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Establishing the trochlear motor axon trajectory: role of the isthmic organiser and Fgf8

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SUMMARY

Formation of the trochlear nerve within the anterior hindbrain provides a model system to study a simple axonal projection within the vertebrate central nervous system. We show that trochlear motor neurons are born within the isthmic organiser and also immediately posterior to it in anterior rhombomere 1. Axons of the most anterior cells follow a dorsal projection, which circumnavigates the isthmus, while those of more posterior trochlear neurons project anterodorsally to enter the isthmus. Once within the isthmus, axons form large fascicles that extend to a dorsal exit point. We investigated the possibility that the projection of trochlear axons towards the isthmus and their subsequent growth within that tissue might depend upon chemoattraction. We demonstrate that both isthmic tissue and Fgf8 protein are attractants for trochlear axons in vitro, while ectopic Fgf8 causes turning of these axons away from their normal routes in vivo. Both inhibition of FGF receptor activation and inhibition of Fgf8 function in vitro affect formation of the trochlear projection within explants in a manner consistent with a guidance function of Fgf8 during trochlear axon navigation.

Key words: Trochlear nerve, Cranial motor nerve, Axon guidance, Fgf8, Isthmus, Organiser, Rhombomere, Chemotropic response

INTRODUCTION

Establishment of correct neuronal connectivity requires precise navigation of growing axons, which depends upon both specific guidance cues in the environment and in the competence of axonal growth cones to read those signals. A growing body of evidence indicates that growth cones are guided by a combination of positive (chemoattractive) and negative (chemorepellent) cues, which may operate in either a manner dependent upon contact with the substratum or via long-range diffusible gradients emitted from a target source (see Mueller, 1999).

Here we investigate the formation of one relatively simple axon pathway: that of the trochlear or IVth cranial nerve. We focus upon the initial projection of trochlear axons as they extend from cell bodies in ventro-anterior rhombomere one (r1) of the hindbrain and fasciculate, growing along a dorsal trajectory that circumnavigates the isthmic organiser at the midbrain-hindbrain boundary (MHB) to a dorsal exit point. At the latter location axons become less tightly associated, before fasciculating once more to project to the eye where they innervate the contralateral superior oblique muscle (dorsal oblique in avian embryos) (Colamarino and Tessier-Lavigne, 1995).

Within the brain (as opposed to the spinal cord), motor neuron organisation is subservient to neuromeric organisation. Thus, the various classes of cranial motor neurons – branchiomotor, visceral motor and somatic motor – are organised within individual neuromeres or in adjacent neuromeric pairs. The oculomotor (III) nucleus is located in the posterior midbrain, the trochlear (IV) nucleus in anterior r1, while the trigeminal (V; r2 and r3), facial (VI; r4 and r5), abducens (VII; r5 and r6) and glossopharyngeal (VIII; r6 and r7) are organised in adjacent pairs of hindbrain segments. The midbrain and each hindbrain segment have their own molecular 'address' reflected by the expression of a unique combination of transcription factors. Current evidence suggests that the transcription factor hierarchy plays a major role in determining the different properties of individual motor nuclei including their axonal projections (Jacob et al., 2001; Lumsden, 1990; Lumsden and Krumlauf, 1996; Lumsden and Keynes, 1989).

Rhombomere 1, within which the trochlear motor nucleus develops, is distinct from the remaining hindbrain segments since its pattern is established through graded signals from the isthmic 'organiser' at the midbrain-hindbrain boundary (MHB), mediated at least in part through the activity of Fgf8 (Irving and Mason, 2000; Meyers et al., 1998; Reifers et al., 1998) (reviewed by Rhinn and Brand, 2001; Wurst and Bally-Cuif, 2001). It is noteworthy that the dorsal projection of trochlear motor axons to exit at the roof plate at the isthmus is unique among motor neurons. Previous studies have shown that the dorsal projection of the trochlear nerve is caused in part by chemorepulsive cues emanating from the floor plate. Those motor nerves with dorsal trajectories (IV, V, VII, IX) are repelled by factors secreted from the floor plate (Colamarino and Tessier-Lavigne, 1995; Guthrie and Pini, 1995; Kennedy et al., 1994). Candidates for chemorepellent cues for trochlear neurons are members of the netrin and semaphorin (Sema) families, since both netrin 1 and Sema3A repel growing trochlear axons in vitro (Colamarino and Tessier-Lavigne, 1995; Varela-Echavarria et al., 1997). netrin 1 is expressed by the floor plate, while Sema3A is expressed by ventral tissues, suggesting that these molecules might govern the dorsal projection of trochlear axons in vivo (Kennedy et al., 1994; Puschel et al., 1995; Varela-Echavarria et al., 1997). In addition, Sema3F, which is expressed in both posterior midbrain and anterior hindbrain in the mouse, also repels trochlear axons and in mice lacking the Sema3F receptor, neuropilin 2, axons fail to exit the neuroepithelium (Chen et al., 2000; Giger et al., 2000).

In this study we explore the relationship of the trochlear motor nucleus and its axonal projection within the neuroepithelium to the isthmic organiser. We show that trochlear axons project towards and extend within the organiser raising the possibility that the isthmus plays a role in guiding trochlear axons. We have examined the role of the isthmus and Fgf8 in trochlear axon navigation and provide direct evidence that Fgf8 acts as a chemoattractant, which guides trochlear axons into the isthmic region and subsequently maintains their axon pathway within it.

MATERIALS AND METHODS

Dissection and collagen gel cultures

E11.5 rat embryos were dissected using tungsten needles and Dispase 1 (Roche) as described previously (Guthrie and Pini, 1995) to isolate the trochlear nucleus in a tissue explant including rostral r1 and the midbrain-hindbrain boundary region. Explants were bilateral and included either the ventral third of the neuroepithelium (axon outgrowth assays) or the entire neuroepithelium (inhibition assays). For certain co-culture assays, explants containing only dorsal isthmic tissue or only dorsal r1 tissue were also taken.

Rat tail collagen gels were prepared as described previously (Guthrie and Lumsden, 1994). MHB explants and dorsal isthmus, dorsal r1 or FGF-soaked beads were placed into gels 100-500μm apart and cultured for 48 hours in media as described previously (Colamarino and Tessier-Lavigne, 1995). Affi-gel blue beads were soaked in Fgf8b (R&D Systems) or PBS (control beads) as described previously (Irving and Mason, 2000; Shamim et al., 1999) and implanted into the collagen matrix. To inhibit Fgf signalling, either the chemical inhibitor of FGF signalling, SU5402 (at 10 μM or 20 μM; CalBiochem), or a neutralising FGF8 antiserum (R&D Systems) at a concentration five times the stated neutralisation dose (ND_{50}), were included in both collagen matrix and cell culture media.

To score the extent of axonal turning towards potential sources of chemotropic cues a simple grid system was used (see Fig. 3B). An inverted T-bar grid was oriented with its stem aligned along the original direction of trochlear axon growth through the explant, perpendicular to the floor plate, as we found that axons did not deviate towards a chemotropic cue until their emergence into the gel, in agreement with previous studies (Colamarino and Tessier-Lavigne, 1995). Axons extended within the collagen gels either singly or in small fascicles and the numbers growing in each sector, either side of the T-bar stem, were scored. Numerical data were analysed using Student's *t*-test.

Implantation of FGF beads in ovo

Heparin acrylic beads were soaked in Fgf8b (R&D Systems) or PBS (control beads) and implanted into HH12 chick embryos as described previously (Irving and Mason, 2000; Shamim et al., 1999). Embryos were incubated for a further 72 hours until HH25.

Immunostaining and in situ hybridisation

Whole embryos were immunostained as described previously (Irving and Mason, 2000) using SC1 antibody (Hybridoma Bank; 1:5 for 5 days) and a horseradish peroxidase-conjugated secondary antibody (Sigma; 1:200). Explants embedded in collagen gel were immunostained using either F84.1 antibody (Prince et al., 1992; Varela-Echavarria et al., 1997) (1:1000) or anti-160 KDa neurofilament antibody (Zymed; 1:10,000) for 3 days and a horseradish peroxidase-conjugated secondary antibody (Sigma; 1:200). Whole-mount in situ hybridisation of embryos was performed using probes reported previously (Irving and Mason, 2000). Embryos were then post-fixed in 4% paraformaldehyde in PBS and immunostaining was performed using anti-Isl1/2 antibody (Thor et al., 1991) as described previously (Mason, 1999).

RESULTS

Trochlear motor neurons develop both within and posterior to the isthmic organiser

Previous studies variously reported the location of trochlear motor neuron cell bodies as being within the midbrain, isthmus or within rhombomere 1 (r1) of the hindbrain (Altman and Bayer, 1981; Bubien-Waluszewska, 1981; Lumsden, 1990; Lumsden and Keynes, 1989; Sohal et al., 1985). *Fgf8* is expressed at the isthmus and can reproduce all of its patterning activities thereby providing the most useful marker for the isthmic organiser. We therefore explored the relationship of trochlear motor neuron cell bodies to the isthmic organiser in the chick embryo using *Fgf8* as a marker of the latter, and an antibody that recognises both Isl1 and Isl2 (Isl+) to distinguish cell bodies of the trochlear motor nucleus (Varela-Echavarria et al., 1996). The LIM homeobox gene, *Isl1*, is an early marker of all differentiated motor neuron cell bodies; the trochlear nucleus additionally expresses *Isl2* (Pfaff et al., 1996; Varela-Echavarria et al., 1996).

In accordance with previous studies, Isl+ motor neuron cell bodies were located ventrally on either side of the floor plate, along the entire rostrocaudal axis of posterior hindbrain segments (r2-7). By contrast, the cell bodies of the trochlear nucleus were detected in only the most rostral part of r1 and also within the *Fgf8*-positive isthmic tissue (Fig. 1A). Small numbers of trochlear motor neurons were first detected at Hamburger and Hamilton stage 17 (HH17; onset of limb bud outgrowth) both within and ventral to the *Fgf8* expression domain (Fig. 1B). *Fgf8* transcripts form a characteristic stripe at the isthmus in all vertebrate classes (Christen and Slack, 1997; Crossley and Martin, 1995; Crossley et al., 1996; Heikinheimo et al., 1994; Mahmood et al., 1995; Ohuchi et al., 1994; Reifers et al., 1998; Shamim et al., 1999). However, the presence of trochlear motor neurons ventral to the *Fgf8*-expressing cells indicated that *Fgf8* was not expressed in isthmic cells closest to the floor plate (Fig. 1B,C). By HH19 many more trochlear motor neurons were detected lying both within the isthmus and immediately posterior to it within anterior r1 (Fig. 1C). At this stage, cell bodies were most closely-packed in the isthmic region. By HH25, cell bodies of the trochlear nerve formed a cluster with a sharp anterior limit exactly coincident with the anterior limit of Fgf8 expression

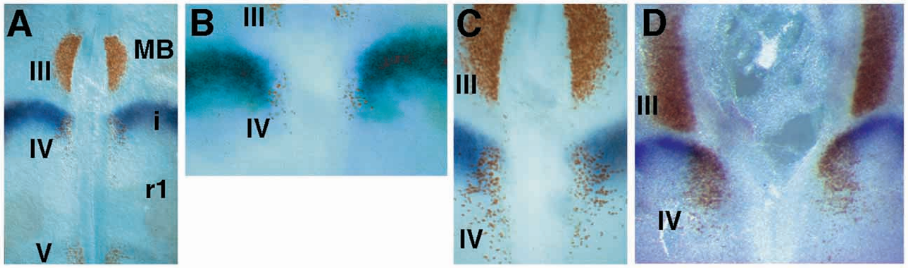

Fig. 1. Trochlear motor neurons are located both within and posterior to the isthmic organiser. (A-D) Chick hindbrains stained for Isl1/2 (brown) and *Fgf8* (blue) by combined immunohistochemistry and in situ hybridisation. All preparations are 'open-book' flat mounts i.e. cut along the dorsal midline and opened such that the floor plate is medial in the preparation and dorsal regions are lateral. (A) HH25 chick embryo showing the position of trochlear motor nuclei within the anterior hindbrain. Cell bodies are detected ventrally in rostral r1, both within and adjacent to the *Fgf8* expression domain. They appear more scattered than oculomotor neurons in the midbrain and most of r1 is essentially devoid of Isl1/2+ motor neurons. (B) The first trochlear motor neurons are detected at HH17 within and ventral to the *Fgf8* expression domain. (C) At HH19 trochlear cell bodies are detected both within and posterior to the isthmus. (D) By HH25 the trochlear nuclei are positioned in a dense cluster in anterior r1 whose anterior extent is coincident with that of *Fgf8* expression: anterior boundary of r1. III, oculomotor nucleus; IV, trochlear nucleus; V, trigeminal nucleus; i, isthmus; MB, midbrain; r1, rhombomere 1.

(Fig. 1D). Posteriorly, a few Isl+ cell bodies were seen in mid to posterior r1 but the majority were located within the anterior half of that rhombomere (Fig. 1A,D). The asymmetric location of trochlear neurons within r1 suggests that their specification may be mediated at least in part by signals from the isthmus.

Trochlear axons extend dorsally both towards and within isthmic tissue

The finding that trochlear motor neuron cell bodies are located both within and posterior to *Fgf8*-positive isthmic tissue prompted us to investigate the relationship of the latter to trochlear axonal projections. We examined trajectories and timing of trochlear axons growth by immunostaining for SC1/DM-GRASP/BEN (hereafter called SC1). SC1 is an axonal surface glycoprotein that is expressed on all hindbrain motor axons and floor plate cells (Burns et al., 1991; Guthrie and Lumsden, 1992; Pourquie et al., 1990). Unfortunately, the SC1 antigen was destroyed when we combined immunohistochemistry with *in situ* hybridisation for *Fgf8*. However the relationship of trochlear axon trajectories to the *Fgf8*-positive tissue was derived by comparison with the Isl/*Fgf8* study.

When the formation of the trochlear projection within the CNS was complete, it was noted that whereas axons from anteriorly located cell bodies extended dorsally (i.e. within the *Fgf8*-positive tissue), those located posteriorly followed an anterodorsal route. The most anterior axons followed a straight trajectory dorsal to the roof plate, eventually forming a single large bundle. By contrast, more posterior axons appeared to fasciculate and defasciculate in smaller bundles as they extended anteriorly towards the isthmus. Within the isthmus these small bundles joined to form larger fascicles and eventually exited the brain at three or four points in the isthmic roof plate (Fig. 2A and data not shown).

We investigated the spatiotemporal formation of the trochlear projection within the CNS. Anterior trochlear cell bodies first extended axons at HH18 (Fig. 2B); prior to this no SC1 staining was detected in r1 (data not shown). Initially axons were short, extending independently of one another and by HH19 the first pioneer axons had reached the roof plate (Fig. 2C). Anterior cell bodies extended axons in a direction

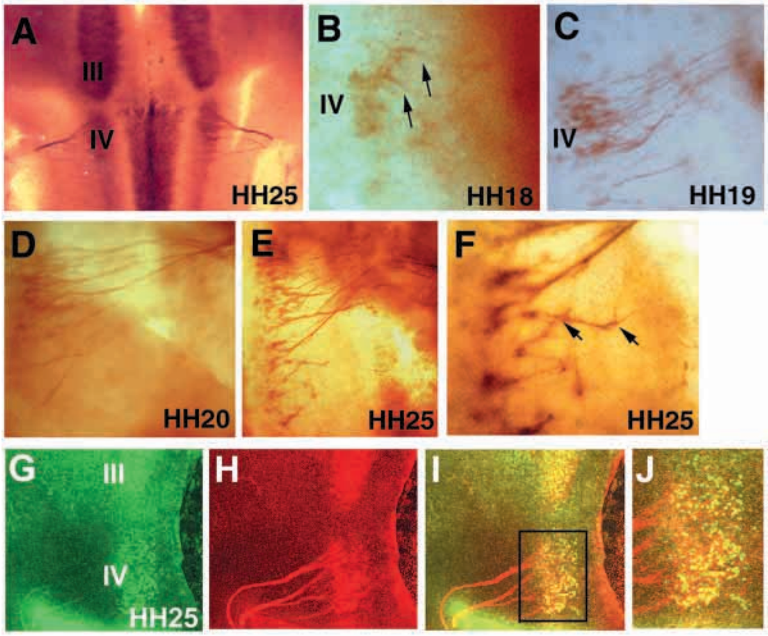

Fig. 2. Trochlear axons project towards and extend within the isthmic organiser. (A-F) Flat mount preparations of chick hindbrains stained for the SC1 antigen. In all cases anterior is towards the top of the image. (A) HH25 chick hindbrain. Trochlear axons project dorsally forming 3-4 main fascicles. (B) Short, SC1-positive trochlear axons (arrows) are first weakly detected at HH18 extending from the anterior cells of the nucleus. (C) By HH19 pioneer axons have reached the roof plate. Axon extension appears to proceed in an anteroposterior wave with axons extending from more anterior cell bodies reach the roof plate before those emerging from posteriorly positioned cell bodies within r1. (D) At HH20 anteriorly positioned cell bodies have reached the roof plate extending dorsally within the isthmus, perpendicular to the floor plate. By contrast, axons from more posterior neurons follow a route that leads them both anteriorly towards the isthmus and dorsally towards the roof plate. (E,F) At HH25 axons are still just forming from posterior trochlear motor neurons and their growth cones are visible at higher magnification (arrows, F). (G-J) Chick hindbrain at HH25 stained for both Isl1/2 (G; green) and SC1 (H; red) and these images are combined in I and J with the trochlear nucleus (boxed in I) being shown at higher magnification in J.

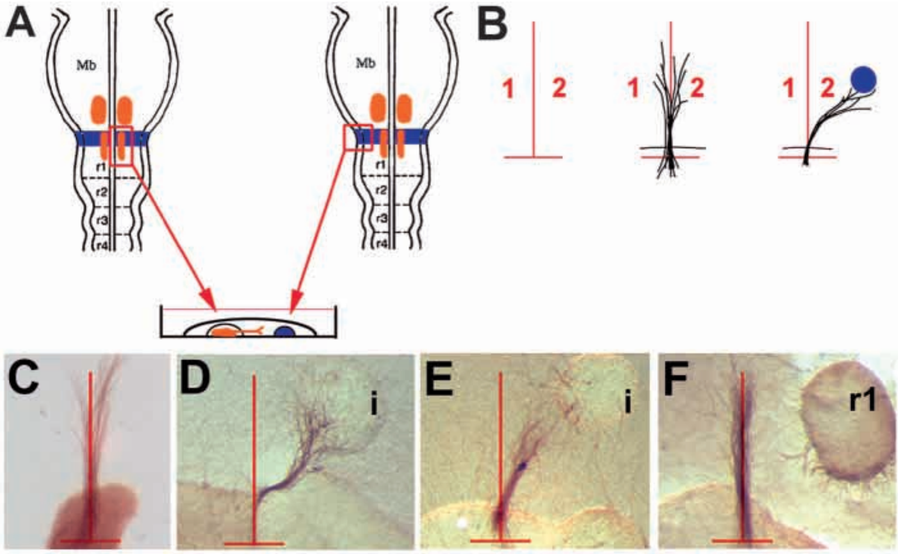

Fig. 3. Isthmic tissue is a source of a diffusible attractant for trochlear motor axons. (A) A schematic representation of how the collagen gel co-culture was assembled, showing the dissected MHB tissue explant containing trochlear neurons (orange) and the dorsal isthmus (blue) explant. Mb, midbrain; r1-r4, rhombomeres 1-4. (B) Scoring system for detecting deflection of axons towards a source (isthmus tissue, blue sphere). The number of axons were counted growing in two sectors (1, 2) of a T-bar placed parallel and central to the initial trajectory of the extending nerve. (C-F) Rat E11.5 explants into collagen gels that were cultured for 48 hours and stained with F84.1 antibody. (C) Trochlear axons grow from an isolated MHB explant defasciculate upon entering the gel but do not deviate greatly from their original trajectories. (D,E) MHB explants cultured at a distance from a piece of isthmus tissue: axons turn towards the isthmus tissue (i) and grow within it. (F) MHB explant cultured at a distance from a piece of posterior rhombomere 1 (r1) tissue: axons are not affected by the r1 tissue.

perpendicular to the floor plate, thereby extending within *Fgf8*-expressing isthmic tissue. By contrast, posteriorly located neurons projected axons at a more acute angle relative to the floor plate, growing rostrally and eventually joining axons from more anterior cells within the isthmic region (Fig. 2D,E). At HH20, axons were becoming organised into fascicles as they extended dorsally. While some fasciculation and defasciculation was observed in ventral r1, axons became organised into several large bundles in the dorsal isthmus (Fig. 2D). By HH25, a large number of anterior axons had reached their exit point, while those extending from posterior cell bodies were much shorter, with growth cones still traversing ventral r1 (Fig. 2E,F). The most mature neurons might be expected to extend axons before those more recently born, suggesting that trochlear neurons might be born in an anteroposterior wave with the youngest cells located posteriorly. Double immunostaining for both SC1 and Isl1/2 confirmed that all SC1-positive axons in r1 were of trochlear origin (Fig. 2G-J) such that direct comparison could be made with the *Fgf8*/Isl1/2 study.

Isthmic tissue acts as a chemoattractant for trochlear axons in vitro

The relationship of trochlear axonal projections, particularly those from posterior cell bodies, towards the isthmic organiser suggested that the latter might play a role in trochlear axon guidance within the CNS. In particular, it raised the possibility that their route might be established by an attractive cue(s) from the isthmus in addition to the established repulsion from the floor plate. We therefore used collagen gel co-cultures (Colamarino and Tessier-Lavigne, 1995; Varela-Echavarria et al., 1997) to test the possible influence of isthmic tissue upon trochlear axons.

A region of ventral r1 and isthmus (mid-hindbrain region) was isolated from embryonic rat brains and cultured for 48 hours at a distance from explants of either dorsal isthmus tissue or posterior r1 tissue (Fig. 3A). Normally, F84.1-immunoreactive trochlear axons extend perpendicular within MHB explants and defasciculate to some extent upon entering the collagen gel, however generally they do not deviate greatly from their original trajectories (Fig. 3C) (Colamarino and Tessier-Lavigne, 1995). To measure deviation towards potential sources of chemotropic cues, an inverted T-bar grid was oriented according to the trajectory of the projection within the explant and numbers of individual axons/fascicles were counted in the sector containing a source and the adjacent sector (Fig. 3B). Explants were not scored if the potential source was located on or near the midline of the grid.

When a piece of dorsal isthmus tissue was placed at a distance from such an MHB explant, axons followed an altered trajectory; turning and growing towards the isthmus tissue (Fig. 3D,E; *n*=20/29; Table 1). By contrast, posterior dorsal r1 tissue did not cause trochlear axons to deviate towards it (Fig. 3F; *n*=7/8; Table 1). In the latter experiments we had anticipated a possible repulsive effect, as Sema3F, which has been shown to repel trochlear axons, is expressed by posterior r1 tissue in the mouse embryo (Chen et al., 2000). Taken together, or data indicate that isthmic tissue, but not posterior r1, contains a diffusible molecule that can influence the direction of growth of trochlear axons at a distance.

Fgf8 is a chemoattractant for trochlear axons in vitro

Our previous studies have shown that Fgf8 secreted by the isthmus patterns r1 and that Fgf8 protein diffuses across the entirety of that segment to position the r1/r2 boundary (Irving

Table 1. Deflection of trochlear axons towards an ectopic isthmic tissue or source of Fgf

Cue	Explant	Number of explant cultures scored	Number of explants with >60% axons in sector 2
Isthmus	MHB	29	20 (*P*<0.001)
Posterior r1 (control)	MHB	8	1
Fgf8 bead	MHB	25	17 (*P*<0.01)
PBS bead (control)	MHB	38	5

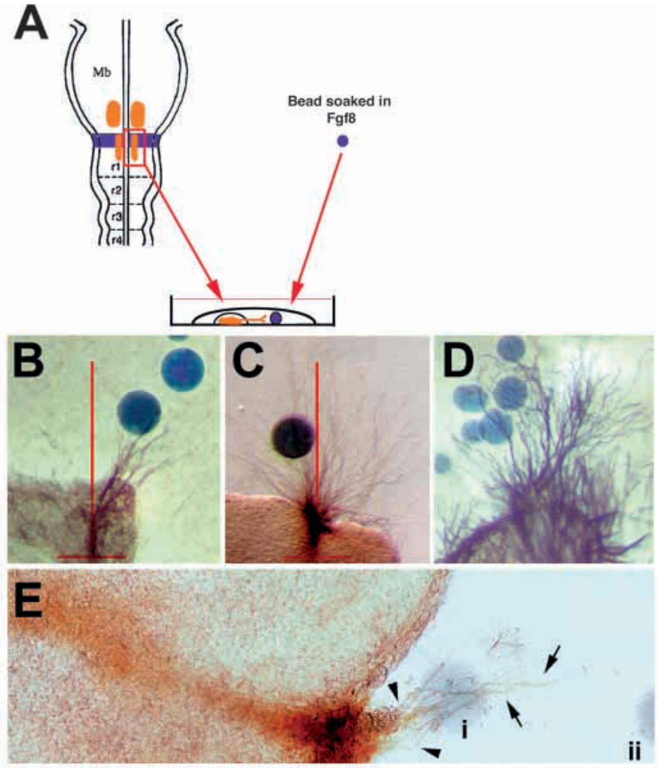

Fig. 4. Fgf8 is a chemoattractant for trochlear motor axons in vitro. (A) Diagramatic representation of the experiment. (B) MHB explants cultured for 48 hours together with an Affi-gel bead soaked in Fgf8b. Trochlear axons turn and grow towards the FGF beads. (C) Trochlear axons are not attracted towards a control bead soaked in PBS. (D) Staining for neurofilament to reveal all axons present shows that growth towards an Fgf8 source is not a general feature of axons from r1 neurons. (E) Turning influences of Fgf8 occur both within and outside the explant. The original trajectory of the trochlear axons extends from the top left corner of the picture (out of focus because of the thickness of the explant tissue above it). As they approach the periphery of the explant, axons turn slightly towards the Fgf8 beads, however, further reorientation towards the closest bead (i) occurs outside the explant (arrowheads). Some of the axons stall at that bead, but others (arrows) extend further and appear to be reorienting towards a second, more distant Fgf8 bead (ii).

and Mason, 2000). Furthermore, studies by several groups have suggested that Fgf8 forms a gradient extending from the isthmus both anteriorly into the midbrain and posteriorly into r1 (for reviews, see Rhinn and Brand, 2001; Wurst and Bally-Cuif, 2001). Thus, Fgf8 is a candidate for the isthmic guidance cue for trochlear axons.

To test whether Fgf8 can act directly to influence the directed growth of trochlear axons we performed co-cultures of MHB explants with a source of Fgf8 provided by a bead loaded with recombinant Fgf8b protein (Fig. 4A). Axonal deflection was scored as described above (Fig. 3B). Trochlear axons reproducibly turned towards the FGF bead (Fig.4B; *n*=17/25; Table 1) and similar results were obtained using Fgf4-soaked beads (data not shown). By contrast, axons rarely turned towards a bead that had been pre-incubated in PBS alone (Fig.4C; *n*=5/38; Table 1) and statistical analysis showed that this effect was not significant when compared to the effects of Fgf8. This response to Fgf8 was not a general feature of all MHB neuronal populations (Fig. 4D). Thus, Fgf8 protein is sufficient to mimic isthmic tissue as a guidance cue for growing trochlear axons in vitro.

It was interesting that in some instances trochlear axons turned towards either isthmic tissue or an Fgf8 bead while still within the explant. This might have been due to either a direct chemotropic influence of ectopic Fgf8 that had entered the periphery of the explant or to its indirect action in inducing an unknown chemoattractant. However, in other cases ectopic Fgf8 promoted turning of axons after they had exited the explant (Fig. 4E) suggesting that Fgf8 can itself act directly to guide trochlear axons, although additional indirect effects within explant tissue cannot be excluded.

Table 2. Deflection of trochlear axons in response to an ectopic Fgf8 source in vivo

Cue	Total number of embryos	Number of embryos with bead in appropriate location	Number of embryos with abnormal trochlear trajectory
Fgf8	105	91	44 ($P<0.001$)
Control	53	50	6

Ectopic Fgf8 redirects trochlear axons in vivo

To investigate whether Fgf8 could influence trochlear axon growth in vivo, we implanted Fgf8-coated beads into chick embryo hindbrains. Beads were inserted unilaterally into dorsal, posterior r1, prior to the onset of trochlear axon outgrowth (Fig. 5A), and embryos were incubated for 72 hours until approximately HH25. The effects of this posterior, “competing” source of Fgf8 were examined by staining with anti-SC1 antibody. Embryos receiving implants of PBS-soaked beads served as controls.

Following insertion of a PBS bead into r1, normal motor axonal trajectories were generally observed (Fig. 5B; *n*=44/50; Table 2). By contrast, embryos that had received Fgf8-coated beads, frequently showed obvious abnormalities in the trochlear projection within r1 (*n*=43/91). Changes in trochlear axon pathfinding could be grouped into 4 classes. The most frequently encountered phenotype (type 1; Fig. 5C; *n*=31/91) was that axons of posteriorly-located cells did not have an anterodorsal trajectory but instead projected dorsally. Moreover, they failed to coalesce into the 3 or 4 large fascicles that normally exit at the isthmus but instead exited dorsal r1 as a series of small parallel-projecting fascicles. Thus, trochlear axons emerged from dorsal r1 over a much broader domain than in control embryos. In addition, in some cases a subset of caudal axons stalled within posterior r1 and did not reach the dorsal neuroepithelium. Thus, in the most frequent phenotype encountered, posteriorly located trochlear axons appeared to have lost their ability to navigate towards the isthmus.

The second phenotype (type 2; *n*=4/91) revealed a dramatic turning of the entire anterior pioneer axon fascicle to project posteriorly towards the ectopic source of Fgf8 (Fig. 5D). Some axons initially extended along a trajectory perpendicular to the floor plate, before making a sharp turn towards the bead. Remaining axons did not turn in this manner but instead generally grew perpendicular to the floor plate (i.e. with a type 1 phenotype). In other instances (type 3 phenotype), implantation of an FGF bead resulted in a complete splitting

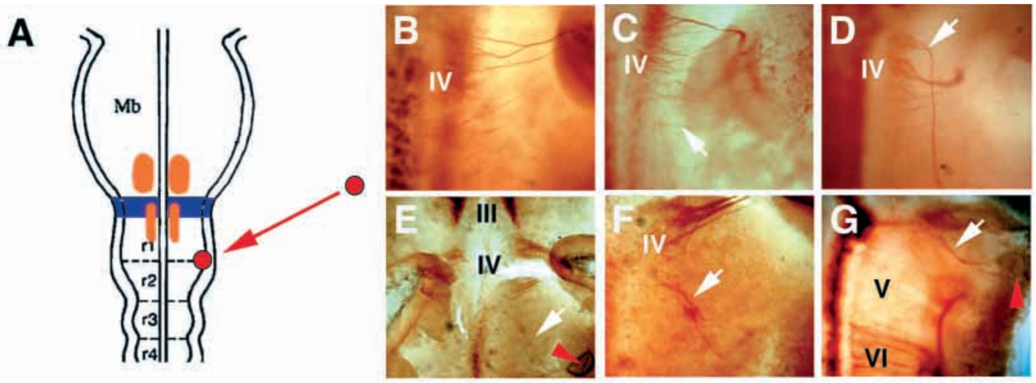

Fig. 5. Ectopic Fgf8 redirects trochlear motor axons in vivo. (A) Diagrammatic representation of the experimental manipulation. (B-G) Flat-mounted hindbrains of HH25 chick embryos stained for SC1 antigen following bead implantation at HH11-12. In all cases anterior is towards the top of the image. Individual motor nuclei are labelled where present on the image: III (oculomotor); IV, trochlear; V, trigeminal (stains only weakly for SC1) and VI, facial. Deflected and misrouted axons or fascicles are indicated by arrows and, where visible, the bead implant is indicated by the red arrowheads. (B) Control embryo showing that implanted PBS beads have no effect on trochlear nerve axon pathways. (C-G) Implanted FGF-soaked beads result in 4 classes of axonal defect. (C) Type 1 phenotype: axons extend from the floor plate dorsally but posterior axons have lost their anterior trajectory towards the isthmus and follow a direct dorsal path. (D) Type 2 phenotype: the most anterior fascicle has turned through 90° within r1 and extends towards the ectopic source of Fgf8 at the r1/2 boundary. (E,F) Low and high magnification images showing the Type 3 phenotype. The trochlear nerve is bisected; the arrow indicates fascicles that have turned towards the ectopic source of Fgf8. (G) Ectopic structures with the morphology of an ectopic isthmic region in posterior r1 induced by an Fgf8 bead are associated with ectopic SC1-positive axons that grow towards the bead.

of the trochlear nerve into 2 main axon groups. The anterior axons followed a normal trajectory to exit the neural tube in the dorsal isthmus region, while posterior axons formed a series of loose fascicles growing caudally and dorsally directly towards the bead (Fig. 5E,F; *n*=2/91).

Implantation of Fgf8 beads into the hindbrain is sufficient to induce gene expression characteristic of the midbrain-hindbrain region, suggesting that the FGF protein was either acting as an ectopic organiser or inducing one. In addition, ectopic motor neurons (Isl+) were present within posterior r1, which is usually devoid of motor neurons (Irving and Mason, 2000). In the present study, we found that a subset of embryos developed a morphology reminiscent of an ectopic isthmus at the level of the bead implant. In these cases, SC1-positive axons were observed projecting from ectopic, ventrally located motor neurons in that region (type 4 phenotype). We believe that these are most likely to represent ectopic trochlear axons, since trigeminal axons in r2 and r3 stain only weakly for SC1 (Fig. 5G) (Chedotal et al., 1995). These ectopic axons extended from cell bodies in the ventral r1/r2 boundary region and grew dorsally towards the ectopic source of Fgf8 (Fig. 5G; *n*=7/91).

Taken together, these data suggested that ectopic Fgf8 can redirect trochlear axons along ectopic pathways. Specifically, it was the anterior component of their pathfinding that was affected, while dorsoventral extension, which is probably largely a product of repulsive cues from the floor plate, seemed unaffected. In addition, in some instances an ectopic morphological isthmic structure appeared to have been generated and was associated with ectopic motor neurons with axonal SC1 staining characteristic of trochlear rather than trigeminal neurons.

Fgf8 is required for guidance of trochlear motor axons

Our in vitro and in vivo studies strongly suggested a role for Fgf8 in navigation of trochlear motor axons towards and within the isthmus during the establishment of their projection within the CNS. To test the idea that Fgf8 was required for trochlear axon projections, we performed a series of inhibition studies using both a pharmacological inhibitor of FGF receptor (FGFR) activation and a neutralising antiserum raised against Fgf8. These studies were undertaken using rat MHB explants in collagen gels and explants included dorsal tissue, since we wished to assay the effects of the inhibitory reagents on axon growth across the entire trochlear axon pathway within the isthmic region (Fig. 6A).

We examined the effect of inhibition of FGFR activity using SU5402, which specifically inhibits signalling through all FGF receptors (Mohammadi et al., 1997). In control explants, trochlear axons extended through the explant as a closely associated bundle of fascicles emerging in the dorsal region of the explant (Fig. 6B) and reproducing the projection pattern observed in vivo (Colamarino and Tessier-Lavigne, 1995). By contrast, inhibition of FGFR activity resulted in fewer axons and fascicles, but those that were present failed to become organised into a single closely organised projection within the explant. Rather, individual axons and fascicles followed diverse pathways through the explant, although a general dorsal direction was maintained (Fig. 6C,D). In only a subset of cases trochlear axons emerged from the explant into the collagen gel (10/20 for SU5402 at 10 μM; 3/12 for SU5402 at 20 μM; 14/25 for SB402451) but in these instances they exited over a much wider region of the explant border than in controls.

These data suggested a role for FGFR activation in establishment and maintenance of the normal trochlear projection, although the severity of the effects may be indicative of other functions for FGF signalling. Moreover, there is a body of evidence indicating that FGFRs can be activated not only by FGF ligands but also by certain members of the CAM and cadherin families of cell adhesion molecules. We therefore used the same explant assay but in combination with a neutralising antiserum raised against Fgf8 (Hunter et al., 2001; Irving and Mason, 2000) to demonstrate a requirement for the latter in the formation of the trochlear projection (Fig. 6E-G). Explants treated with this antiserum did not show the reduction in axon fascicle number or length observed with

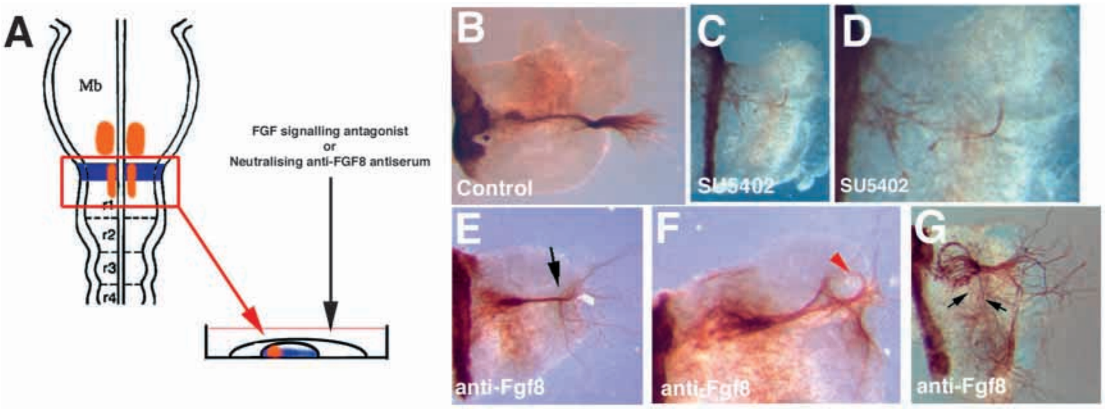

Fig. 6. Fgf8 is required for guidance of trochlear motor axons in vivo. (A) Diagram of explant culture. (B-G) MHB explants cultured for 48 hours and stained with F84.1 antibody. (B) Control explants cultured in the presence of DMSO alone. Note the formation of a single dorsal projection within the explant (C,D). Explant cultured in the presence of the FGFR inhibitor SU5402 at 10 μM; axons fail to navigate correctly within the explant. (E-G) Explants cultured in the presence of a neutralising anti-Fgf8 antiserum. Note that axonal misrouting occurs in both dorsal (arrow in E, arrowhead in F) and ventral (arrows in G) regions of explants. (F) Some appear to follow random or even circular trajectories within the explant (red arrowhead) around the bead. When axons did exit from the explant they did so over a wide region of its dorsal edge (E,G).

SU5402, however the projection of axons within the explant was highly abnormal. In some cases, axons initially began to form a tight bundle projecting dorsally but, in more dorsal regions of explants, extensive defasciculation occurred with axons following rostral and caudal trajectories (Fig. 6E). In other cases, axons appeared misrouted from the time of their initial projections within the ventral-most tissue, with some axons never entering the main axon bundle (Fig. 6F,G). Instead, they followed random abnormal projections within the explant with many projecting posteriorly before exiting over a broad region of the explant. By contrast, control explants cultured in the presence of an antibody that specifically blocks Fgf4 activity (Shamim et al., 1999) did not exhibit any of the above defects (data not shown). These data indicate a requirement for Fgf8 in trochlear axon guidance, both in establishment and maintenance of the projection within the isthmic region.

DISCUSSION

Trochlear neurons develop both within and posterior to the isthmic organiser

The isthmus is the location of an organiser, which tissue grafting studies have shown to pattern both midbrain and anterior hindbrain (Alvarado-Mallart et al., 1990; Alvarado-Mallart, 1993; Marin and Puelles, 1994; Martinez et al., 1995; Martinez et al., 1991; Nakamura et al., 1986; Nakamura et al., 1988). Evidence from ectopic expression or inhibition studies in all vertebrate classes indicates that Fgf8 provides the isthmic patterning signal (Crossley et al., 1996; Irving and Mason, 1999; Irving and Mason, 2000; Lee et al., 1997; Liu et al., 1999; Martinez et al., 1999; Meyers et al., 1998; Picker et al., 1999; Reifers et al., 1998; Shamim et al., 1999). Thus, *Fgf8* expression provides the best marker of the isthmic organiser and current evidence indicates that the *Fgf8*-positive tissue is maintained in the anterior of r1 under the influence of a diffusible signal from the midbrain (Irving and Mason, 1999).

By studying the expression of Isl proteins, some of the earliest molecular markers for differentiated motor neurons (Thor et al., 1991), we have shown that trochlear motor neurons develop within both the *Fgf-8*-positive isthmic tissue and anterior r1. Unexpectedly, we found that *Fgf-8* transcripts did not extend as far ventral as the floor plate and that trochlear motor neurons also developed within this *Fgf-8*-negative region.

It has been shown that Fgf8 acts in concert with sonic hedgehog to regulate the induction of dopaminergic neurons in the posterior midbrain and serotonergic neurons in anterior hindbrain; the differential competence of these two regions being dependent upon further unidentified factors (Ye et al., 1998). It therefore seems likely that *Fgf8* might play a role in the induction of trochlear neurons and also of the oculomotor nucleus located in the posterior midbrain. Indeed preliminary data suggests that Fgf8 is able to induce ectopic Isl-positive motor neurons in posterior r1 and that the axonal projections of these cells is characteristic of the trochlear nucleus (Fig. 5G).

The most anterior trochlear motor neurons (i.e. those within the isthmus) extend axons before those located within r1, suggesting that they are more mature and are probably born first. Thus it is unlikely that cells are born within the isthmus and then migrate posteriorly, but rather there is an anterior-posterior wave of induction of trochlear motor neuron differentiation. Indeed, Isl-positive cells become progressively more sparse as distance from the isthmus increases (Fig. 1) consistent with their induction being regulated by a gradient of signal from the isthmus.

Extension of trochlear axons towards and within the isthmus

Consistent with several earlier studies we found that trochlear axons extended circumferentially in a series of fascicles along a characteristic trajectory to the dorsal midline. This projection is unique among motor neurons and is conserved among all vertebrate classes (Chedotal et al., 1995; Colamarino and Tessier-Lavigne, 1995; Fritzsch and Northcutt, 1993; Fritzsch and Sonntag, 1988; Matesz, 1990; Sinclair, 1958; Szekely and

Matesz, 1993). We investigated the relationship of the trochlear projection to the isthmic organiser cells. We found that while axons from anterior cell bodies took a dorsal trajectory i.e. extending within the *Fgf-8*-positive territory, axons from more posterior motor neurons followed a dorso-anterior path towards the isthmus. Upon arrival within the isthmus, they fasciculated with axons from the anterior cells and projected dorsally to their exit points. Thus, initial axon projection was established within the isthmus by axon pioneers from the most anterior cells, with more posterior cells extending processes only later.

Guidance of trochlear axons: roles of the isthmus and Fgf8

The extension of trochlear axons towards and within the isthmic organiser region suggested that the latter tissue might be a source of guidance cues for their growth cones. This was examined in collagen gel co-cultures, previously used by others to examine chemotropic influences on trochlear axons (Colamarino and Tessier-Lavigne, 1995; Varela-Echavarria et al., 1997). We found that axons extended towards and grew within isthmic tissue, whereas they were neither attracted towards nor repelled by tissue taken from the dorsal part of posterior r1.

These data raised the question of what the isthmic chemoattractant cue might be, and Fgf8 was an obvious candidate. Many studies have shown that FGFs stimulate axon extension in vitro, both from primary neurons and from cell lines with neuronal characteristics (for a review, see Eckenstein, 1994; Mason, 1994). However, there is little data concerning their ability to guide the formation of axonal pathways in vivo, with perhaps the best studies being those on the formation of the retinotectal projection in the frog (for a review, see Dingwell et al., 2000). In this system, initial axonogenesis seems to be dependent upon FGFR activation but via an N-cadherin ligand (Lom et al., 1998). By contrast, signalling regulated by an FGF ligand is required for axon growth (McFarlane et al., 1995) and, significantly, for turning towards and entry into the optic tectum (McFarlane et al., 1996). As yet, it is unclear whether the role of FGF signalling is to promote turning of the growth cone towards the tectum by changing its response to environmental cues, or whether FGF is acting as a chemoattractant. However, there is evidence that FGFs have chemotropic potential in other systems e.g. in migration of neural crest cells and limb myogenic cells in vitro (Murphy et al., 1994; Sieber-Blum and Zhang, 1997; Webb et al., 1997) and in development of the *Drosophila* tracheal system (Affolter and Shilo, 2000).

Our study suggests that Fgf8 is a chemoattractant for trochlear neurons both in vitro and in vivo. Ectopic Fgf8, delivered from beads attracts trochlear axons in vitro, and redirects their growth towards a bead in vivo. In the most severe cases in vivo, the trochlear nerve became split into two with axons extending both anteriorly to the isthmus and posteriorly towards the Fgf8 bead. In addition, the most anterior pioneer axon bundle occasionally turned and projected posteriorly towards the ectopic Fgf8 source. It is not clear why only the most anterior fascicle behaved in this manner, although it may reflect rapid depletion of the Fgf8 protein or the growth of r1, which is considerable at the developmental stages used and might move the bead distant from the site of implantation. In either case, later-extending axons might be expected to be unaffected by ectopic protein.

It remains possible that changes in trajectory of the trochlear motor nerve observed following bead implants in ovo or inhibition studies in vitro may also reflect additional effects of Fgf8-regulated tropic signals. In addition, deflection of trochlear axons towards either isthmic tissue or a source of Fgf8 while within mhb explants in vitro might be due to either direct chemoattractant effects of Fgf8 or its indirect effects in inducing an unidentified chemotropic cue. However, trochlear axons were also found to reorient towards Fgf8 beads after they had left the explant and were extending within the collagen gel. The simplest interpretation of the latter data is that Fgf8 can itself provide a direct chemotropic influence, although although it remains possible that additional, unidentified guidance cues may be regulated by it within MHB tissue following both in vivo and in vitro manipulations.

We further showed that inhibition of FGFR activity disrupts the formation of the trochlear projection within explants in vivo. Most significantly, a specific anti-Fgf8 antiserum causes mis-routing of axons within MHB explants in a manner consistent with a role for Fgf8 in guiding trochlear axons towards the isthmus and maintaining their growth within it. Moreover, a recent study has suggested that higher Fgf8 concentrations are present dorsally in the isthmus (Carl and Wittbrodt, 1999), raising the possibility that Fgf8 might also contribute to dorsal guidance of trochlear axons.

Within the isthmic region, trochlear axons axons come together to form three or four main fascicles that circumnavigate the isthmus. It was notable that there was considerable axon defasciculation as a result of the application of both a pharmacological FGFR inhibitor and an anti-Fgf8 neutralising antiserum, suggesting that Fgf8 might also play a role in inducing or maintaining fasciculation. Indeed, inhibition of FGFR activity promotes defasciculation in other systems (Brittis et al., 1996). However, emergent trochlear axons defasciculate in collagen gel cultures and Fgf8 protein did not noticeably reduce this behaviour.

Multiple chemotropic cues establish the trochlear projection within the CNS

Our observations showed that growing trochlear axons initially extended away from the floor plate in a near-perpendicular direction, presumably reflecting their response to chemorepellents from that tissue. Trochlear axons are repelled by both floor plate tissue and netrin 1 in vitro (Colamarino and Tessier-Lavigne, 1995; Varela-Echavarria et al., 1997), although in netrin-deficient mice the trochlear trajectory is largely normal (Serafini et al., 1996). This presumably reflects the presence of other chemorepellents in the floor plate, such as semaphorins. Sema3A can act as a chemorepellent for trochlear axons in vitro, although its spatio-temporal location within the hindbrain may exclude it from fulfilling this role in vivo (Varela-Echavarria et al., 1997). Sema3F has also been demonstrated as a direct chemorepellent for trochlear axons in vitro, and is expressed in both the anterior midbrain and posterior r1 – these may reflect domains of repulsion that channel trochlear axons on their course around the isthmus. In support of this, mice lacking Neuropilin 2, the preferred Sema3F receptor, show normal positioning of trochlear neuron cell bodies but exhibit a dramatic loss of trochlear axons projecting into the periphery. Instead, axons follow random projections within the CNS (Chen et al., 2000; Giger et al.,

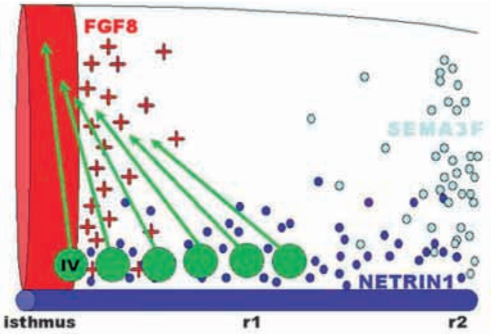

Fig. 7. A model for trochlear axon guidance. Trochlear cell bodies (green) arise in a cluster in ventral anterior r1. Extending axons (green arrows) are exposed to a number of both positive and negative chemotropic signals within the neural tube. Initially, a strong repulsive signal from the floor plate (netrin 1; dark blue) initiates a dorsal trajectory. Additional repulsive signals are present in posterior r1 (Sema3F; light blue). In combination with an attractive signal from the isthmus (Fgf8, red), a net positive signal towards the dorsal isthmic region is produced.

2000). Furthermore, Sema3F is also expressed in tissues surrounding the nervous system, and it has been proposed that, following dorsal decussation and exit from CNS, the trochlear nerve may be guided to the eye by the same molecule acting as a repulsive cue (Giger et al., 2000). However, it should be noted that we found no evidence of a diffusible chemorepellant produced by rat posterior r1 tissue.

Our study developed from the observation that the most anterior trochlear axons followed a simple dorsal trajectory through the isthmic organiser, whereas those located more posteriorly in r1, grew antero-dorsally until they reached the isthmus. We propose that the trochlear projection reflects the sum of repulsive cues (including netrin) from the floor plate, and possibly Sema3F repulsion from posterior r1, and an attractive cue from Fgf8 at the isthmic organiser (Fig. 7).

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