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Inhibition of motor axon growth by T-cadherin substrata

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SUMMARY

As spinal motor neurons project to their hindlimb targets, their growth cones avoid particular regions along their pathway. T-cadherin is discretely distributed in the avoided caudal sclerotome and on extrasynaptic muscle surfaces (B. J. Fredette and B. Ranscht (1994) *J. Neurosci.* 14, 7331-7346), and therefore, the ability of T-cadherin to inhibit neurite growth was tested in vitro. T-cadherin inhibited neurite extension from select neuron populations both as a substratum, and as a soluble recombinant protein. Anti-T-cadherin antibodies neutralized the inhibition. Spinal motor neurons were inhibited only during the stages of axon growth across the sclerotome and muscle innervation. Inhibitory responses corresponded to neuronal T-cadherin expression, suggesting a homophilic binding mechanism. These results suggest that T-cadherin is a negative guidance cue for motor axon projections.

Key words: axon guidance, growth cone repulsion, axonogenesis, T-cadherin, spinal motor neuron, motor axon, hindlimb, neurite, mammal

INTRODUCTION

The intricate network of neuronal projections is established in developing embryos when axons navigate along fixed pathways to their synaptic targets. Evidence has accumulated that axonal pathway selection depends on neuronal growth cone recognition of guidance cues along the axon pathway. Some of these cues have been identified as soluble molecules, secreted from intermediate and final targets, and membrane-bound and extracellular matrix molecules (Goodman and Schatz, 1993). Growth cone recognition of guidance cues results in either the attraction to, or the repulsion from, the regions that express these cues, thus establishing specific axon trajectories (reviewed by Dodd and Schuchardt, 1995).

Somatic motor neurons of the chick spinal cord select their pathways and avoid particular regions as they grow towards and innervate their hind limb muscle targets (Landmesser, 1978a,b; Landmesser and Morris, 1978; Lance-Jones and Landmesser, 1980; Hollyday, 1980). Lumbosacral motor neurons orient their axons away from the ventromedial spinal cord to exit the spinal cord ipsilaterally at fixed ventrolateral positions (Ramon y Cajal, 1929; Tanaka, 1991). The floor plate at the ventromedial spinal cord repels motor neurons in vivo and in vitro, and may thus express an inhibitory signal to direct the ipsilateral projection (Guthrie and Pini, 1995; Tamada et al., 1995). Motor axon growth across the mesenchyme adjacent to the spinal cord occurs in a segmental pattern, with axons projecting selectively across the rostral halves of each sclerotome and avoiding the caudal halves (Keynes and Stern, 1984, 1988; Tosney, 1987, 1988; Dehnbostel and Tosney, 1990). The molecular signals for segmental motor axon growth reside within the sclerotome, as axons project through the appropriate rostral region even after it is experimentally rotated to occupy a caudal position (Keynes and Stern, 1984). Experimental evidence has accumulated for the regulation of segmental axon growth by inhibitory signals emanating from the caudal sclerotomes; membranes from this region cause growth cone collapse (Davies et al., 1990) and axons avoid the caudal sclerotome cells either by branching or turning (Oakley and Tosney, 1993). Once motor axons have entered their target muscles, they begin branching and forming synapses on myotube surfaces in a pattern characteristic for individual muscles (Dahm and Landmesser, 1988; Laskowski and High, 1989). This process appears to involve avoidance of extrasynaptic myotube surfaces, as axonal contacts become restricted to synaptic sites and do not sprout, although filapodia probe outside of the synaptic region (Sanes et al., 1978; Robbins and Polak, 1987; Hill and Robbins, 1987; Balice-Gordon and Lichtman, 1990). Thus, the floor plate, the caudal halves of the somitic sclerotome and the extrasynaptic surfaces of myotubes all express avoidance cues to guide motor axons away from these regions.

T-cadherin, a cadherin cell adhesion molecule that is anchored to the membrane through a glycosyl phosphatidyl inositol (GPI) moiety (Ranscht and Dours-Zimmermann, 1991) and which mediates calcium-dependent, homophilic binding in vitro (Vestal and Ranscht, 1992), delineates the motor axon-hindlimb projection pathway in a pattern that implies a function as an avoidance cue (Fredette and Ranscht, 1994). T-cadherin demarcates the floor plate during axon growth in the spinal cord (Kaneker and Ranscht, unpublished observation), and is therefore a candidate regulator of the ipsilateral motor axon projection out of the spinal cord. In the mesenchyme adjacent to the spinal cord, T-cadherin defines the caudal halves of the somitic sclerotome, and could therefore affect segmental motor axon projections across the sclerotomes' rostral halves (Ranscht and Bronner-Fraser, 1991; Fredette and Ranscht, 1994). In muscle, T-cadherin is distrib-

uted on extrasynaptic surfaces and is specifically excluded from neuromuscular junctions (Fredette and Ranscht, 1994). From this distribution, we have predicted that T-cadherin serves as an avoidance cue to extending motor axons. To test this hypothesis, we have assayed the potential of T-cadherin substrata to promote or inhibit neurite growth in vitro. Consistant with our hypothesis, we demonstrate that T-cadherin acts as an inhibitory substratum for neurite extension. Inhibition of neurite growth on T-cadherin substrata correlates with T-cadherin expression on axon surfaces, which is consistent with the view that homophilic T-cadherin interactions mediate the repulsion of axons in the motor axon pathway.

MATERIALS AND METHODS

Neurite outgrowth assay

Chinese Hamster ovary (CHO) cells stably expressing T-cadherin or N-cadherin were used as substrata for neurite outgrowth assays. CHO-DG44 cells stably transfected with the empty eukaryotic pcDNA1 expression vector (Invitrogen, La Jolla, CA) and the selectable marker plasmid pSV2neo (mock-transfected) served as control substrata. T-cadherin- and mock-transfected cells were previously described (Vestal and Ranscht, 1992). To generate N-cadherin-expressing cells, chicken N-cadherin cDNA (the gift of Dr Chris Kintner, Salk Institute, La Jolla, CA) in SP72 (Promega, Madison, WI) was digested with *Eco*RV and *Xba*I, and the N-cadherin coding region was inserted into the *Eco*RV/*Xba*I-digested pcDNA1 plasmid. The N-cadherin vector construct was co-transfected into CHO-DG44 cells with pSV2 hygro, a plasmid carrying the gene for hygromycin resistance, by calcium phosphate precipitation. Hygromycin-resistant colonies were selected in the presence 400 μg/ml hygromycin and enriched by fluorescence-activated cell sorting using rat monoclonal anti-N-cadherin antibody NCD2 (Sigma, St Louis, MO). Cells were verified to express N-cadherin by western blotting.

In parallel sets of cultures, mock, T-cadherin or N-cadherin transfected CHO cells were grown to confluency in 8-well chamber slides (Lab Tek, Nunc, Inc., Naperville, IL) in α-MEM culture media supplemented with 10% fetal bovine serum (Gemini Bioproducts, Calbasasca, CA), 2 mM L-glutamine (Gibco, Gaithersburg, MD) and 1× HT (Sigma). Sympathetic, ciliary or dorsal root ganglia were removed from stage 36 (E10) chick embryos and the ventral portions of lumbosacral spinal cords were obtained from embryos between stages 21 (E3½) and 30 (E7). The tissue was dissociated by 30 minute incubation (37°C) in 0.05% trypsin diluted in calcium-free Tyrode buffer and the cell densities were determined by counting phase-bright cells with a hemocytometer. Approximately 1000-2500 cells were seeded over each CHO cell monolayer in fresh media containing 50 U/ml partially purified nerve growth factor for the sympathetic and dorsal root ganglia cells or 10 ng/ml rat ciliary neurotrophic factor (Pepro Tech, Inc., Rocky Hill, NJ) for ciliary ganglion cells. The CHO-neuron cocultures were grown overnight (15-18 hours) in a 5% CO_2 incubator at 37°C, fixed overnight with 4% formaldehyde in PBS, rinsed and processed for neurofilament immunofluorenscence with mouse RMO270 monoclonal antibody (Lee et al., 1987) as previously described (Fredette and Ranscht, 1994).

The lengths of neurofilament-labelled neurites were measured by epifluorescence with a Zeiss 405M microscope equipped with an Optronix CCD videocamera and JAVA image analysis system. In each well, adjacent and non-overlapping fields were viewed to select the first 30 singly occuring neurons with visible somata and isolated neurites not contacted by other neurons. The length of each neuron's longest neurite was measured, and the mean length and standard error was determined and tested against control values with an independent *t*-test. The mean neurite lengths were graphed as the percentages of control neurite lengths on mock-transfected CHO cells. Experiments were repeated a minimum of three times on separate days.

In some experiments, the quantification of neurite lengths was performed on ventral spinal cord neurons identified as motor neurons by either DiI backlabelling or SC1 immunofluorescence (Tanaka and Obata, 1984). For DiI backlabelling, stage 24-30 embryos were eviscerated, the ventral spinal cords were exposed by laminectomy and DiI (1.1′-dioctadecyl-3,3,3′,3′-tetraethylindocarbocyanine perchlorate, 0.3% in 100% ethanol, Molecular Probes, Eugene OR) was injected into lumbosacral spinal nerves 1-8. The embryos were incubated in a heated (37°C) and oxygenated Tyrode buffer bath for 3 hours. The lumbosacral region of the spinal cords containing the DiI-backlabelled motor neuron cell bodies were dissociated and grown in culture as described above. However, in place of neurofilament immunofluorescence, the lengths of DiI-labelled neurites was measured directly after fixation. DiI-backlabelling of stage 21-23 motor neurons was not possible due to the short lengths of the spinal nerves, thus preventing specific labelling. Therefore, these young motor neurons were identified instead by immunofluorescence with monoclonal antibody SC1, a marker for motor neurons and floor plate cells in the ventral spinal cord (Tanaka and Obata, 1984). At these early stages, nearly all of the cells that extended neurites in vitro were SC1-positive, and therefore, neurite lengths were measured from the more readily distinguishable neurofilament-labelled cells.

Immunocytochemistry

T-cadherin expression on the surfaces of transfected cells and neurons grown in vitro was tested by T-cadherin immunofluorescence. Neurons plated on mock-transfected CHO cells were grown overnight and fixed for 30 minutes with 4% formaldehyde in PBS. The cultures were rinsed and processed for immunofluorescence with rabbit anti-T-cadherin antiserum (Ranscht and Dours-Zimmermann, 1991) followed by anti-rabbit-FITC IgG (Cappel, Durham, NC). Each antibody was diluted 1:150 in PBS with 2% BSA. The cultures were mounted with Slow Fade (Molecular Probes, Eugene, OR) and examined by epifluorescence with a Zeiss 405M microscope. It should be noted that the dorsal root ganglion sensory neuron and the stage 28 motor neuron cultures contained both T-cadherin-positive and T-cadherin-negative neurons, with a maximum of about 40% negative neurons, but no distinction was made for T-cadherin-negative and T-cadherin-positive neurons when assaying neurite lengths.

The expression of N-cadherin by transfected cells was verified by indirect immunofluorescence with monoclonal antibody GC4 (1:50, Sigma), followed by goat anti-mouse-FITC IgG (1:200, Antibodies, Inc., Davis, CA).

Phospholipase C removal of T-cadherin

Confluent cultures of mock or T-cadherin-transfected CHO cells were incubated for 30 minutes at 37°C with recombinant phosphatidylinositol-specific PLC (Oxford Glycosystems) diluted in α-MEM at 5 U/ml. A parallel set of cultures lacking the enzyme was used as controls. After enzyme treatment, the cultures were rinsed with two changes of α-MEM, followed by a third and final exchange of media containing appropriate growth factors. Neurons were plated and grown on the indicated substrata, and assayed for neurite growth as described above. The efficacy of enzyme treatment was assessed by T-cadherin immunofluorescence immediatly following the enzyme treatment, and thereafter at 5 hour intervals for 28 hours. T-cadherin immunofluorescence was virtually abolished for 15 hours after PI-PLC treatment, and only reappeared substantially by 25 hours. Therefore, T-cadherin was not expressed on the surfaces of the enzyme-treated transfected CHO cells throughout the duration of the neurite outgrowth experiments.

Recombinant T-cadherin proteins

A glutathione-S-transferase fusion protein of T-cadherin EC1-5 was generated in pGEX-2T (Smith and Johnson, 1988). T-cadherin cDNA

(Ranscht and Dours-Zimmermann, 1991) was digested with *Pst*I, endfilled with Klenow, and then digested with *Eco*RI. A 1.7 kb *Eco*RI/blunt end fragment comprising T-cadherin EC 1-5 was purified on agarose gels. After ligation of *Eco*RI linkers and redigestion with *Eco*RI, the DNA fragment was ligated into *Eco*RI-digested pGEX 2T, and transformed into One Shot cells (Invitrogen, La Jolla, CA). The recombinant prepeptide fusion protein was previously described (Vestal and Ranscht, 1992). A 50 ml culture of one bacterial colony containing the desired T-cadherin insert was grown overnight in LB media and then expanded to a volume of 1500 ml. Protein production was induced with 0.1 mM IPTG (isopropyl-β-thiogalactopyranoside) for 3 hours. To harvest the protein, bacteria were pelleted, resuspended in 30 ml TBS (10 mM Tris/HCl, pH 7.6, 150 mM Na Cl) containing 2 mM $CaCl_2$ and 1% Triton X-100, and sonicated twice for 30 seconds. Insoluble protein was removed by centrifugation at 30,000 ***g*** for 20 minutes and discarded. The soluble T-cadherin fusion protein was affinity-purified from the supernatant on glutathione-sepharose 4B sepharose (Pharmacia, Piscataway, NJ). Recombinant T-cadherin protein was cleaved from the fusion protein with thrombin (1 U/ml, Sigma) for 2 hours, eluted from the column with TBS containing $CaCl_2$ and concentrated. Commassie Blue staining and western blot analysis with anti-T-cadherin antibodies confirmed the presence of recombinant T-cadherin protein and the purity of the preparation.

Function-blocking antibodies

Two polyclonal anti-T-cadherin antibodies were produced by immunizing two rabbits, 1748 and 1749, with purified recombinant T-cadherin EC1-5 protein (above). The rabbits were initially immunized with 200 μg of intact fusion protein in Freund's complete adjuvant. Each rabbit received two additional injections consisting of purified recombinant T-cadherin protein in Freund's incomplete adjuvant. The antibody's specificity for T-cadherin was determined by comparing the antisera with the previously characterized anti-T-cadherin antibody (Ranscht and Dours-Zimmermann, 1991) by western blotting (Towbin et al., 1979), immunoprecipitation of chick brain homogenates (Ranscht and Dours-Zimmermann, 1991; Vestal and Ranscht, 1992) and immunocytochemical staining of T-cadherin-transfected CHO cells and chick embryo cryosections (Fredette and Ranscht, 1994). IgGs were purified from the antisera on Protein A Sepharose (Pharmacia, Piscataway, NJ).

RESULTS

T-cadherin substrata inhibit neurite extension from sympathetic neurons

The effect of T-cadherin substrata on neurite growth was assessed in vitro by coculturing dissociated sympathetic neurons on confluent T-cadherin-transfected cells and measuring the lengths of neurofilament-positive neurites (Fig. 1). Chinese hamster ovary (CHO) cells were transfected with T-cadherin cDNA (Vestal and Ranscht, 1992), and clones stably expressing abundant cadherin protein on their cell surfaces were chosen for the experiments (see Fig. 2A). N-cadherin is a known promoter of neurite extension (Matsunaga et al., 1988; Bixby and Zhang, 1990; Doherty et al., 1991) and, thus, N-cadherin cDNA was transfected into CHO cells to provide a positive control substratum. Effects of the CHO cell surface background were assessed with a CHO cell line transfected with the pcDNA1 expression vector alone. Sympathetic neurons from 10-day-old chick embryos were plated onto the cellular substrata at low density. After 15-18 hours in vitro, sympathetic neurons extended long neurites on the mock-trans-

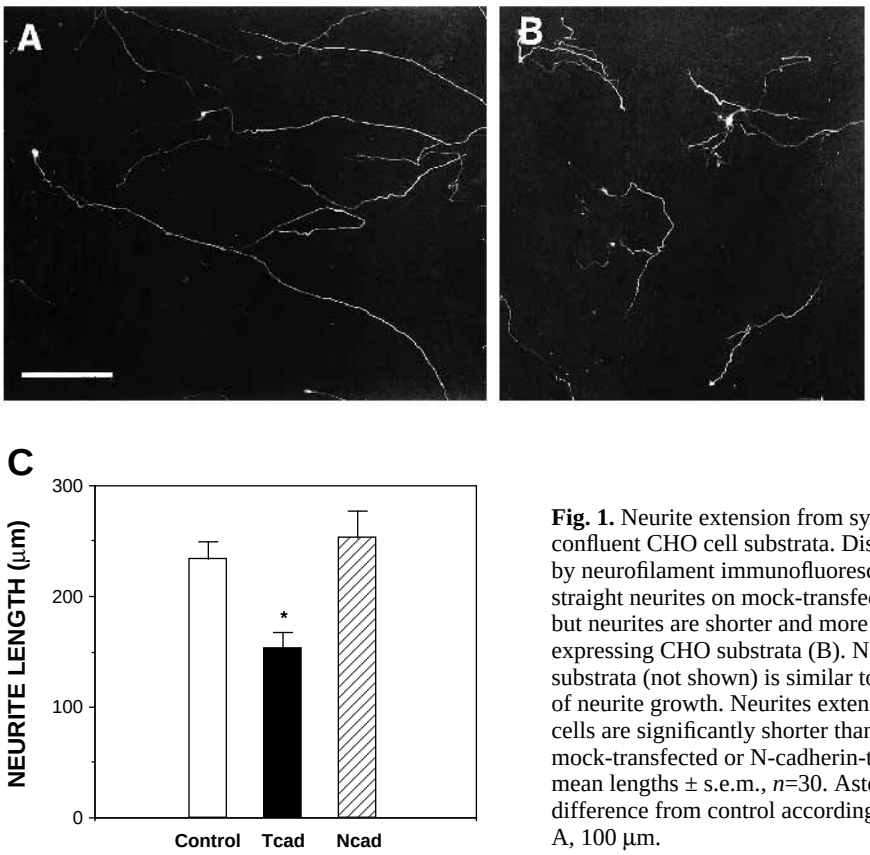

Fig. 1. Neurite extension from sympathetic neurons on confluent CHO cell substrata. Dissociated neurons, visualized by neurofilament immunofluorescence, extent long and fairly straight neurites on mock-transfected (control) CHO cells (A), but neurites are shorter and more irregular on T-cadherin-expressing CHO substrata (B). Neurite growth on N-cadherin substrata (not shown) is similar to controls. (C) Quantification of neurite growth. Neurites extended on T-cadherin-transfected cells are significantly shorter than neurites growing on the mock-transfected or N-cadherin-transfected cells. Values are mean lengths ± s.e.m., n=30. Asterisk indicates significant difference from control according to t-test, $P=2.9\times10^{-4}$. Bar in A, 100 μm.

fected and the N-cadherin-transfected CHO cells (mean lengths ranging between 73 and 670 μm in 13 independent experiments; Fig. 1A and B). In contrast, neurons plated on the T-cadherin substratum extended significantly shorter neurites that were irregular in direction, as though they frequently turned while growing (compare neurites in Fig. 1A and B). Neurite lengths on T-cadherin substrata were shorter by 50-78%, compared to those on mock-transfected control substrata (Fig. 1C).

In order to ensure that these differences are a function of T-cadherin and not an artifact of the CHO cell culture substrata, neurite lengths of sympathetic neurons were assessed on T-cadherin-transfected and mock-transfected L929 cells (Koller and Ranscht, unpublished). Neurites on the L929 T-cadherin substratum were also significantly shorter than those on the control substratum (62.4%±1.7% of control, mean neurite length ± s.e.m.). Thus, the reduction of neurite growth is a function of T-cadherin and not of the cellular background.

Inhibition of neurite growth is specific to T-cadherin

The specificity of T-cadherin substrata on neurite growth inhibition was addressed in three sets of experiments. Neurite outgrowth was assessed (1) after enzymatic cleavage of T-cadherin from substratum cell surfaces with phosphatidyl inositol-specific phospholipase C (PI-PLC), (2) in the presence of anti-T-cadherin antibodies to block T-cadherin function or (3) in the presence of recombinant T-cadherin EC1-5 protein.

To verify the specificity of T-cadherin in neurite growth inhibition, GPI-linked T-cadherin was enzymatically cleaved from transfected cells with GPI-specific PLC (Table 1). Confluent T-cadherin-transfected CHO cell cultures were treated with PI-PLC and removal of T-cadherin from the cell surface was assessed by indirect immunofluorescence. Following PI-PLC-treatment, T-cadherin was efficiently removed from CHO cells for over 18 hours, the duration of the neurite outgrowth assay (Fig. 2B), and was reexpressed at low levels by 24 hours (Fig 2C). Sympathetic neurons plated on PI-PLC-treated substrata extended neurites of the same length as those on the mock transfected cells. No increase, or other change, in neurite outgrowth was detected on enzyme-treated, mock transfected control cells. Therefore, PI-PLC removal of T-cadherin from CHO cell surfaces completely restored neurite outgrowth.

In the second set of experiments, the inhibitory effect of T-cadherin on neurite growth was neutralized by introducing purified anti-T-cadherin IgG into the neuron-CHO cell cocultures. Two function-blocking anti-T-cadherin antisera, 1748 and 1749, were raised by immunizing rabbits with recombinant T-cadherin EC1-5 protein. IgGs from both sera specifically recognized T-cadherin isolated from brain extracts in western immunoblots (Fig. 3), as well as by immunoprecipitation and immunofluorescence (not shown). Anti-T-cadherin IgGs from rabbits 1748 or 1749 were diluted at three 10-fold dilutions ranging from 1 mg/ml to 0.01 mg/ml in supplemented α-MEM, and were added to confluent cultures of mock or T-cadherin-transfected CHO cell. The substrata were preincubated in the

Table 1. Phospholipase C removal of T-cadherin from substratum cells neutralizes T-cadherin-mediated inhibition of neurite growth

	Mock	T-cadherin
Control	100	57.4±2.34*
PLC-treated	98.6±4.52	88.1±7.80

Confluent cultures of mock- and T-cadherin-transfected CHO cells were treated with 5 units/ml PI-PLC prior to adding dissociated sympathetic neurons. Values are mean neurite lengths (± s.d. of 3 experiments, 30 neurites per experiment, and are expressed as a percentage of control (growth on untreated mock-transfected cells). Asterisk indicates significant difference from control length in each of 3 experiments according to *t*-test ($P \leq 4.5 \times 10^{-2}$).

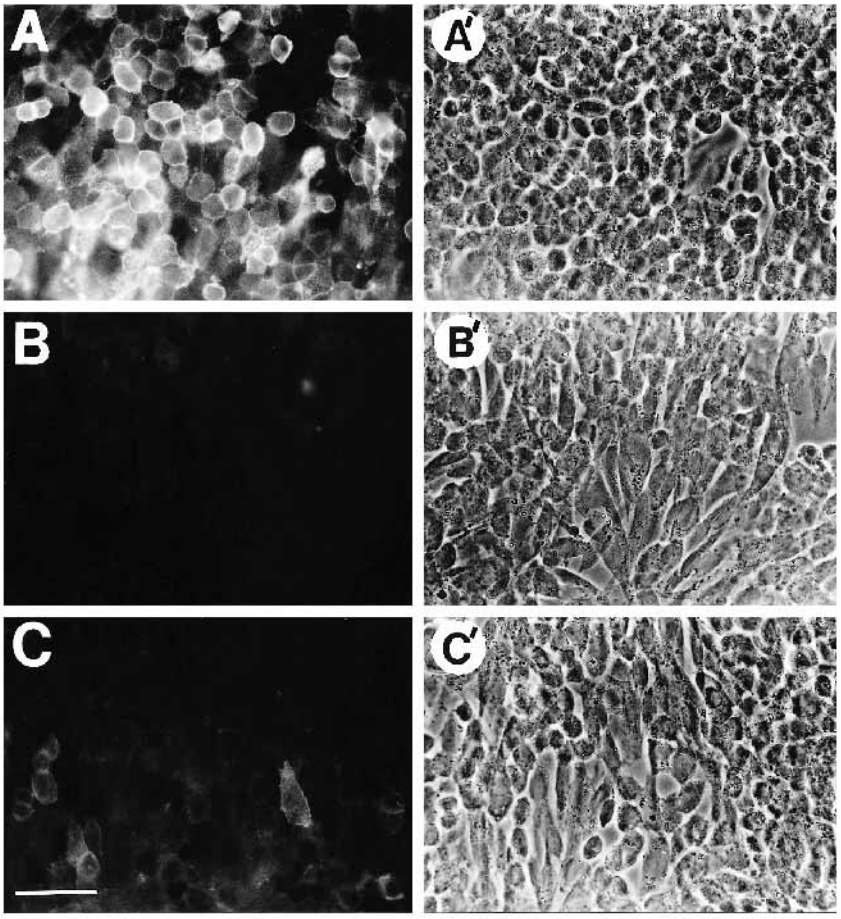

Fig. 2. PI-PLC removal of cell surface T-cadherin from CHO cell-substrata. T-cadherin immunofluorescence (A-C) and corresponding phase (A′-C′) photomicrographs of T-cadherin transfected CHO cell cultures. (A,A′)T-cadherin immunofluorescence of untreated cultures. T-cadherin is abundantly expressed on CHO cell surfaces. (B,B′) Immediately after PI-PLC-treatment, parallel cultures are negative for T-cadherin. (C,C′) 24 hours after PI-PLC treatment, T-cadherin-transfected CHO cells begin to reexpress low levels of T-cadherin on their surfaces. Note that for the duration (18 hours) of the neurite outgrowth assays, T-cadherin is virtually absent from the substratum cells. Bar in C, 50 μm.

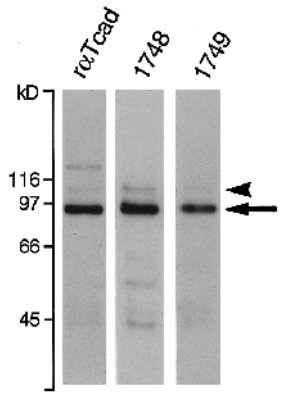

Fig. 3. Rabbit antisera 1748 and 1749 recognize T-cadherin in Western blots. Chick brain homogenates were separated by reducing SDS-PAGE, transferred onto Immobilon-P membranes and probed with rabbit anti-T-cadherin (lane 1, Ranscht and Dours-Zimmermann, 1991), IgG 1748 (lane 2), and IgG 1749 (lane 3), each antibody used at 1:500-1:600 dilutions. Rabbit IgGs 1748 and 1749 specifically recognize the 95×10^3 M_r mature T-cadherin protein (arrow) and the intact 110×10^3 M_r T-cadherin precursor (arrowhead).

antibody solutions for 2 hours. Dissociated sympathetic neurons were then plated onto the CHO cell cultures in the continued presence of the anti-T-cadherin IgG, and the extent of neurite growth was measured after 15-18 hours. Each of the anti-T-cadherin IgGs completely reversed the inhibitory effects of T-cadherin expressed by the substratum cells (Table 2). Neither antibody affected neurite extension on mock-transfected substrata. In the presence of non-immune rabbit IgG, neurite lengths on the T-cadherin substrata remained about 65% of control values even at a concentration of 1 mg/ml, the highest concentration of anti-T-cadherin IgG tested. Thus, rabbit anti-T-cadherin IgG completely and specifically neutralized T-cadherin-mediated inhibition of neurite growth.

In the third set of experiments aimed to demonstrate the specificity of T-cadherin, a soluble recombinant protein comprising the T-cadherin EC1-5 ectodomain was synthesized and introduced into cocultures of sympathetic neurons and mock-transfected CHO cells. Thus, the only variable in this experiment was the presence or absence of T-cadherin protein. Recombinant T-cadherin EC1-5 was diluted to concentrations between 30 μg/ml and 1.5 μg/ml in supplemented α-MEM, and placed over confluent mock- or T-cadherin-transfected CHO cells. Sympathetic neurons cultured overnight in the presence of the recombinant T-cadherin EC 1-5 protein had significantly reduced neurite lengths on the mock-transfected substrata (57.24±3.69% of control at 30 μg/ml, mean neurite length ± s.e.m.). In contrast, recombinant T-cadherin prepeptide used as a control failed to reduce neurite growth (Fig. 4). In one experiment, recombinant T-cadherin EC1-5 protein added to neuron

Table 2. Anti-T-cadherin IgGs neutralize T-cadherin-mediated inhibition of neurite outgrowth

	Mock	T-cadherin
Non-immune IgG	100	64.4±5.16*
Anti-T-cadherin	87.9±10.31	87.0±9.72

Dissociated sympathetic ganglion neurons were grown on mock or T-cadherin-transfected CHO cells in the presence of either non-immune IgG or anti-T-cadherin IgG (1 mg/ml). Values are neurite lengths expressed as a percentage of control (lengths on mock-transfected cells), and are the mean ± s. d. of 5 experiments, 30 neurites per experiment. Asterisk indicates significant difference from control value in each of 5 experiments, according to *t*-test ($P\leq3.6\times10^{-2}$).

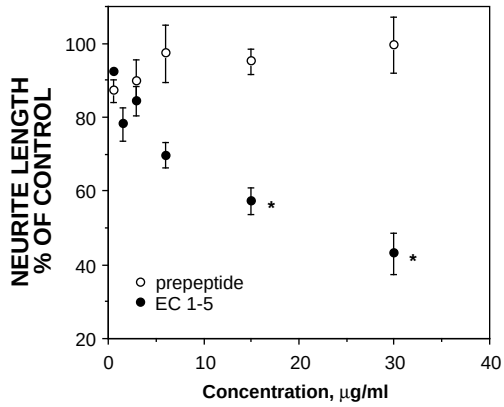

Fig. 4. Recombinant T-cadherin (EC 1-5) protein inhibits neurite outgrowth. In the presence of T-cadherin EC 1-5 protein (filled circles) at concentrations over 30 μg/ml, dissociated sympathetic ganglion neurons cultured on mock-transfected CHO cells extend shorter neurites as compared to controls. Recombinant T-cadherin prepeptide has no effect over controls (open circles). Values are mean neurite lengths + s.e.m., expressed as the percentage of control (growth on mock-transfected CHO cells in absence of added T-cadherin protein). Asterisks indicate significant difference from control for each of 4 experiments, 30 neurites/experiment, *t*-test, $P\leq1.07\times10^{-3}$.

and T-cadherin-transfected CHO cocultures reduced neurite growth a further 27% over the 61% reduction on T-cadherin substrata alone. Thus, the recombinant T-cadherin protein and T-cadherin on the substratum cells additively reduced neurite growth by 88% as compared to the growth on mock-transfected cells alone.

Taken together, these experiments demonstrate that T-cadherin substrata specifically inhibit neurite outgrowth.

T-cadherin inhibits motor neurite growth in a stage specific manner

T-cadherin was previously reported to be expressed in regions that are actively avoided by growing motor neurons during the formation of lumbosacral motor neuron projections to the chick hindlimb (Fredette and Ranscht, 1994). These regions are the posterior somitic sclerotomes and extrasynaptic muscle surfaces. Therefore, it was important to test somatic motor neurons dissociated from the lumbosacral region of ventral spinal cords at various embryonic stages for their sensitivity to T-cadherin substrata. Neurons dissociated from spinal cords between stage 21 and stage 30, and identified in some experiments as motor neurons either by DiI-backlabelling (stage 27-30) or SC1 immunofluorecsence (stage 21-26), exhibited a bitemporal sensitivity to T-cadherin-mediated inhibition. Neurite extension from neurons dissected from embryos at stage 21-23 (when motor axons project through the anterior sclerotome in situ) and stage 27-30 (as motor axons form neuromuscular synapses in vivo) was significantly reduced by T-cadherin substrata. However, neurons were insensitive when they were dissociated from stage 24-26 embryos, which corresponds to the time when T-cadherin is down regulated on motor axons as they sort in the plexus region in vivo, (Fig. 5). As for sympathetic neurons, inhibition of neurite growth was confirmed for stage 23 ventral spinal cord neurons plated on confluent monolayers of T-cadherin-transfected L929 cells,

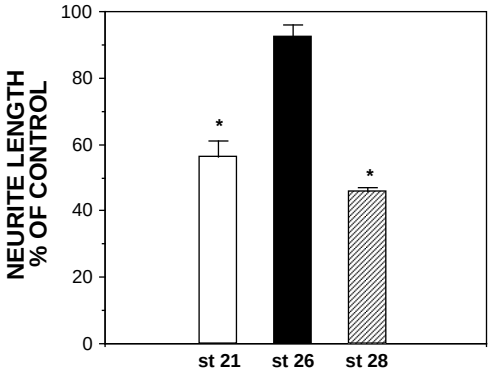

Fig. 5. T-cadherin-mediated inhibition of ventral spinal cord neurite growth is stage-specific. Dissociated spinal cord neurons, identified in several experiments as motor neurons by DiI backlabelling (stage 21 and stage 28) or by SC1 immunofluorescence (stage 21 and stage 26), were grown on T-cadherin-transfected cells, and the lengths of their neurites were measured and expressed as percentages of neurite lengths on mock-transfected substrata. Only neurons from stage 21 and stage 28 ventral spinal cords are inhibited by T-cadherin substrata. No reduction of neurite extension occurs for stage 26 neurons. Values are mean lengths + s.e.m., of 30 neurons from one experiment representative of 3 experiments, all of which produced similar results. Asterisks indicate significant differences from control growth on mock-transfected substrata (*t*-test, $P \leq 2.36 \times 10^{-3}$).

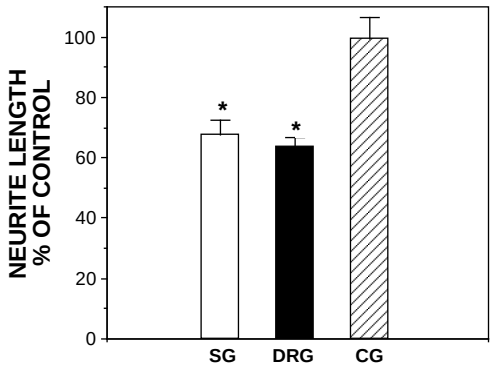

Fig. 6. T-cadherin substrata are inhibitory to neuronal subpopulations. Comparison of neurite outgrowth from sympathetic (SG, open bar), dorsal root (DRG, filled bar) and ciliary ganglion (CG, hatched bar) neurons on T-cadherin-transfected cells. Values are mean + s.e.m. from a representative experiment (n=30 neurites), expressed as a percentage of growth on mock-transfected cells. Asterisks indicate significantly reduced outgrowth on T-cadherin substrata for sympathetic and dorsal root ganglion neurons as compared to outgrowth on control substrata (*t*-test, $P \leq 1.21 \times 10^{-4}$).

(59.03±7.7% of control, mean neurite length ± s.e.m.). Thus, inhibition of motor neurite growth by T-cadherin substrata correlates temporally with the projection of motor axons through the somitic sclerotome and with the innervation of myotubes.

The inhibition of neurite growth by T-cadherin substrata was not only stage specific, but also was restricted to specific neuron types. In addition to sympathetic ganglion neurons and motor neurons, neurite outgrowth from dorsal root ganglion (DRG) sensory neurons was reduced by T-cadherin substrata (Fig. 6). However, ciliary ganglion neurons, like the stage 24-26 motor neurons, were not sensitive to the inhibition, thereby demonstrating that only select populations of neurons respond to T-cadherin as a negative cue.

Neuronal T-cadherin expression correlates with sensitivity to T-cadherin-mediated inhibition

The bimodal sensitivity of ventral spinal cord neurons correlates exactly with the bimodal expression of T-cadherin on motor neuron surfaces in vivo (Fredette and Ranscht, 1994). Therefore the expression of T-cadherin by motor neurons cultured from embryos between stages 21 and 30 was explored by immunofluorescence (Fig. 7). T-cadherin immunofluorescence was assayed on neurons plated on mock-transfected CHO cells, but not on T-cadherin-transfected cells, due to the inability to distinguish substratum T-cadherin from T-cadherin expressed by the neurons. T-cadherin expression on neuronal surfaces correlated with their sensitivity to T-cadherin-mediated inhibition: T-cadherin-sensitive stage 21-23 and many stage 28-30 ventral spinal cord neurons were T-cadherin-positive, but the insensitive stage 24-26 spinal cord neurons were T-cadherin-negative (Fig. 7). This correlation was also observed for other neuronal populations: nearly all sympathetic ganglion neurons and the majority of dorsal root ganglion neurons expressed bright T-cadherin immunofluorescence on their surfaces. In contrast, the vast majority of ciliary ganglion neurons, which did not exhibit an inhibitory response on T-cadherin substrata, were very dim or negative for T-cadherin immunofluorescence. These results demonstrate that inhibition of neurite growth by T-cadherin substrata correlates with T-cadherin expression on neuronal cell surfaces. Thus, homophilic T-cadherin binding between neurons and their environment is likely to mediate the inhibition of neurite growth.

DISCUSSION

One of the mechanisms to specify axonal projection patterns is the repulsion of growth cones from areas delineating boundaries of the axon pathway (Patterson, 1988). Prospective candidates to guide axon trajectories by growth cone repulsion in vivo are molecules that inhibit neurite extension in vitro (reviewed by Dodd and Schuchardt, 1995). A respectable number of these inhibitory molecules have been discovered, including members of the semaphorin family (Luo et al., 1993; Fan and Raper, 1995; Messersmith et al., 1995; Püschel et al., 1995), myelin-associated glycoprotein (Mukhopadhyuy et al., 1994; McKerracher et al., 1994), netrin (Colamarino and Tessier-Lavigne, 1995), a member of the family of ligands for Eph-receptor tyrosine kinases called AL-1/RAGS (Winslow et al., 1995; Drescher et al., 1995), several extracellular matrix molecules (reviewed by Keynes and Cook, 1995) and uncharacterized molecules derived from crude membrane preparations of myelin (Caroni and Swab, 1988), avian tectum (Stahl et al., 1990), rat superior colliculus (Roskies and O'Leary, 1994) and posterior somites (Davies et al., 1990). In this paper, we have identified the first member of the cadherin family, T-cadherin, as an inhibitor of neurite growth.

We suggest that T-cadherin is a specific inhibitor of neurite growth for the following reasons. First, neurite growth is inhibited on T-cadherin-transfected, but not on mock- or N-cadherin-transfected substratum cells. Second, PI-PLC

removal of GPI-linked T-cadherin from substratum cell surfaces fully restores neurite growth to control levels. Third, IgGs from two T-cadherin-specific antisera neutralize T-cadherin's inhibitory effect. Last, recombinant T-cadherin protein comprising extracellular domains EC1-5 inhibits neurite extension on control substrata. Furthermore, the recombinant protein adds to the inhibition of neurite extension produced by T-cadherin substrata.

Somatic motor neurons of the chick lumbosacral spinal cord grow towards and innervate their hindlimb muscle targets by avoiding several regions along their pathway (Landmesser, 1978a,b; Lance-Jones and Landmesser, 1980). T-cadherin is distributed in the motor axon-hindlimb projection path in regions that are repulsive to the extending axons (Fredette and Ranscht, 1994) such as the spinal cord floor plate, the caudal halves of sclerotomes and extrasynaptic muscle surfaces. Consistant with the hypothesis that T-cadherin shapes the motor axon pathway by preventing motor axons from projecting into the T-cadherin-positive regions, we show that T-cadherin substrata indeed inhibit neurite growth. In these in vitro assays, neurons attach to T-cadherin substrata and extend shorter neurites than on control substrata. The convoluted morphology of neurites on T-cadherin substrata indicates that growth cones often alter their direction, resulting in the net reduction of neurite extension. Thus, rather than arresting neurite growth, recognition of T-cadherin substrata by extending growth cones may trigger a signal that results in a trajectory change. Several molecules initially identified as inhibitory by their ability to cause growth cone collapse are now known to elicit more complicated avoidance responses, such as growth cone branching and turning (Oakley and Tosney, 1993; Fan and Raper, 1995). Specifically, during motor axon segmentation, the growth cone filopodia attach to caudal sclerotome cells, but release these contacts and turn to grow across rostral sclerotome cells (Oakley and Tosney, 1993). Thus, the irregular advancement of growth cones on T-cadherin substrata may indicate an active growth cone search for a substratum of higher affinity.

In our previous study, we have established that developing motor neurons express T-cadherin biphasically: first at stage 21-23, when axons project through the sclerotome regions, and second, after stage 27, when motor neurons invade and form synapses on myotubes (Fredette and Ranscht, 1994). During intermediate stages, when axons in the plexus regions sort to form the dorsal and ventral nerve trunks, T-cadherin is not expressed on motor axons. Inhibition of motor neurite growth by T-cadherin substrata parallels the stage-dependent T-cadherin expression on motor neurons: neurite extension is reduced in motor neurons explanted from stage 21-23 and stage 28 embryos, but not in those explanted from stage 24-26 embryos. This correlation between neuronal T-cadherin expression and inhibition of neurite growth is in line with the suggestion that T-cadherin-mediated homophilic interactions direct motor axon avoidance of the floor plate, the posterior sclerotome and extrasynaptic muscle surfaces.

Testing different neuron populations revealed that sympathetic and sensory ganglion neurons, but neither ciliary ganglion neurons nor neurons from the dorsal spinal cord (Kanekar and Ranscht, unpublished observation), were inhibited by T-cadherin substrata. Also, like the motor neurons at different developmental stages, the inhibitory response correlated directly with the expression of T-cadherin on the cultured neuron surface, arguing further that homophilic T-cadherin binding mediates the inhibition. However, unlike the case for motor neurons, T-cadherin expression by sympathetic and dorsal root ganglion neurons in vitro differs from the in

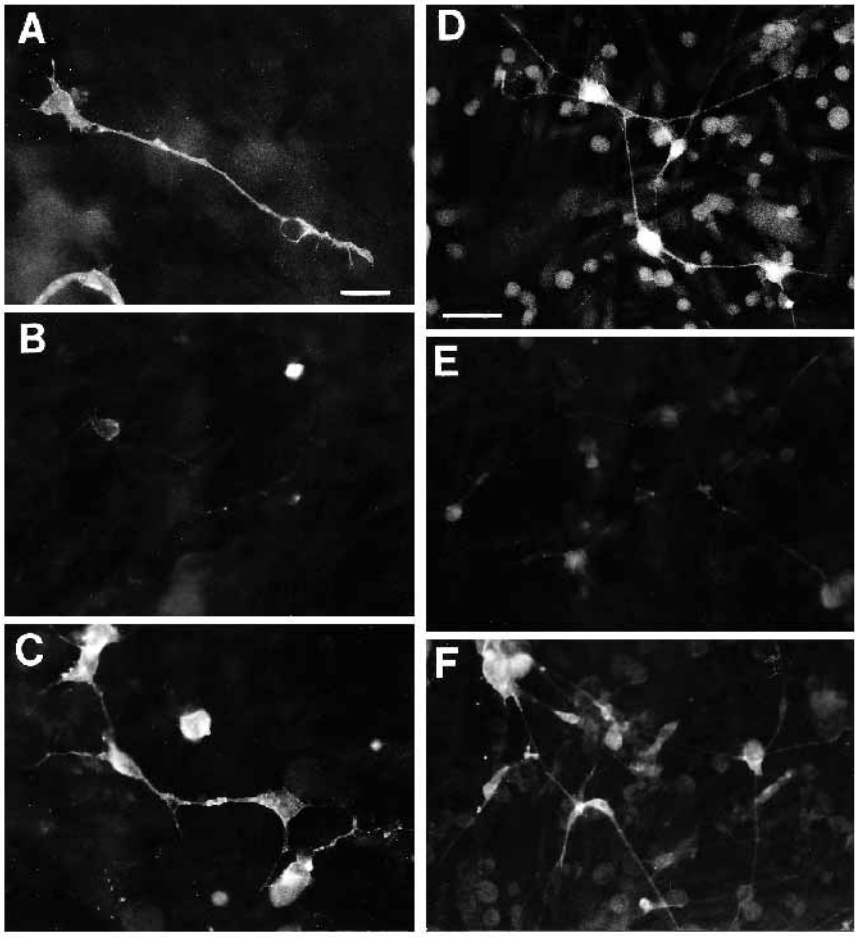

Fig. 7. T-cadherin immunofluorescence of cultured neurons corresponds to their sensitivity to T-cadherin substrata. T-cadherin-sensitive stage 21 (A) and 28 (C) ventral spinal cord neurons, but not the insensitive stage 26 neurons (B), are T-cadherin-positive. Likewise, sympathetic (D) and dorsal root (F) ganglion neurons, both of which are inhibited by T-cadherin substrata, express bright T-cadherin immunofluorescence on their surfaces, but ciliary ganglion neurons (E), which are insensitive, express little or no T-cadherin. Neurons were plated on mock-transfected CHO cells to allow visualization of neuronal T-cadherin. All photographs are from the same experiment, and are exposed for the same amount of time. Bar in A, 20 μm, applies to A-C. Bar in D, 50 μm, applies to D-F.

vivo expression pattern. Cultured sympathetic neurons express abundant T-cadherin, while lacking T-cadherin in vivo. Likewise, virtually all cultured dorsal root sensory ganglion neurons express T-cadherin, yet in vivo, T-cadherin is detected on a small subpopulation of sensory neurons (Fredette and Ranscht, 1994). It is unlikely that only T-cadherin-positive sensory neurons survived in our cultures, as both large and small neurons were represented in vitro, indicating that no discrimination occurred based on growth factor requirements or other culture conditions. Because T-cadherin is not expressed by sympathetic and sensory neurons in vivo, they may not avoid T-cadherin in their pathways.

We have hypothesized that T-cadherin reduces neurite growth by repulsing growth cones, rather than by arresting their growth through increased adhesion, as has been demonstrated for agrin by Campagna et al. (1995). Three observations support our hypothesis. First, while T-cadherin has been demonstrated to mediate calcium-dependent, homophilic binding (Vestal and Ranscht, 1992), these interactions are weak as compared to N-cadherin-induced adhesion. In contrast to N-cadherin, T-cadherin-transfected cells form small and non-campacted aggregates (Ranscht, unpublished obervation). Second, in four independent experiments, adhesion between neurons from either the ventral spinal cord or dorsal root ganglia and T-cadherin-transfected CHO cells was not significantly different from that occuring between neurons and mock-transfected CHO cells (Fredette and Ranscht, unpublished observations). Finally, observations of growth cone behavior by videomicroscopy revealed that 5 of 6 growth cones encountering mock-transfected CHO cells retained filapodial contacts of long durations with the CHO cells (0.08 retractions which occupied 98.94% of the total recording time, mean values), and quickly advanced to pass over the CHO cell surface. In contrast, 7 out of 10 growth cones encountering T-cadherin-transfected cells retained only brief transient filapodial contacts (3.14 mean number of contacts, which occupied 43.34% of the total recording time), and withdrew and grew away from the T-cadherin-positive CHO cells. These time-lapse videomicroscopy recordings, together with the distribution of T-cadherin in regions avoided by extending motor axons, are consistant with the hypothesis that, during the formation of the motor axon pathway, T-cadherin serves as a recognition molecule that causes growth cone avoidance responses.

The results presented in this paper strongly argue that T-cadherin acts as a negative regulator of axon growth for specific neuron populations. In particular, the stage-specific inhibition of motor axon growth suggests a role for T-cadherin in the formation of the motor axon-hindlimb projection pathway. Observation of motor growth cone behavior during axon extension across the sclerotome and during muscle innervation in the presence of function-blocking antibodies or recombinant T-cadherin proteins will be a useful approach to clarify in detail the physiological significance of T-cadherin-mediated cellular interactions in this pathway.

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