Development 126, 2007-2019 (1999)
Printed in Great Britain © The Company of Biologists Limited 1999
DEV8593

Discrete roles for secreted and transmembrane semaphorins in neuronal growth cone guidance in vivo

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Accepted 16 February; published on WWW 6 April 1999

SUMMARY

From the initial stages of axon outgrowth to the formation of a functioning synapse, neuronal growth cones continuously integrate and respond to multiple guidance cues. To investigate the role of semaphorins in the establishment of appropriate axon trajectories, we have characterized a novel secreted semaphorin in grasshopper, gSema 2a. Sema 2a is expressed in a gradient in the developing limb bud epithelium during Ti pioneer axon outgrowth. We demonstrate that Sema 2a acts as chemorepulsive guidance molecule critical for axon fasciculation and for determining both the initial direction and subsequent pathfinding events of the Ti axon projection. Interestingly, simultaneous perturbation of both secreted Sema 2a and transmembrane Sema I results in a broader range and increased incidence of abnormal Ti pioneer axon phenotypes, indicating that different semaphorin family members can provide functionally distinct guidance information to the same growth cone in vivo.

Key words: Secreted semaphorin, Transmembrane semaphorin, Chemorepulsion, Axon guidance, Grasshopper, *Drosophila*, Semaphorin

INTRODUCTION

The establishment of accurate neuronal connectivity is a sequential process involving directed axon initiation, growth cone pathfinding, target selection and finally synapse formation. During each of these stages, neuronal growth cones are guided by many mechanisms, including both attractive and repulsive guidance molecules (reviewed by Goodman, 1996; Tessier-Lavigne and Goodman, 1996).

Several gene families contain members that have been implicated in growth cone guidance, and it is becoming clear that guidance decisions are influenced by a balance of attractive and repulsive signals recognized by migrating growth cones (Stoeckli, 1997; Winberg et al., 1998). Proteins belonging to the semaphorin gene family have been demonstrated to function in both vertebrate and invertebrate nervous systems to mediate axon pathfinding and target selection (Culotti and Kolodkin, 1996; Mark et al., 1997). This large family of guidance molecules, comprising both transmembrane and secreted glycoproteins, is characterized by a conserved ~500 amino acid extracellular semaphorin (sema) domain. The first functionally characterized semaphorins were grasshopper semaphorin I (gSema I, formerly Fasciclin IV), a transmembrane semaphorin shown to be essential for correct neuronal pathfinding in the developing grasshopper limb bud (Kolodkin et al., 1992) and Collapsin-1 (Coll-1), a secreted semaphorin in chick shown to collapse dorsal root ganglion growth cones in vitro (Luo et al., 1993). Since then as many as 30 semaphorins, which can be subdivided into at least 7 structurally distinct classes, have been identified in many animal species from worms to mammals (Mark et al., 1997).

Secreted semaphorins are characterized by the conserved sema domain, an immunoglobulin (Ig) domain C-terminal to the sema domain and, in vertebrates, a carboxy terminal basic domain. Results from a variety of systems have demonstrated that these proteins can function as inhibitory neuronal guidance cues (reviewed by Mark et al., 1997). Evidence implicating secreted semaphorins in chemorepulsion came initially from the finding that chick Coll-1 can cause the collapse of chick dorsal root ganglion growth cones in vitro (Luo et al., 1993). This collapse can direct growth cones to turn away from a source of Coll-1 by inducing a localized reorganization of the actin cytoarchitecture within the growth cone (Fan et al., 1993; Fan and Raper, 1995). Later studies showed that the mammalian orthologues of chick Coll-1, human Sema III/mouse Sem D, could act as repellents to pattern sensory and motor axon projections in the spinal cord and brain (Messersmith et al., 1995; Behar et al., 1996; Puschel et al., 1996; Tanelian et al., 1997; Taniguchi et al., 1997; Varela-Echavarria et al., 1997). Further evidence for an inhibitory function for secreted semaphorins comes from invertebrate studies, where *Drosophila* semaphorin 2a (dSema 2a) has been shown to function as a selective target-derived cue capable of inhibiting synaptic arborization formation (Matthes et al., 1995; Winberg et al., 1998; the original D-Sema II has been renamed dSema 2a, C. Goodman, personal communication). Taken together, the

results from invertebrate and vertebrate studies suggest that the secreted semaphorins are capable not only of inducing growth cone collapse but also of directing growth cone pathfinding, target selection and synaptic terminal arborization.

The role of transmembrane semaphorins in neuronal development is less well characterized. Similar to secreted semaphorins, genetic analysis has shown the transmembrane *Drosophila* semaphorin D-Sema I acts as a repellent and is important for the guidance of CNS and motor neurons (Yu et al., 1998). In vivo antibody perturbation experiments revealed that g-Sema I, a transmembrane semaphorin expressed in the developing grasshopper limb bud, is important for establishing the peripheral Ti pioneer projection into the CNS; however, it remains unclear whether it performs this role as a repellent or an attractant (Kolodkin et al., 1992). In addition, evidence suggests that Sema I acts as an attractive guidance cue for later arising neurons in the limb bud (Wong et al., 1997).

Genetic analysis in *Drosophila* demonstrates that the invertebrate secreted semaphorin dSema 2a acts in combination with a variety of other molecules, including Netrin B and Fasciclin II, to inhibit promiscuous synaptogenesis and to provide a repulsive force for motor neuron growth cones at specific choice points. This clearly demonstrates that growth cones integrate multiple signals during pathfinding and target selection (Winberg et al., 1998). The presence of multiple signaling molecules and guidance cues in the developing grasshopper limb during the period of Ti pioneer axon outgrowth provides a powerful model system for the investigation of functional interactions among these cues (reviewed by Sanchez et al., 1995). Therefore, to elucidate the interaction between different members of the semaphorin family on the guidance of a single well-characterized neuron projection, we have identified and characterized a novel secreted semaphorin in grasshopper, gSema 2a.

During the period of Ti pioneer axonogenesis and extension toward the CNS, gSema 2a is expressed in a striking gradient by the epithelium of the developing limb bud. Our in vivo antibody perturbation results demonstrate this is a chemorepulsive gradient, critical during several key stages of the Ti pioneer pathway. Initially, Sema 2a chemorepulsion is required for the reliable establishment of proximal Ti axonogenesis; thereafter, the gradient of Sema 2a directs growth cone pathfinding, maintains fasciculation and ensures continued proximal extension towards the CNS. Secreted semaphorins can, therefore, contribute to both the initial stages of directed axon outgrowth as well as subsequent growth cone pathfinding events. We further show that in vivo antibody perturbation of both secreted Sema 2a and transmembrane Sema I results in novel Ti axon defects and an increased incidence of defects. This is the first study to demonstrate that different members of the semaphorin family can provide independent guidance information to the same neuronal growth cone in vivo. Therefore, at all stages of outgrowth and pathfinding, growth cone decisions are based on the integration of multiple guidance signals.

MATERIALS AND METHODS

PCR methods, cDNA isolation and sequence analysis

cDNA to poly(A)+ RNA from grasshopper embryos at ~45% of development was prepared and used at 10 ng per 100 μl PCR reaction. PCR was performed using Taq polymerase (Saiki et al., 1988) and the partially degenerate oligonucleotides GCGAATTCTT[CT]TT[CT]-TT[CT]CGNGA[AG]ACNGC (corresponding to an *Eco*RI site and amino acids (aa) FFFRKTA) and GCGAATTCTCCCANGC[GA]-CA[GA]TANGG[GA]TC (corresponding to aa DPYCAW[D/E] and an *Eco*RI site). PCR cycling conditions were: 40 cycles of 94°C for 1.5 minutes, 46°C for 1.5 minutes, 72°C for 2.5 minutes, followed by one cycle of 72°C for 10 minutes. A 700 bp product was obtained, subcloned into pBluescript (Stratagene), sequenced on both strands using a Perkin Elmer Applied Biosystems Division 373a automated DNA sequencer and found to encode a portion of a novel semaphorin domain. This amplification product was used to screen 1.25×10^6 clones from a λgt11 grasshopper embryonic cDNA library (Snow et al., 1988). A single positive cDNA clone was isolated, sequenced in its entirety and found to encode an incomplete open reading frame (ORF) for Sema 2a. Subsequent screening of a second embryonic grasshopper λZAP (Stratagene) cDNA (Seaver et al., 1996) library using this clone resulted in the isolation of ~20 positive clones. One clone was sequenced on both strands over the Sema 2a ORF and immediatly flanking 5′ and 3′ untranslated regions. This clone was used for all subsequent analyses and cloning.

In situ hybridization and northern blot analysis

Nonradioactive, digoxigenin (DIG-11-UTP)-labelled cRNA probes with either sense or antisense orientation were synthesized by run-off in vitro transcription using T3 and T7 RNA polymerases (Boehringer Mannheim). Probes were generated from a 700 base pair region of the conserved sema domain of Sema 2a. In situ hybridization for Sema 2a was performed using the above described digoxigenin-labeled cRNA antisense and sense riboprobes on whole fixed embryos. *Schistocerca gregaria* embryos were obtained from a colony maintained at the University of British Columbia and staged by percentage of total embryonic development (Bentley et. al., 1979). Embryos at developmental stages 30-45% were dissected in phosphate-buffered saline (PBS), fixed for 50 minutes in PEM-FA (0.1 M PIPES [pH 6.95], 2.0 mM EGTA, 1.0 mM $MgSO_4$, 3.7% formaldehyde), washed for 1 hour with three changes in PBT (1× PBS, 0.5% Triton X-100, 0.2% bovine serum albumin), then incubated for 5 hours at room temperature in prehybridization buffer (50% formamide, 5× SSC, 5× Denhardt's, 250 μg/ml Baker's Yeast RNA, 500 μg/ml sheared Herring Sperm DNA). Prehybridization buffer was replaced with hybridization buffer (fresh prehybridization buffer plus 500 ng/ml sodium carbonate hydrolyzed digoxigenin-labeled cRNA probe, which was previously heated to 80°C and placed on ice), and incubated overnight at 53°C in a rotating incubator. Following overnight hybridization, non-hybridized probe was removed by first rinsing in 5× SSC at room temperature for 10 minutes, washed in 0.2× SSC for 60 minutes at 53°C, followed by a final wash in 0.2× SSC at room temperature for 10 minutes. At room temperature, embryos were then rinsed in maleic acid buffer (0.1 M maleic acid, 0.15 M NaCl pH 7.5) for 5 minutes, blocked for 60 minutes (blocking buffer: 1% milk powder in 0.1 M maleic acid, 0.15 M NaCl pH 7.5), and incubated with anti-digoxigenin antibody 1:10000 dilution in blocking buffer for 60 minutes. Embryos were rinsed twice for 30 minutes with maleic acid buffer, equilibrated in Tris saline (100 mM Tris-HCl pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$) for 5 minutes, and incubated overnight in Tris saline plus 0.34 mg/ml NBT and 0.18 mg/ml BCIP. For overnight incubation, Eppendorf tubes were wrapped in aluminum foil and placed on a rocking platform. The reaction was stopped by rinsing 3× with PBS, and then incubated for 30 minutes in PBS. Embryos were cleared with 70% glycerol and mounted in 90% glycerol.

For northern blot analysis, total RNA was extracted from stage 35% embryos using Trizol (Gibco BRL), separated by electrophoresis through a 1.1% agarose-formaldehyde gel, blotted onto Hybond-N+ (Amersham), and hybridized with either the digoxigenin-labeled cRNA probe used for in situ hybridization or a random primed P^{32}-

dATP-labeled DNA probe corresponding to the same 700 base pair region.

gSema 2a antisera production and immunoblot analysis

Anti-grasshopper Sema 2a polyclonal antibodies were produced by immunizing rabbits with 6-histidine-tagged Sema 2a fusion proteins that were expressed in *E. coli*. The bacterial expression constructs were made by PCR amplification of each fragment of Sema 2a and inserted into the *Hin*dIII and *Sph*I sites of pQE30 (Qiagen). Expressed protein was purified by immobilized nickel-chelate affinity chromotography (Qiagen). The fusion proteins of Sema 2a corresponded to the following 5 different regions; amino acids 32-151 (SD1; 13 kDa N-terminal sema domain peptide), amino acids 152-393 (SD2; 26 kDa middle sema domain peptide), amino acids 394-506 (SD3; 13 kDa C-terminal of sema domain peptide), amino acids 516-697 (CT1; 20 kDa C-terminal and Ig domain peptide), and amino acids 525-697 (CT2; 7 kDa C-terminal peptide). In total, 9 rabbits were injected with Sema 2a fusion proteins. Two antibody stocks were generated against SD1, one against SD2, and two against SD3; within the C terminus, two antibody stocks were generated against CT1 and two were generated against the fusion protein CT2. IgG antibodies were purified by protein A-Sepharose chromatography (Pierce) and Fab fragments were generated using ImmunoPure Fab Preparation Kit (Pierce). All antibodies were stored at −20°C. For blocking experiments, antibodies were dialyzed against RPMI and stored at −20°C. For western blot analysis, grasshopper embryonic stage 30-40% whole-cell lysate was probed with the various Sema 2a antibodies (1:1000) and reacted for ECL chemiluminescence as described by the manufacturer (Amersham).

Immunocytochemistry

Schistocerca gregaria embryos at developmental stages 30-40% were dissected, fixed and washed as above for in situ hybridization, and then incubated overnight at 4°C in primary antibody. For Sema 2a staining, whole sera and purified IgGs were diluted 1:500 in PBT; for neuronal staining, rabbit and goat anti-HRP (Jackson Immunoresearch Laboratory) were diluted 1:1000 in PBT. The embryos were washed for 1 hour in PBT with three changes and incubated in the appropriate secondary antibody (1:250 dilution in PBT) for 2 hours at room temperature. Following secondary incubation, embryos were washed for 1 hour with six changes of PBT. Fluorescently labelled embryos were mounted in Slowfade antifade reagent (Molecular Probes) and viewed under fluorescence microscopy.

For Sema 2a/Sema I double-labelling, embryos were incubated simultaneously in the two primary antibodies overnight (1:500 Sema 2a, 1:1 Sema I mAb 6F8), the embryos were washed and the Sema I antibody 6F8 visualized by incubating in secondary antibody Cy3-conjugated goat anti-mouse (1:250) for 2 hours at room temperature, followed by washing and then visualization of Sema 2a antibody by incubating in secondary antibody FITC-conjugated goat anti-rabbit (1:250) for 2 hours at room temperature. The embryos were washed and mounted as above. For Sema 2a and neuronal double labelling, embryos were labeled sequentially for anti-HRP followed by Sema 2a.

Antibody blocking experiments

For all blocking experiments, dialyzed IgGs and Fab fragments were diluted into freshly made supplemented RPMI culture media plus 10% FBS (Wong et. al., 1997). Prior to culturing, embryos from a single clutch were sterilized, dissected and the entire amnion and dorsal membrane removed from the embryo to ensure access of the antibodies during culturing. As embryos within a single clutch typically differ by less than 1% of embryonic development (Bentley et al., 1979), each clutch was randomly divided into experimental and control groups, with several embryos fixed immediately to determine developmental stage at the start of culturing. Embryo culturing began at 30% of development, just prior to Ti pioneer neurons axonogenesis. Following approximately 30 hours in culture, embryos were fixed and immunostained with antibodies to HRP (Jan and Jan, 1982) for visualization of Ti axons and other neurons.

For each culture experiment, limbs from antibody-cultured embryos were compared with limbs from non-antibody-cultured and preimmune-antibody-cultured embryos. For embryos incubated in Sema 2a alone, the Ti pathway was scored as abnormal for one or more of the following observed characteristics: defasciculation for a minimum distance of 50 μm anywhere along the pathway, dorsal projection of one or both axons within the femur for a minimum distance of 25 μm, distal projection of one or both axons, failure of one or both axons to turn ventrally at the trochanter-coxa segment boundary typically characterized by continued proximal extension towards the CNS, or dorsal extension toward the Cx2 cells. For embryos incubated in Sema I alone, the Ti pathway was scored as abnormal for one or more of the following observed characteristics (Kolodkin et al., 1992): defasciculation for a minimum distance of 25 μm anywhere along the pathway, multiple axon branches that extended ventrally within the trochanter, axon branches crossing the trochanter-coxa boundary dorsal to the Cx1 cells, and axon branches that crossed the trochanter-coxa segment boundary, did not turn ventrally, but continued proximally toward the CNS. For embryos incubated in both Sema 2a and Sema I, the Ti pathway was scored as abnormal if it met any one of the criteria described above for either Sema 2a or Sema I. The novel hybrid phenotype defects observed were characterized by defasciculation anywhere along the pathway accompanied by both extensive axonal branching and distal or dorsal projection abnormalities. In the hybrid phenotypes, a variety of dorsal projection defects were observed including continued proximal extension in the dorsal compartment, extensive fasciculation with the Cx2 cells, or a ventral turn within the coxa to contact the Cx1 cells. For each antibody tested, the data are presented as a percentage of the abnormal Ti pathways observed. Error bars = s.e.m., calculated using the number of experiments and their average observed aberrance; *n* = number of limbs scored for each treatment.

RESULTS

Identification of a novel secreted grasshopper semaphorin gene

The conservation of the 500 amino acid sema domain among members of the semaphorin gene family enabled us to use a degenerate PCR-based strategy to isolate a new secreted grasshopper semaphorin, called gSema 2a (see Experimental Procedures). Sema 2a is a secreted protein that is most similar to *Drosophila* semaphorin 2a (dSema 2a), with which it shares 67% amino acid identity within the sema domain and 63% within the C terminus (Fig. 1). Grasshopper Sema 2a contains a semaphorin domain and an Ig domain; however, similar to dSema 2a, gSema 2a does not have the basic C-terminal domain observed in the vertebrate homologues. Grasshopper Sema 2a shares approximately 34% and 23% amino acid identity with human Sema III/mouse Sem D/chick Coll-1 within the sema and C-terminal domain, respectively. A comparison of the secreted semaphorin gSema 2a and the transmembrane grasshopper semaphorin Sema I reveals the two grasshopper semaphorins share 37% amino acid identity within the sema domain.

Initial characterization of Sema 2a by northern blot hybridization revealed a single transcript of approximately 5 kb (data not shown). Western blot analysis, using antibodies generated against three regions of the sema domain (SD1-3) and two regions of the C-terminal domain (CT1 and CT2) (Fig.

2A), detected a protein at approximately 80 kDa in grasshopper lysate (Fig. 2B,C). Duplicate antibodies from independent rabbits were generated against all regions except SD2, and all antibodies detected the same 80 kDa band. Preimmune sera revealed no bands at 80 kDa (Fig. 2C) and none of the Sema 2a antibodies cross react with Sema I (Fig. 2D).

Grasshopper semaphorin expression is developmentally regulated

Sema 2a is expressed in the embryo prior to axonogenesis (<29%) and persists through 40% embryonic development, the latest stage that we examined. At approximately 30% of development, a gradient of Sema 2a is evident in the limb bud epithelium with the highest protein distribution occurring in the distal and dorsal-most aspect of the limb (Fig. 3A). This gradient of Sema 2a expression coincides with the differentiation and subsequent axonal projection of the well-characterized Ti pioneer neurons (which occurs between 30 and 35% of development), which establishes the first peripheral projection into the CNS from the limb (Fig. 4A,B,F). Sema 2a does not appear to be expressed by the Ti neurons or other limb pre-axonogenesis neurons during this stage of development. By approximately 32% of development, as the Ti growth cones approach the trochanter segment epithelium, transmembrane Sema I expression is also evident within the developing limb bud and is localized to a circumferential band in the trochanter (Fig. 4B,C). While Sema 2a and Sema I are both expressed in the dorsal trochanter epithelium, little or no coexpression is observed elsewhere in the limb at this stage (Fig. 4C).

At approximately 33% of development, Sema 2a expression remains high in the distal and dorsal limb bud; however, increasing expression is also observed in the ventral epithelium distal to the trochanter Sema I band (Fig. 3B). By this stage (33%), the Ti growth cones have migrated proximally out of the Sema 2a gradient and have contacted the Sema I-expressing trochanter epithelium, where they make a stereotyped ventral turn (Fig. 4F). Sema 2a expression at developmental stages after the completion of the Ti projection (>35%) is localized to epithelial bands in the distal femur, mid to distal half of the tibia and the distal tip of the tarsus (Figs 3C-E, 4D-F). Interestingly, the circumferential epithelial expression pattern of Sema 2a is, for the most part, complementary to the circumferential epithelial expression of transmembrane Sema I (Fig. 4D-F). In general, within each limb segment Sema 2a is expressed in circumferential bands in the mid to distal region of each segment, while Sema I is expressed in the proximal region (Fig. 4D,E). The spatial and temporal distribution of Sema I and 2a suggests that these molecules may be involved in the guidance of a variety of neurons that arise during limb bud development (Wong et al., 1997).

In addition to the epithelial expression, Sema 2a is expressed by presumptive muscle precursor cells within the developing limb bud and throughout the entire embryo (Fig. 3D), laminar cells within the developing eye (Fig. 3H) and in circumferential epithelial bands of the antennae (Fig. 3I). In particular, high levels of Sema 2a expression are evident in the precursor muscle cells of the extensor tibiae and the levator and depressor tarsus, two large muscle groups in the developing limb bud. Interestingly, pan muscle expression of secreted dSema 2a was observed in *Drosophila* embryos, and here it was shown to act as an inhibitory guidance cue necessary for accurate motor neuron target selection and synapse formation (Winberg et al., 1998).

At all stages examined, the distribution of Sema 2a protein detected with the different antisera corresponds identically to the sites of Sema 2a gene expression as detected by in situ hybridization (Fig. 3F,G). Furthermore, all antibodies generated against the different regions of Sema 2a produced the same immunocytochemistry results, including experiments using duplicate sera generated from independent animals and C-terminal antibodies.

Antibodies directed against the conserved sema domain of gSema 2a disrupt Ti pioneer axon pathfinding

The striking gradient of Sema 2a protein in the developing limb bud during the period of Ti pioneer neuron axonogenesis and

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gSema 2a                                    MAAKLWNLLLVAASVHLVGSVEQLHQDLIHE    31
dSema 2a    MSLLQLSPLLALLLLLCSSVSETAADYENTWNFYYERPCCTGNDQGNNNYGKHGADHVRE    60

                                            #
gSema 2a    FSCGHKYYRTFHLDEKRESLYVGALDKVYKLNLTNISLSDCERDSLTLEPTNIAN--CVSK    90
dSema 2a    FNCGKLYYRTFHMNEDRDTLYVGAMDRVFRVNLQNISSSNCNRDAINLEPTRDDVVSCVSK   121

gSema 2a    GKSADFDCKNHIRVIQPMGDGSRLYICGTNAHSPKDWVVYSNLTHLQRHEYVPGIGVGIAK   151
dSema 2a    GKSQIFDCKNHVRVIQSMDQGDRLYVCGTNAHNPKDYVIYANLTHLPRSEYVIGVGLGIAK   182

gSema 2a    CPFDPEDSSTAVWVENGNPGDLPGLYSGTNAEFTKADTVIFRTDLYNLTTGRREYSFKRTL   212
dSema 2a    CPYDPLDNSTAIYVENGNPGGLPGLYSGTNAEFTKADTVIFRTDLYNTSAKRLEYKFKRTL   243

gSema 2a    KYDSKWLDNPNFVGSFDVGEYVLFFFRETAVEYINCGKSVYSRVARVCKKDVGGKNILSQN   273
dSema 2a    KYDSKWLDKPNFVGSFDIGEYVYFFFRETAVEYINCGKAVYSRIARVCKKDVGGKNLLAHN   304

gSema 2a    WATFLKARLNCSIPGEFPFYFNEIQGVYK-MPNTDKFFGVFSTSVTGLTGSAICSFTLKDI   333
dSema 2a    WATYLKARLNCSISGEFPFYFNEIQSVYQLPSDKSRFFATFTTSTNGLIGSAVCSFHINEI   365

gSema 2a    QEVFSGKFKEQATSSSAWLPVLPREVPDPRPGECVNDTELLPDTVLNFIRSHPLMDGAVSH   394
dSema 2a    QAAFNGKFKEQSSSNSAWLPVLNSRVPEPRPGTCVNDTSNLPDTVLNFIRSHPLMDKAVNH   426

gSema 2a    EGGKPVFYKRDVLFTQLVVDKLKVNLVGKNMEYIVYYAGTSTGQVYKVVQWYDSGGLPQSL   455
dSema 2a    EHNNPVYYKRDLVFTKLVVDKIRID-IL-NQEYIVYYVGTNLGRIYKIVQYYRNGESLSKL   485

gSema 2a    LVDIFDVTPPEPVQALHLSKEYKSLYAASDNIVRQIELVMCHHRYSNCLQCARDPYCGWDR   516
dSema 2a    L-DIFEVAPNEAIQVMEISQTRKSLYIGTDHRIKQIDLAMCNRRYDNCFRCVRDPYCGWDK   545

                                                #
gSema 2a    DSNSCKSYNPGLLQDVTN-TSANLCEHSVMKKKLIVTWGQSIHLGCFLKVPEVLSSQTISW   576
dSema 2a    EANTCRPYELDLLQDVANETS-DICDSSVLKKKIVVTYGQSVHLGCFVKIPEVLKNEQVTW   605

gSema 2a    VHYTKDKGRYPIVYRPDKYIETSEHGLVLISVTDSDSGRYDCWLGGSLLCSYNITVDAHRC   637
dSema 2a    YHHSKDKGRYEIRYSPTKYIETTERGLVVVSVNEADGGRYDCHLGGSLLCSYNITVDAHRC   666

gSema 2a    SAPGGSNDYQKIYSDWCHEFERSKIAMKTWERKQAQCSTKQNNS-NQKTHPNDIFHSNPVA   697
dSema 2a    TPPNKSNDYQKIYSDWCHEFEKYKTAMKSWEKKQGQCSTRQNFSCNQ--HPNEIFRKPNV   724
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Fig. 1. Amino acid comparison of gSema 2a and dSema 2a. Amino acid sequence alignment between gSema 2a and dSema 2a. Identical amino acids are shown in bold, signal sequences in italics and the Ig domains are underlined. The beginning and end of the semaphorin domains are indicated by a # symbol. Database accession no.: AF134904.

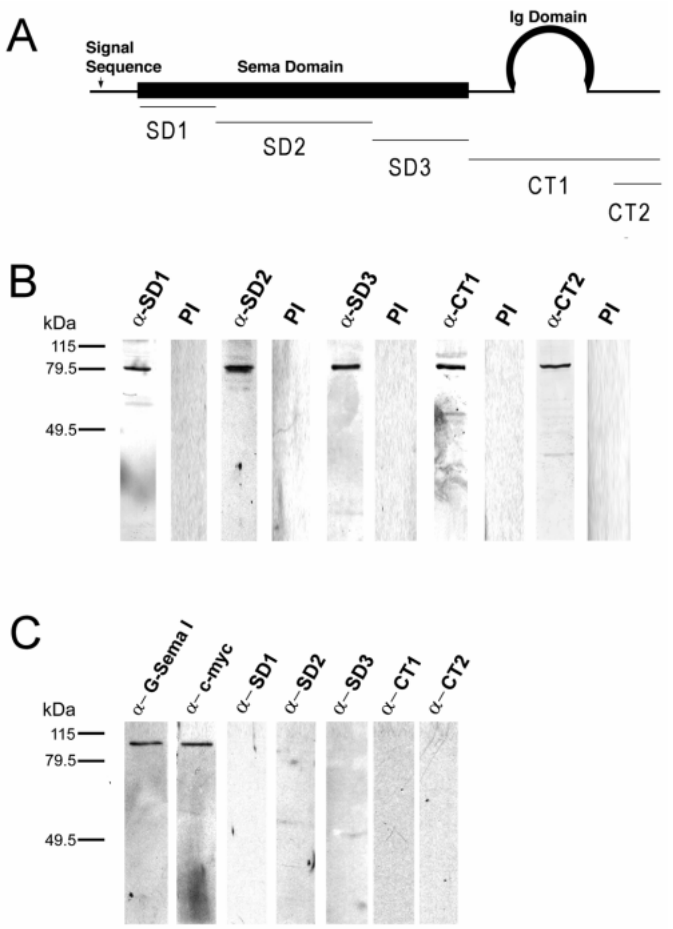

Fig. 2. Recombinant peptides generated for antibody production. (A) Schematic representation of the regions of Sema 2a used to generate antigenic fusion proteins; Sema Domain 1 (SD1), Sema Domain 2 (SD2), Sema Domain 3 (SD3), C terminus 1 (CT1) and C terminus 2 (CT2). See Experimental Procedures for amino acid number. (B) Western blot analysis of whole-cell lysate from grasshopper embryos at 31% of development using the antibodies generated against the various regions of Sema 2a. A molecular weight of 80 kDa was consistently observed. PI, preimmune. (C) Western blot detection of a myc-tagged Sema I fusion protein with Sema I monoclonal antibody 6F8 (α-gSema I), monoclonal c-myc antibody and polyclonal antibodies to Sema 2a. None of the Sema 2a antibodies recognized Sema I.

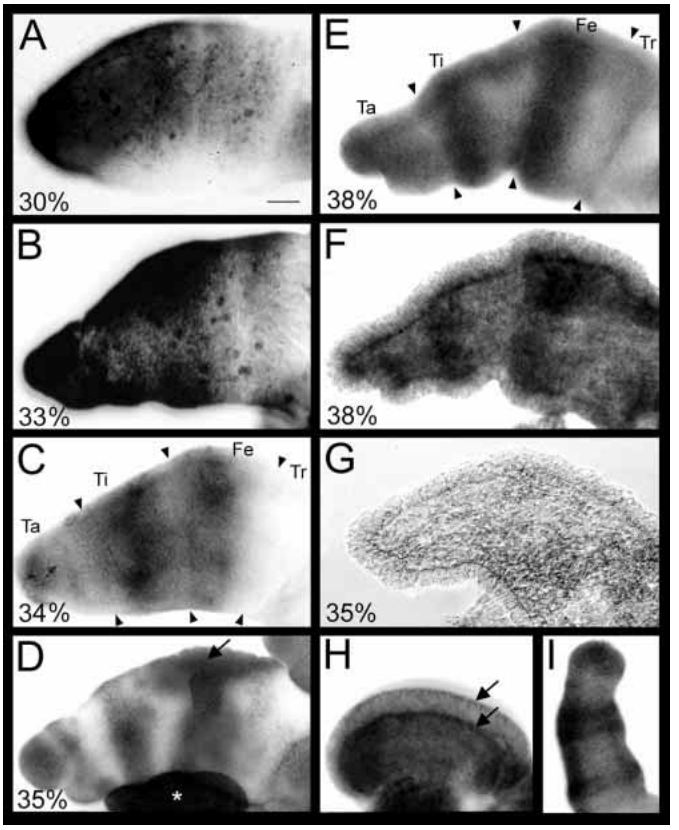

Fig. 3. Sema 2a is expressed in the developing limb bud during Ti pioneer axon outgrowth. (A) Immunocytochemistry at 30% of embryonic development reveals that Sema 2a is expressed throughout much of the limb bud epithelium. A gradient is evident, with the highest concentration of Sema 2a localized to the distal and dorsal aspect of the limb bud. (B) At approximately 33% of development, Sema 2a expression is still high in the dorsal and distal regions of the limb. An increase in expression is also apparent in the ventral region of the limb. (C) By 34% of development, Sema 2a becomes restricted to circumferential bands of epithelial staining. The most proximal edge of Sema 2a expression ends in the proximal femur, near the femur-trochanter segment boundary. (D) By 35% development, epithelial bands are evident in the mid to distal femur, distal half of the tibia and the distal tip of the tarsus. At this stage presumptive muscle staining is apparent in the developing limb (arrow demarcates extensor tibiae in dorsal femur). Sema 2a is heavily expressed in pluripodia (asterisk). (E) Circumferential epithelial band expression pattern is still apparent at 38% of development. (F) Dig-RNA antisense labeling of a limb at 38% of development revealed Sema 2a mRNA localization is identical to protein expression pattern in the developing limb bud. Circumferential bands are apparent in the mid to distal femur, tibia and tarsus, similar to the antibody labeling (E). (G) Dig-RNA sense strand in situ hybridization of a limb at approximately 35% of development. (H,I) In situ hybridization with Dig-RNA antisense shows Sema 2a expression in laminar cells of the eye (arrows) and circumferential epithelial bands in the antennae. For all limb figures, distal is to the left and dorsal is up. Immuncytochemistry using antibodies generated against SD1 is shown (A-E); however, the same staining pattern was seen with all antibodies. Arrowheads demarcate segment boundaries. Ta, Tarsus; Ti, Tibia; Fe, Femur; Tr, Trochanter. Scale bar is 50 μm in A, 60 μm in B,C, 65 μm in D,G, 70 μm in E,F,H,I.

outgrowth suggests that Sema 2a directs growth cone guidance at this location. To investigate the function of Sema 2a, we cultured grasshopper embryos in the presence of Sema 2a antibodies during the 5% of development necessary for the establishment of Ti pioneer projection into the CNS. For each culture experiment, we compared the percentage of aberrant pathways observed following incubation in RPMI media alone with that observed following the addition of Sema 2a-purified IgG, Fab fragments or purified IgG isolated from preimmune sera. Given that all the Sema 2a antibodies generated (including duplicate sera from independent animals) demonstrated the same high degree of specificity for Sema 2a protein on western blots and the same immunocytochemistry results, all antisera were tested for function blocking activity.

Under normal conditions, the Ti neurons establish a stereotyped projection through the limb bud into the CNS. Typically the growth cones of the Ti neurons extend proximally

along the femur epithelium until they contact and reorient toward a pre-axonogenesis neuron (Tr cell) located in a circumferential band of Sema I-expressing epithelium (Fig. 4A-C,F). The growth cones then extend ventrally along the Sema I-expressing epithelium before contacting and reorienting toward another pre-axonogenesis neuron (Cx1) located in the coxa. After contact with the Cx1 cells, the growth cones extend proximally into the CNS. The two Ti axons typically remain fasciculated along the entire length of the projection. For the majority of antibody perturbation experiments, we dissected and cultured embryos at approximately 30% of development, the stage just prior to Ti pioneer neuron axonogenesis (Figs 4A, 5A).

Embryos cultured in the presence of culture media alone, preimmune antiserum IgG or 1 μM CT2 antiserum IgG or Fab fragments, had no observable effect on Ti axon pathfinding (Figs 5B,G,H, 6). In contrast, embryos cultured in the presence of antibodies directed against the semaphorin domain (SD1-3) exhibited a number of aberrant projections which typically fell into three classes. These include: (1) the direct distal growth of one of the Ti axons (Fig. 5C), (2) defasciculation of the two pioneer neuron axons along the entire length of the femur, followed by the dorsal projection of at least one of the axons (Fig. 5D) and (3) dorsal and distal growth of at least one of the axons (Fig. 5E,F). Often the dorsal projecting axons would loop

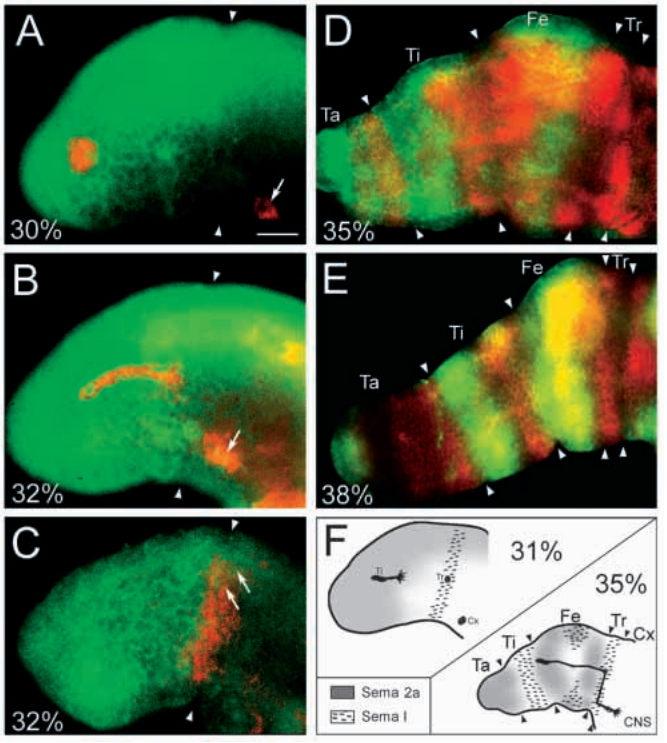

Fig. 4. Sema 2a and Sema I expression during Ti pioneer axon outgrowth. (A,B) Sema 2a and anti-HRP double staining at 30% and 32% development, respectively (green: Sema 2a protein; red: HRP-stained neurons; arrowheads demarcate the region of the trochanter limb segment; arrows indicate the Cx1 cells located proximal to the Tr limb segment). (C) Sema 2a and Sema I double staining at 32% embryonic development. Sema 2a and Sema I are both expressed in the dorsal trochanter (arrows). (green: Sema 2a; red: Sema I; arrow heads demarcate the region of the trochanter limb segment). (D,E) Sema 2a and Sema I double staining at 35% and 38% embryonic development, respectively. (green: Sema 2a; red: Sema I; arrowheads demarcate limb segment boundaries; Ta, tarsus; Ti, tibia; Fe, femur; Tr, trochanter). (F) Schematic of the Sema 2a and Sema I expression pattern during Ti outgrowth (31% panel: Ti, Ti pioneer neurons; Tr, trochanter guidepost neuron; Cx, coxa 1 guidepost neurons; 35% panel, arrowheads demarcate segment boundaries; Ta, tarsus; Ti, tibia; Fe, femur; Tr, trochanter; Cx, coxa). Scale bar is 40 μm in A, 50 μm in B,C, 55 μm in D, and 100 μm in E.

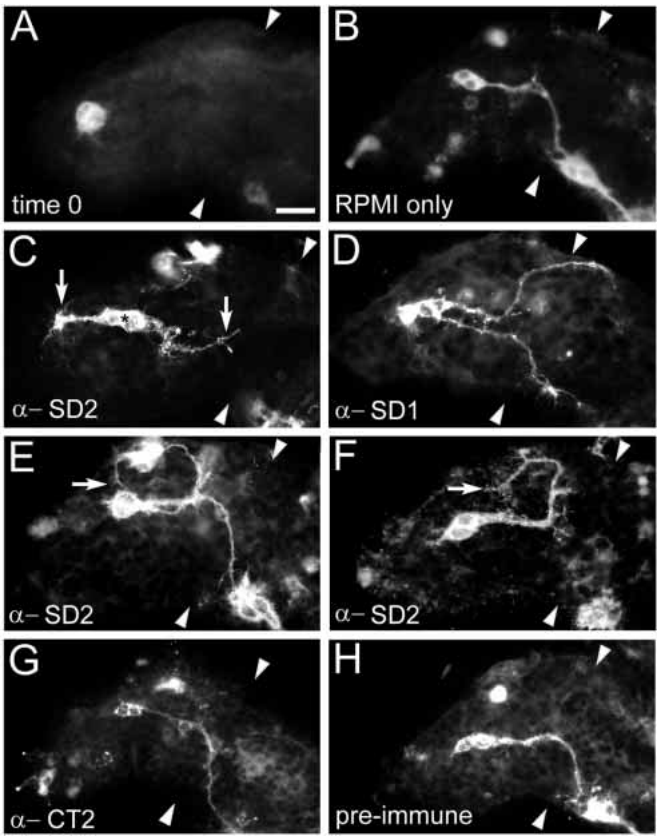

Fig. 5. Antibodies directed against the semaphorin domain of Sema 2a disrupt Ti pioneer pathfinding. (A) Embryos fixed at the start of the experiment, (Time 0: approximately 30% of development), reveal Ti pioneer neurons have just differentiated from the underlying epithelium. (B) The Ti pioneer projection is normal following 30 hours incubation in RPMI culture media. (C-F) Four representative examples of limb buds following 30 hours of culturing in RPMI media in the presence of antibodies directed against the semaphorin domain of Sema 2a (1 μM). (C) This limb shows a distal projecting phenotype following antibody perturbation of the SD2 region. Asterisk indicates the Ti cell bodies, arrows demarcate distal and proximal projecting growth cones. (D) Early defasciculation and dorsal projection seen in a limb following incubation with SD1 antibodies. One growth cone has crossed the trochanter into the dorsal coxa and the second growth cone has projected correctly to the Cx1 cells in the ventral coxa. (E,F) Two examples of the dorsal looping axon phenotype. Arrows demarcate growth cones that have wrapped around dorsally and are in contact with (E), or near (F), the Ti cell bodies. Both limbs are from SD2 antibody blocking experiments. (G) Antibodies generated against the last 7 kDa of Sema 2a, the region C-terminal to the Ig domain, do not disrupt Ti axon pathfinding (1 μM). (H) A typical limb following incubation in the presence of 1 μM SD2 preimmune, demonstrating preimmune antibodies do not disrupt Ti axon pathfinding. In all panels, dorsal is up and distal is to the left; arrowheads indicate the region of the trochanter segment. Scale bar is 50 μm in A,C-F, is 60 μm in H, and is 65 μm in G,B.

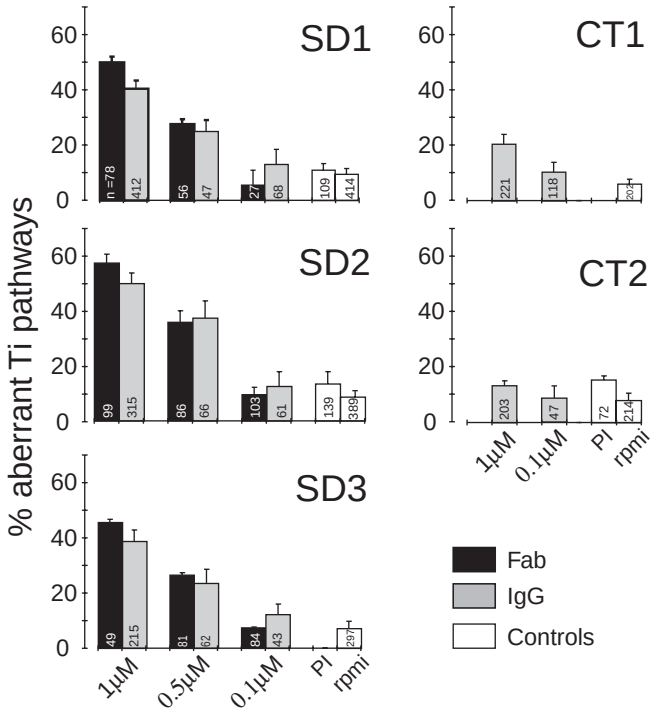

Fig. 6. Summary of Sema 2a antibody perturbation experiments. Embryos cultured in the presence of antibodies generated against the sema domain of Sema 2a exhibit a dose-dependent increase in the frequency of aberrant Ti projections over both control and C-terminal perturbation experiments. Furthermore, perturbation of the SD2 region of the sema domain, at both 1 μM and 0.5 μM, led to a significantly higher incidence of defects compared to either SD1 or SD3 antibody perturbation experiments. Data are pooled from 18, 20, 16, 10 and 11 experiments using antibodies directed against SD1, SD2, SD3, CT1 and CT2, respectively. *n* = number of limbs scored for each treatment.

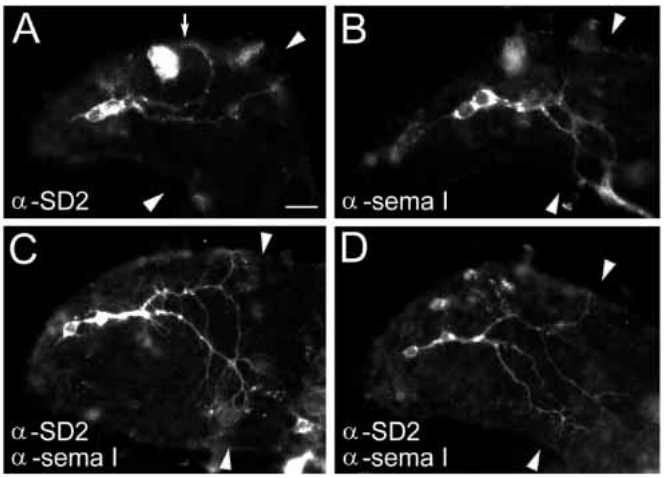

Fig. 7. Sema 2a and Sema I act in combination to guide Ti axon pathfinding. (A) A typical Sema 2a antibody-blocking phenotype, illustrating early axon defasciculation and dorsal projections. The growth cone of one of the Ti neurons has contacted the heavily labeled femoral chordontal organ (arrow). (B) Typical Sema I antibody blocking phenotype, illustrating axon defasciculation, branching within and near the Sema I trochanter epithelium, and proximal extension into the coxa. (C,D) Representative aberrant projections observed in combined Sema 2a and Sema I antibody blocking experiments. Note both early defasciculation and dorsal projections, extensive axon branching along the entire projection and proximal extension into the coxa. Phenotypes characteristic of the independent blocking of Sema 2a or Sema I were also observed in the combination blocking experiments, see inset in Fig. 8. In all panels, arrowheads indicate region of trochanter segment. The scale bar is 50 μm.

completely around and the growth cones would be in close proximity to their cell bodies (Fig. 5E,F). Considering the high expression of Sema 2a in the dorsal and distal tip of the limb bud during the period of Ti pioneer neuron axonogenesis, the nature of the guidance errors are consistent with a repulsive role for the gradient of Sema 2a. Interestingly, although dorsal projection abnormalities were common, we rarely observed axons aberrantly projecting into the ventral limb compartment. This suggests that, normally, the chemorepulsive gradient of Sema 2a is critical for counterbalancing an attractive distal/dorsal cue or possibly a repulsive ventral cue.

In control cultures, aberrant projections were observed in approximately 10% of limbs, establishing the baseline level of Ti projection errors for these cultures (Fig. 6). These control projection abnormalities were subtle and typically included defasciculation and aberrant dorsal projections within the trochanter limb segment. Furthermore, increasing the concentration of the CT2 antibodies up to 2 μM (data not shown), or the preimmune antiserum to 2 μM (Fig. 8), had no effect on the number or type of guidance errors. Embryos cultured in the presence of antisera generated against the entire C terminus including the Ig domain (CT1) did, however, lead to increased frequency of Ti axon projection abnormalities (Fig. 6). Yet, even using the highest concentration of CT1 antibody (1.0 μM), the frequency of errors was 50% lower than observed in the semaphorin domain perturbation experiments. Moreover, the type of abnormalities observed following the 1.0 μM CT1 blocking experiments were similar to control abnormalities, typified by defasciculation and dorsal projection within or near the trochanter limb segment.

In contrast, addition of sema domain antiserum IgG or Fab fragments to the culture medium at concentrations between 0.1 and 1.0 μM resulted in a dose-response disruption of the normal Ti axonal projection (Fig. 6). Of the three general classes of defects, defasciculation and dorsal projection abnormalities were most commonly observed; however, all three classes of malformations occurred significantly more often than in the control experiments. For example, we rarely observed the distal or dorsal looping projections in the control experiments, or in the experiments employing antibodies that recognize the C terminus (CT1 and CT2). In addition, following sema domain perturbation, the defasciculation and aberrant dorsal projection phenotypes often occurred distal to the trochanter limb segment; whereas, following blocking of the C terminus or in the control experiments, these phenotypes were observed most often near or within the trochanter segment. Although the frequency of pathfinding errors varies with concentration, it remains undetermined whether the distribution of abnormal phenotypes is also dose dependent. Culturing in the presence of Fab or IgG antibodies showed similar results with no significant variation in frequency of malformed Ti pathways or the type of defects observed (Fig. 6). Furthermore, perturbation studies

using the duplicate sera showed statistically similar results, reflected in the calculated frequency of pathfinding errors illustrated in Fig. 6. Interestingly, the distal and dorsal looping projection phenotypes were observed more often following perturbation of the SD2 domain of Sema 2a, and the frequency of aberrant projections was significantly greater for both the IgG and Fabs at all concentrations tested than was observed following perturbation using antibodies directed against other regions of Sema 2a ($P<0.05$).

Western analysis and immunocytochemistry results are identical for all the antibodies generated against Sema 2a. However, the CT1 and CT2 antibodies, including duplicate sera generated in independent animals, display little or no axon guidance blocking activity. These results suggest that the C terminus of Sema 2a is less critical for mediating the guidance function of the molecule or, alternatively, that none of the polyclonal antisera that we have generated against the C terminus recognizes a functionally important region. Our results demonstrate that the semaphorin domain, in particular the central region, plays an important role in mediating the axonal guidance activity of Sema 2a.

gSema 2a and gSema I guide discrete regions of the Ti pioneer projection

At approximately 33% of development, the Ti growth cones have migrated out of the gradient of Sema 2a in the proximal femur and have contacted the Sema I-expressing trochanter epithelial band, where they make a stereotyped ventral turn (Fig. 4F). By this stage of development, Sema 2a and Sema I are both expressed by the dorsal trochanter epithelium (Fig. 4C) and, although Ti growth cones frequently extend branches into this region, they normally retract, reorient and grow ventrally along the Sema I-expressing epithelium (O'Connor et al., 1990). In order to test whether Sema 2a in the dorsal trochanter directs the ventral turn of the Ti growth cones, we repeated the antibody perturbation experiments at 32% of development. Surprisingly, following incubation in the presence 1 μM Sema 2a IgG or Fabs, we observed control levels of projection abnormalities in the embryos. The Ti sibling axons remained fasciculated and the growth cones reliably made the ventral turn within the Sema I epithelial band, contacted the Cx1 cells and entered the CNS (data not shown). Therefore, it appears that once the Ti growth cones have successfully migrated into the Sema I-expressing trochanter epithelium, Sema 2a signaling is not critical for Ti pathfinding to the CNS.

Although perturbation of Sema 2a function after the Ti growth cones have reached the Sema I-expressing trochanter epithelium has no effect on pathfinding, antibody perturbation of transmembrane Sema I function at this later developmental stage does lead to Ti projection abnormalities (Fig. 7B; Kolodkin et al., 1992). Typical Sema I perturbation abnormalities include defasciculation and multiple branching of the two sibling pioneer axons within or near the Sema I-expressing trochanter epithelia, and also results in the formation of axon branches that extend proximally across that trochanter-coxa boundary. Therefore, it is likely that the chemorepulsive gradient of Sema 2a determines the initial direction of Ti1 axon outgrowth, maintains fasciculation and ensures accurate projection to the trochanter; however, once the Ti growth cone contacts the trochanter epithelium, Sema I is responsible for maintaining axon fasciculation and preventing branching and extension into the dorsal regions of the coxa limb segment. It remains unclear whether Sema I is acting as a repulsive guidance cue, restricting branching and defasciculation, or a permissive/attractive cue, preventing proximal extension past the trochanter. Regardless of the nature of Sema I signaling in the trochanter, however, the results from blocking each semaphorin independently demonstrate that the two semaphorins guide discrete regions of the same axon pathway. Interestingly, neither the perturbation of Sema 2a or Sema I signaling affects the ventral turn within the trochanter, suggesting the presence of additional guidance cue(s) in the Ti pathway.

Although the two semaphorins appear to have temporally, spatially and functionally distinct roles in Ti axon guidance, at approximately 32-33% of development the Ti growth cones interact with both the Sema 2a chemorepulsive gradient and the Sema I-expressing trochanter epithelium. To investigate the role of different semaphorin family members acting simultaneously on a single well-characterized axon projection, we cultured embryos in the presence of both Sema 2a and Sema I antibodies: thus blocking both semaphorins during the period of Ti axon pathfinding.

Similar to the Sema 2a perturbation experiments, stage 30-31% embryos were cultured for the duration of Ti growth cone pathfinding into the CNS. Consistent with our previous experiments, we observed the typical early defasciculation and distal or dorsal axon projection in 56% of limbs from embryos cultured in the presence of 1 μM Sema 2a SD2 IgG (Figs 7A, 8). Incubation with 1 μM Sema I monoclonal antibodies led to

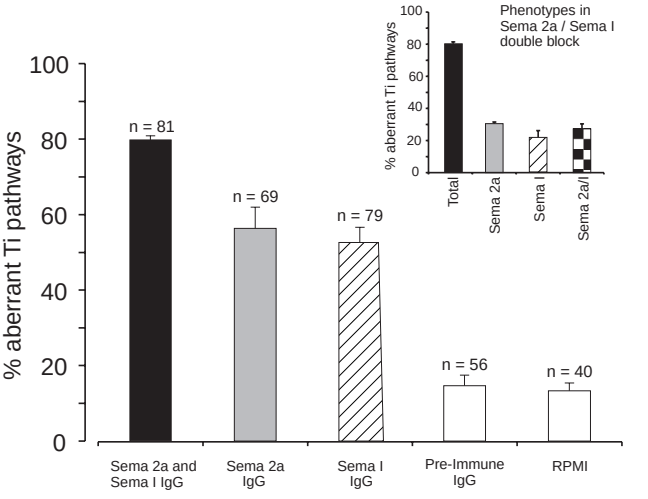

Fig. 8. Summary of combined Sema 2a and Sema I antibody perturbation experiments. Graph illustrates percentage of aberrant Ti pathways following culture either in the presence of antibodies directed against the SD2 region of Sema 2a (56%), gSema I (52%), both Sema I and the SD2 region of Sema 2a (80%), SD2 preimmune (13%) or RPMI culture media alone (14%). Inset shows the breakdown of phenotypes observed in combined Sema 2a/Sema I blocking experiments. Of the 80% aberrant Ti pathways, 30% exhibited abnormalities characteristic of the independent perturbation experiments of Sema 2a, 23% exhibited the independent Sema I antibody perturbation phenotypes and 27% exhibited hybrid phenotypes. Data are pooled from 3 experiments in which 1 μM SD2 Sema 2a IgG, 2 μM SD2 preimmune IgG and 1 μM monoclonal antibody 6F8 were used as blocking reagents. *n* = number of limbs scored for each treatment.

defects in 52% of limbs, again consistent with earlier experiments, these defects were typified by defasciculation and multiple branching of the two sibling pioneer axons within or near the Sema I-expressing trochanter epithelia and axon branches that extend proximally across that trochanter-coxa boundary (Figs 7B, 8) Unlike the Sema 2a perturbation phenotype, distal or dorsal looping abnormalities were not observed following Sema I perturbation. Also, unlike the typical Sema I phenotype, multiple branch formation was not observed following Sema 2a perturbation experiments. Control cultures (RPMI alone or 2 μM pre-immune) showed few aberrant projections and the phenotypes were characteristic of baseline defects (Fig. 8).

Following overnight culture in the presence of both 1 μM Sema 2a SD2 antibodies and 1 μM Sema I antibody, embryos exhibited malformed Ti pioneer pathways in 80% of the limbs, a frequency substantially greater than observed following perturbation of either semaphorin alone (Fig. 8; $P<0.05$). The phenotypes included axon abnormalities characteristic of the independent Sema 2a and Sema I perturbations experiments, such as embryos displaying either early defasculation, distal and dorsal projection (30% of limbs; Fig. 8), or defasciculation within the trochanter and extensive axon branching (23% of limbs; Fig. 8). However, in addition to these previously characterized malformations, we observed new aberrant phenotypes that resembled hybrid forms of Sema 2a and Sema I defects (Fig. 7C,D). Such Ti pathways exhibited both early axon defasciculation, dorsal or distal projections, and extensive branching (27% of limbs; Fig. 8). Although we tested only one concentration of antibody (1 μM), it is noteworthy that the errors in the double-block experiment appear to be nearly additive. For example, following perturbation of both semaphorins 30% of limbs displayed Sema 2a phenotypes and 27% displayed a hybrid, this combined 57% is close to the 56% error observed following blocking Sema 2a alone. Similarly, 23% of limbs displayed Sema I phenotypes and 27% displayed a hybrid, this combined 50% is close to the 52% error observed following blocking Sema I alone. The typical phenotypes observed in the independent and combined antibody perturbation experiments are summarized in Fig. 9. These results demonstrate that the perturbation of both Sema 2a and Sema I within the developing limb bud leads to an increased frequency and range of abnormal Ti pioneer axon projections, illustrating that the combined functions of a secreted and transmembrane semaphorin can guide individual growth cones in vivo.

DISCUSSION

Growth cone steering is a complex, highly integrated process involving the reception and transduction of multiple guidance cues into motile forces. The semaphorin gene family has been implicated in neuronal development in organisms ranging from *Drosophila* to human, with members displaying remarkable conservation

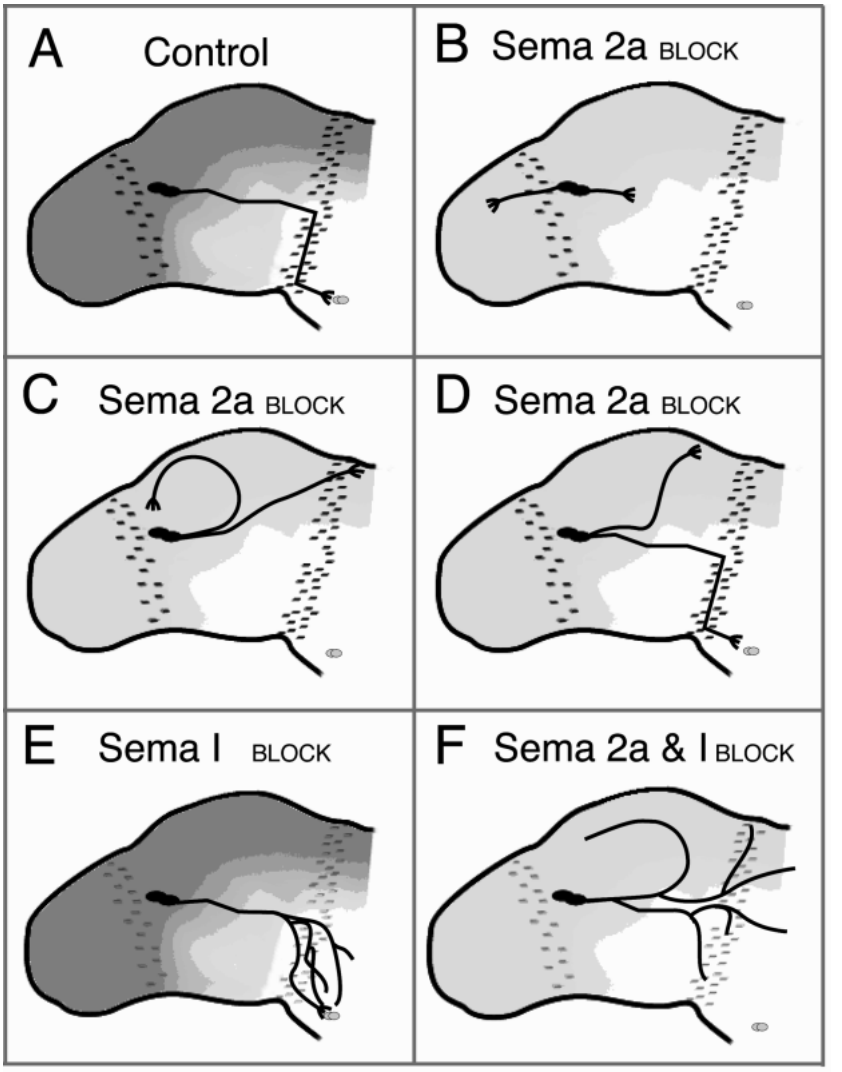

Fig. 9. Relationship of the Ti projection in control and Semaphorin antibody blocking experiments. (A) Control (RPMI media, preimmune or CT2 antibodies): shading represents the graded distribution of Sema 2a. Dashed areas represent Sema I distribution. The Ti axons extend proximally from the Ti cell bodies, turn ventrally in the Sema I-expressing trochanter epithelium and subsequently contact the Cx1 cells and turn proximally toward the CNS. (B-D) Schematics illustrating the range of observed phenotypes in the presence of Sema 2a function blocking antibodies. Axons defasciculate and migrate toward areas that exhibit high Sema 2a levels. Lighter shading represents blocking of Sema 2a function. (E) Schematic illustration of typical phenotype observed in the presence of Sema I function blocking antibody. Axons migrate normally to the trochanter, but branch and defasciculate in and near the region expressing Sema I, as well axon branches often project aberrantly into the coxa. However, aberrant Ti growth cones often eventually contact Cx1 guidepost cells and project successfully to the CNS. Lighter dashes represent antibody blocking of Sema I. (F) Schematic representation of a typical phenotype observed in the presence of both Sema 2a sema domain and Sema I function blocking antibodies. Ti axons exhibit a range of aberrant phenotypes including early defasciculation with distal and dorsal projections (characteristic of Sema 2a blocking), defasciculation and branching within the trochanter and proximal extensions into the coxa (characteristic of Sema I blocking) and combinations of these phenotypes. Lighter shading of Sema 2a and Sema I represents the blocking of function.

phylogenetically both in sequence and function (Mark et al., 1997). In this study, we have characterized Sema 2a, a novel secreted semaphorin in grasshopper. We have used the embryonic grasshopper Ti pioneer pathway to demonstrate that this secreted semaphorin acts both independently and in combination with the transmembrane semaphorin Sema I to ensure accurate axonal outgrowth and pathfinding in vivo. Our results illustrate that growth cones in vivo are influenced by the input from multiple guidance cues and steering decisions are based on the relative balance of these cues.

A chemorepulsive role for gSema 2a in establishing the Ti pioneer projection

The establishment of the stereotypic Ti pioneer projection requires a series of growth cone pathfinding events beginning with the decision to extend proximally along the limb axis. This may be mediated, in part, by the plane of division of the epithelial cell that gives rise to the Ti neurons and to the expression of fasciclin II in the surrounding epithelium (Lefcort and Bentley, 1989; Diamond et al., 1993). During Ti pioneer neurons axonogenesis, there is a graded distribution of Sema 2a, with the highest expression in the distal and dorsal compartments of the limb. Antibody blocking of Sema 2a at this stage results in defects consistent with the Ti pioneer growth cones requiring Sema 2a to direct and maintain their proximal extension toward the CNS. For example, Sema 2a antibody perturbation during the early stages of Ti axonogenesis and outgrowth results in a significant number of axons aberrantly projecting into the distal tip and dorsal regions of the limb, areas expressing high levels of Sema 2a. Therefore, Ti growth cones prefer to migrate down this gradient of Sema 2a protein; we believe this to be the first demonstration of an observable in vivo chemorepulsive gradient.

Though repelled by areas of high expression, the Ti growth cones are still able to grow on a substratum of Sema 2a. This is similar to the finding that *Drosophila* Sema II does not prevent growth cones from exploring their environment, but establishes a threshold that specific attractive signals must overcome in order to permit synapse formation (Winberg et al., 1998). The repulsion of Ti growth cones by Sema 2a is also analogous to the observation that cultured temporal retinal axons are inhibited from branching on an incorrect target consisting of caudal superior colliculus membranes, yet they are still able to extend across these membranes (Roskies and O'Leary, 1994).

In addition to initiating and maintaining directed axon outgrowth, Sema 2a promotes Ti axon fasciculation. Following Sema 2a perturbation, we typically observed that the sibling axons were not fasciculated and that they often extended independent aberrant projections. It is likely that Sema 2a provides a surround repulsion that encourages the Ti axons to fasciculate with each other, presumably a less inhibitory interaction. This preference to interact with neuronal substrata is also illustrated by the close apposition of Ti growth cones with the Tr and Cx1 cells, two preaxonogenesis neurons normally encountered along the Ti projection to the CNS (Caudy and Bentley, 1986). Furthermore, following Sema 2a perturbation, aberrant dorsal projecting growth cones are often observed to form close interactions with the neuronal Cx2 cells and femoral chordontal organ in the dorsal limb compartment, or even loop around to fasciculate with their own cell bodies. These results suggest that aberrantly projecting Ti growth cones continue to prefer interactions with neuronal substrata rather than limb epithelium, indicating even severely misguided growth cones continue to assess the relative attractiveness of their environment.

When embryos were cultured in the presence of antibodies that perturbed the function of Sema 2a, we rarely observed aberrant projections into the ventral compartment of the limb. One explanation for this is the possibility of an attractive cue in the dorsal and distal region of the limb, or a ventral repulsive cue, that Sema 2a is required to counter. In the absence of Sema 2a repulsion, the Ti growth cones project dorsally and/or distally, possibly in response to these cues. While the presence of these additional attractive or repulsive signals may be important for the pathfinding of other axons such as the distally projecting motor neurons, the Ti neurons must usually ignore these cues. Thus, under normal developmental conditions, we suggest that it is the balance between these signals that maintains proximal Ti axonal extension along the limb axis towards the Tr cell. We also found that the frequency of Ti projection defects was dependent on the amount of Sema 2a antibody included in the culture media. This dose-sensitivity provides further evidence for a fine balance among guidance signals and suggests that growth cones assess relative levels of attraction and repulsion during pathfinding. Our findings are similar to the observation that changes in gene dosage of dSema 2a and *Drosophila* Netrin B alter growth cone target selection by shifting the relative balance of inhibitory and attractive signals (Winberg et al., 1998). Taken together, our axon initiation and pathfinding results and the genetic analysis of guidance molecule interactions during motor neuron target selection illustrate that all stages of axonal development are dependent on the reception and comparison of multiple guidance cues.

gSema 2a similarity with other secreted semaphorins

Sequence analysis reveals Sema 2a is most closely related to the *Drosophila* secreted semaphorin, dSema 2a and, similar to gSema 2a, during embryogenesis dSema 2a is expressed by muscles (Kolodkin et al., 1993; Winberg et al., 1998). Loss-of-function dSema 2a mutants have revealed specific targeting errors in which neurons make ectopic contacts with inappropriate muscles (Winberg et al., 1998). Correspondingly, gain-of-function dSema 2a mutants exhibit stalling of specific growth cones and failure to innervate target muscle (Matthes et al., 1995; Winberg et al., 1998). Therefore, it appears that normally the low level of dSema 2a on muscles is sufficient to repel inappropriate axon branches and confines synaptic arborization to specific muscles. This genetic analysis demonstrates dSema 2a can act selectively as a target-derived inhibitory synaptic arborization cue and a local repellent for extending motor neurons. Given the high levels of gSema 2a protein in muscle during motor neuron outgrowth, it will be interesting to investigate whether Sema 2a is involved in determining neuromuscular synaptic specificity in grasshopper.

The chemorepulsive role for Sema 2a in growth cone pathfinding is similar to the functions described for the vertebrate secreted semaphorins, Collapsin-1/Sema III/D, which have also been shown to act as repulsive guidance cues,

patterning sensory projections in the developing spinal cord and brain (Mark et al., 1997). Thus, despite the wide evolutionary divergence among the many species that express secreted semaphorins, there appears to be a high degree of conservation of semaphorin function.

Functional specificity within semaphorin domains

In an effort to determine the structural elements necessary for Sema 2a function, we conducted function blocking experiments using antibodies generated against five different regions of Sema 2a. We found blocking the semaphorin domain had severe effects on the pathfinding of Ti growth cones, increasing axon pathfinding errors from approximately 10% in controls to 60% following perturbation of the central region of the sema domain. These defects were characterized by aberrant projections into areas of high expression of Sema 2a, indicating that the semaphorin domain is necessary for the chemorepulsive function of Sema 2a. Similarly, in vitro studies have demonstrated that the sema domain does confer functional specificity to secreted semaphorins. For example, the region of specificity within the sema domain of Coll-1 was narrowed to a 70 amino acid region which, when transplanted into the backbone of any other secreted semaphorin family member, was sufficient to determine the biological activity of the new chimeric molecule (Koppel et al., 1997). In Sema 2a, this 70 amino acid stretch spans both fusion proteins SD1 and SD2, the N-terminal and central regions of the sema domain respectively, with all but the first amino acid lying within SD2 region. Interestingly, we found a significantly higher number of axonal malformations following perturbation of the central region of the sema domain compared to following perturbation of other regions of Sema 2a, suggesting this region is also of particular functional importance for invertebrate secreted semaphorins.

If the semaphorin domain is responsible for the chemorepulsive activity of Sema 2a, what is the function of the C-terminal domain? In vitro studies have shown that members of the vertebrate secreted semaphorins need to be dimerized to be functional and that dimerization is dependent on cysteine residues in the C terminus (Eickholt et al., 1997; Koppel et al., 1997; Klostermann et al., 1998; Koppel and Raper, 1998). In addition, it appears that secreted collapsin family members bind and activate their receptors as preformed dimers (Koppel and Raper, 1998). Therefore, the absence of axon abnormalities following perturbation with antibodies directed against the last 172 amino acids of the C terminus of Sema 2a may be a consequence of Sema 2a having been secreted as a covalent dimer. Alternatively, since neither gSema 2a and dSema 2a contain the carboxy-terminal basic domain characteristic of the vertebrate secreted semaphorins, a region proposed to be necessary for regulating the repulsive activity (Adams et al., 1997; Klostermann et al., 1998), the C-terminal portion of Sema 2a may not be required for chemorepulsion. In vitro studies using sema domain-Fc fusion proteins of vertebrate secreted semaphorins have also indicated that the C terminus is not absolutely necessary for the repulsive activity; however, the activity of these chimeric proteins is considerably lower than full-length Coll-1 (Eickholt et al., 1997; Koppel and Raper, 1998).

In addition to promoting dimerization, the Ig and basic domains of vertebrate secreted semaphorins bind strongly to Neuropilin family members, components of the secreted semaphorin receptor complex (Feiner et al., 1997; He and Tessier-Lavigne, 1997; Giger et al., 1998). The partial attenuation of Sema 2a repulsion that we observed following perturbation with antibodies directed against the entire C-terminal region, including the Ig domain, may result from a disruption of receptor complex interactions. However, insect neuropilins have not been identified, therefore suggesting the secreted semaphorins may signal through a different mechanism in invertebrates. Nevertheless, it is possible that the conserved sema domain mediates the biological activity of secreted semaphorins, while the Ig and basic domains are involved in dimerizing and binding the ligand to the receptor complex and thereby potentiating its activity. Taken together, our in vivo function blocking data and the vertebrate studies indicate biological activity is domain specific and highly conserved among secreted semaphorins.

Semaphorins act independently and in combination to guide growth cone pathfinding in vivo

During the period of Ti growth cone pathfinding, the gradient expression of Sema 2a in the ventral limb compartment ends at the trochanter, a region where Sema I is expressed, and only the dorsal region of the limb bud continuing to express Sema 2a. The Ti growth cones typically halt their proximal migration within the Sema I-expressing trochanter epithelium and reorient ventrally, migrating away from the region of coexpression of Sema I and Sema 2a in the dorsal trochanter. Our results demonstrate that the chemorepulsive gradient of Sema 2a effectively guides Ti growth cones to the Tr1 guidepost cell located within the band of Sema I-expressing epithelium. Following contact with the trochanter epithelia, however, Sema I signaling appears to be critical for accurate Ti growth cone pathfinding. Given that contact of the Ti growth cones with the Cx1 neuron is also necessary for accurate pathfinding to the CNS (Bentley and Caudy, 1983), then the Ti growth cones must respond to at least three distinct cues during development: Sema 2a, Sema I, and an unknown neuronal cell surface molecule. Thus, even in the relatively simple grasshopper model system, accurate axon outgrowth and pathfinding is dependent on the integration of multiple guidance cues by growth cones as they explore their environment.

We have shown here that the secreted semaphorin Sema 2a acts as a repellent, however the nature of Sema I signaling at the trochanter epithelial band remains unclear. While axon defasciculation and additional branch formation following antibody perturbation is consistent with an inhibitory function of Sema I, the normal restriction of growth cone migration into the dorsal coxa, the adhesivity of Ti growth cones to the trochanter epithelium (Condic and Bentley, 1989) and examination of Sema I function on other neurons in the developing limb (Wong et al., 1997) supports an attractive/permissive function for Sema I. Although our experiments do not address this issue, it seems likely that the simple characterization of semaphorins as attractive or repulsive cues may not accurately reflect their function in vivo. In fact, it has become evident that single semaphorins may exert a wide range of functions, depending on the downstream signaling cascades stimulated in a neuronal growth cone. For example, Song et al. (1998) have recently shown that the

vertebrate secreted semaphorin, Sema III/D, can be switched from a repulsive guidance molecule to an attractive cue depending on the level of cGMP within the growth cone.

The expression patterns of Sema 2a and Sema I during the period of Ti pioneer outgrowth and throughout limb during development lead us to hypothesize that different members of the semaphorin family may act both independently and in combination to guide individual growth cones. Indeed, we observe that blocking the function of each semaphorin alone leads to spatially, temporally and phenotypically discrete axon malformations, demonstrating that these two semaphorins independently guide regions of Ti growth cone pathfinding. However, since the Ti pioneer growth cones contact regions of Sema 2a and Sema I as they approach the trochanter epithelium, there are also periods when the Ti growth cone must be receiving guidance information from both semaphorins. Consistent with this hypothesis, following simultaneous perturbation of both semaphorins, we observed a significant increase in both frequency and range of axonal malformations. In these double-blocking experiments, single axon projections displayed abnormalities resembling hybrids of the individual blocking experiments, suggesting that not only do the two different semaphorin members provide discrete guidance information to an individual growth cone, the growth cone must continuously integrate and compare this incoming information to pathfind correctly.

In this study, we provide direct evidence for an in vivo chemorepulsive gradient that is necessary for determining both the initial direction of axon outgrowth as well as subsequent growth cone pathfinding events. Furthermore, our in vivo results demonstrate that different classes of semaphorins can act discretely and simultaneously at the level of an individual growth cone, supporting the notion that it is the balance of their downstream signaling cascades that determines pathfinding decisions.

This work was supported by a Rick Hansen Institute studentship to C. M. I., by Medical Research Council MT-13246 grant to T. P. O'C. and by NIH-NS3165 and Whitehall Foundation grants to A. L. K.

REFERENCES

Adams, R. H., Lohrum, M., Klostermann, A., Betz, H. and Puschel, A. W. (1997). The chemorepulsive activity of secreted semaphorins is regulated by furin-dependent proteolytic processing. *EMBO J.* **16**, 6077-6086.

Behar, O., Golden, J. A., Mashimo, J., Schoen, F. J. and Fishman, M. C. (1996). semaphorin III is needed for normal patterning and growth of nerves, bones and heart. *Nature* **383**, 525-528.

Bentley, D. and Caudy, M. (1983). Pioneer axons lose directed growth after selective killing of guidepost cells. *Nature* **304**, 62-65.

Bentley, D., Keshishian, H., Shankland, M. and Toroian-Raymond, A. (1979). Quantitative staging of embryonic development of the grasshopper Schistocerca nitens. *J. Embryol. Exp. Morphol.* **54**, 47-74.

Caudy, M. and Bentley, D. (1986). Pioneer growth cone steering along a series of neuronal and non-neuronal cues of different affinities. *J. Neurosci.* **6**, 1781-1795.

Condic, M. L. and Bentley, D. (1989). Pioneer growth cone adhesion in vivo to boundary cells and neurons after enzymatic removal of basal lamina in grasshopper embryos. *J. Neurosci.* **9**, 2687-2696.

Culotti, J. G. and Kolodkin, A. L. (1996). Functions of netrins and semaphorins in axon guidance. *Curr. Opin. Neurobiol.* **6**, 81-88.

Diamond, P., Mallavarapu, A., Schnipper, J., Booth, J., Park, L., O'Connor, T. P. and Jay, D. G. (1993). Fasciclin I and II have distinct roles in the development of grasshopper pioneer neurons. *Neuron* **11**, 409-421.

Eickholt, B. J., Morrow, R., Walsh, F. S. and Doherty, P. (1997). Structural features of collapsin required for biological activity and distribution of binding sites in the developing chick. *Mol. Cell. Neurosci.* **9**, 358-371.

Fan, J., Mansfield, S. G., Redmond, T., Gordon-Weeks, P. R. and Raper, J. A. (1993). The organization of F-actin and microtubules in growth cones exposed to a brain-derived collapsing factor. *J. Cell. Biol.* **121**, 867-878.

Fan, J. and Raper, J. A. (1995). Localized collapsing cues can steer growth cones without inducing their full collapse. *Neuron* **14**, 263-274.

Feiner, L., Koppel, A. M., Kobayashi, H. and Raper, J. A. (1997). Secreted chick semaphorins bind recombinant neuropilin with similar affinities but bind different subsets of neurons in situ. *Neuron* **19**, 539-545.

Giger, R. J., Urquhart, E. R. Gillespie, S. K. H., Levengood, D. V., Ginty, D. D. and Kolodkin, A. L. (1998). Neuropilin 2 is a receptor for semaphorin IV: Insight into the structural basis of receptor function and specificity. *Neuron* **21**, 1079-1092.

Goodman, C. S. (1996). Mechanisms and molecules that control growth cone guidance. *Annu. Rev. Neurosci.* **19**, 341-477.

He, Z. and Tessier-Lavigne, M. (1997). Neuropilin is a receptor for the axonal chemorepellent semaphorin III. *Cell* **90**, 739-751.

Jan, L. Y. and Jan, Y. N. (1982). Antibodies to horseradish peroxidase as specific neural markers in Drosophila and grasshopper embryos. *Proc. Natl. Acad. Sci. USA* **79**, 2700-2704.

Klostermann, A., Lohrum, M., Adams, R. H. and Puschel, A. W. (1998). The chemorepulsive activity of the axonal guidance signal semaphorin D requires dimerization. *J. Biol. Chem.* **273**, 7326-7331.

Kolodkin, A. L., Matthes, D. J., O'Connor, T. P., Pate, N. H., Admon, A., Bentley, D. and Goodman, C. S. (1992). Fasciclin IV: sequence, expression and function during growth cone guidance in the grasshopper embryo. *Neuron* **9**, 831-845.

Kolodkin, A. L., Matthes, D. J. and Goodman, C. S. (1993). The semaphorin genes encode a family of transmembrane and secreted growth cone guiance molecules. *Cell* **75**, 1389-1399.

Koppel, A. M., Feiner, L., Kobayashi, H. and Raper, J. A. (1997). A 70 amino acid region within the semaphorin domain activates specific cellular response of semaphorin family members. *Neuron* **19**, 531-537.

Koppel, A. M. and Raper, J. A. (1998). Collapsin-1 covalently dimerizes, and dimerization is necessary collapsing activity. *J. Biol. Chem.* **273**, 15708-15713.

Lefcort, F. and Bentley, D. (1989). Organization of cytoskeletal elements and organelles preceding growth cone emergence from an identified neuron in situ. *J. Cell Biol.* **108**, 1737-1749.

Luo, Y., Raible, D. and Raper, J. A. (1993). Collapsin: a protein in brain that induces the collapse and paralysis of neuronal growth cones. *Cell* **75**, 217-227.

Luo, Y., Shephard, J. L. Renzi, M. J., Chang, S. and Raper, J. A. (1995). A family of molecules related to Collapsin in the embryonic chick nervous system. *Neuron* **14**, 1131-1140.

Mark, M. D., Lohrum, M. and Puschel, A. W. (1997). Patterning neuronal connections by chemorepulsion: the semaphorins. *Cell & Tiss. Res.* **290**, 299-306.

Matthes, D. J., Sink, H., Kolodkin, A. L. and Goodman, C. S. (1995). semaphorin II can function as a selective inhibitor of specific synaptic arborizations. *Cell* **81**, 631-639.

Messersmith, E. K., Leonardo, E. D., Shatz, C. J., Tessier-Lavigne, M., Goodman, C. S. and Kolodkin, A. L. (1995). semaphorin III can function as a selective chemorepellent to pattern sensory projection in the spinal cord. *Neuron* **14**, 949-959.

O'Connor, T. P., Duerr, J. S. and Bentley, D. (1990). Pioneer growth cone steering decisions mediated by single filopodial contacts in vivo. *J. Neurosci.* **10**, 3935-3946.

Puschel, A. W., Adams, R. H. and Betz, H. (1996). The sensory innervation of the mouse spinal cord may be patterned by differential expression of and differential responsiveness to semaphorins. *Mol. and Cell. Neurosci.* **7**, 419-431.

Roskies, A. L. and O'Leary, D. D. (1994). Control of topographic retinal axon branching by inhibitory membrane-bound molecules. *Science* **265**, 799-803.

Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B. and Ehrlich, H. A. (1988). Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* **239**, 487-494.

Sanchez, D., Ganfornina, M. D. and Bastiani, M. J. (1995). Contributions of an orthopteran to the understanding of neuronal pathfinding. *Immun. Cell. Biol.* **73**, 565-574.

Seaver, E. C., Carpenter, E. M. and Bastiani, M. J. (1996). REGA-1 is a GPI-linked member of the immunoglobulin superfamily present on restricted regins of sheath cell processes in grasshopper. *Development* **122**, 567-578.

Snow, P. M., Zinn, K., Harrelson, A. L., McAllister, L., Schilling, J., Bastiani, M. J., Makk, G. and Goodman, C. S. (1988). Characterization and cloning of fasciclin I and fasciclin II glycoproteins in the grasshopper. *Proc. Natl. Acad. Sci. USA* **85**, 5291-5295.

Song, H-j., Ming, G-L., He, S., Lehmann, M., McKerracher, L., Tessier-Lavigne, M. and Poo, M-m. (1998). Conversion of neuronal growth cones responses from repulsion to attraction by cyclic nucleotides. *Science* **281**, 1515-1518.

Stoeckli, E. T. (1997). Molecular mechanisms of growth cone guidance: stop and go? *Cell Tiss. Res.* **290**, 441-449.

Tanelian, D. L., Barry, M. A., Johnston, S. A., Le, T. and Smith, G. M. (1997). semaphorin III can repulse and inhibit adult sensory afferents in vivo. *Nature Med.* **3**, 1398-1401.

Taniguchi, M., Yuasa, S., Fujisawa, H., Naruse, I., Saga, S., Mahina, M. and Yagi, T. (1997). Disruption of semaphorin III/D gene causes severe abnormality in peripheral nerve projection. *Neuron* **19**, 519-530.

Tessier-Lavigne, M. and Goodman, C. S. (1996). The molecular biology of axon guidance. *Science* **274**, 1123-1133.

Varela-Echavarria, A., Tucker, A., Puschel, A. W. and Guthrie, S. (1997). Motor axon subpopulations respond differentially to the chemorepellents Netrin-1 and semaphorin D. *Neuron* **18**, 193-207.

Winberg, M. l., Mitchel, K. J. and Goodman, C. S. (1998). Genetic analysis of the mechanisms controlling target selection: Complementary and combinatorial functions of Netrins, semaphorins, and IgCAMs. *Cell* **93**, 581-591.

Wong, J. T. W., Yu, W. T. C. and O'Connor, T. P. (1997). Transmembrane grasshopper semaphorin I promotes axon outgrowth in vivo. *Development* **124**, 3597-3607.

Yu, H-H., Araj, H. H., Ralls, S. A. and Kolodkin, A. L. (1998). The transmembrane semaphorin Sema I is required in Drosophila for embryonic motor and CNS axon guidance. *Neuron* **20**, 207-220.