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Zebrafish Semaphorin Z1a collapses specific growth cones and alters their pathway in vivo

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SUMMARY

The *semaphorin/collapsin* gene family encodes secreted and transmembrane proteins several of which can repulse growth cones. Although the in vitro activity of Semaphorin III/D/Collapsin 1 is clear, recent analyses of two different strains of *semaphorin III/D/collapsin 1* knockout mice have generated conflicting findings. In order to clarify the in vivo action of this molecule, we analyzed *sema Z1a*, a zebrafish homolog of *semaphorin III/D/collapsin 1*. The expression pattern of *sema Z1a* suggested that it delimited the pathway of the growth cones of a specific set of sensory neurons, the posterior ganglion of the lateral line, in zebrafish. To examine the in vivo action of this molecule, we analyzed (1) the pathways followed by lateral line growth cones in mutants in which the expression of *sema Z1a* is altered in an interesting way, (2) response of lateral line growth cones to exogenous Sema Z1a in living embryos, and (3) the pathway followed by lateral line growth cones when Sema Z1a is misexpressed by cells along their normal route. The results suggest that a repulsive action of Sema Z1a helps guide the growth cones of the lateral line along their normal pathway.

Key words: Semaphorin, Zebrafish, Growth cone guidance, Lateral line, Cell signalling

INTRODUCTION

During development, neuronal growth cones extend along specific pathways to their targets by utilizing both repulsive and attractive cues (Tessier-Lavigne and Goodman, 1996). The repulsive activity, upon growth cones, of a variety of factors has been extensively analyzed with in vitro assays (Walter et al., 1987; Raper and Kapfhammer, 1990; Fitzgerald et al., 1993; Drescher et al., 1995; Fan and Raper, 1995). The *semaphorin/collapsin* family of molecules has recently been of interest in this regard, because several of the members are thought to be repulsive for specific growth cones (Kolodkin, 1996). These proteins are either secreted or transmembrane, may have an Ig domain or thrombospondin type 1 domains, and all share a large, conserved sema domain. The first member of this family that was identified to be repulsive for growth cones was chick Collapsin 1, a secreted protein that causes the collapse of specific subsets of growth cones in vitro including those of the dorsal root ganglion (DRG) cells (Luo et al., 1993). Explant experiments showed that Sema III/D, which is the mammalian homolog to chick Collapsin 1, selectively repelled the axons of the cutaneous DRG cells but not the muscle afferent DRG cells (Messersmith et al., 1995; Puschel et al., 1995). This made sense since Sema III/D is normally expressed by the ventral spinal cord, and cutaneous but not muscle afferent DRG axons are restricted to the dorsal cord. This suggested that Sema III/D secreted by the ventral spinal cord cells normally acts to restrict the cutaneous DRG axons to the dorsal cord. Recent generation and examination of *sema III/D* knockout mice suggest that the requirement of *sema III/D* for this restriction depends upon the genetic background of the mice. In one strain some cutaneous DRG axons appear to extend aberrantly into the ventral cord (Behar et al., 1996), while in another strain cutaneous DRG axons remained within the dorsal cord (Taniguchi et al., 1997). In the latter strain, a subset of peripheral nerves were abnormally defasciculated but apparently extended to their correct target regions. The reason for the difference in phenotype between the two different knockout mice is presently unclear.

To better understand the in vivo role of *sema III/D/collapsin 1*, we focused on the role of a zebrafish Semaphorin, Sema Z1a, that is homologous to Semaphorin III/D/Collapsin 1, for pathfinding by a specific set of sensory growth cones, those of the posterior lateral line ganglion. Zebrafish embryos are useful for examining the behavior of growth cones because the embryos are transparent and features are accessible at all stages of development (Kimmel et al., 1995). Furthermore, the zebrafish nervous system is relatively simple and contains numerous, well characterized neurons, including the neurons of the posterior lateral line (Metcalfe, 1985; Metcalfe et al., 1985; Collazo et al., 1994; O'Malley et al., 1996).

The lateral line is a sensory structure that detects water-born vibrations, found in fishes and amphibians. It consists of 10-11 sensory organs that are located periodically along the anterior/posterior axis midway between the dorsal and ventral margins of the trunk and tail in larval zebrafish. The posterior

lateral line ganglion contains the 15-20 sensory neurons that innervate the sensory organs. This is located posterior to the otocyst and anterior to the axial muscles. The sensory organs develop from cells derived from a mass of cells called the migrating primordium of the lateral line (Metcalfe, 1985; Metcalfe et al., 1985). The primordium migrates posteriorly, starting from the posterior lateral line ganglion at approximately 20 hours and reaching the posterior end of the tail by 42 hours. As the primordium migrates it periodically deposits small clusters of cells that differentiate into the sensory organs. Interestingly the growth cones of the neurons of the posterior lateral line ganglion that innervate the sensory organs extend together with the migrating primordium. The primordium and growth cones migrate posteriorly to the first myotome. Once they reach the axial muscles they migrate between the skin and the axial muscles of the horizontal myoseptal region.

Our analysis demonstrates that Sema Z1a is expressed by the axial muscles in a pattern that suggests it may guide the growth cones of the posterior lateral line by delimiting the pathway these growth cones can follow on the muscles. We further show that application of Sema Z1a can induce collapse of lateral line growth cones in vivo, and that the growth cones will deviate from their normal pathway when confronted with cells that are misexpressing Sema Z1a. These results strongly suggest that Sema Z1a normally guides posterior lateral line growth cones via a repulsive mechanism.

MATERIALS AND METHODS

Zebrafish embryos

Zebrafish (*Danio rerio*) embryos were collected and allowed to develop at 28.5°C, and staged as described by Westerfield (1995). In some cases pigmentation was inhibited by exposure to 0.2 mM phenylthiourea (Sigma) at about 20 hpf (hours postfertilization; Burrill and Easter SS, 1994). *floating head* (*flh*) carrier fish were obtained from M. Halpern. *you-too (yot)* embryos were supplied by P. Haffter.

In situ hybridization and cryostat sectioning

Antisense *sema Z1a* digoxigenin (DIG)-labeled riboprobes were synthesized and then adjusted to approximately 300 bps fragment length by limited alkaline hydrolysis (Cox et al., 1984). Hybridization on wholemounted embryos was performed as described by Westerfield (1995). For sectioning, fixed embryos were immersed in 30% sucrose, embedded in OCT compound (Miles, Elkhart, IN), and cut using a Reichert-Jung 2800 cryostat. Sections were air dried and mounted in 70% glycerol under a coverslip.

Immunohistochemistry

To label cells expressing Sema Z1a^{myc} and growth cones expressing the HNK1 antigen differentially, embryos were first stained using anti-myc then anti-HNK1 antibodies. Briefly, embryos were fixed in 4% paraformaldehyde in PBS, incubated with a 1:10 dilution of anti-myc mAb (Evan et al., 1985), incubated with a 1:100 dilution of anti-mouse IgG-HRP, and treated with 0.3 mg/ml diaminobenzidine (DAB), 0.08% $NiCl_2$, and 0.08% $CoCl_2$ which gives a black peroxidase reaction product . Embryos were then re-fixed, incubated with 1:4000 dilution of mAb zn12 (anti-HNK-1) (Metcalfe et al., 1990; Trevarrow et al., 1990), incubated with anti-mouse IgG-HRP, and then with 0.5 mg/ml DAB, which results in a brown peroxidase reaction product.

Labeling of growth cones and axons by photo-oxidation of DiI fluorescence

To mark DiI-labeled growth cones with a brown DAB reaction product, embryos were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, embedded in 0.7% agar in 0.1 M phosphate buffer on glass slides, and under visual control through DIC optics of a compound microscope 2 mg/ml DiI in n,n-dimethylformamide was passed from a micropipette into the posterior lateral line ganglion iontophoretically. Embryos were then held overnight at 4°C in a dark humid chamber to allow for spread of the DiI into axons and growth cones and the fluorescently labeled cells were marked with a brown reaction product by following the photo-oxidation

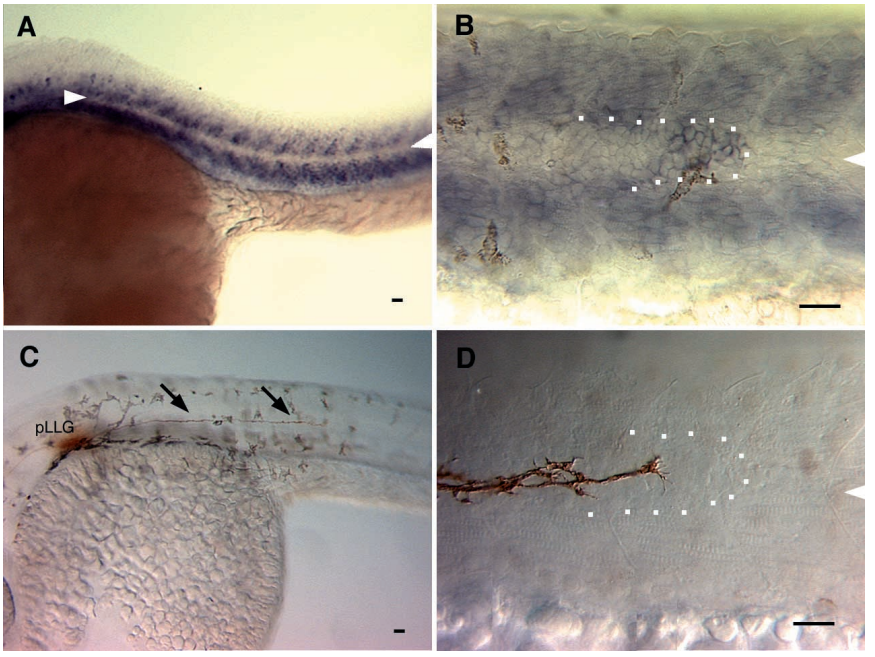

Fig. 1. *sema Z1a* expression by the myotomes suggests it may delimit the pathway of the growth cones of the posterior lateral line ganglion. All panels are sideviews showing wholemounted 24 hpf embryos with anterior to the left and dorsal up. (A) The trunk of an embryo hybridized to an antisense *sema Z1a* riboprobe showing that *sema Z1a* is expressed by the axial muscles in the dorsal and ventral portions but not the horizontal myoseptal region (arrowheads) of the myotomes. (B) A higher magnification view of another embryo hybridized to an antisense *sema Z1a* riboprobe showing that *sema Z1a* is also expressed by the cells at the leading edge of the migrating primordium (leading edge is outlined by square dots here and in D). Here the alkaline phosphatase reaction was over-reacted to better show expression by the leading edge cells. Arrowhead denotes the midway point along the dorsal-ventral axis of the horizontal myoseptal region in this panel and in D. (C) The posterior lateral line axons extend caudally along the horizontal myoseptal region of the myotomes. Axons (arrows) of the posterior lateral line ganglion (pLLG) were labeled by injection of DiI into the ganglion and then marking the DiI-labeled neurons with a brown reaction product via photo-oxidation. (D) Higher magnification view of several labeled pLLG growth cones in another embryo. The migrating primordium can be seen with DIC optics. Scale bars 20 μm.

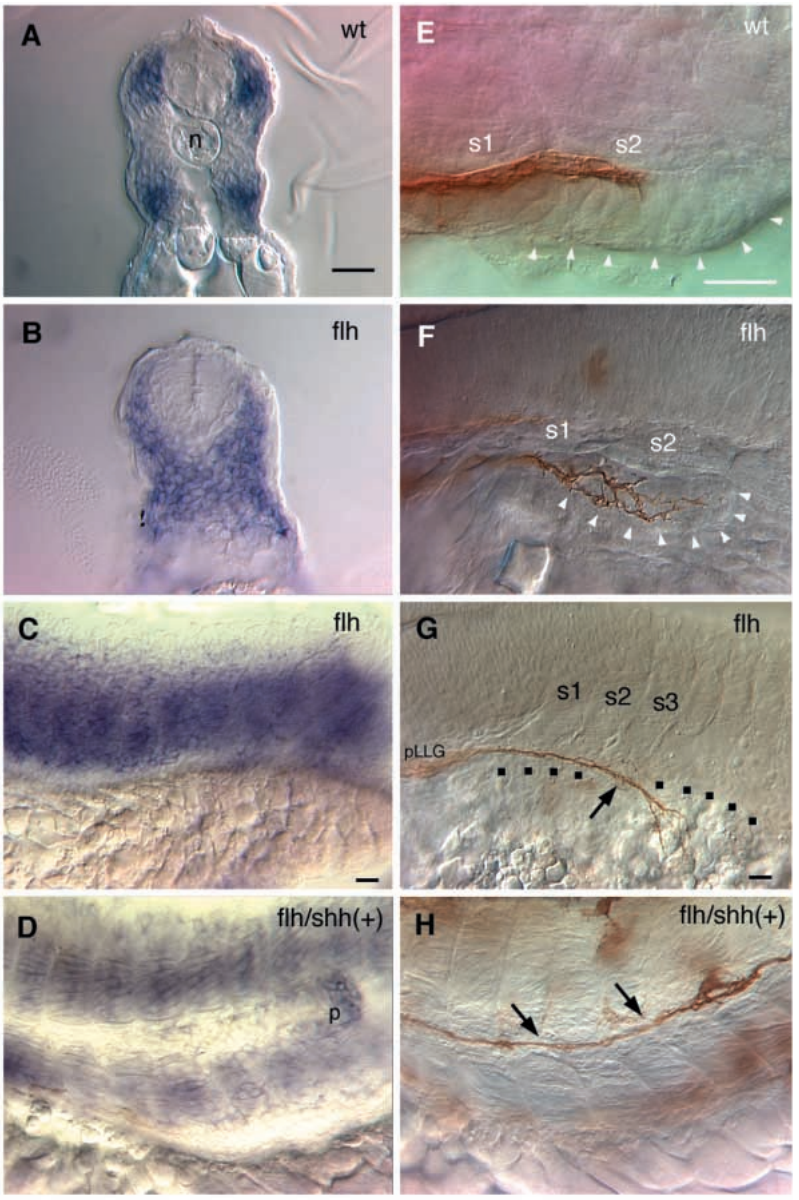

Fig. 2. *sema Z1a* expression and the pathway followed by the lateral line axons are coordinately altered in *floating head* (*flh*) homozygous embryos and *flh* embryos overexpressing *sonic hedgehog* (*shh*). Expression of *sema Z1a* was assayed by in situ hybridization (A-D), and the lateral line axons and growth cones by labeling them with DiI followed by photo-oxidation (E-H). (A) Transverse section of the trunk of a 24 hpf wild-type embryo showing that the dorsal and ventral portions of the myotome express *sema Z1a* but not the horizontal myoseptal region. The spinal cord can be seen dorsal to the notochord (n). In this panel and in B, dorsal is up. (B) Transverse section of the trunk of a 24 hpf *flh* embryo showing that *sema Z1a* is expressed by the entire myotome with no *sema Z1a*-free zone. Note that the notochord is missing in *flh* embryos as previously reported (Halpern et al., 1995). (C) Sideview of the trunk of a 24 hpf *flh* embryo showing that the longitudinal *sema Z1a*-free stripe is missing. The yolk tube can be seen ventral to the myotomes. Dorsal is up and anterior left. (D) Overexpression of *shh* downregulates *sema Z1a* and can restore a relatively normal *sema Z1a* expression pattern in the myotomes of *flh* embryos. Sideview of the trunk of a 26 hpf embryo showing that *sema Z1a* expression is reduced in the middle portion of the myotomes. Note that cells at the leading edge of the migrating primordium (p) express *sema Z1a* and appear to be migrating along an appropriate pathway. (E) Dorsal view of the trunk of a 22 hpf wild-type embryo showing that the lateral line growth cones extend between the lateral surface of the myotomes in segment 1 (s1) and segment 2 (s2) in the horizontal myoseptal region and the medial portion of the migrating primordium (lateral border outlined by arrowheads). Note that the growth cones appear to be in contact with the surface of the horizontal myoseptal cells, and that the axons are fasciculated. (F) Same view as in E but of a 22 hpf *flh* embryo showing that the growth cones are not fasciculated and appear to be extending within the body of the migrating primordium rather than in contact with the horizontal myoseptal cells as in E. (G) Sideview of the trunk of a 27 hpf *flh* mutant, showing that the lateral line axons (arrow) and growth cones extend aberrantly ventral to the myotomes and upon the yolk tube. The border between trunk and yolk tube is indicated by dots. (H) Sideview of the trunk of a 36 hpf *flh* embryo overexpressing *shh* showing that now the lateral line axons follow a relatively normal pathway within the middle region of the myotomes. Scale bars (A,B) (C,D) (E, F) (G,H) 20 μm.

procedure with 0.5 mg/ml DAB and epifluorescence illumination (Lubke, 1993).

Time lapse analysis

Growth cones were labeled within 30 minutes by ejecting DiI iontophoretically from a micropipette inserted into the posterior ganglion of the lateral line in living embryos that were anesthetized with 0.01% tricane (3-aminobenzonic acid ethylester). The embryos were then embedded in 0.7% agar. Following labeling, the growth cones were exposed to Sema Z1a-containing medium, Sema Z1a^{myc}-containing medium, or control conditioned medium as described below. The embryos were mounted onto a modified plastic dish with a coverslip bottom (Kapfhammer and Raper, 1987), and growth cone behavior was monitored on a epifluorescence microscope (Zeiss) equipped with a SIT camera (DAGE-MTI) and a temperature controlled stage (Medical Systems Corp.). Images were processed using Image-1 software (Universal Imaging Corp.) and transferred onto optical disks. Sixteen frames were averaged per image (exposure=1.03 second), and images were captured every 2 minutes for up to 2 hours 18 minutes. Time lapse records started approximately 15 minutes after the ejection of the conditioned medium due to the time required for transferring embryos from the injection microscope to the modified recording chamber on the microscope dedicated to time lapse recordings.

Preparation and application of conditioned medium onto growth cones

Culture medium containing recombinant Sema Z1a was generated by transfecting COS-1 cells grown at 37°C, 5% CO_2 in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal calf serum, 50 U/ml penicillin, and 50 mg/ml streptomycin. COS-1 cells were transfected with DNA expression constructs pBK-CMV-sema Z1a,

pBK-CMV-sema Z1a^{myc}, or as a control pBK-CMV (Stratagene) using the calcium phosphate method (Ausubel et al., 1991). After 48 hours the conditioned media were collected and centrifuged at 5000 ***g*** for 10 minutes to remove debris. To apply the conditioned media onto the growth cones, embryos were anesthetized, embedded in 0.7% agar on a slide, and mounted onto a compound microscope with DIC optics. A sharp micropipette filled with the conditioned medium was inserted, under visual control, into the leading edge of the migrating primordium, and the medium was ejected with 10 pressure pulses (10 mseconds, 5-20 psi) over a period of 10 minutes.

RNA and DNA injection into eggs

Approximately 1 nl of 200 ng/μl of capped RNA or 50 ng/μl of DNA that was suspended in water containing 0.1% phenol red was pressure-injected from a sharp micropipette into a single blastomere of zebrafish embryos at the 1-8 cell stage as described previously (Ekker et al., 1995). In all cases, more than 80% of injected embryos developed normally. To induce expression of *sema Z1a* or *sema Z1a^{myc}* driven by the zebrafish *hsp70* promoter, embryos in a Petri dish were transferred into a water bath at 40°C and incubated for 1 hour. Following induction, embryos were allowed to develop for 2-4 hours at 28.5°C and fixed for analysis.

RESULTS

sema Z1a expression by axial muscles delimit the pathway of lateral line growth cones

To clarify the in vivo role of semaphorins in zebrafish embryos, we cloned *sema Z1a*, a homolog of *semaphorin D/III/collapsin 1*, and analyzed its expression pattern (Yee and Kuwada, unpublished data). The zebrafish possesses a second gene referred to as *sema Z1b* that is also highly homologous to *semaphorin III/D/collapsin 1* (Roos, Bernhardt and Schachner, personal communication; see Discussion). Interestingly, *sema Z1a* is expressed in a striped pattern by the metamerically arranged axial muscles in the trunk and tail (Figs 1A,B, 2A). Each myotome consists of a dorsal and ventral region separated by the horizontal myoseptal region (Eisen et al., 1986). *sema Z1a* is expressed by the dorsal and ventral regions but not by the horizontal myoseptal region at times when axons are extending within and upon these myotomes. This *sema Z1a* mRNA free region is 5-6 muscle cells wide (20-25 μm) and centered upon the horizontal myoseptum that separates the myotome into dorsal and ventral halves. Furthermore, *sema Z1a* is not expressed by the epidermis overlying the horizontal myoseptal region (Fig. 2A). Thus, *sema Z1a* expression delineates a longitudinal stripe in the trunk and tail that is free of *sema Z1a* mRNA.

Interestingly, the growth cones of the posterior lateral line ganglion extend posteriorly upon the muscles of the horizontal myoseptal region that do not express *sema Z1a*. The growth cones are first projected at about 20 hpf, and after reaching the axial muscles extend caudally between the epidermis and axial muscles of the horizontal myoseptal region (Metcalfe, 1985) (Figs 1C,D, 2E). The coincidence between the pathway of the posterior lateral line growth cones and the Sema Z1a free stripe suggests that Sema Z1a guides these growth cones along their pathway via a repulsive action.

sema Z1a is also expressed by cells at the leading edge of the migrating primordium of the posterior lateral line in front of the growth cones (Fig. 1B). As previously mentioned, the migrating primordium moves caudally together with the lateral line growth cones. Although the nature of the relationship between the primordium and growth cones is presently unclear, the growth cones are never observed in advance of the leading edge of the migrating primordium (Metcalfe, 1985). Thus expression of *sema Z1a* by the leading edge of the primordium may act to regulate the motility of growth cones so that the growth cones do not overtake the primordium and, therefore, act to keep the two components of the lateral line system in register.

sema Z1a expression by the axial myotomes and the pathway of the posterior lateral line axons are coordinately altered in several mutant embryos

As a first step in examining the function of Sema Z1a, several different mutant embryos that were likely to exhibit alterations in the myotome expression of *sema Z1a* (see Discussion) were analyzed. *sema Z1a* expression was examined in the myotomes of *floating head* (*flh*) (Halpern et al., 1995), and *you-too* (*yot*) (van Eeden et al., 1996) mutant embryos. Both mutant embryos are characterized by a reduction or lack of the horizontal myoseptum and specialized muscle cells called muscle pioneer cells normally found in the horizontal myoseptal region. In both embryos, *sema Z1a* is uniformly expressed by the entire myotome, and, therefore, there is no longitudinal Sema Z1a free zone in the myotomes (Fig. 2B,C for *flh*; data not shown for *yot*).

Since *flh* and *yot* embryos lacked the longitudinal *sema Z1a* free zone that the posterior lateral line growth cones normally follow, the pathways followed by these growth cones were assayed in these mutant embryos by labeling them with the HNK-1 antibody, which labels the lateral line axons and growth cones, or with DiI. In 22 hpf wild-type embryos, the growth cones form tight bundles and appear to be extending sandwiched between and in contact with the lateral border of myotomes and the medial border of the migrating primordium when viewed from a dorsal perspective (Fig. 2E). In contrast, in *flh* embryos most of the growth cones are defasciculated and appear not to be in contact with the myotome border, having shifted laterally into the migrating primordium (Fig. 2F). Later the lateral line growth cones extended both ventrally and posteriorly onto the yolk sack and tube, and then extended posteriorly ventral to the myotomes (Fig. 2G) rather than upon the myotomes as in wild-type embryos. In each case the aberrantly extending growth cones remained associated with the migrating primordium. The lateral line growth cones followed similar aberrant pathways in *yot* mutant embryos (not shown). Thus, in *flh* and *yot* mutants a change in *sema Z1a* expression within the myotomes is correlated with alteration of the pathway that lateral line growth cones follow.

Since both mutations likely affect signalling from the axial mesoderm required for the differentiation of the horizontal myoseptal region (Halpern et al., 1995; van Eeden et al., 1996), we next tried to rescue the normal *sema Z1a* expression pattern in mutant embryos. The myotome phenotype of *flh* embryos is attributed to the lack of an inductive signal from the notochord since the embryos are missing the notochord (Halpern et al., 1995). One candidate signal is sonic hedgehog (shh) which is a factor secreted by the notochord that is normally involved in patterning of nearby tissues such as the spinal cord (Tanabe and Jessell, 1996). Therefore, overexpression of *sonic*

hedgehog (*shh*) in *flh* mutants might induce axial muscles to adopt a horizontal myoseptal fate. This in turn may lead to an inhibition of *sema Z1a* expression by axial muscles and, therefore, change the pathways followed by lateral line growth cones. To test this, *shh* RNA was injected into 1- to 8-cell stage *flh* embryos and they were assayed for *sema Z1a* expression at 27 hpf or the pathways followed by the lateral line axons at 36 hpf. 68% of injected embryos assayed for *sema Z1a* expression (*n*=31) exhibited various degrees of reduction of *sema Z1a* expression by the myotomes, predominantly by the muscle cells midway between the dorsal and ventral borders of the myotomes *flh* embryos (Fig. 2D). The putative effect of shh on *sema Z1a* expression indicated some specificity since expression in many other regions of the embryos was not changed (not shown). In 66% of injected *flh* embryos in which the lateral line axons were assayed (*n*=32), the axons extended upon the myotomes along a longitudinal pathway approximately midway between the dorsal and ventral borders of the myotomes (Fig. 2H). So the lateral line axons extended posteriorly in the same region of the myotome in which *sema Z1a* was inhibited. In contrast, neither the *sema Z1a* expression pattern (*n*=28) nor the pathways of the lateral line axons (*n*=30) were affected in *flh* embryos injected with *green fluorescent protein* (*gfp*) RNA. The examination of *flh*, *yot*, and *shh* overexpressed *flh* embryos demonstrates that the expression pattern of *sema Z1a* in the myotomes is tightly correlated with the pathways followed by the lateral line growth cones.

Sema Z1a can induce transient collapse of growth cones of the posterior lateral line in vivo

To directly examine the effect of Sema Z1a on the lateral line growth cones, recombinant Sema Z1a was applied directly onto the growth cones in vivo. Since the lateral line growth cones co-migrate with the migrating primordium of the lateral line, conditioned medium containing recombinant Sema Z1a protein or control medium was pressure ejected from a micropipette inserted into the leading edge of the migrating primordium under visual guidance in 26-27 hpf embryos. Embryos were fixed 30 minutes after application of the medium and the morphology of the lateral line growth cones were assayed by labeling them with DiI. Previously we had shown that the medium conditioned for 48 hours by COS cells transiently transfected with *cmv-sema Z1a* or *cmv-sema Z1a*^{myc} collapses chick DRG growth cones just as chick Collapsin 1, and confirmed by western blotting that the conditioned medium contained the Sema Z1a^{myc} protein (Yee and Kuwada, unpublished data).

The Sema Z1a- or Sema Z1a^{myc}-conditioned medium but not medium conditioned by COS cells transfected with the expression vector alone had dramatic effects on the lateral line growth cones. Following application of control medium, lateral line growth cones were complex with numerous lamellopodia and filopodia (26-27 hpf embryos, *n*=11; Fig. 3A). However, after application of Sema Z1a (26-27 hpf embryos, *n*=8) or Sema Z1a^{myc} (26-27 hpf embryos, *n*=7) conditioned media, the growth cones exhibited simple, stick-like morphology (Fig. 3B,C). When recombinant Sema Z1a was injected into the tectum at stages when many retinal growth cones have reached the tectum (50 hpf embryos; *n*=7), these growth cones appeared unperturbed and similar in morphology to retinal growth cones following injection of control medium (*n*=6; data not shown).

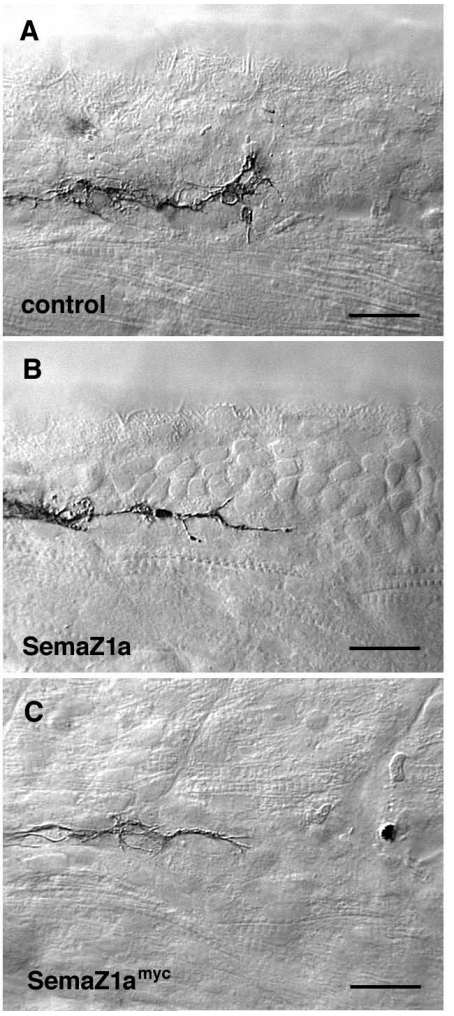

Fig. 3. Lateral line growth cones collapse in response to application of recombinant Sema Z1a containing medium. Several lateral line growth cones were labeled with DiI and marked with a brown reaction product from photo-oxidation following injection of media into migrating primordia located at s7-10 in 26-27 hpf embryos. (A) Sideview of the trunk showing that the morphology of lateral line growth cones is complex with numerous filopodia and lamellipodia following application of control medium. (B,C) Sideviews of the trunk showing that lateral line growth cones are collapsed following application of Sema Z1a- (B) or Sema Z1a^{myc}- (C) containing media. Dorsal is up and anterior left. Scale bars, 20 μm.

Thus, direct application of recombinant Sema Z1a induces the collapse of a subset of growth cones.

The above static analysis of the effect of recombinant Sema Z1a upon the morphology of the lateral line growth cones was corroborated and extended by a dynamic analysis of the response of the growth cones to applied Sema Z1a. For these experiments a single lateral line growth cone was labeled with DiI, Sema Z1a (*n*=3) or control medium (*n*=3) was injected onto the growth cone, and the behavior of the growth cone was recorded with time-lapse video microscopy in 26-27 hpf embryos. By 15 minutes following the ejection of recombinant Sema Z1a, growth cones were already collapsed and for the next 15 minutes the main body of the collapsed growth cone

was immotile (Fig. 4). The collapsed growth cones had short filopodia extending from their tips. During this 15 minutes period these filopodia retracted. Thirty minutes following Sema Z1a exposure, the growth cones resumed their extension at a rate comparable to that of control growth cones (Fig. 4C), but the complex morphology of the growth cones recovered much more slowly. This demonstrates that the inhibitory effects of Sema Z1a are transient, and suggests that Sema Z1a may affect the mechanisms that control growth cone morphology and the mechanisms that control axon extension differentially.

Lateral line growth cones appear to avoid cells that ectopically express *sema Z1a* in vivo

The collapse activity of recombinant Sema Z1a upon the lateral line growth cones suggests that these growth cones will change their pathway to avoid cells in the horizontal myoseptal region that misexpress *sema Z1a*. To test this hypothesis an inducible DNA expression construct controlled by a zebrafish *hsp70* promoter (Warren and Kuwada, unpublished data) was used to heat activate the expression of *sema Z1a*^{myc}. This construct is useful for examination of axon pathfinding since exposure to elevated temperatures used to induce expression does not by itself induce errors in axonal outgrowth (Warren and Kuwada, unpublished data). The *hsp70-sema Z1a*^{myc} DNA construct was injected into 1- to 8-cell stage embryos, embryos were transiently placed at elevated temperatures at 21-23 hpf, and the pattern of *sema Z1a*^{myc} expression assayed with a mAb against the c-myc epitope, and the lateral line axons with the HNK-1 antibody, 3 hours later. Control embryos were injected with a *hsp70-myc* epitope DNA expression construct.

When lateral line growth cones encountered ectopic Sema Z1a^{myc}-expressing muscle cells in the horizontal myoseptal region (*n*=27 embryos), they appeared to change their normal pathway to avoid these cells in all cases. Usually (19/27 cases) the growth cones avoided the misexpressing cells but remained within the horizontal myoseptal region (Fig. 5A,B). Occasionally (8/27 cases) the growth cones avoided the misexpressing cells but traversed more dorsal or ventral regions of the myotomes that express *sema Z1a* (Fig. 5C). Often the axons made sharp turns away from a cell expressing Sema Z1a^{myc} suggesting that the growth cones were repulsed by the Sema Z1a$^{myc.}$ Interestingly, the axons often appeared to have extended quite close to the misexpressing cells before turning away. This might signify that effective amounts of Sema Z1a do not diffuse much from the secreting cell. Consistent with this possibility, labeling of Sema Z1a^{myc} with the mAb against the c-myc eptope was not detected beyond the expressing cells. Growth cones never took such aberrant routes when there was no exogenous Sema Z1a^{myc} expression by cells in the horizontal myoseptal region (*n*=35) or when they encountered horizontal myoseptal cells that expressed the myc epitope

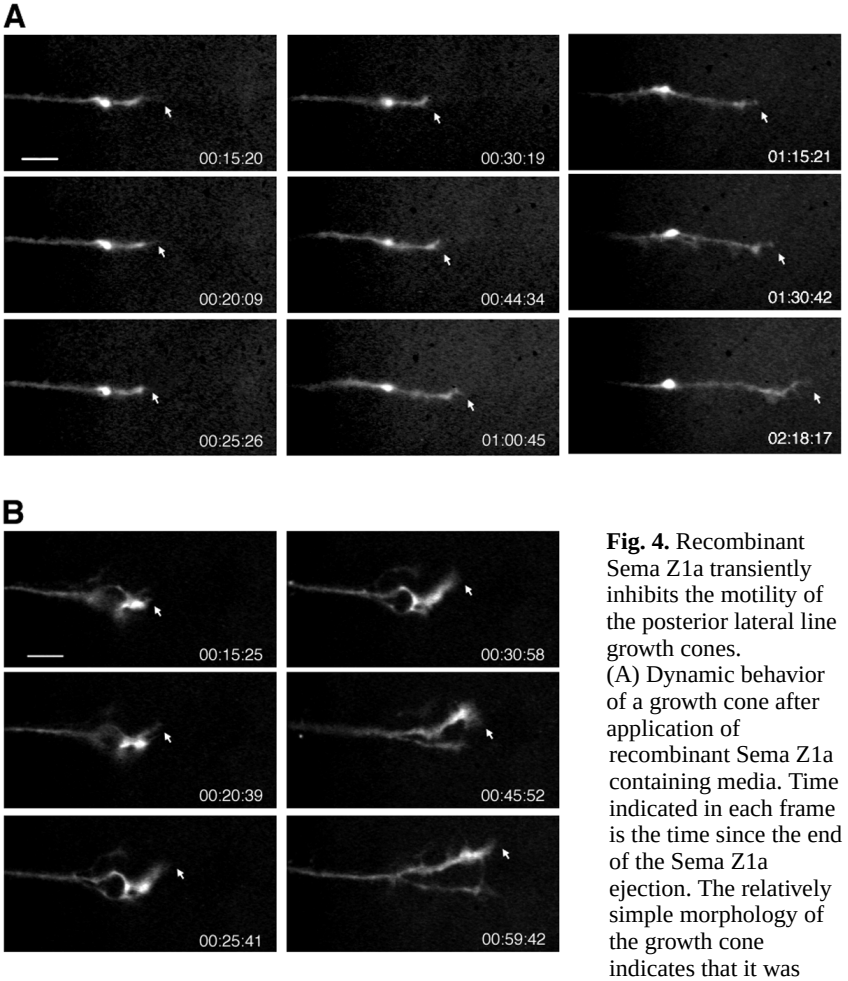

Fig. 4. Recombinant Sema Z1a transiently inhibits the motility of the posterior lateral line growth cones. (A) Dynamic behavior of a growth cone after application of recombinant Sema Z1a containing media. Time indicated in each frame is the time since the end of the Sema Z1a ejection. The relatively simple morphology of the growth cone indicates that it was already collapsed by 15 minutes 20 seconds. Note the short leading filopodum (arrow denotes the distal tip) retracts between 15 and 30 minutes. After 30 minutes the growth cone begins to extend posteriorly again even though it is still collapsed. The growth cone slowly recovers its normal complex morphology over the next several hours. (B) Dynamic behavior of a growth cone after application of control medium. The growth cone is complex and highly motile. Arrow indicates the leading tip of the growth cone. Scale bars, 20 μm. (C) Quantification of the rate of extension by lateral line growth cones following application of Sema Z1a, Sema Z1a^{myc}, or control medium. Shown are the mean and standard error of the distance extended by 3 growth cones sampled at 15, 30, 45, and 60 minutes after medium application in each condition. Note that after 30 minutes growth cones extended at their normal rates.

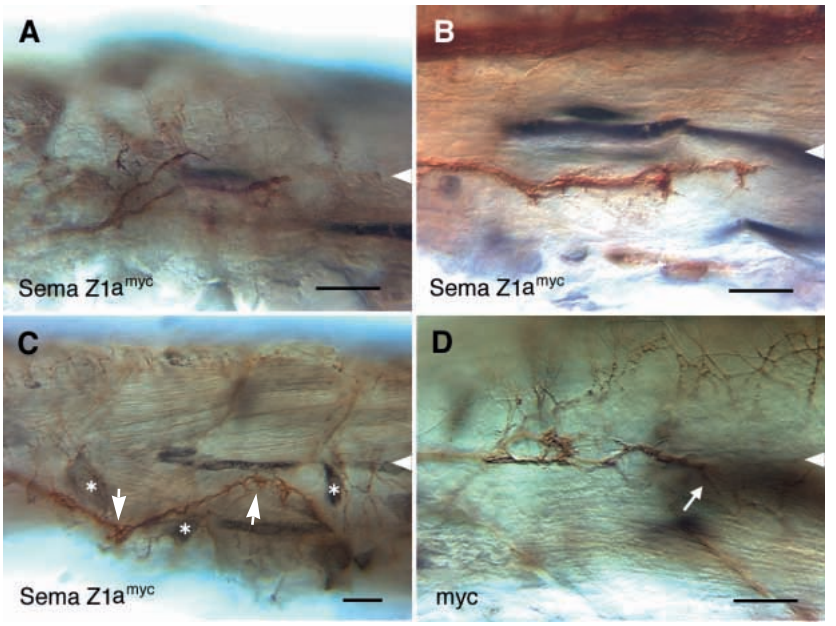

Fig. 5. Lateral line growth cones appear to avoid cells along their normal pathway that were induced to misexpress *sema Z1a*^{myc}. Sideviews of the trunk of 24-27 hpf embryos that were heat induced to express *sema Z1a*^{myc} 3 hours earlier. Sema Z1a^{myc}-expressing cells were labeled with anti-myc (black) and lateral line axons labeled with anti-HNK1 (brown). (A) A lateral line nerve branch with one arm extending dorsal and the other branch ventral to a horizontal myoseptal cell expressing Sema Z1a^{myc} in the first somite. (B) Lateral line growth cones extending ventral to Sema Z1a^{myc}-expressing horizontal myoseptal cells. (C) Lateral line axons appeared to have made several turns (arrows) to dodge epidermal cells (asterisks) and horizontal myoseptal cells expressing Sema Z1a^{myc}. Anti-HNK1 labeled peripheral axons of Rohon-Beard cells can also be seen in this panel as well as in D. (D) Lateral line axons are not perturbed by myc-epitope-expressing horizontal myoseptal cell (arrow) and follow their normal pathway along the midpoint of the horizontal myoseptum (white arrowhead in this and all other panels). Scale is 20 μm for all panels.

alone (*n*=15; Fig. 5D). These results strongly suggest that when posterior lateral line growth cones encounter cells ectopically making Sema Z1a, they will change their pathway to avoid the cells.

DISCUSSION

Role of Sema Z1a in guidance of the posterior lateral line growth cones

In the past several years the role of molecules that act to inhibit the complexity and motility of growth cones have been intensively studied (Tessier-Lavigne and Goodman, 1996). For the most part these molecules have been reported to be involved in the formation of specific neuronal projection patterns and maps (Drescher et al., 1995; Matthes et al., 1995; Messersmith et al., 1995; Puschel et al., 1995; Behar et al., 1996; Nakamoto et al., 1996). This study presents in vivo evidence that an inhibitory growth cone signal may also play an important role in navigating growth cones over long distances in embryos. First, the pathway followed by the growth cones of the posterior lateral line ganglion correlates with the expression pattern of *sema Z1a* by the axial muscles and is consistent with a repulsive action of Sema Z1a. In wild-type embryos the growth cones extend upon a pathway that is delimited by cells expressing *sema Z1a*. In *flh* and *yot* homozygous embryos *sema Z1a* expression in the muscles is altered and the pathway followed by the growth cones is concomitantly altered. Furthermore, when a relatively normal expression pattern of *sema Z1a* is restored in the muscles of *flh* embryos by overexpression of *shh*, the growth cones follow a relatively normal pathway. Second, direct application of recombinant Sema Z1a onto the growth cones in living zebrafish embryos demonstrates that Sema Z1a can collapse these growth cones in vivo. Interestingly, following collapse, axons began to extend at relatively normal rates prior to the recovery of a complex morphology by the growth cones. This suggests the possibility that Sema Z1a may in some cases act primarily to control the morphology of the growth cones rather than regulate the rate of axon extension. Third, the growth cones appeared to avoid muscle cells within their normal pathway when these cells are induced to misexpress *sema Z1a*. In most cases the growth cones veered around the misexpressing cell but stayed within the otherwise *sema Z1a* free zone. However, in some cases growth cones actually extended beyond their normal pathway onto muscles that normally express *sema Z1a*. In these cases it is possible that the misexpressing horizontal myoseptal cells generated high enough levels of Sema Z1a to cause the growth cones to actually extend upon muscles that normally express *sema Z1a*. This suggests that growth cones can detect local differences in Sema Z1a concentration. Supporting this idea, it has been reported that temporal retinal growth cones can habituate to a repulsive signal upon continuous exposure and can respond to small gradients of the signal (Walter et al., 1990a,b).

Role of *sema Z1a* expressed by the leading edge of the migrating primordium

In addition to the myotome, *sema Z1a* is expressed at relatively low levels by cells at the leading edge of the migrating primordium. The migrating primordium and the lateral line growth cones comigrate with the growth cones always found in the proximal portion of the primordium (Metcalfe, 1985). Since Sema Z1a is repulsive for these growth cones, it seemed odd that the leading edge of the primordium would secrete Sema Z1a in advance of the growth cones. However, the presumed relatively low amounts of Sema Z1a protein that is secreted may act to primarily regulate the motility of the growth cones so that the growth cones do not overtake the primordium. This would ensure that the primordium and the growth cones retain their spatial relationship as they advance. Although little is really known about the mechanisms that control the formation of the posterior lateral line sensory system, it may rely upon the close association between growth

cones and the primordium cells that will give rise to sensory organs.

sema Z1a expression is regulated by a notochord derived factor

Our experiments took advantage of the finding that *sema Z1a* is aberrantly expressed by the entire myotome in *flh* and *yot* homozygous embryos, in order to correlate changes in expression of *sema Z1a* with pathfinding by the lateral line growth cones. These mutants possess abnormal myotomes that are missing the muscle pioneers and horizontal myoseptum (Halpern et al., 1995; van Eeden et al., 1996). Both the muscle pioneers and the horizontal myoseptum can be rescued by transplanting wild-type notochord cells into *flh* embryos signifying that notochord-derived signals normally induce these structures (Halpern et al., 1995; Odenthal et al., 1996). Thus, the myotome defect including the aberrant expression of *sema Z1a* is likely a consequence of the missing notochord function in these mutants. This suggests that downregulation of *sema Z1a* by the horizontal myoseptal region cells is induced by notochord derived signals.

The hedgehog family of secreted molecules are attractive candidates for regulation of *sema Z1a*. These molecules have been demonstrated to be essential for normal patterning of the ventral neural tube (Chiang et al., 1996; Ericson et al., 1996; Tanabe and Jessell, 1996). In zebrafish *shh* and *echidna hedgehog* (*ehh*) are expressed by the notochord (Kraus et al., 1993; Ekker et al., 1995; Currie and Ingham, 1996). Furthermore, injection of *ehh* RNA can induce muscle pioneers (Currie and Ingham, 1996). Consistent with the purported role of hedgehogs for regulation of *sema Z1a*, we found that overexpression of *shh* leads to downregulation of *sema Z1a* in the myotomes of *flh* mutants.

Does Sema Z1a act directly upon the lateral line growth cones?

Our experiments clearly demonstrate that Sema Z1a can affect the behavior of the lateral line growth cones. However, it is possible that Sema Z1a may exert its effects upon the growth cones indirectly by regulating migration of the migrating primordium. In fact, the primordium and growth cones remain associated and are affected in identical fashion in the *flh* and *yot* embryos and in *flh* embryos overexpressing *shh*. The most extreme version of this hypothesis would have Sema Z1a guiding the primordium with the growth cones towed along passively. This seems unlikely since our dynamic analysis demonstrates the active, motile nature of the growth cones. Furthermore, application of recombinant Sema Z1a dramatically reduces the motility of these growth cones. We cannot rule out the possibility that Sema Z1a may act upon the migrating primordium cells which in turn secrete another factor that actually collapses the growth cones. However, the fact that recombinant Sema Z1a can collapse growth cones from chick DRG explants grown on laminin substrates (Yee and Kuwada, unpublished data) suggests that the effect of Sema Z1a upon the lateral line growth cones is likely to be a direct one.

Our experiments demonstrate that Sema Z1a can collapse lateral line growth cones, and that the growth cones will avoid Sema Z1a in vivo. Whether Sema Z1a is necessary for normal pathfinding is still an open question. The recent finding that the phenotypes of *sema III/D* knockout mice varies with strain suggests that some strains are better able to compensate for the loss of Sema III/D (Behar et al., 1996; Taniguchi et al., 1997). One intriguing possibility is that a different semaphorin may be the basis for this compensation. Interestingly, the zebrafish appears to have two cDNAs, *sema Z1a* and *sema Z1b*, that share high sequence homology with *semaphorin D/III/collapsin 1* (Yee and Kuwada, unpublished data; Roos, Bernhardt and Schachner, personal communication). *sema Z1b* is expressed by newly generated myotomes but is then down-regulated so that the anterior myotomes no longer express *sema Z1b* by 20 hpf (Roos, Bernhardt, and Schachner, personal communication) when the growth cones are first being extended from the posterior lateral line ganglion. Although the distribution of the Sema Z1b protein is not known, the down-regulation of *sema Z1b* by the anterior myotomes, determined by in situ hybridization, prior to extension of lateral line growth cones upon the myotomes suggests that *sema Z1b* unlike *sema Z1a* may not play a significant role in guiding posterior lateral line growth cones.

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