lamina in the *Xenopus* optic tract since retinal axons are also immunopositive. The integral membrane HSPGs, syndecans, are potential candidates because syndecans 2 and 3 have recently been shown to be expressed in a complementary pattern in the developing *Xenopus* brain with syndecan 2 defining the diencephalic/midbrain boundary (Teel and Yost, 1996). In addition, syndecans can support FGF-2-FGFR1 interactions and signaling (Steinfeld et al., 1996).

Our results suggest a crucial role for HSPGs in the establishment of the retinotectal projection, both during axon elongation through the diencephalon and subsequent target recognition in the tectal area. HS activity appears to stem, at least in part, from its participation in FGF-2 signaling. In the future it will be important to identify the individual HSPG species involved and to determine their specific actions in the development of the visual projection.

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