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Transmembrane grasshopper Semaphorin I promotes axon outgrowth in vivo

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SUMMARY

Members of the Semaphorin family of glycoproteins play an important role in axonal pathfinding by functioning as inhibitory guidance cues. Here we provide evidence that a transmembrane form of Semaphorin (Semaphorin I), which is expressed by bands of epithelial cells in the developing grasshopper limb bud, functions as an attractive/permissive cue for the growth cones of the subgenual organ. In addition, we demonstrate that Semaphorin I is needed for initial axonal outgrowth from the subgenual organ. These results are consistent with an alternative function for a transmembrane form of Semaphorin and may explain the previously reported arrest of the proximal extension of the subgenual organ growth cones in the absence of the Ti1 pioneer pathway.

Key words: Semaphorin I, neurite outgrowth, grasshopper, subgenual organ

INTRODUCTION

Axonal growth cones are guided to their targets during development by a wide variety of guidance cues. Generally, guidance molecules act as permissive/attractive cues, or as repulsive/inhibitory cues to effectively steer growth cones towards or away from a specific region (reviewed by Tessier-Lavigne, 1994; Goodman, 1996; Tessier-Lavigne and Goodman, 1996). While some of these cues are diffusible and mediate their effects from a distance, other cues are membrane bound and have a short range contact-mediated effect. The Semaphorin family of glycoproteins (Kolodkin et al., 1993), also referred to as the Collapsin family (Luo et al., 1993), have been implicated to function as guidance molecules in a number of systems (Messersmith et al., 1995; Matthes et al., 1995; Kolodkin et al., 1992; Luo et al., 1993; Adams et al., 1996). Both cell surface and secreted forms of Semaphorin have been identified in several species and it is thought that both forms of Semaphorin act as inhibitory cues (Kolodkin et al., 1993; Luo et al., 1993, 1995; Messersmith et al., 1995; Puschel et al., 1995). While most guidance molecules that have been identified fall into a specific functional category, at least one family of molecules, Netrin/Unc 6 exhibits both attractive and repellent activity (Ishii et al., 1992; Colamarino et al., 1995; Wadsworth and Hedgecock 1996). Whether any members of the Semaphorin family are able to function as bifunctional guidance cues is unknown.

Members of the Semaphorin family of glycoproteins are characterized by a ˜500 aa domain which is highly conserved between both invertebrates and vertebrates (Kolodkin et al., 1993; Kolodkin, 1996; Luo et al., 1993; Adams et al., 1996). The first Semaphorin gene identified was grasshopper *Semaphorin I* (*Sema I*, formerly *Fasciclin IV*), which encodes for a transmembrane glycoprotein found on subsets of fasciculating axons in the central nervous system (CNS) as well as on bands of epithelial cells in the developing grasshopper embryo (Kolodkin et al., 1992). One of these bands of epithelial expression was shown to play a role in the development of the peripheral nervous system (Kolodkin et al., 1992). Specifically, a pair of neurons, the Ti1 pioneer neurons, establish the first projection toward the CNS in the developing limb bud. Their growth cones make a stereotyped ventral turn that is coincident with a band of epithelial cells in the trochanter limb segment that express Sema I. Antibody blocking experiments resulted in more variable turning patterns of Ti1 growth cones, resulting in the formation of multiple branches that often crossed the Sema I band (Kolodkin et al., 1992). These results are consistent with the Sema I band playing a role in stalling the growth cones prior to making the ventral turn.

Molecules similar to Sema I have been identified in insects, mouse, human and chick (Kolodkin et al., 1993; Luo et al., 1995; Adams et al., 1996). While little is known about the function of the transmembrane forms of Semaphorin in these systems, secreted Semaphorins appear to act as chemorepulsive guidance cues (Luo et al., 1993; Messersmith et al., 1995; Puschel et al., 1995; Matthes et al., 1995). In the mammalian nervous system, dorsal root ganglion neurons project axons to characteristic termination sites in the spinal cord. Messersmith et al. (1995) demonstrated that Semaphorin III, which is normally expressed at high levels in the ventral spinal cord but not dorsally, inhibited the growth of cutaneous sensory axons. These results suggest that Semaphorin III may have a role in the patterning of cutaneous sensory projections by preventing axons from entering certain target regions. Fan and Raper (1995) demonstrated that dorsal root ganglion growth cones could be induced to turn away from collapsin (chick homologue of Sema III) coated beads, suggesting that Semaphorin III may act to direct growth cone steering by causing local collapse of regions of the growth cone. In *Drosophila*, during embryonic development, a secreted form of Semaphorin (D-Sema II) is expressed by a subset of neurons in the CNS

(Kolodkin et al., 1993). Loss-of-function mutations resulted in mutant flies with increased lethality and behavioral abnormalities, which may reflect defects in the underlying neural circuits (Kolodkin et al., 1993). This indicates that D-Sema II may control the development of neural circuits by functioning in growth cone guidance or aiding the formation of specific synaptic connections. In addition, missexpression of D-Sema II in identifiable ventral body wall muscles inhibited contact and synaptogenesis of the appropriate motoneuron (Matthes et al., 1995). These results suggest that secreted Semaphorins may act as chemorepulsive guidance cues during neuronal pathfinding.

In the present study, we have used the developing grasshopper limb bud to further study the role of Sema I in vivo. In the embryonic limb bud, the Ti1 pioneer neurons extend axons through the limb and establish the first projection to the CNS. A number of neurons which arise distal to the Ti1 neurons later in embryogenesis reach the CNS by fasciculating with the pathway pioneered by the Ti1 neurons (Keshishian and Bentley, 1983). Klose and Bentley (1989) showed that elimination of the Ti1 pathway resulted in the arrest of the proximal extension of growth cones from the subgenual organ (SGO) which arises later in development. They proposed that the presence of pioneer neurons is necessary for the successful migration of distal neurons over regions of peripheral tissue in which it is difficult to travel successfully, due possibly to chemical or mechanical properties of the tissue. Since Semaphorins have been implicated to have growth cone inhibitory properties, we examined the possibility that the arrest of the SGO axon is mediated by a band of epithelial cells expressing Sema I. We demonstrate that SGO axons extend into a band of Semaphorin I expressing epithelial cells located just distal to the Ti1 cell bodies at approximately 40% of embryonic development. In the absence of the Ti1 pathway, the SGO growth cones extended into the band of Sema I and typically stopped growing at the proximal edge of the Sema I band. This suggested that the Sema I band may have a permissive/attractive function. To test this hypothesis, antibodies were used to block the function of Sema I. Blocking the function of Sema I inhibited axonal growth from the SGO into the Sema I expressing band. These results suggest that the transmembrane semaphorin G-Sema I may have an alternative function, acting as an attractive or permissive guidance cue in vivo.

MATERIALS AND METHODS

Immunocytochemistry

Shistocerca gregaria embryos were obtained from a colony maintained at the University of British Columbia. Embryos were staged by percentage of total embryonic development (Bentley et al., 1979). To visualize neurons and Sema I expressing epithelium, embryos were dissected in saline, and fixed in PEM-FA (0.1 M PIPES, 2 mM EGTA, 1.0 mM $MgSO_4$, 3.7% formaldehyde) for 1 hour, washed for 45 minutes with 6 changes of PBT (1× PBS, 0.1% BSA, 0.1% Triton X-100), and incubated in primary antibody overnight at 4˚C. The following primary antibodies were diluted in PBT: mAb 6F8 (anti-Semaphorin I antibody from a hybridoma cell line kindly provided by Dr C. Goodman; 1:1) and rabbit anti-HRP (Jackson Immunoresearch Laboratories; 1:1250). After primary incubation, the embryos were washed for 45 minutes with 6 changes of PBT and incubated for 2 hours in secondary antibody. The following secondary antibodies were diluted in PBT: HRP-conjugated goat anti-mouse IgG, HRP-conjugated goat anti-rabbit IgG, FITC-conjugated goat anti-rabbit IgG, and Cy3-conjugated goat anti-mouse IgG (Jackson Immunoresearch Laboratories, all 1:250). Following secondary incubation, embryos were washed for 45 minutes with 6 changes of PBT. Fluorescently labeled embryos were mounted in Slowfade antifade reagent (Molecular Probes) and viewed under fluorescence microscopy. The HRP-labeled embryos were reacted in 0.5% diaminobenzidine in PBT, and the reaction was stopped by 5 serial washes in PBT, cleared in 50% glycerol and mounted in 70% glycerol.

For double labeling, embryos were incubated simultaneously in the two primary antibodies overnight, washed, and the 6F8 antibody labeling was visualized first by incubating in secondary antibody HRP-conjugated goat anti-mouse for 2 hours, then washing and reacted in diaminobenzidine solution containing 0.06% nickel chloride. Embryos were then incubated in the HRP-conjugated goat anti-rabbit secondary antibody for 2 hours and the second antibody was visualized with a diaminobenzidine reaction as described above. For fluorescent secondary antibodies, embryos were incubated in the first secondary antibody for 2 hours, washed and then incubated in the fluorescence-conjugated second secondary antibody for 2 hours. The embryos were washed and mounted as above for fluorescence labeled embryos.

Heat shock

Clutches of grasshopper embryos at 27% of development were positioned in 1.5 ml Eppendorf tubes and immersed in a hot water bath at 45-47˚C for 30 minutes as described previously (Klose and Bentley 1989). Heat shocked eggs and control eggs (no heat shock) were then incubated at 30˚C until 45% development (approximately 4 days), fixed, and the neurons and Semaphorin-expressing epithelium were labeled as above.

Limb fillet

Grasshopper embryos at 38-43% of development were anchored ventral side down on a poly-L-lysine coated glass coverslip. The posterior side of the T3 limb was cut longitudinally with a glass needle and spread apart on the coverslip to flatten the epithelium. A suction pipette was used to remove the mesodermal cells overlying the neurons. The pioneer neuron cell bodies which are located on the basal, upward-facing surface of the epithelium were located with Nomarski optics. To remove neurons, a glass needle was used to remove only the Ti1 cell bodies, or both the Ti1 and Fe1 cell bodies without disturbing other cells in the limb. The limbs were then cultured in freshly made RPMI culture medium (supplemented with 5% heat inactivated fetal bovine serum). At the end of the culture period, the embryos were fixed and processed as above. For DiI labelling experiments, fillets were lightly fixed for 10 minutes and a small crystal of DiI (Molecular Probes) was placed on the SGO projection at its point of contact with the Ti1 neurons. After labeling, the fillets were fixed for an additional 30 minutes and then processed for anti-HRP fluorescence immunocytochemistry as described above, with the exception that the solutions did not contain Triton X-100.

For blocking experiments, the mAb reagents were prepared as follows. Hybridoma cell lines secreting 6F8 mAb or anti c-myc mAb were cultured in serum free medium. Ammonium sulphate saturated H_2O was added to the tissue culture supernatant bringing it to a 25% solution, incubated on ice for 1 hour and spun at 15,000 ***g*** at 4˚C for 1 hour. The supernatant was then brought to 55% with H_2O-saturated $(NH_4)_2SO_4$, incubated overnight at 4˚C and spun as above. The pellet was resuspended in PBS using approximately 1/40 volume of the original hybridoma supernatant, dialyzed against 1× PBS overnight with three changes, filtered through a 0.2 μm filter, and further concentrated using centricon filters (Amicon) to a final concentration of 600 μM. The concentrated antibody was then dialyzed overnight in RPMI with three changes. For blocking experiments, each antibody reagent was diluted with three portions of RPMI supplemented with

5% heat inactivated fetal bovine serum resulting in an antibody concentration of 200 μM. Limb fillets were cultured in either mAb 6F8 or mAb anti c-myc reagent overnight and then fixed and stained as above.

RESULTS

Axons of the SGO extend into a band of Sema I expression by 40% of embryonic development

To address the possibility that Sema I plays a role in the outgrowth of axons from the SGO, we first examined whether Sema I was expressed in the developing limb bud in a manner consistent with a role in influencing the SGO projections. Kolodkin et al. (1992) had previously reported that from 34.5 to 35.5% of development, bands of Sema I are localized in circumferential stripes of limb epithelium in the tibia, femur, trochanter and coxa. Since the SGO does not arise until approximately 38% of development, we extended these observations by examining the expression pattern of Sema I at later stages of development.

We used the anti-Sema I monoclonal antibody 6F8 to detect Sema I expression in the developing limb bud (Kolodkin et al., 1992). The first detectable expression of Sema I in the limb bud is at approximately 30% of development and this expression persists at least through 50% of development, the latest stage that we examined. At 38% of development, corresponding to the time that the SGO first arises, the limb epithelium stains intensely for multiple bands of Sema I located in the coxa, trochanter, femur, tarsus and a very distinct band just distal to the approximate location of the tibia-femur (Ti-Fe) segment boundary (Figs 1A, 2A). This pattern of expression is similar to that previously reported by Singer et al. (1995) for a 35% embryo. By 40% of development (Figs

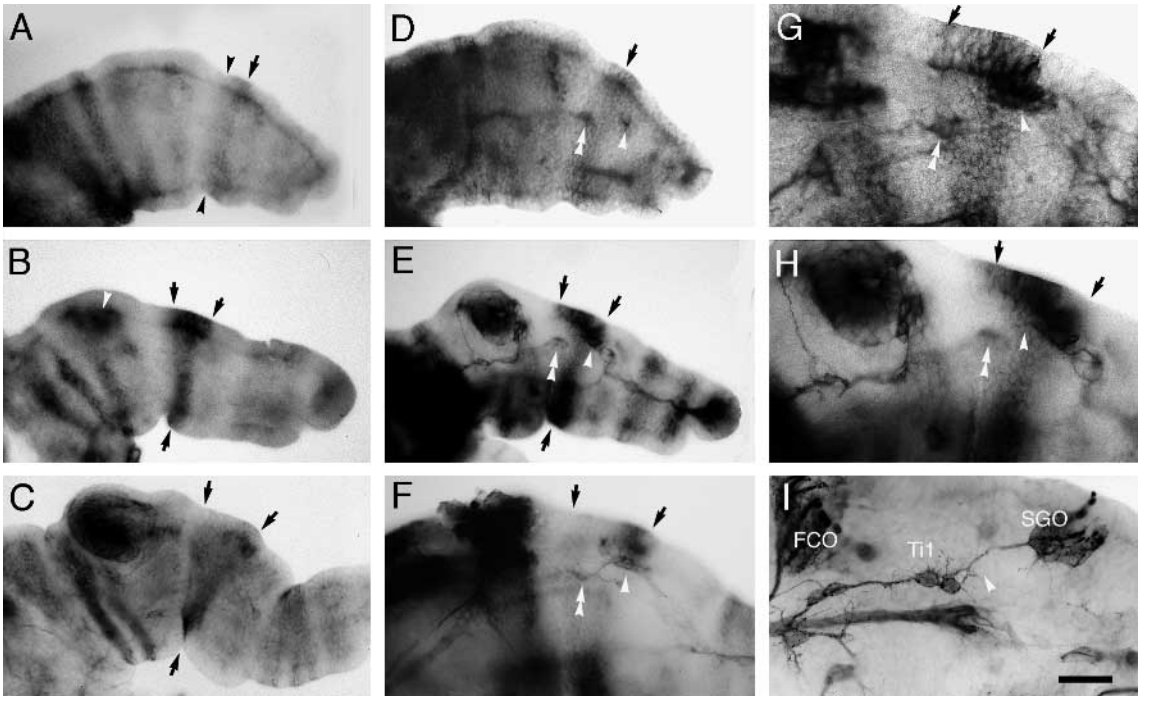

Fig. 1. Embryonic expression of grasshopper Semaphorin I in the developing limb bud. Sema I is expressed in bands of epithelial cells as well as by the femoral chordotonal organ (FCO) in the developing grasshopper limb bud. (A-C) The pattern of Sema I expression in the limb bud at three different stages of development (38%, 40%, and 42% respectively) as visualized with HRP immunohistochemistry using mAb 6F8. (D-H) Embryos double stained with mAb 6F8 and anti-HRP antibody to visualize the position of the epithelial bands of Sema I expression in relation to neuronal cell bodies and processes. Proximal is to the left, and dorsal is up. (A) Limb bud at 38% of development showing bands of Sema I expression. A band of Sema I expression (arrow) is apparent at this stage just distal to the approximate location of the tibia-femur segment boundary (Ti-Fe; arrowheads). (B) At 40% of development, the Sema I band at the Ti-Fe region is broader and staining is more intense (arrows). Also the FCO staining is more apparent (arrowhead). (C) At 42% of development, the band of Semaphorin at the Ti-Fe region is still present and has become very broad at the dorsal part of the band (arrows). (D,G) Limb bud at 38% of development showing the location of the SGO (arrowhead), which is beginning to differentiate at the distal border of a band of Sema I expression. The Ti1 cell bodies are located on the proximal edge of the same band (double arrowheads). (E,H) At 40% of the development, the SGO (arrowhead in E) has begun to send a proximal projection into the band of Semaphorin (arrows). The band of Semaphorin is still located between the Ti1 cell bodies (double arrowheads) and the cells of the SGO, and the growth cones of the SGO have extended into the band (arrowhead in H). (F) By 42% of development, the SGO (arrowhead) axons have contacted the Ti1 cell bodies (double arrowheads) and have fasciculated with the pioneer pathway, exiting the band of Sema I (arrows) at the Ti-Fe boundary. (I) High magnification of a limb at 42% of development single labeled with anti-HRP shows that at this stage, the SGO axons (arrowhead) have contacted the Ti1 cell bodies, and fasciculated with the Ti1 pathway. Scale bar, 200 μm (A,C,D,F); 175 μm (B,E); 100 μm (G,H,I).

1B, 2B), the limb continues to express Sema I, and the band distal to the Ti-Fe boundary becomes more prominent, extending approximately 6-8 cells wide near the dorsal midline of the limb. Expression of Sema I is still high at 42% of development (Figs 1C, 2C), and the band of expression at the Ti-Fe boundary becomes very broad and still stains intensely for Sema I. Other than the limb epithelial expression of Sema I, the neurons of the femoral chordotonal organ (FCO) also express Sema I (Figs 1B, 2). The FCO neurons express Sema I as soon as it differentiates (approximately 35%) and continues to express the protein to the latest developmental stage that we have examined (approximately 50%).

If a band of Sema I is responsible for preventing the proximal extension of the axons of the SGO in the absence of the Ti1 neurons, it is necessary to determine whether Sema I is expressed in the region between the Ti1 cell bodies and the SGO. To test this, we used antibody double labeling to examine the pattern of Sema I expression in relation to the identified neurons. The SGO is first identifiable at approximately 38% of development, typically along the distal edge of the band of epithelial cells expressing Sema I at the Ti-Fe boundary (Figs 1D,G, 2A). The Ti1 cell bodies are located on the opposite side along the proximal edge of the same band of Sema I. At approximately 40% of development (Figs 1E,H, 2B), the axons of the SGO have extended into the Sema I band, but the growth cones have not yet reached the Ti1 cell bodies. At this stage the SGO growth cones are within filopodial contact of the Ti1 pioneer neuron cell bodies. By 42% of development, the axons of the SGO have contacted the Ti1 cell bodies and have fasciculated with the Ti1 pioneer pathway (Figs 1F,I, 2C). During this stage, the band of Sema I at the Ti-Fe boundary is approximately 8-10 cells wide. The Ti1 cell bodies are still located on the proximal edge of the band of Sema I, but the SGO is now partially within the band. These results indicate that the spatial and temporal expression of Sema 1 are consistent with Sema I playing a role in the guidance of the SGO growth cones.

The SGO projection is pioneered by the most proximal 1-3 neurons

While the SGO matures into a large collection of sensory neurons (>20 neurons), its initial projection to the Ti1 cell bodies appears to be pioneered by only one or a few growth cones (see Fig. 1I). In order to determine the number of growth cones that establish the projection from the SGO to the Ti1 neurons we used the limb fillet preparation to label the first axons that contact the Ti1 neurons. Using the lipophillic tracer DiI, we found that the initial projection from the SGO to the Ti1 neurons is pioneered by the most proximal 1-3 neurons (Fig. 3A,B), and as more neurons differentiate in the SGO they extend along the established projection (Fig. 3C,D).

The SGO growth cones are arrested within a band of Sema I after elimination of the Ti1 neurons by heat shock

Previous work by Klose and Bentley (1989) demonstrated that the Ti1 pathway provides a scaffold upon which the SGO axons extend along into the CNS. They showed that heat shock of embryos at 27% of development (the Ti1 neurons start to differentiate at approximately 28% of development) results in the failure of the Ti1 neurons to differentiate in a fraction of the embryos, but the differentiation of other cells which arise later in the limb is not affected. Elimination of the Ti1 neurons by heat shock resulted in the inability of the SGO growth cones to migrate past the region of the Ti-Fe boundary, indicating that the presence of the Ti1 pathway is necessary for the development of the SGO projection (Klose and Bentley, 1989).

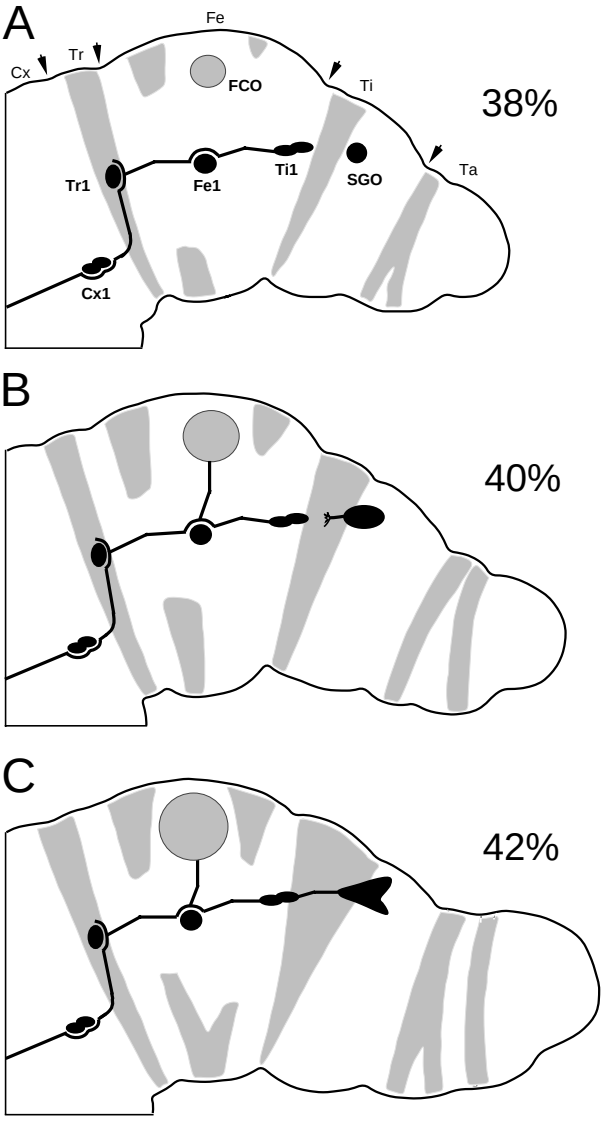

Fig. 2. Location of the SGO and Ti1 neurons in relation to Sema I expression in the developing limb bud. Schematic diagrams of embryonic limb buds at 38, 40, and 42% of development (A-C respectively), showing the location of epithelial bands of Sema I expression (patterned stripes) in relation to the locations of identified neurons. (A) At 38% of development, the Ti1 neurons, which have already established a projection to the CNS, are located on the proximal edge of a band of Sema I near the tibia-femur segment boundary (segment boundaries are marked by an arrow). The cells of the SGO are just beginning to differentiate at this stage at the distal edge of the same band of Sema I. The cells of the FCO are also apparent at this stage and stain positively for Semaphorin expression. (B) By 40% of development, axons from the SGO extend proximally within the band of Sema I. Axons from the FCO have contacted the Fe1 by this stage and have fasciculated with the Ti1 pioneering pathway. (C) Sema I expression in the limb bud at 42% of development. The SGO axons have left the band of Sema I and have extended along the Ti1 pioneer pathway towards the CNS.

Although some circumferential spreading of the SGO processes along the Ti-Fe boundary was observed, none of the SGO growth cones extended past the region of the Ti-Fe segment boundary in the Ti1 free limb buds even by 55% of development (Klose and Bentley, 1989). Since we have demonstrated that Sema I is expressed in a band of epithelial cells in the region of the Ti-Fe segment boundary at the time of axonal outgrowth from the SGO, we further examined the possibility that this band of Sema I expression may play a role in arresting the proximal extension of the SGO growth cones by determining the location of arrest of the SGO growth cones in relation to the Sema I band.

We first repeated the heat shock experiments of Klose and Bentley (1989). Similar to the previous report, we found that the Ti1 neurons failed to differentiate in a fraction of the heat shocked embryos. Approximately 18% of the embryos subjected to heat shock at 27% of development had at least one limb bud lacking Ti1 pioneer neurons. The limb morphology and differentiation of later arising neurons were not affected. In the absence of the Ti1 cells, the Fe1 and Tr1 guidepost neurons established the initial projection toward the CNS and the later arising FCO extended along this projection (Fig. 4). However, as reported in the previous experiments, elimination of the Ti1 neurons prevented the proximal extension of the SGO growth cones in all of the limbs examined, both at 45% of development (Fig. 4A,B), and at 50% of development (Fig. 4C,D). The growth cones did not appear collapsed in any of these limbs, even as late as 50% of development (Fig. 4D), indicating that the arrest of the SGO growth cones was not due to growth cone collapse.

To obtain a better understanding of the possible effects of Sema I on the growth cones of the SGO, we used fluorescence double labeling to determine the location of SGO growth cone arrest in relation to the band of Sema I. We heat shocked 361 embryos and examined 22 limbs that did not contain Ti1 neurons and were successfully double labeled. In the absence of the Ti1 neurons, the growth cones of the SGO extended into the Sema I band and typically reached the proximal edge of the band by 42% of development (Fig. 5A,B) and remained at this location throughout later stages examined (Fig. 5D,E). In each case, the growth cones of the SGO extended well into the band of Sema I expression, usually to the proximal edge of the band, but were never observed to leave the band. The growth cones of the SGO did not appear collapsed while within the band of Sema I (Fig. 5C). The ability of the SGO growth cones to migrate within the band of Sema I during normal development and in the absence of the Ti1 cells suggests that it is unlikely that Sema I may be preventing the proximal extension of the SGO growth cones by acting as an inhibitory or repulsive guidance molecule. However, since the SGO growth cones are able to extend within the Sema I band until they reach the edge of the band, but are not able to leave the band, these results strongly suggest that Sema I may contribute to the arrest of the SGO growth cones by acting as an attractive or adhesive guidance cue. In order to test this, we used an alternative approach to remove the Ti1 cell bodies from the developing limb bud.

Proximal extension of the SGO growth cones is arrested when the Ti1 cell bodies are removed from a limb fillet preparation

As an alternate approach to the heat shock experiments, the Ti1 cell bodies were physically extracted from the developing limb bud prior to the extension of the SGO axons. Using the limb fillet preparation (O'Connor et al., 1990), the Ti1 cell bodies were removed using a sharp glass needle. We selectively extracted the Ti1 cell bodies from limbs at 38% of development, a stage at which the SGO is first beginning to differentiate, but does not yet have any processes (Fig. 6A). Similar to the heat shock experiments, we found that after removal of the Ti1 neurons the SGO axons extended proximally for a short distance and then ceased to grow (Fig. 6C-F). In the absence of the Ti1 cell bodies, no proximal extension of the SGO growth cones past the region that was previously occupied by the Ti1 cell bodies was observed, but the growth cones often exhibited complex morphologies, typically extending dorsal and ventral branches circumferentially (Fig. 6C,E,F). This indicates that the SGO growth cones are able to migrate within the band of Sema I, but are not able to grow out of the band in the absence of the Ti1 cell bodies as observed in the heat shock experiments. The axons of the SGO of the contralateral control limb fillets with intact Ti1 cell bodies fasciculated normally with the pioneer pathway and extended proximally toward the CNS (Fig. 6B). Also, in all experiments where only the Ti1 cell

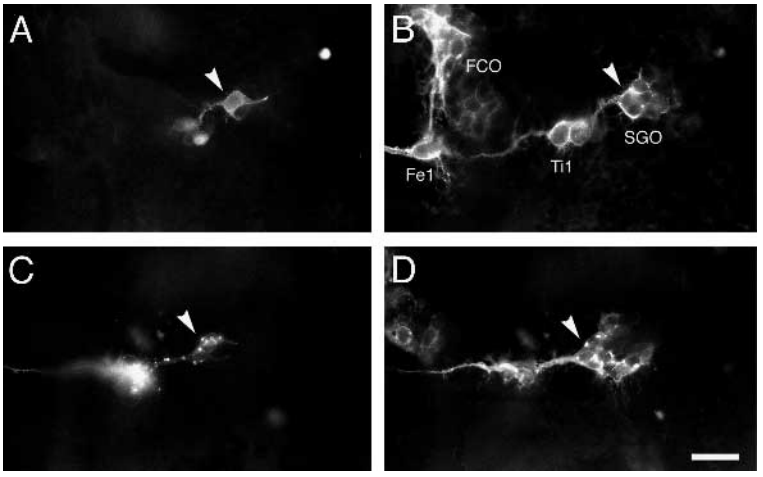

Fig. 3. Number and location of SGO neurons which extend growth cones onto the Sema I-expressing band of epithelium in the developing limb bud. The lipophillic dye, DiI, was applied to the entire bundle of SGO axons at the point of contact with the Ti1 cell bodies, allowing visualization of the individual SGO neurons which have extended growth cones to the Ti1 neurons. (A) At 42% of development, the growth cones, which had initially migrated across the Sema I band to fasciculate with the Ti1 cell bodies, consists of the growth cones of one to two SGO neurons (arrowhead). (B) HRP labeling of the same limb immediately following DiI labeling indicates that the first SGO neurons to extend axons to the Ti1 neurons are located at the proximal tip (arrowhead) of a large group of SGO neurons, consisting of at least 15 cells. (C) DiI labeling of the entire SGO axon bundle at approximately 43% of development indicates that at least three SGO neurons (arrowhead) have extended axons to the Ti1 neurons. (D) HRP labeling of the neurons in the limb shown in C indicates that the SGO consists of numerous cells, and the neurons which have extended axons to the Ti1 neurons are located at the tip of the group of SGO neurons (arrowhead). Scale bar, 100 μm.

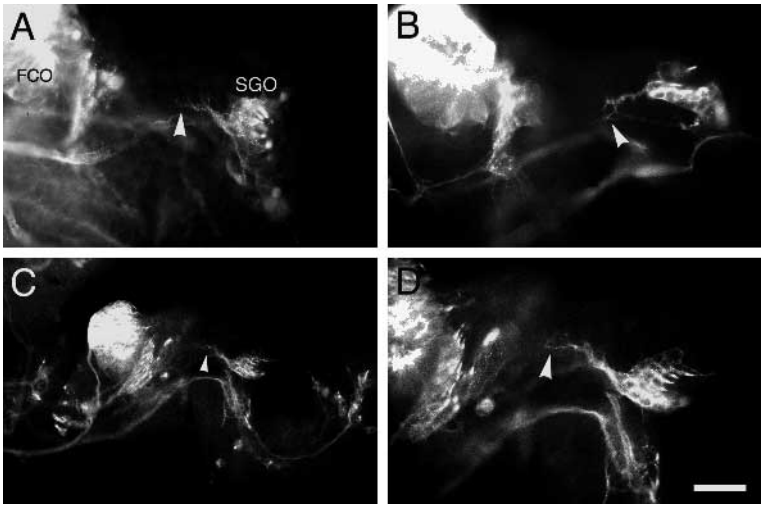

Fig. 4. Elimination of the Ti1 pioneer pathway by heat shock prevents the proximal extension of the SGO growth cones. Heat shock treatment of embryos at 27% of development prevented the differentiation of the Ti1 cell bodies as visualized by anti-HRP immunocytochemistry of heat shocked embryos at 45% and 50% of development. (A,B) Heat shocked embryos at 45% of development. In the absence of the Ti1 neurons, the axons of the SGO are arrested from proximal migration in the approximate location of the Fe-Tr segment boundary. Note that the growth cones of the SGO are not collapsed (arrowhead). (C) By 50% of development, the axons of the SGO (arrowhead) are still arrested at the location of the Fe-Tr segment boundary. (D) Higher magnification of C, the growth cones of the SGO (arrowhead) do not appear collapsed even at 50% of development. Scale bar, 100 μm (A,B,D); 200 μm (C).

bodies were removed, the FCO extended its normal projection ventrally and fasciculated with the Fe1 guidepost cell. In some cases, as a consequence of the removal of the Ti1 neurons, the Fe1 guidepost neurons were removed. This resulted in the removal of the entire pioneer pathway through the femur allowing us to test whether removal of the pathway had any effects on the extension of axons from the FCO. In limbs where both the Ti1 and Fe1 cells were removed, the SGO growth cones were arrested; however, in contrast to the SGO, removal of the Ti1 and Fe1 cell bodies did not arrest the ventral extension of the FCO growth cones (Fig. 6D). During normal development the axons of the FCO extend ventrally, contact the Fe1 neuron and then extend along the pioneer pathway into the CNS (Keshishian and Bentley 1983). However, in the absence of the Ti1 and Fe1 cells the axons of the FCO extended past the region normally occupied by the missing Fe1 cell body, finally making a sharp proximal turn at the ventral midline region of the limb. These results indicate that the removal of neurons from the limb does not result in cessation of neurite outgrowth in all the other cells in the limb, suggesting that the cessation of growth of the SGO axons is due to the expression of Sema I.

6F8 mAb blocking of Sema I inhibits SGO axon outgrowth and extension

The monoclonal antibody 6F8 had previously been shown to cause aberrant Ti1 pathway formation by blocking the function of Sema I in the trochanter limb segment (Kolodkin et al. 1992). To examine whether Sema I plays a role in SGO outgrowth, we cultured limb fillets at 38% of development in the presence of mAb 6F8. Prior to culturing, the Ti1 neurons were removed from contralateral limb fillets and limbs were cultured for 30 hours until approximately 43% of development. Control cultures were bathed in a monoclonal antibody to the c-myc protein. As we have indicated above, the SGO has differentiated by 38% but does not yet have any axons, allowing us to examine the role of Sema-I in axon outgrowth. Outgrowth from

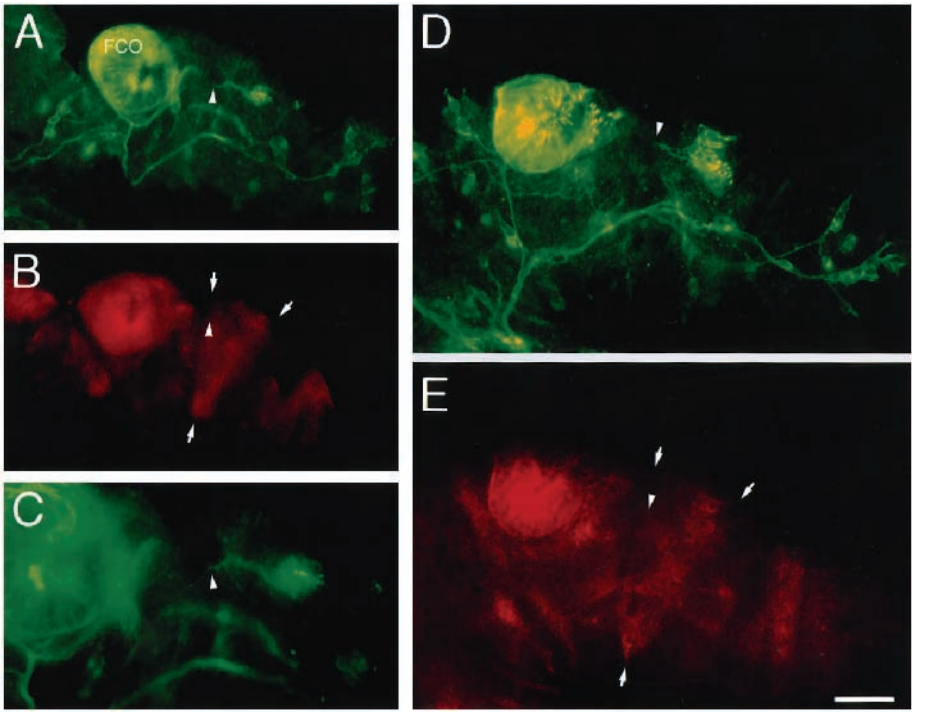

Fig. 5. The growth cones of the SGO are arrested within a band of Sema I expression. Heat shocked embryos were double labeled to reveal neuronal projections (A,C,D) and the bands of Sema I expression in the epithelium (B,E). (A,B) The growth cones of the SGO (arrowhead) are arrested at the proximal edge of a band of Sema 1 (arrows) at 45% of development in the absence of the Ti1 neurons. (C) Higher magnification of A shows that the growth cones of the SGO (arrowhead) do not appear collapsed. Numerous filopodia and branches extend from the growth cones. (D,E) A different limb showing the arrest of the axons of the SGO (arrowhead) within the proximal edge of a band of Semaphorin (arrows) at 42% of development. Scale bar, 200 μm (A,B); 150 μm (D,E); 100 μm (C).

the SGO neurons was completely prevented in the majority of the limbs which were cultured in medium that contained mAb 6F8, both in the absence (Fig. 7A; *n*=27/32), and presence (Fig. 7B; *n*=17/21) of the Ti1 cell bodies, indicating that Sema I is necessary for initial axon outgrowth from the SGO. All other neurons in the limb differentiated and extended normally, and the FCO axons fasciculated normally with the Fe1 cells (Fig. 7). In the presence of the monoclonal antibody against the c-myc protein, the axons of the SGO extended normally, fasciculating with the Ti1 neurons in control limbs (Fig. 7D; *n*=30/30), and in the absence of the Ti1 cell bodies, the growth cones were arrested (Fig. 7C; *n*=37/37).

To examine the role of Sema I in axon extension after outgrowth from the SGO has occurred, the same experiment was performed on embryos at a later stage, at approximately 40-41% of development. The precise extent of outgrowth from an individual SGO prior to antibody addition was difficult to accurately ascertain since variations in developmental age between animals from the same pod is typically ±1%. During this period of development there is extensive variation in the extent of SGO outgrowth, ranging from little to no axon at 40.5% of development, to crossing of the Sema I band and fasciculation with the Ti1 pathway by 42% of development. Limb fillets at approximately 40-41% of development with axon lengths ranging from less than 50 μm to more than 100 μm were cultured as above in the presence of mAb 6F8 or anti c-myc antibody. After 30 hours in culture (44% of development) in the presence of mAb 6F8, approximately 33% (20/62) of the limbs that were missing Ti1 neurons still had axons less than 50 μm in length, while 94% (47/50) of the limbs with intact Ti1 pathways had axons that extended further than 50 μm, the majority of which had fasciculated with the Ti1 pathway. In the presence of anti c-myc antibody, 91% (31/34) of the axons in limbs missing Ti1 neurons extended further than 50 μm, stopping at the Sema I boundary, and 100% (30/30) of the limbs with an intact Ti1 pathway had axons which extended greater than 50 μm, and had fasciculated normally with the Ti1 pathway. Although there is great variability in the lengths of the axons in this set of experiments, the key finding is that in the absence of the Ti1 neurons, many of the SGO axons were still less than 50 μm in length after culturing in the presence of 6F8, indicating that in addition to stimulating axon outgrowth, Sema I may also be needed for the proximal extension of the SGO axons on the limb epithelium after outgrowth has occurred. Although Sema I seems to be absolutely required for SGO axon initiation regardless of the presence or absence of the Ti1 neurons, the presence of the Ti1 neurons in the limb after outgrowth has occurred allowed the proximal extension of the majority of the SGO axons onto the Ti1 pathway in the presence of mAb 6F8. The ability of the Ti1 neurons to allow proximal extension of the SGO axons onto the Ti1 pathway is likely due to the establishment of filopodial contact of the SGO growth cones with the Ti1 neurons prior to, or soon after culturing in the presence of mAb 6F8. This would allow the growth cones to extend to the Ti1 neurons. To further address the role that Sema I and the Ti1 neurons play in SGO axon extension will require a careful analysis of individually labeled SGO axons using videomicroscopy.

The results of these experiments and the heat shock experiments suggest that Sema I may act as a high affinity substrate for the SGO growth cones (Fig. 8). These results also suggest that the SGO growth cones have a higher affinity for the Ti1

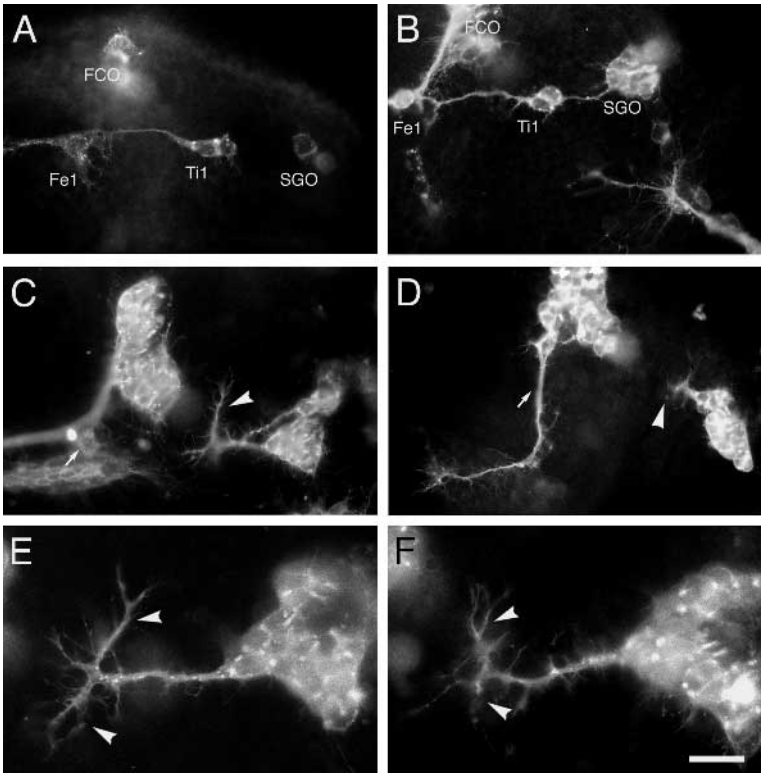

Fig. 6. Removal of Ti1 cell bodies in the limb fillet preparation inhibits proximal extension of the SGO growth cones. Limb fillets at 38% of development were prepared, allowing the Ti1 neuronal cell bodies to be selectively extracted. The limb fillets were cultured until 43% of development and neurons were subsequently visualized using standard immunohistochemistry. (A) At 38% of development, the cell bodies of the SGO and FCO are beginning to differentiate but do not yet have any axonal processes. (B) A control limb fillet following 30 hours in culture, showing that the SGO axons have contacted the Ti1 cell bodies and have fasciculated with the Ti1 pioneer pathway. Note also that the FCO has fasciculated with the Fe1 cell and has extended along the Ti1 pathway. (C) Removal of the Ti1 neurons at 38% of development prevented the proximal extension of the SGO growth cones resulting in a dorsal turn within the Sema 1 domain (arrowhead) when visualized 30 hours after culturing. The FCO axons have fasciculated with the Fe1 neuron (arrow) and have extended along its axon. (D) Both the Fe1 and Ti1 neurons were removed from a 38% limb fillet before culturing. After 30 hours in culture, the SGO axons have arrested their proximal extension (arrowhead) while the FCO axons have extended ventrally past their normal turning point at the region previously occupied by the Fe1 cell (arrow). The FCO axons extended to approximately the ventral midline of the limb before turning proximally toward the CNS. (E,F) Higher magnification of the arrested SGO growth cones in limbs where the Ti1 neurons were removed indicates that although the SGO axons have arrested their proximal migration, their growth cones do not appear collapsed, and they have extended multiple branches both dorsally and ventrally (arrowheads). Scale bar, 100 μm.

neurons and that the Ti1 neurons are necessary for continuous proximal extension out of the Sema I band. This scheme of affinities is consistent with previous reports on the hierarchy of affinities in the developing grasshopper limb bud (Caudy and Bentley, 1986b).

DISCUSSION

Intermediate targets are essential for the patterning of the developing nervous system in a variety of systems (Klose and Bentley, 1989; Ghosh et al., 1990; Marcus et al., 1995; Serafini et al., 1996; Tessier Lavigne and Goodman, 1996). In the developing grasshopper limb bud, the first projection towards the CNS is established by the Ti1 pioneer neurons between 30-34% of development (Keshishian and Bentley, 1983). Later in development, neurons arising in distal regions of the limb bud fasciculate with and extend along the Ti1 pathway into the CNS. One such groups of neurons, the mechanoreceptor neurons of the subgenual organ (SGO), arise at approximately 38% of development, distal to the Ti1 cell bodies. Klose and Bentley (1989) have shown that the Ti1 neurons are necessary to mediate the extension of axons from the SGO into the CNS. Removal of the Ti1 cell bodies by heat shock prevented proximal extension of the SGO axons past the approximate region of the Ti-Fe segment boundary of the limb, suggesting that unique mechanical or chemical properties of the limb epithelium in the region between the Ti1 cells and the SGO were preventing the proximal migration of the SGO growth cones in the absence of the Ti1 neurons. Using a monoclonal antibody directed against Grasshopper Semaphorin I (Sema I), we found that Sema I is expressed in a band of limb epithelium just distal to the Ti-Fe limb segment boundary directly between the Ti1 cell bodies and the SGO. During development, the axons of the developing SGO extend through the band of Sema I and fasciculate with the Ti1 pioneer pathway to enter the CNS. In limb buds where the Ti1 neurons were not present, the growth cones of the SGO were prevented from extending beyond the band of Sema I and were arrested within the proximal edge of the band, consistent with Sema I mediating this effect. However, unlike the functions of other Semaphorins, this effect was not mediated by collapse or growth cone repulsion.

Chick collapsin (the chick homologue of mouse Sema III) has previously been shown to prevent the extension of chick sensory growth cones in vitro by inducing growth cone collapse (Luo et al., 1993). This involves filopodial and lamellipodial retraction as the actin network disassembles in affected growth cones (Fan et al., 1993). In contrast, the SGO growth cones do not appear collapsed while contacting the band of Sema I and readily extend on the Sema I substrate. Presently, it is unclear what role growth cone collapse may play during development as there have been few observations of collapse in vivo. This is not unexpected as it would be unusual for the entire growth cone to come into contact with an inhibitory environment. Growth cones are likely deflected by high concentrations of collapsing activity due to localized collapse of a small region of the growth cone which first contacts the source of inhibitory cues, effectively steering the growth cone extension away from the source of inhibitory cues before complete growth cone collapse occurs. Consistent with this, Fan and Raper (1995) have shown that collapsin immobilized onto beads induces the turning of growth cones away from the bead following contact, rather than resulting in the complete collapse of the growth cone. Alternatively, a few filopodial contacts can result in the collapse of sympathetic neuron growth cones when they contact retinal neurites in vitro (Kapfhammer et al., 1987), suggesting that under the right conditions, a small number of contacts is sufficient for growth cone collapse. Unlike the in vitro results with chick collapsin, the ability of the SGO growth cones to migrate within the band of Sema I suggests that Sema I is not an inhibitory cue for the SGO axons, and the absence of growth cone collapse, even after the arrest of the SGO growth cones within the band, indicates that the arrest of the proximal extension of the SGO growth cones is not mediated by growth cone collapse.

Sema I is an attractive/permissive cue for the growth cones of the SGO

Members of the Semaphorin family have previously been shown to function as inhibitory guidance cues. However, our results are more consistent with Sema I being an attractive/adhesive cue for the SGO growth cones for a number of reasons. First, the SGO growth cones are able to extend well into the band of Sema I expression both during normal development and in the absence of the Ti1 cell bodies. Second, in

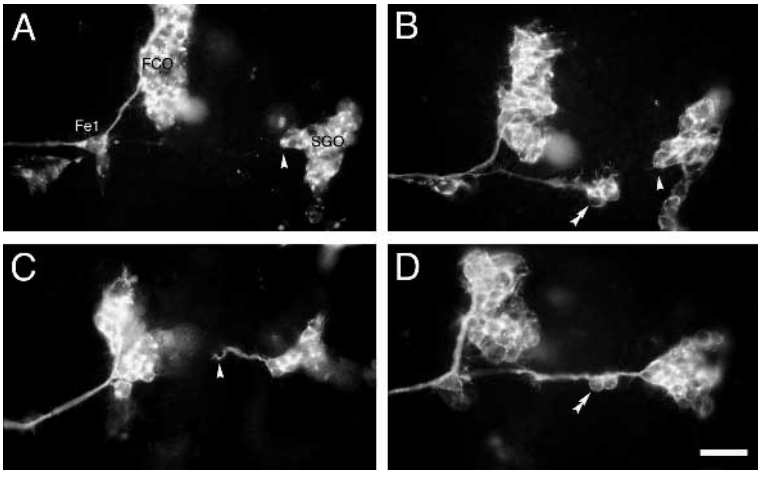

Fig. 7. mAb 6F8 blocking of Sema I prevents axon outgrowth from the SGO. Limb fillets at 38% of development were incubated until 43% of development in the presence of either 6F8 mAb or anti-cmyc mAb and then stained using standard immunocytochemistry. (A) Following incubation in mAb 6F8, the SGO axons were completely absent (arrowhead) in the limb fillets where the Ti1 cell bodies were removed prior to culture. Note that the FCO has differentiated and extended axons and fasciculated with the Fe1 cell. (B) In the presence of mAb 6F8, no axon outgrowth from the SGO was apparent (arrowhead) in limb fillets with intact Ti1 neurons (double arrowheads), while the FCO projection extended normally. (C) Incubation of limb fillets without the Ti1 cell bodies in the presence of anti-cmyc mAb did not prevent axon outgrowth from the SGO, but the axon was arrested from proximal migration (arrowhead) as seen in the absence of antibody. (D) In the presence of anti-cmyc mAb, and with the Ti1 neurons intact (arrowheads), the axons of the SGO were able to fasciculate normally with the Ti1 pathway. Scale bar, 100 μm

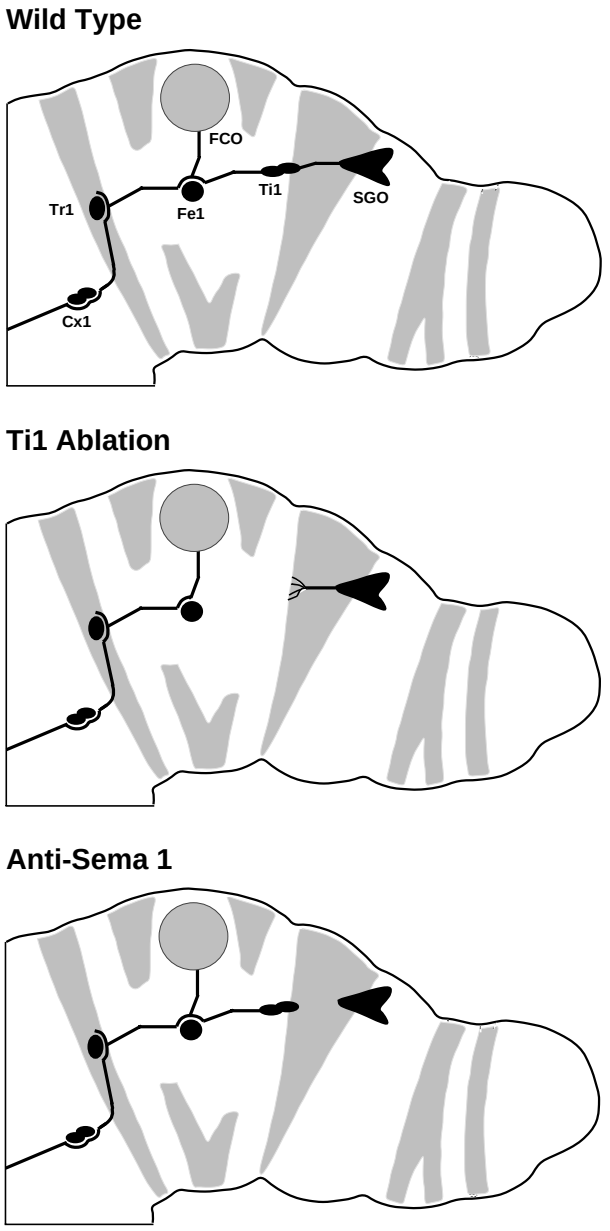

Fig. 8. Schematic summary of heat shock and limb fillet experiments. Top: The SGO axon normally fasciculates with the Ti1 pathway to grow towards the CNS. Middle: When the Ti1 pathway is eliminated by heat shock, the SGO axon is prevented from extending proximally to the CNS, and is arrested within a band of Sema I. The growth cones are not collapsed and typically extend to the proximal edge of the Semaphorin band. Bottom: The SGO axon is missing in experimental limb fillets cultured in media containing mAb 6F8 in the absence (or presence) of the Ti1 neurons.

the absence of the Ti1 cell bodies the SGO growth cones typically extend to the proximal edge of the Sema I band but do not extend further into the femur intrasegmental epithelium. It is therefore likely that the epithelium outside the Sema I band is not as permissive for axonal growth, thereby preventing the extension of the SGO growth cones out of the band of Sema I. The inability of the SGO growth cones to leave the Sema I band until contacting the Ti1 cells ensures that the SGO growth cones establish a projection into the CNS along the Ti1 pathway. Third the growth cones of the SGO are complex and highly branched while within the band of Sema I, typical of growth cones extending along an adhesive substrate (Payne et al., 1992; Burden-Gulley, 1995). Similarly, observations of Ti1 pioneer growth cone morphology (Caudy and Bentley, 1986a,b) and adhesive interactions (Condic and Bentley, 1989) have suggested that growth cones in the vicinity of segment boundaries, corresponding to the location of Sema I expression, exhibit a high affinity for the epithelium. Caudy and Bentley (1986a) have shown that Ti1 growth cones extend more lamellae, filopodia and branches at these locations. Fourth, antibody blocking experiments demonstrated that Sema I is necessary for initial axon outgrowth from the SGO. In the presence of anti-Sema I monoclonal antibody 6F8, axonal outgrowth was completely prevented in the majority of subgenual organs both in the presence and in the absence of the Ti1 cell bodies. In addition, in the absence of the Ti1 cell bodies, growth was also arrested in a significant number of subgenual organs that had initiated an axon at the time of 6F8 antibody addition.

A role for Sema I in the patterning of the grasshopper PNS

Our results indicate that Sema I is a guidance molecule which plays an important role in the proper development of the grasshopper PNS. We have found that Sema I is needed for initial axon outgrowth and extension from a subset of afferent neurons in the limb, the SGO neurons. However, while Sema I is needed for outgrowth from the SGO, the absence of Sema I-expressing epithelium adjacent to other neurons in the limb indicates axon initiation from other neurons, such as the Ti1 pioneers does not require Sema I. Additionally, although the proximal migration of the Ti1 axons is prevented by a band of Sema I at the Fe-Tr segment boundary of the limb, addition of 6F8 mAb to block the function of Sema I did not prevent the extension of the Ti1 growth cones onto the band of Sema I expression, but instead allowed the growth cones to extend across the band (Kolodkin et al., 1992), indicating the requirement of Sema I for axon extension differs between different neurons. These observations suggest that Sema I expressed on bands of limb epithelium functions to pattern afferent projections in the limb by stalling or stopping the proximal projection of growth cones, possibly to allow other guidance cues to direct further growth. Resumption of proximal migration of growth cones out of a Sema I band requires contact and fasciculation of the growth cones with other neurons at the proximal edge of the band. We have shown that the proximal extension of the SGO growth cones is arrested within a band of Sema I expression at the Ti-Fe segment boundary in the absence of the Ti1 neurons. The ability of the SGO growth cones to leave the band of Sema I in the presence of the Ti1 neurons is likely due to a preference of the SGO growth cones for the Ti1 neurons. Similarly, a band of Sema I at the Fe-Tr segment boundary has previously been demonstrated to prevent the proximal extension of the Ti1 growth cones. The Ti1 growth cones make a sharp ventral turn when they reach the Tr1 guidepost cell in a band of Sema I expressing epithelium and grow ventrally within the Sema I band. The growth cones do not leave the band until they contact and turn proximally toward the Cx1 guidepost cells located at the proximal edge of the band. Laser

ablation of the Cx1 cells prevents the Ti1 growth cones from making this proximal turn (Bentley and Caudy, 1983). The SGO neurons seem to respond to the Ti1 neurons in a similar manner. Thus, the Ti1 cells likely play an analogous role to the Cx1 cells in allowing growth cones to exit regions of G-Sema 1 expression.

The Semaphorins are a growing family of axon guidance molecules with multiple functions during development

Our results indicate a permissive role for Sema I in neuronal guidance. This is in distinct contrast to previously known functions of the secreted members of the Semaphorin family, suggesting that the transmembrane form of Semaphorin may have a contrary function. Previous antibody blocking experiments in developing grasshopper limb bud indicated that Sema I in the trochanter limb segment may provide a preferred substrate for the Ti1 growth cones (Kolodkin et al., 1992). Although alternative interpretations are consistent with this data (Kolodkin et al., 1993), a permissive/attractive role for Sema I is supported by observations that Ti1 growth cones exhibit a preference for epithelium in the region of limb-segment boundaries (Caudy and Bentley, 1986a, b; Condic and Bentley, 1989). In addition, a transmembrane form of Semaphorin isolated on T lymphocytes (CD-100) has been shown to stimulate lymphocyte aggregation (Hall et al., 1996) and new transmembrane members of the mouse Semaphorin family have thrombospondin repeats, a motif known to promote neurite outgrowth (Adams et al., 1996). However, in light of the homology to the secreted forms of Semaphorin, an alternative explanation is that Sema I molecules may act as bifunctional guidance cues. This has been demonstrated for members of the netrin family. Netrin 1 and Unc-6 (a nematode homologue of netrin) are bifunctional molecules, acting as chemoattractants and chemorepellents (Kennedy et al., 1994; Colamarino and Tessier-Lavigne, 1995; Hedgecock et al., 1990). UNC 6, a secreted protein which is believed to form a gradient in the extracellular environment, plays a role in circumferential guidance in the nematode. Null mutations disrupt both dorsal and ventral migrations while partial loss-of-function mutations disrupt either ventral or dorsal migrations, suggesting that different domains of the protein direct dorsal or ventral growth cone guidance (Hedgecock et al., 1990). In addition, axons extend either dorsally or ventrally along the presumptive UNC 6 gradient depending on the particular UNC-6 receptor expressed (Hamelin et al., 1993; Chan et al., 1996). In chick, netrin 1 secreted by the floor plate of the neural tube has been shown to attract the growth cones of commissural neurons whose axons extend towards the floor plate, and repel trochlear motor axons which originate near the floor plate and extend dorsally (Kennedy et al., 1994; Colamarino and Tessier-Lavigne, 1995). Further experiments will be necessary to determine the functional capabilities of secreted and transmembrane forms of Semaphorins.

The Semaphorins are a growing family of molecules with multiple functions during development. Initial studies have indicated that members of the Semaphorin family act as repulsive guidance cues and are essential in the patterning of the developing nervous system. Here, we have presented evidence for an alternative function for a transmembrane member of the Semaphorin family, Sema I, in acting as a cell surface attractive or permissive guidance cue in neuronal development. Additionally, recent evidence has also suggested that members of the Semaphorin family may have various functions in development other than its known function as a neuronal guidance molecule. Mouse knockout experiments have revealed that Semaphorin III is essential in the normal development of heart, bones and muscle (Behar et al., 1996). The finding that two Semaphorins are encoded in viral genomes (Kolodkin et al., 1993), as well as its expression on T lymphocytes (Hall et al., 1996) indicates that Semaphorins may play a role in the immune system. Although Semaphorins were initially implicated as chemorepellent cues, it is very likely that members of this family have diverse functions during development.

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