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Sema3a1 guides spinal motor axons in a cell- and stage-specific manner in zebrafish

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In order for axons to reach their proper targets, both spatiotemporal regulation of guidance molecules and stepwise control of growth cone sensitivity to guidance molecules is required. Here, we show that, in zebrafish, Sema3a1, a secreted class 3 semaphorin, plays an essential role in guiding the caudal primary (CaP) motor axon that pioneers the initial region of the motor pathway. The expression pattern of Sema3a1 suggests that it delimits the pioneer CaP axons to the initial, common pathway via a repulsive action, but then CaP axons become insensitive to Sema3a1 beyond the common pathway. Indeed, *nrp1a*, which probably encodes a component of the Sema3a1 receptor, is specifically expressed by CaP during the early part of its outgrowth but not during later stages when extending into *sema3a1*-expressing muscle cells. To examine this hypothesis directly, expression of *sema3a1* and/or *nrp1a* was manipulated in several ways. First, antisense knockdown of Sema3a1 induced CaP axons to branch excessively, stall and/or follow aberrant pathways. Furthermore, dynamic analysis showed they extended more lateral filopodia and often failed to pause at the horizontal myoseptal choice point. Second, antisense knockdown of Nrp1a and double knockdown of Nrp1a/Sema3a1 induced similar outgrowth defects in CaP. Third, CaP axons were inhibited by focally misexpressed *sema3a1* along the initial common pathway but not along their pathway beyond the common pathway. Thus, as predicted, Sema3a1 is repulsive to CaP axons in the common region of the pathway, but not beyond the common pathway. Fourth, induced ubiquitous overexpression of *sema3a1* caused the CaP axons but not the other primary motor axons to follow aberrant pathways. These results suggest that the repulsive response to Sema3a1 of the primary motor axons along the common pathway is both cell-type specific and dynamically regulated, perhaps via regulation of *nrp1a*.

KEY WORDS: Axon guidance, Growth cone, Filopodia, Branching, Pausing, Zebrafish

INTRODUCTION

Functional connections of neurons require the guidance of growth cones to their proper targets. To date, several families of molecules have been identified that can act as guidance factors by either attracting or repelling the motile growth cone at the tip of the growing axon. One family of guidance molecules is the semaphorin family, a diverse gene family with eight subclasses that is conserved from invertebrates to humans. These proteins are secreted or transmembrane, may have an Ig domain or thrombospondin type 1 domain, and all share a large, conserved Sema domain (reviewed by Tessier-Lavigne and Goodman, 1996; Kolodkin, 1998; Raper, 2000). The first vertebrate member of the family, chick collapsin 1 (now called Sema3a), is a secreted protein that repels specific subsets of growth cones (Luo et al., 1993). More recent reports have revealed that its repulsive activity can be converted to an attractive one by manipulating cyclic nucleotide levels within growth cones (Song et al., 1998; Polleux et al., 2000) and modulated by neurotrophic factors (Tuttle and O'Leary, 1998). Thus, regulation of the responsiveness of a growth cone to semaphorins could be important for proper pathfinding. However, whether this method of regulation, in fact, occurs in vivo remains unclear.

Zebrafish spinal motor system is an excellent system for analysis of the molecular and cellular mechanisms controlling axon guidance (Eisen et al., 1986; Myers et al., 1986; Westerfield et al., 1986). Each somitic hemisegment is typically innervated by three identifiable primary motoneurons; CaP (caudal primary), MiP (middle primary) and RoP (rostral primary) (Fig. 1). They first exit the spinal cord and extend ventrally on the medial surface of the somite until they reach the horizontal myoseptal region. This region of the pathway is pioneered by the CaP growth cone and is referred to as the common pathway, as initially all three motoneurons extend their axons along it. At the distal end of the common pathway, the growth cones pause to contact a group of specialized cells called muscle pioneers and then follow divergent pathways to extend to the ventral, dorsal and horizontal myoseptal muscles within the myotome (Eisen et al., 1986).

Semaphorins participate in guiding motor growth cones in zebrafish. The zebrafish contains two copies of the *sema3a* gene, *sema3a1* and *sema3a2* (Yee et al., 1999; Roos et al., 2000). Expression of these genes is dynamic. Initially, *sema3a2* is transiently expressed in the posterior half of each somite followed by expression of *sema3a1* in the posterior half of each somite (Shoji et al., 2003). Subsequently, *sema3a1* expression changes so that it is expressed by the dorsal and ventral regions of each somite, but not in the horizontal myoseptal region in between by the time motor growth cones are being projected (Shoji et al., 1998; Yee et al., 1999). As the expression pattern of *sema3a1* is changing, somitic expression of *sema3a2* is downregulated (Bernhardt et al., 1998). Overexpression of Sema3a2 by RNA injections suggested that Sema3a2 can affect outgrowth by spinal motor axons (Roos et al., 1999) and focal misexpression of Sema3a1 suggested that Sema3a1 can repulse motor axons (Halloran et al., 2000).

The demonstration that Sema3a proteins may serve as repulsive guidance factors for motor growth cones raised an inconsistency. CaP growth cones are repelled by Sema3a1, yet beyond the choice point this growth cone extends into the *sema3a1*-expressing ventral myotome. Thus, it was hypothesized that CaP growth cones are

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initially restricted to the common pathway by *sema3a1* expression in the dorsal and ventral myotomes; however, once at the choice point, they lose their responsiveness to Sema3a1, allowing them to enter the ventral myotome (Halloran et al., 2000).

To test the hypothesized role of Sema3a1 for guidance of motor growth cones, we examined the expression of a component of the Sema3a receptor neuropilin 1 (Nrp1) (Kolodkin et al., 1997; He and Tessier-Lavigne, 1997), and examined outgrowth by motor growth cones under a variety of conditions that manipulated the expression of *sema3a1/nrp1a*. Our results demonstrate that the spatiotemporal pattern of *nrp1a* expression correlates with sensitivity to Sema3a1 by CaP but not MiP or RoP axons along the common pathway, but then is downregulated once at the choice point. Furthermore, CaP but not MiP or RoP axons are repulsed by Sema3a1 along the common pathway and repulsion of CaP axons by Sema3a1 is dynamically regulated to allow the CaP axons to extend along ventral muscle that express *sema3a1*. Thus, our results suggest that changes in sensitivity to Sema3a1 conferred by the dynamic regulation of *nrp1a* are an important mechanism for guidance of the CaP growth cone to their ventral myotome target.

MATERIALS AND METHODS

Fish colony

Zebrafish (*D. rerio*) were maintained in a laboratory breeding colony at 28.5°C on a 14/10 hour light/dark cycle. Embryos collected from breeding fish were allowed to develop at 28.5°C and staged as hours post-fertilization (hpf), or by the number of somites (somite-stage) (Kimmel et al., 1995). The transgenic zebrafish strain *hsp:gfp-sema3a1-mt* was generated previously (Shoji et al., 2003).

RNA in situ hybridization and immunohistochemistry

Digoxigenin (DIG) labeled riboprobes for *sema3a1* and *neuropilin 1a* (*nrp1a*) were synthesized by in vitro transcription and hydrolyzed to an average length of 200-500 bp by limited alkaline hydrolysis (Cox et al., 1984). The procedure for hybridization to whole-mounted embryos has been described by Schulte-Merker et al. (Schulte-Merker et al., 1992).

Whole-mount immunostaining with a 1/50 dilution of mAb (monoclonal antibody) Znp1 which recognizes primary motor axons (Trevarrow et al., 1990; Melancon et al., 1997), a 1/10 dilution of anti-myc mAb (Evan et al., 1985) and a 1/400 dilution of a polyclonal anti-GFP antibody (Clontech) was performed following procedures previously described (Shoji et al., 1998; Halloran et al., 2000; Shoji et al., 2003). To detect biotin-labeled cells, VECTASTAIN Elite ABC (peroxidase) kit (Vector labs) and diaminobenzidine (DAB) substrate were used. $NiCl_2$ (0.08%) and $CoCl_2$ (0.08%) were added to 0.3 mg/ml DAB to obtain the blue-black peroxidase reaction product. For in situ hybridization/antibody double-labeling with mAb Znp1 and *sema3a1* or *nrp1a* probes, fixed embryos were first processed for mAb Znp1 followed by re-fixation and processing for in situ hybridization.

Sections were cut with a microslicer (DTK-3000W, Dosaka EM) after whole-mount hybridization or immunostaining. Embryos were embedded in 30% albumin, 0.5% gelatin, 0.8% glutaraldehyde in PBS and cut into 40-50 μm sections.

Morpholino oligonucleotide injection

Morpholino oligonucleotides (MO) were obtained from Gene Tools. The antisense morpholino sequence (25 mer) was complimentary to a sequence of the 5′UTR of *sema3a1* or *nrp1a*. The control morpholino sequence had five bases mismatched compared with the antisense morpholino sequence. Sequences were as follows: *sema3a1* antisense, 5′-CTTGTAGCCCACA-GTGCCCAGAGCA-3′; *sema3a1* control, 5′-CTTCTAGCCGACAGAG-CCCAGTGCA-3′; *nrp1a* antisense, 5′-GAATCCTGGAGTTCGGAGTG-CGGAA-3′; *nrp1a* control, 5′-GAATGCTCGACTTCGGAGTCCGCAA-3′.

MOs were solubilized in 1× Danieau Solution [58 mM NaCl, 0.7 mM KCl, 0.4 mM $MgSO_4$, 0.6 mM $Ca(NO_3)_2$, 5 mM HEPES, pH 7.6] and were injected into recently fertilized eggs (~3 ng for each embryos, as calculated from estimating the volume of injected solution). The injected embryos were fixed at 26- to 29-somite stage and stained with mAb Znp1 to label the primary motor axons. CaP axons in segments 7-15 were analyzed.

DNA injection

Approximately 1 nl of a 50 ng/ml solution of DNA in water containing 0.1% phenol red was pressure injected from a micropipette into a single blastomere of zebrafish embryos at the one- to four-cell stage as described previously (Shoji et al., 1998). The amount of DNA injected was determined by estimating the volume of the Phenol Red containing solution by visual inspection. To induce expression of *hsp70:sema3a1-myc* or *hsp70:myc*, embryos were incubated at 38°C for 30 minutes starting at 15 hpf. Following induction, embryos were allowed to develop at 28.5°C and fixed for analysis at 28 hpf. This method gives rise to embryos that mosaically express the construct. CaP axons were analyzed in segments where ectopic Sema3a1-myc or control Myc was expressed by muscle fibers along the common pathway.

Laser induction

Individual muscle fibers were laser induced to express Sema3a1 in *hsp70:gfp-sema3a1-myc* embryos at the 16-18 somite-stage (17-18 hpf). A dechorionated embryo was mounted inside a small thin Teflon ring (1 mm diameter) on a glass slide filled with a ringer solution, and held in place with a coverslip. Single muscle fibers were heat induced using a Micro Point dye-laser (Photonic Instruments, Arlington Heights, IL) as described previously (Halloran et al., 2000). Briefly, individual muscle cells were visualized with DIC optics, and heat induced with a burst of dye-laser pulses (Coumarin 440) delivered at a frequency of 4 Hz. The laser beam was focused onto a single muscle fiber by focusing a helium/neon laser that was collinear with the dye-laser using a 63× objective on a Zeiss Axioscope microscope.

Prediction of axonal extension according to segment and developmental stage

To facilitate laser activation of single muscle fibers, we determined the average status of CaP axons from each mid-trunk segment at each stage between 22 and 29 somites. Seven to ten embryos immunostained with the monoclonal Ab Znp1 were examined at each stage between 22 and 29 somites (20-23.5 hpf) to determine when CaP axons from each segment were initially projected and when they arrived at the horizontal myoseptal choice point (muscle pioneers) (Fig. 1C). The timing of initial outgrowth and arrival at the choice point corresponded with that from direct analysis of living CaP growth cones in *nrp1a:gfp* transgenic zebrafish (see below). This information enabled us to predict the location of a CaP growth cone in a given segment at a given developmental stage in living embryos.

In vivo imaging of CaP axons in living embryos

The behavior of CaP growth cones was examined by using *nrp1a:gfp* transgenic zebrafish in which GFP is expressed by CaP from the onset of axonogenesis (W.S., unpublished). The timing of axon extension and the pathway followed by the GFP-labeled CaP axons in the transgenic embryos corresponded to that inferred from a time line established from static images of mAb Znp1 labeled CaP axons taken at different stages. Images of GFP-labeled axons were periodically recorded with confocal microscopy (Zeiss Axiovert with LSM5 Pascal) every 10 to 15 minutes. Duration of extension along the common pathway was quantified as the time between initial axonal protrusion and arrival at the nascent horizontal myoseptum. Pausing at the choice point was determined as the time between arrival of the growth cone at the horizontal myoseptum and re-extension into the ventral myotomes.

Chimeric embryos

Chimeric embryos were generated by transplanting wild-type donor blastomeres from 1K-stage embryos into 1K-stage hosts (Myers et al., 1988; Zeller and Granato, 1999). Newly fertilized donor embryos were injected with a mixture of 2.5% biotin-dextran (M_r 10K; Molecular Probes) and 2.5% rhodamine-dextran (M_r 10K; Molecular Probes) in 0.1 M KCl. At 1K-cell stage cells from donor embryos the were sucked up into a pipette and ejected into unlabeled, host embryos (either wild-type controls or *hsp70:gfp-sema3a1-myc* transgenics) at the same stage. At the 20-somite stage, the

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chimeric embryos were heat-induced as described previously (Shoji et al., 2003), and were fixed 2-4 hours later with 4% paraformaldehyde and processed to visualize the biotin-labeled donor cells.

RESULTS

Expression pattern of *sema3a1* and *neuropilin-1* correlates with cell-specific and stage-specific guidance of CaP motor axons

High expression of *sema3a1* by the dorsal and ventral but not horizontal myoseptal myotomes suggested that Sema3a1 delimits motor axons to the common pathway along the horizontal myoseptal myotome and that some motor axons become insensitive to Sema3a1 beyond the choice point within the horizontal myoseptal region (Halloran et al., 2000). One potential mechanism for such dynamic responsiveness to Sema3a1 would be pathway dependent regulation of *nrp1* expression by motoneurons. To examine this possibility the expression of *sema3a1* and *nrp1* were analyzed to see how they correlate with outgrowth by the primary motor axons.

We have previously reported that *sema3a1* is expressed by the posterior half of early somites followed by a change to expression predominantly by the dorsal and ventral regions of the myotome with little expression in the horizontal myoseptal region in between (Shoji et al., 1998; Yee et al., 1999; Shoji et al., 2003). This change in the *sema3a1* expression pattern takes place when the CaP axons are pioneering the initial, common portion of the motor pathways. When viewed in transverse sections, CaP axons can be seen to extend along the common pathway in the horizontal myoseptal region where expression of *sema3a1* was much lower and along their specific pathway in the ventral region where expression of *sema3a1* was higher (Fig. 2A). Although the most medial myotome that make up the CaP-specific ventral pathway express less *sema3a1* compared with the more lateral ventral myotome, we presume that the level of the secreted Sema3a1 is higher along the ventral pathway compared with the horizontal myoseptal region. In addition, although there is some expression of *sema3a1* in the horizontal myoseptal region the most medial muscle fibers expressed less *sema3a1* than the more lateral ones. When viewed in a horizontal section, the inverse relationship between the CaP axon and *sema3a1* expression was apparent with the region of the myotome immediately adjacent to the CaP axon not expressing much *sema3a1* (Fig. 2B). Thus, the initial pathway followed by the CaP axon is correlated with a region of the myotome where *sema3a1* was reduced, suggesting that the expression pattern of *sema3a1* acts to channel the CaP axon to the common pathway.

As the receptor for Sema3a consists of neuropilin 1 (Nrp1) and plexin A1 (*plxna1* – Zebrafish Information Network) (Kolodkin et al., 1997; Kitsukawa et al., 1997; Tamagnone et al., 1999; Takahashi et al., 1999), we examined expression of *nrp1* and *plxna1* during axogenesis by primary motor neurons. Zebrafish have two copies of the *nrp1* gene, with *nrp1a* dynamically expressed in a segmental pattern by cells in the ventral spinal cord (Bovenkamp et al., 2004; Yu et al., 2004). The location of the *nrp1a*-positive cells within each spinal segment, their axon trajectory and early time of axon outgrowth suggests that the CaP motor neurons are likely to express *nrp1a* (Fig. 2C). Interestingly, expression of *nrp1a* correlated with axon extension along the common pathway with strong expression when the CaP axon was extending along the common pathway to the choice point (Fig. 2D) and subsequent downregulation of expression when the axon was beyond the choice point (Fig. 2E). *nrp1a* message was detected in the CaP cell bodies but not the axons. There is a possibility, however, that our methods may have failed to detect axonally localized *nrp1a* mRNA. CaP motoneurons also appeared to express the other copy, *nrp1b,* in a similar pattern, but expression was much weaker (not shown). Unlike the specific expression of *nrp1a*, *plxna1* was expressed broadly in the ventral spinal cord during primary motor axogenesis (not shown). Thus, components of the Sema3a1 receptor are specifically expressed by CaP and expression of *nrp1a* correlates with guidance of their axons by Sema3a1 along the common motor pathway.

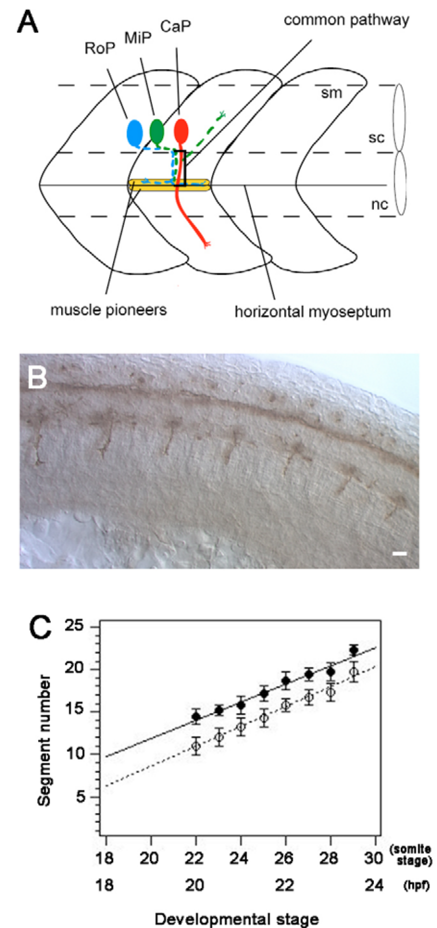

Fig. 1. CaP axons extend sequentially starting with those from anterior segments. (**A**) Schematic representation of the three primary motoneurons (CaP, MiP and RoP) showing their axonal trajectories. CaP axons are projected first to establish the common pathway. The common pathway ends at the muscle pioneers located at the horizontal myoseptum that divides the myotome into dorsal and ventral halves. This point is a choice point where the three axons diverge and follow cell specific pathways to innervate the ventral (CaP), dorsal (MiP) and horizontal myoseptal (RoP) myotomes. sc, spinal cord; nc, notochord; sm, somite. (**B**) Side view of the trunk of a 28-somite stage (23 hpf) embryos showing that CaP axons labeled with mAb znp1 are more developed anteriorly. Scale bar: 20 μm. (**C**) The compilation of the stage of initial CaP axon projection (black circle) and the stage when they arrive at the horizontal myoseptum (white circle) by CaPs located in specific segments. CaP axons were projected earlier in anterior segments, e.g. stage 22 for CaPs in segment 14 and stage 29 for those in segment 22. Most CaP axons reached the horizontal myoseptum 1.5 hours after time of initial projection. Each data point represents the mean of at least seven CaPs that were assayed from embryos at 22- to 29-somite stages (20-23.5 hpf). Bars in indicate standard deviations.

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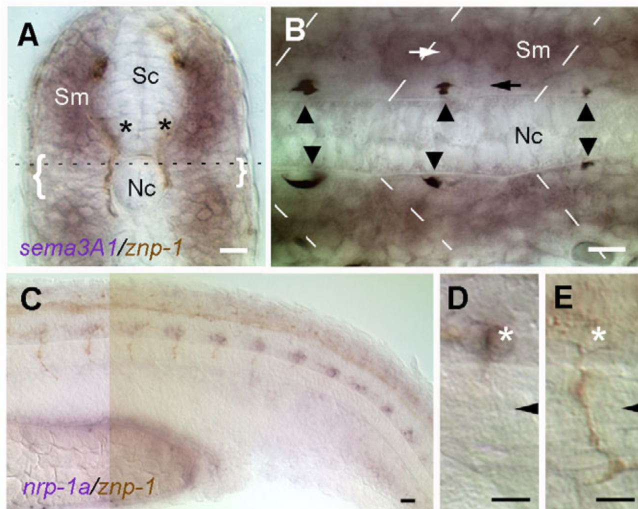

Fig. 2. The expression patterns of *sema3a1* and *nrp1a* correlate with extension of CaP axons along the common pathway. In situ hybridization (purple) for *sema3a1* (A,B) or *nrp1a* (C-E) with immunostaining (brown) by mAb Znp1 that labels primary motor axons in 26-somite stage (22 hpf) embryos. Unless otherwise noted, embryos are oriented with rostral leftwards and dorsal upwards. (**A**) A transverse section of the trunk with dorsal upwards showing that *sema3a1* is expressed in the dorsal and ventral regions of the myotome and less so in the horizontal myoseptal region (brackets). Asterisks indicate CaP motoneurons whose axons extend along the medial surface of the myotome. Sm, somite; Nc, notochord; Sc, spinal cord. Broken line indicates the level of the horizontal section shown in B. (**B**) A horizontal section with rostral leftwards, showing that the myotome cells immediately adjacent to the notochord, which CaP axons (arrowheads) extend upon, express little to no *sema3a1* (black arrow). However, the more lateral cells express higher levels of *sema3a1* (white arrow). Broken lines indicate somite borders. (**C**) Lateral view of the trunk showing that *nrp1a* is expressed segmentally in ventral spinal neurons that, based upon their axon trajectory, correspond to CaP neurons. Presumptive VaP (variable primary) neurons that arise in about half of the hemisegments as equivalent pair of CaP, but later die, may also express *nrp1a*. The expression of *nrp1a* declines in more anterior and developed CaPs. (**D**) *nrp1a* is expressed by CaP motoneurons (asterisk) while they are extending along the common pathway. (**E**) *nrp1a* expression is much reduced in CaP neurons (asterisk) with axons (mAb Znp1 immunostained in brown) extending onto the specific ventral pathway. Arrowheads in D,E indicates the position of the horizontal myoseptum. Scale bars: 20 μm.

Antisense knockdown of Sema3A/Neuropilin-1 signaling results in abnormal extension by CaP axons

To determine whether Sema3a1 is required for normal outgrowth by primary motor axons, we injected antisense morpholino oligomers (MO) (Nasevicius and Ekker, 2000) against *sema3a1* into recently fertilized embryos and assayed primary motor axons with mAb Znp1 following further development. Previously, we showed that this *sema3a1* antisense MO efficiently knocked down translation of Sema3a1 in *hsp:gfp-sema3a1-myc* transgenic embryos following heat induction (Shoji et al., 2003). CaP axons were aberrantly branched or had not extended as far in Sema3a1 morphant embryos compared with control morphant embryos (Fig. 3A-C, Table 1). Furthermore, for five of 30 CaP axons that were abnormally short, transverse sections showed CaP axons extending into more lateral myotome rather than along the medial surface of the myotome (compare Fig. 3D and Fig. 2A). Recall that the most medial muscle fibers normally do not express *sema3a1* while the more lateral ones do during the time that CaP axons are pioneering the common motor pathway (see above). Thus, it appears that in the absence of Sema3a1, CaP axons branched excessively, did not extend as far and sometimes extended into lateral muscle fibers that normally express *sema3a1*.

We also examined the requirement of Nrp1a for proper outgrowth by CaP axons by injection of antisense *nrp1a* MOs to knockdown Nrp1a (Lee et al., 2002). As with the *sema3a1* morphants, CaP axons branched aberrantly and/or were shorter compared with control *nrp1a* morphants (Table 1; Fig. 3E). As expected, injection of antisense MOs against *sema3a1* and *nrp1a* together also induced CaP axons to branch abnormally and/or to extend less (Table 1). Thus, it appears that Sema3a/Nrp1 signaling is necessary for proper outgrowth by CaP axons.

CaP axons extended more lateral filopodia and often failed to pause at the horizontal myoseptal choice point in *sema3a1* morphants

To investigate how a decrease in Sema3A/Nrp1 signaling leads to aberrant outgrowth by CaP axons, we examined the dynamic behavior of CaP growth cones with time lapse microscopy. To do this we used transgenic zebrafish (*nrp1a:gfp*) in which the *nrp1a* promoter regulated expression of *gfp* so that CaP axons are labeled by GFP from the beginning of axogenesis (W.S., unpublished). A complete analysis of the dynamic behavior of CaP growth cones will be reported elsewhere. Here, we present findings pertinent to the role of Sema3A/Nrp1 signaling for guidance of CaP growth cones.

Table 1. Abnormal CaP axons in *sema3a1* and *nrp1a* morphant embryos

MO		Total embryos	Aberrant branches*	Short axons†	Embryos with either defect‡
sema3a1 (3 ng/fertilized egg)	Antisense	16	6	4	9 (P<0.1)
	Control	13	3	1	3
sema3a1 (9 ng/fertilized egg)	Antisense	30	15	7	18 (P<0.05)
	Control	23	5	3	7
nrp1a (3 ng/fertilized egg)	Antisense	26	10	6	12 (P<0.05)
	Control	20	3	0	3
nrp1a (7 ng/fertilized egg)	Antisense	21	10	6	14 (P<0.05)
	Control	19	4	0	4
sema3a1+nrp1a antisense (each 3 ng/fertilized egg)		26	12	6	15

*Embryos that in which two or more CaP axons exhibited aberrant branches in segments 7-15.
†Embryos with at least one short CaP axons in segments 7-15.
‡Fisher's test

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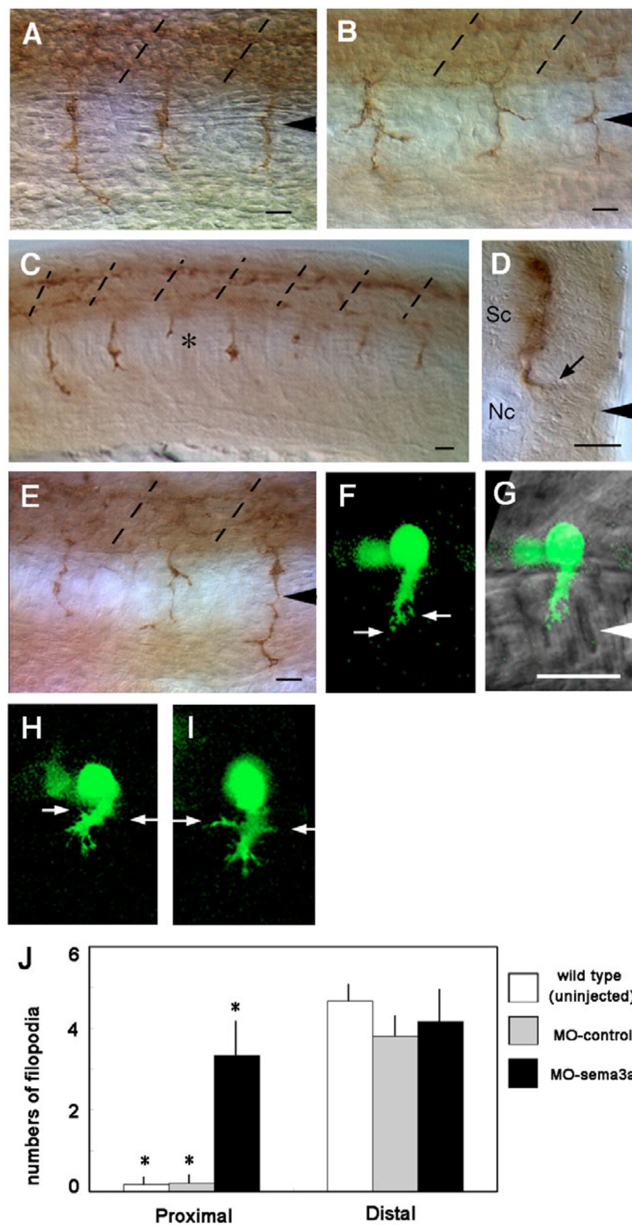

Fig. 3. Extension by CaP axons is aberrant following knockdown of Sema3a1. (**A**) CaP axons extend normally in a control *sema3a1* MO-injected embryo (24 hpf) seen in a lateral view. Arrowhead indicates the nascent horizontal myoseptum and broken lines indicate somitic segmental borders. (**B**) CaP axons often branch aberrantly in an antisense *sema3a1* MO-injected embryo (24 hpf). (**C**) CaP axons can stall (asterisk) in an antisense *sema3a1* MO-injected embryo (24 hpf). (**D**) Transverse section showing a CaP axon extending abnormally into the lateral myotome in the horizontal myoseptal region (arrow) in an antisense *sema3a1* MO-injected embryo (24 hpf). Sc, spinal cord; Nc, notochord. (**E**) Aberrantly branched CaP axons in an antisense *nrp1a* MO-injected embryo (24 hpf). (**F**) GFP-labeled CaP neurons in a *nrp1a:gfp* transgenic embryo showing filopodia from the leading edge of the growth cone (arrows) but not the axon behind the growth cone. (**G**) Nomarski bright-field image is overlaid to show the location of the CaP growth cone. White arrowhead indicates the horizontal myoseptum. (**H**,**I**) Two independent examples showing the same feature. Filopodia extending from the growth cone and trailing axon (arrows) of CaP neurons in a *nrp1a:gfp* transgenic embryo following injection of antisense *sema3a1* MO. (**J**) Histogram showing an increase in lateral filopodia in CaP axons in the proximal half of the axon in Sema3a1 morphant embryos compared with uninjected embryos and control morphants. Filopodia were quantified from 10 μm CaP axons that were divided into proximal and distal halves. Bars indicate s.d. **P*<0.002 for Student's *t*-test between CaP axons in antisense *sema3a1* morphants (*n*=6) versus control morphants (*n*=5) and uninjected embryos (*n*=6). Scale bars: 20 μm.

In uninjected and *sema3a1* control MO-injected wild-type embryos, CaP growth cones emerged from the cell bodies, extended ventrally along the common pathway to reach the horizontal myoseptal choice point, paused at the choice point and then resumed extending ventrally along the ventral myotome (Fig. 4) (Eisen et al., 1986). In *sema3a1* antisense MO-injected embryos, all CaP growth cones emerged from the cell bodies correctly and most of the growth cones reached the choice point with normal timing (Fig. 4A,B). However, 3/24 growth cones took 30-80 minutes longer to reach the choice point and the duration of extension to the choice point was more dispersed in antisense morphants compared with controls (*P*<0.003, F-test). This is reminiscent of some of the CaP growth cones that appeared to have stalled following interference of Sema3a1/Nrp1a signaling described in the previous section. Furthermore, the CaP axons were more complex, with more filopodia emerging from the axon (lateral filopodia) behind the growth cone in addition to filopodia emanating from the growth cone compared with uninjected and control MO injected wild-type embryos (Fig. 3F-J). Some of the lateral filopodia thickened and developed into branches (Fig. 5). Surprisingly, 10/24 CaP growth cones in *sema3a1* morphants failed to pause at the choice point, while all 27 CaP growth cones in control morphant or uninjected control embryos paused at the choice point (Fig. 4C). When growth cones paused they decelerated and stopped for between 20 minutes and more than 2 hours. In the 10 growth cones that failed to pause, the growth cones extended through the choice point with no deceleration (not shown). This suggests that Sema3a1 regulates the complexity of CaP axons and is required for pausing at the muscle pioneers.

To examine whether the changes in CaP growth cone behavior were directly due to a decrease in Sema3a1 or indirectly via other changes induced by a decrease in Sema3a1, we analyzed specification of muscles and motoneurons in morphant embryos that could potentially account for the observed changes. First, the gross morphology of the axial muscles examined with differential interference contrast optics and the expression of the slow twitch muscle marker F59 (Crow and Stockdale, 1992; Devoto et al., 1996) were normal in the antisense morphants (not shown). This suggests that many aspects of the muscles are not obviously perturbed. Second, the pattern of *islet1* and *islet2*-expressing cells in the ventral spinal cord (Appel et al., 1995; Segawa et al., 2001) is normal, suggesting that motoneurons are unperturbed in the antisense morphants (not shown). Thus, it appears that knocking down Sema3a1 directly affects CaP axons rather than indirectly via interference with muscle development or motoneuron specification.

CaP axons are repulsed by muscle cells focally misexpressing Sema3a1 within the common pathway but not beyond the choice point

The expression pattern and knock down studies of *sema3a1* and *nrp1a* suggested that Sema3a1 is required for normal outgrowth by CaP axons. To see if Sema3a1 can repulse CaP growth cones, we

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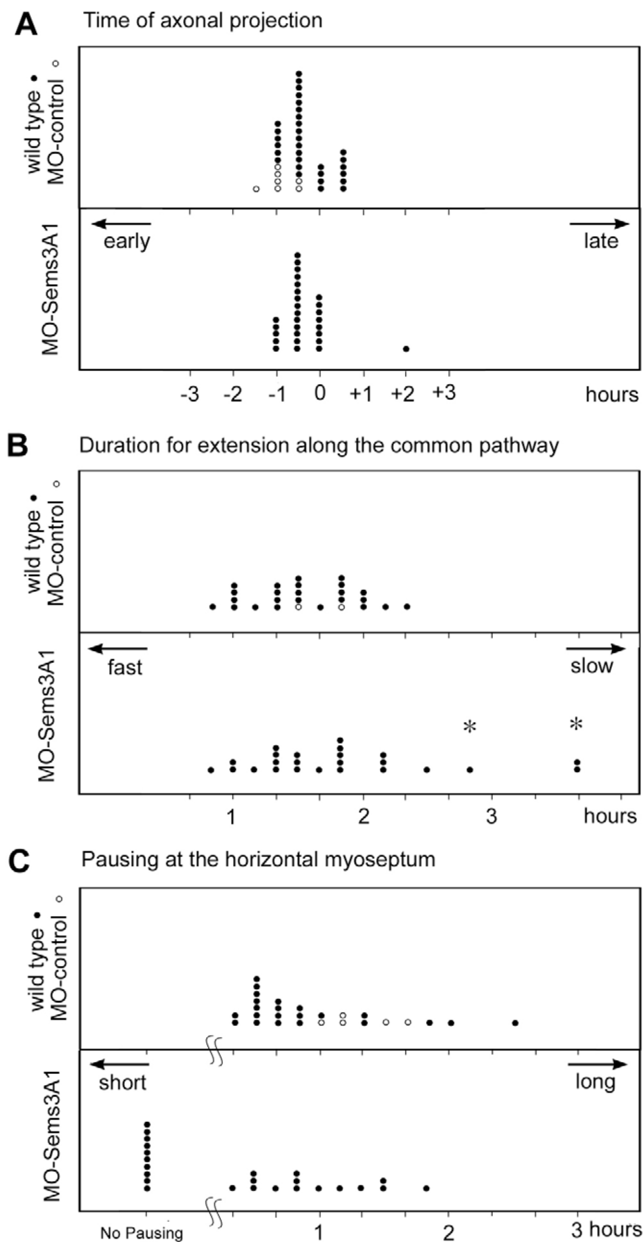

Fig. 4. Timing of extension by CaP axons on the common pathway in control and antisense *sema3a1* morphants. Three steps of axonal development, initial projection (A), extension along the common pathway (B) and pausing at the horizontal myoseptum (C), were assayed in living *nrp1a:gfp* transgenic embryos. (**A**) Time of axonal projection was comparable between control morphants/uninjected embryos and antisense morphants (F-test and *t*-test). Time when the axon was initially projected occurred within a window ±1 hour from the time measured from mAb Znp1-labeled CaPs (see Fig. 1C) in uninjected embryos/control morphants and antisense morphants. (**B**) The duration for extension by CaP axons along the common pathway from the cell body to the horizontal myoseptum was generally similar, but more dispersed in antisense morphants than in controls (P<0.003, F-test). Asterisks indicate three cases in which the time required to reach the horizontal myoseptum was significant longer compared with control CaPs. (**C**) A significant number of CaP axons failed to stop and pause at the horizontal myoseptum in antisense morphants (P<0.0002, Fisher's exact probability test). White dots in the upper part of each panel indicate CaP axons in control *sema3a1* morphants and black dots indicate axons in uninjected embryos.

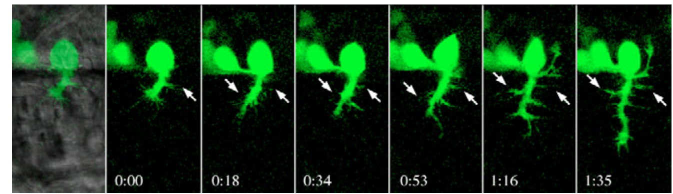

Fig. 5. Some lateral filopodia extended from CaP axons develop into aberrant branches in antisense *sema3a1* morphants. Sequential images of an extending CaP axon in a *nrp1a:gfp* embryo (starting at 22 hpf) showing that some of the lateral filopodia enlarge into branches (arrows). Time of each frame is shown below each micrograph.

employed two strategies to focally express Sema3a1 in individual muscle fibers. First, recently fertilized embryos were injected with *hsp70:sema3a1-myc* constructs, heat induced at 15 hpf, and assayed at 28 hpf. In these embryos, a random mosaic of cells expressed exogenous Sema3a1 following heat induction. When CaP axons encountered muscle fibers expressing exogenous Sema3a1 along the common pathway in these embryos, the axons stalled or turned away from the muscle fiber in 72% of cases (Fig. 6B,C; n=65) but ignored all control myc epitope expressing fibers in embryos injected with *hsp70:myc-tag* constructs and continued extending along their pathway (Fig. 6A; n=20). These results ($P<5\times10^{-9}$, Fisher's test) suggest that Sema3a1 is repulsive to CaP axons during the common pathway.

Second, we induced individual muscle cells within the common pathway to express exogenous Sema3a1 by focusing a laser microbeam onto a single muscle fiber in *hsp70:gfp-sema3a1-myc* transgenic embryos (Halloran et al., 2000; Shoji et al., 2003). The response of CaP axons to focal induction of Sema3a1 on the common pathway was analyzed by laser induction of Sema3a1 in muscle pioneer cells located at the distal end of the common pathway. Muscle pioneers were laser-induced at 17-18 hpf and CaPs assayed at 20-24 hpf. In control experiments, where GFP was laser induced in muscle pioneers in *hsp70:gfp* transgenic embryos, CaP axons extended normally in all cases (Fig. 6D; n=16). However, CaP axons stalled in the vicinity of the Sema3a1-expressing muscle pioneers when assayed up to 1.5 hours beyond the time CaP growth cones should have reached the muscle pioneers (Fig. 6E and Table 2; n=9). In cases in which CaP axons were stalled, MiP axons extended normally (Fig. 6F; open arrowhead). Thus, both mosaic expression and laser induction of exogenous Sema3a1 along the common pathway appear to repulse CaP axons.

The fact that CaP axons are repulsed by Sema3a1 while on the common pathway but then extend into Sema3a1-positive ventral muscles beyond the choice point suggests that CaP axons are insensitive or less sensitive to Sema3a1 during the CaP-specific ventral pathway. To confirm this, the response of CaP axons to exogenous Sema3a1 on the CaP specific pathway beyond the muscle pioneers was examined by laser inducing muscle fibers along the medial surface of the myotomes two or three fibers ventral to the presumptive choice point to express Sema3a1 in *hsp70:gfp-sema3a1-myc* transgenic embryos. CaP axons extended normally in all cases (n=8), despite encountering ventral muscle cells expressing exogenous Sema3a1 (Fig. 6G). These results demonstrate that the CaP axon is sensitive to Sema3a1 on the common pathway but insensitive or less sensitive on the specific ventral pathway.

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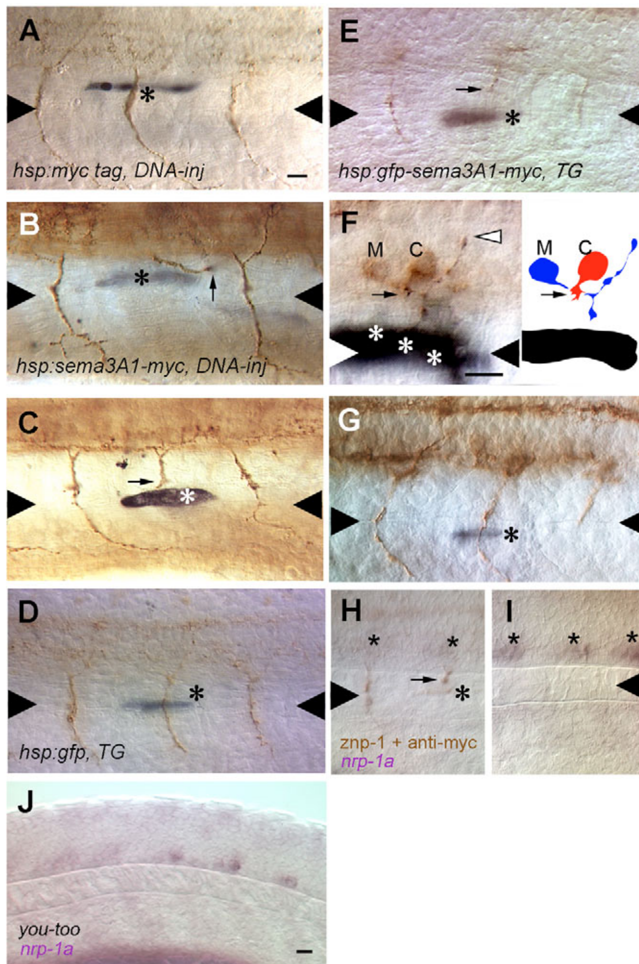

Fig. 6. CaP axons are repulsed by myotome cells that express ectopic Sema3a1 along the common pathway but not by those along the CaP specific ventral pathway. (**A**) A CaP axon is not perturbed by a myotome cell (asterisk) along the common pathway that expresses the Myc epitope following heat induction of a *hsp70:myc* injected embryo. Triangles denote the horizontal myoseptum. (**B**) A CaP axon (arrow) appears to have turned away to avoid a myotome cell (asterisk) along the common pathway that expresses ectopic Sema3a1 following heat induction of a *hsp70:sema3a1-myc*-injected embryo. CaP axons in the segment anterior and posterior to the repulsed CaP follow a normal trajectory. The myotome cell expressing ectopic Sema3a1 in the posterior segment is out of the focal plane of the CaP axon and is located lateral to the medial myotome cells lining the common pathway. (**C**) A CaP axon (arrow) appears to have stalled when encountering a myotome cell (asterisk) along the common pathway that expresses ectopic Sema3a1 following heat induction of a *hsp70: sema3a1-myc*-injected embryo. (**D**) A CaP axon is not perturbed by a muscle pioneer cell (asterisk) laser induced to express GFP in a *hsp70:gfp* transgenic embryo. (**E**) A CaP axon (arrow) is stalled in the vicinity of a muscle pioneer (asterisk) laser induced to express ectopic Sema3a1 in a *hsp70:gfp-sema3a1-myc* transgenic embryo. (**F**) A CaP axon (arrow) is stalled in the vicinity of three myotome cells (asterisks) along the common pathway laser induced to express ectopic Sema3a1 in a *hsp70:gfp-sema3a1-myc* transgenic embryo, but a presumptive MiP axon (white arrowhead) is normal. Right panel shows a camera lucida drawing of the CaP and MiP motoneurons. C, the CaP cell body; M, the MiP cell body. (**G**) A CaP axon appears normal despite encountering a ventral muscle fiber beyond the horizontal myoseptum laser-induce to express ectopic Sema3a1 in a *hsp70:gfp-sema3a1-myc* transgenic embryo. The CaP axon immediately posterior to the experimental CaP axon maybe shorter because it is paused at the choice point. (**H**) *nrp1a* expression is downregulated in CaP neurons, despite not having reached the horizontal myoseptum because of stalling in the vicinity of a myotome cell (asterisk) laser induced to express ectopic Sema3a1 in a *hsp70:gfp-sema3a1-myc* transgenic embryo. Stars indicate CaP cell bodies. (**I**) CaPs (stars) in more caudal segments in the embryo shown in H have not yet projected axons but do express *nrp1a*. (**J**) *nrp1a* is downregulated in CaP neurons in *you-too* mutants (26 somite-stage; 22 hpf) in which CaP axons fail to extend properly along the common pathway. Scale bars: 20 μm.

As downregulation of *nrp1a* expression in CaP motoneurons correlates with extension into the Sema3a1-positive ventral pathway, we wondered if downregulation of *nrp1a* was a consequence of normal extension of the CaP axon to the choice point. This appears not to be the case as downregulation of *nrp1a* by CaPs occurred despite inhibition of their axons along the common pathway owing to laser induction of exogenous Sema3a1 in muscle pioneers (Fig. 6H, I; *n*=5). To examine this issue further, we analyzed *nrp1a* expression by CaPs in *you-too* mutants, where muscle pioneers are missing and motor axons failed to extend properly (van Eeden et al., 1996). If proper pathfinding along the common pathway to the choice point is required for downregulation of *nrp1a* by CaP motoneurons, then one might predict that CaPs would not downregulate *nrp1a* in *you-too* embryos. However, *nrp1a* was downregulated in mutant CaPs (Fig. 6J) corroborating the finding that downregulation of *nrp1a* occurs in CaPs, despite inhibition of their axons by targeted misexpression of Sema3a1. Thus, downregulation of *nrp1a* by CaPs appears to be independent of proper pathfinding along the common pathway.

Table 2. CaP axons are inhibited by laser-induced ectopic Sema3a1, but may re-extend at later stages

Amount of time following initial outgrowth	Total axons	Inhibited by the misexpressing cells	Branched near the misexpressing cells	Extended into ventral myotomes†
1.5-2.0 hours	4	4	0	0
2.5-3.0 hours	5	5	0	0
3.5-4.0 hours	9	4	2	3
4.5-5.0 hours	6	1	1	4

The proportion of CaP axons that extended into the ventral myotome beyond the horizontal myoseptal choice point increased over time from the time of initial outgrowth.
*Muscle pioneers were laser induced to express Sema3a1 in *hsp70:gfp-sema3a1-myc* embryos at 17-18 hpf, which was at the predicted time of initial outgrowth or earlier for each CaP assayed (see Fig. 1C), and were allowed to develop for 1.5-5.0 h before fixation to assay CaP axons. CaP axons normally reach the muscle pioneers 1.5 h after initial projection of axons.
†The increase in axons extending into the ventral myotomes 3.5-4.0 h and 4.5-5.0 h after initial outgrowth compared with 1.5-2.0 h and 2.5-3.0 h was significant ($P<0.02$, Fisher's test).

Interestingly, when the muscle pioneers were laser induced to express Sema3a1 at or before the time of initial outgrowth by CaP axons and assayed at various times later, the proportion of CaP axons that extended into ventral myotome beyond the horizontal myoseptal choice point increased with time (Table 2). For example 1.5-2.0 hours after the predicted time of initial outgrowth, 4/4 CaP axons were inhibited and none had extended to or beyond the horizontal myoseptum into ventral myotome. Normally CaP takes about 1.5 hours to reach the horizontal myoseptum after initial outgrowth (see Fig. 1C). However, 4.5-5.0 hours later, 2/6 were inhibited or abnormally branched and 4/6 had extended into the ventral myotome. The percent of CaP axons extending into ventral myotome increased from 0% at 1.5-2.0 and 2.5-3.0 hours later to 33% at 3.5-4.0 hours later to 67% at 4.5-5.0 hours later. Thus, it is possible that inhibition of CaP axons by the induced muscle fibers along the common pathway is temporary and that they re-extend following downregulation of *nrp1a*. These findings are consistent with the hypothesis that expression of *nrp1a* by CaP motoneurons regulates responsiveness to Sema3a1 but downregulation of *nrp1a* by CaP motoneurons can occur independently of normal extension along the common pathway.

CaP axons but not MiP nor RoP axons are responsive to Sema3a1

The MiP and RoP primary motor axons follow the pioneer CaP axon along the common pathway (Eisen et al., 1986). Labeling primary axons with mAb Znp1 suggested that MiP axons were unaffected in Sema3a1 antisense morphant embryos and when they encountered muscle pioneers induced to express exogenous Sema3a1 (see previous sections). Thus, it is possible that repulsion of primary axons by Sema3a1 may be cell type specific. However, low levels of *nrp1a* expression are visible in the ventral spinal cord region that contains MiP and RoP (Fig. 2C), and so these motoneurons may be sensitive to Sema3a1 on the common pathway. To test directly whether repulsion by Sema3a1 is cell specific, we examined primary motoneurons labeled with biotin-dextran/TRITC-dextran in embryos overexpressing Sema3a1. Mosaic embryos were generated by transplanting biotin-dextran/TRITC-dextran labeled wild-type cells into unlabeled *hsp70:gfp-sema3a1-myc* transgenic hosts at the blastomere stage (see Materials and methods). These embryos were heat induced to misexpress *sema3a1* at the 20-somite stage and embryos in which primary motoneurons were derived from labeled wild-type cells were assayed 2-4 hours after heat induction.

Table 3. Wild-type CaP but not MiP or RoP extend aberrantly when Sema3a1 is ubiquitously misexpressed

Wild-type donor cell	Total neurons	Trajectory of axons: Normal	Trajectory of axons: Aberrant*
CaP	18	3	15[†]
MiP	11	11	0
RoP	12	12	0
CaP (control)*	10	10	0

Wild-type motoneurons were transplanted into *hsp70:gfp-sema3a1-myc* transgenic hosts and then heat induced after further development (see Materials and methods).
Axons with aberrant branching and/or misrouting (P<0.003 versus control, Fisher's test).
[†]In control chimeras, wild-type cells were transplanted into wild-type embryos and treated with the heat-induction protocol.

As before, labeled CaP axons (15/18 cases) branched abnormally or followed inappropriate pathways (Fig. 7, Table 3). Transverse sections revealed that some CaP axons extended laterally into the myotomes, rather than along the medial surface of the myotome as they normally do (Fig. 7C). These abnormalities resembled what was observed in the *sema3a1* morphant embryos. This could be due to masking of a gradient of Sema3a1 or to desensitization of the CaP growth cones following exposure to high ubiquitous levels of Sema3a1. Similarity of gain- and loss-of-function phenotypes has also been observed for semaphorin and other axon guidance molecules (Walter et al., 1990; Polleux et al., 1998; Liu et al., 2003). Interestingly, the other primary motor axons, MiP (n=11) and RoP (n=12), extended normally along the common pathway. Spinal interneurons, CoPA and VeLD neurons, also extended axons normally (not shown). All three primary motoneurons projected axons normally following heat induction in control embryos in which labeled wild-type cells were transplanted into wild-type hosts. Thus, the action of Sema3a1 on the primary motor axons along the common pathway is specific to CaP among the three primary motoneurons.

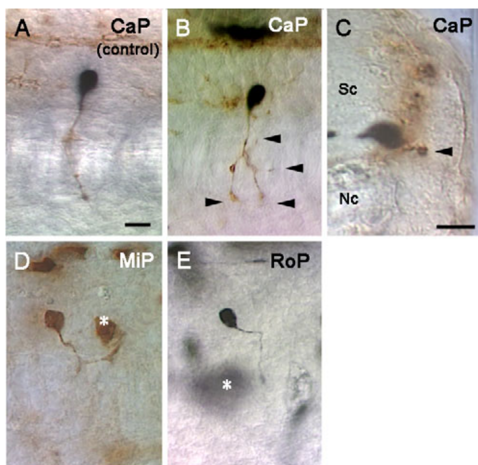

Fig. 7. Ubiquitous expression of ectopic Sema3a1 induces abnormal outgrowth by CaP axons but not MiP or RoP axons. Wild-type biotin-labeled donor cells (black) were transplanted into unlabeled *hsp70:gfp-sema3a1-myc* transgenic hosts. Hosts were heat induced and donor motor axons assayed with mAb Znp1 (brown). (**A**) Wild-type donor CaP in a heat induced wild-type host extended normally. (**B**) Wild-type CaP neuron in a *hsp70:gfp-sema3a1-myc* transgenic host branched (arrowheads) aberrantly following heat induction of Sema3a1. (**C**) Transverse section showing a donor CaP axon extending aberrantly into lateral regions of the myotome (arrowhead) in a *hsp70:gfp-sema3a1-myc* transgenic host following heat induction of Sema3a1. (**D**) A donor MiP axon extended normally in a *hsp70:gfp-sema3a1-myc* transgenic host following heat induction of Sema3a1. Asterisk indicates a non-motoneuron donor cell. (**E**) A donor RoP axon extended normally in a *hsp70:gfp-sema3a1-myc* transgenic host following heat induction of Sema3a1. Scale bars: 20 μm.

DISCUSSION

In vivo roles of Sema3a1 for development of the CaP axon

Our previous investigations of pathfinding by CaP axons suggested that initially Sema3a1 restricts CaP growth cones to the common pathway by a repulsive mechanism (Halloran et al., 2000). Here, we have shown that the loss of Sema3a1/Nrp1a signaling or ubiquitous misexpression of Sema3a1 can lead to aberrant morphology and behavior by CaP growth cones but not that of other neurons. Furthermore, we confirmed by laser induction of Sema3a1 in individual muscle fibers that CaP axons are sensitive to Sema3a1 along the common pathway up to the horizontal myoseptal choice

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point but not along the specific pathway beyond the choice point. This change in sensitivity of CaP axons to Sema3a1 is mirrored by the transient expression of *nrp1a* by CaP motoneurons.

Following knockdown of Sema3a1/Nrp1a signaling, CaP axons exhibited two distinct responses along the common pathway. First, analysis of living axons in *nrp1a:gfp* embryos demonstrated a significant increase in the number of lateral filopodia extended from the axon behind the growth cone with some of these lateral filopodia thickening into aberrant branches. This presumably accounts for the increased branching noted when CaP axons were examined statically in fixed morphant embryos. As semaphorin signaling might lead to a transient increase in intracellular Ca^{2+} (Behar et al., 1999; Sakai et al., 1999) and low levels of Ca^{2+} promotes filopodial extension while higher levels inhibit filopodia formation (Gomez et al., 2001; Lohmann et al., 2005), the increased lateral filopodia in CaP axons observed in Sema3a1 morphants could be a consequence of decreased Ca^{2+} resulting from decreased Sema3a1 signaling. The increased filopodial activity could signify that there is increased exploration of the local environment when Sema3a1/Nrp1a signaling is decreased. One interesting possibility suggested by this result is that Sema3a1 diffused from nearby myotome cells may normally act to limit exploratory behavior to keep outgrowth on target and prevent aberrant branch formation by CaP axons.

Second, CaP axons sometimes extended into more lateral muscle fibers within the horizontal myoseptal region when Sema3a1/Nrp1a signaling was knocked down. Normally CaP axons extend on the medial surface of the most medial muscle fibers that make up the common pathway within the horizontal myoseptal region. The medial fibers express little to no *sema3a1*, while the more lateral fibers express more. Thus, Sema3a1 produced by the lateral fibers may normally act to keep CaP growth cones on the medial surface of the medial cells where the concentration of Sema3a1 should be the lowest. In this regard, Sema3a1 may be acting in concert with the *diwanka* gene product. In zebrafish *diwanka* mutants, CaP axons fail to extend along the common pathway, and it has been hypothesized that *diwanka* may be required for a short range cue localized to the medial surface of the myotome that promotes axon extension (Zeller and Granato, 1999). Therefore, a combination of an attractive cue on the medial surface and repulsive cues from more lateral myotome cells may act to guide CaP growth cones along the common pathway.

At the choice point, CaP growth cones often failed to decelerate and pause when Sema3a1/Nrp1a signaling was decreased. This may signify that a low level of Sema3a1 derived by the more lateral muscle cells in the horizontal myoseptal region acts as a pause signal. This might be achieved by a combination of low level inhibitory activity of Sema3a1 and potential adhesive interactions at or near the muscle pioneers or some other as yet unknown function of semaphorins. Although how Sema3a1/Nrp1a signaling serves this action is unclear, the lack of pausing in the absence of Sema3a1/Nrp1a does suggest that semaphorins may regulate temporal aspects of axon extension. At this point, it is unclear what consequences, if any, a failure to pause may have. However, the finding that MiP and RoP are unperturbed on the common pathway in antisense morphants and/or in transgenics following ubiquitous misexpression of Sema3a1 suggests that there is no consequence for axonogenesis by these axons of the failure of CaP axons to pause at the choice point.

Genetic studies have identified a variety of cues for guidance of CaP axons. As mentioned above, *diwanka* function is needed for initial axonal extension on the common pathway, and *unplugged* is necessary for correct pathway choice at the horizontal myoseptal choice point (Zeller and Granato, 1999; Zhang and Granato, 2000). These two signals are derived from adaxial cells that are initially located at the medial edge of the somite, but later migrate laterally when the CaP axons are extending along the common pathway. *stumpy* and *topped* are required for ventral outgrowth from the choice point, and *topped* may function as a short-range attractive cue derived from the ventromedial myotome (Beattie et al., 2000; Rodino-Klapac and Beattie, 2004). Our results demonstrate that Sema3a1 signaling is also involved in the guidance of CaP axons. In fact, the stalled axons and increased branching observed when Sema3a1/Nrp1a signaling is decreased is reminiscent of the stalling and increased branching observed in *unplugged* embryos in which a MuSK-like gene is mutated (Zhang et al., 2004), suggesting that these two signaling systems may work together to guide CaP axons. Thus, Sema3a1/Nrp1a signaling is part of a complex network of guidance cues that guides CaP axons from the cell bodies to their target muscles.

Regulation of Sema3a1 sensitivity of CaP axons

CaP axons extend along the common pathway to the choice point at the horizontal myoseptum. During outgrowth along the common pathway, CaP axons are sensitive to Sema3a1 but then lose this sensitivity beyond the choice point. The loss of sensitivity to Sema3a1 is presumably important for CaP axons as they extend into Sema3a1-expressing ventral myotome after pausing at the choice point. How do CaP axons lose their sensitivity to Sema3a1? Contact with the muscle pioneers that are located at the choice point appear not be necessary for this change. CaP axons can enter the ventral myotome despite the elimination of muscle pioneers (Melancon et al., 1999). We found that the expression of one component of the Sema3a receptor, Nrp1a, correlates with the decrease in responsiveness to Sema3a1. Thus, downregulation of the Sema3a1 receptor by CaP axons could account for the decrease in sensitivity to Sema3a1. The finding that muscle pioneers are dispensable for extension onto the ventral myotome suggests that downregulation of Nrp1a may be independent of interactions with the muscle pioneers. In fact, when CaP axons were stalled along the common pathway because of encounters with a Sema3a1 misexpressing myotome cell, downregulation of *nrp1a* still occurred, even though the axons had not reached the choice point. Thus, the downregulation of *nrp1a* by CaP motoneurons is not a consequence of normal axon extension along the common pathway. Some other mechanism, perhaps a cell-autonomous one, may regulate *nrp1a* expression and thus sensitivity of CaP axons to Sema3a1. Interestingly, the offset of the Tag1 cell-adhesion molecule on spinal commissural axons coincides with the arrival of the axons at the floor plate, but this downregulation can occur independently of the floor plate (Dodd et al., 1988; Karagogeos et al., 1991), as it can with Nrp1a.

Regulation of responsiveness to several other guidance factors have been analyzed. Netrin/DCC signaling on commissural axons is silenced by Slit/Robo signaling once at the floor plate (Stein and Tessier-Lavigne, 2001; Sabatier et al., 2004; Long et al., 2004). In *Drosophila*, sensitivity of commissural axons to Slit is regulated by midline Comm expression by keeping Robo in intracellular compartments rather than the axonal surface (Keleman et al., 2002). Synthesis of guidance receptors can also regulate sensitivity for Epha2, the mRNA of which is transported to and translated in distal segments of commissural axons as they contact the floor plate to presumably mediate sensitivity to ephrins once the axons cross the midline (Brittis et al., 2002).

Other mechanisms for regulation of responsiveness to Sema3a1 by CaP axons, besides potential regulation of Nrp1a, may also be important for pathfinding by CaP axons. In *Xenopus*, retinal growth cones adapt to Sema3a via endocytosis-mediated desensitization followed by protein synthesis-dependent resensitization (Piper et al., 2005). Signaling that modulates the levels of cGMP can convert the Sema3a response of *Xenopus* spinal growth cones from repulsion to attraction (Song, 1998). Similarly, neurotrophins and chemokines can regulate the response of DRG growth cones to Sema3a (Tuttle et al., 1998; Dontchev et al., 2002; Chalasani et al., 2003). In fact, the secreted chemokine Sdf1 can inhibit the repulsive response of growth cones to Sema3a (Chalasani et al., 2003). Interestingly, Sdf1a is expressed by the horizontal myoseptum (Li et al., 2004) and motoneurons express the Sdf1 receptor, Cxcr4b (Chong et al., 2001) in zebrafish embryos, making them potential modulators of repulsion induced by Sema3a1. Thus, it is possible that several mechanisms, including the downregulation of *nrp1a* may participate in insuring proper guidance of CaP axons along the common and specific pathways.

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