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Intercellular Adhesion and Pathfinding Molecule T-cadherin in the Development of the Nervous System

A dissertation submitted in partial satisfaction of the requirements for the degree

Doctor of Philosophy

in

Neurosciences

by

Harper C VanSteenhouse

Committee in Charge:

Professor Barbara Ranscht, Chair
Professor Nicholas Spitzer, Co-Chair
Professor Samuel Pfaff
Professor Eric Turner
Professor Anthony Wynshaw-Boris

2007

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Co-Chair

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Chair

University of California, San Diego

2007

Try to learn something about everything
and everything about something.

Thomas Henry Huxley

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Chapter 3 in part is being prepared for publication as *Untitled* by Ciato C, VanSteenhouse H, Ranscht B and Shapiro L. The dissertation author is the primary investigator on this portion of the collaborative paper.

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Publications

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Ciatto C, VanSteenhouse HC, Ranscht B, Shapiro L. Currently untitled. *In preparation.*

VanSteenhouse HC, Ranscht B. Cadherins as Regulators of Specific Motor Neuron Connectivity. Program No. 723.7. *Abstract Viewer and Itinerary Planner.* Washington, DC: Society for Neuroscience, 2004. Online.

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VanSteenhouse HC, Horton ZA, Goodman MB, O'Hagan R, Tai M-H, Zipser B. Cell type-specific glycosylations in C. elegans. Society for Neuroscience 32nd Annual Meeting. Orlando, FL. Nov. 7, 2002.

Baker M, VanSteenhouse HC, Tai M-H, Huang L, Johansen J, Johansen KM, Xu Y, Hollingsworth RI, Zipser B. Constitutive and Developmentally Regulated Glycosylations of CAMs Mediate Sequential Steps in Synaptic Targeting. 4th International Symposium on Organogenesis: Molecular Control of Neuronal Organogenesis. Ann Arbor, MI. Oct. 6, 2001.

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ABSTRACT OF THE DISSERTATION

Intercellular Adhesion and Pathfinding Molecule T-cadherin

in the Development of the Nervous System

by

Harper C VanSteenhouse

Doctor of Philosophy in Neurosciences

University of California, San Diego, 2007

Professor Barbara Ranscht, Chair

Professor Nicholas Spitzer, Co-chair

Adult animals exhibit an amazing array of behaviors controlled by an exquisitely complex nervous system. Axon pathfinding is an essential component to the development of a fully functioning nervous system. Axon pathfinding cues are molecules in the tissue surrounding growing axons that instruct directionality of axonal outgrowth leading to their proper trajectory from cell body to target. Cell adhesion molecules are one class of proteins that function as contact attractant or repellent pathfinding cues. This dissertation examines the contribution to axon pathfinding of T-cadherin, a member of the cadherin family of adhesion molecules.

Both in vitro and in vivo, T-cadherin is found to be a contact repellant pathfinding cue. Not only is T-cadherin a cue in the environment, it signals to T-cadherin on growth cones in a homotypic manner. Soluble T-cadherin collapses growth cones of motor neuron explants. T-cadherin substrates inhibit neurite outgrowth of wildtype—but not knock-out—spinal neurons. T-cadherin expressed on motor neurons and in posterior sclerotome of chicken embryos directs the outgrowth of motor neurons exclusively through the anterior sclerotome. T-cadherin's properties of homophilic adhesion and inhibition of neurite outgrowth require dimerization, and both functions can be blocked by a single point mutation disrupting this dimer formation as predicted by structural studies. The presence of the pro-domain of T-cadherin also appears to disrupt normal T-cadherin function.

T-cadherin acts in a dominant negative manner over N-cadherin function in several in vitro assays. T-cadherin co-expression negates N-cadherin induced cellular morphology, causes abnormal cell aggregation and segregation, and abrogates strong

N-cadherin homophilic adhesion. T-cadherin may have dominance by inducing the cleavage of N-cadherin. When T- and N-cadherin are coexpressed in the same cell, a portion of the N-cadherin is detected as a smaller species than when expressed alone.

Thus, T-cadherin is shown to be a homotypic repulsive axon pathfinding cue, which may be functioning though an interaction with other cadherins. This interaction may explain T-cadherin's signaling abilities—in spite of being GPI-anchored—by modulating the signal transduction abilities that normally induce neurite outgrowth as a function of the other cadherin's interactions.

Chapter 1: Introduction

Axon Pathfinding

The adult nervous system displays a high order of structural specificity that is generated during development. The specific positioning of neurons, and the specific connections they make to their target cells is essential for a properly functioning nervous system. In an attempt to explain the high degree of specificity, R. W. Sperry put forth a theory of chemoaffinity over 4 decades ago (Sperry, 1963). The essence of this theory is that chemical cues guide neuronal connectivity due to specific molecular interactions of neurons with their environment. This line of thought has received support through the identification of molecular cues recognized by receptors on growth cones at intermediate and final targets of their trajectory. For example, retinal ganglion cell axons make terminal arbors at specific locations in the tectum as a result of their interpretation of the coordinate system produced by ephrin and Eph concentration gradients (reviewed in McLaughlin and O'Leary, 2005). A hypothesis stemming from this chemoaffinity theory is that cell recognition molecules on extending growth cones regulate axon pathfinding and target cell recognition.

An initial naïve motor axon can grow essentially without error directly to its target as opposed to a regressive removal of incorrect connections (Landmesser and Morris, 1975; Landmesser, 1978a; Lance-Jones and Landmesser, 1981). Such precision requires three conditions: *information* in the environment and ability to *interpret* and *act* on the information by the axon. The units of environmental

information are termed pathfinding cues. Axon pathfinding cues are typically grouped into five classes: extracellular matrix (e.g. Laminin, Fibronectin), diffusible chemoattracttion (e.g. Netrin-DCC), diffusible chemorepulsion (e.g. secreted Semaphorins), cell surface adhesion (e.g. NCAM, Cadherins) and contact inhibition (e.g. Ephrins) (Tessier-Lavigne and Goodman, 1996). Each of the exemplary environmental pathfinding cues have a cognate receptor present on the axon, such as Eph receptors in the case of ephrin ligands. As discussed further below, the interaction could also be symmetrical, in which the receptor and ligand are the same class of molecule, such as the case with NCAM or Cadherin based cell surface adhesion. Given environmental information in the form of a pathfinding cue, and the ability to interpret this cue in the form of a receptor molecule, the last piece of the system is the cell's ability to act based on the information received. In the case of axon pathfinding, the ultimate output is a change in movement of the growth cone. The growth cone is the sensory organ at the leading tip of the axon. The growth cone expresses a compliment of receptor molecules, thus allowing it to directly sense the environment as the axon is growing (Wen and Zheng, 2006). The cytoskeleton of the growth cone is in a state of dynamic flux, which allows it to react quickly and bi-directionally to environmental signals. A signal that tips the cytoskeletal equilibrium toward retrograde actin flow will result in retraction, and a tip toward polymerization or stabilization will result in outgrowth. Directionality of entire growth cone movements (as opposed to all or nothing whole-growth cone collapse and cessation of growth) is

provided by tightly localized signaling and cytoskeletal dynamics within the growth cone compartment (Zhou and Cohan, 2004).

The series of continual and ubiquitous pathfinding signaling interactions and microadjustments in the growth of the axon growth cone during development leads to the directed and organized growth of an axon from its cell body to its target on a macro scale in the developed organism.

Adhesion molecules as guidance cues

Cell adhesion proteins are integral membrane proteins that affect the cohesion of cells to juxtaposed cells or to extracellular matrix. Dynamic protein expression levels and the specificity of adhesion conferred by expression of myriad adhesion proteins drives complex organization of groups of cells during development. The organizational effects of cellular adhesion in creating multi-cellular tissues and organisms has long been recognized (Steinberg, 1963). Steinberg concluded after showing that mixed dissociated heart and retina cells aggregate together, but segregate from each other completely and in a reproducible pattern that:

> Differences in cellular adhesiveness which may be built into a system of tissues to bring about the spreading of one tissue over another, or the penetration of one tissue into another, would incidentally (and coincidentally) provide all the conditions required, in an artificial mixture of cells, for sorting out to occur, and for its morphological result to imitate the anatomy normally produced by mass tissue movements.

Cell adhesion proteins expressed on growth cones can subserve the role of receptors for pathfinding cues by encouraging or discouraging the formation of cell-

cell adhesive contacts. This intercellular molecular "adhesive" interaction can also function as a signal-receptor interaction leading to the transduction of a signal into the cell, thus affecting cellular processes such as magnitude and direction of axonal growth (Kiryushko et al., 2004). For example, upon homophilic binding, N-cadherin associates with FGFR and activates the MAPK-ERK signaling cascade leading to neurite outgrowth (Perron and Bixby, 1999; Suyama et al., 2002).

There is also a possibility of higher order organizational effects attributable to adhesion molecules. The diversity of adhesion molecules expressed in the nervous system, as well as their varying interaction specificities lead to the idea that cell adhesion molecules could constitute a sort of functional code by which an axon could be labeled and this label could also recognize its cognate label in the environment leading to attraction or repulsion, an idea much like Sperry originally put forth.

Cadherin family of cell adhesion molecules

One family of cell adhesion proteins, Cadherins, are of particular interest for their possible role in molecular "coding" of neurons important for processes leading to establishment of proper specific neural function—spatial arrangement and functional connection (Shapiro and Colman, 1999; Ranscht, 2000; Huntley, 2002). Cadherins make attractive characters for a code since the numerous family members are abundantly present in the nervous system in a highly spatially and temporally regulated manner (Redies et al., 1992; Redies et al., 1993; Fredette and Ranscht, 1994; Redies, 1995; Obst-Pernberg et al., 2001; Price et al., 2002); they show differential

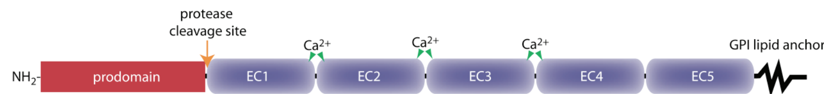
binding strengths in different combinations with a predominant specificity for homophilic binding (Takeichi, 1995); and they are able to affect neurite growth (Bixby and Zhang, 1990). Thus, the organization of functionally similar neurons could be directed by the combinatorial cadherin surface expression into nuclei and tracts with other self-similar coded neurons. In fact, it was shown by collaborative work between Dr. Ranscht's and Dr. Jessell's labs that spinal motor neurons segregate into distinct pools according to a combinatorial cadherin code (Price et al., 2002). Furthermore, propriosensory neurons, which complete the simple reflex circuit from the muscle targets to the spinal neurons, also express the same specific ETS family transcription factors as the motor neurons in each individual circuit (Fredette and Ranscht, 1994; Lin et al., 1998; Price et al., 2002) and appear to be delieated by the same cadherins as motor neurons, suggesting the possibility that the cadherin code may extend to the whole circuit. These findings give credence to the possible power of a cadherin code in establishing functional pathways and connections. My doctoral continues the examination of the hypothesis that cadherins form a code leading to functional connections in the nervous system. To this end, I have undertaken the following experimental approaches to show how this code is physically actualized by one particular cadherin family member, T-cadherin.

T-cadherin

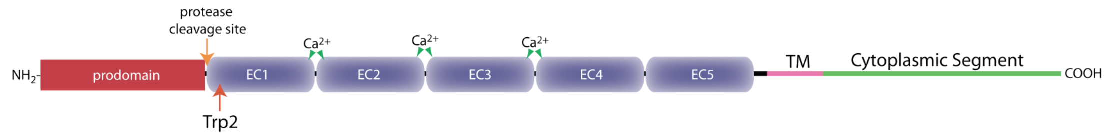
T-cadherin was first described by Barbara Ranscht and colleagues as a member of the Cadherin family of calcium dependant cell adhesion molecules (Ranscht and

Dours-Zimmermann, 1991). T-cadherin is a classical cadherin with 5 extracellular cadherin domains. Although it is classified as such, and has high sequence similarity with other classical cadherins (43% amino acid identity between chicken T- and N-cadherin), T-cadherin exhibits some strikingly unique structural features (depicted in Figure 1-1.) First, T-cadherin is the only classical cadherin that is GPI-anchored. Secondly, all other classical cadherins have a conserved tryptophan residue in position 2 (Type-II cadherins also have a tryptophan at residue 4), which has been shown to be integral for homophilic adhesion (Shapiro et al., 1995; Tamura et al., 1998); T-cadherin lacks this tryptophan. Thirdly, although all classical cadherins are originally translated as a pro-protein, T-cadherin is the only cadherin shown to be expressed normally on the cell surface in both pro- and mature forms.

T-cadherin



N-cadherin (Type-I)



MN-cadherin (Type-II)

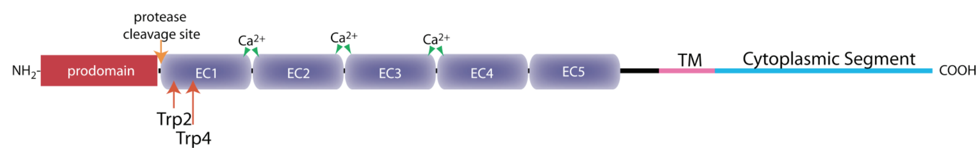


Figure 1-1: Domain structure of T-cadherin and comparison with other classical cadherins

T-cadherin (top) is a classical cadherin with five extracellular cadherin repeat domains (EC1-EC5) like all Type-I and Type-II classical cadherins exemplified here by N-cadherin (middle) and MN-cadherin (bottom), respectively. T-cadherin structure diverges from other cadherins by its lack of intracellular domain—instead being GPI-anchored to the plasma membrane—and by its lack of conserved Trp residues at the N-terminus of both Type-I and Type-II cadherins.

T-cadherin is also functionally unique among cadherin family members studied in that it participates in repulsive interactions. For example, N-cadherin coated substrates strongly induce neurite outgrowth of ciliary ganglion neurons (Bixby and Zhang, 1990). On the other hand, T-cadherin substrates are repulsive to growing axons in vitro (Fredette et al., 1996). In this prior study, repulsiveness was measured as a decrease in the length of neurite outgrowth. The repulsive response seems to be due to a homotypic interaction between T-cadherin on the axon and the substrate because the repulsive effect is observed only with axons cultured from the chick embryo at stages when T-cadherin is expressed, but not with axons cultured at stages when T-cadherin expression is downregulated. Since T-cadherin is GPI-anchored to the membrane and lacks transmembrane and intracellular domains, we hypothesize that T-cadherin functions via a laterally associated protein of unknown nature to transduce signals or interacts with and modulates the function of other membrane integral proteins, such as other cadherins.

Patterned development of motor pathways

The motor axon pathway is a good model system for elucidating the function of T-cadherin as an axon guidance cue both because of the carefully studied anatomy of the system (Nakao and Ishizawa, 1994) and the already described spatial and temporal regulation of T-cadherin expression (Ranscht and Bronner-Fraser, 1991; Fredette and Ranscht, 1994).

Motor neurons' axons grow in a highly stereotyped pattern (see Figure 1-2) from their cell bodies located in the ventral lateral neural tube (nt) to musculature of the trunk and limbs (Krull and Koblar, 2000). Motor axons grow as a tightly fasciculated group of axons through the body in distinct pathways. Along the entire pathway are several defined discrete choice points where the group of axons bifurcate into separate nerve bundles, such as in the limb to form ventral and dorsal branches.

The pathfinding of motor axons is directed by a number of known and yet-to-be-discovered cues present in specific tissues that act to either exclude entry or promote entry of axons. Motor neurons in the ventral-lateral neural tube extend axons as ventral roots to eventually innervate the muscles. There is a complex but stereotypic pattern of axon pathways and step-wise bifurcation points between the neural tube and various muscles. First, all motor axons grow specifically through the rostral half of the sclerotome—completely avoiding the caudal half sclerotome—during st. 21-23 in the chick hindlimb levels. The sclerotome is the ventral-medial compartment of the somite, a segmentally repeating paraxial block of tissue positioned just lateral to the neural tube (Christ et al., 2000). Within the sclerotome is a specific example of tissue that axons are excluded from. All motor axons grow exclusively through the anterior half sclerotome, and completely avoid the posterior half (Figure 1-2b) (Keynes and Stern, 1984; Rickmann et al., 1985). Experiments have implicated an unidentified repulsive cue in the caudal sclerotome directing this pattern. When a somite is experimentally rotated anterior-to-posterior, axons still grow through the originally rostral half sclerotome (Keynes and Stern, 1984). Membrane fractions isolated from

caudal half sclerotome cells cause growth cone collapse in vitro (Davies et al., 1990), and axons avoid caudal sclerotome cells in vitro (Oakley and Tosney, 1993). This highly ordered specific growth across a series of repeating sclerotomes along the length of the trunk ultimately leads to the segmental pattern of spinal nerves seen in the adult organism.

At limb levels, after emerging from the lateral side of the sclerotome, the dorsal ramus deviates from this main pathway and eventually innervates the dermomyotome—the presumptive axial trunk muscles. The remaining major bundle of axons progress toward the base of the limb where they pause forming a plexus. At the plexus, axons defasciculate and re-sort to form new patterns of nearest-neighbor relationships and fasciculation. The newly reorganized fascicles of axons then grow into the limb and bifurcate into one ventral and one dorsal pathway. These fascicles later branch into bundles of motor axons that will each innervate one limb muscle.

T-cadherin shows an intriguing pattern of expression in the developing motor axon system. Within the sclerotome of the developing chicken embryo T-cadherin is expressed exclusively in the caudal half. Mouse embryonic sclerotomes, on the other hand, do not show caudal-exclusive T-cadherin expression. T-cadherin is initially expressed on all motor neurons and their axons as axons are crossing the sclerotome. T-cadherin expression is later down-regulated in all motor neurons just before and during axon sorting in the plexus. Then expression is again upregulated before the time of neuromuscular synapse formation. This second upregulation is motor-pool specific. For example, in the upper hind limb, T-cadherin expression is detected

exclusively on motor neurons innervating the adductor and external femorotibialis (Fredette and Ranscht, 1994; Price et al., 2002).

T-cadherin is one of several candidate repulsive molecules located in the posterior sclerotome. Some of these molecules have been studied, but none have been shown to be essential to the patterned pathfinding of motor axons across the anterior sclerotome. Ephrins expressed in caudal sclerotome were shown to be essential to the migration pattern (similar to the motor neuron outgrowth pattern) of neural crest cells, but not to the outgrowth of motor neurons (Koblar et al., 2000). As reviewed by Krull, F-spondin, Collagen IX, two PNA-binding glycoproteins, Tenascin and Versican are all also expressed as T-cadherin is, in a caudal-only pattern, and have been shown to have inhibitory effects in vitro yet none have been shown regulate motor neuron patterning in the sclerotome exclusively (Krull and Koblar, 2000; Krull, 2001).

To determine whether T-cadherin has a role in the pathfinding pattern of the motor system, I have experimentally perturbed the normal in vivo pattern of T-cadherin expression in order to study the effect of T-cadherin on axon patterning. I have ectopically expressed T-cadherin when it is normally downregulated and decreased T-cadherin from its normal levels of expression. With these techniques, I conducted the experiments described in Chapter 2 to examine the hypothesis that T-cadherin mediated signaling is necessary for the pathfinding of motor axons from the ventral neural tube to their muscle fiber targets.

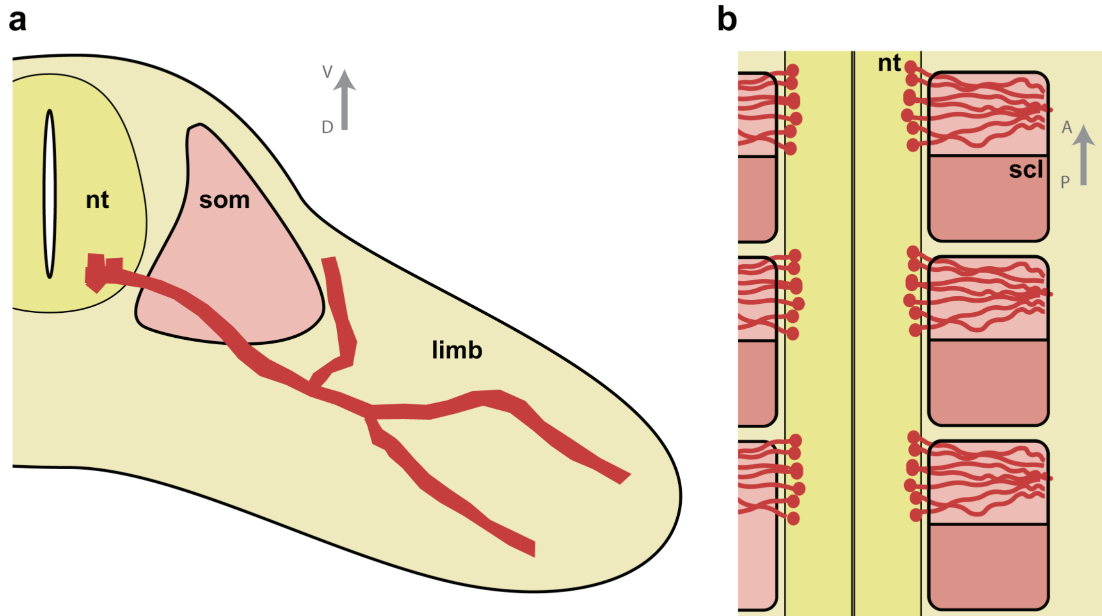


Figure 1-2: Motor axon pathways from neural tube to muscle targets in limb and body wall

Schematic diagram of one half of a cross-section of chicken embryo at the limb level (a) and of a horizonal section of three ventral root segments of chicken embryo. Motor neuron cell bodies reside in the ventral neural tube (nt) and send axons (red) out the ventral root through the sclerotome compartment (scl) of the somite (som) and form various pathways through the body and limb to ultimately innervate the musculature.

In ovo electroporation

A powerful technique that I used extensively in the experiments described below was creating transgenic developing chicken embryos by in ovo electroporation (Muramatsu et al., 1997; Itasaki et al., 1999; Momose et al., 1999; Swartz et al., 2001a; Krull, 2004; Scaal et al., 2004). The initial step of in ovo electroporation consists of injection of a DNA solution into the living chicken embryo within its shell. An electric field—in the form of a series of square pulses of current driven through electrodes surrounding the embryo—is then created across the embryo in line with the site of injection. This current electroporates—destabilizing the plasma membrane forming transient pores (Tieleman, 2004)—the neighboring cells allowing entry of the injected DNA into the cell. This current also electrophoreses the DNA—directing the DNA to move in the direction toward the anode—thus controlling which cells are transfected by their relative position to the injection site. Thus, this technique enables the delivery of specific DNA to induce long-lasting protein expression at a point of time into specific groups of cells in the living organism. In order to create transgenic embryos, I have electroporated DNA encoding an active promoter element upstream of T-cadherin cDNA and a marker gene to achieve ectopic expression of those genes (Figure 1-4a) or double stranded RNA in order to use post-transcriptional interference to specifically silence gene expression (Figure 1-4b). After the DNA is electroporated into the cells of a developing embryo, the egg can be re-closed and the chicken embryo will continue to develop further when placed in an incubator. The result is an embryo that has developed under the experimental condition of an altered level of a

single molecule in a specific region. I repeatedly used variations of this technique in order to thoroughly examine the role of T-cadherin by observing the resulting changes in the developmental program after experimental upregulation or downregulation of T-cadherin in neurons or in their environment.

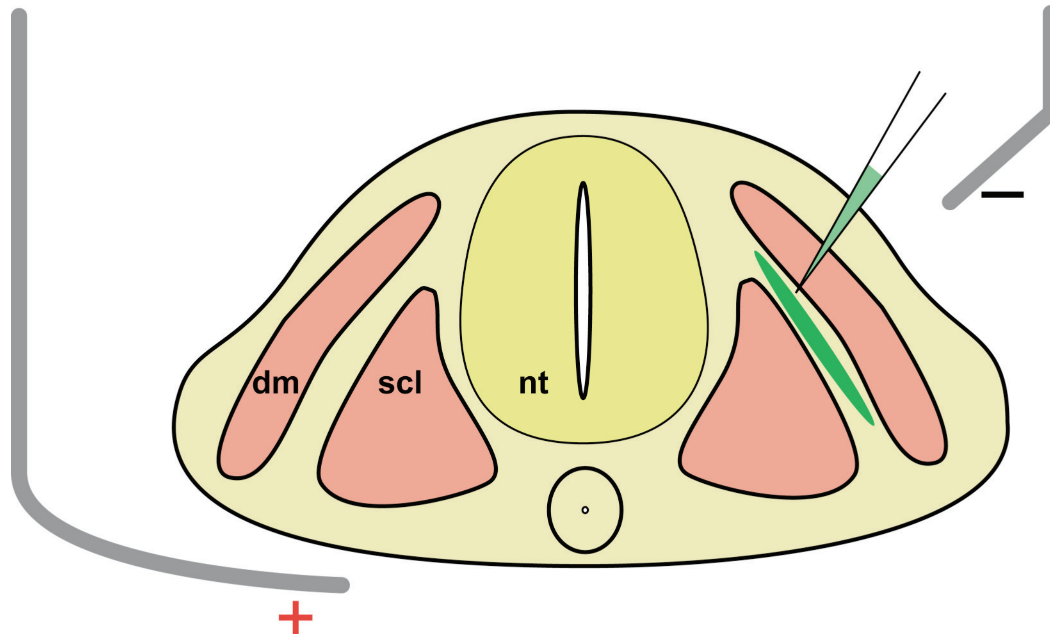


Figure 1-3: In ovo electroporation into embryonic chicken sclerotome

Expression vector DNA in solution (green) is injected with a finely drawn glass pipette into the relatively less dense tissue between the dermomyotome (dm) and sclerotome (scl) compartments of the somite. Platinum electrodes are positioned to apply a square pulse current across the embryo in the ventro-medial direction of the sclerotome from the site of injection (+ and -).

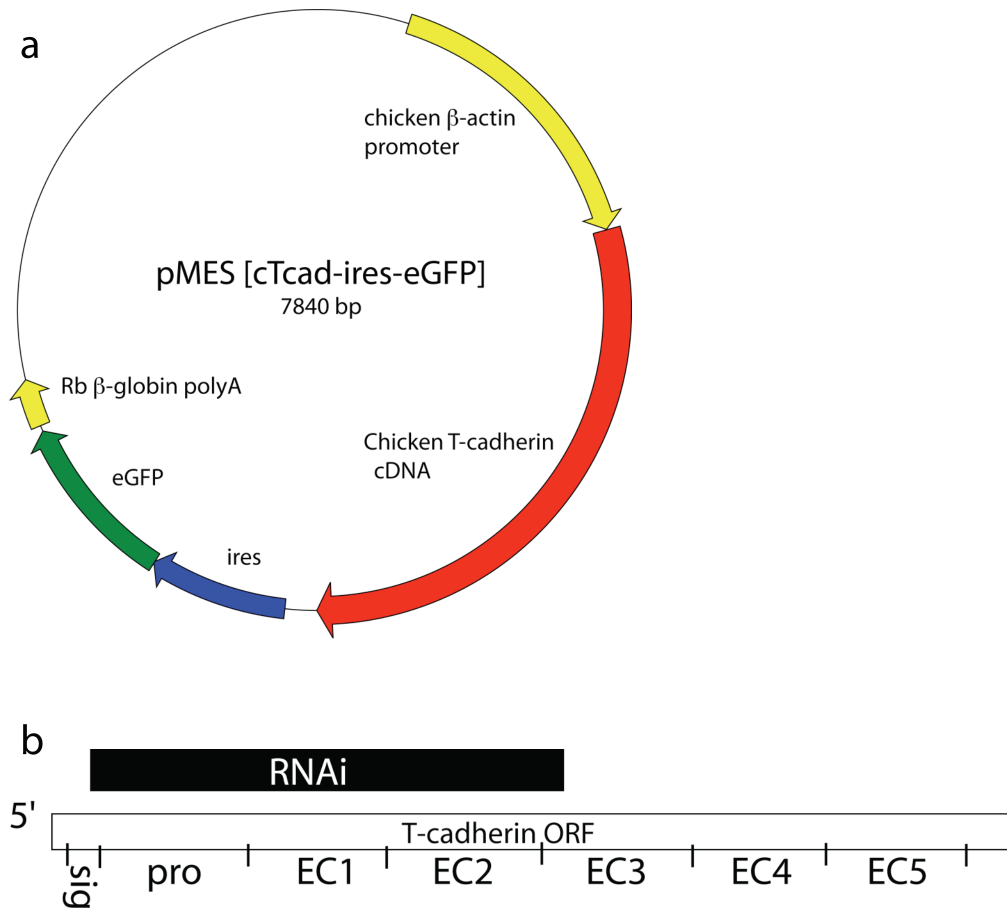


Figure 1-4: Expression vector and RNAi used for in ovo electroporation

Schematic diagram of pMES circular DNA expression vector with bicistronic cassette used for expression of both chicken T-cadherin and a eGFP marker (a). Location of complementarity between T-cadherin cDNA and RNAi used for knockdown (b) is indicated by the position of the black bar on a schematic of T-cadherin open reading frame.

Experimental strategy

In order to fully examine the role of T-cadherin in the developing nervous system, as described in the following dissertation, I used three related and somewhat parallel approaches: the in vivo dissection of motor neuron pathfinding function (Chapter 2), and in vitro dissection of T-cadherin function in terms of its own structure (Chapter 3) and its interactions with other cadherins (Chapter 4).

I attempted to extend and expand upon our lab's previous in vitro based assays showing that T-cadherin is a repulsive cue to neurons. To further determine what function T-cadherin expression has in the organization of a developing embryo, experiments needed to be done in the developing embryo. For this, the model system of motor neuron pathway development in chicken embryos was chosen. As described above and further in Chapter 2, this was a good choice of model system because the system is well characterized, and T-cadherin is expressed in such a pattern that made it a likely candidate for a pathfinding cue, and the system is amenable to experimental perturbation as well as observation and analysis. We wished to examine the homophilic nature of a putative T-cadherin pathfinding cue, so perturbation of expression in both motor neurons as well as the environment was required. Thus, in this system, the expression of T-cadherin was perturbed in both a gain-of-function manner by ectopically expressing T-cadherin transgenically in a region of the environment where T-cadherin is usually absent, and a loss-of-function manner by knocking down expression of T-cadherin in motor neurons. In combination, these

experiments show the necessity and sufficiency of T-cadherin expression for the specific patterning of T-cadherin-positive motor neuron outgrowth.

One of the most noteworthly properties of cadherins, including T-cadherin, is their propensity to form homotypic dimers—although weaker heterophilic binding has been seen as well (Redies, 2000). This homophilic propensity produces cell sorting and separate aggregation when two populations of cells expressing different cadherins are mixed (Nose et al., 1988; Duguay et al., 2003). The homophilic propensity conferred onto cells by cadherins is highly specific in classical aggregation experiments (Miyatani et al., 1989). Chapter 3 describes experiments used to examine the relationship between the structure of T-cadherin and dimerization or adhesive function. A series of various in vitro cell adhesion assays were employed to compare the adhesive function of wildtype T-cadherin to a series of mutant versions of T-cadherin. Mutants were designed in order to test hypotheses about various structural elements' contributions to adhesive function.

Although cadherins exhibit strong and predominantly homophilic adhesive interactions, they have also been shown on many occasions to have some level of promiscuity in heterophilic interactions with fellow members of the cadherin family. This type of interaction could have significance to the understanding of T-cadherin function in the developing organism where many other cadherins are expressed in specific temporal and cell-type specific patterns. Chapter 4 describes a series of in vitro experiments that measure and describe the possible heterotypic interaction of T-cadherin with other cadherins.

The following chapters describe experiments designed and undertaken to explore the function of T-cadherin in a robust manner—in order to determine T-cadherin function in the developing nervous system by integrating various levels of observation from structural, biochemical, cellular, developmental and embryological points of view.

Chapter 2: Role of T-cadherin in development of segmentation of spinal motor nerves

Earlier experiments in our lab (which will be expanded upon in this dissertation) suggested that, at least in vitro, T-cadherin may be a homotypic repulsive axon pathfinding cue (Fredette et al., 1996). In vitro assays of axon pathfinding, such as the ones described here that measure neurite outgrowth under different (but homogeneous) conditions are very simplified. They intentionally minimize the number of signals reaching neurons, and different experiments control for all but one variable, the factor in question (T-cadherin in this case). The neurons in these experiments, therefore, experience very different conditions than neurons in a developing embryo. In an organism, there are many different cell types in the environment of any given neuron, all of which are potential sources of pathfinding signals that are all continuously bombarding the neuron. Unlike in vitro assays where single processes and growth cones can be studied, in an intact embryo axons are bundled tightly their with neighbors, so there are also confounding population effects. Thus, in an effort to examine the role of T-cadherin as a pathfinding cue in the living developing embryo, it is essential to choose the right system to minimize the level of complexity that can confound or mask experimental results. In the case below, we have chosen to examine the developing motor neuron system of the embryonic chicken. The fact that T-cadherin is expressed both on neurons and in the environment allows for the study of the homotypic nature of the putative pathfinding signal. Although we cannot limit the

number of confounding molecules in the environment of outgrowing motor neurons, this system offers a fairly simple topography. Motor neurons grow through a block of tissue (sclerotome) where they are confronted with T-cadherin-positive and T-cadherin-negative regions. During the time of axon outgrowth, neurons interact with a limited number of cell types. This system is relatively simple yet robust enough to study the impact of a single molecule amongst many on a single cell type amongst many.

Thus, given a good model system in which to study T-cadherin function, we sought to alter the pattern of T-cadherin and then monitor the impact of such perturbations on the pattern of motor axon trajectory formation. The finding that altering the locations of T-cadherin expression alters the normal pattern of axon growth is strong evidence supporting the hypothesis that T-cadherin is a repulsive pathfinding cue in the developing embryo.

Introduction

The proper concerted function of the adult nervous system is a result of a coherent network of connections between neurons. Thus, the proper consistent and orderly formation of these connections in the developing embryo is essential. Motor axons extend from neuronal cell bodies to their muscle targets across distances up to thousands of times greater than the cell body diameter. The great scales and low error tolerance require sophisticated and robust mechanisms for directing the axons. A general mechanism by which axons pathfind toward their target is by reacting to attractive or repulsive signals expressed on the surfaces of cells in their path (Dickson, 2002). An axon is directed to its ultimate target by successive step-wise short-range and short-term attractive or repulsive signals along its whole pathway. Many such axon pathfinding cues and their axonal receptors have been described. LMC_l motor neurons that express EphA4 are repelled by EphrinA-2 and -5 expressed on cells in the ventral limb and are thus directed properly into the dorsal limb (Eberhart et al., 2000; Helmbacher et al., 2000; Eberhart et al., 2002). Tectal projection neurons forced to misexpress the attractive cue N-cadherin selectively join the N-cadherin expressing tectothalamic and tectobulbar pathways rather than the N-cadherin negative tectoisthmic pathway (Treubert-Zimmermann et al., 2002). The diversity of pathfinding cues providing specific directional signals to the multitude of specific axon-types in a developing embryo remains to be elucidated.

The cadherin family of intercellular adhesion molecules fit the criteria of being diverse and specific membrane localized signal-receptor systems. At least 80

molecules with cadherin domains have been described, and individual cadherin family members show specificity for homophilic adhesion in general (Ranscht, 2000). Their diversity in the nervous system and their expression by neurons and growth cones makes cadherins excellent candidates as axon guidance cues. Specific cadherins expressed by an axon confer the ability to specifically interpret the milieu of cadherin signals in the environment confronting many types of axons as exemplified by selective fasciculation in the tectal projection neuron system (Treubert-Zimmermann et al., 2002). Classical cadherins with a cytoplasmic domain are able to impact cell motility by virtue of their connection to the actin cytoskeleton through interactions with α- and β-catenins (Huber et al., 1996).

One particular cadherin, T-cadherin, is an interesting candidate as a unique homotypic repulsive cue for axon pathfinding. Our previously described in vitro assays have shown that T-cadherin is repulsive to neurite outgrowth (Fredette et al., 1996) and confers prodominantly homophilic intercellular adhesion to heterologous cells (Vestal and Ranscht, 1992).

The chicken embryonic spinal motor system is an ideal model for examining the role of T-cadherin signalling during in vivo axon pathfinding. The pattern of motor neuron outgrowth through somites is highly specific—exclusively though the anterior half of the sclerotome compartment of each somite (Keynes and Stern, 1984). We previously described T-cadherin protein expression specifically on cells of the posterior halves of sclerotomes, and on motor axons during their growth through the anterior halves (Ranscht and Bronner-Fraser, 1991). Studies by others showed that

motor neurons were directed through the anterior half by an unknown repulsive activity in the posterior half (Keynes and Stern, 1984; Davies et al., 1990; Oakley and Tosney, 1993). Candidate repulsive activities expressed specifically in posterior sclerotomes are: T-cadherin, EphrinB-1(Wang and Anderson, 1997; Koblar et al., 2000), F-spondin (Debby-Brafman et al., 1999; Tzarfati-Majar et al., 2001), PNA-binding molecules (Davies et al., 1990; Oakley and Tosney, 1991) and Versican (Landolt et al., 1995; Perissinotto et al., 2000). Thus amongst others, T-cadherin was shown to have negative action in vitro and patterned expression consistent with being a repulsive cue to motor axons. However no role for any of these molecules has yet been demonstrated conclusively for motor neuron axon guidance in vivo.

To determine whether T-cadherin functions in vivo as a motor axon pathfinding cue, we perturbed the expression pattern of T-cadherin on motor neurons and on the sclerotome. We used the technique of in ovo electroporation to ectopically express, up-regulate or down-regulate T-cadherin expression. A combination of ex vivo assays and examination of resultant in vivo pathfinding patterns were used to determine T-cadheirn's role. Our experiments revealed that T-cadherin is a homotypic repulsive cue to motor neurons both in vitro and in vivo. T-cadherin expressed by substrates or in the media causes stunted neurite outgrowth and growth cone collapse of cultured primary spinal neurons. This T-cadherin repulsion is dependent upon T-cadherin expression by the neurons; loss of T-cadherin from motor neurons causes a loss of repulsive response. Conversely, T-cadherin-positive motor axons in vivo avoid ectopic clusters of T-cadherin expressing cells. When T-cadherin is down-regulated in

motor neurons, they aberrantly grow into a normally restrictive T-cadherin-positive zone of somites.

Results

T-cadherin is a repulsive cue to motor axons in vitro

Embryonic chicken neurons cultured in vitro on T-cadherin-expressing substrates extend shorter neurites after 15-18 hours than when cultured on substrates lacking T-cadherin (Fredette et al., 1996). Likewise, neurite outgrowth inhibition by T-cadherin expressing substrates is seen with primary cultures of cells from dissociated embryonic mouse spinal cords (Figure 2-4). Spinal neurons grown on a cell-monolayer substrate not expressing any T-cadherin extend Tuj-1-positive neurites an average length of 8.74µm after 21 hours. When spinal neurons are grown on a monolayer of cells transfected with and expressing T-cadherin, the average length is 6.48µm, 25.8% (±2.52) shorter than those grown on control substrates ($n=4$, $p<0.05$). Thus, an environment rich in T-cadherin is a negative environment for neurite outgrowth from spinal cord-derived neurons.

To further characterize T-cadherin as a repulsive axon pathfinding cue, we wished to determine whether this long-term inhibition of neurite outgrowth corresponded to a short-term repulsive response by individual neuronal growth cones. The growth cone is located at the very distal end of the axon, and therefore the cell's leading process to encounter a new environment during outgrowth. The growth cone is endowed with a wide array of molecular receptors and signaling machinery able to sense and transduce extracellular signaling molecules into cell cytoskeleton rearrangements (Cooper, 2002). Thus, the growth cone serves as a sensor to new

environments controlling the direction and magnitude of the growth of an axon, and its response to a stimulus is a key indicator of the axon's behavior. T-cadherin is expressed on filopodia and lamellipodia of motor neuron growth cones of spinal neurons explanted from st. 21 chicken embryos (Figure 2-1c).

To examine the responses by individual motor axon growth cones to T-cadherin, ex vivo ventral spinal cord explants were grown and soluble recombinant T-cadherin was applied to the media. Ex vivo cultures for growth cone collapse assays were prepared from the ventral third of thoracic and lumbar spinal cords of st. 21-22 chicken embryos to ensure a high proportion of T-cadherin-positive motor neurons cultured. Explants of approximately 100µm diameter were cultured for 3 days, during which time axons extended radially away from the explant. After 3 days, soluble T-cadherin protein (final concentration 10µg/ml) was applied to the media. Cultures were fixed after 30 minutes incubation, and membranes were labeled with DiI for visualization and morphological analysis.

To determine whether T-cadherin has a significant effect on the proportion of collapsed growth cones, each growth cone was classified into three categories: spread filopdia and lamellopodia (fully spread), collapsed lamellopodia but with some persistent filopodia (partially collapsed) or fully collapsed. Application of soluble T-cadherin in the media caused a 46.1% decrease in the proportion of fully spread growth cones and a corresponding increase in collapsed growth cones compared to controls ($p<0.001$). Most of the increased collapsed growth cones were of the partial

collapse class, the background level of fully collapsed growth cones was relatively constant over both conditions.

Thus, T-cadherin exhibits, in vitro, functional qualities essential for a repulsive axon pathfinding cue. Previous studies showed that T-cadherin substrates inhibit neurite outgrowth. The current study confirms that finding and shows that T-cadherin causes acute growth cone collapse.

Figure 2-1: T-cadherin induces motor neuron growth cone collapse

Axons growing radially away from ventral spinal cord explant immunostained against neurofilament (a). Neurofilament immunostaining of axons and growth cones growing in ventral spinal cord ex vivo culture (b). The growth cone of a spinal neuron cultured ex vivo defined by DiI membrane staining (c). T-cadherin immunostaining shows punctate expression of T-cadherin on the surface of the entire growth cone. Growth cones (stained with lypophilic DiI) are predominantly of a fully spread morphology in control ventral spinal cord explant conditions (d, ctrl). When T-cadherin is added to the media, the predominant morphology is collapsed (d, +T-cadherin). Summary of growth cone morphology classification of spinal neuron explant cultures in control conditions compared to with application of T-cadherin to the media for 45 minutes (e).

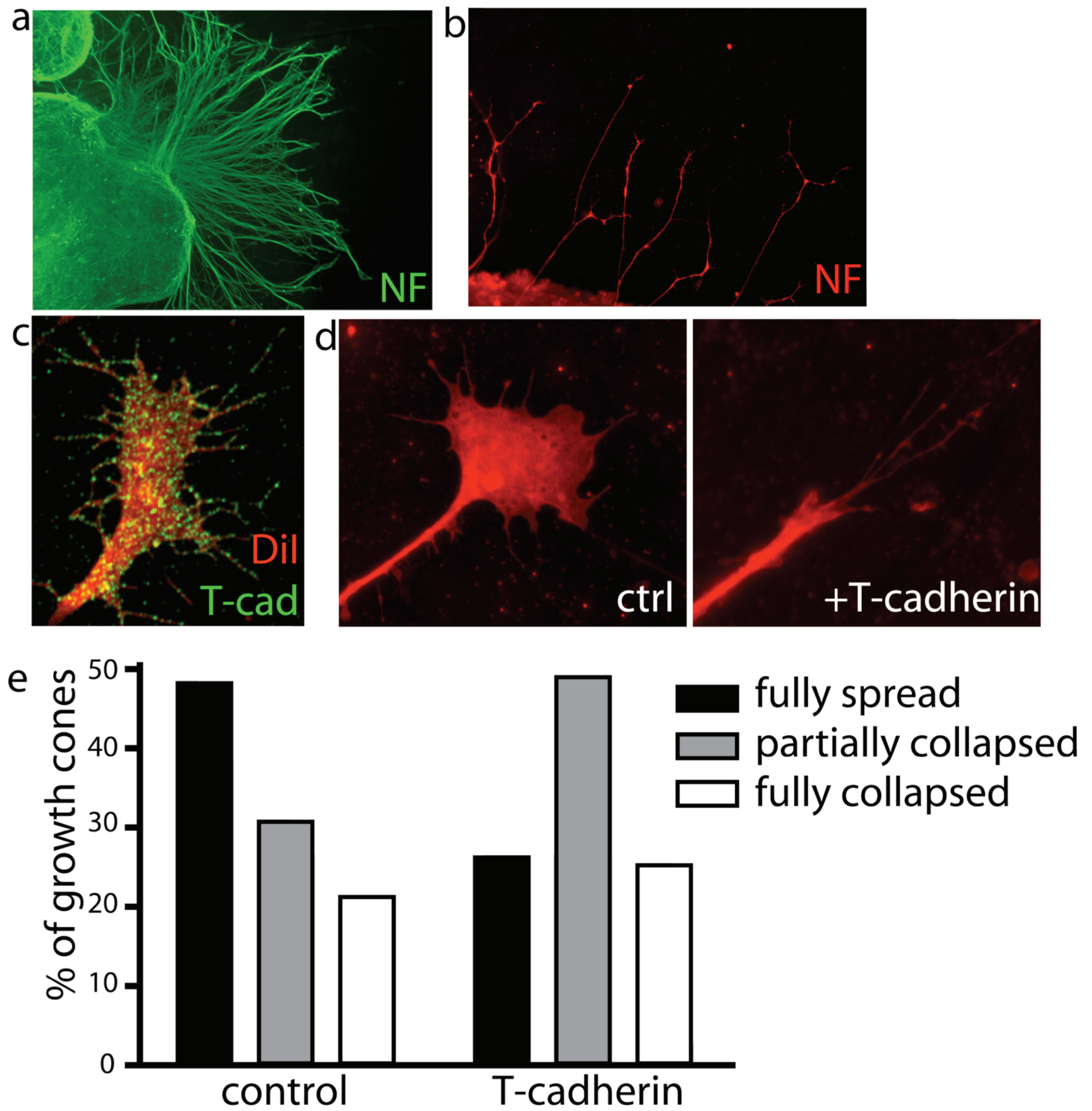


Figure 2-1: T-cadherin induces motor neuron growth cone collapse

Transgenic T-cadherin expression by chicken embryo sclerotome cells in ovo

To determine whether T-cadherin has a role in directing axon pathfinding in vivo, we sought a physiological model for possible T-cadherin-influenced axon pathfinding. This in vivo model would then be tested by manipulating T-cadherin expression patterns while axons grew. The system we chose to examine T-cadherin's role in axon pathfinding was the developing chicken embryo motor axon projection. Chicken motor neuron pathways are well characterized (Jacob et al., 2001; Schneider and Granato, 2003), readily accessible to experimental genetic manipulation (Swartz et al., 2001a), and T-cadherin is expressed on motor neurons and their enviroment in a temporal and spatially-regulated manner (Ranscht and Bronner-Fraser, 1991; Fredette and Ranscht, 1994; Fredette et al., 1996). In order to test T-cadherin's role in the patterned axon growth thorough the sclerotome, we used in ovo electroporation as a method to ectopically express T-cadherin, which is selectively expressed in the posterior sclerotome. To co-express T-cadherin and a GFP marker, we generated an expression vector (pMES-Tcad) that would transcribe a bicistronic cassette with T-cadherin and eGFP genes downstream of the chicken β-actin promoter. By virtue of the IRES element in the pMES expression cassette, each gene is translated individually, yielding two separate proteins expressed by the electroporated cell. The co-expression of cytosolic eGFP and membrane bound T-cadherin was verified by liposomal transfection into CHO cells followed by indirect immunofluoresence with anti-T-cadherin antibodies (Figure 2-2a). Transfected, GFP-positive, cells displayed punctate surface T-cadherin immunostaining typical for T-cadherin expressing cells.

To verify co-expression of T-cadherin and the eGFP marker in cells of a living chicken embryo, pMES-Tcad was injected and electroporated into sclerotomes of st. 13 embryos. Injected and electroporated embryos were re-sealed in their eggs and allowed to continue development in an incubator. At 24 hours post-electroporation, embryos were dissected, fixed and sectioned. T-cadherin immunohistochemistry confirmed that GFP-positive cells ectopically expressed T-cadherin in the normally T-cadherin-negative anterior sclerotome, and over-expressed T-cadherin at greater than normal levels in posterior sclerotome cells (Figure 2-2b).

Specific targeting—limited to the sclerotome—and efficiency of transduction of electroporated cells was confirmed by histologial examination (Figure 2-2c-e). In cross sections, eGFP positive cells are found strictly lateral to the neural tube and ventral to the dermomyotome in the sclerotome (Figure 2d), the tissue motor axons grow through in this region after emerging from the ventral root of the spinal cord (Figure 2-2d). Longitudinal sections show widespread transgene expression in the anterior sclerotome defined by the absence of the posterior sclerotome marker EphrinB-1 (Figure 2-2e).

Figure 2-2: In ovo electroporation and ectopic co-expression of T-cadherin and eGFP in sclerotome

CHO cells transfected with pMES-Tcad expression vector show co-expression of cytoplasmic eGFP fluorescence and surface T-cadherin by immunofluorescence (a). T-cadherin and eGFP co-expression in chicken embryonic sclerotome cells electroporated with pMES-Tcad (b). Electroporated anterior (A) cells ectopically express surface T-cadherin, and posterior (P) cells overexpress surface T-cadherin. Electroporated cells marked with eGFP fluorescence overlaid onto a phase contrast image of an embryo cross section (c). Transfected cells are located only in the sclerotome, between the neural tube (nt), notochord (nc), and dermomyotome (dm). Motor neurons immunostained against neurofilament grow out of the ventral root and through the sclerotome on both untransfected side (left) and eGFP electroporated and expressing side (right) of the embryo (d). Coronal section of sclerotome-electroporated embryo (e). Electroporation transfected cells marked by pCAX-driven eGFP expression are found in both anterior and posterior (defined by Ephrin-B1 immunostaining) sclerotome compartments.

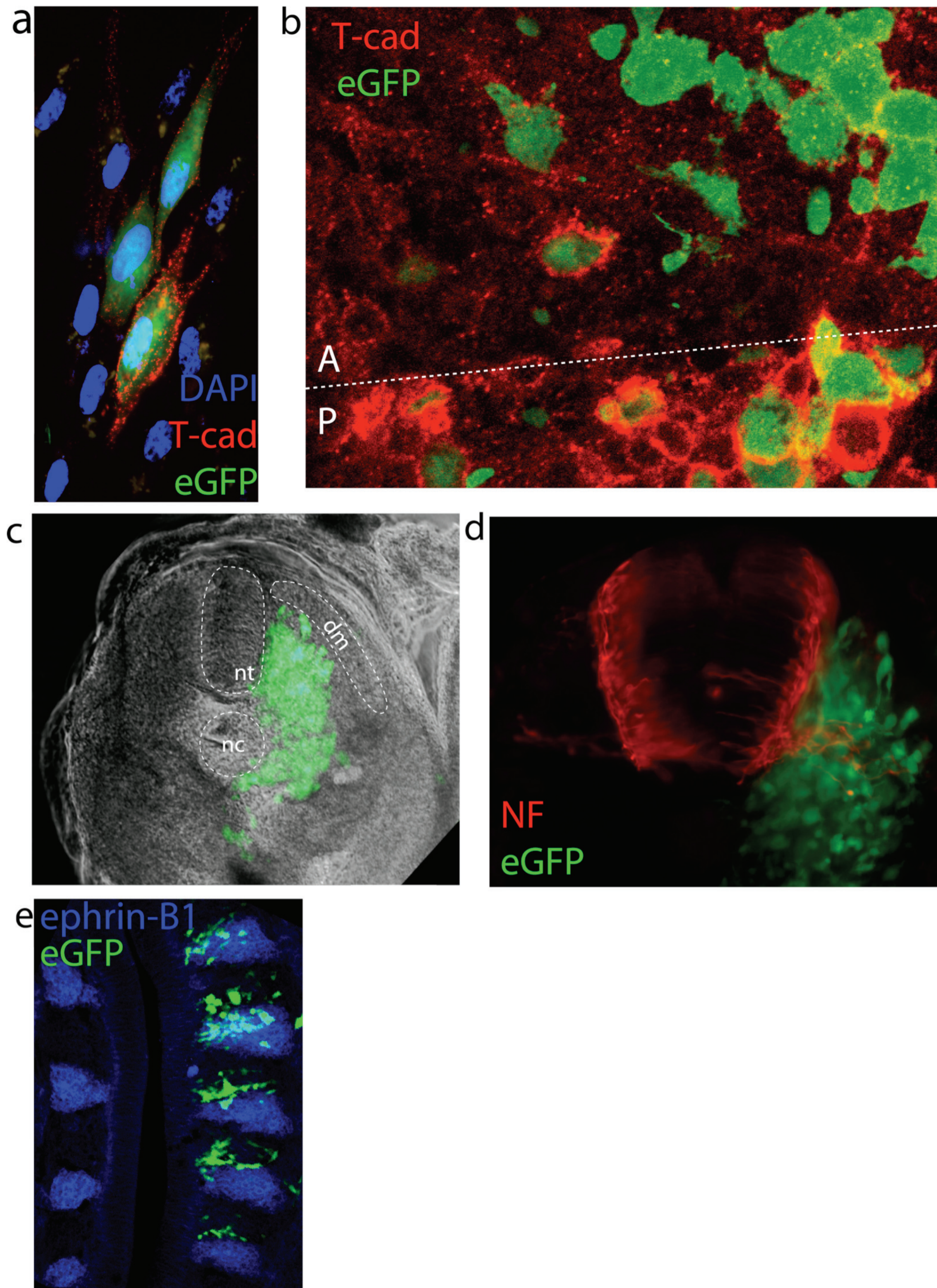


Figure 2-2: In ovo electroporation and ectopic co-expression of T-cadherin and eGFP in sclerotome

Ectopic T-cadherin in anterior sclerotome establishes an ectopic repulsive zone to motor neurons

To determine whether T-cadherin is sufficient as a repulsive axon pathfinding cue in vivo, we used a gain-of-function experiment in the motor neuron model system described above. T-cadherin's expression pattern in the environment of the motor neuron is consistent with its being a repulsive cue to motor axons. T-cadherin is present in the posterior sclerotome where axons are restricted from growing, but is absent in the anterior sclerotome that axons normally grow through.

Ectopic expression of eGFP-alone control vector (pCAX) by electroporation into sclerotomes did not perturb axonal outgrowth patterning. Neurofilament-immunostained axons grew across the Ephrin-B1-negative anterior sclerotomes in an identical manner through a cluster of GFP-positive cells as through eGFP-negative cluster (Figure 2-2e, 2-3a) and as through a non-electroporated sclerotome (data not shown). In contrast, in T-cadherin electroporated embryos, motor neurons grew in an abnormal pattern, avoiding areas of sclerotome with ectopic T-cadherin expression. Ventral roots were seen as compressed, comprising a smaller proportion of the anterior sclerotome when electroporated cells were present at the margins of the anterior sclerotome (Figure 2-3b, top). Ventral roots bifurcated around T-cadherin expressing sclerotome cells in central anterior sclerotomes (Figure 2-3b, bottom).

To compare the results between treatments across embryos with different transfection levels and cell positions of chimeric electroporated sclerotomes, we calculated a colocalization index (CI) for each electroporated sclerotome. Each CI was

determined from a confocal micrograph optical section exhibiting eGFP-marking of transfected cells and neurofilament immunostaining of axons, after using EphrinB-1 immunostaining to isolate one anterior sclerotome. The CI for a given sclerotome was determined by measuring the number of colocalized GFP and neurofiliment pixels normalized to the total amount of neurofilament staining and GFP expression. The CI of T-cadherin transfected sclerotomes was significantly decreased (48%) compared to control GFP-alone transfected sclerotomes (Figure 2-3c). Thus, motor axons specifically avoid clusters of anterior sclerotome cells that ectopically express T-cadherin (Figure 2-3b).

Figure 2-3: Motor axons avoid anterior sclerotome cells ectopically expressing T-cadherin

Coronal section of chicken embryonic NF immunostained ventral root growing laterally (up) from neural tube (along bottom) in control eGFP electroporated sclerotome (a). Motor neurons grow normally through a cluster of eGFP transfected sclerotome cells (yellow). Motor neurons avoid clusters of T-cadherin transfected sclerotome cells (b). Ventral roots are compressed or bifurcated depending on relative location of transfected sclerotome cells. Summary and comparison of colocalization index of motor neurons and transfected sclerotome cells in control or T-cadherin electroporated embryos (c). T-cadherin electroporated sclerotomes had a 48% reduction in colocalization. Mean±SEM, N=13 (control), 17 (T-cadherin) $p<0.05$.

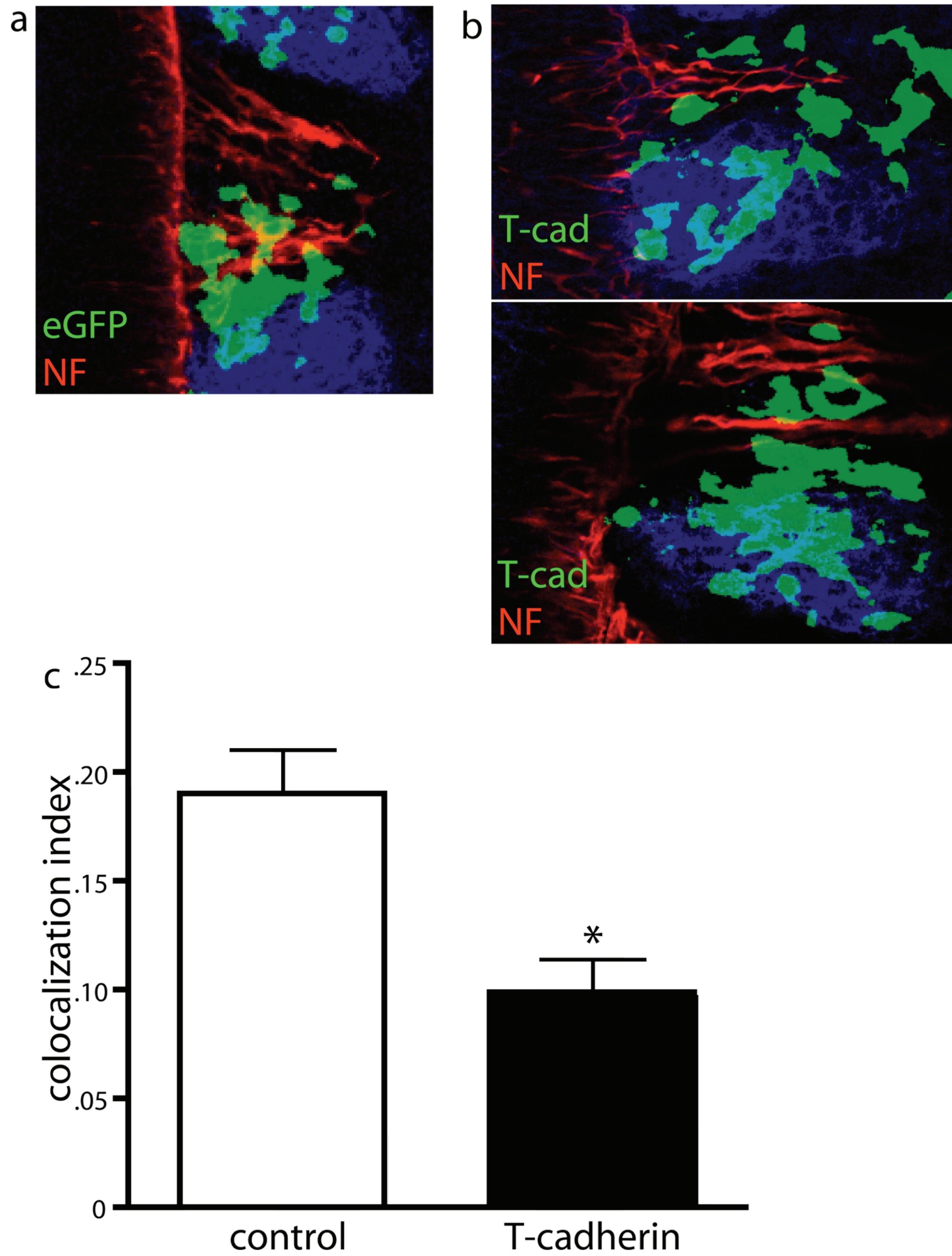


Figure 2-3: Motor axons avoid anterior sclerotome cells ectopically expressing T-cadherin

T-cadherin repulsion is mediated by a homotypic interaction with axonal T-cadherin

T-cadherin is expressed on both motor neurons and the posterior sclerotome at the stage of motor neuron outgrowth through the sclerotome (Ranscht and Bronner-Fraser, 1991). T-cadherin, like other cadherin family members (Redies, 2000), is known to homotypically interact in trans with T-cadherin expressed on neighboring cells. This intercellular homotypic interaction has been demonstrated by the selective homophilic aggregation of cells expressing T-cadherin (Vestal and Ranscht, 1992). This event could also be considered as an interaction between both intercellular signal and receptor. The question of whether T-cadherin expressed on motor neurons mediates the repulsive signaling of T-cadherin expressed on posterior sclerotomes is raised by the diametrically opposed pattern of T-cadherin expression and its previously shown homophilic adhesive nature.

To determine whether T-cadherin on motor neurons is required to receive the repulsive T-cadherin signal in the environment, we first examined the neurite outgrowth properties of spinal neurons cultured from wild-type and T-cadherin null mice. The spinal neurons used in these assays are defined by Tuj-1 expression. Of these Tuj-1-positive neurons, 77.2% (n=448, data not shown) also express T-cadherin (Figure 2-4a). As shown in Figure 2-4 b-f, wildtype spinal neurons have a 25.8% (±2.52) reduction of average neurite length on T-cadherin-positive substrates compared to T-cadherin-negative control substrates. To determine whether this T-cadherin substrate–induced neurite inhibition requires T-cadherin on motor neurons,

we created spinal neuron cultures from T-cadherin null mice. Neurons lacking T-cadherin showed no difference in neurite outgrowth length on either T-cadherin-positive or –negative substrates. The measured 0.6% (±3.0) shorter average neurite length on the T-cadherin-positive versus control substrates (Figure 2-4e) is not statistically different than zero by 1-sample t-test, but is significantly different than the 25.8% reduction of wildtype neurons ($p<0.001$). Thus, spinal neurite outgrowth inhibition by T-cadherin in the environment depends upon homotypic signal reception by T-cadherin on the neurites.

Figure 2-4: T-cadherin is a homotypic repulsive cue in vitro

Cultured spinal neuron explants co-immunostained against Tuj-1 and T-cadherin show that most Tuj-1 positive neurons also express T-cadherin (a). Micrographs of spinal neurite outgrowth of wild-type neurons (b and c) or T-cadherin knockout neurons (d and e) on non-transfected control (b and d) or T-cadherin expressing (c and e) substrata. Average relative reduction of neurite lengths of spinal neurons grown on T-cadherin expressing versus control substrates (f). Spinal neurons were cultured from either wildtype (left bar) or T-cadherin knockout (right bar) mouse embryos. Mean±SEM, *$p<0.005$

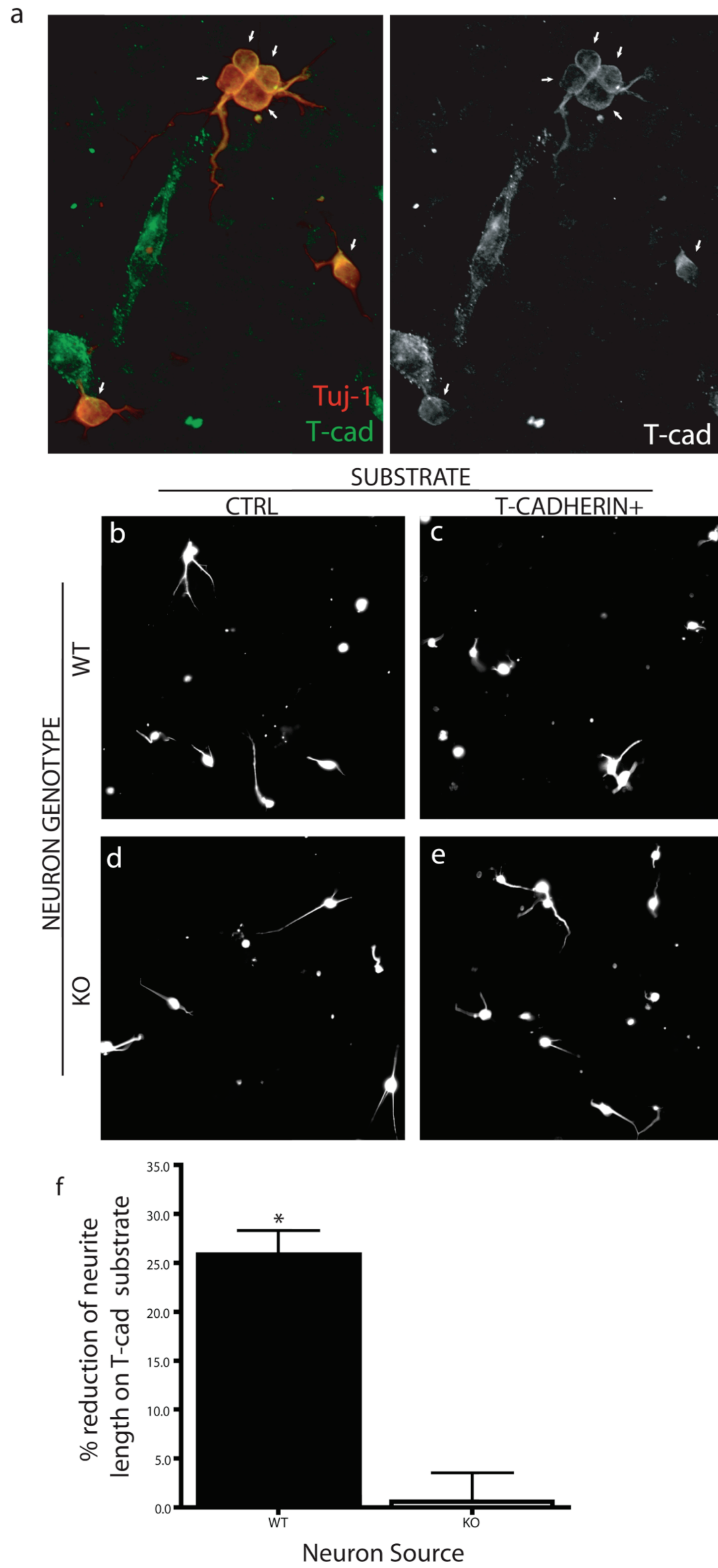


Figure 2-4: T-cadherin is a homotypic repulsive cue in vitro

RNAi induced knockdown of T-cadherin in chicken embryo motor neurons in ovo results in aberrant axons entering the posterior sclerotome

Since cultured murine motor neurons require T-cadherin to respond negatively to a repulsive T-cadherin–rich environment, we next asked whether motor neurons in vivo require normal T-cadherin expression in order to be repelled by the posterior sclerotome and be properly routed through the anterior sclerotome. We chose the chicken motor neuron pathway to address this question, as T-cadherin is not expressed in the sclerotome in the mouse at corresponding stages. We used in ovo electroporation to perturb the normal pattern or T-cadherin in the developing chicken embryonic motor neuron system. Specific reduction of T-cadherin expression was achieved by using RNA interference (RNAi). Electroporation of double-stranded RNA into chicken embryo cells results in post-translational gene silencing specific to the gene complimentary to the RNAi used (Bourikas and Stoeckli, 2003; Pekarik et al., 2003; Rao et al., 2004). Thus, we created RNAi homologous to a 900bp region of chicken T-cadherin with the least sequence identity to other cadherins, the prodomain and the two N-terminal cadherin repeats (Figure 2-5a). Knockdown of T-cadherin expression levels in individual axons was confirmed by culturing neural tube explants derived from embryos after they had been electroporated in ovo at st. 13 with T-cadherin RNAi. After 3 days culture in vitro on laminin and poly-lysine coated glass substrates, axons are seen extending radially away from the explants. Non-electroporated (data not shown) as well as eGFP-alone electroprated control explants express T-cadherin—as shown by indirect immunostaining—on all axons (Figure

2-5b) (Fredette and Ranscht, 1994). On the other hand, axons extending from neurons that had been electroporated in ovo with RNAi—marked as GFP-positive resulting from a co-electroporated vector—are not positive for T-cadherin immunostaining (Figure 2-5c).

Having verified the RNAi-induced knockdown of T-cadherin in spinal neuron explants, we asked whether knockdown of T-cadherin on motor neurons has an effect on motor neuron outgrowth in vivo. We co-electroporated RNAi and eGFP marker, or control eGFP vector alone, into st. 13 embryo neural tubes. Embryos were then allowed to develop in ovo for 24 hours after which time embryos were dissected, fixed, and sectioned coronally. Sections were immunostained with anti-EphrinB-1 to differentiate posterior sclerotome from anterior sclerotome, and neurofilament antibody to stain all motor axons. Axons originating from electroporated motor neurons were marked with eGFP expression. No axons in any control electroporated embryo were found progressing more than 10% of the distance across the posterior sclerotome from the neural tube to dermomyotome (Figure 2-5d). In contrast, axons that received RNAi were found that had grown across the entire posterior sclerotome (Figure 2-5e) (11 embryos, 3-9 electroporated roots per embryo). Two to seven ventral roots with eGFP-positive axons were examined from each of ten embryos that had received RNAi electroporation into the ventral neural tube. Out of the tens of thousands of axons represented in this sample (10 embryos, 2-7 electroporated roots per embryo), at least 18 separable units were seen in the posterior sclerotome, having progressed further than 10% of the distance from the neural tube to the

dermomyotome. There is no reliable way to differentiate between a bundle of multiple axons versus a single axon in our sections, so we are only able to put a lower bound on the number of deviant axons. In fact, in many cases the deviant units were likely to be bundles since they bifurcated at some point into multiple units.

Figure 2-5: T-cadherin is a homotypic repulsive cue to motor axons in ovo

Schematic showing location of homology between T-cad RNAi and T-cadherin pro-domain and EC1-2 (a). Axons in ventral spinal cord explant cultured from embryonic neural tubees electroporated with eGFP (b). All axons, both untransfected and eGFP electroporated, show T-cadherin immunostaining (red left and right). RNAi and eGFP co-electroporated spinal axons in culture 3DIV exhibit greatly reduced T-cadherin immunofluorescence (c). Coronal section of sclerotome from chicken embryo that had been electroporated with RNAi into the motor neurons (d). Most axons (stained by NF-antibodies) grow normally through the anterior sclerotome and avoid the posterior half. A bundle of RNAi-transfected (eGFP-positive) motor axons grow through the middle of the posterior sclerotome.

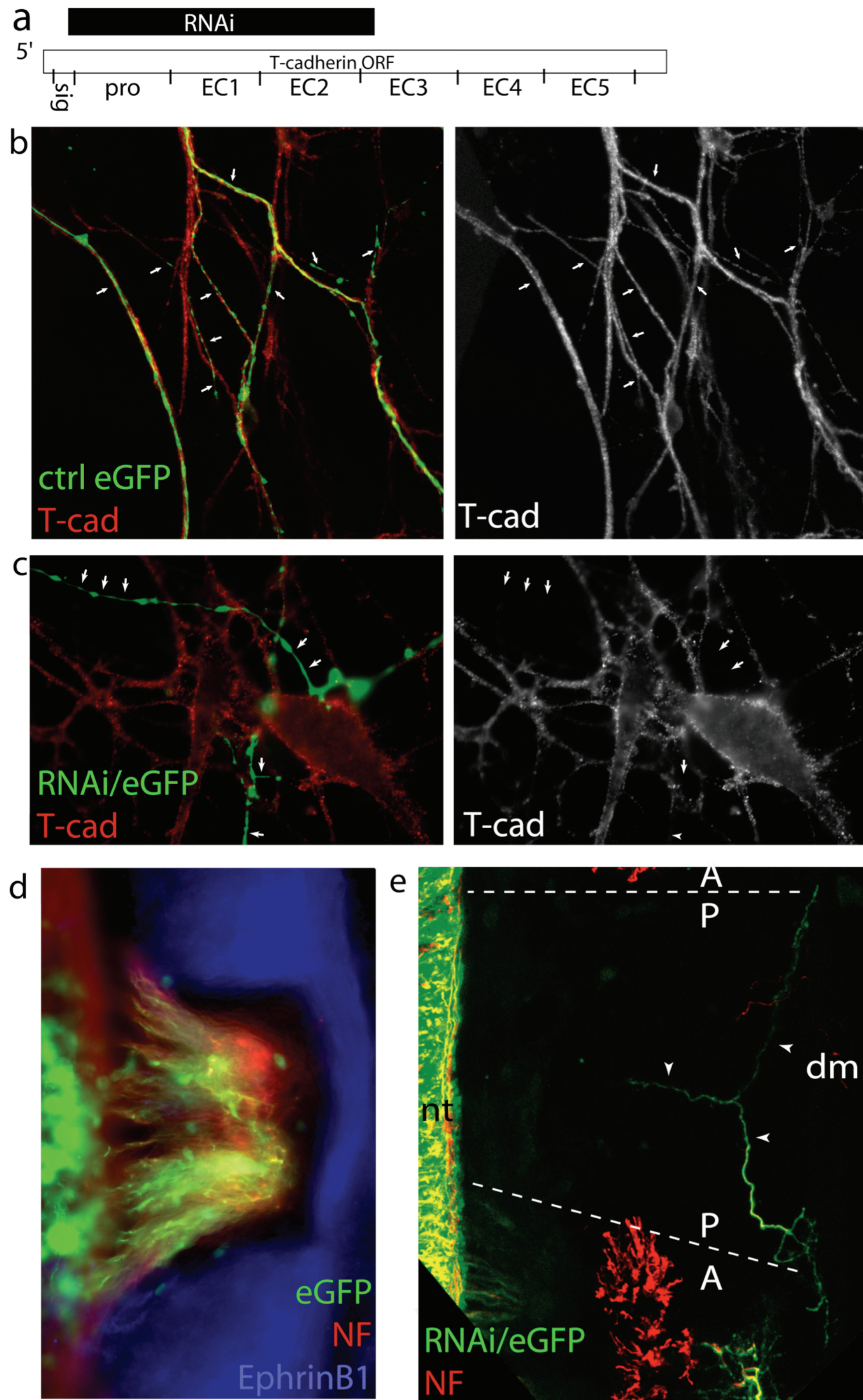


Figure 2-5: T-cadherin is a homotypic repulsive cue to motor axons in ovo

Normal T-cadherin expression pattern is not required for normal gross pathfinding of motor neurons into the limb and to major muscle groups

Lastly, we questioned whether the pathfinding defects seen above were the result of a general inability of axons to progressively grow through the embryo properly or were due to the loss of specific pathfinding decision signals. To answer this question, we examined the necessity for T-cadherin expression patterning after motor neurons navigated through the somite and continued their paths though the plexus and into their specific muscle targets. Unlike in the sclerotome, there is no T-cadherin expression gradient surrounding the motor neuron pathway in the limb. Therefore, axons do not confront a binary choice between T-cadherin positive or negative regions of mesenchyme while they are growing through the limb (Fredette and Ranscht, 1994). T-cadherin expression on motor axons is downregulated during plexus sorting, but then is re-upregulated on a subset of axons as they reach their muscle targets (Fredette and Ranscht, 1994). Thus, it was thought unlikely that T-cadherin plays a specific role in the decision-making of these pathways. Ephrin expression, on the other hand, does create a binary choice point with Ephrin–A2 and –A5 being expressed in the ventral limb bud, which prevents EphA4-expressing LMC_l motor neurons from entering this zone (Eberhart et al., 2000; Helmbacher et al., 2000; Eberhart et al., 2002). To examine potential pathfinding defects when T-cadherin is constitutively overexpressed in motor neurons, we examined motor neuron projections at the plexus in chicken embryos after electroporation into the spinal cord. To determine whether gross pathfinding is altered when T-cadherin is completely absent

from motor neurons we also examined the motor neuron pathway in T-cadherin knockout mice. Neither of these manipulations resulted in aberrant pathways at a relatively gross level indicating that T-cadherin does not exert a major role during later stages of motor axon growth.

T-cadherin expression is downregulated in all motor neurons during plexus formation at the mRNA level and upregulated on specific motor neuron pools and their axons thereafter (Sacristan et al., 1993; Fredette and Ranscht, 1994). To determine whether this downregulation is required for proper plexus sorting and axonal pathfinding thereafter, we asked whether constitutive over-expression of T-cadherin in motor neurons causes improper or failed routing of axons to major muscle groups. Constitutive overexpression of T-cadherin was achieved by electroporating pMES-Tcad into motor neurons of st.18 embryos. All motor axons were detected by indirect neurofilament immunofluorescence in sections of the embryos. Electroporated motor neurons and their axons were labeled with eGFP by virtue of the pMES IRES-EGFP bicistronic expression cassette. T-cadherin electroporated axons were seen in all major pathways (Figure 2-6b)—ramus visceralis (not shown), dorsal ramus, and dorsal and ventral limb nerve trunks—just as control eGFP electroporated axons do not (Figure 2-6a). Thus, simply overexpressing T-cadherin in some axons does not alter their trajectories, either by preventing axons from entering or instructing entry to a specific projection. Furthermore, control and T-cadherin electroporated axons were always found within the group of non-transfected axons, not deviated away from established pathways.

Since T-cadherin expression is upregulated in some motor neurons after plexus sorting, we asked whether this upregulation was required for proper pathfinding of neurons to their specific muscle groups. We used T-cadherin knockout mice for these experiments since all axons would be T-cadherin-deficient, rather than the chimeric partial knockdown resulting from RNAi in ovo electroporation. To determine whether axon routing depends on T-cadherin expression, we backlabeled pathways from specific muscle groups of E12.5 mice. Rhodamine-labeled dextran was injected into muscles of the dermomyotome, ventral limb, or dorsal limb. The labeled dextran is taken up by axon terminals in these muscle groups, and actively transported retrogradely to the corresponding cell bodies in the ventral spinal cord. After dye transport, cross-sections show the positioning of labeled neurons within the spinal cord. The location of labeled neurons within defined motor neuron columns (Landmesser, 1978a, b; Hollyday, 1980) indicates the identity of the motor neurons, and their expected muscle group innervation (Sharma et al., 2000; Huber et al., 2005). In both T-cadherin knockout (Figure 2-6c) and wild-type mice (not shown), labeled cell bodies form distinct and tight clusters in specific positions in the ventral spinal cord. Dermomyotome injections labeled ventral-medial neurons (MMC), ventral limb injections labeled ventral-lateral neurons (LMC_m), and dorsal limb injections labeled lateral neurons slightly more dorsal than those of vental limb injections (LMC_l). Thus, absence of T-cadherin in murine motor neurons does not cause gross pathfinding errors from motor columns to these three main muscle groups.

These experiments show that perturbing T-cadherin expression does not cause a general defect in axon outgrowth or pathway progression. Instead, T-cadherin specifically affects an axon's ability to make proper pathfinding decisions based upon differential expression in the axons' environment such as the sclerotome.

Figure 2-6: T-cadherin expression perturbation doesn't cause any gross motor axon pathfinding errors during growth through the limb.

Horizontal section of chicken embryo at hindlimb level previously electroporated with eGFP in ventral neural tube motor neurons (a). eGFP labeled axons (arrows) are found in all motor neuron pathways (shown with NF-immunostaining).Motor neurons electroporated with T-cadherin and eGFP are found in all motor axon pathways (b). Spinal cord cross-sections of T-cadherin null mice with retrograde labeled motor columns (c). Axial muscle injections result in MMC cell body labeling. Dorsal limb injections result in LMC_l cell body labeling. Ventral limb injections result in LMC_m cell body labeling.

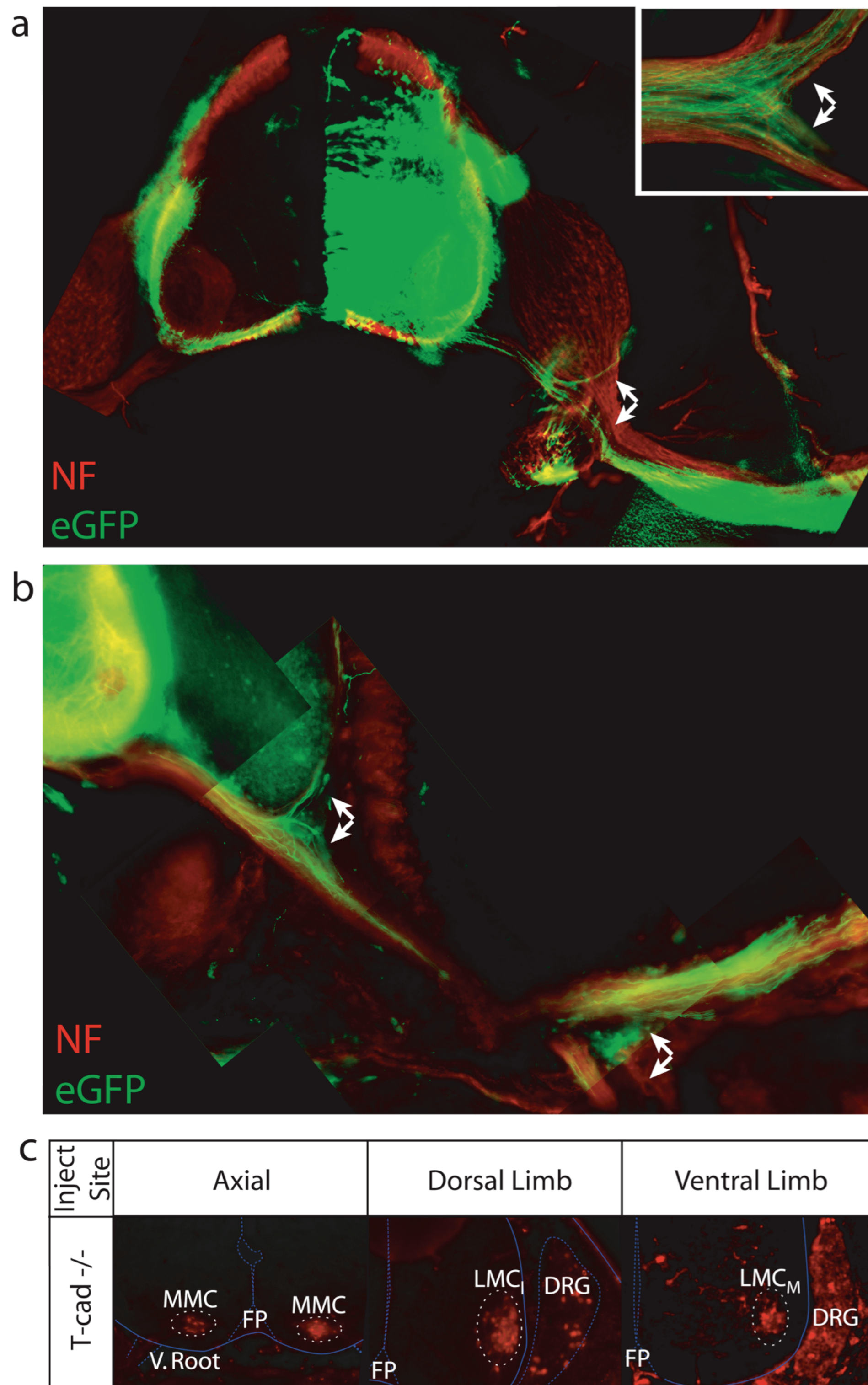


Figure 2-6: T-cadherin expression perturbation doesn't cause any gross motor axon pathfinding errors during growth through the limb.

Discussion

Axon guidance cues are essential for proper pathfinding of extending axons during embryonic development. Myriad cues act as serial step-wise traffic signals directing axons along stereotypic pathways to ultimately reach their targets, e.g., other neurons, glands, or muscle fibers. Numerous attractive and repulsive signals are integrated by axons along their pathways. We show here that T-cadherin is a repulsive cue for extending motor axons in vivo and in vitro. This role of T-cadherin makes it unique in two aspects. Other classical cadherins examined, such as N-cadherin, are attractive to axons, and other repulsive cues described, such as Ephrins, are comprised of a signal-receptor set, as opposed to the symmetrical homotypic T-cadherin interaction.

T-cadherin is a negative cue to neurons in vitro

Hallmark properties of repulsive cues are the abilities to inhibit neurite outgrowth and to collapse growth cones. We had shown previously that the presence of T-cadherin on substrates onto which T-cadherin expressing neurons were grown causes a reduction in neurite lengths as compared to control substrates. These previous studies correlated this effect with the presence of T-cadherin on the neuron surface. Neurite outgrowth inhibition was completely abrogated when neurons were cultured from embryos at a time when they have normally down-regulated T-cadherin expression. To confirm this finding in a more controlled system, we tested the ability

for T-cadherin substrates to inhibit neurite outgrowth of mouse spinal neurons explanted from wild-type or T-cadherin knockout mice of the same age. We revealed that wildtype neurons had 28% shorter neurites on T-cadherin vs. control substrates whereas knockout neurons showed no difference between the two substrates. This confirms the homotypic nature of T-cadherin-mediated repulsion. Additionally, motor axons of chicken ventral spinal cord explants show a higher proportion of collapsed growth cones when T-cadherin is applied to the media compared to controls. This finding implies that T-cadherin can function as a pathfinding cue by affecting the axonal cytoskeleton. Applied to the situation in vivo, a discrete point-interaction of T-cadherin on the growth cone and with a T-cadherin substrate would cause a localized cytoskeletal collapse, and result in the turning away of the growth cone from the T-cadherin substrate.

One obvious problem for a homotypic repulsive molecule that is able to both bind in trans or in cis—as cadherins are generally thought to (Boggon et al., 2002)—is that such a molecule could short circuit and negatively signal in *cis*, leading to auto-repulsion on the axon. Constitutive and non-specific repulsion would prevent sensitive reactions to the axon's environment. This must not be the case, however, since as we have shown T-cadherin-positive axons are still able to respond to environmental, *trans*, T-cadherin. Segregation is one mechanism by which neurons have solved a similar problem when Ephrins and Eph receptors are both expressed by a single neuron. The ligand and receptor are prevented from signaling in *cis* by physical separation into distinct membrane domains (Marquardt et al., 2005). It

would be unlikely for every cadherin molecule on the cells surface to be physically separated in the same way. However, a similar effect would be seen if the efficiency of *cis* interactions was very low. So far, examinations of cadherin binding strength has been in solution where *cis* and *trans* dimerizations both appear possible, but these results may be different if the molecules are constrained to a planar arrangement, as in a plasma membrane. Furthermore, since T-cadherin does not have a cytoplasmic tail, a possible partner that provides for signal transduction may also provide steric hindrance preventing *cis*-interactions. Thus, inclusion of T-cadherin into a hetero-complex would serve to physically separate T-cadherin molecules.

T-cadherin is a repulsive pathfinding cue in vivo

After showing that T-cadherin has sufficient properties for a repulsive cue by general in vitro testing, we sought to examine whether T-cadherin would serve this role at a specific pathfinding decision point in an intact organism. We decided to take advantage of the chicken embryonic motor system to answer this question. We used in ovo electroporation to ectopically express T-cadheirn in specific tissues in the embryo. Motor axons avoided ectopic regions of T-cadherin in the anterior sclerotome where T-cadherin is normally not expressed and axons normally grow through. Conversely, electroporating ventral neural tubes with RNAi yielded down-regulation of T-cadherin expression in motor neurons. T-cadherin-negative axons were found growing through posterior sclerotomes, from where they were normally completely restricted.

Interestingly, only a very small proportion of RNAi electroporated axons were misrouted into the normally restrictive posterior sclerotome. There are three possible explanations for this result. First, RNAi knockdown inherently leads to inconsistent levels of gene silencing between cells. Thus, only a small proportion of motor neurons would be expected to have the highest levels of knockdown. If there is a threshold of T-cadherin expression above which axons are able to respond to T-cadherin repulsion, then only axons with levels below this threshold will misroute into the normally restrictive zone. Second, in ovo electroporation results in chimeric transfection. Since in a population of outgrowing neurons only some neurons are transfected whereas others remain wild type, it is possible that the repulsion signal received by wildtype neurons is able to direct the whole population. In fact, populations of motor axons are tightly fasciculated by the presence of cell adhesion molecules, such as NCAM and L1 (Landmesser et al., 1988) suggesting that the whole fascicle could function as a single unit. Thus, knocked down neurons although unable to be repelled by T-cadherin could "go with the flow" and end up pathfinding correctly anyway. These two experimental shortfalls resulting from electroporation of RNAi cannot be overcome by using the knockout mice to answer these questions. Although T-cadherin is expressed by motor neurons in the mouse embryo, the expression pattern of T-cadherin in the motor neuon pathway is different than in chicks and T-cadherin is not present in mouse sclerotomes.

In addition, T-cadherin is unlikely the only factor that regulates the specific growth through the anterior sclerotome. Likewise, Koblar et al. used function

blocking antibodies to prevent ephrin–B1 signaling and did not drastically perturb neuron pathfinding in the anterior sclerotome (2000). There could have been redundant signaling (T-cadherin and/or other) that compensated for the loss of ephrin signaling. Other possible repulsive cues present in the posterior sclerotome, such as F-spondin (Debby-Brafman et al., 1999; Tzarfati-Majar et al., 2001), PNA-binding molecules (Davies et al., 1990; Oakley and Tosney, 1991) and Versican (Landolt et al., 1995; Perissinotto et al., 2000). No evidence has been shown that any of these molecules are necessary and sufficient alone for spinal motor neuron segmental patterned outgrowth. Then, only rarely would T-cadherin knockdown alone be sufficient to overcome the redundant repulsive receptor mechanism.

It is unlikely that the pathfinding errors seen in our in ovo perturbations are merely the result of a general dysfunction of axonal growth caused by T-cadherin mis-regulation. If this were the case, we would expect motor neurons that constitutively overexpress T-cadherin or are null for T-cadherin would have pathfinding errors other than those seen during growth across the anterior sclerotome. However, when we examined motor neuron axon projections at later time points in either knockout mice, or motor neuron electroporated chicks, no gross differences were detected from the normal conditions. Importantly, it is beyond the scope of these experiments to detect fine pathfinding differences (e.g. individual motor pools to individual muscles) or minor fasciculation differences resulting from T-cadherin mis-expression. We are only able to conclude that axons do not require normal T-cadherin expression patterns to establish the motor neuron–hindlimb projection.

Axonal repulsion by adhesion molecules

Classification of T-cadherin as a cell adhesion molecule continues to be reasonable in light of its function as an axon repellant. In fact, T-cadherin's adhesive strength, although substantial, has been shown to be significantly less than that of other cadherins, such as N-cadherin (Vestal and Ranscht, 1992). Adhesion assays typically make use of heterologous cells expressing single cadherins to quantify the strength of adhesion or the magnitude of aggregation induced by expression of a given cadherin. These cells, which normally do not express cadherins, may lack the full compliment of transduction machinery to respond to T-cadherin interaction in a repulsive manner as primary motor neurons in culture do. Thus, in vitro adhesion experiments may measure initial T-cadherin–T-cadherin binding that in a physiological system would result in a signal transduction event and cytoskeleton rearrangement. Indeed, the recognition of a signal by a receptor must require contact formation between the two proteins. This interaction could be thought of as adhesion. Obviously, the initial bonds must be broken if the repulsion signal is to be effective in redirecting the incoming axon. ADAM10 is a protease that cleaves ephrin–A2 after its binding to EphA3 receptors thus allowing the axon to retreat due to the repulsion signal, rather than remain bound (Hattori et al., 2000). Another example of a mechanism by which a similar conflict can be resolved is the endocytosis of EphB-Ephrin–B complexes, thus terminating adhesion and allowing repulsion (Zimmer et al., 2003). There might, therefore, be an unknown analogous system for the

homophilic T-cadherin interaction to be interrupted after the repulsion signal is received by the axon to facilitate retreat. It will be interesting for future studies to elucidate how T-cadherin adhesion is dissociated to allow for repulsion, as well as what mechanism is downstream of activation of GPI-anchored T-cadherin to link it to the cytoskeletal changes that comprise growth cone collapse and axonal turning.

Materials and Methods

Chicken embryos

Embryos from fertilized White Leghorn chicken eggs (McIntyre Farms, Lakeside, CA) were incubated in a humidified forced-air incubator at 39° until the desired developmental stages. Stages were determined according to the criteria of Hamburger and Hamilton (Hamburger and Hamilton, 1951).

In Ovo expression vector and RNAi

T-cadherin and eGFP co-expressing bicistronic electroporation vector (pMES-Tcad) was created by inserting the full-length chicken T-cadherin cDNA (accession number M81779) XbaI/StuI fragment into empty pMES (gift from S Pfaff, Salk Institute, La Jolla, CA) opened with XbaI and SmaI. pMES drives transgene expression with a chicken β-actin promoter fused to CMV-IE enhancer, and the transgene is followed by an IRES-EGFP (Swartz et al., 2001b). The expression vector

pCAX-eGFP was used for control (β-actin/CMV-IE–driven eGFP alone) expression (Osumi and Inoue, 2001) (gift from C Krull, University of Michigan, Ann Arbor, MI).

T-cadherin specific RNAi was created following the protocol provided for Ambion MEGAscript transcription kit (Austin, TX) using Promega ribonucleotide triphosphates (Madison, WI). Transcription template with T7 priming sites was created using PCR mutagenesis with primers containing T7 primer and homologous sequence to T-cadherin cDNA at nucleotides 91-109 and 998-1010 (5'-UAAUACGACUCACUAUAGGGAGAGGUUCUGUUGCUUGCGUGUGC-3' and 5'-UAAUACGACUCACUAUAGGGAGAGGCGUUUCCAUUGUCUCACGA-3'). Double-stranded RNA was purified using Roche mini Quick Spin RNA columns after annealing.

Immunohistochemistry

Motor neuron axons were detected using indirect immunostaining using anti-neurofilament antibodies 3A10 for chicken (Developmental Studies Hybridoma Bank) and Tuj-1 (Covance Resesarch Products) for mouse. Posterior half sclerotomes were identified using Ephrin–B1 immunohistochemistry (gift from Elena Pasquale, Burnham Institute).

Targeted in ovo electroporation

Sclerotome electrepration was accomplished essentially as described for dermomyotome electroporation (Chen et al., 2004), except using reversed electrode

polarity. pMES-Tcad was injected at a concentration of 2.7μg/μl, pCAX was injected at 3.0μg/μl. Injections were performed at st. 13 into 6 of the 7 caudal-most somites.

Neural tubes were electroporated as previously described (Krull, 2004). For knockdown experiments, RNAi (350ng/μl final) was mixed with pCAX eGFP expression vector (460ng/μl) for injection and in ovo electroporation at st. 15. For constitutive overexpression of T-cadherin in motor neurons, pMES-Tcad or pCAX control were injected into neural tubes and electroporated at st. 18.

After electroporation, embryos were overlaid with 2ml L-15 and eggs were resealed using cellophane tape and replaced in incubator for further development until the indicated stages.

Ventral spinal cord explant cultures

Stage 23 embryos non-electroporated or previously electroporated with pCAX or RNAi/pCAX were removed from their eggs. Embryos were decapitated and sectioned just anterior to lower limb. Trunk sections were rinsed in dissection media (25%FBS, 21mM Glucose, 10mM HEPES, 100U/ml Penicillin (Gibco), 100μg/ml Streptomycin (Gibco), DME/F12 (Gibco)). Embryo trunks were treated with 1U/ml Dispase II (Roche Applied Science, Indianapolis, IN) in dissection media for 30 minutes at room temperature. Embryos were then dissected in ice cold dissection media. First, the epidermis and body wall were removed, then the notocord and somites were separated from the spinal cord. Finally, DRG and dura were removed from the spinal cord. Spinal cords were prepared into open-book configuration by

sectioning at the dorsal midline. The dorsal half of the spinal cords and the floorplate were removed, and the ventral halves were collected. Ventral spinal cords were rinsed in 2 changes of dissection media. These strips were then sectioned into small (approximately 100µm) pieces, which were triturated and washed in 2 changes of dissection media. 4-6 pieces were placed onto a glass coverslip coated with 200µg/ml poly-L-lysine and 20µg/ml laminin and overlayed with explant culture media (10%FBS, 21mM glucose, 22mM glutamine, 1x Insulin-Transferrin-Selenium (Gibco), 6ng/ml Progesterone (Sigma) 1.6µg/ml putrescine (Sigma), 100U/ml Penicillin, 100µg/ml Streptomycin, DME/F12).

Growth cone collapse assay

Chicken embryo ventral spinal cord explants were cultured for 52 hours before initiation of assay. Column-purified soluble his-and flag-tagged T-cadherin produced in HEK293 cells (final concentration 10µg/ml) or control buffer were added to explant culture medium (described above). Cultures were incubated for 45 minutes at 37° in test media. Then, most media was removed and explants were fixed in 500µl 4% PFA for 30 minutes at room temperature. Explants were washed with PBS and stained with 30 minute incubation at 37° in 2.5µg/ml DiI (Molecular Probes). Explants were destained with three 10 minute washes of PBS at 37° and coverslips bearing explants were mounted onto slides using DakoCytomation Fluorescent Mounting Medium (Carpenteria, CA). Photomicrographs were taken at 60x. Image files were blinded and growth cones were scored as fully spread (defined by full lamellipodia and filopodia),

partly collapsed (some persistent filopodia but collapsed lamellipodia) or fully collapsed. Results were analyzed with chi-squared test, n=3 experiments, 170 growth cones (control); 4 experiments, 127 growth cones (T-cad).

Spinal cord primary cultures and neurite outgrowth assay

Spinal neuron primary cultures were prepared from E14 mice. Spinal cords were removed from 4-6 mouse embryos and cells were dissociated in 30U/ml Papain for 45 minutes at 37°. Neurite outgrowth assays were performed essentially as described (Domeniconi et al., 2005) by depositing ~7.5 x10^4 dissociated neurons onto confluent monolayers of CHO cells in 8-well chamber slides (Nunc). Cultures were incubated for 21 hours at 37° in 50% DMEM/F-12 and 50% Neurobasal media (Invitrogen) supplemented with 2% horse serum, 1x B27, 1x N2 (Invitrogen), 100pg/ml GDNF , 10ng/ml CNTF, and 1ng/ml BDNF (Sigma). Cultures were then fixed for 30 minutes with 2% PFA. Neurons and neurites were observed using secondary immunohistochemistry with antibodies against Tuj-1 (Covance). 50 full-field images using a 40x objective were saved using identical exposure and gain settings. The lengths of 150-600 neurites were measured using ImageJ; identical contrast and brightness settings were used for each condition. Percent neurite reduction induced by T-cadherin expression by the substrate was calculated by dividing the difference between average length in ctrl and T-cadherin substrates by ctrl. Differences between wild-type and T-cadherin knockout neuronal explants were tested by student's t-test, n=3 independent experiments each. To determine the number

of T-cadherin positive neurons, some cultures were also immunostained using 1749 anti-T-cadherin primary antibody.

Image analysis and Colocalization Index calculation

Image J software (U. S. National Institutes of Health, Bethesda, MD) was used for image analysis. Single sclerotomes were digitally isolated by masking a trapezoidal region of EphrinB-1 negative sclerotome, less a 15μm lateral border. Confocal images (z-step 3μm) were background-subtracted and thresholded (intensity=50) and the number of pixels were counted for each channel (α-neurofiliment immunostaining and eGFP expression) and for colocalized pixels, calculated using Colocalization plug-in (Bourdoncle, 2004). Colocalization index was calculated as the number of colocalized pixels normalized for overall quantity of axons and efficiency of sclerotome transfection, defined by the geometric mean of pixel counts from the two individual channels.

Retrograde motor column labeling

Mouse E12.5 embryos were dissected and superfused during retrograde transport. Motor neurons innervating specific muscle groups were retrogradely labeled with rhodamine-conjugated dextran (Sigma) as previously described (Sharma et al., 2000).

A portion of this chapter is being prepared for publication as *T-cadherin is a homotypic repulsive axon pathfinding cue that directs segmented motor neuron outgrowth* by H VanSteenhouse and B Ranscht. The dissertation author is the primary investigator on this paper.

Chapter 3: Structure and function of T-cadherin adhesive domain

Introduction

Finding that T-cadherin has the function of a repulsing pathfinding cue in vitro and in vivo led us to turn toward the question of how T-cadherin's unique structural features are important to its unique function. The features that make T-cadherin stand-out as different compared to the rest of the classical cadherins are its lack of a cytoplasmic domain, absence of critical residues in EC1 required for homophilic binding of other cadherins, and the conspicuous presence of the pro-protein form expressed on cell surfaces. Examination of the function of T-cadherin in light of being GPI-anchored, and thus unable to signal directly into the cell will be taken up in Chapter 4. This chapter will examine the differences between T-cadherin and other cadherins in terms of their dimer binding interface, and between the pro- and mature forms of T-cadherin.

The exact structural mechanism for cadherin-mediated adhesion is undetermined. There are several models supported by data in the literature, and the matter is far from being settled (Troyanovsky, 2005). Many studies have identified different conserved regions in classical cadherins that are important for adhesion. Different studies have looked for and found varying sizes of conserved regions of different classical cadherins to be important, namely, several EC domains required for interdigitation (Chappuis-Flament et al., 2001; Zhu et al., 2003), EC1-2 (Perret et al.,

2002), the region flanking the HAV motif in EC1 (Shapiro et al., 1995), a tryptophan at position 2 (Tamura et al., 1998; Boggon et al., 2002), and a pair of alanine residues thought to construe a binding pocket for Trp^2 at positions 78 and 80 (Tamura et al., 1998). T-cadherin, however, does not share the conserved HAV motif or Trp^2 residue. Thus, the structure of T-cadherin that affords its conserved adhesive function while lacking conserved elements was of great interest. Fortunately, the laboratory of Lawrence Shapiro at Columbia University has recently solved the crystal structure of T-cadherin (personal communications). The structure solved for a T-cadherin dimer predicts a dimer interface unique amongst known classical cadherins, owing to T-cadherin's unique amino terminus that was indeed expected to prevent normal classical cadherin dimer formation. Our lab has collaborated with the Shapiro lab in order to integrate functional studies with the structural information obtained. The experiments in the first half of the chapter below describe our efforts toward confirming prediction made by the structural studies that amino acid residues at positions 14 and 140 form crucial elements of the dimer interface. To confirm these findings, the Shapiro lab made a series specific point mutations of T-cadherin that were predicted to interrupt this dimer formation. I used CHO cells transfected with and expressing these dimer interface T-cadherin mutants in a series of cellular functional assays in order to test the hypothesis put forth by the structural data.

Another unique aspect of T-cadherin's amino terminus (the region thought important for homophilic binding) is that the pro-domain remains in an unprocessed state to some extent even after being expressed on the cell surface. Previous studies

have shown that although E-cadherin was always fully processed by the time it was expressed on the cell surface, when experimentally expressed on the cell surface with its pro-domain intact it conferred no intercellular adhesion (Ozawa and Kemler, 1990). The experiments described in the second half of this chapter were undertaken in order to determine whether the pro-domain of T-cadherin acts in an analogous manner—as expected from structural data as well—to abrogate homophilic adhesion. Since the pro-domain containing form is normally expressed on the cell surface, such a finding could indicate that the expression is functionally relevant in the context of cells or tissues potentially as a form of regulation of adhesion.

Results

T-cadherin dimer interface mutants are expressed normally on the cell surface

Wild-type T-cadherin is expressed on the surface of the cell, attached to the plasma membrane via a GPI-anchor in vivo (Ranscht and Dours-Zimmermann, 1991). T-cadherin is also attached to the membrane of Chinese hampster ovary (CHO) cells via a GPI anchor that can be cleaved with phosphatidylinositol-specific phospholipase C (Vestal and Ranscht, 1992), making CHO cells a good heterologous cell line to model T-cadherin induced intercellular interactions. CHO cells do not normally express T-cadherin (Figure 1a). In order to use CHO cells transfected with T-cadherin dimer interface mutants as a model in functional studies, we first confirmed that the T-cadherin mutant molecules are normally expressed—targeted and attached to the

plasma membrane—in CHO cells. Four mutated T-cadherin molecules were used for this study. Mutations R14E and R14S were two different mutations designed to test the necessity of residue 14, and mutations R14E/D140S and R14S/D140S have the same first-site mutations as the previous two, respectively, along with a second-site mutation at residue 140. Cell lines stably transfected with plasmids encoding mutant T-cadherin were indirectly immunostained live in the presence of azide. T-cadherin immunostaining of all four mutant cell lines (Figure 3-1c-f) appeared identical to wild-type T-cadherin expressing cells (Figure 3-1b). Staining was located on the cell surface, as a ring around the cell in a single confocal optical section. The fluorescent signal was punctate in distribution, indicating that the mutant T-cadherin—like wildtype—is located in discrete lipid raft membrane domains due to the GPI anchoring.

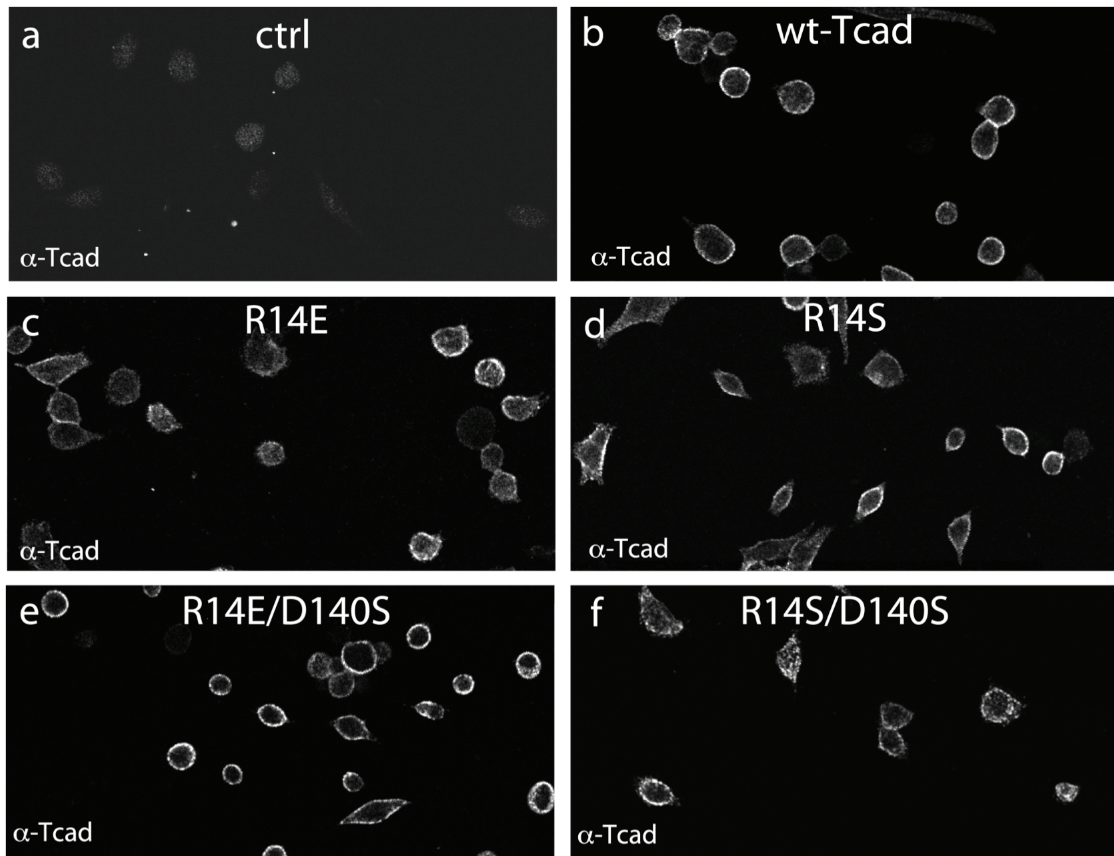


Figure 3-1: Surface expression of T-cadherin dimer interface mutants

Non-transfected CHO cells are not immunoreactive to T-cadherin anti-sera (a). CHO cell lines transfected with wildtype T-cadherin (b) or various mutant T-cadherins (c-f) exhibit T-cadherin immunoreactivity on the cell surface.

T-cadherin dimer interface mutants do not confer intercellular homophilic adhesion

The hallmark of in vitro cellular function of T-cadherin is that it confers homophilic intercellular adhesion between two cells expressing T-cadherin. We hypothesize this intercellular adhesion as being the summation of multiple individual dimer formations between two T-cadherin molecules oriented in *trans* on two opposing cell surfaces. Thus, the first two functional assays we subjected the T-cadherin dimer interface mutants to were designed to test homophilic aggregation and homophilic cell-substrate adhesion.

CHO cells are normally substrate attached, but can be dissociated into single cells by treatment with cell dissociation buffer and maintained in suspension in normal media for short periods of time. Thereafter, populations of dissociated CHO cells incubated in a revolving vessel can be used as a model for cellular aggregation. Non-transfected parental CHO cells express no cadherins, and thus exhibit very little background calcium dependent aggregation over the span of this assay (Figure 3-2a-b). CHO cells transfected with wild-type T-cadherin form large aggregates after 45 minutes (Figure 3-2c). Like other cadherins, T-cadherin requires the presence of calcium for adhesive function (Vestal and Ranscht, 1992). Thus, aggregation levels remain at background levels when T-cadherin adhesion is negated by supplementing the media with 1mM EDTA (Figure 3-2d). Unlike wildtype T-cadherin, both the single and the double T-cadherin point mutant cell lines showed near-background absence of aggregation in the presence of 5mM Ca^{2+} (Figure 3-2e-h) or 1mM EDTA

(not shown). These results establish that a single amino acid mutation at R14 is sufficient for abrogating T-cadherin-based cellular aggregation.

To quantify T-cadherin-mediated adhesive interactions, we measured the binding of radioactively labeled CHO cells in suspension to monolayers of the same cell type. Test cells were metabolically radiolabeled with S-35 and labeled with DiI and then dissociated. Test cells in low density suspension were then incubated with non-radioactive cellular monolayer substrata in the presence of 5mM Ca^{2+} or 1mM EDTA control for 30 minutes. Assays were visually monitored by DiI fluorescence, and quantitated using the adhesion index derived from radioactivity counts for each pair of substrate cells and test cells. Cells expressing wildtype T-cadherin show a significant 25.8% (±6.63) increase in adhesion over non-transfected control cells (Figure 3-2i, bar 2). Cells expressing wildtype T-cadherin do not significantly adhere to non-transfected cells (Figure 3-2i, bar 1) indicating the adhesion is due to cadherin homophilic binding. Dimer interface mutants R14E, R14S, R14E/D140S and R14S/D140S have adhesion indices that are not statistically different than non-transfected control (Figure 3-2i, bars 3-6). Thus, residue R14 is necessary for normal T-cadherin based homophilic adhesion.

Figure 3-2: T-cadherin mediated homophilic intercellular adhesion is abrogated when critical dimer interfaces residues are mutated

Homophilic aggregation of cells in suspension. T-cadherin expressing cell lines form large aggregates in the presence of Calcium (c), but not EDTA (d). Cell lines expressing dimer mutations of T-cadherin show minimal aggregation (e-h) similar to background levels in non-transfected cells (a-b). Summary quantification of cell-substrate adhesion assay (i). T-cadherin homophilic adhesion (bar 2) is statistically greater than control homophilic adhesion (defined as 100%) or T-cadherin to control cell heterophilic adhesion (bar 1). Homophilic adhesion of cells expressing dimer mutations of T-cadherin (bars 3- 6) are statistically equal to controls. N=3 for all, except 4 for T. (* $p<0.05$)

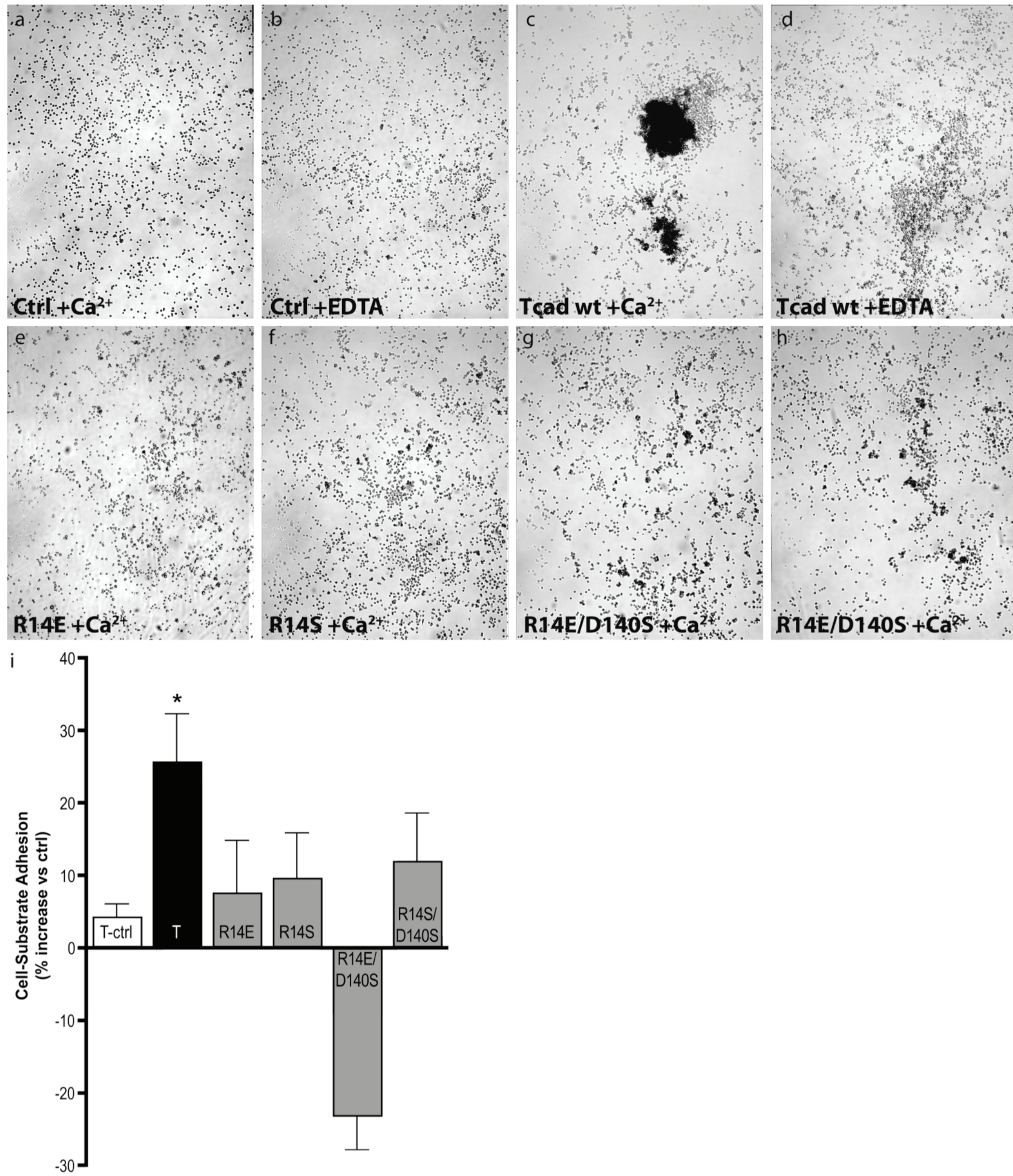


Figure 3-2: T-cadherin mediated homophilic intercellular adhesion is abrogated when critical dimer interfaces residues are mutated

T-cadherin dimer interface mutants do not inhibit the outgrowth of neurites in vitro

Having shown above in two different assays that T-cadherin dimer interface mutants lack the ability to confer intercellular adhesion, we then questioned whether these mutants can serve as a homotypic repulsive cue to neurite outgrowth as wildtype T-cadherin can. We thus cultured dissociated mouse embryonic spinal neurons on substrates composed of monolayers of CHO cells expressing either wildtype T-cadherin, one of the four dimer interface mutants described above, or non-transfected control cells. After 21 hours in vitro, we fixed and immunostained spinal neurons using Tuj-1 neurofilament antibody (Figure 3-3a-c). We measured and compared the length of Tuj-1–positive neurites in each condition. Spinal neurons explanted from wildtype mice showed the expected reduction of neurite length on wild-type T-cadherin substrates, but neurons grown on each dimer interface mutant substrate showed statistically identical neurite length to non-transfected control substrates (Figure 3-3d). Spinal neurons explanted from T-cadherin knock-out mice had statistically similar neurite lengths on all substrates. Thus, T-cadherin–mediated inhibition of neurite outgrowth requires homotypic T-cadherin *trans* dimer formation. Furthermore, this dimer cannot form without the critical residue R14.

Figure 3-3: T-cadherin dimerization is required for homotypic neurite outgrowth inhibition

Neurofiliment immunostaining of neurite outgrowth on various substrates (a-c). Wildtype mouse spinal neurons exhibit shorter neurites when grown on monolayers of CHO cells expressing T-cadherin (b) compared to control substratum (a). Neurite length of neurons grown on any mutant T-cadherin expressing substrata is not inhibited (exemplified by cell line expressing R14S/D140S in c), similar to controls. Quantification of neurite outgrowth assays (d). Wildtype but not knockout neurons exhibit inhibited neurite outgrowth on T-cadherin-positive compared to control substratum. Substrata expressing any of the dimer mutant T-cadherin do not inhibit neurite outgrowth of wildtype or knockout neurons. N=3 independent experiments. (* $p<0.01$)

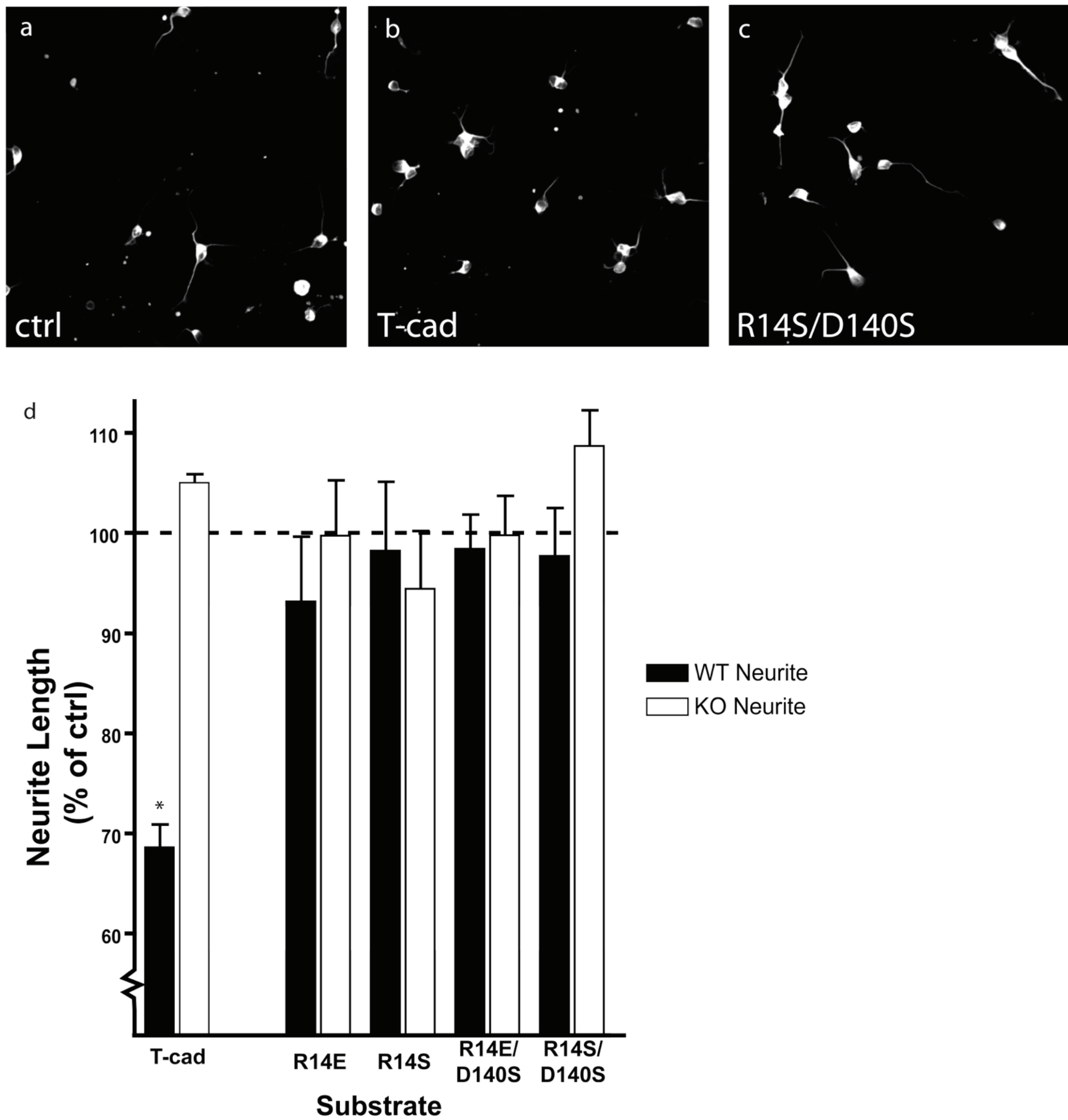


Figure 3-3: T-cadherin dimerization is required for homotypic neurite outgrowth inhibition

Pro-T-cadherin expressed on the cell surface is susceptible to convertase proteolytic cleavage

One unique feature of T-cadherin expression is that it is present in two forms on the cell surface. The two forms represent the effects of differential proteolytic processing. T-cadherin and all other classical family members of cadherins are initially transcribed as a pre-pro-protein. In the case of other, the pro-domain is removed by a proprotein covertase before the molecule is transported to the plasma membrane. Thus, only the shorter mature form of other cadherins is detectable at the surface of the cell. T-cadherin, on the other hand, is expressed on the surface of cells as two different sized species, corresponding to the unprocessed pro-protein and the mature processed form of the protein. These two differentially processed forms are seen to be present in different ratios on a variety of tissues in the developing organism (B.R., unpublished data). The two forms are also seen when T-cadherin is transfected and expressed in heterologous cells. Figure 3-4 is a Western blot of cellular lysates showing two bands corresponding to two different forms of membrane-bound T-cadherin present in transfected cells, but not in untransfected controls. Figure 3-5, lane 1 is a Western blot showing two bands corresponding to the two different forms of soluble flag-tagged T-cadherin expressed by HEK293 cells. T-cadherin primary sequence has an RQKR consensus cleavage site for the proprotein convertase, Furin, at the N-terminus of the first cadherin repeat (Denault and Leduc, 1996; Posthaus et al., 1998). Thus, it is expected that Furin is the convertase that produces mature T-cadherin from its pro-protein precursor. A majority of the T-cadherin is present as the

immature pro-protein size in the purified and concentrated supernatant of HEK293 cells transfected with the soluble T-cadherin expression vector. Pre-incubation of the soluble purified T-cadherin with increasing concentrations of recombinant Furin yields an increasing proportion mature T-cadherin and concomitantly decreasing proportion of pro-T-cadherin (Figure 3-5 lanes 2-7). Thus, Furin is able to process the immature proprotein fraction of T-cadherin expressed by cells.

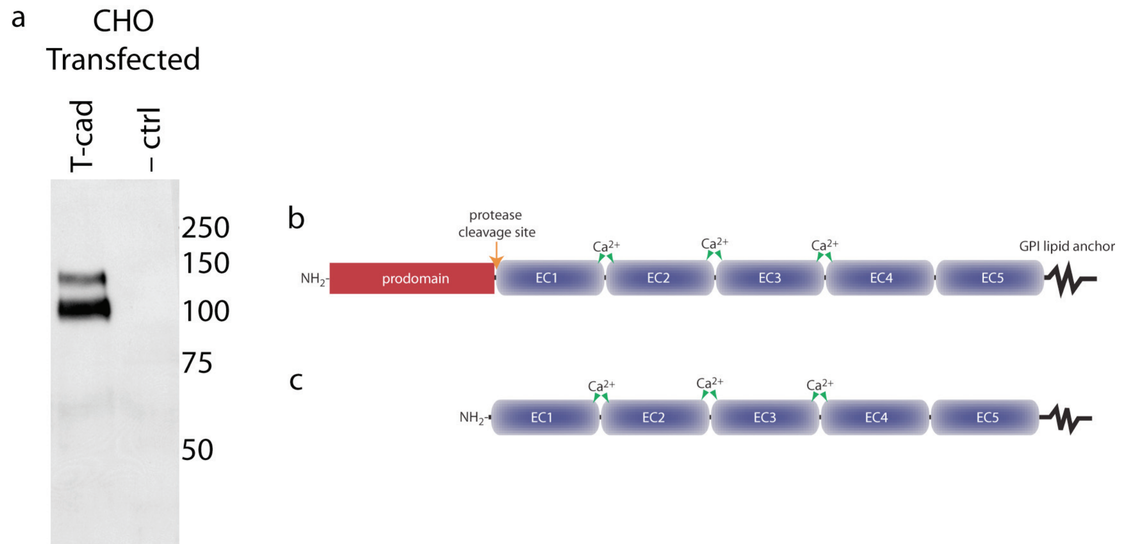


Figure 3-4: Western blot of T-cadherin.

CHO cells transfected with T-cadherin cDNA express two different sized species of T-cadherin (a, lane1). The approximately 130kD band corresponds to the unprocessed T-cadherin, schematized in (b) and the ~105kD band corresponds to the fully processed mature T-cadherin (c). Untransfected control CHO cells do not express any T-cadherin (a, lane2).

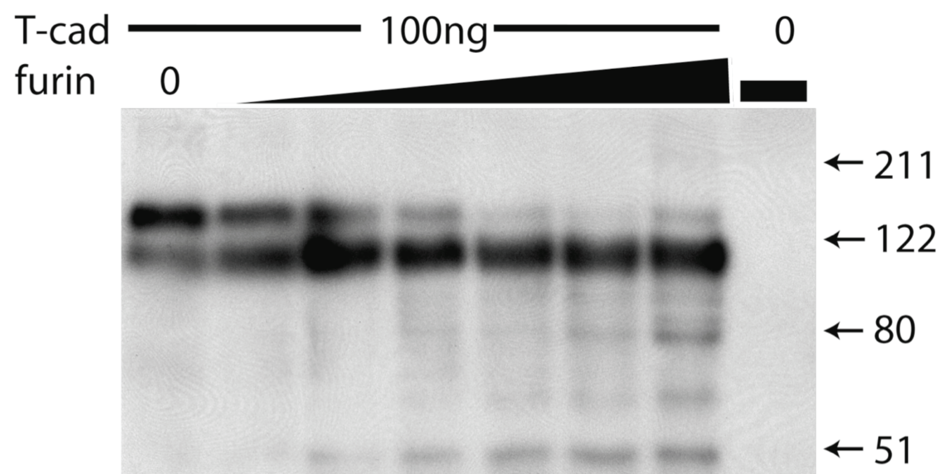


Figure 3-5: T-cadherin processing by the proprotein convertase Furin.

Purified Flag-tagged mouse T-cadherin expressed by transfected HEK293 cells is detected as two different sized species (lane 1) on SDS-PAGE western blot immunostained for T-cadherin using antibody 1810. Incubation of T-cadherin with Furin protease results in a dose-dependent increase in the ratio of mature form to immature pro-protein (lanes 2-7). There is no 1810 immunoreactivity when T-cadherin soluble protein is omitted from the furin reaction sample (lane 8).

Role of alternative proteolytic processing of T-cadherin for adhesivity and intermolecular interaction

The fact that the remaining pro-T-cadherin expressed by cells is susceptible to processing by a ubiquitous convertase, yet this "immature" form persists on the surface of cells expressing T-cadherin raises the question of whether the presence of the "immature" form serves some physiological role. One possibility is that the balance between the processed and the unprocessed forms regulates T-cadherin–induced adhesivity. The possible acute availability of an increased amount of mature T-cadherin quickly cleaved from of a pro-protein form may be important for the development of tissues. Developmental processes are known to regulated by dynamic intercellular adhesion, in part to allow for the rapid morphological changes and cell migrations that occur. One such example is NCAM, which is poly-sialyated early in development thus converting the adhesion molecule to an anti-adhesive form (Rutishauser and Landmesser, 1996; Fujimoto et al., 2001). Later in developmental, the proportion of PSA-NCAM decreases corresponding to a time when its physiological significance is thought to shift from plasticity to stability.

Comparison with earlier studies of E-cadherin adhesivity suggest the possibility that the pro-T-cadherin would not have the adhesive function of mature T-cadherin. When E-cadherin was mutated so that it would be expressed only an unprocessed pro-protein, it lost the ability to confer intercellular adhesion between cells expressing it (Ozawa and Kemler, 1990). Thus, it is plausible that pro-T-cadherin would have a similar non-adhesive function.

To determine the contribution to T-cadherin adhesivity of pro- and mature forms on the surface of the cell, I attempted an experiment analogous to the one Ozawa and Kemler used for studying E-cadherin. I thus created multiple point mutations in the T-cadherin cDNA to encode an uncleaved protein. This mutant T-cadherin (TcadXmut) has a substitution in the proprotein cleavage site (RQKR) that renders it uncleavable by endogenous proprotein convertases, such as furin. Instead, the replaced cleavage site IEGR is sensitive to cleavage by FactorXa, an activated form of a human blood clotting factor not present in the in vitro heterologous cell system used for aggregation assays. The resultant amino acid sequence of the cleaved form of TcadXmut is identical to that of wildtype cleaved T-cadherin. Transforming cells in culture with TcadXmut was expected to create a heterologous system in which intercellular adhesivity could be assayed while controlling the relative amounts of pro- and mature forms of T-cadherin by application of various concentrations of exogenous FactorXa.

TcadXmut could be expressed in cells by transient transfection. As expected, TcadXmut is present on the surface of transfected L929 cells, shown by punctate circumferential staining in live cell immunocytochemistry using anti-mouse T-cadherin antibody 1810 (Figure 3-6 b-c) in a similar pattern to cells transfected with wildtype T-cadherin (Figure 3-6 a).

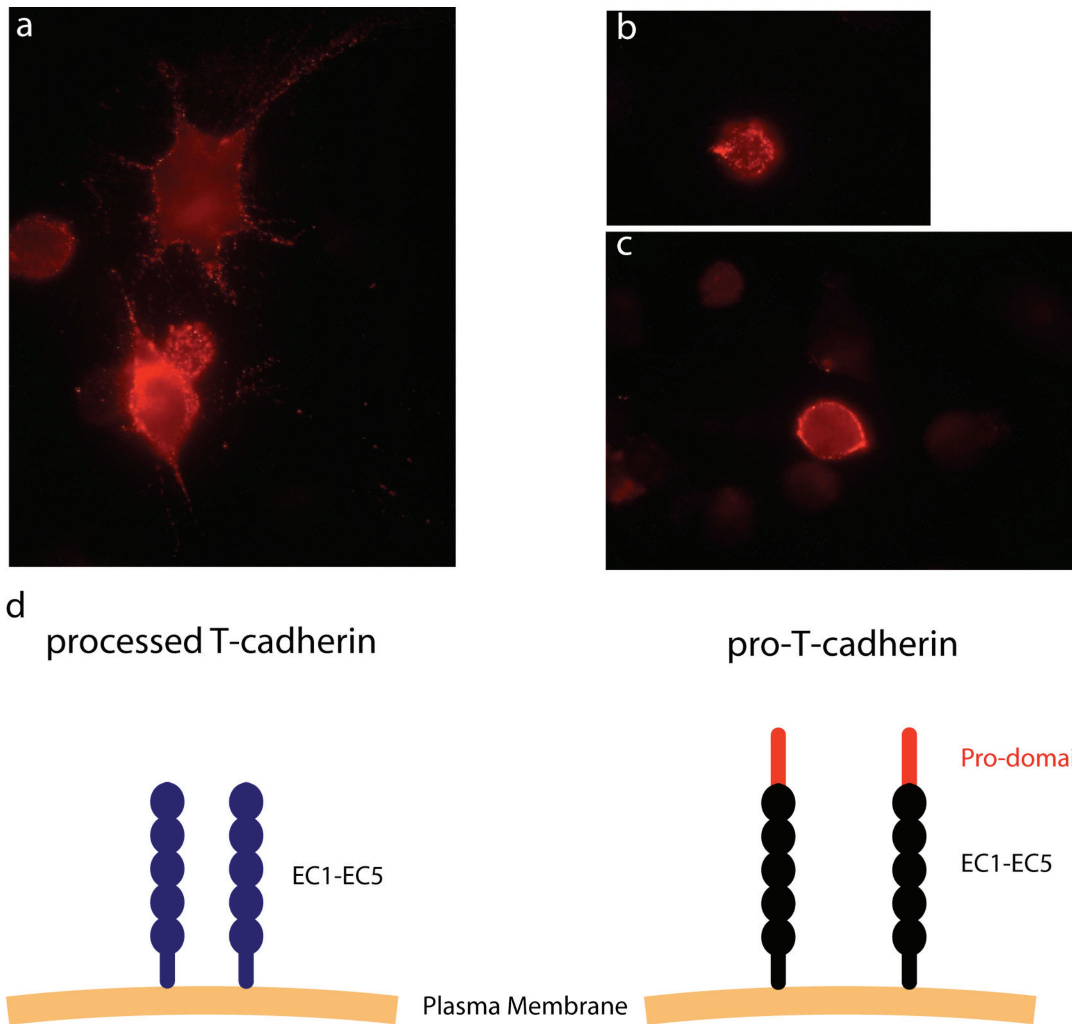


Figure 3-6: L929 cells transfected with wildtype T-cadherin or TcadXmut expression vector.

Cultured L929 cells transiently transfected with wildtype (a) or TcadXmut (b-c) expression vector are immunopositive for T-cadherin on the surface of some cells. Schematic diagram of processed and pro-domain-containing T-cadherin expressed on the surface of cells (d).

However, subsequent selection for stable TcadXmut-expressing cell lines was not possible. Expression of TcadXmut apparently results in dis-adhesion of such cells from the substrate. These dis-adhered cells are visible as non-adherent GFP+ cells suspended in the media when T-cadherin is co-transfected with a marker eGFP expression vector. When GFP is co-transfected with wildtype T-cadherin essentially zero green cells are found floating in the media, whereas when GFP is co-transfected with TcadXmut then approximately 5-10% of the cells floating in the media fluoresce green. This observed dis-adhesion due to TcadXmut expression may be either a result or a cause of cell death, making it impossible to cultivate TcadXmut expressing cells required for standard cell aggregation assays. Additionally, as a practical matter, it is also impossible to stably select for transfected cells of an adherant cell culture when those same transfected cells are selectively washed away in the media. As implied by the inability of TcadXmut expressing cells to remain adherent to a culture surface, I hypothesize that the TcadXmut expression confers a generalized anti-adhesive or non-adhesive property to the cells. This would indicate that the presence of pro-T-cadherin on cell surfaces could act in an opposing manner to adhesive fully processed T-cadherin, implying a possible regulatory mechanism. This experiment is not a conclusive test of this hypothesis, but it lends corroborative evidence to suggest that the same non-adhesive property of pro-E-cadherin is found with pro-T-cadherin.

The suggestion from the solved structure of T-cadherin that the extreme N-terminus of mature T-cadherin is located in an essential dimerization surface confirmed by abrogation of function resulting from amino-terminal point mutations

suggests a mechanism by which the presence of a pro-domain attached to the N-terminus would disrupt T-cadherin adhesion by sterically blocking dimerization.

Methods

Aggregation assay

Short term aggregation assays were performed essentially as described previously except incubation time was 45 minutes (Patel et al., 2006).

Cell-substrate adhesion assay

Substrate cells were grown to 90% confluency in 24-well plates. Probe cells were grown to 60% confluency in 6-well plates. Probe cells were cys/met-starved for 45 minutes then metabolically radiolabeled by incubation with 20µCi/ml Redivue Pro-mix L-[^{25}S] in vitro cell labeling mix (Amersham Biosciences) and 2.5µg/ml DiI (Molecular Probes) in cys/met-free media (Gibco) for 4 hours. Cells were then washed and dissociated in Enzyme-free Cell Dissociation Buffer (Gibco) and resuspended in 25mM HEPES buffered HBSS / 10µg/ml DNaseI / .01% BSA. 500µL of approximately 5 x 10^5 cells/ml probe cells suspension was added to substrate wells and allowed to settle for 10 minutes at 25°C. The assay was then started by addition of 5mM Ca^{2+} or 1mM EDTA to individual wells and cells were incubated at 37° for 30 minutes. Unbound cells were washed twice with .5ml HHBSS. The remaining cells were lysed and collected in .1%SDS/1N NaOH and counted in a scintillation counter (Beckman LS6500). Adhesion indices were calculated by subtracting EDTA control condition from Ca^{2+} condition and dividing by input probe cell radioactivity. Each condition for a given experiment was normalized to a non-transfected internal control.

Each condition was conducted in duplicate. N=3 independent experiments for each substrate-probe combination, except N=4 for T-cadherin. Statistical analyses consisted of one-way ANOVA and Dunnett's Multiple Comparison post-test, using Prism software (GraphPad, San Diego, CA). All graphs show mean ±SEM.

Spinal cord primary cultures and neurite outgrowth assay

Spinal neuron primary cultures were prepared from E14 wildtype (C57/BL6, Harlan–Sprague–Dawley, Indianapolis, IN) and T-cadherin knockout mice. Spinal cords were removed from 4-6 mouse embryos and cells were dissociated in 30U/ml Papain for 45 minutes at 37°. Neurite outgrowth assays were performed essentially as described (Domeniconi et al., 2005) by depositing ~7.5 x10^4 dissociated neurons onto confluent monolayers of CHO-FLP cells transfected with wildtype T-cadherin, or T-cadherin having single or double point mutations in 8-well chamber slides (Nunc). Cultures were incubated for 21 hours at 37° in 50% DMEM/F-12 and 50% Neurobasal media (Invitrogen) supplemented with 2% horse serum, 1x B27, 1x N2 (Invitrogen), 100pg/ml GDNF, 10ng/ml CNTF, and 1ng/ml BDNF (Sigma). Cultures were then fixed for 30 minutes with 2% PFA. Neurons and neurites were observed using immunohistochemistry with antibodies against Tuj-1 (Covance Research Products). Neurites were measured using ImageJ using identical contrast and brightness settings for each condition. N=3 independent experiments, 200-600 neurites were measured for each condition for each experiment. Statistical analyses of each neuron genotype consisted of one-way ANOVA and Tukey post-test, using Prism.

Discussion

The above experiments confirm that T-cadherin does make an unusual cadherin dimer interface, as suggested by structural studies. Further, this dimer interface is required for T-cadherin to confer intercellular adhesion and aggregation and also for T-cadherin's function as a negative cue to inhibit axonal outgrowth. In particular, a single residue, R14, is necessary for all of these functions in wildtype T-cadherin. These findings answer some basic questions about how T-cadherin functions as an adhesion molecule and pathfinding cue. This new understanding of T-cadherin structure allows for the further examination of the molecular function, as begun in the experiments described in Chapter 4. In particular, determination of the similarities and differences between T-cadherin and other classical cadherins allows for more thorough predictive models to be created and tested.

Experiments also showed that T-cadherin is expressed on the cell surface as both pro-T-cadherin and the mature protein. The pro-domain containing version is labile to proteolysis by the proprotein convertase Furin, suggesting that the pro-domain containing version is left unprocessed by some mechanism extrinsic to the molecule itself. It is unlikely to be protected by an alteration of the cleavage site structure or differential glycosylation that blocks proteolysis. Instead, the pro-form may be protected by the presence of another associated protein, or simply the result of incomplete processing.

In order to further study any potential functional role of pro-T-cadherin, I created and expressed a mutant form of pro-T-cadherin that cannot be processed by

the murine cells it is expressed in. Unfortunately, this experiment was unable to live up to expectations as cells over-expressing pro-T-cadherin only were not amenable to stable selection. However, this result was somewhat instructive. Unlike wildtype T-cadherin expressing cells, the pro-T-cadherin expressing cells were observed to detach from their culture substrates, indicating that the pro-form was functionally different than the mature form. And indeed, the pro-form seems to have a function in the cell. At least at the high levels of expression produced in this system, the pro-form appears to confirm anti-adhesion, or toxicity to cells.

A portion of this chapter describing the functional analysis of the T-cadherin dimer interface point mutations is being prepared for publication as part of *Untitled* by C Ciato, H VanSteenhouse, B Ranscht and L Shapiro. The dissertation author is the primary investigator on this portion of the collaborative paper.

Chapter 4: Functional interaction of T-cadherin with other cadherin family members

Introduction

Due to T-cadherin's unique structural features—such as the lack of conserved Trp residues in the first cadherin repeat critical for homophilic binding, and the lack of an intercellular domain—it is difficult to extrapolate T-cadherin's physiological function from other, more extensively studied cadherins. Since T-cadherin's function also seems to be unique amongst cadherins in that it is primarily repulsive, it is interesting to explore how T-cadherin's unique structural features may endow it with a unique function. I have examined one particular hypothesis of how T-cadherin functions at the cell surface, specifically the possibility that T-cadherin functions by interaction with and modulation of the function of other cadherins. Cells, such as motor neurons, express multiple cadherins. Thus, the question arises of how multiple cadherins interact. Preliminary results of T-cadherin crystal structure determination indicate that T-cadherin may be able to form dimers with other cadherins in a dissimilar yet mutually exclusive binding surface compared to typical homophilic cadherin EC1 domain strand swapping (L. Shapiro, personal communications). Thus, it is possible that T-cadherin is able to interact physically with other cadherins with an adhesion-like dimerization.

I have used a series of in vitro cell-based assays to examine this possibility of interactions. First, intercellular adhesion and aggregation can be measured by incubating a suspension of cells that express a given cadherin at 37° while shaking. The cells enter the assay as single units, but depending on the strength of their intercellular adhesive interactions, form increasingly larger aggregates. The level of intercellular adhesivity is measured by counting the decrement in total number of separable particles (either aggregates or single-cells) as well as the size of representative cell aggregates. Second, differential adhesion, as well as cell sorting and segregation can be detected with a similar assay by observing over time the relative locations of two labeled and mixed populations of cells in the resultant aggregates. Third, adhesion can be measured by quantifying the number of radio-labeled test cells that remain adhered to a substratum comprised of unlabeled cadherin-expressing cells after an incubation period followed by gentle washing. The test reagents in all cases are CHO cells stably expressing one of the various cadherins under study, or pairs of cadherins. The assays also take advantage of the property of cadherins being trypsin resistant in the presence of calcium. Thus, careful use of limiting concentrations of trypsin in the presence of Ca^{2+} yields detached and separated cells that have lost the majority of their non-cadherin intercellular adhesion molecules (those not calcium protected) but still have intact cadherins on the cell surface (Takeichi and Nakagawa, 1998). Cadherin-mediated adhesion is calcium dependant, so EDTA in the media as well as untransfected CHO cells that normally express no cadherin molecules can be used as negative controls to account for

background (non-cadherin induced) adhesion. Examining the effects of cadherin expression in these heterologous cells that normally do not express any cadherins is instructive to how T-cadherin may be functioning. Indeed, differences in the morphologies of cells resulting from various cadherins as well as alternative proteolysis of cadherins depending on the expression profile are observed and described in the following pages.

Many neurons in an organism, such as chicken or mouse, express multiple cadherins. As examples, adductor motor neurons express Cadherins MN, T, 8, and 6b whereas external femorotibialis motor neurons express Cadherins T, 8, and 6b (Price et al., 2002), and developing retinal ganglion cell axons express both T- and N-cadherin (Redies and Takeichi, 1993). The cadherin code hypothesis posits that this combinatorial pattern of expression of cadherins is instructive during the development of the embryo. Such a combinatorial code can yield many more theoretically discernable codes than if each character in the code were independent. Supporting this idea, in vitro, cells expressing both T- and N-cadherins segregate from cells expressing only N-cadherin (see below). Thus, cadherins have the power to specifically label cells destined to be neighbors in a simple system, but it is not fully understood how the combinatorial expression observed in an organism affects its development. To elucidate the function of combinatorial expression of T-cadherin and other cadherins, I have used a similar simplified in vitro model of intercellular adhesion. I began this inquiry by examining T-cadherin's possible interaction with N-cadherin because N-cadherin is an archetypal Type-I classical cadherin that has been

thoroughly studied, is expressed along with T-cadherin in the nervous system, and exhibits strong homophilic adhesion allowing for sensitive analyses of possible interactions.

Analysis of the assays when cadherins are combined on one cell surface can shed light on whether the interaction implied by the combinatorial code hypothesis exists, as opposed to simple independent actions of each cadherin. Independent action would yield properties resulting from a combination of cadherins of what would be expected from a simple average of the two cadherins' properties, or alternatively, the properties of the stronger cadherin would mask the weaker cadherin's properties. Any deviation from the expected results derived from these assumptions about independence is suggestive of an interaction. In the experiments described below, many significant deviations are observed from the expected properties given the assumption that N- and T-cadherins function fully independently, indicating that T- and N-cadherin interact when expressed together.

Role of T-cadherin expression in combinatorial modulation of other cadherins: CHO cell morphology

Figure 4-1 shows DIC micrographs of the cells expressing various cadherins used for these in vitro assays. Morphological observations of these normally bipolar CHO-DG44 cells expressing both T- and N-cadherins gave initial support to the hypothesis that T-cadherin interacts with (rather than behaves independently of) N-cadherin. Specifically, measurements made of the shape of such CHO cells indicate

that T-cadherin is a dominant negative to N-cadherin function. In order to compare the morphological shape of a population of individual isolated cells, the ratios of lengths of major axis to minor axis of single cells not touching other cells were used as a measure of cell morphology. This ratio decreases to 1.0 with increasing roundness or rotational symmetry and increases with increasing cellular linearity. Wildtype CHO cells have a mean axis ratio of 5.9 indicating that they are normally moderately oblong (Figure 4-1a). Cells expressing T-cadherin are significantly more round (Figure 4-1b) (mean ratio = 2.0, n>120 for each condition, p<0.001, ANOVA and Tukey post-test). Cells expressing N-cadherin are significantly more long and narrow (Figure 4-1c) (mean ratio= 7.4, p<0.05) than either untransfected control cells or T-cadherin expressing cells. Interestingly, when T- and N-cadherin are co-expressed in CHO cells (Figure 4-1d), the mean ratio is 3.0, closer to the roundness measured for T-cadherin expression alone than the value measured for any of the other cell lines. Importantly, the value of 3.0 is less than that of N-cadherin alone, and less than the average of values measured for the two cell lines expressing N-cadherin and T-cadherin alone (Figure 4-1e). As shown in Figure 4-4 by immunoblotting of the respective cellular lysates, the levels of expression of N- and T-cadherin are similar to each other, and expression levels of N-cadherin is even greater in the dual-expressing cells than either unitary-expressing cell lines. Thus, even with abundant N-cadherin expression, the morphology of dual-expressers is much closer to that of T-cadherin alone, than to N-cadherin alone or to the simple average of N-cadherin alone and T-cadherin alone cells. These data indicate that T-cadherin is effectively a “roundness factor” and that

N-cadherin is a “skinniness factor” in CHO cells. Futhermore, the results indicate that this effect of T-cadherin on cellular morphology is dominant to those of N-cadherin. The cell morphologies measured may be an indication of adhesive strengths or cytoskeletal rearrangement resulting from cadherin expression.

Following the suggestion that T-cadherin works in a dominant negative manner over N-cadherin in this assay of cell morphology, I explored the possibility that a similar dominant negative interaction occurs between T-cadherins and other cadherins in other assays that more directly measure the functional significance in question, intercellular adhesion.

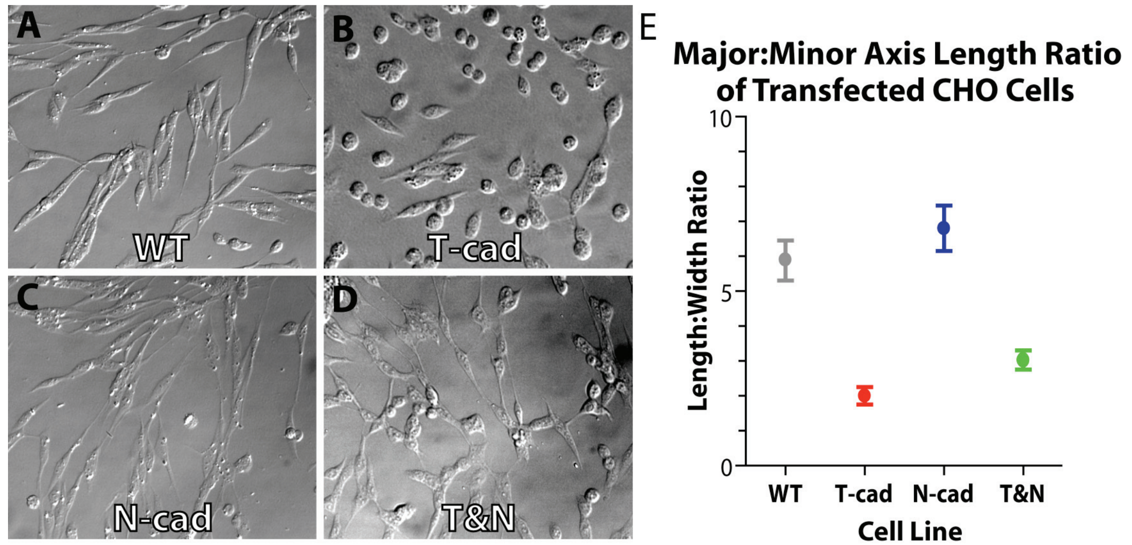


Figure 4-1: T-cadherin expression makes CHO cells rounder, and is dominant to elongation due to N-cadherin

Untransfected CHO cells are bipolar and moderately elongated (a). CHO cells expressing T-cadherin are primarily spheroid (b). CHO cells expressing N-cadherin are very elongated and narrow (c). CHO cells expressing N- and T-cadherin are predominantly spheroid (d), similar to (b).

Quantification and summary of morphological measurements (e) of CHO cells expressing T-cadherin, N-cadherin, or both T- and N-cadherins compared to untransfected controls. Y-axis is the average ratio of major axis length to minor axis length of at least 120 cells for each condition. Statistical analysis using one-way ANOVA and Tukey post-hoc test confirms that each condition yields statistically different shapes of cells ($p<0.05$).

One of the most striking structural differences between T-cadherin and the rest of the family of cadherins is its lack of an intracellular domain. This feature is, in fact, how T-cadherin was originally named—as Truncated Cadherin (Ranscht and Dours-Zimmermann, 1991). Other classical cadherins, such as N- and E-cadherin, possess a fairly conserved intracellular domain necessary for strong homophilic adhesion (Nagafuchi and Takeichi, 1988). GPI-anchored T-cadherin, on the other hand apparently has a weaker homophilic adhesion in heterologous cell assays than N-cadherin (Figure 4-2). And, as was shown in Chapter 2, T-cadherin is repulsive in nature to axons both in vitro and in vivo. Thus, it is possible that this particular structural difference contributes to the fact that T-cadherin is also functionally different from the other classical cadherins. Specifically, I hypothesize that T-cadherin can interact with other cadherins and thus act as an endogenous dominant-negative. In this model, T-cadherin would interact either in *cis* or *trans* with another cadherins' extracellular domains, and decrease strong *cis* or *trans* homo-dimerization and instead confer a repulsive signal. This hypothesis is first supported by the observation that T-cadherin acts in a dominant fashion to N-cadherin in terms of cellular morphology. It is likely that the cellular morphology differences seen above result from different levels of cell–substrate traction or even more direct effects on the cellular cytoskeleton depending on the particular cadherin expression in the cells. These morphological observations are at a gross cellular level, however, and more specific examination of molecular function is required to determine how T-cadherin interacts with N-cadherin. Intercellular adhesion conferred by cadherin expression, moreso than morphological

differences, are of interest to this study since it is the adhesive (or intercellular interaction) function that is most related to the question of how cadherins influence axonal pathfinding.

Role of T-cadherin expression in combinatorial modulation of other cadherins: adhesivity

Previous experiments in our lab had raised the possibility of interesting interactions between T-cadherin and N-cadherin adhesive function (B.R., personal communications) that I have examined closer, described below. In these studies I have primarily used the cell-substrate adhesion assay described in detail in Chapter 3 that allows for measurement of adhesivity between homophilic or heterophilic combinations of cellular substrates and probe cells in suspension.

It should be noted that during this assay probe cells are suspended in media at a very low density. The low density is important to minimize the aggregation of probe cells while they are in solution, and to instead measure their adhesion to the substrate. Pre-aggregation of the probe cells would exacerbate differences in adhesion measured in this assay and confound measurements of heterophilic adhesion with homophilic adhesion. Additionally, probe cell suspensions are applied and allowed to settle for 10 minutes in calcium free conditions so that cells are near the substrate when the assay begins with the addition of Ca^{2+} or EDTA. These assumed conditions were confirmed by using DiI-labeled cells in mock assays and observing nearly all cells that adhered to substrates were independent and not part of an aggregate.

This cell-substrate assay allows for the measurement of relative levels of adhesive interaction between various combinations of cadherins expressed on cell surfaces. It has already been shown in other aggregation and adhesion assays that cells expressing N-cadherin have strong levels of intercellular adhesion (Miyatani et al., 1989). Such N-cadherin cells form large aggregates when incubated in suspension with calcium (Figure 4-3). Our lab has shown that similar aggregation occurs with a population of cells expressing T-cadherin (Vestal and Ranscht, 1992). Unlike the nearly complete aggregation of N-cadherin cells, T-cadherin expressing cells form many smaller aggregates and still many cells are left unaggregated. This finding suggested that T-cadherin homophilic adhesion is weaker relative to homophilic N-cadherin adhesion. The substrate adhesion assay described here (Figure 4-2) confirms this inference. T-cadherin to T-cadherin adhesion has an adhesion index of 1.240 ± 0.0480, indicating that 24% more probe cells adhere to the substrate due to homophilic cadherin adhesion compared to untransfected controls and thus that the strength of adhesion is stronger than controls. The N-cadherin homophilic adhesion index is 1.600 ±0.140, significantly greater than either untransfected control or T-cadherin homophilic adhesion ($p<0.05$ ANOVA, Tukey post-hoc test).

Beyond confirming previous homophilic adhesion measurements, this cell-substrate adhesion assay is able to measure the heterophilic adhesion between two different cadherins. Aggregation experiments (described below) using two different populations of cells expressing different cadherins indicate that N- and T-cadherin have a strong primary propensity for homophilic adhesion and indicate that

heterophilic adhesion exists but is weaker than homophilic adhesion. There is relatively little intermixing of T- and N-cadherin expressing cells within an aggregate (Figure 4-3). However, in mixed aggregation assays, there is no way to discern whether heterophilic binding is weaker than both cadherin's homophilic binding (and by how much), or if one cadherin's homophilic binding is sufficiently strong to result in a low probability of heterophilic binding and thus complete segregation. To this point, the present cell-substrate adhesion measurements confirm that N-homophilic binding is significantly stronger than N-to-T heterophilic binding (adhesion index = 1.367 ±.0991). T-T homophilic binding is statistically similar to T-N heterophilic binding, but mean heterophilic binding tended to be slightly stronger than mean T-cadherin homophilic adhesion.

Given these baseline measurements of T- and N-cadherin homophilic and heterophilic adhesion strengths we were then in the position to ask how co-expression of T-and N-cadherins affected a cell's adhesive properties. Thus, we repeated the above assay using T- and N-cadherin co-expressing cells as the probe cells, and measured their adhesion index to substrates comprised of N-cadherin or T-cadherin solo-expressing cell monolayers. The dual-transfected cells express each cadherin at levels similar or slightly greater than in the respective solo-transfected cells (Figure 4-4). If both cadherins acted completely independently, we would expect the dual-expressing cells to adhere to each substrate with a strength equal to that of the respective solo-expressing cells. The presence of a unilateral additional cadherin would not (under the hypothesis of independence) influence the homophilic adhesion,

and as such would be irrelevant for this assay. This was not the result, however. The dual-expressing cells adhere to both N-cadherin and T-cadherin expressing substrates significantly less strongly than N-cadherin homophilic adhesion (adhesion indices = 1.265 ±0.0625 and 1.280 ±0.106, respectively). This finding that the co-expression of T-cadherin along with N-cadherin fully abrogates the strong N-cadherin homophilic binding and results in binding strengths equivalent to that of T-to-N heterophilic adhesion indicates that T-cadherin is dominant to N-cadherin. Thus, T-cadherin interacts with the function of N-cadherin present on the cell rather than functioning independently.

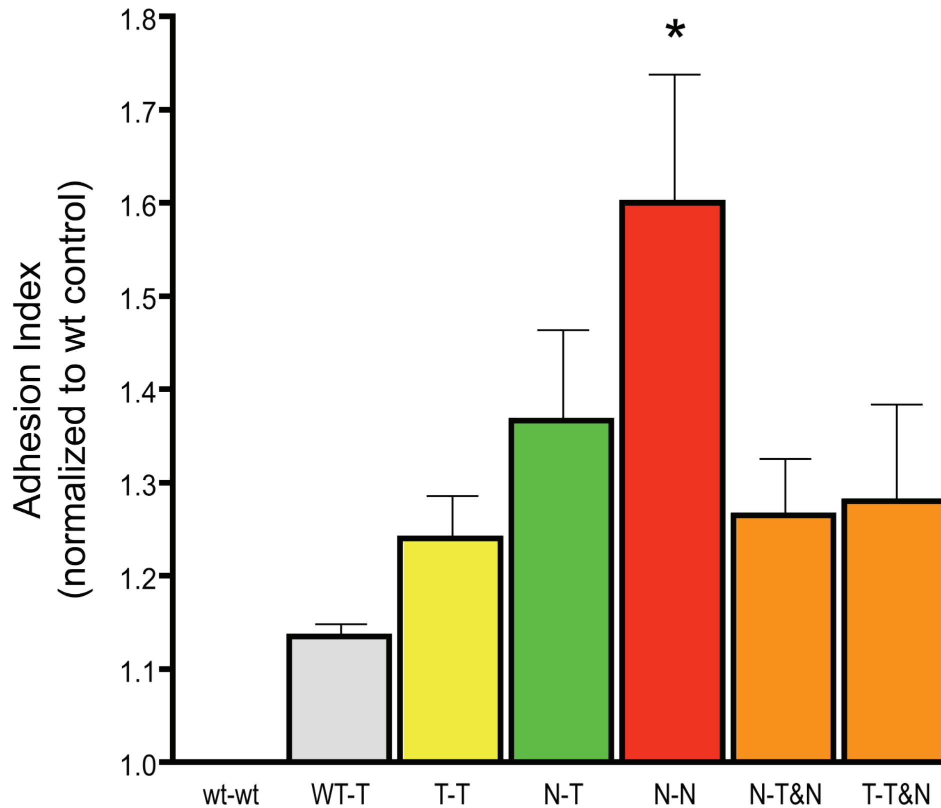


Figure 4-2: Summary results of cell-substrate adhesion assays between various combinations of T- and N-cadherin

Adhesion index values are calculated for each cell-substrate combination indicated along the X-axis by comparison to an internal untransfected control (wt-wt, bar 1). Bar 2 shows the background level of binding of T-cadherin cells to untransfected control cells. Bars 3 and 5 show the strength of T and N homophilic binding, respectively. N to T heterophilic binding is shown with bar 4. The strength of binding between N- and T-cadherin co-expressing cells to N- or T-cadherin expressing substrates is shown with bars 6 and 7. (N=2-4 for each condition *$p<0.01$ ANOVA, Dunnett's Multiple Comparison Test)

Role of T-cadherin expression in combinatorial modulation of other cadherins: Aggregation and Segregation

Like the cell-substrate assays above, in vitro aggregation is a simplified model system for observing the interaction of single cadherins and cadherin combinations. In such assays, cells expressing different cadherins are dissociated then mixed in a well at moderately low density. These cell populations are then allowed to aggregate. Revolving the whole assay provides for bringing the individual cells together at the center of a well, as well as gently mixing the suspension of cells giving individual cells the opportunity to adhere (or not) to a variety of other cells. Using only a single cell-type population in such an assay will give an indication of the strength of homophilic binding, since stronger binding will result in more cells remaining adhered to each other after physically coming into contact and thus larger aggregates will form (in a shorter amount of time). Mixing two different populations of cells will allow for determination of the differential aggregation (i.e. segregation) of like versus different cells. To determine the aggregation and segregation properties conferred on a cell by expression of various cadherins, we have used populations of CHO cells that differ only by the type of cadherin they express in aggregation and segregation assays.

N-cadherin is used in these adhesion assays as an archetypal strong homophilic type-I classical cadherin in order to examine and compare with the aggregation and segregation induced by T-cadherin expression, but we are also interested in how T-cadherin expressing cells interact with populations of Type-II cadherin expressing cells (such as MN-cadherin, cadherin-6b). This interest arises because aggregation or

segregation of cells expressing T-cadherin and Type-II cadherin has particular physiologic significance. Specific combinations of T-cadherin and Type-II cadherins are expressed in motor neurons in a pool-specific manner in the ventral spinal cord. The precise combination of cadherins expressed by individual motor neurons is essential for the proper segregation of motor pools. For example, Price and colleagues showed that neurons ectopically misexpressing MN-cadherin do not properly segregate (2002).

A long-term version of the standard aggregation assay (described in Chapter 3) is used as an in vitro surrogate for cellular segregation. After 18-30 hours of incubation after trypsinization, cells expressing a cadherin have formed aggregates whose size depends upon the magnitude of adhesivity. A population of N-cadherin expressing cells is nearly completely aggregated with a smooth perimeter, indicating a tight packing of cells, after 24 hours. Whereas a population of T-cadherin expressing cells is predominantly, but significantly more incompletely, aggregated—many loose cells remain unaggregated and the perimeter is loose and unorganized—compared to N-cadherin aggregates, indicating less tight compaction due to lower homophilic adhesive strength. Type-II cadherins exhibit even lower levels of aggregation, with many smaller aggregates resulting as opposed to one large aggregate, after even longer incubation times.

To measure the inherent cell-segregation conferred onto a population of cells due to cadherin expression, two populations of cells expressing different cadherins were labeled with fluorescent dyes and then mixed for the aggregation assay. The

long-term nature of this assay obviates the need for close control of the differential sensitivities to trypsin cleavage by different cadherins expressed on a single cell. As a control, when both populations express the same cadherin the distribution of labeled cells is completely randomly intermixed (Figure 4-3a). I have repeated assays previously published showing that Type-I cadherins (such as N-cadherin) segregate from Type-II cadherins (such as MN-cadherin and Cadherin-6b) but that Type-II cadherins do not segregate from each other (Patel et al., 2006). Interestingly, T-cadherin cells segregate both from Type-I and from Type-II cadherin expressing cells. When T-cadherin cells are mixed with N-cadherin cells, the two populations completely segregate, but then appear to form super-aggregates composed of aggregates of both types of cells (Figure 4-3b), similar to the case seen in mixtures of R- and N-cadherin expressing cells (Inuzuka et al., 1991). N-cadherin expressing cells are always at the core of the super-aggregates, surrounded by T-cadherin cells. This topology is likely to indicate the higher binding strength of N-cadherin compared to T-cadherin, in agreement with the adhesivity index measurements described above. Likewise, in agreement with the adhesivity measurements of N-to-T heterophilic adhesion, aggregates of T-cadherin cells are able to adhere to the cells exposed at the perimeter of N-cadherin aggregates, forming the hetero-super-aggregate seen. The segregation seen in mixtures of T-cadherin with Type-II expressing cells is less absolute than that seen with N-cadherin. In the case with Type-II cadherins, such as MN (Figure 4-3e) and 6b (Figure 4-3f), there are homogenous aggregates of each cadherin as well as some intermingling of the Type-II cadherin expressing cells in T-

cadherin aggregates. There is no large super-aggregate as is seen in combination with N-cadherin, suggesting relatively lower heterophilic binding strength of T-cadherin to Type-II cadherins compared to T-to-N binding.

To further examine the hypothesis raised above of T-cadherin function being dominant to N-cadherin adhesive function, aggregation assays were conducted using populations of cells co-expressing both T-cadherin and N-cadherin. If T- and N-cadherin functioned independently of each other, we would expect that expression of T-cadherin would not alter the ability of dual expressing cells to randomly intermix with N-cadherin solo-expressing cells since both populations of cells would have N-cadherin expression. Contrarily, T-cadherin co-expression with N-cadherin results in segregation of those cells from N-cadherin solo-expressing cells (Figure 4-3d). Segregation is also seen between dual expressing cells and T-cadherin solo-expressing cells (Figure 4-3c). Thus, rather than the second cadherin being an independent bystander in this assay, the two cadherins interact in combination yielding a cell type different from either cadherin alone. The resultant aggregates are not identical to those composed of mixtures of N and T solo-expressing cells, however. The individual units of the super-aggregates are smaller, possibly due to there being less of a difference in adhesion strength (as was measured above) between solo and dual expressing cells, both in terms of homophilic and heterophilic adhesion. This is consistent with the adhesion indices measured for the cell-types (Figure 4-2).

Another interesting observation is that resultant aggregates of dual-expressers with either solo-expresser are always oblong, as opposed to the nearly symmetrical

and spherical T-cadherin or N-cadherin homogenous aggregates. Additionally, the dual expressing cells are always at the poles of the oblong aggregates. The implications of this observation are unclear, but may be a result of how the super-aggregates form. The oblong super-aggregate may be created as a result of the relatively smaller building-block primary aggregates that coalesce to form the super-aggregate. The piece-wise construction may create a vertical cylindrical structure in the well, since larger building-block aggregates are forced more centrally in the well compared to unaggregated cells due to revolution of the cells. The relative strength (or weakness) of heterophilic adhesion compared to homophilic adhesion of the dual-expressing cells compared to solo-expressing cells may be the cause of the dual expressers being found at the poles, similar to the manner in which T-cadherin aggregates are adhered to the perimeter of the N- and T- cadherin expressing super-aggregates.

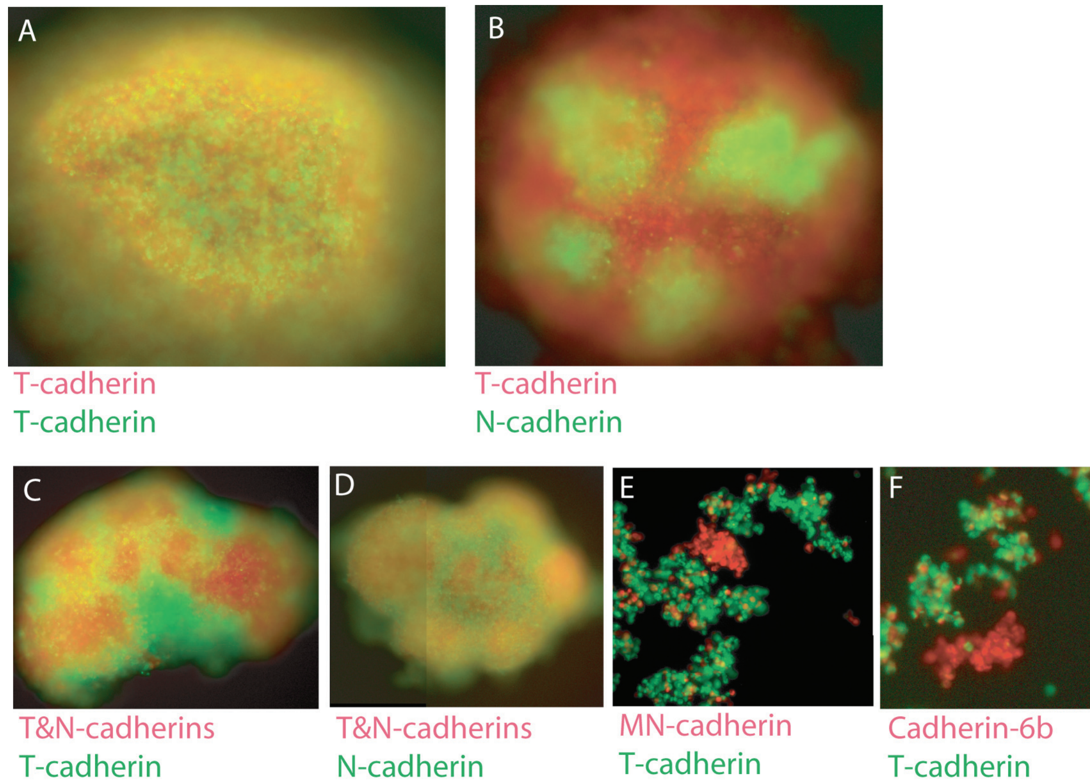


Figure 4-3: T-cadherin expressing cells segregate from both Type-I and Type-II cadherin expressing cells in vitro

Differently labeled T-cadherin cells randomly intermix (a). T-cadherin cells segregate from N-cadherin cells (b). T-cadherin cells segregate from Cadherin-6b cells (e). T-cadherin cells segregate from T- and N-cadherin coexpressing cells (c). N-cadherin cells moderately segregate from T- and N-cadherin coexpressing cells (d). T-cadherin cells segregate from MN-cadherin cells (f). N=3 independent experiments in quadruplicate for each condition.

Role of T-cadherin in the cleavage of N-cadherin

The above functional assays describing the adhesive role of T-cadherin alone or in combinatorial interaction with N-cadherin have raised the possibility that T-cadherin affects cellular adhesion by a dominant negative relationship with other cadherins, such as N-cadherin. A possible mechanism by which T-cadherin could act as a dominant negative in homophilic cadherin binding is suggested by the interesting observation originally made by Debora Vestal in the Ranscht lab (unpublished data) that on cells co-expressing T-cadherin approximately one third of the N-cadherin is present as a shorter, approximately 95kD, species as well as the expected 130kD species. This is in stark contrast to N-cadherin only expressing cells, the lysates of which have only one N-cadherin immunoreactive band at a size of 130kD (Figure 4-4, lanes 6-7). This fragment is also immunoreactive to antibodies recognizing the cytoplamic domain, indicating that it is the extracelluar amino terminus of the molecule that is cleaved and released. We hypothesize that an interaction between T-cadherin and N-cadherin results in proteolytic cleavage of N-cadherin. This interaction appears to be cell autonomous. If T-cadherin expressing cells are co-cultured with N-cadherin expressing cells, the N-cadherin is only detected as the full-length form. Thus, T-cadherin must be on the same cell membrane, not a juxtaposing membrane for the interaction to occur and result in cleavage of N-cadherin.

To further explore this hypothesis, I sought to determine the site of cleavage of N-cadherin by sequence identification of the product. N-cadherin was immunoprecipitated from the lysates of T- and N-cadherin co-expressing cells in order

to purify for the two N-cadherin species. As shown in Figure 4-5, the cleaved fragment is confirmed to be immunoprecipitated along with the full-length N-cadherin. In fact, the immuoprecipitation appears to enrich for the cleaved fragment in the final sample. This immunopurified sample was then fractionated with SDS-PAGE and transferred to a PVDF membrane. From the immobilized sample, the 95kD band representing the cleaved form of N-cadherin was isolated and submitted for Edman chemistry sequencing by the UCSD protein sequencing facility. Amino-terminus sequencing was chosen because the proteolytic cleavage of N-cadherin is expected to be at the N-terminus of the molecule, since antibodies raised against the C-terminus of N-cadherin also detect both full and cleaved species of N-cadherin (data not shown). Once the amino terminal residues remaining on the cleaved N-cadherin were identified, the intention was to use this cleavage site information to attempt to identify the protease, and to determine whether other cadherins may be susceptible to cleavage by the same proteolytic mechanism. Unfortunately, each of three sequencing attempts using this same procedure and increasing sample sizes were unable to yield any usable sequence information. Thus, it is likely that the cleaved N-cadherin in our sample is amino-terminal blocked and unavailable to react with the sequencing chemistry. Alternatively, the cleavage of N-cadherin may not be at one clean, discrete site. If there were many possible sites yielding many species with different amino terminal residues, then no one single residue would be detectable from the mixture at each sequencing cycle. If this were the case, the cleavage sites would have to be a relatively limited number all of which are located in a small region of the molecule, since the

western blot shows a single fairly well defined band for the cleavage product. In fact, although not definitive evidence for such a rough cleavage event, the HPLC traces for each cycle of sequencing showed many small peaks and no overwhelming primary signal. Because of the importance of elucidating the mechanism of N-cadherin cleavage by T-cadherin co-expression, finding alternative methods of determining the cleavage site continues to be an important goal. Even though this study was unable to determine the site of cleavage directed by the presence of T-cadherin, the existence of such a cleavage event dependent upon T-cadherin co-expression continues to be an important finding that may explain why the presence of T-cadherin is found to abrogate normal N-cadherin induced cell morphology (Figure 4-1), N-to-N-cadherin homophilic adhesion (Figure 4-2), and aggregation (Figure 4-3).

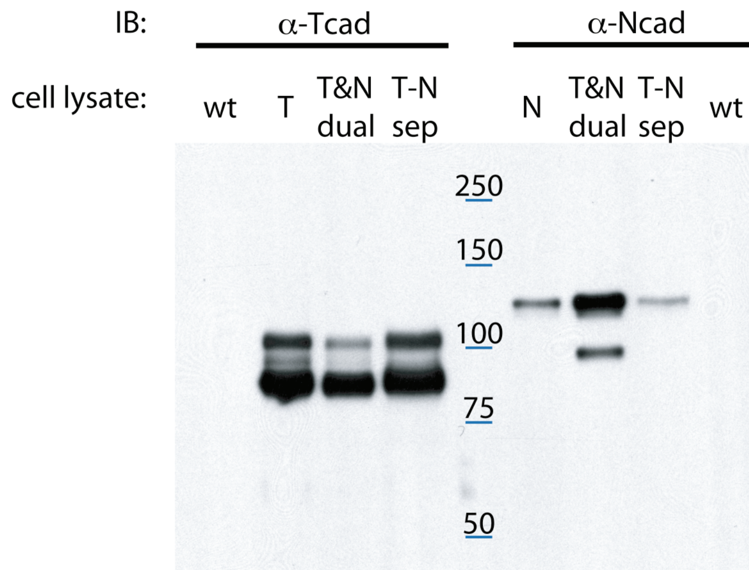


Figure 4-4: N-cadherin is present as a shorter form when co-expressed with T-cadherin in CHO cells

A western blot of lysates prepared from various cadherin-expressing and untransfected control CHO cell lines, detected for T-cadherin immunoreactivity (left) or N-cadherin immunoreactivity (right). T-cadherin is not expressed by wild-type CHO cells (Lane 1). T-cadherin immunoblotting is identical between lysates from cell lines expressing only T-cadherin (lane 2) or coexpressing T- and N-cadherin (lane 3) or in cultures of mixed populations each expressing T and N-cadherin alone (lane 4). Sizes are indicated by the molecular weight markers (lane 5). N-cadherin expressed alone is a single band species (lane 6), but when co-expressed with T-cadherin a smaller fragment of N-cadherin is also detected (lane 7). Lysates prepared from a mixed culture of T-cadherin and N-cadherin solo-expressing cell populations show only the normal N-cadherin single band (lane 8). N-cadherin is not expressed by wild-type CHO cells (lane 9).

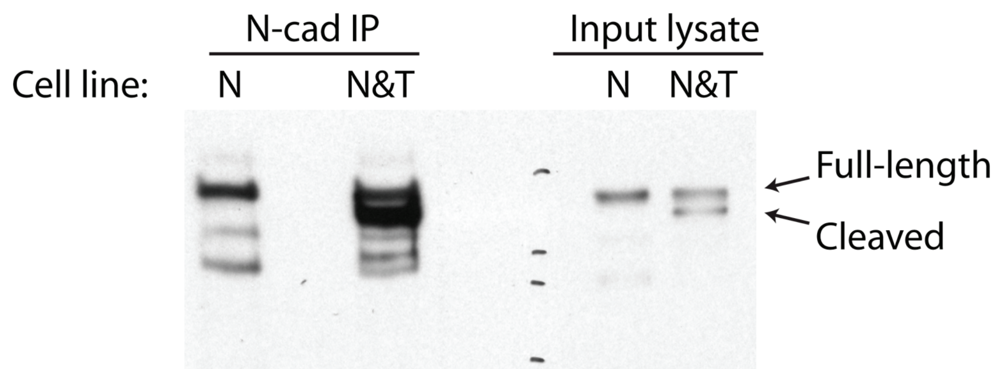


Figure 4-5: Western blot of N-cadherin immunoprecipitates

Full length and cleaved forms of N-cadherin are present in N-cadherin immuoprecipitates from N- and T-cadherin expressing CHO cells (lane 2). The N-cadherin banding patterns of immunoprecipatated samples (lanes 1-2) are similar to that of lysates that were the input to the immunoprecipitation (lanes 3-4).

Materials and Methods

Cell lines

T-cadherin and N-cadherin expressing cells are CHO (DG-44) transfected with pCDNA1 containing the respective cDNA. Cells were stable selected then sorted using flow cytometry. The top decile of expressing cells were used in this experiment. All cell lines were tested for continued expression by immunocytochemistry before experiments began. Type-II cadherin expressing cells were CHO-FLP cells, provided by S. Price.

Morphological measurements

CHO cells were grown on uncoated acid-washed glass coverslips for 48 hours. Coverslips of cells were fixed with 4% PFA for 30 minutes and mounted onto slides. DIC images were captured, and cell dimensions (in two dimensions) were measured using ImageJ software package.

Long-term aggregation assay

Nearly confluent cells were split 1:3 18 hours before the experiment. Populations of cells were labeled with 5μg/ml DiI or DiO (Molecular Probes) in 10%FCS/DMEM for 4 hours, followed by 3 washes for 10 minutes each with HBSS at 37°. Cells were released and dissociated by treatment of 0.05% Trypsin followed by trituration. Cell suspensions were spun down and resuspended at a concentration of 1

x 10^5 cells/ml in 70U DNAseI/10%FCS/DMEM. 500µl total labeled cells were placed in each well of a prepared 24-well ultra attachment low plate (Corning). Each condition was tested in quadruplicate in three independent experiments. Cell aggregation plates were placed on a revolving platform at 75RPM in a 37° 5% CO2 humidified incubator. Assays involving only T- and N- cadherins were incubated for 18 hours. Assays involving Type-II cadherins were incubated for 36 hours.

Cell-substrate adhesion assay

Performed essentially as described in Chapter 3. N=2-4 independent experiments conducted in triplicate for each condition.

N-cadherin fragment sequencing

Lysates were prepared from each CHO cell line described by solubilization of a confluent 10cm plate of cells with 1mL RIPA lysis buffer (150mM NaCl, 50mM Tris, 1%NP-40, .5% Deoxycholate, .1% SDS) on ice for 20 minutes after washing with PBS. After centrifugation, lysate supernatants were collected and run on SDS-PAGE or pre-cleared with ProteinG-sepharose beads and immunoprecipiated with NCD2 antibody (Developmental Studies Hybridoma Bank) conjugated proteinG-sepaharose beads overnight at 4°. IP samples were heated to 75° in SDS-PAGE sample buffer for 15 minutes and electrophoresed, followed by transfer to Problott (Applied Biosystems) membrane in CAPS transfer buffer for sequencing analysis.

Discussion

All four of the experimental approaches described above to measure the function of T-cadherin in combination with other cadherins reach the same conclusion. The presence of T-cadherin in combination with N-cadherin abrogates N-cadherin's function as measured when expressed without T-cadherin. T-cadherin coexpression causes an abrogation of the elongated morphology of N-cadherin expressing CHO cells (Figure 4-1). T-cadherin coexpression causes an abrogation of the particularly strong N-N homophilic intercellular adhesion (Figure 4-2). T-cadherin coexpression causes an abrogation of random intermixing of two populations expressing N-cadherin and causes the co-expressing cell population to segregate from N-alone cells (Figure 4-3). T-cadherin coexpression causes abrogation of the exclusive expression of full-length 130kD N-cadherin and causes the cleavage of N-cadherin (Figure 4-4).

Although all of the in vitro cell-based assays are slightly different in what aspects of T-cadherin function they measure, they all allow for the specific determination of some aspect of cell-cell adhesion and interaction as would occur within a complex organism. Using them in combination allows for a more complete picture of T-cadherin function both alone and in combination with other cadherins while still using a simplified system where carefully controlled experimental measurements can be made.

It is very exciting in this case to have clues as to the molecular event that is underpinning the functional interaction between molecules measured in terms of intercellular adhesion. The induced cleavage of N-cadherin only in the presence of coexpressed T-cadherin is consistent with and therefore adds strength to the conclusion from the adhesion assays that T-cadherin acts as a dominant negative. These two sets of findings together allow us to expand our hypothesis to suggest that the dominant negative action of T-cadherin function over N-cadherin function is due, at least in part, to the physical interaction (direct or indirect) of T-cadherin and N-cadherin which has the ultimate result of the cleavage and removal of the N-terminus of N-cadherin. Studies have shown that N-cadherin like other cadherins owe much of their homophilic adhesion function to the N-terminal region. For example, N-cadherin homophilic adhesion can be completely abrogated by single point mutations at amino acid residues 2, 78 or 80 (Tamura et al., 1998). It has also been shown for the very similar C-cadherin that removal of the EC1 and EC2 domains completely abrogates adhesion (Chappuis-Flament et al., 2001). Thus, such shortened N-cadherin could still be attached to its normal intercellular signaling machinery, but no signals could be received through N-cadherin. The finding that N-cadherin remains on the cell and therefore may still be occupying intercellular signaling molecules suggests that N-cadherin based signaling could be inhibited for relatively long-term by this interaction, since even newly expressed full length N-cadherin that would be able to receive an intercellular signal is unable to transduce this signal since the downstream players are occupied.

Another important finding from the above aggregation assays is that T-cadherin expressing cells segregate from cells expressing all three other cadherins examined here, including Type-I N-cadherin, Type-II cadherins MN and 6b. This in vitro finding suggests that the in vivo findings of Price, et. al. that mis-segregation caused by misexpression of MN-cadherin may be an example of a widespread mechanism for instructing cell migration and proper settling position of motor neurons. Thus, the presence or absence of T-cadherin as well as a number of other possible Type-II cadherins on a given motor neuron may collectively contribute to a combinatorial cadherin coding system for the proper sorting out and segregation of specific groups of motor neurons, termed pools. Thus, neurons of a given motor pool, which are functionally defined by all innervating the same single muscle could be genetically defined by their expression of a particular set of cadherins. Simply by virtue of their code expression profile and the inherent adhesion and segregation functions conferred by the cadherins' expression cells could be marked for co-localization in a given pool very early on in development.

Chapter 5: Concluding Remarks

Summary

The experiments described in this dissertation have been undertaken to answer two broad questions:

(1) Is T-cadherin a repulsive axon pathfinding cue?

(2) How does T-cadherin, an adhesion molecule, confer a repulsive signal to neuronal growth cones?

The results of the described experiments indicate that T-cadherin is a repulsive signal to motor axons growing into their typical segmental pattern of ventral roots. First, T-cadherin was shown to be a homotypic repulsive cue, causing growth cone collapse of motor axons in culture and inhibition of neurite outgrowth of axons that express T-cadherin, but not of those null for T-cadherin. As summarized in Figure 5-1, it was then shown that ectopic expression of T-cadherin in the rostral sclerotome creates an ectopic restrictive zone is created that motor axons avoid while growing through the normally T-cadherin-negative rostral sclerotome. Then it was shown that electroporating motor neurons with RNAi complimentary to T-cadherin resulted in decreased T-cadherin expression levels. Motor neurons with experimentally reduced T-cadherin expression in live embryos pathfind through the normally restrictive—T-cadherin-positive—caudal sclerotome.

Although T-cadherin is a cue that guides motor neurons in their growth across the first intermediate target tissue—the sclerotome—T-cadherin was shown to be a specific guidance cue, rather than having general effects on motor axon outgrowth. This was concluded after observing that T-cadherin does not grossly affect axonal growth at other timepoints and locations during motor neurons pathfinding into the the limb musculature. The loss of function experiment using T-cadherin knockout mice showed no difference in gross pathfinding of motor neurons from specific columns to specific muscle groups. The gain of function experiment in chicken embryos by electroporation of T-cadherin expression vector into motor neurons also showed no general pathfinding errors, as ectopically expressing motor neurons were able to grow into all possible major branches of the motor neuron pathway.

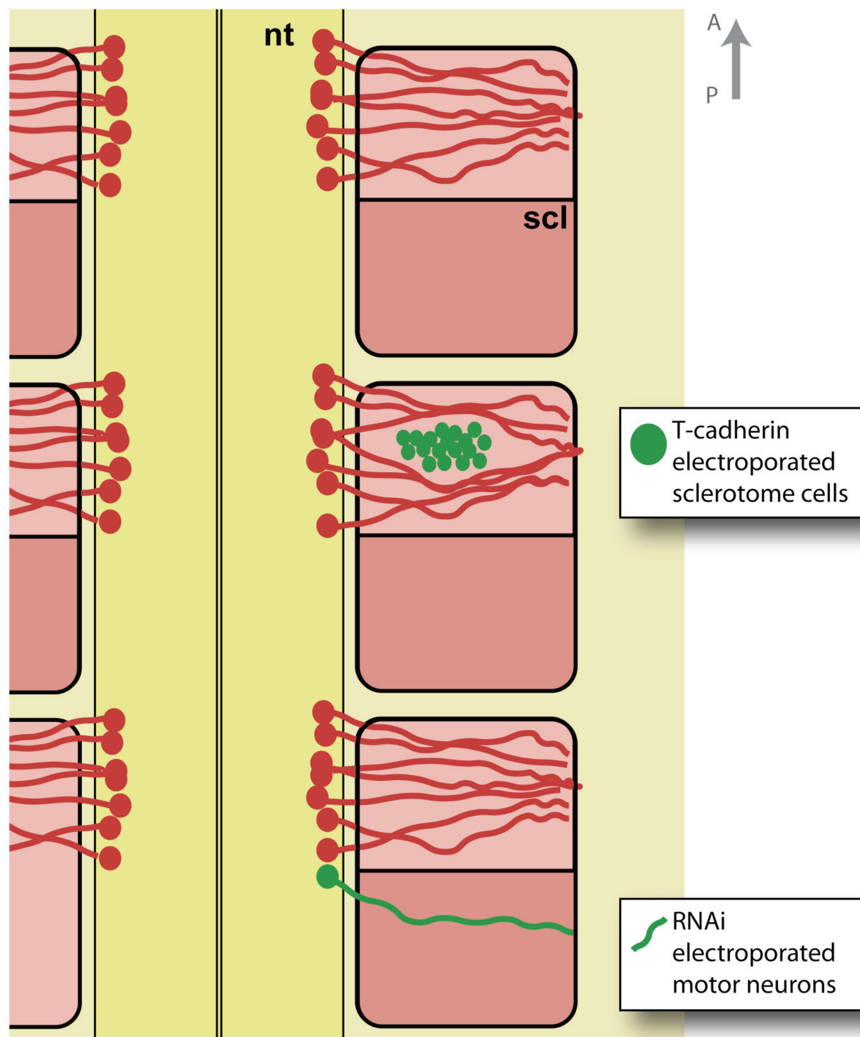


Figure 5-1: Summary of electroporation experiments results.

Schematic diagram of a horizontal section of chick embryo showing motor axons growing laterally out of midline neural tube (nt) though sclerotomes (scl) in normal or experimental conditions. Normally (top), motor axons (red lines) grow exclusively through anterior sclerotome (lighter half). However the segmental pattern of outgrowth is disrupted in gain-of-function (middle) and loss-of-function (bottom) experiments. When anterior sclerotome cells ectopically misexpress T-cadherin (green), these cells create a new zone in the anterior sclerotome avoided by motor neurons (middle). When motor neurons express lowered levels of T-cadherin resulting from RNAi electroporation, some such axons (green) are misrouted through the normally restrictive posterior sclerotome (bottom).

After seeing that T-cadherin is a repulsive pathfinding cue in the model case of chicken embryonic motor neuron development, several experiments were undertaken in an effort to understand how T-cadherin may be serving such a role. Several aspects of T-cadherin structure and adhesive function were thus examined.

The adhesive function of several mutated T-cadherin molecules were examined. The point mutations were expected to disrupt homophilic dimer formation, as discerned from the crystal structure determination completed by L. Shaprio's lab. Adhesion assays confirmed the structural implication that T-cadherin residues R14 and D140 are essential for normal homophilic T-cadherin dimerization and intercellular adhesion. Furthermore, these mutants do not exhibit the neurite outgrowth inhibiting effects of wildtype T-cadherin indicating that homophilic dimerization is essential to the pathfinding function as well.

In order to compare the adhesive function of "mature" T-cadherin, versus the "immature" pro-T-cadherin also expressed on the cell surface, a mutant form of T-cadherin was created that would not be cleaved to create the mature form in heterologous cells. Instructively, cells expressing such a mutant did not survive under conditions of long term selection, indicating that the pro-form of T-cadherin indeed has a different function than processed T-cadherin. Although the resultant cell death made further study difficult, it can be inferred that exclusive overexpression of the pro-T-cadherin interrupts normal cell-substrate adhesion or otherwise causes toxicity.

Since T-cadherin is expressed in the motor neuron in conjunction with other cadherins, one cadherin—N-cadherin—was expressed together with T-cadherin in

heterologous cells to observe possible cadherin crosstalk. Comparison of cells expressing T- and N-cadherin together with those expressing each cadherin alone indicates that T-cadherin interacts with N-cadherin in a dominant negative manner. To determine whether T-cadherin expression alters the adhesive function conferred by other cadherins, in vitro adhesion assays compared homophilic N-cadherin–mediated intercellular adhesion in the absence or presence of T-cadherin. These assays confirmed a dominant negative–like action of T-cadherin in N-cadherin expressing cells. Dual expressing cells segregated from N-cadherin only expressing cells, similar to cells expressing only T-cadherin. Dual expressing cells showed modest levels of cell-substrate adhesion, rather than the strong adhesion observed in N-N homophilic conditions. Moreover, very similar magnitudes of N-T heterophilic or T-T homophilic adhesion conditions were measured in the same assay.

T-cadherin was shown to form mild heterophilic adhesive interactions with N-cadherin in heterophilic cell-substrate assays and in long-term aggregation assays were T-cadherin expressing cells surrounded N-cadherin expressing cells. Besides a heterophilic binding mechanism with N-cadherin, T-cadherin could be interacting by inducing the proteolytic cleavage of co-expressed N-cadherin. Lysates from dual-expressing cells contain a substantial fraction of abnormally short N-cadherin.

In the chicken embryonic ventral spinal cord, T-cadherin is expressed in combination with Type-II cadherins in a motor pool specific manner, and specific cadherin combinatorial patterned expression is required for proper pool sorting. Aggregation assays with mixed populations of various cadherin-expressing cells were

used to determine whether the perturbation of pool sorting due to altering cadherin expression could result strictly from the biophysical adhesive properties of T-cadherin and Type-II cadherins. Results from these experiments confirm that T-cadherin expressing cells segregate away from Type-II expressing cells in vitro, mimicking the specific cadherin code–dependent segregation seen in the chicken spinal cord. Together with the finding that T-cadherin interacts with and induces proteolytic cleavage of other cadherins, this provides evidence that the combinatorial cadherin code affects axon guidance.

T-cadherin as a pathfinding cue: simultaneous "adhesion" and "repulsion"

T-cadherin as well as the rest of the cadherin family are classically known as intercellular adhesion molecules. This is a perfectly valid functional classification as shown in numerous studies, including this one. On the other hand, this classification may understate intracellular signaling as another major function of T-cadherin—and indeed other cadherins already studied as such, for example N-cadherin. It is easy to imagine how an adhesion molecule could have a contact attractant effect on neuronal passers-by. A neuron expressing a cadherin would come into contact with a substrate also expressing the same cadherin and the homophilic adhesion thus ensued would prevent the neuron from growing away from this substrate. As shown in the studies above, however, T-cadherin causes a repulsive response in growing neurites. The two functions of one molecule at one time being adhesive and repulsive are seemingly at odds. However, there is only contradiction if the adhesion is permanent. Instead, the

adhesion can be thought of more as a general intermolecular interaction. First, this interaction will cause both molecules to undergo conformational change. Second, this interaction requires physical contact between both molecules for a certain amount of time. Third, this interaction will occur with a certain kinetic rate between the on and off states. The above list of properties is general enough to describe any signaling molecule-receptor interaction, but also to describe the adhesive interactions between two cadherins. The fact that cells expressing cadherins “adhere” simply means that the intermolecular interaction is relatively long lived and the kinetics favor the on-state versus the off-state, compared to other more transient signaling interactions (Figure 5-2). T-cadherin has a lower strength of homophilic adhesion compared to N-cadherin. That implies that T-cadherin interactions favor the on-state less so than N-cadherin interactions. The existence of an on-state also allows for a signal to be passed, by virtue of the bimolecular conformation change, from one cell surface to another. And, the existence of an off-state allows for the “adhesion” to be broken and allows the cell to respond to the signal—in the case of T-cadherin by repulsion and avoidance.

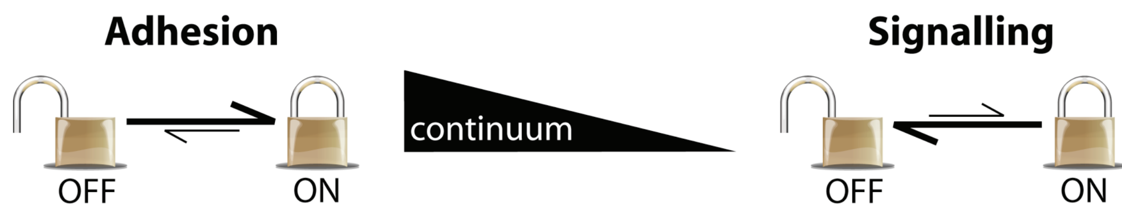


Figure 5-2: Schematic of continuum of kinetics between adhesive and signaling interactions

In this respect, T-cadherin homophilic adhesion is similar to ephrin-Eph receptor interactions. In vitro molecular assays show dimerization of ephrins to their congnate Eph receptors (Himanen et al., 2001; Himanen and Nikolov, 2003), whereas in vivo a repulsive response is usually elicited (Pasquale, 2005). In the case of EphA7, the adhesion and signaling functions can be physically separated. Expression of a splice variant of EphA7 that lacks the kinase domain required for signalling shifts the cellular response from repulsion as seen with full-length EphA7 expression to adhesion (Holmberg et al., 2000).

Additionally, it is important to interpret the results of cell-based functional experiments in the context of the time course of the cellular response being measured. For example, intercellular cadherin adhesion can induce nearly complete aggregation of tens of thousands of cells over a span of tens of minutes, whereas inhibition of neurite outgrowth occurs over a span of at least tens of hours.

Another important aspect of the interpretation of cadherin-based adhesion assays is the usage of simplifying in vitro model systems. The heterologous cells in which cadherins are transfected are by design not cells that normally express cadherins. Thus, there may not be the normal compliment of the signal interpretation machinery that a more naturally cadherin-expressing cell-type would have. The cells used to show an adhesive interaction of T-cadherin may not be *able* to exhibit a repulsive response. Indeed, this is an essential factor in order to allow for measurement of the strength of adhesion without confounding effects, but it should

not be taken as a strict example of physiological relevance of the molecules in question.

The question should not be whether T-cadherin (or any other adhesion molecule) is strictly a signaling molecule *or* an adhesion molecule; it can be and almost certainly is *both*. T-cadherin's function, however, needs to be interpreted in conjunction with the finding of possible interacting molecules, especially other cadherins. The questions should be how does the adhesion or signaling occur, and what is the relationship between the two functions?

T-cadherin interaction with other cadherin family members

The above findings in combination lead to one possible model of T-cadherin function via interactions with other cadherins on the same cell. Proteins that physically interact with the EC1 of E-cadherin have been found in cross-linking experiments (Troyanovsky, 2003). These interacting proteins have been hypothesized to modulate homophilic intercellular adhesion in a positive manner (Troyanovsky, 2005). T-cadherin could likewise be a negative modulator to homophilic adhesion by destabilizing homo-dimers.

The need for a complex intermolecular interaction in an explanation of how T-cadherin works arises from the finding that T-cadherin is expressed alongside other strong intercellular adhesion proteins, but is by itself a repulsive cue. Thus, on the surface of one cell, there is a conflict that must be resolved. Figure 5-3 (a) is a depiction of a growth cone expressing a variety of adhesion molecules (T- and N-

cadherin are shown in red and green, respectively) in contact with a substrate also expressing various adhesion molecules. In this model there is a static, stable situation where the growth cone adheres to the substrate. This could be an endpoint of growth for this axon; barring any signals instructing it to grow further. This could also be the point where the axon tip undergoes final differentiation and forms a synapse on the substrate (e.g. a muscle fiber). However, as panel (b) shows, when T-cadherin is active, this is not to be the final destination for this axon, but rather a midway pathway choice and signaling point. T-cadherin expressed in the environment is a repulsive signal, sending the axon away and toward its final destination. The T-cadherin on the substrate eventually interacts (forms a *trans* dimer) with the T-cadherin on the growth cone. This interaction sends a signal into the cell that causes repulsion. Now there is a case of contradiction between stable adhesion on the surface of the growth cone to the substrate and intracellular signal for a repulsive response. How can the growth cone cytoskeleton collapse while the membrane is adhered? The resolution could be a simple tug-of-war with the intercellular adhesion giving way eventually. Alternatively, as the data above suggest, there may be a more elegant resolution of controlled regulation. The finding that T-cadherin can bind to N-cadherin with at least equal strength to T-cadherin homophilic adhesion suggests that there could be a partner swap in the intercellular intermolecular interactions. As depicted in (c), the T-cadherin could interrupt the *trans* homophilic adhesive N-cadherin dimers by creating *cis* heterophilic dimers. The model put forth by L. Shapiro and colleagues suggests that *cis* and *trans* cadherin dimers are thermodynamically equivalent (Shapiro et al., 1995;

Boggon et al., 2002) making such a swap between *trans* and *cis* plausible. Thus, the intercellular adhesion would be broken and the cell cytoskeleton would be free to collapse locally at the site of the original T-cadherin repulsion signal. However, now of course the cadherins on the cell's surface are all bound up in cis heterophilic interactions. Axon pathfinding is a continuously ongoing contact-formation and signaling process and the growth cone will encounter a new substrate as it moves away from the current substrate. To enable the growth cone to convert back to a state of readiness to incoming signals, N-cadherin could be cleaved by a proteolytic event initiated by the presence of T-cadherin (d), as seen in CHO cell co-expression studies. As in panel (e) this cleavage would shear apart the heterotypic dimers, and the cell would be left with individual molecules on its cell surface. Indeed, a body of evidence is mouting that indicate metalloproteases operate with high specificity to regulate axon guidance (McFarlane, 2003). A metalloprotease is responsible for the cleavage of N-cadherin from retinal organotypic cultures, producing a soluble 90kD fragment that is functionally adhesive when experimentally susbstrate-bound (Roark et al., 1992; Paradies and Grunwald, 1993). However, this shed 90kD fragment is too large to be the shed fragment of N-cadherin due to T-cadherin coexpression seen in the present study. Additionally, two specific metalloproteases were found to cleave E-cadherin also releasing a soluble 80kD fragment with some retained function (Noe et al., 2001).

This model of cessation of an adhesive interaction by proteolytic removal of a functional domain—hypothesized in this dissertation to break N-cadherin homophilic dimers and allow repulsion—is highly analogous to the finding that hippocampal cells

could only withdraw from ephrinA-2 expressing cells if the metalloprotease ADAM10 could cleave the ectodomain of ephrinA-2 (Hattori et al., 2000). Without the proteolytic cleavage, growth cones collapsed but were left adhered to the repulsive substrate. Interesting, when probing the N-cadherin fragment seen in our assays with antibodies against the cytoplasimic domain of N-cadherin we found that N-cadherin remained reactive, suggesting cleavage releases the EC1/EC2 adhesive domains.

As depicted in (e), normal turnover would replace cleaved N-cadherin with fresh N-cadherin. Importantly, in this model, the T-cadherin remains on the surface after N-cadherin is removed from it, rather than the whole dimer being endocytosed for turnover. The repulsion by the substrate is mediated by T-cadherin, and this signal must remain to prevent the growth cone from returning in order to make a decisive turn away. Thus, it is important that the homotypic receptor, T-cadherin, remain present and vigilant on the growth cone.

Figure 5-3: Model of T-cadherin interaction with other cadherins

Schematic depicting a growth cone in a relatively stable situation of background adhesion to a substrate (a). A contradiction occurs when T-cadherin on the substrate signals repulsion to the growth cone while the growth cone is adhered to the same substrate (b). The contradiction is resolved by cis heterodimerization of T-cadherin with the adhesive cadherin (c) thus interrupting intercellular adhesion. The growth cone surface is returned to its original state by the proteolytic cleavage (d) of the adhesive molecule (N-cadherin in this case) and turnover of the resulting cleaved fragment (e). (See text above for full explanation).

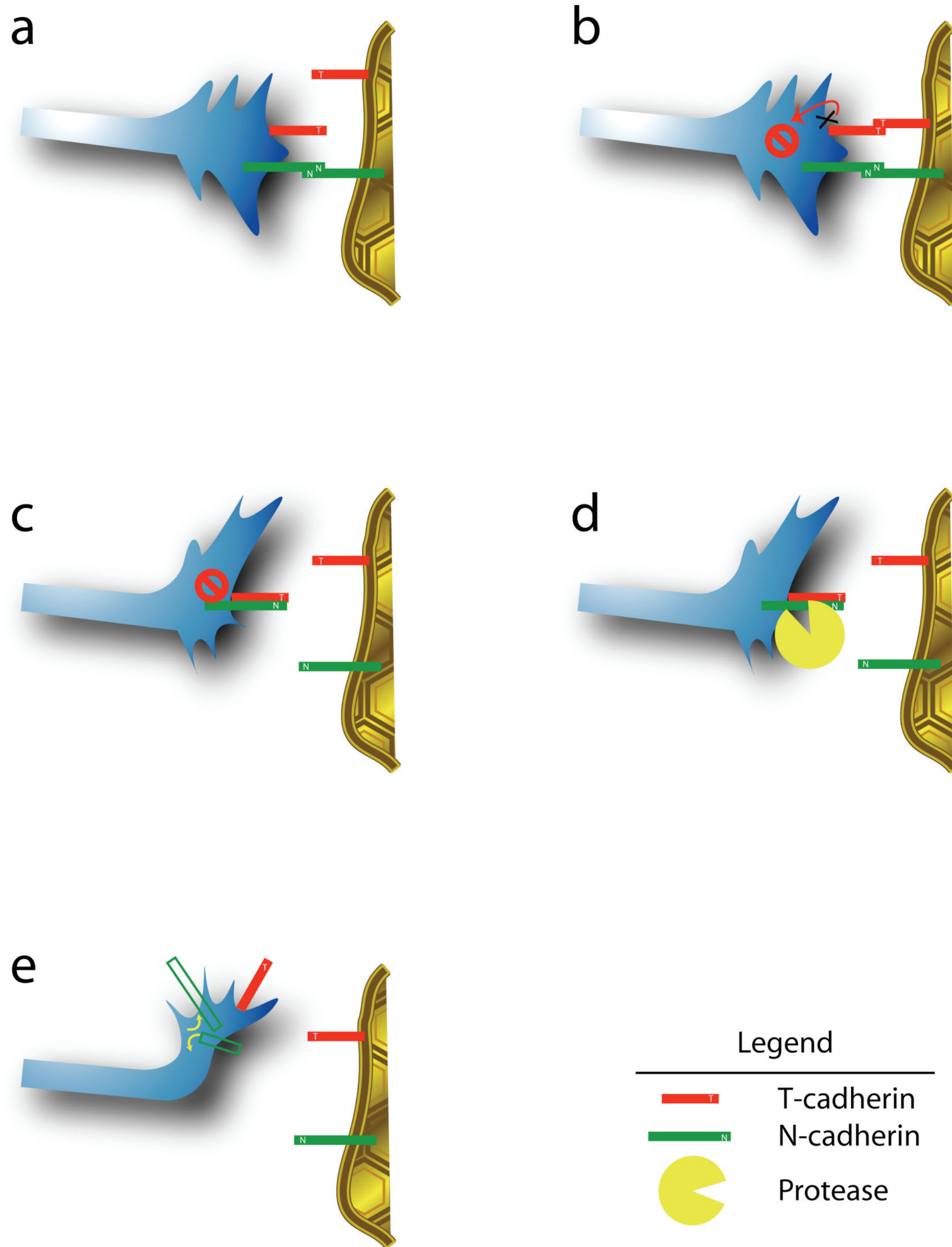


Figure 5-3: Model of T-cadherin interaction with other cadherins on a growth cone surface

Another layer of complexity is brought onto this model of T-cadherin function and interaction by recognizing that other cadherins—N-cadherin, for example—have signaling functions as well as adhesive functions (Doherty et al., 2000; Zhou and Snider, 2006). It has been shown in neuronal and non-neuronal systems that N-cadherin has signaling properties that activate ERK and PLCγ pathways resulting from an interaction of N-cadherin with FGF receptors (Saffell et al., 1997; Lom et al., 1998; Suyama et al., 2002). Specifically in primary neurons, Perron and Bixby found that N-cadherin substrates induced short-term ERK activation and membrane localization, which was required for normal N-cadherin–induced short-term induction of neurite outgrowth as well as longer-term extension of neurites (Perron and Bixby, 1999). Axonal outgrowth downstream of ERK activation may be due to a signaling pathway to the cytoskeleton via GSK-3 and MAP-1b (Del Rio et al., 2004; Goold and Gordon-Weeks, 2005). Combining the finding that T-cadherin inhibits N-cadherin homophilic binding with the pathway known for N-cadherin signaling in neurite outgrowth, yields the model in Figure 5-4.

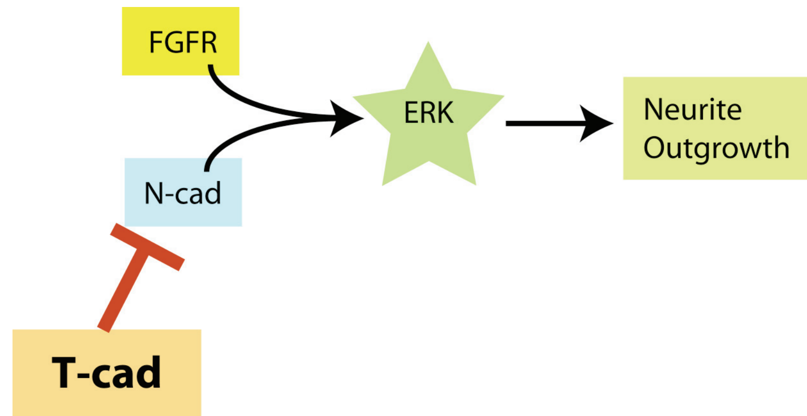


Figure 5-4: Possible pathway for T-cadherin to control N-cadherin induced neurite outgrowth

Superposition of T-cadherin's role as dominant negative regulator of N-cadherin onto the already known pathway downstream of N-cadherin homophilic binding leading to an increase in neurite outgrowth.

It is possible that a similar pathway leading to cytoskeletal rearrangements through ERK explains the lengthening effects of N-cadherin expression in CHO cells, compared to wildtype CHO cells and why T-cadherin blocks the N-cadherin–induced elongatated cell morphology, resulting in significantly rounder dual-transfected CHO cells (see Figure 4-1). Additionally, this is a potential model for how T-cadherin could function in vivo as an inhibitor of neurite outgrowth in neurons that express both T and N-cadherin. Since N-cadherin is only expressed on a subset of T-cadherin expressing neurons, and other cadherins are co-expressed with T-cadherin in various cell types, it is important to determine whether this model only applies to interactions with N-cadherin, or other cadherins as well. It remains to be seen how general this action of T-cadherin is in regulating the function of other cadherins. It is known that

the ERK pathway being downstream of N-cadherin is shared by other cell adhesion proteins, such as L1 and NCAM, but it is unknown how common this signaling pathway is in other cadherins.

Species differences in T-cadherin expression pattern

A major consideration in the interpretation of results as well as in the initial selection of a model system in which to study T-cadherin was that the expression pattern of T-cadherin which diverges between mouse versus chicken. Unlike the chicken sclerotome, T-cadherin is not compartmentalized in the mouse sclerotome by high-level expression in the posterior half and the absence in the anterior half. Expression patterns in motor neurons appear identical, however, in both species. The absence of a choice point for motor neurons between T-cadherin–positive and –negative zones in the sclerotome means that in mouse development, T-cadherin is not a likely cue to be guiding the pathfinding of motor neurons through the anterior sclerotome. That this particular system is not completely generalizable across species does not weaken the conclusions of T-cadherin function. In vitro assays are inherently more sensitive than studies in vivo where confounding and redundant cues are present. Thus, even if T-cadherin on mouse motor neurons is not observed to affect pathway choices, differential responses can be recognized in vitro. In fact, the *function* does appear generalizable across species, as shown by identical neurite outgrowth inhibiting responses of both mouse and chicken neurons in the presence of environmental T-cadherin. Thus, the analysis of chicken motor system development is used as a model

for T-cadherin function, rather than for motor system development across species. Although probably not applicable to mouse motor neuron growth through the sclerotome, anywhere in an organism where T-cadherin-positive neurons face a choice between T-cadherin-positive and T-cadherin-negative zones the finding that T-cadherin is a homophilic repulsive cue is likely to apply.

Interestingly, there are also differences in the expression between mouse and chick in the caudal sclerotome with respect to ephrins, another contact repellant axon guidance cue. Chicken posterior sclerotome shows specific expression of EphrinB-1 (Krull et al., 1997) whereas mouse posterior sclerotome has analogous specific expression of EphrinB-2 but not EphrinB-1 (Wang and Anderson, 1997). Thus, it is possible there may be an analogous interspecies difference for motor pathfinding cues as is found for repulsive cues directing neural crest migration. In this case, it is the functional activity that is conserved, not the specific molecule. Thus, another repulsive molecular cue (related or unrelated to T-cadherin) could be directing axonal guidance in the mouse sclerotome.

Implications for regeneration and neural repair

Many important scientific discoveries made in the research effort to improve recovery from damage to the nervous system have been in the areas of promoting neural growth by providing positive growth factors (Blesch et al., 2002) and by neutralizing inhibitory factors (Yiu and He, 2006) to repair lesions. Driving axon re-growth is certainly essential, but we must also recognize that unrestrained and

uncontrolled axon outgrowth is not the ultimate goal. Neural regeneration, if it is to reproduce original function of the nervous system, must couple growth promotion with growth control and organization and correct innervation. Herein lies the importance of understanding the control mechanisms directing highly ordered axonal outgrowth during the natural period of exuberant growth. A literature survey of 50 guidance factors and receptors found that none maintained their developmental-stage distribution into adulthood (Harel and Strittmatter, 2006). Such a major rearrangement of pathfinding cues makes it unlikely that axons would find their way back to their original targets after injury without any intervention. Constraining axons to ensure correct synaptic re-targeting will certainly require the concerted actions of many pathfinding cues. Elucidation of the roles of each pathfinding cue contributes a piece to the puzzle of functional recovery from nervous system injury. In this dissertation I have laid out roles for T-cadherin in neurite inhibition, axonal guidance, neuronal migration and interaction with other cadherins' activities. Hopefully, this improved understanding will contribute to the field of axon pathfinding in development and disease.

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