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Slit proteins are not dominant chemorepellents for olfactory tract and spinal motor axons

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SUMMARY

Members of the Slit family are large extracellular glycoproteins that may function as chemorepellents in axon guidance and neuronal cell migration. Their actions are mediated through members of the Robo family that act as their receptors. In vertebrates, Slit causes chemorepulsion of embryonic olfactory tract, spinal motor, hippocampal and retinal ganglion cell axons. Since *Slits* are expressed in the septum and floor plate during the period when these tissues cause chemorepulsion of olfactory tract and spinal motor axons respectively, it has been proposed that Slits function as guidance cues. We have tested this hypothesis in collagen gel co-cultures using soluble Robo/Fc chimeras, as competitive inhibitors, to disrupt Slit interactions. We find that the addition of soluble Robo/Fc has no effect on chemorepulsion of olfactory tract and spinal motor axons when co-cultured with septum or floor plate respectively. Thus, we conclude that although *Slits* are expressed in the septum and floor plate, their proteins do not contribute to the major chemorepulsive activities emanating from these tissues which cause repulsion of olfactory tract and spinal motor axons.

Key words: Chemorepulsion, Axon guidance, Slit, Lateral olfactory tract, Spinal motor axon, Rat

INTRODUCTION

The concept that axons may be guided by diffusible repellents arose from the demonstration that some regions of the developing nervous system create exclusion zones that actually cause axons to turn away from them. Thus, the septum, olfactory cortex and neocortex are all structures avoided by early olfactory tract axons in vivo and are all structures that secrete chemorepellents for these axons in vitro (Pini, 1993). Similarly, those primary sensory axons that are restricted to the dorsal horn of the spinal cord are also repelled during development by diffusible activity arising from the basal plate (Fitzgerald et al., 1993). Subsequently it emerged that factors secreted from the floor plate cause chemorepulsion of spinal motor (Guthrie and Pini, 1995), trochlear (Colomarino and Tessier-Lavigne, 1995) and mesencephalic axons of the alar and basal plates in vitro (Tamada et al., 1995). A genetic screen of *Drosophila* led to the identification of the *roundabout (robo)* mutant in which axons that do not normally cross the midline now do so with the result that many axons grow back and forth across the midline (Seeger et al., 1993). In contrast, the *slit* mutant is characterised in having both crossing and non-crossing axons, which enter the midline and remain there (Kidd et al., 1999). These observations led to the hypothesis that Robo/Slit interactions are required for midline crossing. Robo is a transmembrane glycoprotein (Kidd et al., 1998a) that functions as a receptor for a secreted protein, Slit (Brose et al., 1999; Kidd et al., 1999). A function for *commissureless* (*comm*) is also suggested in this process, at least in *Drosophila*, since *comm* gain-of-function mutants show defects in axon guidance that are similar to those found in *robo* mutants (Tear et al., 1996; Kidd et al., 1998b).

In vertebrates, three *Slit* genes (Itoh et al., 1998; W. Yuan et al., 1999) and three *Robo* genes (Brose et al., 1999; Li et al., 1999; Sundaresan et al., 1998; S. Yuan et al., 1999) have been identified. In vitro, Slit1 and Slit2 can cause chemorepulsion of olfactory tract axons (Ba-Charvet et al., 1999; Li et al., 1999; W. Yuan et al., 1999) while Slit2 causes chemorepulsion of spinal motor (Brose et al., 1999), hippocampal (Ba-Charvet et al., 1999) and retinal ganglion cell axons (Erskine et al., 2000; Niclou et al., 2000; Ringstedt et al., 2000). Additionally, Slit1 and Slit2 repel migrating neuronal precursors in vitro (Hu, 1999; Wu et al., 1999; Zhu et al., 1999) and Slit2 can promote the formation of axon collaterals (Wang et al., 1999).

Since the septum expresses *Slit1 and Slit2* (Ba-Charvet et al., 1999; Brose et al., 1999; Li et al., 1999; W. Yuan et al., 1999) which cause chemorepulsion of olfactory tract axons (Ba-Charvet et al., 1999; Li et al., 1999; W. Yuan et al., 1999) and the floor plate expresses *Slit2* (Brose et al., 1999; W. Yuan et al., 1999) which causes chemorepulsion of spinal motor neurons (Brose et al., 1999), it has been proposed that these *Slits* may be developmental guidance cues. However, there are

two observations that are at odds with the hypothesis that Slit1 and Slit2 mediate both axon guidance and neuronal migration. First, Hu and Rutishauser (Hu and Rutishauser, 1996) demonstrated that the activity secreted by the neonatal caudal septum for migrating olfactory interneurone-precursors must be different from the embryonic septal activity that causes chemorepulsion of olfactory tract axons because the caudal septum does not cause axonal chemorepulsion. Secondly, expression of *Slits* within the septum (Ba-Charvet et al., 1999; Li et al., 1999; Wu et al., 1999) far outlasts the limited period, E14.5-E17, during which the septum secretes chemorepulsive activity for olfactory tract axons (Pini, 1993; Hu and Rutishauser, 1996).

We have used a soluble receptor for Slit proteins in an attempt to block the activities secreted from the septum and floor plate that cause chemorepulsion of olfactory tract and spinal motor axons respectively. We report here that chemorepulsion of both olfactory tract and spinal motor axons by the septum and floor plate are unaffected by soluble Robo/Fc constructs and conclude that other signalling mechanisms are more likely to predominate during development.

MATERIALS AND METHODS

Animals

Tissue was obtained from Sprague Dawley rats (UCL, London); the day of vaginal plug was designated as E0.

Cloning of recombinant Slits and Robo1

The complete open reading frames for human *Slit1*, *Slit2* and *Slit3* were amplified by PCR, and subcloned into pcDNA3.1/his-myc vector to allow expression of C-terminus epitope-tagged proteins. Similarly, the extracellular domain of human *Robo1* and *Robo2* were amplified by PCR and subcloned into the pIGplus vector to allow expression of soluble Robo/Fc chimeras. The sequence and orientation of the constructs were confirmed by DNA sequencing.

Cells

A CHO cell line, expressing polyoma large T antigen (CHOP), was obtained from Dr J. Dennis (Ontario, Canada) and grown in Dulbecco's modification of Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 20 mg/ml L-proline, 100 U/ml penicillin and 100 μg/ml streptomycin. 9E10 hybridoma cells (obtained from the American Type Culture Collection, Rockville, MD), secreting an antibody to the c-myc epitope, were grown in RPMI-1640 supplemented with 2 mM L-glutamine, 100 μg/ml streptomycin, 100 U/ml penicillin and 10% fetal calf serum. Cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2.

Lipofection

CHOP cells were transfected with plasmid DNA using Lipofectamine Plus reagent (Life Technologies, Scotland) according to the manufacturer's instructions. To make Robo/Fc-conditioned medium, the lipofection medium was replaced after 3 hours with complete DMEM and then allowed to become conditioned for 5 days. Medium was harvested and clarified before use in explant cultures. To make aggregates, cells were re-suspended in 1.5% low-melting agarose at 3×10^7 cells/ml and 15 μl droplets of the cell suspension were allowed to set before being trimmed into blocks of around 400 μm^2 for use in explant co-cultures. For each experiment, expression of Slits and Robo/Fc proteins were confirmed by western blotting using monoclonal antibody 9E10 and anti-human immunoglobulin (Fc-specific) antibody respectively.

Explant cultures

The olfactory bulbs and septa from E15 embryos were dissected into pieces of around 200-400 μm^2 using fine tungsten needles. Olfactory bulb explants were co-cultured with either E15 septal explants or CHOP cell aggregates expressing Slit1, Slit2 or Slit3 in rat tail collagen gels (Lumsden and Davies, 1983). Cultures were incubated at 37°C in complete DMEM in a humidified atmosphere containing 5% CO_2. Embryonic spinal motor neurons were grown from E12 basal plate explants that were co-cultured with E12 floor plate explants as described previously (Guthrie and Pini, 1995). After 24-48 hours, the cultures were examined by phase contrast microscopy. For quantitative analysis, axons of the proximal and distal segments were counted to determine whether outgrowth was radial or asymmetrical. For qualitative analysis, scoring was always carried out blind by two independent observers. In order to test the effects of soluble Robo, either Robo1/Fc, Robo2/Fc or mock-conditioned medium was added to the co-cultures.

Immunoprecipitation

48 hours after lipofection, conditioned medium was harvested and clarified by centrifugation. Immunoprecipitation of Slits was carried out at 4°C for 1 hour by incubating 1 ml of conditioned medium with monoclonal antibody 9E10. The immune complexes were precipitated with Protein-G Sepharose (Pharmacia) and then separated by SDS-PAGE under reducing conditions and transferred to nitrocellulose membranes. The membranes were then probed with monoclonal antibody 9E10 and anti-mouse immunoglobulin-horseradish peroxidase (HRP) conjugate using diaminobenzidine (DAB)/nickel as substrate (Vector Laboratories).

Similarly, Robo1/Fc and Robo2/Fc were detected in the medium using Protein-G Sepharose for immunoprecipitation and following separation by SDS-PAGE and transfer, the blots were probed with an anti-human immunoglobulin (Ig)-biotin conjugate, streptavidin/HRP and DAB/nickel substrate.

RESULTS

Expression and secretion of recombinant Slits and Robo/Fc in CHOP cells

Slit proteins can be differentially processed in a cell-dependent manner. The full-length Slit2 (~190 kDa) is proteolytically processed into N-terminal and C-terminal fragments of 140 kDa and 55-60 kDa, respectively. The C-terminal fragment appears to be more diffusible whereas the N-terminal fragment and full-length Slit2 are predominantly membrane-associated. The collapsing activity for growth cones of olfactory tract axons resides in the N-terminal fragment and full-length Slit2 (Ba-Charvet et al., 1999). We first determined the expression and processing of Slits by CHOP cells that had been transiently transfected with the epitope-tagged *Slit* constructs. Conditioned medium was harvested from transfected cells and subjected to immunoprecipitation and western blotting using monoclonal antibody 9E10. The supernatants from cells transfected with *Slit1*, *Slit2* or *Slit3* constructs contained a ~200 kDa band (Fig. 1A) corresponding to the full length Slit protein, which was not detected following mock-transfection. In addition, Slit2 and Slit3 were proteolytically cleaved, since fragments at between 55-60 kDa corresponding to the C terminus were detected (Fig. 1A, lanes 3 and 4). In our studies we could not detect the N-terminal fragment because the constructs were tagged only at the C terminus and we were without antibodies to detect both N- and C-terminal fragments.

A similar approach was used to confirm the expression and

secretion of the Robo1/Fc chimera. After lipofection, Robo1/Fc from the conditioned medium was immunoprecipitated with Protein G-Sepharose and blotted with an anti-human immunoglobulin Fc-specific antibody. A band of 116 kDa corresponding to the extracellular domain of Robo1 fused to human immunoglobulin Fc portion was secreted from Robo1/Fc but not mock-transfected cells (Fig. 1B). A similar approach was employed to confirm the expression and secretion of Robo2/Fc (data not shown).

Soluble Robo/Fc abolishes chemorepulsion of olfactory tract axons by Slit2 but not by the septum

Slit2 has been shown to cause growth-cone collapse and chemorepulsion of olfactory bulb axons in vitro and has recently been proposed as a candidate for the septal activity that causes chemorepulsion of olfactory tract axons (Ba-Charvet et al., 1999; Li et al., 1999). We have tested this hypothesis as follows. First, we demonstrated that soluble Robo1/Fc was able to block chemorepulsion of olfactory tract axons by Slit2-expressing CHOP cell aggregates. In the absence of soluble Robo1/Fc, Slit2-expressing aggregates caused robust chemorepulsion of olfactory tract axons in 86% of cases (44 of 51 explants). The mean number of axons emerging from the proximal segment was 9.60±1.68 (mean ± standard error of the mean, s.e.m.) and from the distal half was 31.70±3.35 (Table 1; Table 2; Fig. 2A). However, in the presence of soluble Robo1/Fc, chemorepulsion of these axons occurred in only 13% of cases (6 out of 48 explants; $P<0.001$, χ^2 test). The remainder showed radial outgrowth (Fig. 2B) with 30.20±6.56 axons emerging from the proximal half and 33.80±2.62 axons emerging from the distal half of the explant (Table 2; $P<<0.01$, χ^2 test). Thus, our soluble Robo1/Fc chimera both binds Slit2 and neutralises its effect in co-culture.

We next carried out co-cultures with E15 septal and olfactory bulb explants in the presence and absence of the soluble Robo1/Fc chimera. As expected (Pini, 1993; Hu and Rutishauser, 1996), septal explants caused chemorepulsion of olfactory tract axons (Fig. 2D) in 86% of cases (42 out of 49 explants) with 8.69±1.14 and 35.35±1.5 axons emerging from the proximal and distal halves respectively (Table 1; Table 2). However, in the presence of soluble Robo1/Fc robust chemorepulsion still occurred in 80% of cases (37 of 46 explants; $P>0.2$, χ^2 test) with 9.88±1.68 and 34.50±1.09 axons emerging from the proximal and distal halves of the explant (Table 1; Table 2; Fig. 2E; $P>0.5$, χ^2 test).

Since *Robo2* is expressed in olfactory bulb (Ba-Charvet et al., 1999) and Slits bind Robo2 with higher affinity (Brose et al., 1999), we carried out co-culture experiments to determine whether chemorepulsion of olfactory tract axons by the septum could be inhibited by the presence of soluble Robo2/Fc. We found that Robo2/Fc could inhibit chemorepulsion of olfactory tract axons in co-culture with Slit2-expressing CHOP cells (Fig. 2C) resulting in 21.25±2.32 and 28.75±3.50 axons emerging from the proximal and distal halves of the explant (Table 2; $0<0.05$, χ^2 test). In contrast chemorepulsion of olfactory tract axons by the septum was unaffected by the presence of Robo2/Fc (Fig. 2F) since 6.20±1.69 and 32.60±1.42 axons emerged from the proximal and distal halves of the explant (Table 2; $P>0.5$, χ^2 test).

We ruled out the possibility that the addition of Robo/Fc could lead to an increase in axon outgrowth by culturing olfactory bulb explants in the absence and presence of Robo/Fc. We found that addition of either Robo1/Fc or Robo2/Fc did not affect axonal outgrowth from olfactory bulb explants (Fig. 2G-I).

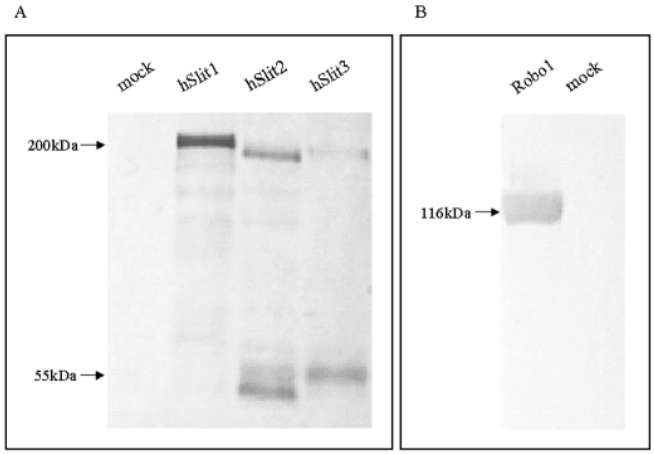

Fig. 1. Expression and processing of c-myc-tagged human Slit proteins and human Robo1/Fc chimera in CHOP cells. (A) Immunoprecipitation of c-myc-tagged human Slit1, Slit2 or Slit3 from conditioned medium was carried out with monoclonal antibody 9E10. The proteins were separated by SDS-PAGE and following transfer to nitrocellulose membranes, the blots were probed with monoclonal antibody 9E10. (B) Immunoprecipitation of human Robo1/Fc chimera from conditioned medium was carried out with Protein G-Sepharose followed by SDS-PAGE and western blotting with an antibody to human immunoglobulin. Molecular mass markers: myosin (220 kDa), phosphorylase b (97.4 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20 kDa), lysozyme (14.3 kDa).

Since we have demonstrated that the Robo1/Fc and Robo2/Fc constructs block chemorepulsion of olfactory tract axons mediated by Slit2 but not by the septum, it is unlikely that this protein is the major determinant of chemorepulsion mediated by the septum.

Slit1 and Slit3 cause chemorepulsion of olfactory tract axons which is abolished in the presence of soluble Robo/Fc

However, it is possible that Robo1/Fc and Robo2/Fc do not bind Slit1, which is known to cause chemorepulsion of olfactory tract axons (W. Yuan et al., 1999) (although data not shown) and whose mRNA is also expressed in the septum (Ba-Charvet et al., 1999). We tested this possibility by setting up

Table 1. Chemorepulsion of olfactory tract axons and spinal motor axons caused by septum and floor plate is independent of Slit/Robo interactions

Co-culture condition	Without Robo/Fc (%)	With Robo/Fc (%)
Slit1 vs olfactory bulb	90	8
Slit2 vs olfactory bulb	86	13
Slit3 vs olfactory bulb	83	13
Septum vs olfactory bulb	86	80
Floor plate vs basal plate	87	90

Co-culture experiments were carried out in the absence and presence of Robo1/Fc. Test explants (olfactory bulb or basal plate) showing chemorepulsion were counted and expressed as a percentage of the total number of explants.

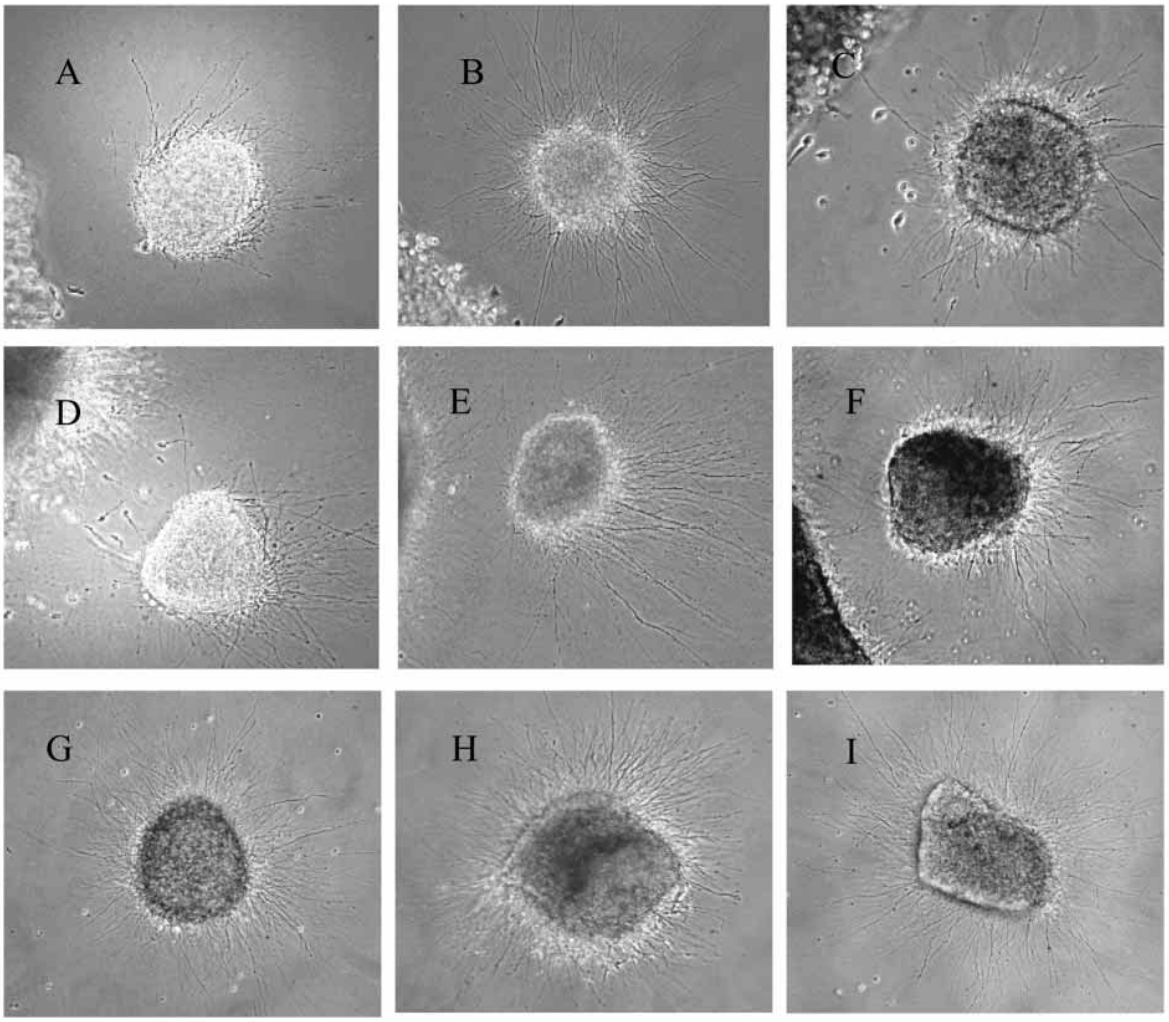

Fig. 2. Chemorepulsion of olfactory tract axons caused by Slit2 but not by the septum is inhibited in the presence of soluble Robo/Fc. E14.5-15 olfactory bulb explants were co-cultured with either aggregates of Slit2-expressing cells (A-C), E14. 5-15 septal explants (D-E) or alone (G-I) in collagen gels in the absence (A,D,G) or presence of Robo1/Fc conditioned medium (B,E,H) or Robo2/Fc conditioned medium (C,F,I). The cultures were incubated at 37°C for 24-48 hours and examined by phase contrast microscopy for axonal outgrowth of olfactory tract axons.

co-cultures of Slit1-expressing cell aggregates and olfactory bulb explants in the presence of soluble Robo1/Fc or Robo2/Fc. Slit1 caused chemorepulsion of olfactory tract axons in 90% of cases (22 out of 24 explants; Fig. 3A) with 10.0±1.73 and 28.00±3.98 axons emerging from the proximal and distal halves (Table 1; Table 2) whereas in the presence of Robo1/Fc, chemorepulsion occurred in only 8% of cases (1 out of 13 explants) leading to radial axon-outgrowth (Fig. 3B) with 23.75±2.00 and 30.25±2.98 axons emerging from the proximal and distal halves (Table 1; Table 2; $P<0.05$, χ^2 test). Similar studies showed that Slit1-mediated chemorepulsion of olfactory tract axons could be inhibited by Robo2/Fc (data not shown).

Although *Slit3* expression data in the septum have not been reported and it has not previously been tested for chemorepellent activity, we found that Slit3 did cause chemorepulsion of olfactory tract axons (Fig. 3C) in 83% of cases (15 out of 18 explants) with 12.20±2.47 and 28.80±1.99 axons in the proximal and distal halves (Table 1; Table 2). Furthermore, in the presence of soluble Robo1/Fc, chemorepulsion occurred in only 13% of cases (1 out of 8 explants); radial growth occurred in the rest of the cases (Fig. 3D) with 19.25±2.33 and 20.25±3.25 axons in the proximal and distal halves (Table 1; Table 2; $P<0.05$, χ^2 test). Similar studies showed that Slit3-mediated chemorepulsion of olfactory tract axons could be inhibited by soluble Robo2/Fc (data not shown). Thus, our results show that soluble Robo1/Fc and Robo2/Fc can bind Slit1, Slit2 and Slit3 and overcome their chemorepellent effects on olfactory tract axons yet they fail to abolish chemorepulsion of these axons mediated by the septum (Table 1; Table 2).

Soluble Robo1/Fc does not abolish chemorepulsion of embryonic spinal motor neurons mediated by the floor plate

The floor plate mediates chemorepulsion of spinal motor neurons in vitro (Guthrie and Pini, 1995) and at E12, all three *Slits* are expressed by the floor plate (Brose et al., 1999; W. Yuan et al., 1999). In vitro, Slit2 causes chemorepulsion of embryonic spinal motor neurons (Brose et al., 1999) raising the

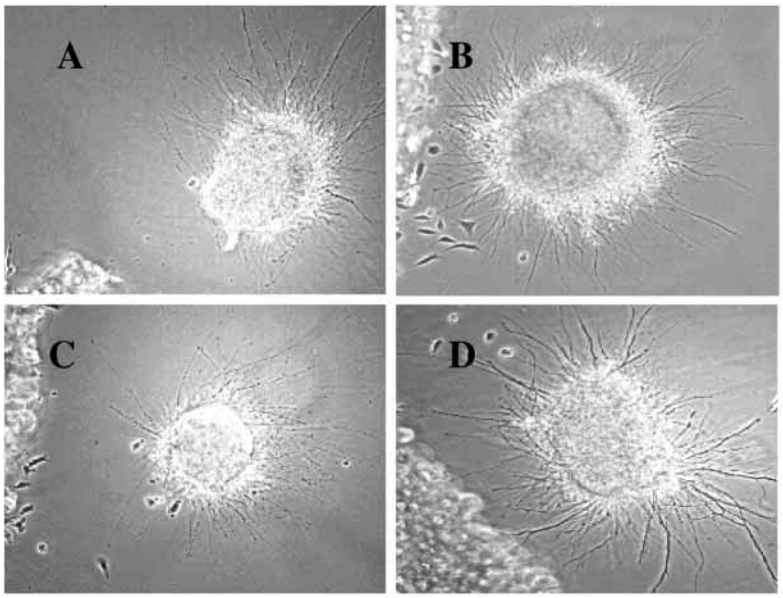

Fig. 3. Chemorepulsion of olfactory tract axons caused by Slit1 or Slit3 is inhibited in the presence of soluble Robo1/Fc. E14.5-15 olfactory bulb explants were co-cultured with aggregates of cells expressing either Slit1 (A,B) or Slit3 (C,D) in collagen gels in the absence (A,C) or presence (B,D) of Robo1/Fc conditioned medium. The cultures were incubated at 37°C for 24-48 hours and examined by phase contrast microscopy for chemorepulsion of olfactory tract axons.

possibility that Slit2 or another member of Slit family may contribute to the chemorepulsive activity of the floor plate. We tested this hypothesis by conducting co-culture experiments with E12 floor plate and basal plate explants in the presence and absence of soluble Robo1/Fc. Again, chemorepulsion of spinal motor axons by the floor plate was demonstrated in 87% of cases (13 out of 15 explants; Fig. 4A) which was not blocked by soluble Robo1/Fc in 90% of cases (9 out of 10 explants; Fig. 4B). Data are summarised in Table 1.

Thus, we conclude that although *Slits* are expressed in the septum (Ba-Charvet et al., 1999; Li et al., 1999) and floor plate (Brose et al., 1999; W. Yuan et al., 1999) and their proteins can cause chemorepulsion of olfactory tract (Ba-Charvet et al., 1999; Li et al., 1999) and spinal motor axons (Brose et al., 1999) in vitro, they are unlikely to be the effective guidance cues for these axons during development.

Table 2. Quantitative evaluation of axonal outgrowth from olfactory bulb explants cocultured under differing conditions

	Axonal outgrowth	
	Proximal	Distal
Septum vs OB		
Mock	8.69±1.14	35.35±1.5
Robo1/Fc	9.88±1.68	34.50±1.09
Robo2/Fc	6.20±1.69	32.60±1.42
hSlit1 vs OB		
Mock	10.0±1.73	28.0±3.98
Robo1/Fc	23.75±2.0	30.25±2.87
Robo2/Fc	n.d.	n.d.
hSlit2 vs OB		
Mock	9.60±1.68	31.70±3.35
Robo1/Fc	30.2±6.56	33.80±2.62
Robo2/Fc	21.25±2.32	28.75±3.50
hSlit3 vs OB		
Mock	12.20±2.47	28.80±1.99
Robo1/Fc	19.25±2.33	20.25±3.25
Robo2/Fc	n.d.	n.d.

Olfactory bulb (OB) explants were co-cultured with either septal explants or CHOP cells transfected with hSlits in the absence and presence of Robo/Fc conditioned medium. Axon numbers emerging from the proximal and distal halves of olfactory bulb explants were counted. Data are expressed as mean±standard error of mean (s.e.m.).

n.d.=not determined.

DISCUSSION

We had shown the existence of activities emanating from the septum and floor plate which cause chemorepulsion of olfactory tract (Pini, 1993) and spinal motor axons (Guthrie and Pini, 1995). Cells transfected with *Slit1* or *Slit2* cDNA expression vectors cause chemorepulsion of olfactory tract axons in vitro (Ba-Charvet et al., 1999; Li et al., 1999; W. Yuan et al., 1999). Since *Slit1* and *Slit2* mRNAs are expressed in the septum (Ba-Charvet et al., 1999; Li et al., 1999), we have

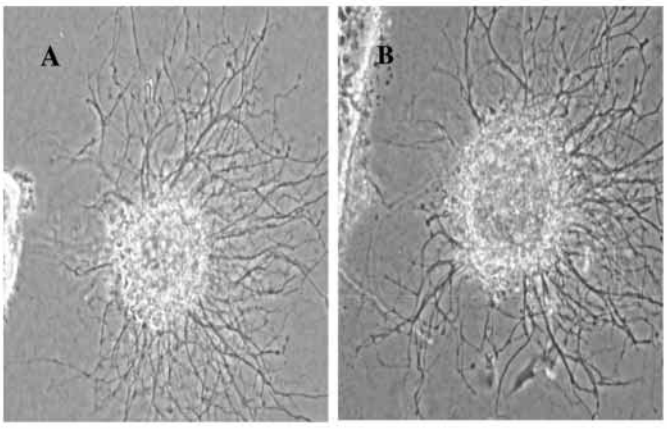

Fig. 4. Chemorepulsion of spinal motor axons caused by floor plate-derived chemorepulsive activity is not inhibited in the presence of soluble Robo1/Fc. E12 basal plate explants were co-cultured with E12 floor plate explants in collagen gels in the absence (A) or presence (B) of Robo1/Fc conditioned medium. The cultures were incubated at 37°C for 24-48 hours and examined by phase contrast microscopy for chemorepulsion of spinal motor axons growing out of basal plate explants.

extended these studies by determining whether endogenously expressed Slits from the septum cause chemorepulsion of olfactory tract axons in the presence of soluble Robo1/Fc or Robo2/Fc. We have found that soluble Robo1/Fc or Robo2/Fc have no effect on chemorepulsion of olfactory tract axons caused by the septum. It is unlikely that Robo1/Fc and Robo2/Fc do not bind all three Slits as our co-culture studies demonstrate that they readily inhibit Slit1, Slit2 or Slit3-mediated chemorepulsion of olfactory tract axons when these proteins are secreted from mammalian cells transfected with their respective cDNA clones.

This suggests that either endogenous Slits are not the key mediators or are minor components of the septal chemorepulsive activity. It is, however, possible that Slits may mediate effects on very early olfactory tract axons since *Slit2* is expressed when the first mitral and tufted cells are born at around E14 in the rat (Ba-Charvet et al., 1999; Bayer, 1983), but this remains to be determined. In any event, between E14.5 and E17, when the majority of olfactory tract axons are developing, chemorepulsion by the septum is not abolished in vitro by blocking the functioning of Slits. Hirata et al. (Hirata et al., 2001) have reported that RoboN (the hemagglutinin-tagged extracellular domain of Robo) did not affect formation of the lateral olfactory tract and that an apparently normal pathway developed in the absence of the septum in organotypic cultures. However, in the absence of axon counts it is difficult to know whether the tract that developed in the absence of the septum contained equivalent numbers of axons. Those axons deriving from the lateral aspects of the olfactory bulb might be expected to arrive in the tract in the absence of chemorepulsion mediated by the septum. Furthermore, these experiments do not address the possibility that lateral olfactory tract axons never innervate the septum because they are actively prevented from doing so by chemorepulsion.

The expression patterns of *robo* receptors on olfactory tract axons are as yet unresolved. In some studies, *robo1* but not *robo2* was found in the olfactory bulb whereas in other studies the converse appeared to be the case (Ba-Charvet et al., 1999; Li et al., 1999). We have, therefore, tested the effects of soluble Robo2/Fc on chemorepulsion of olfactory tract axons. Again, as with Robo1/Fc, we found that chemorepulsion of olfactory tract axons by the septum was unaffected by the presence of either Robo2/Fc or a combination of the two Robos (n=4; data not shown).

We conclude that while the Slits are very effective chemorepulsive molecules for olfactory tract and spinal motor axons in vitro (Ba-Charvet et al., 1999; Brose et al., 1999; Li et al., 1999; W. Yuan et al., 1999), they may not be responsible for the chemorepellent activities described elsewhere (Pini, 1993; Guthrie and Pini, 1995; Hu and Rutishauser, 1996). Other observations are consistent with our conclusions on the contribution of Slits in chemorepulsion of olfactory tract axons. The most relevant are those of Hu and Rutishauser (Hu and Rutishauser, 1996) who demonstrated the presence of a repellent migratory factor, for subventricular precursor cells, emanating from the caudal septum. In contrast to the chemorepulsive activity that is present during E14.5-E17 (Pini, 1993; Hu and Rutishauser, 1996), the septal-derived migratory factor is present throughout embryogenesis and in the postnatal period up to P7 (Hu and Rutishauser, 1996). Slit1 and Slit2 have recently been proposed as candidate septal-derived migratory factors (Hu, 1999; Wu et al., 1999) because of their ability to cause asymmetric migration of neuronal precursors from subventricular zone explants in vitro. Crucially, soluble Robo neutralises the effects of endogenous Slit in whole-mount telencephalon studies consistent with its function as a migratory factor for olfactory bulb interneurone precursors (Wu et al., 1999).

Further evidence that Slits may not be the major chemorepellents for olfactory tract axons comes from expression data. Both *Slit1* and *Slit2* are expressed at E15 and at E18 in the septum (Ba-Charvet et al., 1999), yet the septal-derived activity declines from E14.5 and is absent at E18 (Pini, 1993). Secondly, the expression of *Slit1* (Ba-Charvet et al., 1999; W. Yuan et al., 1999) in neocortex is inconsistent with our observation that E14.5 but not E18/P0 neocortex causes chemorepulsion of olfactory tract axons (Coutinho, 1999) although *Slit1* is expressed at both these developmental stages (W. Yuan et al., 1999). This suggests that although many classes of axons are susceptible to chemorepulsion by Slits in vitro, this is not necessarily indicative of a developmental function.

Our observations also demonstrate that chemorepulsion of spinal motor axons by the floor plate is not blocked by the presence of soluble Robo1/Fc and thus we also suggest that Slits may not be major determinants for axon guidance of spinal motor neurons. However, the contribution of Slits as migratory factors secreted from the septum and acting on olfactory bulb interneurone precursors is consistent with all of the available data.

We do not dogmatically exclude a contribution of Slits, but our results suggest that Slit/Robo interactions are not the dominant factor in chemorepulsion of olfactory tract and spinal motor axons. It is possible that having been bound by the soluble Robo/Fc, Slits could still bind to some, as yet, unidentified receptor but this remains speculation. In addition, redundancy between different Slits is an unlikely explanation since the Robo/Fc constructs we use actually bind all three Slits. Thus, one explanation for our results is that there is redundancy between Slits and other as yet unidentified guidance molecules. They are unlikely to be secreted semaphorins because these either do not cause chemorepulsion of olfactory tract axons in vitro or, if they do, they are not expressed in the septum (DeCastro et al., 1999; Hu and Rutishauser, 1996; Li et al., 1999). It is also unlikely that netrins are strong candidates since neither netrin1- nor netrin2-expressing cells cause chemorepulsion of olfactory tract axons (Hu and Rutishauser, 1996; Li et al., 1999) and olfactory tract development is normal in netrin1-deficient animals (Serafini et al., 1996). Our results provide data consistent with the existence of an unidentified guidance molecule whose activity is expected to be dominant over that of Slit/Robo interactions.

The generation of mice deficient in *Slit1* or *Slit2* should help to resolve the contribution of Slit in the guidance of olfactory tract and spinal motor axons.

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