Molecular analysis of axon repulsion by the notochord

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Accepted 29 November 2002

SUMMARY

During development of the amniote peripheral nervous system, the initial trajectory of primary sensory axons is determined largely by the action of axon repellents. We have shown previously that tissues flanking dorsal root ganglia, the notochord lying medially and the dermamyotomes lying laterally, are sources of secreted molecules that prevent axons from entering inappropriate territories. Although there is evidence suggesting that SEMA3A contributes to the repellent activity of the dermamyotome, the nature of the activity secreted by the notochord remains undetermined. We have employed an expression cloning strategy to search for axon repellents secreted by the notochord, and have identified SEMA3A as a candidate repellent. Moreover, using a spectrum of different axon populations to assay the notochord activity, together with neuropilin/Fc receptor reagents to block semaphorin activity in collagen gel assays, we show that SEMA3A probably contributes to notochord-mediated repulsion. Sympathetic axons that normally avoid the midline in vivo are also repelled, in part, by a semaphorin-based notochord activity. Although our results implicate semaphorin signalling in mediating repulsion by the notochord, repulsion of early dorsal root ganglion axons is only partially blocked when using neuropilin/Fc reagents. Moreover, retinal axons, which are insensitive to SEMA3A, are also repelled by the notochord. We conclude that multiple factors act in concert to guide axons in this system, and that further notochord repellents remain to be identified.

Key words: Axon guidance, Neural development, Chick embryo, Notochord, Spinal nerves, Dorsal root ganglia, Semaphorin

INTRODUCTION

During development, growing axons navigate towards their targets under the influence of multiple guidance cues present in the embryonic environment. These cues, which are diffusible or bound to plasma membrane and/or extracellular matrix, promote or prevent axon growth by attractive or repulsive mechanisms, respectively (Goodman, 1996; Mueller, 1999). The overall balance of such cues determines, therefore, the path that an axon follows. A simple anatomical system for assessing the role of different axonal guidance cues concerns the development of the vertebrate peripheral spinal nervous system (Tannahill et al., 2000). In this system, the initial trajectory of spinal axons in the periphery is largely imparted by repulsive cues that surround outgrowing axons in three dimensions. Insights into axon repulsive mechanisms have come from considering the influences that establish the initial trajectory of peripheral spinal nerves in different embryonic axes. For example, along the anteroposterior axis, peripheral spinal nerve segmentation is imparted by the neighbouring somites, which act to limit sensory dorsal root ganglia (DRG) and motor axon trajectories to the anterior half-sclerotome of each somite (Keynes and Stern, 1984; Rickmann et al., 1985). This segmental restriction is mediated by repulsive molecules expressed on posterior half-sclerotome cells that prevent growing axons from invading the posterior half-somite (Davies et al., 1990). Although Ephin-Bs and F-spondin have been implicated in peripheral spinal nerve segmentation (Koblar et al., 2000; Tzarfati-Majar et al., 2001; Wang and Anderson, 1997), an unidentified peanut agglutinin-binding molecule is also likely to play an important role (Davies et al., 1990; Vermeren et al., 2000).

The initial trajectory of DRG and motor axons within the somites is further restricted within the dorsoventral plane, because growing axons avoid the notochord lying medially to the DRG and the dermamyotome which lies laterally (Fig. 1A). Insights into the mechanisms that influence DRG axon trajectory in the periphery have come from experiments involving tissue explants cultured in three-dimensional collagen gels (Keynes et al., 1997; Nakamoto and Shiga, 1998). It was found that the notochord and dermamyotome, and to a lesser extent ventral neural tube, secrete repellents acting on DRG axons over considerable distances. It is probable, therefore, that tissues lying either side of the DRG channel axon trajectories by repulsion in vivo. It has also been found that the notochord has axon-repulsive activity at the appropriate developmental stages for early outgrowing DRG axons (Keynes et al., 1997; Nakamoto and Shiga, 1998).
Notochord removal experiments in ovo have lent further support for such a role of the notochord, together with its surrounding matrix, in axon repulsion (Stern et al., 1991; Tosney and Oakley, 1990). In contrast, surgical removal of dermamyotome has proved less informative as residual ectoderm, which is also a source of repulsive cues, regenerates in situ to maintain a source of repulsion (Keynes et al., 1997; Tosney, 1987).

Although the molecules mediating axon repulsion by the notochord and dermamyotome remain to be identified, dermamyotome-mediated repulsion may be explained, in part, by Sema3A (collapsin 3) and Sema3D (collapsin 2) (Luo et al., 1995). Sema3C, whose activity is thought to be mediated through heterodimers of neuropilin 1 and 2 (Chen et al., 1998; Raper, 2000; Renzi et al., 1999; Takahashi et al., 1998), has been reported to induce the collapse of sympathetic chain ganglia (SCG) but not DRG axons (Adams et al., 1997; Chen et al., 1998; Koppel et al., 1997; Takahashi et al., 1998). Moreover, Sema3D does not induce collapse of either DRG or sympathetic growth cones (Koppel et al., 1997; Raper, 2000). It seems unlikely, therefore, that Sema3C or Sema3D have a direct influence on DRG axon trajectory. In mice, expression of the neuropilin 1-dependent semaphorin, Sema3e, has been found in the perinotochordal mesenchyme (Miyazaki et al., 1999a; Miyazaki et al., 1999b). Although the biological activity of chick Sema3E protein has yet to be reported, mouse Sema3E protein appears to repel DRG axons (Miyazaki et al., 1999a; Miyazaki et al., 1999b). As described above, the phenotype of neuropilin 1 knockout mice, which has brought into question the role of neuropilin 1-dependent semaphorins, also raises questions concerning the role of other semaphorins, such as Sema3C, which are thought to act through neuropilin 1 together with neuropilin 2. The role of secreted semaphorins acting through neuropilin 2, such as Sema3F (Chen et al., 1998; Raper, 2000; Renzi et al., 1999), also remains unclear. Although defasciculation of peripheral spinal nerves has been reported in neuropilin 2 knockout mice, defects in initial axon trajectory consistent with neuropilin 2-dependent repulsion by the notochord and dermamyotome have not been described (Giger et al., 2000).

Recently, it has been shown that slit genes are expressed in the notochord, raising the possibility that they could contribute to repulsion of peripheral spinal axons. Consistent with this is the observation that slit2 expression precedes, and continues beyond, the initial outgrowth of DRG axons (Holmes and Niswander, 2001; Li et al., 1999). Expression of slit3 has also been reported in the notochord at similar stages (Holmes and Niswander, 2001). Slit2 protein has been shown to repel many different axon types, including olfactory bulb, hippocampal, spinal motor column and retinal axons (Brose et al., 1999; Erskine et al., 2000; Li et al., 1999; Nguyen Ba-Charvet et al., 1999; Niclou et al., 2000; Ringstedt et al., 2000; Yuan et al., 1999). DRG axons, however, are not repelled by Slit2 in collagen gel assays, and DRG growth cones do not collapse in response to Slit2 (Nguyen Ba-Charvet et al., 1999; Niclou et al., 2000). Further evidence against a role for Slit2 in motor axon repulsion has come from an examination of the repulsive properties of the floor plate, which expresses high levels of Slit2 (Holmes and Niswander, 2001; Holmes et al., 1998; Yuan et al., 1999). When soluble robo/Fc receptor fusion proteins are used to inhibit Slit2, motor axon repulsion by the floor plate is

**Fig. 1.** (A) Surround repulsion of peripheral DRG axons in the chick embryo trunk. Schematic diagram through the trunk in transverse section showing tissues that are sources of secreted repulsive signals (red arrows). These guidance cues operate in concert to constrain DRG axon paths in a typical bipolar trajectory (black arrows). (B) Expression of Sema3A in the chick embryo notochord. Following whole-mount in situ hybridisation on stage 19 embryos, transverse sections (30 µm) were cut and mounted. The section shown is through the brachial region and Sema3A expression is seen in the notochord and dermamyotome. Note that the neural tube split open during the hybridisation procedure. dm, dermamyotome; drg, dorsal root ganglion; fp, floor plate of neural tube; nt, neural tube; n, notochord; sc, sclerotome. Scale bar: ~100 µm.
unaffected (Patel et al., 2001). At first sight, this argues against a role for Slit2 in peripheral spinal axon guidance, but recent studies have suggested that a membrane-bound N-terminal Slit2 fragment is not a preferred substratum for DRG axon growth (Nguyen-Ba-Charvet et al., 2001). Thus, the role of Slit proteins in peripheral spinal axon guidance remains unclear.

In order to investigate the molecular nature of peripheral spinal axon repulsion by the notochord, we undertook a screen for axon repellents secreted by the notochord and identified SEMA3A as a candidate repellent. Furthermore, experiments in which notochord-derived SEMA3A repulsion is inhibited indicate that, although SEMA3A contributes to the notochord activity, other unidentified factors are also critical in determining spinal sensory axon trajectories in the periphery.

**MATERIALS AND METHODS**

**Tissue dissection**

Chick embryos were staged according to Hamburger and Hamilton (Hamburger and Hamilton, 1992). Stage 26-27 DRGs, stage 36 DRGs and stage 35-36 SCG were dissected between thoracic and upper lumbar regions. Olfactory bulb and retinal explants were dissected from stage 36 and stage 26-29 embryos, respectively. Notochords were dissected from stage 17-21 embryos.

**Collagen gel cultures**

Tissue explants were co-cultured in collagen gels essentially as described previously (Keynes et al., 1997; Ohta et al., 1999). Tissues were cultured in 15 mm four-well dishes (Nunc) and rat-tail type VII collagen was obtained from Sigma or Roche. Unless otherwise stated, DRG and SCG explants were positioned 250-500 µm from notochord or COS cell explants, whereas retinal and olfactory bulb explants were positioned 200-400 µm away. DRG, SCG and olfactory bulb cultures were incubated for 24-30 hours and retinal explants for 48-72 hours. All tissues were cultured in DMEM (Sigma) containing gentamycin (50 µg/ml, Sigma) with either 50 ng/ml NGF (Alomone) or 50 ng/ml NT3 (a gift from Regeneron Pharmaceuticals) for DRG and SCG cultures; ITS (Sigma) for olfactory bulb cultures, or 3% chick embryo extract (Ohta et al., 1999) for retinal explants. Neuruplin 1/Fc and neuruplin 2/Fc fusion proteins were obtained from R&D Systems. After incubation, cultures were fixed in 4% paraformaldehyde, 15% sucrose, phosphate-buffered saline pH 7.4.

**Evaluation of axon growth**

Axon outgrowth in the quadrant proximal to the notochord or COS cell co-explant was compared with the distal quadrant and scored as described previously (Keynes et al., 1997). A score of zero represents no outgrowth towards the co-explant; two, a weak and/or patchy outgrowth; four, more consistent outgrowth which does not extend closer to the co-explant than ~50 µm; six, growth approaches the co-explant closer than ~50 µm, but does not contact it; eight, axons contact the co-explant, but some asymmetry between proximal and distal remains; ten, no difference in outgrowth in the proximal versus distal quadrants, with axons growing into and/or past the co-explant. At least three independent experiments were performed for each assay. Photography was performed using an Axiovert microscope with dark-field and phase-contrast optics (Zeiss). Statistics were performed using a Kruskal-Wallis non-parametric test followed by a Bonferroni/Dunn post-hoc ANOVA test at the 1% significance level.

**COS cell transfection**

COS-7 cells were transfected with a pCAGGS (Niwa et al., 1991) vector containing SEMA3A, SEMA3C or vector control using LipofectAMINE (Invitrogen) as described previously (Ohta et al., 1999). Full-length chick SEMA3C was cloned from a chick notochord cDNA expression library based on published sequences (accession number AF022946). COS cell aggregates were made by the hanging drop method (Kennedy et al., 1994). SEMA3A-expressing COS cells were mixed with vector-transfected cells to moderate the amount of SEMA3A secreted by the aggregate. Aggregates were sub-divided before placing in collagen gels. To control for SEMA3A expression levels, the same COS cell aggregate was used in comparing the effects of neuruplin 1/Fc with controls.

**RESULTS**

**SEMA3A as a candidate for mediating axonal repulsion by the notochord**

In order to identify candidate axon repellents secreted by the notochord, we screened supernatants from pools of a notochord cDNA expression library expressed in COS cells for growth cone collapse-inducing activity. After screening ~1000 pools containing 500-1000 clones each, we identified one positive pool that was subsequently found to contain SEMA3A. Consistent with previous studies (Shepherd et al., 1996), SEMA3A expression in the notochord was confirmed by in situ hybridisation (Fig. 1B). Expression of other class 3 semaphorins has also been reported at low levels in the notochord (Luo et al., 1995). By PCR, we confirmed the presence of SEMA3C, SEMA3D and, to a lesser extent, SEMA3E in the notochord library (data not shown). Given the low levels of SEMA3E, and that SEMA3C and SEMA3D are not known to repel DRG axons (Adams et al., 1997; Chen et al., 1998; Koppel et al., 1997; Takahashi et al., 1998), SEMA3A represents the best candidate for a notochord-derived semaphorin mediating peripheral spinal axon guidance.

To explore the potential contribution of SEMA3A to notochord-mediated repulsion, we exploited the sensitivity of different DRG axon populations, selected by growth medium containing different neurotrophins (Farinas et al., 1998; Martin-Zanca et al., 1990; Phillips and Armanini, 1996), to repulsion by SEMA3A. Older DRG axons grown in NGF, but not NT3, are repelled by SEMA3A in vitro (Messersmith et al., 1995; Pond et al., 2002; Puschel et al., 1996; Shepherd et al., 1997). This is thought to limit the extension of NGF-, but not NT3-, responsive axons in vivo to the dorsal spinal cord, as they are repelled by SEMA3A expressed in the ventral spinal cord (Messersmith et al., 1995; Pond et al., 2002; Puschel et al., 1996; Shepherd et al., 1997). The differential SEMA3A sensitivity also correlates with the expression of neuruplin 1, which is required for SEMA3A-mediated repulsion. NGF-responsive axons maintain neuruplin 1 expression, whereas NT3-responsive axons lose neuruplin 1 expression around the time that they project into the spinal cord (Fu et al., 2000; Pond et al., 2002).
We therefore employed collagen gel co-cultures to assay repulsion by the notochord of different populations of stage 36 (day 10) DRG axons. Our results indicate that stage 36 DRG axons cultured in the presence of NGF, but not NT3, are repelled by stage 17-21 (day 3) notochords (Fig. 2A,B,F; mean score ± standard error of the mean is 1.2±0.1 versus 7.9±0.2). Although this is consistent with the action of SEMA3A, it does not exclude the action of other semaphorins acting through neuropilin 1, or of other unidentified repellents. In contrast to the differential responsiveness of stage 36 DRG axons to SEMA3A when cultured in NGF versus NT3, it has been noted that earlier DRG axons are responsive to SEMA3A when cultured in either neurotrophin (Pond et al., 2002; Puschel et al., 1996; Shepherd et al., 1997). We therefore examined repulsion by the notochord of earlier DRG axons, grown from stages closer to the times of initial axon outgrowth in vivo.

Stage 26-27 (day 5) DRGs were used, being the earliest stages at which DRGs can be isolated and cultured reproducibly (Masuda et al., 2000). Similar to the response of stage 29-31 DRG axons to SEMA3A (Pond et al., 2002; Shepherd et al., 1997), earlier DRG axons (stage 26-27) cultured in either neurotrophin are strongly repelled by the notochord, consistent with a role for SEMA3A (Fig. 2C,D,F; mean scores are 0.8±0.2 and 1.6±0.2 for NGF and NT3 cultures, respectively).

Similar to peripheral DRG axons, axons from SCGs, lying ventral to the DRGs, avoid the notochord and do not cross into the contralateral side. SCG axons are known to be sensitive to repulsion by several semaphorins, including SEMA3A, SEMA3C and SEMA3F (Adams et al., 1997; Chen et al., 1998; Koppel et al., 1997; Takahashi et al., 1998). We therefore tested whether SCG axons are sensitive to notochord repulsion. Stage 36 SCG axons were strongly repelled by the notochord (mean score 0.3±0.1), and were significantly more sensitive to notochord repulsion than similar stage DRG axons cultured in NGF (Fig. 2E,F; P<0.001). To examine whether the differential sensitivity of SCG and DRG axons to notochord repulsion could be accounted for by differences in SEMA3A responsiveness, we compared the repulsion of SCG and DRG axons to SEMA3A-expressing cells within the same collagen gel. Qualitatively, SCG and DRG axons of both stages showed a similar extent of repulsion to SEMA3A secreted from COS cell aggregates (Fig. 3). The finding that SCG axons are more repelled by the notochord than stage 36 DRG axons (Fig. 2F) is consistent with the secretion of a repellent additional to SEMA3A (see below). Taken together, these results implicate a neuropilin 1-binding semaphorin, probably SEMA3A, as a candidate molecule that contributes to notochord-derived axon repulsion.
Inhibition of notochord repulsion by soluble neuropilins

To test whether neuropilin 1-binding semaphorins, including SEMA3A, are secreted by the notochord, we employed soluble neuropilin receptor proteins, comprising the extracellular domains of rat neuropilin 1 or neuropilin 2 fused to the human IgG complement-binding fragment (Fc), to titrate out semaphorin activity in collagen gel assays. To control for the effectiveness of the neuropilin 1/Fc receptor reagent, we first used neuropilin 1/Fc to inhibit SEMA3A repulsion derived from COS cells expressing SEMA3A. In these experiments we used SEMA3A-expressing cell aggregates that matched the extent of repulsion obtained with notochord explants. Using vector-transfected cells, with or without neuropilin 1/Fc, no significant change in the growth of stage 36 DRG axons was observed (Fig. 4A,E). However, in co-cultures of stage 36 DRGs and SEMA3A-expressing cells, axon repulsion was significantly reduced in the presence of the neuropilin 1/Fc ($P<0.0001$), and many more axons approached and occasionally contacted the cell aggregate (from a mean score of 1.4±0.2 to 5.0±0.8; compare Fig. 4B,C,E). As a control, the addition of 10 μg/ml neuropilin 2/Fc did not significantly reduce repulsion (Fig. 4D,E), consistent with the finding that SEMA3A binds neuropilin 2 only with low affinity (Chen et al., 1997).

When stage 36 DRGs were cultured in NGF with stage 17-21 notochords in the presence of neuropilin 1/Fc, repulsion of DRG axons was significantly reduced in a dose-dependent manner ($P<0.0001$). In the presence of neuropilin 1/Fc, the mean score increased from 1.6±0.2 in control co-cultures to 3.2±0.2 and 5.7±0.4 for 3 μg/ml and 10 μg/ml neuropilin 1/Fc, respectively (Fig. 4F,G). In these experiments, the reduction of repulsion was equivalent to that seen using neuropilin 1/Fc to block SEMA3-mediated repulsion (see above). The observation that neuropilin 2/Fc does not cause a similar reduction in repulsion (Fig. 4H-J; mean score 1.6±0.2 and 2.1±0.2 for 3 μg/ml and 10 μg/ml neuropilin 2/Fc, respectively) indicates that the Fc domain is not responsible for reducing notochord-induced repulsion, and also suggests that neuropilin 2-binding semaphorins are unlikely to be involved. Taken together, these results are in keeping with the proposition that the repulsive activity secreted by the notochord has a major component based on SEMA3A.

We next tested whether the neuropilin/Fc receptor reagents could reduce the repulsion by the notochord of earlier (stage 26-27) DRG axons as well as SCG axons. Overall, in these experiments a reduction in repulsion was evident, but this was significantly less than for stage 36 DRG axons ($P<0.0001$) and was more variable (Fig. 5). At 10 μg/ml neuropilin 1, the mean score for DRG and SCG axon repulsion increased from 0.6±0.2 to 2.3±0.3, and from 0.2±0.1 to 2.2±0.2, respectively (Fig. 5A,D,G). At 10 μg/ml neuropilin 2, an apparent reduction in repulsion was observed for DRG and SCG axons, but this was not statistically significant at the 1% level (Fig. 5B,E,G; the mean score increased from 0.6±0.2 to 1.0±0.3, and from 0.2±0.1 to 1.2±0.4, respectively). When combinations of neuropilin 1/Fc and neuropilin 2/Fc were used, the reduction in repulsion was not significantly different from that for neuropilin 1/Fc alone (Fig. 5C,F,G; at 5 μg/ml of each neuropilin/Fc the mean score increased from 0.6±0.2 to 2.6±0.3, and from 0.2±0.1 to 2.2±0.2, respectively (Fig. 5A,D,G). At 10 μg/ml neuropilin 2, an apparent reduction in repulsion was observed for DRG and SCG axons, but this was not statistically significant at the 1% level (Fig. 5B,E,G; the mean score increased from 0.6±0.2 to 1.0±0.3, and from 0.2±0.1 to 1.2±0.4, respectively). When combinations of neuropilin 1/Fc and neuropilin 2/Fc were used, the reduction in repulsion was not significantly different from that for neuropilin 1/Fc alone (Fig. 5C,F,G; at 5 μg/ml of each neuropilin/Fc the mean score increased from 0.6±0.2 to
2.4±0.5, and from 0.2±0.1 to 2.0±0.3, for DRG and SCG axons, respectively; at 10 μg/ml of each neuropilin/Fc the respective values were 2.7±0.5 and 1.7±0.3).

Because neuropilin 1/Fc receptor reagents were significantly less effective in inhibiting notochord repulsion of early DRG and SCG axons compared with later DRG axons, even though they appear equally sensitive to SEMA3A (see above), it is probable that multiple repellents are secreted by the notochord. Although much of the repulsive activity on early DRG axons appears not to be mediated by neuropilin-binding proteins, our results support a role for SEMA3A nonetheless. Neuripilin 1-binding molecules are secreted by the notochord, as there is a significant reduction in repulsion of DRG and SCG axons with neuropilin 1/Fc. A role for neuropilin 2 is not revealed by these experiments, but the expression of neuropilin 2 in mouse (Chen et al., 1997) and chick DRGs (Fig. 5) could indicate a potential contribution of neuropilin 2.

Expression of SEMA3C and neuropilin 2 in the chick embryo trunk

SCG neurons express both neuropilin 1 and neuropilin 2, and respond to semaphorins that bind either receptor (Chen et al., 1998; Giger et al., 1998). The finding that SCG axons are more repelled by the notochord than stage 36 DRG axons raises the possibility that SCG axons also respond to a neuropilin 2-binding semaphorin, such as SEMA3C. We therefore confirmed the expression of SEMA3C in the notochord by wholemount in situ hybridisation (Fig. 6A,B), in keeping with previous data (Luo et al., 1995). SEMA3C expression was also noted in the trunk at sites similar to SEMA3A, such as the posterior half-sclerotome of more anterior somites (Fig. 6B), consistent with expression of the mouse homologue (Puschel et al., 1995).

Although it has been reported that DRG axons are insensitive to SEMA3C (Adams et al., 1997; Chen et al., 1998; Koppel et al., 1997; Takahashi et al., 1998), very early DRG axons have not been tested. If these axons are SEMA3C-sensitive, they should express neuropilin 2. We examined the expression of neuropilin 2 in the trunk and found strong expression in the anterior but not posterior half-sclerotome of the earliest forming somites, with expression being maintained in more anterior somites in regions where neural crest cells coalesce to form DRGs (Fig. 6C-E). By stage 26, neuropilin 2 expression had decreased in the DRGs, but was detectable in both DRGs and their axons (Fig. 6F). The spinal motor columns of the ventral neural tube were also seen to express neuropilin 2 (Fig. 6D). This expression pattern is reminiscent of mouse neuropilin 2 (Chen et al., 1997).

These results are consistent with a possible role for a neuropilin 2-binding semaphorin in notochord-mediated repulsion, but the inability of neuropilin 2/Fc to prevent repulsion (Fig. 5G) argues against this. As a further test we examined whether DRG axons taken from early stages are able to respond to a source of SEMA3C (Fig. 7). As a control, we found that SCG axons were strongly repelled by cells expressing SEMA3C (Fig. 7B,E; mean score 1.7±0.3); however, DRG axons taken from stage 26-27 were not repelled (Fig. 7D,E; mean score 8.8±0.2). Therefore, although the notochord may secrete SEMA3C, it is probable that SEMA3C does not account for the repulsion of these DRG axons, as they do not respond to the repulsion of these SEMA3C and express neuropilin 2 only weakly.

The notochord repels retinal axons

Several other axon populations have well-characterised responses to known axon repellents. These can therefore be employed in assays of notochord repulsion to explore the nature of the repellents secreted by the notochord. For example, retinal axons are known to be insensitive to SEMA3A (Luo et al., 1993; Vermeren et al., 2000). When stage 26-29 retinal explants were cultured together with notochord, retinal axons were repelled (Fig. 8; mean score 2.4±0.3). Furthermore, when retinal and notochord explants were cultured together in the presence of soluble neuropilins, there was no significant reduction in repulsion (Fig. 8; mean scores 2.1±0.5 and 0.9±0.3 in the presence of 10 μg/ml neuropilin 1 or neuropilin 2,
Expression of SEMA3C and neuropilin 2 in the developing chick embryo trunk. (A,C) Lateral views of whole-mount in situ hybridisation for SEMA3C at stage 18 (A) and for neuropilin 2 at stage 20 (C). Somites anterior to the wing bud are shown (anterior is up, dorsal is right). SEMA3C expression in (A) is seen in the notochord (white arrows) and posterior halves of consecutive sclerotomes (white arrowheads). Neuropilin 2 expression in (C) is seen in the anterior half-sclerotomes (white arrowheads); black arrowheads indicate somite boundaries. Scale bars: ~100 μm. (B,D) Transverse sections (30 μm) through the trunk of stage 19 embryos after whole-mount in situ hybridisation. (B) SEMA3C expression at the level of a posterior half-sclerotome. Expression is seen in the notochord, posterior half-sclerotome and ventral half of the neural tube. (D) Neuropilin 2 expression at the level of the anterior half-sclerotome. Expression is seen in the anterior half sclerotome in the region of DRG formation, and in the ventral neural tube. Scale bar: ~100 μm. (E) Lateral view of whole-mount in situ hybridisation for neuropilin 2 at stage 24. Expression is seen in the anterior half-sclerotomes of recently formed somites (arrow) and in the DRGs forming more anteriorly (arrowheads). Scale bar: ~1 mm. (F) Whole-mount in situ hybridisation for neuropilin 2 on a stage 26 embryo trunk. After in situ hybridisation, the trunk was hemisected and viewed from the neural tube side to help visualise expression. Weak neuropilin 2 expression is seen in the DRGs (arrows) and axons leaving the DRGs (arrowheads). Scale bar: ~100 μm. as, anterior half-sclerotome; dm, dorsal mesenchyme; drg, dorsal root ganglion; nt, notochord; ps, posterior half-sclerotome.

DISCUSSION

SEMA3A as a notochord-derived axon repellent

Using an expression cloning strategy to isolate axon repellents secreted by the notochord, we have identified SEMA3A as a likely candidate. First, it is expressed by the notochord at the appropriate stages. Second, DRG and motor axon populations express the SEMA3A receptor component, neuropilin 1 (Chen et al., 1997), and are repellled by SEMA3A (Luo et al., 1993; Messersmith et al., 1995; Varela-Echavarria et al., 1997; Vermeren et al., 2000) and by the notochord (Keynes et al., 1997; Nakamoto and Shiga, 1998). Our results using stage 36 DRG axons are also in keeping with the maintenance of neuropilin 1 expression in NGF-selected axon populations (Fu et al., 2000; Pond et al., 2002). Finally, neuropilin 1/Fc but not neuropilin 2/Fc treatment reduces repulsion by the notochord. This reduction was statistically significant, and the addition of higher concentrations of neuropilin 1/Fc did not reduce repulsion further (data not shown). Using stage 36 DRGs, the repulsion score with SEMA3A (or notochord) was reduced to levels equivalent to that of a weak repulsive source.

Several factors may explain why blocking repulsion from SEMA3A-expressing cells did not reduce the score to that of control cells. There was individual variability in the scores; some cultures were completely disinhibited whereas others were only partially so. This may reflect the difficulty in standardising the amount of SEMA3A being secreted by the cell aggregates. Also, the semi-quantitative scoring system that we use is particularly sensitive to low levels of repulsion and scores of five and above (which represents half the numerical scale) are in the realms of weak to no repulsion. Finally, as the neuropilin receptor reagents are from rat sequences, they may have less efficacy. Despite these concerns, we conclude that neuropilin 1/Fc can be used as an effective tool in collagen gel assays to detect the presence of repulsive molecules such as SEMA3A.

Taken together, our results strongly suggest that SEMA3A is secreted by the notochord and repels DRG axons. However, although it is probable that a major repellent for stage 36 axons respectively), again suggesting that the notochord secretes axon repellents that act independently of neuropilins.

$slit2$ and $slit3$ are expressed by the notochord in vivo (Holmes and Niswander, 2001; Li et al., 1999), and Slit2 can act as a repellent for retinal axons (Erskine et al., 2000; Niclou et al., 2000; Ringstedt et al., 2000), raising the possibility that Slit2 is secreted by the notochord to repel retinal axons. We examined this possibility by exploiting the observation that olfactory bulb axons are repelled by Slit2 (Li et al., 1999; Nguyen Ba-Charvet et al., 1999; Yuan et al., 1999). When stage 36 olfactory bulb explants were cultured together with notochord, we found that axons were not repelled (Fig. 8; mean score 7.4±0.4), suggesting that Slit2 does not mediate notochord repulsion of retinal axons. As olfactory bulb axons can be repelled by SEMA3F and not SEMA3A (de Castro et al., 1999), this further suggests that neuropilin 2-binding semaphorins do not contribute to the repulsive activity of the notochord. Overall, our results strongly suggest that unidentified axon repellents, additional to semaphorins, mediate peripheral spinal axon repulsion by the notochord.
is SEMA3A, our results indicate that other repellents contribute to the activity of the notochord on stage 26-27 DRG and stage 36 SCG axons. In particular, we noted that neuropilin 1/Fc treatment reduces notochord repulsion of stage 26-27 DRG axons to a much lesser extent than that of stage 36 DRG axons.

Other semaphorins as notochord-derived axon repellents

Although our results are consistent with SEMA3A being secreted by the notochord, they do not exclude the possibility that other semaphorins act in concert, because in our inhibition experiments neuropilin 1/Fc could bind to semaphorins other than SEMA3A. Several other semaphorins, including SEMA3C and SEMA3D have been shown to be expressed by the notochord in chick embryos (Luo et al., 1995; Shepherd et al., 1996), and it remains to be determined whether any further semaphorins are also expressed here. It is unlikely that SEMA3D directly repels DRG axons because it does not induce collapse of their growth cones (Koppel et al., 1997). SEMA3E is also expressed at low levels in the perinotochordal region, and it may contribute to repulsion by the notochord as it has the appropriate repulsive activity for DRG axons (Miyazaki et al., 1999a; Miyazaki et al., 1999b).

Our results raise the possibility of a role for SEMA3C, because it is expressed at moderate levels by the notochord. SEMA3C is thought to operate through a combination of neuropilin 1 and 2 (Chen et al., 1998). Although we find that DRGs express neuropilin 2, our inhibition experiments suggest that signalling through this receptor is not functionally significant. This is also supported by our direct tests of early DRG axon repulsion by SEMA3C, in which we find no evidence to support a role for this semaphorin. As we find higher expression of neuropilin 2 in stage 17-20 DRGs, it remains possible that SEMA3C plays a role in the earliest stages of axon outgrowth.

SEMA3C, in addition to SEMA3A, may also contribute to repulsion of SCG axons by the notochord, as it has the appropriate activity on SCG axons and is expressed in the notochord. We also found a diminution of repulsion when notochord-SCG co-cultures were treated with neuropilin 2/Fc.
(repulsion score increased from 0.2±0.1 to 1.2±0.4), although this was not statistically significant at the 1% level (P<0.012). This may reflect the difficulty in scoring small differences in repulsion in cultures showing strong repulsion.

It has also been reported that SEMA3F, a neuropilin 2-binding semaphorin, is expressed in the perinotochordal region (Eckhardt and Meyerhans, 1998). However, olfactory bulb axons are not repelled by the notochord but are repelled by SEMA3F (de Castro et al., 1999), further suggesting that neuropilin 2 does not mediate notochord repulsion. Again, this is consistent with the absence of defects in the early guidance of peripheral spinal nerves in mice lacking neuropilin 2 (Giger et al., 2000). The role of SEMA3F in vivo remains somewhat unclear as it has been recently reported that mouse DRG axons are repelled by Sema3F in vitro (Dionne et al., 2002). It is also of particular note that both SEMA3A and SEMA3C are expressed in posterior half-somites (Eickholt et al., 1999; Shepherd et al., 1996), a well-characterised repulsive barrier to peripheral spinal nerves (Tannahill et al., 2000). As it is thought that peripheral nerve segmentation is based on contact repellents, rather than secreted repellents, any contribution of semaphorins to this repulsive mechanism will probably be short- rather than long-range.

The role of other molecules in axon repulsion by the notochord

The apparently normal pathfinding of peripheral spinal axons with respect to the notochord in mice lacking Sema3a, neuropilin 1 or neuropilin 2 would also suggest that factors other than semaphorins mediate axon repulsion by the notochord. Consistent with this, our results show that neuropilin 1/Fc is unable to inhibit a large proportion of the repulsion of stage 26-27 DRG axons by the notochord. In these experiments, it is probable that the neuropilin 1/Fc reagent is not saturated as it can almost completely inhibit repulsion by the notochord of stage 36 DRG axons. Furthermore, retinal axons, which do not express neuropilins (Takagi et al., 1995; Takahashi et al., 1998), are still repelled by the notochord. This latter result might be explained by secretion of molecules from the notochord that do not normally function as DRG axon repellents. For example, although we have previously shown that sonic hedgehog, which is strongly expressed by the notochord, is unable to repel DRG axons in collagen gel experiments (Keynes et al., 1997), in some situations it can interfere with the growth of retinal axons (Trouche et al., 2001). Similarly, Slit proteins can repel retinal but not DRG axons (Erskine et al., 2000; Niclou et al., 2000; Ringstedt et al., 2000), and they are also expressed by the notochord (Holmes and Niswander, 2001; Li et al., 1999). Our finding that retinal but not olfactory bulb axons are repelled by the notochord suggests, however, that notochord repulsion is not mediated by Slit2.

Clearly, the simplicity of axon repulsion by the notochord at the anatomical level is underlain by a considerable complexity at the molecular level. Although our results implicate SEMA3A, the precise identity and relative contributions of the relevant molecules remain to be elucidated. If many different molecules are involved, the use of multiple knockouts may be required to assess the individual contribution of each molecule in the absence of others. In addition to long-range axon repellents described in this paper, a full characterisation of midline repulsion by the notochord will also need to consider molecules that act at short-range, in the immediate vicinity of the notochord. For example, the perinotochordal matrix is rich in extracellular matrix components, such as chondroitin sulphate proteoglycans (Landolt et al., 1995; Newgreen et al., 1986; Pettway et al., 1990; Ring et al., 1996), which are known to inhibit axon growth in other systems (Anderson et al., 1998; Becker and Becker, 2002; Chung et al., 2000; Dou and Levine, 1994; Fichard et al., 1991; Silver, 1994).

We thank the Wellcome Trust and MRC for support. D. T. is a Royal Society University Research Fellow.

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