

## A sensory axon repellent secreted from ventral spinal cord explants is neutralized by antibodies raised against collapsin-1

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### SUMMARY

During embryogenesis, different subclasses of sensory neurons extend central projections to specific locations in the spinal cord. Muscle and cutaneous afferents initially project to the same location in the dorsal cord. Later, specific muscle afferents leave other afferents behind and project into the ventral cord. Previous studies have shown that ventral spinal cord explants secrete a repellent for sensory neurites. We now find that antibodies to collapsin-1 neutralize this repellent activity. Additional data suggest that all afferents respond to collapsin-1 when they are first confined to the dorsal cord, but that ventrally projecting

muscle afferents become collapsin-1 insensitive as they project into the ventral cord. Our results suggest that the transient dorsal expression of collapsin-1 prevents all efferents from entering the cord early and sustained ventral expression prevents dorsally terminating afferents from entering the ventral cord later.

Key words: collapsin-1, semaphorin-D, semaphorin-III, spinal cord, sensory afferent, trk-C, antibody, repulsion, repellent, growth cone collapse, guidance, pathfinding, chick

### INTRODUCTION

The correct wiring together of the nervous system requires that specific axonal growth cones respond appropriately to a variety of inhomogeneously distributed guidance cues in their environment. Some of these cues are likely to act as attractants, while others are likely to act as repellents. Dorsal root ganglion (DRG) neurons provide a particularly advantageous system for the study of axonal guidance and the characterization of possible guidance cues. Each DRG is composed of many anatomically and physiologically distinct classes of primary sensory neurons, which terminate centrally in well-defined and distinct target regions (Brown, 1981; Willis and Coggeshall, 1991).

The early development of central projections of chick muscle and cutaneous sensory afferents has been well described (Davis et al., 1989; Mendelson et al., 1992). They first reach the spinal cord within lumbar regions at E4 (stage 23). The axons initially extend both rostrally and caudally in the nascent dorsal funiculus. Not until E6 (stage 28) do branches from these axons begin to invade the spinal gray matter of the dorsal horn. For the next 2 days, the growing branches of the cutaneous and muscle afferents are confined to the dorsal gray matter. Around E8.5 (stage 34), ventrally projecting muscle afferents begin to leave the cutaneous afferents behind in the dorsal horn, extending through the spinal gray matter until they reach the ventral horn laminae around E10.

The mechanisms responsible for the patterning of DRG

afferents in the spinal cord are largely unknown; however, there is some evidence from studies using mouse and rat tissues that suggests a role for inhibitory or repellent guidance cues. DRG neurites selectively fail to invade ventral regions of mouse spinal cord explants, but do invade dorsal cord regions (Peterson and Crain, 1982). More recently, rat ventral spinal cord explants, but not dorsal cord explants, have been shown to secrete a diffusible factor that repels sensory neurites in culture (Fitzgerald et al., 1993). A strong candidate for this repellent cue is collapsin, a protein in chick brain tissue that induces collapse and paralysis of sensory neurite growth cones (Luo et al., 1993). Collapsin, which has subsequently been designated collapsin-1, is a member of a family of proteins, the semaphorins/collapsins (Kolodkin et al., 1993; Püschel et al., 1995; Luo et al., 1995). Species homologues of chick collapsin-1 include human semaphorin III and mouse semaphorin D, both of which are approximately 90% identical to collapsin-1 in their amino acid sequences (Püschel et al., 1995; Messersmith et al., 1995).

The distribution of collapsin-1 mRNA in the chick spinal cord suggests that it could be the ventral cord repellent (Shepherd et al., 1996). Collapsin-1 mRNA is first seen in the chick cord at E4 (stage 24). By E6 (stage 28), the time at which the very first sensory axons have just begun to enter the gray matter of the dorsal horn, collapsin-1 mRNA is strongly expressed in two locations in the ventral half of the spinal cord: in cells near the spinal canal and in the ventral horns. There is a lower level of expression in the dorsal half of the cord, prin-

cially in a group of cells directly adjacent to the area where sensory afferents will first enter the spinal gray matter (Fig. 1). Over the next 3 days, a collapsin-1 free zone enlarges in the location where the dorsal horn laminae are developing. By E9/10 (stage 35/36), collapsin expression is more diffuse but less intense throughout the cord and remains absent from the dorsal horn. Other studies using RNA probes to the rat and mouse homologues of collapsin-1 have also reported a much higher level of collapsin-1 message in the ventral as compared to dorsal cord at the age when sensory afferents first project into the dorsal cord (Püschel et al., 1995; Messersmith et al., 1995; Wright et al., 1995; Püschel et al., 1996; Giger et al., 1996).

A recent paper by Messersmith et al. (1995) repeated the Fitzgerald et al. (1993) experiments and, in addition, showed that rat DRG neurites do not grow towards COS cells expressing the human homologue of collapsin-1, sema-III, in collagen gel cocultures. They inferred that collapsin-1 could be the sensory axon repellent secreted by the ventral cord. Messersmith et al. (1995) further showed that the responsiveness of rat DRG neurites to the spinal cord and to the collapsin-1 repellent activities depends on which neurotrophin is used to supplement the media in which explants are cultured. They reported that DRG neurites grown in media containing NT-3 do not avoid ventral cord or collapsin-1-secreting COS cells, while neurites grown in media containing NGF do avoid ventral cord or collapsin-1-secreting COS cells. Evidence from other studies suggests that NT-3 supports the survival of muscle afferents that terminate in the ventral horn while NGF supports many other sensory neurons that send projections only to dorsal laminae (Klein et al., 1994; Oakley et al., 1995). Because of this selective dependence of different classes of sensory axons on NT-3 and NGF, the results of Messersmith et al. (1995) are consistent with the hypothesis that collapsin-1 released from cells in the ventral cord prevents collapsin-1 sensitive afferents from invading ventrally, but does not interfere with the normal ventral termination of collapsin-1 insensitive afferents.

The primary objective of this study was to determine if collapsin-1 is the sensory axon repellent activity secreted by the ventral spinal cord. We raised polyclonal antibodies against pure recombinant collapsin-1 that block the collapsing activity of recombinant protein in a tissue culture assay. Here we report that these antibodies neutralize a sensory axon repellent secreted by ventral spinal cord explants. This result provides strong support for the identification of collapsin-1 as a sensory axon repellent secreted by the ventral spinal cord. In addition, our results suggest that all sensory afferents may be sensitive

to collapsin-1 around the time that they first enter the spinal gray matter. Ventrally terminating muscle afferents are confirmed to become insensitive to collapsin-1 later, around the time that they invade collapsin-1 rich regions in the ventral cord. These results are consistent with the hypothesis that dorsally produced collapsin-1 acts as a repellent for all sensory afferents early, preventing their entry into the dorsal gray matter. Ventrally produced collapsin-1 may act later to prevent the entry of dorsally terminating sensory afferents into the ventral gray matter.

## MATERIALS AND METHODS

### RNA in situ hybridization

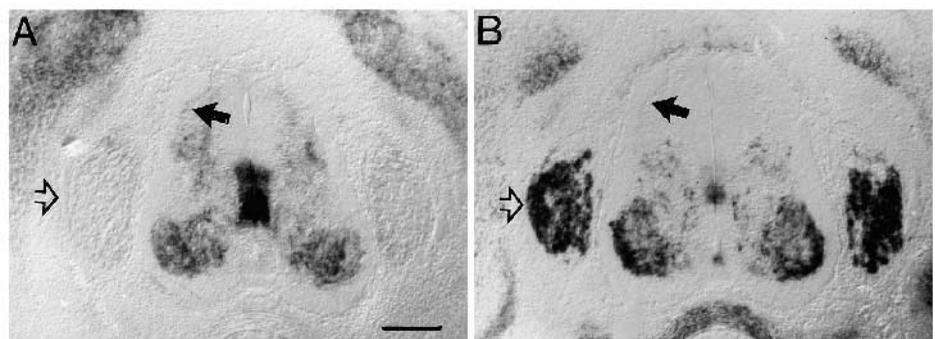
Digoxigenin-labeled RNA probes complementary to the entire coding sequences of collapsin-1 and collapsin-2 were generated using a procedure derived from that of Schaeren-Wiemers and Gerfin-Moser (1993) as reported in Luo et al. (1995). Chick tissue was prepared and hybridized as described in Shepherd et al. (1996).

### Anti-collapsin-1 antiserum production

To obtain a sufficient amount of collapsin-1 protein for antibody production, we engineered a myc-epitope tagged version of collapsin-1 into the baculovirus transfer vector pVL1392. The c-myc epitope (EQKLISEEDL) was inserted just before the stop site at the carboxy-terminal end of the molecule. Virus capable of directing the expression of a recombinant collapsin-1 protein in insect cells was generated using the instructions supplied by PharMingen. Recombinant protein can be detected by the anti-c-myc monoclonal antibody 9E10 (Evan et al., 1985). The myc-tagged collapsin-1 secreted into 3 day postinfection media was enriched on a S-Sepharose cation exchange column (Luo et al., 1993) and further purified to homogeneity on an anti-myc 9E10 antibody affinity column. Approximately 2-5 mg of myc-tagged collapsin-1 can be purified from one liter of insect cell culture medium. The myc-tagged collapsin-2 protein was produced in a similar manner.

Rabbits were injected with 100 µg of myc-tagged collapsin-1 a total of four times at 2-week intervals before collection of their serum. Antisera were tested for their ability to recognize recombinant collapsin-1 on western blots. Those that recognize collapsin-1 on western blots were tested for their ability to neutralize the collapsing activity of recombinant collapsin-1. For the functional blocking experiments, antibodies are purified from the best antiserum (rabbit #475) with a protein A affinity column according to Harlow and Lane (1988). To deplete the purified antibodies of anti-collapsin-1 activity, 500 µl of antibodies (5.6 mg/ml) were incubated with 10 µg of purified myc-tagged collapsin-1 for 2 hours at room temperature, loaded into an anti-myc affinity column, incubated for an additional 3 hours and then run off the column with PBS that included an additional 0.1 M NaCl.

**Fig. 1.** Comparison of collapsin-1 and collapsin-2 mRNA expression in E6 chick spinal cord. The expression patterns of collapsin-1 (A) and collapsin-2 (B) mRNAs in adjacent fixed and frozen cross sections of a stage 28 (E6) chick spinal cord made at the lumbosacral level and visualized with digoxigenin-labeled RNA probes. A DRG (open arrow) and what will later be the entry point for sensory axons into the dorsal gray matter of the cord (black arrow) are indicated. Scale bar, 200 µm.



### Western blot analysis

Purified myc-tagged collapsin-1, collapsin-2 and highly enriched collapsin-1 preparation from adult chicken brain were separated on a 7% reducing SDS-PAGE gel. The native collapsin-1 from adult chicken brain was enriched through S-sepharose and WGA affinity columns as described by Luo et al. (1993). The resulting western blot was probed with anti-c-myc monoclonal antibody, anti-collapsin-1 antiserum from rabbit #475, antiserum depleted of anti-collapsin-1 antibodies and preimmune serum from rabbit #475. Appropriate anti-mouse or anti-rabbit alkaline-phosphatase-conjugated secondary antibodies (Jackson) were used and reacted according to standard protocols.

### Neural explants and cell culture for collapse assay

Chick DRG explants were prepared and cultured as described in Fan et al. (1993). Experiments in which the collapsin-1 responsiveness of DRGs grown in NGF or NT-3 were compared the same base medium as described in Fan et al. (1993) was utilized, but with 40 ng/ml NT-3 (Alamone labs) or 7S NGF (Coll. Res.) instead of 20 ng/ml NGF.

### Collapse assay

The procedure for the collapse assay was essentially the same as that previously described (Raper and Kapfhammer, 1990). In brief, small aliquots of recombinant collapsin-1 or collapsin-2 were added to DRG explant cultures. The added material was gently mixed into the culture medium, the cultures were incubated at 37°C in 5% CO<sub>2</sub> for 1 hour, and then fixed for 1 hour with 4% paraformaldehyde in PBS containing 10% sucrose. The fixed preparations were then scored as to whether their growth cones had normal spread morphology (i.e. had lamellipodia and filopodia) or were collapsed. Experiments in which the morphology of TrkC-expressing growth cones was assessed were processed as follows. Fixed cultures were washed several times in PBS, incubated in a blocking solution of 3% BSA, 2.2% polyvinylpyrrolidone, 0.3% Triton X-100 in PBS for 30 minutes, and then incubated overnight at 4°C in polyclonal rabbit anti-TrkC antiserum diluted 1:2000 in blocking solution. The following day, cultures were washed several times in PBS, incubated 1-2 hours at room temperature in Cy<sup>3</sup>-conjugated anti-rabbit secondary antibody (Jackson) diluted 1:500 in blocking solution, washed several times in PBS and then scored as described, previously. All incubations were at room temperature unless otherwise stated. TrkC-positive and TrkC-negative growth cone morphologies were scored separately.

The neutralizing activity of anti-collapsin-1 antibodies was determined by incubating 1 µl of buffer containing various amounts of collapsin-1 with 5 µl of 5 mg/ml anti-collapsin-1 antibodies for 15-

20 minutes at room temperature prior to their addition to DRG cultures.

### Agarose gel cocultures

DRGs from lumbar cords of E7 embryos were explanted onto laminin-coated filming dishes (MatTek 35mm) and cultured at 37°C in 5% CO<sub>2</sub> for 5 hours in 150 µl of our standard media (Fan et al., 1993). The laminin coating was applied by pipetting 100 µl of 40 ng/ml laminin onto the glass coverslip and incubating for 1 hour. The laminin was removed and replaced with 150 µl of media and a single DRG explant was placed on the coverslip. After 5 hours, the media was replaced with 50 µl of fresh media warmed to 37°C containing 1% melted agarose (Sigma Type VII low gelling temperature). After letting the media partially solidify at room temperature for 1-2 minutes, a single piece of either E6 dorsal or ventral lumbar spinal cord approximately 500×900 µm across was explanted on the surface of the solidified medium approximately 800 µm from the DRG explant. An additional 100 µl of warm 1% agarose media was added to the culture and allowed to solidify. Cultures were incubated overnight at 37°C at 5% CO<sub>2</sub>. The following day, patterns of DRG neurite outgrowth were observed with phase optics on a Zeiss Axiovert 35. Patterns of neurite outgrowth were recorded on an optical laser disc for analysis at a later time. Either 5 µl or 15 µl of 5 mg/ml collapsin-1 antibodies, antiserum depleted of collapsin-1 antibodies or preimmune serum were added to 1 ml of melted agarose culture medium prior to its addition to the explant cultures.

### Scoring of the cocultures

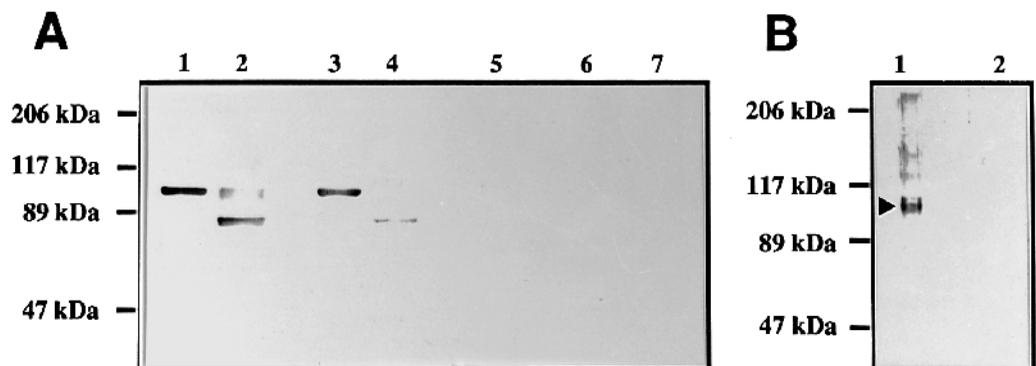
Stored images of all explant cultures were coded at random and then scored blind by a single individual. Cultures where the average length of DRG neurites was less than 900 µm (i.e. less than the distance from the DRG to the distal side of the ventral cord explant) were not scored. If DRG neurites were seen to turn away from a spinal cord explant, and no more than five neurites were seen to extend underneath it to the far side of the explant, the culture was scored as one where avoidance was seen. A culture that did not meet these criteria was scored as one where no avoidance was seen.

## RESULTS

### Production of a polyclonal antiserum to collapsin-1 that neutralizes its functional activity

A rabbit polyclonal antiserum was raised to pure recombinant chick collapsin-1. Antiserum from rabbit number 475 recog-

**Fig. 2.** Characterization of rabbit anti-chick collapsin-1 antibodies. (A) 2 µg of recombinant myc-epitope-tagged collapsin-1 (lanes 1,3,5,6) or recombinant myc-epitope-tagged collapsin-2 (lanes 2,4,7) were run on an SDS-PAGE gel and blotted onto nitrocellulose. Lanes 1 and 2 were probed with a monoclonal antibody to the myc epitope, lanes 3 and 4 were probed with anti-collapsin-1 antiserum from rabbit #475, lanes 5 and 6 were probed with preimmune serum from rabbit #475, and lanes 6 and 7 were probed with protein-A purified, collapsin-1-depleted antibodies prepared from the same serum. (B) A western blot of collapsin-1 enriched by S-sepharose and WGA chromatography from adult chick brain. 2 brain equivalents of WGA eluate were added per lane. Lane 1 was probed with affinity-purified anti-collapsin-1 antibody from rabbit #475, and lane 2 was probed with affinity-purified preimmune antibody from the same rabbit. The arrowhead indicates the collapsin-1 band in lane 1.

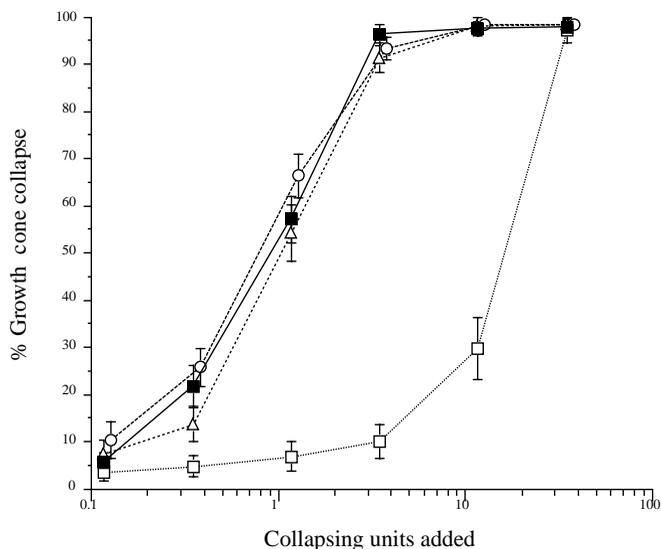


gized the antigen. The antiserum was purified by protein-A affinity chromatography and found to be specific for chick collapsin-1. The antiserum was found to be neutralizing in coculture assays (data not shown).

nizes recombinant collapsin-1 protein on western blots (Fig. 2A, lane 3), while preimmune serum from the same rabbit fails to recognize the recombinant protein (Fig. 2A, lane 7). Anti-collapsin-1 antiserum also recognizes to a lesser degree a related molecule, collapsin-2, on western blots (Fig. 2A, lane 4). The antibodies bind a form of recombinant collapsin-2 that includes the full peptide sequence, as judged by the presence of a myc-epitope tag that was engineered onto the C-terminal end of the molecule, but which is shorter (Fig. 2A, lane 4, low band) than what is presumably the normal glycosylated form (Fig. 2A, lane 2, upper band). Anti-collapsin-1 antiserum recognizes a band of appropriate molecular weight for native collapsin-1 on western blots of adult chick brain derived collapsing activity (Fig. 2B, lane 1). This band can only be detected if the collapsing activity is first enriched by S-sepharose and WGA affinity chromatography as described by Luo et al. (1993). Weaker additional higher bands were also evident that were not detected by the preimmune serum from the same rabbit (Fig. 2B, lane 2).

Collapsin-1-binding antibodies were removed from the anti-collapsin-1 antibody preparation to generate a 'depleted' antibody pool for control studies. Anti-collapsin-1 antibodies were reacted with myc-epitope-tagged recombinant collapsin-1 and then removed from solution by passing the mixture over an anti-myc column. This depleted antibody preparation did not recognize either recombinant collapsin-1 or collapsin-2 on western blots (Fig. 2A, lanes 5,6).

Growth cone collapse is elicited in a dose-dependent manner when varying amounts of partially purified recombinant collapsin-1 are added to explanted E7 DRG cultures. Varying

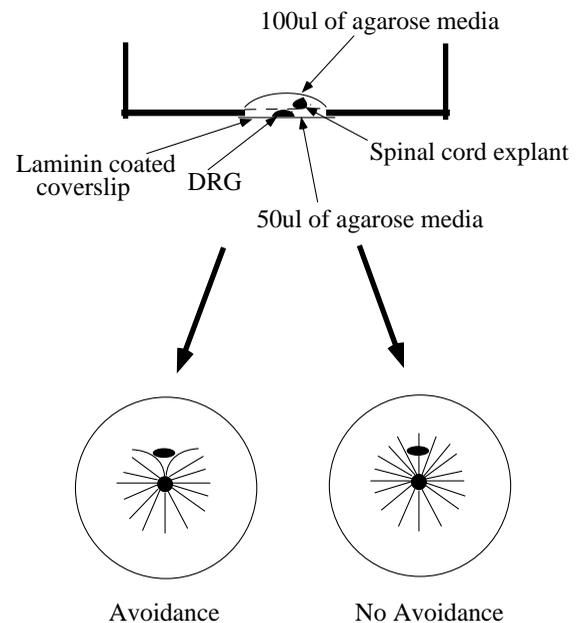


**Fig. 3.** The neutralization of collapsin-1 activity by anti-collapsin-1 antibodies in vitro. The relative responsiveness of E7 DRG growth cones when treated with various concentrations of partially purified recombinant collapsin-1 protein preincubated with a fixed amount of rabbit protein-A purified anti-collapsin-1 antibodies (open squares); protein-A purified antibodies from preimmune serum (open triangles); protein-A purified antibodies depleted of anti-collapsin-1 antibodies (open circles); or no antibodies (closed squares). See Methods for details. Error bars indicate 95% confidence limits of the means estimated using the assumption that the data are distributed according to a binomial distribution.

amounts of recombinant collapsin-1 were preincubated with a fixed amount of protein-A purified anti-collapsin-1 polyclonal antibodies prior to adding the mixture to DRG explant cultures. A comparison of this dose-response curve with a curve generated in the absence of antibodies is shown in Fig. 3 (no antibodies, filled squares; with antibodies, open squares). Approximately 20-fold more collapsin-1 is required to give the same collapsing response in the presence of 25  $\mu$ g of anti-collapsin-1 antibodies than is required without antibodies present. Presumably the neutralizing effect of a fixed amount of activity-blocking antibodies is nullified when they are all bound up to excess collapsin-1. The addition of protein-A purified antibodies prepared from preimmune serum (Fig. 3, open circles), or anti-collapsin-1-depleted antibodies (Fig. 3, open triangles) do not block activity as judged by their failure to alter collapsin-1 dose-response curves.

### Chick ventral spinal cord explants secrete a sensory axon repellent that is blocked by antibodies to collapsin-1

E7 chick DRGs were cocultured with E6 chick dorsal or ventral spinal cord halves to confirm that chick ventral spinal cord explants, like those of rat, secrete a repellent for sensory axons. The DRGs were explanted onto laminin-coated glass coverslips upon which their axons grew. Spinal cord explants were



**Fig. 4.** Schematic diagram illustrating the agarose coculture experimental arrangement. The upper schematic shows a stylized cross section through an agarose coculture. Neurites extend from a DRG explant on laminin-coated glass. Approximately 800  $\mu$ m away, and 200  $\mu$ m above the glass, a spinal cord explant is suspended in agarose-containing medium. The lower schematics illustrate the two principle results obtained after approximately 18 hours in culture as seen from above. On the left is a culture that would be scored as one in which avoidance has occurred. The normal radial pattern of DRG neurite outgrowth is disrupted by neurites turning away from the spinal cord explant. The schematic on the right illustrates a culture in which the radial pattern of DRG neurites is unperturbed by the spinal cord explant; it would be scored as one in which avoidance has not occurred.

**Table 1. The effects of preimmune, anti-collapsin-1 and anti-collapsin-1-depleted antibodies on the avoidance of ventral spinal cord explants by DRG neurites**

Explant type and condition	n	Avoidance	
		%	No avoidance %
Dorsal cord only	24	21	79
Ventral cord only	45	73	27
Ventral cord + 15 $\mu$ l preimmune	21	71	29
Ventral cord + 15 $\mu$ l antibody	26	23	77
Ventral cord + 5 $\mu$ l antibody	20	45	55
Ventral cord + 5 $\mu$ l depleted antibody	19	74	26

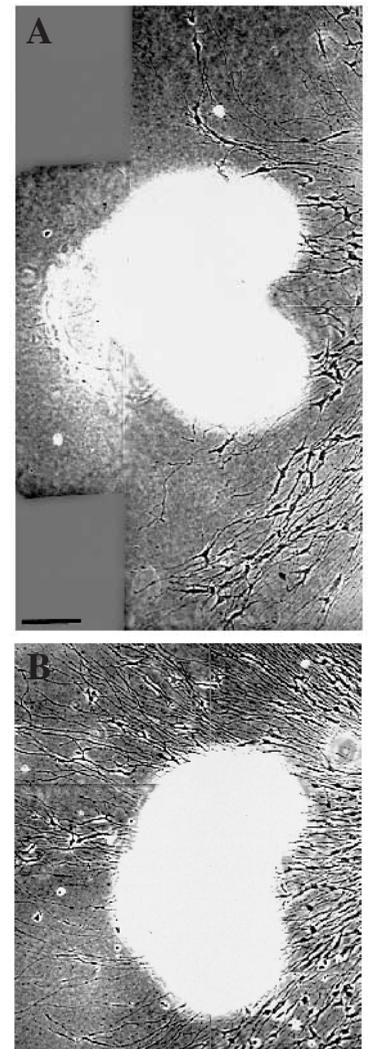
E7 DRGs were explanted next to the tissues indicated in the left-hand column. No antibodies, antibodies purified from preimmune serum, antibodies purified from antiserum against collapsin-1, or anti-collapsin-1-depleted antibodies were added to the cocultures. The number of explant pairs analyzed are indicated along with the percentage that showed avoidance.

suspended in agarose stabilized medium approximately 200  $\mu$ m above the glass surface and 800  $\mu$ m away from the DRGs (Fig. 4). The agarose prevents convective currents from disrupting gradients of secreted proteins generated by the spinal explants. Importantly, axons cannot grow from spinal explants embedded in agarose medium. Previous studies have utilized collagen-stabilized cultures for the same purpose, which, in contrast, provide a substratum upon which spinal axons can grow. These axons would be expected to disrupt the assay since previous work indicates that sensory axons collapse and turn away when contacting a variety of CNS axons (Kapfhammer and Raper, 1987a,b). The agarose gelled medium thereby allowed us to better focus our assay on exclusively diffusible repellent activities. Sensory axons were scored as avoiding spinal explants if they turned away and did not extend underneath them (see Methods). All scoring was done blind from videoimages stored on a laser disk.

In agreement with previous studies using rat tissues, neurites extending from E7 chick DRGs avoid E6 ventral spinal cord explants (73%) significantly more often than they avoid dorsal cord explants (21%) (Table 1; Fig. 4A). When preimmune serum is included in the media of test cultures, the proportion of sensory axons that avoid ventral cord explants is essentially unaffected. However, if anti-collapsin-1 antibodies are included in the agarose media, the avoidance of ventral cord by sensory axons is reduced to a level comparable to that seen for axons approaching dorsal cord explants (Table 1; Fig. 4B). Smaller concentrations of anti-collapsin-1 antibodies reduce avoidance without completely abolishing it. Concentrations of anti-collapsin-1-depleted antibodies equal to these lower concentrations do not neutralize avoidance. In pilot experiments, higher concentrations of depleted antibodies partly neutralized avoidance, presumably because not all anti-collapsin-1 antibodies were removed from the preparation (data not shown).

### The sensitivity of ventrally terminating muscle afferents to recombinant collapsin-1 protein is age dependent

Previous work suggests that ventrally terminating muscle afferents, unlike cutaneous afferents, are insensitive to collapsin-1. In an effort to confirm this result, and to determine if this is true at early developmental stages, we performed a series of growth cone collapse assays using E7 and E10 DRG explants cultured in either NGF- or NT-3-sup-

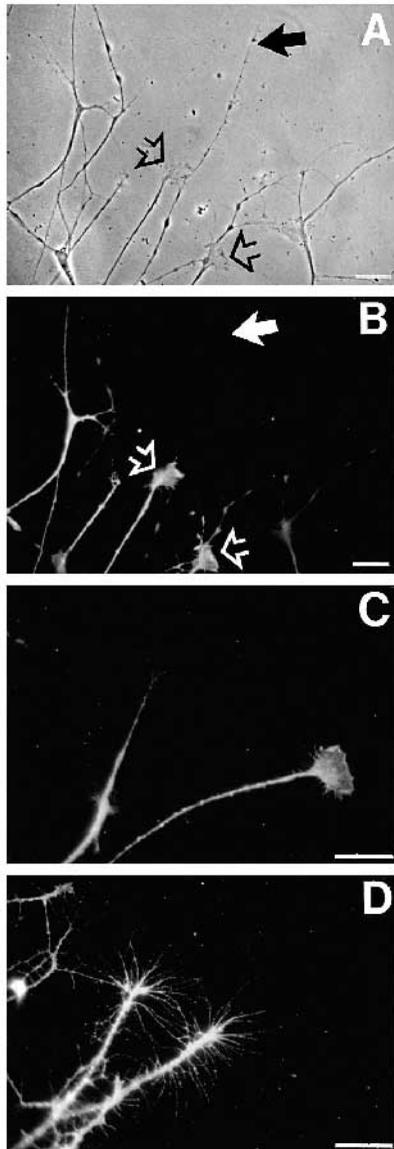


**Fig. 5.** Anti-collapsin-1 antibodies neutralize a sensory neurite repellent secreted by ventral spinal cord explants. Phase-contrast photographs of E7 DRGs and E6 ventral spinal cord explants cocultured overnight. In the absence of anti-collapsin-1 antibodies, DRG neurites turn away from a ventral spinal cord explant (A). In the presence of anti-collapsin-1 antibodies, avoidance is neutralized, allowing neurites to pass underneath a ventral spinal cord explant (B). Scale bar, 200  $\mu$ m.

plemented media (Fig. 7). A polyclonal antibody to chicken TrkC was used to identify sensory axon subtypes expressing the extracellular domain of the chick TrkC receptor (Lefcort et al., 1996). At later developmental stages, these are likely to consist mostly of group Ia and II muscle afferents, since it is these subtypes that are missing from TrkC knock out mice (Klein et al., 1994).

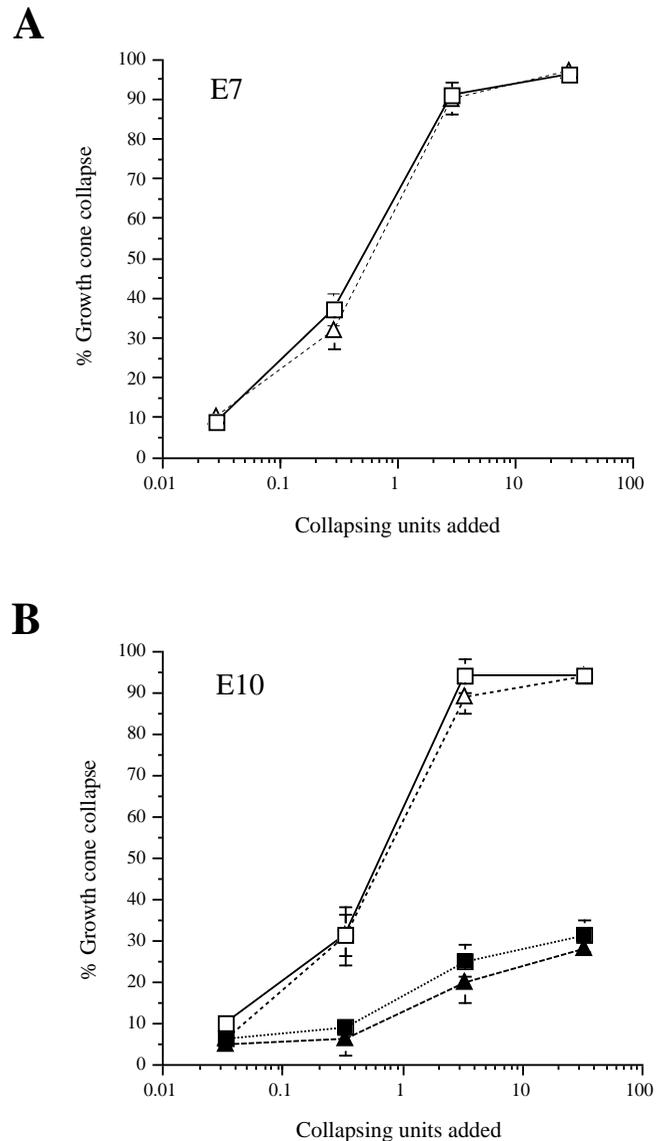
In E10 DRG explants, a clearly identifiable subset of TrkC-positive neurites is observed when the explants are grown in either NGF- or NT-3-supplemented cultures. Between 50 and 70% of all neurites are TrkC-positive in NT-3, while only 20-30% are TrkC-positive in NGF. There is also a marked difference in responsivity to recombinant collapsin-1 between TrkC-positive growth cones as compared to TrkC-negative growth cones. If a collapsing unit (CU) is defined as the amount of collapsin-1 required to collapse 50% of E7 DRG growth cones, then 4 CU causes over 90% of TrkC-negative E10 growth cones to collapse and only about 20% of E10 TrkC-positive growth cones to collapse (Figs 6A,B, 7A,B). Similar results are seen in both NT-3- and NGF-containing cultures. TrkC-positive muscle afferents are significantly less sensitive to collapsin-1 than TrkC-negative afferents, although up to a third of all TrkC-positive growth cones can collapse under these con-

**Fig. 6.** The effects of collapsin-1 on the growth cones of TrkC-positive E10 DRG neurons. A subset of E10 DRG growth cones express TrkC as recognized by a rabbit polyclonal antiserum. (A) A phase-contrast view of E10 growth cones grown in NT-3-supplemented media with 30 c.u. of added recombinant collapsin-1. Both spread (open arrows) and collapsed (closed arrow) growth cones are apparent. (B) TrkC expression in the same field of view as in A. The position of the spread (open arrows) and collapsed (closed arrow) growth cones are indicated. The majority of spread growth cones are TrkC-positive while the majority of collapsed growth cones are not. (C) An example of both spread and collapsed E10 DRG TrkC-positive growth cones after treatment with 30 c.u. of recombinant collapsin-1. (D) Treatment with 100 c.u. of recombinant collapsin-1 induces the loss of lamellipodia in all E10 DRG TrkC-positive growth cones. Scale bar, 20  $\mu$ m.



ditions (Fig. 6C). When 100 CU are added to E10 cultures, total collapse of all TrkC-positive growth cones is observed in either NGF- or NT-3-containing cultures. These collapsed neurites, however, cannot be scored according to our normal criteria since, in spite of the withdrawal of their lamellae, they still have filopodia (Fig. 6D).

Recombinant collapsin-1 causes similar amounts of growth cone collapse in E7 DRG explants cultured in either NGF- or NT-3-supplemented media (Fig. 7A). Staining E7 DRG explants with the TrkC antibody reveals that, at this age, as *in vivo* (Eric Frank, unpublished observations), nearly all axons are TrkC immunopositive, regardless of whether they are cultured in NGF- or in NT-3-containing media (data not shown). It is therefore impossible to positively identify ventrally terminating muscle afferents in these early cultures. Never the less, the fact that all E7 growth cones collapse at low concentrations of collapsin-1, and that they are equally responsive whether grown in NGF or NT-3, suggests that ventrally terminating muscle afferents are fully responsive at this early age.



**Fig. 7.** Collapsin-1 collapses all E7 chick DRG growth cones but only collapses TrkC-negative E10 DRG growth cones. (A) The relative responsiveness of E7 DRG growth cones when treated with various concentrations of partially purified collapsin-1 protein. The DRGs were cultured overnight in base media supplemented with 40 ng/ml NGF (squares) or NT-3 (triangles). (B) The relative responsiveness of E10 DRG growth cones when treated with various concentrations of partially purified collapsin-1 protein. The DRGs were cultured overnight in base media supplemented with 40 ng/ml NGF (squares) or NT-3 (triangles). All cultures were stained after fixing with an anti-TrkC antibody and TrkC-positive (closed symbols) and TrkC-negative (open symbols) were scored separately for collapse. Error bars indicate 95% confidence limits of the means estimated using the assumption that the data are distributed according to a binomial distribution.

### Recombinant collapsin-2 fails to collapse of DRG growth cones

As reported previously, collapsin-2 mRNA is also expressed in the ventral spinal cord. Since the antibodies that we raised against collapsin-1 cross react with collapsin-2, we tested sensory growth cones for responsiveness to recombinant

collapsin-2. Collapse assays with recombinant collapsin-2 were carried out on E7 and E10 DRG explants cultured in either NT-3- or NGF-supplemented media. These cultures were also stained with the anti-TrkC antisera after fixation. A range of collapsin-2 protein concentrations was tested for collapse activity corresponding to concentrations from 1- to 100-fold of what is required to obtain 50% collapse with collapsin-1 protein. No collapse is obtained in TrkC-positive or -negative growth cones in either E7 or E10 DRG cultures grown in NT-3- or in NGF-supplemented media (data not shown). Similar experiments with recombinant collapsin-3 indicates that it also does not collapse E7 DRG growth cones (data not shown).

## DISCUSSION

### Ventral cord repellent activity

If collapsin-1 constitutes the sensory axon repellent produced by the ventral spinal cord: (1) soluble collapsin-1 should repel sensory axons, (2) collapsin-1 should be produced in the ventral cord and (3) antibodies capable of neutralizing collapsin-1 activity should prevent sensory axons from being repelled by ventral spinal cord explants.

Collapsin-1 was initially purified on the basis of its ability to inhibit the motility of sensory axon growth cones (Luo et al., 1993). Consistent with this motility-inhibiting activity, sensory axons will not approach transfected COS cells secreting recombinant collapsin-1/Sema-D/Sema-III in collagen stabilized medium (Püschel et al., 1995; Messersmith et al., 1995). Collapsin-1 can therefore act as a repellent of sensory axons. The production of collapsin-1 can be tentatively inferred by the prominent expression of collapsin-1 mRNA in the ventral spinal cord (Fig. 1A) at appropriate developmental times (Püschel et al., 1995; Messersmith et al., 1995; Wright et al., 1995; Shepherd et al., 1996). Although some collapsin-1 mRNA is present in the dorsal cord as well, perhaps consistent with the small amount of repellent activity that we observed with dorsal cord explants, the great preponderance of collapsin-1 mRNA is found in the ventral cord. We have thus far been unsuccessful in confirming the presence of collapsin-1 protein within the ventral spinal cord. The polyclonal antibody that we produced does not detect an appropriately sized band on western blots of crude extracts from whole or ventral cord, nor does it detect a ventrally localized protein in fixed and frozen spinal cord sections. We tentatively attribute our failure to visualize the collapsin-1 protein to the extremely high potency of its activity and the concomitantly low levels of collapsin-1 likely to be physiologically produced. Our antibody may not be sensitive enough to detect physiological protein levels.

The avoidance of ventral cord explants by sensory axons is greatly reduced in the presence of anti-collapsin-1 antibodies. Sensory axons behave as if they were approaching dorsal cord explants instead of ventral cord explants and generally do not turn away. Antibodies prepared from preimmune serum do not neutralize the ventrally produced repulsive activity for sensory axons. These are the expected results if the repellent activity is collapsin-1. Consistent with this interpretation, a recent brief description of a sema-D/collapsin-1 knockout mouse indicates that at least one class of sensory axons terminates in abnormal

ventral locations in the absence of collapsin-1 (Bahar et al., 1996).

As a further control for our studies, anti-collapsin-1 antibodies were reacted with myc-epitope-tagged recombinant collapsin-1 and then removed from solution by passing the mixture over an anti-myc column. The antibodies that flowed through presumably should be those that do not bind collapsin-1. This depleted antibody preparation does not neutralize the collapsing activity of recombinant collapsin-1 protein and is also significantly less effective in blocking the ventrally produced sensory axon repellent activity than the original, non-depleted antibodies. However, these depleted antibodies still have some residual ability to neutralize the ventrally produced repellent. We attribute this remaining neutralizing activity to a failure of our procedure to remove all anti-collapsin-1 antibodies, particularly those that bind collapsin-1 at lower affinities. Even these poorly binding antibodies may be sufficient to retard the diffusion of collapsin-1 from the ventral explant through the agarose, thereby preventing sensory axon avoidance of the explant at a distance.

It is important to note that, although the antibodies were raised against pure recombinant collapsin-1, on western blots they cross react with the related molecule collapsin-2, and on blots of native material they recognize higher molecular weight bands that could correspond to other semaphorin family members. Collapsins 1 and 2 have identical domain structures but are only about 50% identical in their amino acid sequences (Luo et al., 1995). Like collapsin-1, collapsin-2 mRNA is also predominantly localized to the ventral cord (Fig. 1B) while sensory axons are making their central connections (Luo et al., 1995). The distributions of these two relatives suggest that collapsin-2 produced in the ventral cord could contribute to a repellent activity for sensory axons, and that anti-collapsin-1 antibodies cross-reacting with collapsin-2 might neutralize this additional activity.

It is unlikely that collapsin-2, or another ventrally localized family member, collapsin-3, contribute to the repellent activity for sensory axons secreted by ventral cord explants. Recombinant collapsins 2 or 3, produced in either insect cells or in mammalian cells, do not inhibit sensory axon motility even at concentrations more than 10 times higher than those required to obtain a strong response with collapsin-1. The possibility remains, however, that some other perhaps as yet undiscovered relative of collapsin-1 could contribute to the sensory repellent activity produced in the ventral cord. The ability of anti-collapsin-1 antibodies to neutralize the ventral-cord-produced repellent strongly implies, however, that any additional repellent activities are likely to be a members of the collapsin/semaphorin family of proteins.

Messersmith et al. (1995) found that neurites from rat E14 DRG sensory ganglia are not repelled by ventral cord explants when grown in NT-3, a trophic factor that promotes the survival of ventrally projecting muscle afferents *in vitro* (Horey-Lee et al., 1993). They interpreted their results as indicating that NT-3 caused only ventrally projecting muscle afferents to extend from explanted sensory ganglia, and that these afferents are insensitive to the repellent effects of collapsin-1. Our finding that TrkC-positive afferents growing from E10 DRGs are significantly less sensitive to collapsin-1 than TrkC-negative axons is further confirmation of their interpretation. TrkC is the trophic factor receptor for NT-3 and is expressed by ventrally

projecting muscle sensory afferents and a few cutaneous afferents in rat and chick (McMahon et al., 1994; Oakley et al., 1995). In knockout mice, the absence of TrkC clearly leads to a loss of the group Ia and group II muscle afferents, a result consistent with the pattern of Trk C mRNA expression DRGs (Klein et al., 1994; McMahon et al., 1994; Oakley et al., 1995). By E10 in the chick, TrkC-positive muscle afferents have penetrated into ventral regions of the spinal cord, leaving the other classes of TrkC-negative sensory axons behind (Eric Frank, personal communication; Davis et al., 1989; Mendelson et al., 1992). The relative insensitivity of most TrkC-positive E10 afferents to collapsin-1 therefore correlates well with the behavior of this subclass of afferents in vivo.

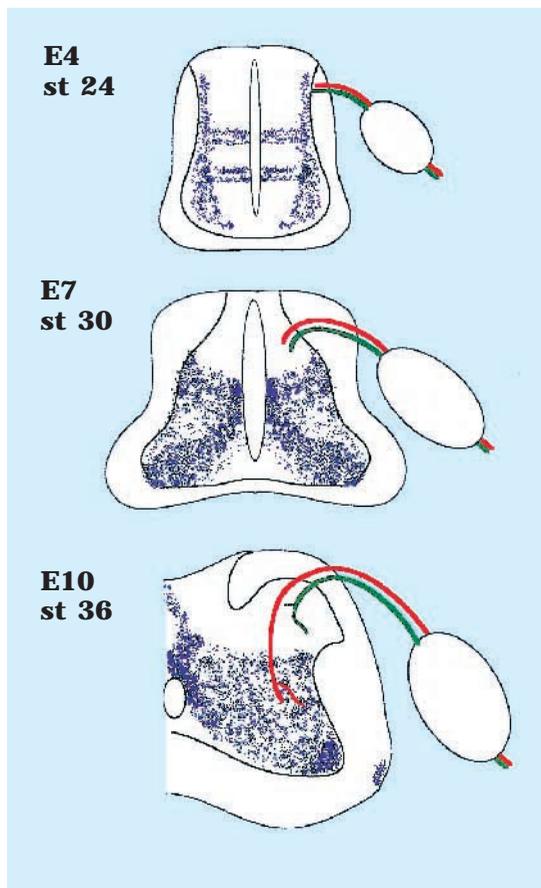
In contrast to the results obtained with E10 DRGs, all axons growing from E7 DRGs cultured in either NGF- or NT-3-containing media, collapse fully when exposed to low concentrations of recombinant collapsin-1. The expression of TrkC does not provide a useful basis by which to discriminate between subclasses of sensory axons extending from E7 DRGs. However, neurons that give rise to ventrally terminating muscle afferents are certain to be present in explanted E7 DRGs (Sharma et al., 1994). We therefore conclude that all sensory subclasses, including the muscle afferents that later will

terminate ventrally, are likely to be highly responsive to collapsin-1 at E7. There is evidence that the same is true for early (E 12.5) DRG afferents in the mouse (Puschel et al., 1996). In the chick, all sensory afferents enter the spinal cord but fail to invade the dorsal gray matter between stages 23 and 27 or E4-E6 (Davis et al., 1989; Mendelson et al., 1992). Collapsin-1 mRNA is present in the gray matter just beneath the dorsal root entry zone during this waiting period (Shepherd et al., 1996). Beginning at stage 28, a collapsin-1-free zone emerges in the dorsal gray area which sensory afferents are just beginning to invade.

If all sensory afferents are responsive to collapsin-1 at early stages, as we infer, then the waiting period prior to sensory afferent invasion may be enforced by collapsin-1-expressing cells in the dorsal gray matter. If the loss of collapsin-1 expression controls the timing of dorsal invasion, then premature invasion would be expected in collapsin-1 knockout animals and delayed invasion would be expected in animals with prolonged ectopic collapsin-1 expression in the dorsal horns.

These results suggest that all sensory afferents may be responsive to collapsin-1 when they first enter the cord, and that the dorsal-most expression of collapsin-1 may therefore have to be down-regulated before the afferents can enter the dorsal horn (Fig. 8). Further, these results support the identification of collapsin-1 as a ventral cord repellent for sensory axons once they have entered the spinal cord. High levels of ventral expression may at first prevent all sensory afferents from invading ventral regions. Later, as muscle afferents lose much of their responsiveness to collapsin-1, they may thereby be permitted to invade ventrally.

We would like to thank Eric Frank for his very helpful advice, Jainli Lu for expert technical assistance, Adam Koppel for his blind scoring, Len Feiner for providing recombinant collapsin-3 protein and all the members of the Raper and Chang laboratory for their help. L. F. R. is an investigator of the Howard Hughes Medical Institute. This work was supported by grants to J. A. R. from the NIH and the McKnight Foundation.



**Fig. 8.** Trajectory of cutaneous sensory afferents and muscle Ia afferents in relation to *collapsin-1* expression at different embryonic ages in the chick spinal cord. The green line represents the central projection of the cutaneous sensory afferents. The red line represents the central projection of the Ia sensory afferents. The blue speckled pattern represents *collapsin-1* mRNA distribution.

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(Accepted 19 January 1997)