Neuron, Vol. 17, 979–990, November, 1996, Copyright ©1996 by Cell Press

Cadherin Function Is Required for Axon Outgrowth in Retinal Ganglion Cells In Vivo

**Rebecca Riehl,*§ Karl Johnson,* Roger Bradley,†
Gerald B. Grunwald,‡ Elsa Cornel,*
Alain Lilienbaum,*‖ and Christine E. Holt***
*Department of Biology
University of California at San Diego
La Jolla, California 92037-0366
†Molecular Neurobiology Laboratory
Salk Institute for Biological Sciences
P. O. Box 85800
San Diego, California 92138-9216
‡Department of Pathology, Anatomy, and Cell Biology
Thomas Jefferson University
Philadelphia, Pennsylvania 19107

Summary

The cell–cell adhesion molecule N-cadherin strongly promotes neurite outgrowth in cultured retinal neurons. To test whether cadherins regulate process outgrowth in retinal neurons in vivo, we have blocked cadherin function in single cells by expression of a dominant negative N-cadherin mutant. We report that when cadherin function is inhibited, axon and dendrite outgrowth are severely impaired, particularly in retinal ganglion cells. Laminar migration and cell type specification, by contrast, appear unaffected. Further, expression of the catenin-binding domain of N-cadherin, which blocks cadherin-mediated adhesion in early embryos, does not affect axon outgrowth, suggesting that outgrowth and adhesion are mediated by distinct regions of the cytoplasmic domain. These findings indicate that cadherins play an essential role in the initiation and extension of axons from retinal ganglion cells in vivo.

Introduction

A key step in the differentiation of a neuron is the genesis of an axon and dendrites. Not only must the appropriate number of these processes be generated, but also their growth and guidance must be finely regulated. Retinal ganglion cells (RGCs), for example, normally elaborate four or five dendrites and a single axon, which extends along a defined pathway to the optic tectum (Holt, 1989). Studies in vitro have shown that certain cell adhesion molecules stimulate the initiation and elongation of retinal neurites (Reichardt et al., 1989; Drazba and Lemmon, 1990; Bixby and Harris, 1991). Whether this class of molecules plays a similar role in vivo is not known. In the current study, we have used an in vivo transfection approach to determine which aspects of RGC differentiation are regulated by the cell adhesion molecule N-cadherin.

N-cadherin is a Ca^{2+}-dependent homophilic cell–cell adhesion molecule that promotes retinal neurite outgrowth in vitro, either as a purified protein substrate or when expressed in transfected fibroblasts (Matsunaga et al., 1988a; Bixby and Zhang, 1990; Payne et al., 1992). In addition, function-blocking N-cadherin antibodies inhibit neurite outgrowth from chick retinal neurons on astroglia (Neugebauer et al., 1988). When given a choice, retinal neurons show no preference for N-cadherin over laminin or L1 as a culture substrate, suggesting that these adhesion molecules are permissive for axon outgrowth (Lemmon et al., 1992). N-cadherin is expressed widely in the embryonic CNS, including the eye primordia, and thus could provide a substrate for axon growth in vivo (Hatta et al., 1987; Matsunaga et al., 1988b; Inuzuka et al., 1991; Simonneau et al., 1992). Neurites track along a pattern of N-cadherin expressed on the surface of cultured nonneuronal cells, and N- and R-cadherin are expressed in restricted and complementary patterns in the developing chick CNS, suggesting that cadherins may impart directional information to growing axons (Redies et al., 1992, 1993; Redies and Takeichi, 1993). Thus, although there is evidence that N-cadherin acts to promote and possibly guide the growth of retinal neurites in vitro, it is not known whether N-cadherin plays a role in either of these processes in vivo.

The functional importance of cadherins in early embryogenesis has been demonstrated in vivo by inhibiting cadherin-mediated adhesion through overexpression of a truncated form of N-cadherin (Kintner, 1992). This mutant (N-cadΔE) lacks most of the extracellular domain through which cadherins interact homophilically, and is thought to exert its dominant negative effect by competing with endogenous cadherins for cytoplasmic effectors such as the catenins (Kintner, 1992; Fujimori and Takeichi, 1993). Cadherin-mediated adhesion requires that the cytoplasmic tail of cadherin be linked with the cytoskeleton through the catenins (Aberle et al., 1994; Nagafuchi et al., 1994). Because the cytoplasmic domain is highly conserved between the different classical cadherins, the N-cadΔE dominant negative N-cadherin mutant is thought to inhibit the function of several endogenous cadherins in expressing cells. Indeed, overexpression of N-cadΔE causes complete loss of cell–cell adhesion in early Xenopus embryos (Kintner, 1992), which is mediated by XB/U- and EP-cadherin (Muller et al., 1994).

We have inhibited cadherin function in developing retinal cells by lipofecting the N-cadΔE dominant negative N-cadherin construct into the embryonic Xenopus eye. This approach gives rise to mosaic expression of the truncated cadherin within the retinal neuroepithelium and has allowed us to test the functional role of cadherins in axon and dendrite extension in single neurons as they develop in an unperturbed environment. Our results demonstrate that cadherin function is important for axon and dendrite outgrowth in RGCs in vivo. Further, experiments with two additional deletion mutants, including one comprising the catenin-binding region alone, suggest that cadherin-mediated neurite outgrowth, in contrast to cell–cell adhesion, involves

§ Present address: Department of Cell Biology and Physiology, Washington University Medical School, St. Louis, Missouri 63110-8228.
‖ Present address: Institut Pasteur, Biologie Moleculaire de l'Expression Genique, 25 Rue du Dr. Roux, 75015 Paris.

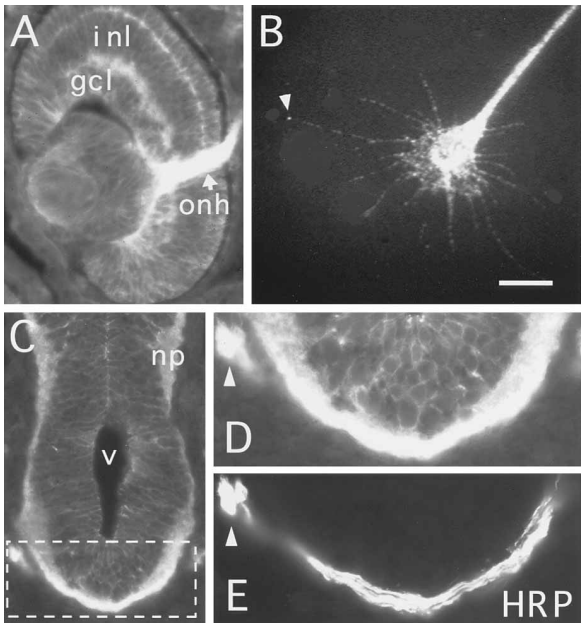

Figure 1. N-Cadherin Expression in the Embryonic Optic Pathway

(A) Transverse section of a stage 39 eye showing immunostaining with an anti-Xenopus N-cadherin antibody. Immunoreactivity is strong in the optic nerve head (onh) and is present throughout the retina in the plexiform and cell body layers.

(B) Tip of neurite extended from a cultured retinal neuron showing intense N-cadherin staining in the growth cone and neurite shaft; staining appears punctate in lamellipodia and filopodia and extends out to the tips of filopodia (arrowhead).

(C)–(E) Transverse section of the diencephalon at the level of the chiasm at stage 39 showing extensive N-cadherin staining in both the cell body and neuropil (np) regions. The optic fibers of one eye were anterogradely filled with HRP and the tissue was double labeled for N-cadherin (C and D) and HRP (E). (D) and (E) show higher magnification views of the boxed region in (C). Retinal axons grow through an N-cadherin-rich region in the ventral diencephalon. Arrowheads in (D) and (E) point out the optic nerve. Ganglion cell layer (gcl), inner nuclear layer (inl), ventricle (V). Scale bars, 10 μm in (B); 20 μm in (D) and (E); and 50 μm in (A) and (C).

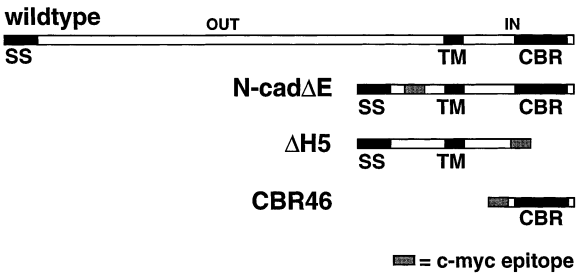

Figure 2. Schematic Representation of the N-Cadherin Deletion Mutants

The dominant negative N-cadherin (N-cadΔE) has a large deletion in the extracellular domain. The signal sequence remains intact, as does the transmembrane region and the full cytoplasmic domain. Also shown are the myc tag inserted at the deletion site, and the region of catenin binding (CBR) at the C-terminus. The ΔH5 mutant comprises the 105 membrane proximal cytoplasmic amino acids, the transmembrane domain, and signal sequence, lacks the catenin-binding region, and is myc-tagged at the C-terminus. The CBR46 mutant comprises the 70 C-terminal amino acids of the cytoplasmic domain, including the catenin-binding region. It lacks a transmembrane domain and is myc-tagged at the N-terminus. CBR, catenin-binding region; SS, signal sequence; TM, transmembrane domain.

interactions that are distinct from cadherin/catenin interactions.

Results

N-Cadherin Expression in the Developing Visual Pathway

To characterize N-cadherin expression in the early visual pathway, an anti-Xenopus N-cadherin antibody was used to stain sections of stage 39 embryos, when the first population of RGC axons are navigating the pathway and arriving at the tectum. N-cadherin is widely expressed in the developing CNS, including the retina and optic pathway (Figure 1). All retinal cell layers express N-cadherin, as well as the undifferentiated cells, in the marginal zone (Figure 1A). The fiber layers, including the ganglion cell fiber layer, are also strongly N-cadherin positive. The optic nerve head is intensely labeled, probably owing to the high density of RGC axons in this area. In the diencephalon, N-cadherin immunolabeling is most abundant in the neuropil regions where retinal and nonretinal axons are located. The outlines of cell bodies are also visible with anti-N-cadherin immunofluorescence, indicative of cell surface expression (Figures 1C and 1D). To determine if both the substrate pathway and RGC axons express N-cadherin, RGC axons were anterogradely labeled with horseradish peroxidase (HRP), and the embryos were double immunolabeled for HRP and N-cadherin. Overlap of the two staining patterns indicates that N-cadherin is present on RGC axons and on neuroepithelial substrate cells of the optic tract (Figures 1D and 1E).

To examine the expression pattern of N-cadherin on retinal axons and growth cones, cultured retinal explants were immunolabeled for N-cadherin. N-cadherin is expressed in a punctate pattern over the entire surface of growth cones, including lamellipodia and filopodia (Figure 1B). The most intense staining occurs in the central region of the growth cone, which may reflect either the greater cell volume in this area or a nonuniform distribution of N-cadherin over the growth cone. Thus, RGC axons and growth cones, and the surrounding cellular environment, express N-cadherin during the formation of the optic projection.

Adhesion and Cell Fate Decisions Not Disrupted by Loss of Cadherin Function

To examine whether cadherin plays a role in early retinal neurogenesis, the dominant negative N-cadherin (N-cadΔE; Figure 2) or the reporter gene luciferase was introduced into the retinal neuroepithelium by injecting the eye primordia of late neurula embryos (stage 19) with a mixture of DNA and the lipofection reagent DOTAP (Lilienbaum et al., 1995). At this stage, the eye primordium is composed of proliferating neuroepithelial cells. Our observations suggest that only cells that are mitotic at the time of lipofection efficiently express introduced genes (R. Dorsky, S. McFarlane, and C. E. H., unpublished data). The first neurons are born 6–8 hr later in

the central retina, at stage 25 (Holt et al., 1988), and the first RGC axons begin to extend roughly 4 hr after this, at stage 28 (Grant and Rubin, 1980). Exogenously introduced proteins begin to be expressed ~8 hr after lipofection (Holt et al., 1990), coincident with the birthdates of the first differentiating neurons. After injection, the embryos were allowed to develop for ~2 days until stage 41, when normally the majority of RGC axons have grown through the optic pathway to the optic tectum. Transfected cells were visualized by immunolabeling with either an antibody to the myc tag on the N-cadΔE protein or an anti-luciferase antibody. The majority of transfected cells were located in the retina; however, some expression was observed in regions outside the eye, including the brain and the epithelial lining of the pharyngeal cavity. Expression outside the retina probably results from diffusion of the DNA/DOTAP mixture.

Cells expressing N-cadΔE remained integrated in the retinal neuroepithelium, although their somata were sometimes more rounded than luciferase-expressing cells, suggesting that some adhesive contacts had been lost (Figure 3). Hoechst stain of retinal neurons expressing the dominant negative cadherin revealed that their nuclei were intact and identical in appearance to untransfected cells and cells expressing luciferase (data not shown). Thus, loss of cadherin function in individual retinal cells does not severely disrupt adhesive contacts, showing that cell–cell adhesion in the differentiating retina is not entirely cadherin dependent.

The serendipitous transfection of cells in the gut epithelium allowed a direct comparison of N-cadΔE-expressing cells in two epithelial cell types in the same embryo. In striking contrast to the retinal neuroepithelium, all of the N-cadΔE-expressing cells in the gut epithelium exhibited abnormal rounded morphologies and delaminated from the epithelial layer ($n = 28$; Figure 4F). This endodermally derived tissue is typically comprised of elongated and cuboidal cells that lie flush with the epithelial surface and expresses E- and B-cadherin (Boller et al., 1985; Murphy et al., 1994; Hermiston and Gordon, 1995). Expression of a mutant β1 integrin that impairs integrin function (Lilienbaum et al., 1995) does not disrupt cell adhesion in the gut epithelium (Figure 4E), supporting the idea that adhesion is cadherin specific. This result demonstrates that expression of the N-cadΔE protein at this relatively late stage of development effectively blocks cell–cell adhesion in the gut epithelium.

Laminar migration and neuronal differentiation in the retina were apparently unaffected by the inhibition of cadherin function. Cells expressing the N-cadΔE mutant protein exhibited the entire range of differentiated retinal cell types (photoreceptor, amacrine, bipolar, horizontal, Muller, and ganglion cells; Figures 3 and 4). Furthermore, N-cadΔE-expressing cells distributed between the three retinal layers in approximately the same ratio as luciferase-expressing cells (data not shown). Finally, while undifferentiated neuroepithelial cells with endfeet were observed in younger stage embryos (35/36 and 37/38) transfected with either N-cadΔE or luciferase, cells with this morphology were not seen at later stages (41 and older). These results indicate that retinal neurons can migrate and differentiate while expressing the dominant negative N-cadherin.

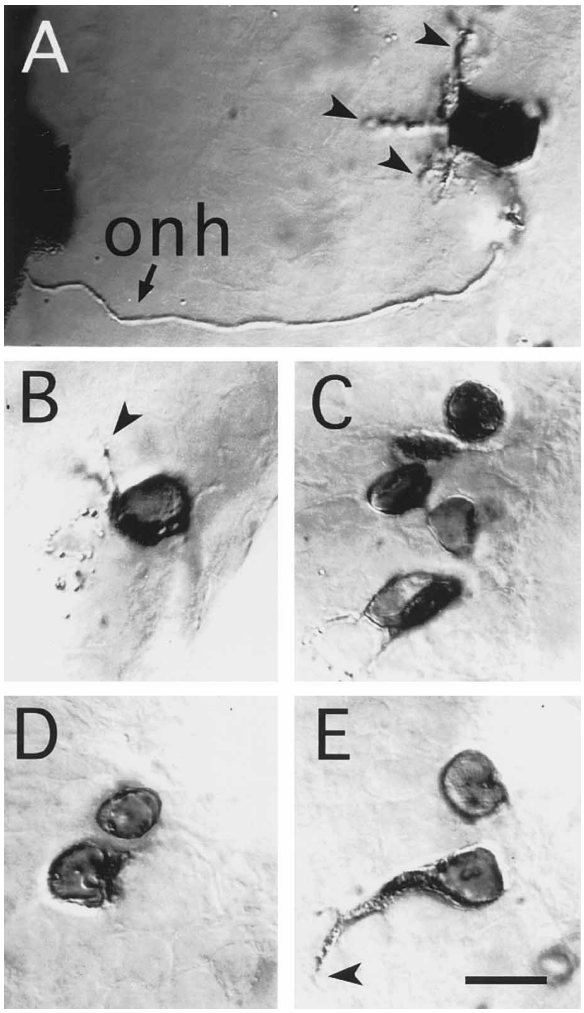

Figure 3. Dominant Negative N-Cadherin-Expressing RGCs Fail to Extend Processes

Stage 41 retinae immunostained with anti-luciferase (A) and anti-myc (B–E) antibodies in wholemount and sectioned transversely at 50 μm.

(A) Control luciferase-expressing RGC has several dendrites (arrowheads) and extends an axon into the optic nerve head (onh; arrow). The axon of this cell could be followed into the tectum (data not shown).

(B)–(E) RGCs expressing the dominant negative N-cadΔE possess few or no processes. The RGC in (B) has sparse dendrites but no axon; the RGCs shown in (C) and (D) have neither axons nor dendrites; one of the cells in (E) has a process (arrowhead), which is either a dendrite or a remnant of its basal endfoot. Anti-myc staining is concentrated at the edges of cell somata, indicating that the mutant N-cadherin localizes to the cell surface. Luciferase staining is uniformly distributed through the cell body, indicative of cytoplasmic localization. All labeled cell somata are positioned in the ganglion cell layer. Vitreal surface is to the right. Scale bar, 10 μm.

Axon Initiation Is Inhibited in Dominant Negative N-Cadherin-Expressing RGCs

To test the function of cadherin in axon outgrowth, the morphologies of RGCs transfected with dominant negative N-cadherin or luciferase constructs were compared. Transfected cells were identified as RGCs if their somata were positioned in the ganglion cell layer. This layer is

populated mainly by RGCs, although displaced amacrine cells occasionally reside here (Dunlop and Beazley, 1984; Chng and Straznicky, 1992).

The majority of luciferase-expressing RGCs exhibited normal morphologies for this stage of development (Holt, 1989). Cells had several dendrites and an axon that exited the eye (see Figure 3A) and fully extended along the pathway into the tectum. In striking contrast, RGCs expressing N-cadΔE often completely lacked an axon and dendrites (see Figure 3; Figure 5). Axonogenesis, in particular, was strongly inhibited: 70% of mutant cadherin–expressing RGCs failed to send out an axon compared with 14% of luciferase-expressing ganglion cells. Dendrite formation was also markedly reduced with 51% of mutant cadherin–expressing RGCs completely lacking dendrites compared with only 10% of the luciferase-expressing cells. Also, the number of dendrites that formed on dominant negative N-cadherin-expressing RGCs was significantly lower than luciferase-expressing cells (mean = 1 and 4.5, respectively; Figure 5). Surprisingly, the RGCs without dendrites were not always the same cells that failed to initiate an axon. For example, RGCs possessing only an axon or only dendrites were observed, as were cells that lacked all processes. No luciferase-expressing RGC was devoid of all processes, while 28% of N-cadΔE-expressing cells exhibited this extreme phenotype. These results indicate that inhibition of cadherin function impairs process initiation in developing RGCs.

Since our method of visualizing cell morphology relies on immunodetection of the foreign protein, the concern arose that the lack of processes in the dominant negative N-cadherin-expressing RGCs might simply reflect a failure to transport the mutant protein from the soma into axons and dendrites rather than an actual loss of these processes. To address this possibility, cells were cotransfected with luciferase, known to fill axons and fine processes (Holt et al., 1990), and the N-cadΔE mutant. Double immunolabeling showed that the majority (~90%) of cells coexpressed the two proteins, and that they were codistributed throughout individual neurons (Figure 6). Axons of these coexpressing cells, for example, were always positive for both luciferase and the myc-tagged N-cadΔE along their entire extent, indicating that the mutant cadherin was transported into processes as efficiently as the luciferase protein (Figure 6). Moreover, a similar reduction in process outgrowth was seen when RGCs cotransfected with the two constructs were visualized using luciferase immunostaining alone or anti-myc staining alone (65% of RGCs lack an axon, 35% lack dendrites, 2.8 ± 0.8 dendrites per RGC; n = 26). Thus, impaired process outgrowth in RGCs appears to be a functional consequence of expression of the dominant negative N-cadherin.

RGC Axon Elongation Is Retarded by Dominant Negative N-Cadherin Expression

N-cadherin promotes not only the initiation of neurites but also their continued growth in vitro (Neugebauer et al., 1988). In the few cases where RGCs expressing the dominant negative cadherin did elaborate axons and

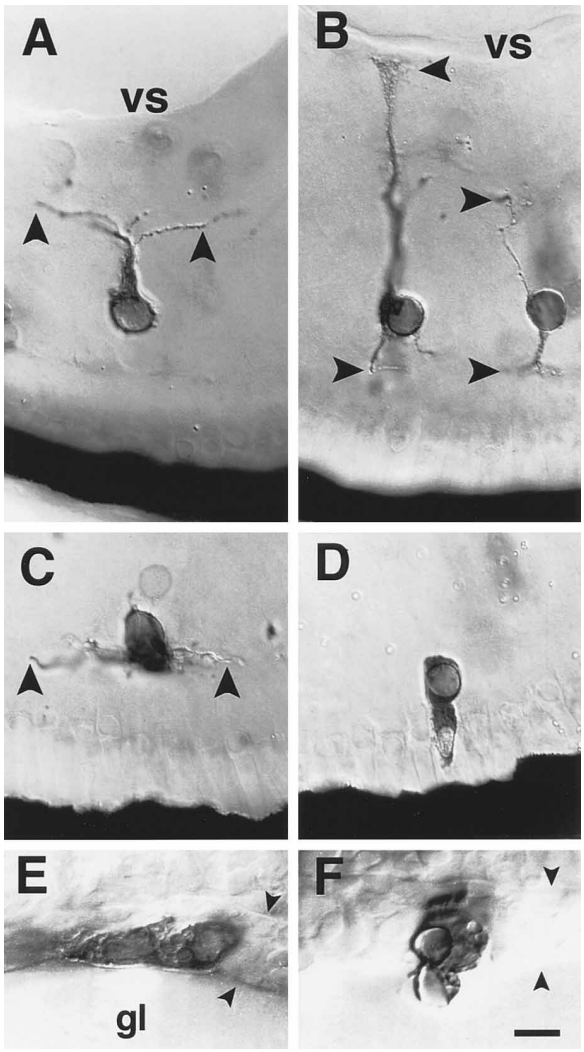

Figure 4. N-CadΔE-Expressing Cells Differentiate into Multiple Cell Types in the Retina but Delaminate from the Gut Epithelium

(A)–(D) Sections of transfected retinae at stage 41 showing examples of different retinal cell types expressing N-cadΔE and labeled with the myc antibody.

(A) Amacrine cell with its soma in the INL and processes in the inner plexiform layer (arrowheads).

(B) Bipolar cell (right) and Muller glial cell (left) with an endfoot attached at the vitreal surface (vs). Arrowheads indicate the laminar extent of processes from these cells.

(C) Horizontal cell with processes in the outer plexiform layer (arrowheads).

(D) Photoreceptor in the outer nuclear layer. Based on morphology, this cell appears to be a cone. In (A)–(D), the retina is oriented with the dark pigment epithelium at the bottom and vitreal surface at the top.

(E) and (F) Transverse sections of the gut epithelium lining the buccal cavity at stage 41.

(E) Two gut epithelial cells expressing a mutant form of the chicken β1 integrin subunit and labeled with an antibody to chicken β1 (Lilienbaum et al., 1995). The cells lie flush with the surrounding epithelium and maintain close contact with neighboring untransfected cells. Gut lumen, gl.

(F) Cells expressing N-cadΔE are rounded, lose contact with their neighbors, and protrude into the lumen. Arrowheads delineate the epithelial layer. Scale bar, 10 μm.

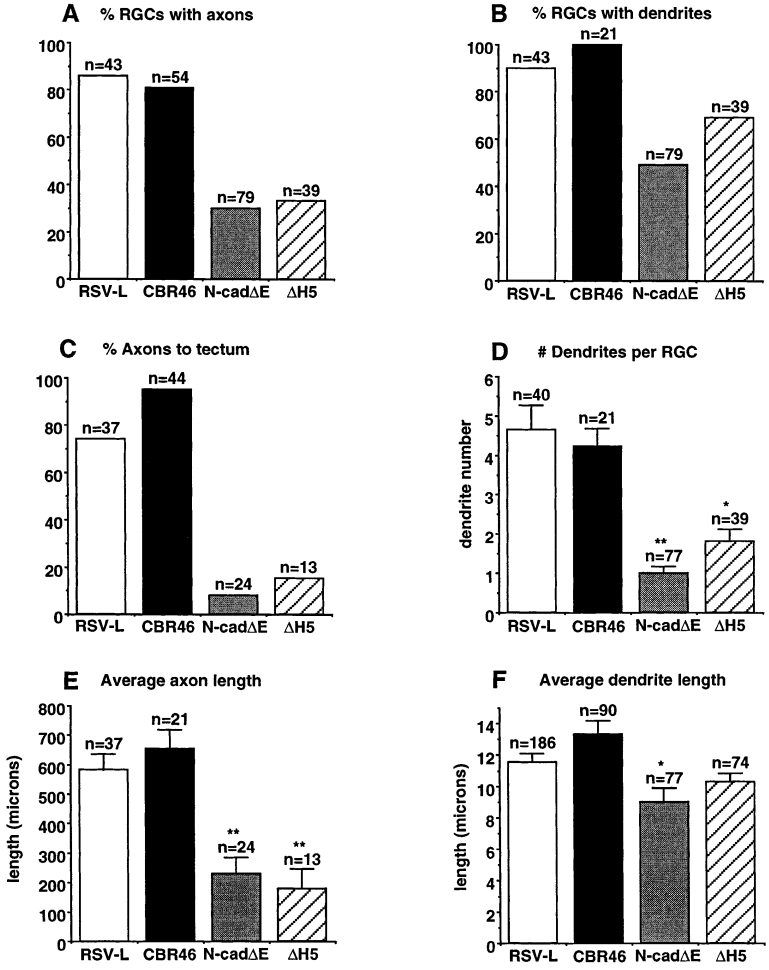

Figure 5. Inhibitory Effect of Dominant Negative N-Cadherin on Axon and Dendrite Genesis in RGCs

Quantitative analysis of N-cadΔE-, ΔH5-, CBR46-, and luciferase-expressing RGCs at stage 41.

(A) Percentage of RGCs with axons is reduced in cells expressing N-cadΔE and ΔH5, while cells expressing CBR46 are at control levels.

(B) Percentage of RGCs with dendrites is reduced in cells expressing N-cadΔE and ΔH5.

(C) The majority of axons expressing CBR46 or luciferase reach the tectum, whereas the percentage of axons that reach the tectum is small in cells expressing N-cadΔE and ΔH5. For (D)–(F), data is mean ± SEM.

(D) The average number of dendrites per cell is significantly reduced in cells expressing N-cadΔE and ΔH5.

(E) and (F) The average length of axons and dendrites, respectively: axon length is strongly reduced and dendrite length is slightly decreased in RGCs expressing N-cadΔE and ΔH5. n, the number of cells analyzed. Single asterisk, $p < .05$; double asterisk, $p < .0001$, unpaired *t* test.

dendrites, the length of their processes was measured to determine whether growth was affected. The majority of luciferase-expressing RGC axons exited the eye, and many could be followed to the tectum (27 of 37; see Figure 5). RGC axons expressing the dominant negative N-cadherin, however, exhibited retarded growth with very few reaching the tectum (2 of 24). The average length of N-cadΔE-expressing axons was reduced 69% when compared with axons of luciferase-expressing cells (see Figure 5). The dendrites of RGCs expressing N-cadΔE were also shorter on average than those expressing luciferase (see Figure 5). The effect on dendrite length, however, was not as pronounced as on axon length. Similar results were observed with RGCs that had been cotransfected with luciferase and the N-cadΔE mutant and visualized with anti-luciferase immunolabel (average axon length 270.8 ± 100.5 μm; n = 9).

The majority of RGC axons that expressed the N-cadΔE mutant lacked growth cones at their tips, terminating instead in unexpanded stumps along the optic pathway (78%, n = 45 [includes data not used for length analysis], Figure 6). Most of the luciferase-expressing RGC axons reached the tectum, where they began to arborize and therefore lacked growth cones. An absence of growth cones was also observed in cotransfected RGCs visualized with a luciferase antibody, demonstrating that the effect was not due to a failure of N-cadΔE to localize to growth cones (Figure 6). Lack of growth cones suggests that the dominant negative N-cadherin-expressing axons were not growing at the time of fixation.

The trajectories of N-cadΔE-expressing axons were indistinguishable from luciferase-expressing axons, and no pathfinding errors were evident. However, because the N-cadΔE mutant may arrest axon growth, the normal trajectories may reflect axon extension that had occurred before expression of the mutant protein.

Axon Growth Inhibited at Later Developmental Stages

The decrease in average length for axons expressing N-cadΔE could reflect either an inhibition of axon growth or a delay in RGC development. RGCs in older embryos (stages 43–45) were therefore examined to determine if those expressing the dominant negative mutant had elaborated longer processes. Intact immunopositive cells were observed 3 days after transfection and no evidence of cell death, such as pycnotic nuclei or cellular debris from labeled cells, was seen, suggesting that neurons remain viable while maintaining expression of N-cadΔE. Luciferase-expressing RGCs continued to grow, developing larger, more highly branched arbors in both the retina and the tectum. In contrast, dominant negative N-cadherin-expressing RGCs had not developed further than was seen at stage 41: approximately

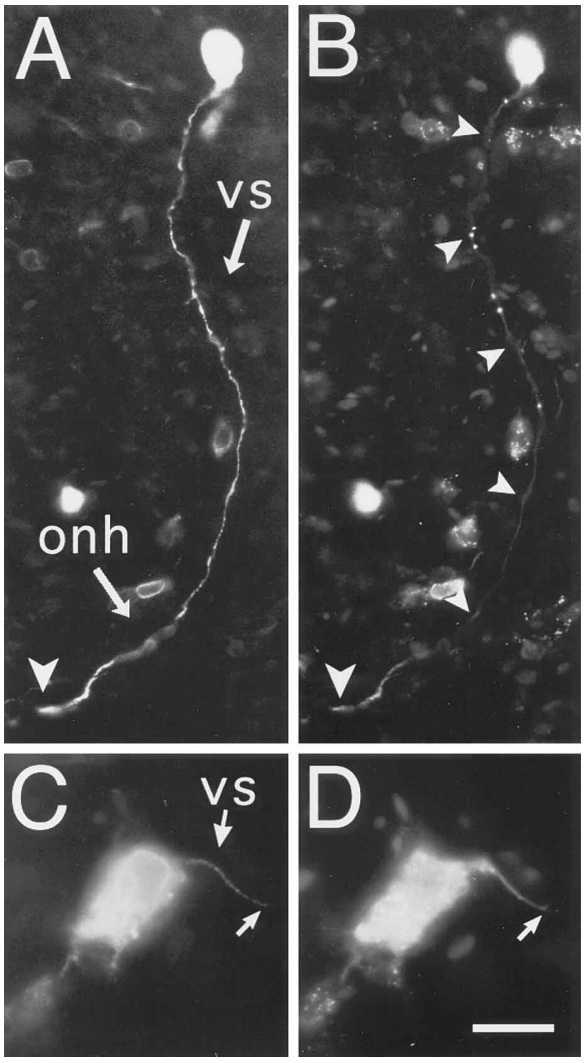

Figure 6. Fine Morphology of Cells Revealed by Immunostaining for Dominant Negative N-Cadherin

RGCs cotransfected with luciferase and N-cadΔE and double labeled with anti-luciferase and anti-myc antibodies.

(A) and (C) Anti-myc staining reveals the distribution of the myc-tagged N-cadΔE protein. (B) and (D) show anti-luciferase staining. The axon in the cell shown in (A) and (B) coexpresses both proteins along its full extent. Note the growth cone (arrowhead) in the optic nerve head (onh). The cell in (C) and (D) has a short thin axon visualized similarly by both antibodies and lacks a growth cone at its tip (arrow). Dorsal is up. VS, vitreal surface. Scale bar, 10 μm in (A) and (B); 20 μm in (C) and (D).

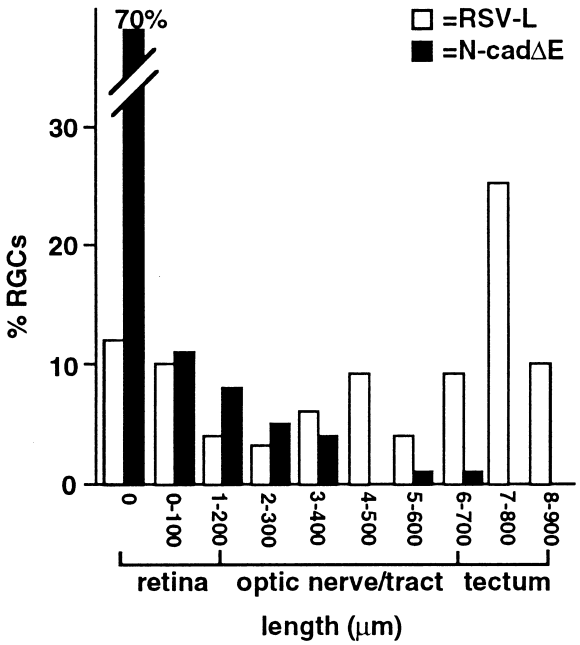

Figure 7. Axon Extension within the Pathway Severely Impaired by Dominant Negative N-Cadherin

N-cadΔE-expressing cells are represented as solid bars; luciferase-transfected controls as open bars. No N-cadΔE axons of over 700 μm were observed.

the same percentage of RGCs lacked axons (71%, n = 75) and the average length of existing axons was not changed significantly (148.4 ± 37 μm, n = 22). The lack of continued axon elongation demonstrates that the growth retardation observed in N-cadΔE-expressing RGCs is not due to a delay in differentiation and supports the idea that the N-cadΔE protein causes growth cone arrest.

Axon Growth Inhibited throughout the Optic Pathway

Cadherin function might be required for RGC axon growth at specific points in the optic pathway. If this were the case, we would expect to see that a large fraction of the dominant negative N-cadherin-expressing RGCs had axons of similar length as they accumulated at a particular point. To address this possibility, axon lengths were grouped by 100 micron intervals, and their distribution examined. A histogram of the lengths of luciferase-expressing axons revealed a bimodal distribution (Figure 7). The majority of luciferase-expressing cells had axons of about 800 microns (the approximate length of the pathway from retina to tectum in a stage 41 embryo); a few cells had either no axon or a very short one, and the remainder extended between 200 and 700 microns. Luciferase-expressing cells with no axon may be displaced amacrine cells rather than RGCs, and those with short axons could represent later-born RGCs with still-developing axons. In contrast, the majority (70%) of N-cadΔE-expressing RGCs had no axon. The percentages of N-cadΔE-expressing cells with axons of a given length decrease steadily at greater distances from the ganglion cell layer (Figure 7). The lack of a sharp drop-off in axon length indicates a general retardation of axon growth owing to loss of cadherin function rather than a block at a particular point in the pathway.

N-CadΔE Inhibits N-Cadherin-Mediated Outgrowth In Vitro

To test whether N-cadΔE specifically blocks cadherin-mediated neurite outgrowth, retinal cells from dissociated eye primordia were transfected with N-cadΔE or green fluorescent protein (GFP) and plated on laminin

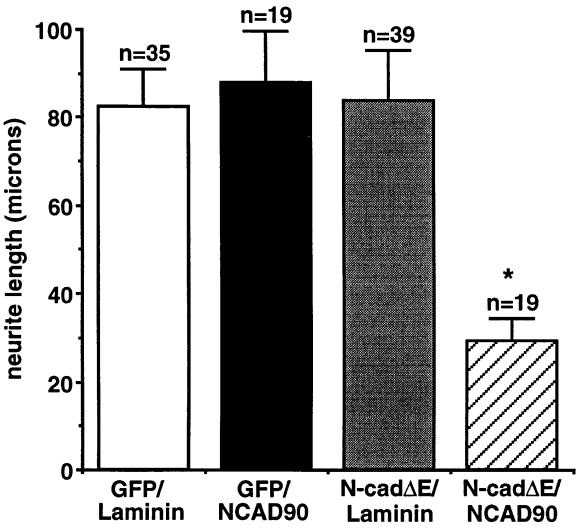

Figure 8. Specificity of N-CadΔE In Vitro

Neurites from N-cadΔE-expressing retinal neurons on N-cadherin (NCAD90) are significantly shorter than neurites from GFP-expressing control neurons on N-cadherin ($p = .0001$) and N-cadΔE-expressing neurons on laminin ($p = .0024$), while GFP-expressing neurons extend neurites of approximately equal lengths on both substrates.

and N-cadherin (NCAD90) substrates. NCAD90 is a naturally occurring proteolytic turnover product of N-cadherin that retains the ability to promote neuronal adhesion and neurite outgrowth (Paradies and Grunwald, 1993). Neurites from N-cadΔE-expressing neurons were ~60% shorter on NCAD90 than on laminin, whereas GFP-expressing neurites were of similar length on both substrates (Figure 8). Thus, the dominant negative N-cadΔE mutant appears to specifically inhibit cadherin-mediated neurite outgrowth.

Catenin-Binding Region of N-Cadherin Does Not Impair Process Outgrowth

To determine which intracellular region of the N-cadherin molecule is important for neurite outgrowth, retinae were first transfected with a myc-tagged cadherin mutant that consists of the C-terminal 70 amino acids of the cytoplasmic domain containing the catenin-binding region (CBR46; see Figure 2). Following RNA injection in early embryos, this mutant blocks cell–cell adhesion and appears to sequester β-catenin (R. B. and C. Kintner, unpublished data). CBR46-expressing gut cells in the present study were typically rounded in morphology and delaminated from the epithelium (data not shown) like the N-cadΔE-expressing gut cells (see Figure 4). This severe impairment of gut cell adhesion shows that the CBR46 mutant effectively competes for cytoplasmic effectors in the absence of a membrane localization sequence. By contrast, retinal cells expressing CBR46 were indistinguishable from control neurons and exhibited normal process extension. Most of the CBR46-expressing RGCs had an axon (81%), the majority of which reached the tectum (95%; see Figure 5). The CBR46-expressing RGCs also exhibited robust dendrite outgrowth that was quantitatively similar to control luciferase-expressing cells (see Figure 5). Thus, the CBR46 mutant does not appear to impair RGC differentiation, suggesting that the catenin-binding portion of cadherin is not essential in stimulating axon outgrowth.

To map further the neurite-promoting activity of the cadherin cytoplasmic domain, we used a myc-tagged cadherin mutant comprising the membrane-proximal portion of the cytoplasmic domain, which excludes the catenin-binding region (ΔH5; see Figure 2). This ΔH5 mutant, and similar ones used previously (Kintner, 1992), impairs cell adhesion in early embryos (Bradley and Kintner, unpublished data), but does not appear to disrupt cell adhesion in the gut epithelium in lipofected embryos (data not shown). ΔH5-expressing RGCs exhibited a marked reduction in axon outgrowth similar to that seen with N-cadΔE (see Figure 5). Less than 40% of ΔH5-positive RGCs initiated an axon, and these axons were, on average, ~60% shorter than luciferase- and CBR46-expressing axons. Dendrite genesis was also inhibited by the ΔH5 mutant, but to a slightly lesser extent than the N-cadΔE mutant (see Figure 5). ΔH5-expressing RGCs extended significantly fewer dendrites per cell than luciferase- or CBR46-expressing cells, and 20% fewer RGCs possessed dendrites. There was little effect on dendrite length, however (see Figure 5). Thus, the results with the ΔH5 mutant in the retina closely mimic those of N-cadΔE and suggest that the ΔH5 portion of the cadherin molecule, rather than the catenin-binding domain, is critical for axonogenesis in RGCs.

Non-RGC Neurons Are Less Severely Affected by the Loss of Cadherin Function

As N-cadherin is expressed in all layers of the retina (see Figure 1), we examined transfected cells in the inner nuclear layer (INL) to determine if non-RGC retinal neurons are affected by loss of cadherin function. A reduction in process initiation from INL cells expressing the dominant negative N-cadΔE mutant was observed (Table 1). Process length, however, was not significantly affected and the percentage of cells that had at least one process was nearly at control levels. These results indicate that cells in the INL are at least partially dependent on cadherin function for process initiation, but that the continued extension of those processes does not require functional cadherins.

Since N-cadherin is widely expressed in the embryonic brain (see Figure 1), transfected neurons in the mesencephalon and diencephalon were also examined to determine if their development was affected by expression of N-cadΔE. Most of the transfected neurons in the brain at the stages examined had several processes of short to medium length (10–50 μm) rather than a distinct axon, although a few did have long axons. Quantitative analysis revealed no significant differences between N-cadΔE- and luciferase-expressing neurons (Table 1). Process outgrowth from forebrain and midbrain neurons, therefore, appears unaffected by overexpression of a dominant negative mutant cadherin.

Discussion

This study examines the role of cadherins in axon outgrowth in vivo. We show that N-cadherin is expressed on

Table 1. Effects of N-CadΔE on Other Neurons

Cell Type/Construct	Cells with Processes				
	Percentage	Number/Cell[a]	n	Length[a]	n
INL/N-cadΔE	95	2.9 ± 0.4[b]	37	19.4 ± 1.8	108
INL/Luciferase	100	6.0 ± 0.8	13	22.2 ± 0.6	78
Brain/N-cadΔE	98	3.5 ± 0.4	44	20.4 ± 1.2	154
Brain/Luciferase	88	3.3 ± 0.5	24	20.7 ± 2.2	79

[a] Data shown represent the mean ± SEM.
[b] $p < 0.005$.

RGC axons and growth cones and in the optic pathway during formation of the retinotectal projection in Xenopus. RGCs in which cadherin function was blocked by expression of a dominant negative N-cadherin mutant are strongly inhibited in the initiation and extension of axons and dendrites, while other CNS neurons expressing the same mutant were affected to a lesser degree or not at all. RGCs expressing only the catenin-binding domain of N-cadherin sent out axons that grew normally to the tectum, whereas those expressing the ΔH5 non-catenin-binding region were inhibited in axon outgrowth. These results indicate an important role for cadherins in promoting axon outgrowth, in particular for neurons such as RGCs, which project their axons over long distances and through complex and varied environments.

Once initiated, axons must receive both growth and guidance signals to reach their targets. Our data suggest that functional cadherins are required early in RGC development for axon initiation, and then subsequently act to stimulate axon growth in the pathway. An alternative explanation is that RGCs expressing the dominant negative N-cadherin are delayed in differentiation, and hence have fewer and shorter axons. However, this possibility is unlikely for several reasons. It does not agree with the observation that axon number and length did not significantly increase in transfected embryos analyzed at later stages of development. Moreover, in younger retinae, the transition from a neuroepithelial to a neuronal phenotype occurred at the same developmental stage in luciferase- or dominant negative N-cadherin-expressing cells. Finally, of the few axons observed that expressed the dominant negative N-cadherin, most lacked growth cones at their tips, suggesting that extension was arrested.

It is unlikely that RGCs expressing the dominant negative cadherin fail to send out axons because they are unhealthy or that the inhibitory effect is not specific. Cells expressing the dominant negative cadherin have healthy nuclei, as visualized by Hoechst stain. In addition, RGCs are able to survive for several days while expressing the mutant N-cadherin, and, in a previous study using the same dominant negative mutant, it was shown that cells that express it are viable (Holt et al., 1994). Moreover, the finding that RGCs expressing the mutant cadherin can extend neurites on laminin but are impaired on NCAD90 indicates that the inhibition of axon growth is due to a specific block in cadherin function. Finally, the severity of the effect of the dominant negative N-cadherin varies with cell type. Within the retina, RGC process outgrowth is strongly inhibited, while cells in the INL are impaired more in process initiation than in extension. Neurons in the diencephalon and mesencephalon appear unaffected by the loss of cadherin function. The cell-type specificity of the effect indicates that the expression of the dominant negative N-cadherin does not compromise the survival or health of transfected neurons.

During normal RGC development, dendrites are usually elaborated shortly after axonogenesis (Holt, 1989). This process is inhibited in RGCs in which cadherin function is impaired. Dendrite initiation was strongly inhibited, while dendrite extension was somewhat reduced. Dominant negative–expressing RGCs have fewer dendrites on average than control RGCs, but those dendrites reach close to normal lengths. This result suggests that axons and dendrites have different requirements for growth stimulatory molecules during outgrowth. The evidence from both axons and dendrites indicates that blocking cadherin function in RGCs results in a severe inhibition of their ability to initiate processes.

Because the dominant negative N-cadherin inhibits the function of all cadherins with the conserved cytoplasmic domain, it cannot be determined from this study if N-cadherin in particular is required for retinal axon outgrowth. Different cadherins are expressed in the retina of other species (Inuzuka et al., 1991; Suzuki et al., 1991). This question could be addressed through similar experiments with an N-cadherin-specific dominant negative mutant, as has been done for E-cadherin (Levine et al., 1994).

Our results are consistent with the idea that cadherins are required for promoting RGC axon outgrowth but not for navigational decisions (Lemmon et al., 1992). Given the severe inhibition of axon growth caused by the dominant negative N-cadherin, however, this study cannot distinguish whether cadherins are involved in navigational aspects of growth. The lack of pathfinding errors in N-cadΔE-positive axons may simply reflect appropriate navigation that occurred before expression of the mutant cadherin. Thus, any requirement for cadherin in axon guidance would be masked by arrested growth.

The lack of an effect on forebrain and midbrain neurons that express the mutant N-cadherin suggests that these cells either have no requirement for N-cadherin in process outgrowth or that they are able to compensate for loss of cadherin function by relying on other neurite outgrowth–promoting molecules, such as the integrins. In contrast, the severity of the inhibitory effect on RGCs suggests that RGC axons, which must navigate over long distances through a variety of different environments, may be more sensitive to a perturbation of

their cytoskeletal outgrowth machinery or pathfinding signals. RGCs, and their axons in particular, respond differently than other retinal neurons to certain growth and guidance cues. RGCs, rod photoreceptors, and amacrine cells have cell type–specific responses to different adhesion molecules, including N-cadherin (Kljavin et al., 1994). Additionally, RGC axons avoid the deeper layers of the retina owing to the presence of a repulsive signal that RGC dendrites and INL cell processes apparently ignore (Stier and Schlosshauer, 1995). RGC axons may carry specific receptors for this and other inhibitory molecules, and therefore may become growth inhibited when deprived of stimulatory signals from N-cadherin or other cadherins.

The extracellular deletion dominant negative mutant of N-cadherin has been used in other systems to investigate the role of cadherins in early development (Kintner, 1992; Fujimori and Takeichi, 1993; Dufour et al., 1994). In these studies, the dominant negative cadherin inhibited Ca^{2+}-dependent adhesion and interfered with morphogenesis of Xenopus embryos during gastrulation and eyebud formation. The dominant negative protein affected the behavior of endogenous cadherins, either depleting them of associated cytoplasmic proteins such as α-catenin (Kintner, 1992) or altering their localization from concentration at sites of cell–cell contact to a diffuse cell-surface distribution (Fujimori and Takeichi, 1993). Interestingly, the catenin-binding domain mutant (CBR46), despite lacking a transmembrane domain, inhibits cell adhesion in the gut epithelium and in gastrulating embryos almost as well as the full length cytoplasmic domain dominant negative mutant (R. B., unpublished data). Our finding that the catenin-binding domain mutant does not inhibit axon growth suggests that the catenins, in contrast with their crucial role in cadherin-dependent cell–cell adhesion, are not important in stimulating cadherin-mediated axon outgrowth. The ΔH5 partial cytoplasmic domain, however, was almost as effective as N-cadΔE (full cytoplasmic domain) at inhibiting process outgrowth in RGCs, indicating that the N-cadΔE mutant acts by competing with endogenous cadherins for cytoplasmic effectors that bind this region. At present, it is not known what molecules interact with this region of the cadherin cytoplasmic domain. One possibility is that this region regulates N-cadherin-induced changes in Ca^{2+} needed for neurite outgrowth (Bixby et al., 1994).

Retinal axons rely on several adhesion molecules to promote extension. In culture experiments, a cocktail of antibodies to several different adhesion molecules is required to block completely neurite outgrowth (Neugebauer et al., 1988). In vivo, β1 integrin subunit mutants that perturb integrin function inhibited axon outgrowth from Xenopus RGCs, primarily within the retina (Lilienbaum et al., 1995). β1 integrin mutant–expressing axons that managed to exit the retina extended normally to the tectum. In contrast, axons expressing the dominant negative N-cadherin are inhibited at all regions of the pathway. The variant profiles of axon inhibition observed with the β1 integrin or N-cadherin mutants demonstrate that the requirements for these two molecules are different during formation of the optic projection.

In addition to promoting neurite outgrowth from retinal cells, N-cadherin plays a role in the earlier developmental events of retinal formation and lamination. Expression of a similar dominant negative N-cadherin in the anterior region of Xenopus embryos inhibits eyecup formation (Dufour et al., 1994), and N-cadherin antibodies perturb normal histogenesis and lamination in cultured chick retina (Matsunaga et al., 1988b). These early processes of retinal morphogenesis were unaffected by expression of the dominant negative N-cadherin in single cells in vivo: mutant-expressing retinal neurons migrated and differentiated normally and remained integrated in the neuroepithelium. This observation agrees with the previous finding that the importance of cadherin-dependent adhesion in the retina decreases at later stages (Matsunaga et al., 1988b). Other cell adhesion systems, such as the integrins or the Ca^{2+}-independent cell adhesion molecules, assume a larger role in maintaining tissue integrity and may be sufficient to allow the dominant negative cadherin-expressing cells to migrate normally and maintain close neighbor contacts.

While catenins have been shown to be crucial for cadherin-mediated adhesion and signaling, our results suggest that cadherins have multiple intracellular interactions with different functions in neurite outgrowth or cell adhesion. This finding offers a possible explanation for the apparently contradictory role of N-cadherin as an adhesive anti-migratory molecule (Akitaya and Bronner-Fraser, 1992; Barami et al., 1994) that also serves to promote axonal elongation. Our results demonstrate that cadherin function is required in vivo for retinal process initiation, and in particular for RGC axon growth in the optic pathway. Different types of neurons, however, exhibit different levels of redundancy in which molecules they use to promote process outgrowth. Thus, the in vivo analysis of the behavior of individual genetically altered cells has revealed structural and cell-type specificity in cadherin function. Further elucidation of the mechanism by which N-cadherin promotes neurite outgrowth will require the identification of the effector proteins that interact with the ΔH5 region.

Experimental Procedures

Frog Embryos

Fertilized eggs were obtained from hormone-induced matings of adult Xenopus pairs or by in vitro fertilization. Embryos were reared in 10% Holtfreter's (Holtfreter, 1943) with antibiotics at 14°C–21°C and manually removed from their vitelline membranes before injection. Embryos were staged according to Nieuwkoop and Faber (1967). Motile embryos were anesthetized with .04% tricaine (ethyl 3-aminobenzoate methanesulfonic acid; Aldrich) before surgery or fixation.

Plasmids

The dominant negative N-cadherin extracellular deletion mutant (N-cadΔE) was a gift from C. Kintner of the Salk Institute (Kintner, 1992). The dominant negative was myc-tagged by synthesizing and annealing two complementary oligonucleotides, creating a short fragment of DNA coding for ten amino acids of human c-myc with overhanging BamHI ends and an internal HindIII site. This fragment was then inserted in the BamHI deletion site of the dominant negative cadherin.

Three different expression vectors were used in the in vivo transfection of the dominant negative N-cadherin, pRSV-SVneo, CS2+, and CS2+MT. First, the N-cadΔE/myc cDNA was subcloned from the sp72 vector into the pRSV-SVneo expression vector (Reszka et

al., 1992). The N-cadΔE/myc cDNA was later subcloned into CS2+, a more efficient expression vector for Xenopus containing the CMV promoter (the gift of D. Turner). The CS2+MT/CBR46 construct codes for the 70 C-terminal amino acids of Xenopus N-cadherin, which includes the catenin-binding region, and has six myc epitopes inserted at the N-terminus. The CS2+MT/ΔH5 construct contains the membrane-proximal 105 amino acids (without the catenin-binding region) and transmembrane and signal sequences and has six myc epitopes at the C-terminus. Luciferase was used as a control exogenous gene and has previously been shown to reveal the fine morphology of expressing cells (Holt et al., 1990; Lilienbaum et al., 1995). The RSV-luciferase (RSV-L) expression plasmid was a gift from S. Subramani (de Wet et al., 1987) and the CS2+ vector encoding GFP was a gift of D. Turner.

Antibodies

A polyclonal anti-Xenopus N-cadherin antibody was the gift of C. Kintner (Detrick et al., 1990). Conditioned media from the 9E10 anti-human c-myc hybridoma cell line (Evan et al., 1985) was used for immunolabeling the myc-tagged mutant N-cadherins. On a Western blot of N-cadΔE-transfected fibroblasts, the myc antibody binds to a band of 45 kD, the approximate size of the extracellular deletion N-cadherin mutant. A polyclonal guinea pig antibody was used to detect luciferase expression. Secondary antibodies were conjugated to HRP (for wholemount anti-myc staining), fluorescein, or rhodamine (for cryostat and culture staining).

In Vivo Lipofection Injections

Lipofections were targeted to the eye primordia by injection at stage 19 (late neurula) using previously published methods (Lilienbaum et al., 1995). Embryos were positioned anterior end up in indentations molded into plasticene clay in 100% modified Ringer's solution (MR; Gimlich and Gerhart, 1984). Glass micropipettes used for injections were pulled on an electrode puller (Sutter Instruments), broken to a tip diameter of less than 50 μm, and filled with a mixture of DNA (Plasmid Maxi Kit, Qiagen) and DOTAP (Boehringer-Mannheim) at 1:3 w/w (Holt et al., 1990). Each embryo received 5–10 air pressure injections of 10–20 nl from a Picospritzer II (General Valve Corporation) into the neuroepithelium of both presumptive eyebuds. The embryos were incubated in 100% MR for an additional 30–60 min to allow healing after injection, then transferred to 10% Holtfreter's, and raised at room temperature for 1–3 days.

Visualization of the Optic Projection

RGC axons were labeled using HRP (type VI; Sigma) as described previously (Cornel and Holt, 1992). The lens of the right eye was surgically removed, and HRP dissolved in 1% lysolecithin was placed in the eye cavity. After allowing time for anterograde filling of the axons (25 min), embryos were fixed in 4% paraformaldehyde (Sigma) in 0.1 M phosphate buffer overnight at 4°C.

Immunohistochemistry

Embryos were fixed as above. For cryostat sections, embryos were sunk in 30% sucrose, embedded in optimal cutting temperature (OCT; Miles, Inc.), quick frozen at −20°C, cut in 12 μm sections, and collected on gelatin-coated microscope slides (Fisher). For wholemount embryos, the skin epidermis was removed from the brain and eyes and the lenses were dissected out to give increased accessibility for the antibody. The embryos were permeabilized in methanol (Fisher) overnight at −20°C, then rehydrated in phosphate-buffered saline (PBS).

Standard immunohistochemical procedures were used for both cryostat and wholemount preparations (Cornel and Holt, 1992; Lilienbaum et al., 1995). Nonspecific antibody binding was blocked with 5% goat serum (Sigma). 9E10 conditioned media was used at 1:5 for cryostat sections or undiluted for wholemounts; anti-luciferase antibody was used at 1:100 for both. Secondary antibodies were fluorophore or HRP-coupled goat anti-mouse or anti-guinea pig used at 1:500 (Jackson Laboratories and Molecular Probes). Cryostat sections were mounted in glycerol with the anti-bleaching agent p-phenylenediamine (pPD-glycerol; 1 mg/ml pPD in 9 parts glycerol, 1 part 1 M Tris–HCl [pH 8.5]; Sigma).

After primary and secondary antibody incubation, wholemount embryos were reacted with diaminobenzidine (DAB; Sigma) as described previously (Lilienbaum et al., 1995). Embryos were post-fixed in 1% gluteraldehyde (Sigma) for 1 hr, embedded in gelatin/albumin (1.5% gelatin [Fisher], 45% albumin [Sigma] in 0.9% saline), set with gluteraldehyde, and sectioned at 50 μm on an Oxford vibratome. Sections were collected in series on 1% gelatin on microscope slides, and allowed to dry at the edges to adhere to the gelatin. The samples were then dehydrated through a graded alcohol series, cleared in xylenes (Fisher), and mounted in Permount (Fisher).

Retinal Cultures

Retinal explant cultures for growth cone immunohistochemistry were cultured on polyornithine/laminin (PO/LN; Sigma) coated glass coverslips as described previously (Harris and Messersmith, 1992), and were fixed and immunostained as for cryostat sections. Dissociated retinal cultures for testing N-cadΔE specificity were prepared as described previously (Harris and Messersmith, 1992), except that the eye primordia (stage 22) were transfected with N-cadΔE and GFP cDNAs before dissociation. Eye primordia were treated with a Ca^{2+}-free solution containing trypsin (ATV solution) for 2 min to loosen cell–cell contacts (Holt et al., 1990) before transfer to transfection media containing DNA:DOTAP at a ratio of 1:3 in L15 with 10% fetal bovine serum (FBS) and 5% fungibact (FB). Eyes were incubated in transfection medium for 3–4 hr, then dissociated and cultured in L15 with 10% FBS and 5% FB for 24 hr. For cell culture substrates, the purified extracellular domain of chicken N-cadherin (NCAD90; Paradies and Grunwald, 1993) and laminin were deposited onto polyornithine-coated glass coverslips at 10 μg/ml and 15 μg/ml, respectively. Cultures were fixed in 2% paraformaldehyde and stained with an anti-myc antibody (9E10) and a rhodamine-conjugated secondary.

Cell Analysis

Embryo Sections

Fluorescent samples were photographed either with a camera attachment on a Zeiss Axioskop or using a cooled CCD camera (Spectrasource) attached to a Nikon optiphot-2 microscope. CCD images were captured using a Gateway 2000 PC and processed with Adobe Photoshop software. HRP-reacted immunopositive neurons in vibratome sections were examined with Nomarski optics and drawn at 16× and 40× using a camera lucida attachment. Drawings were scanned into a computer (Apple) using the ScanJet IIc (Hewlett-Packard), and the axons and dendrites were analyzed for length and number per cell using NIH Image 1.45, Excel (Microsoft), and Statview II (Abacus Concepts).

A process from an RGC was identified as an axon if it grew along the vitreal surface toward the optic nerve head, and as a dendrite if it was oriented toward the inner plexiform layer. In the cases where an axon could be followed through two or more serial sections, all such sections were drawn, and the partial axon lengths obtained were added to give the total axon length. Axons that terminated in the retina or optic nerve usually extended over one or two sections. Axons that reached the tectum often extended over several sections, as the optic tract grows caudally within the brain. Therefore, the lengths of the axons to the tectum are probably underestimated by this method. The fact that an axon terminated within a section and had not been cut off at the section edge was confirmed by focusing through the entire depth of the tissue, and by closely examining the adjacent sections for continuation of the process.

In analyzing dendrite number and length, no distinction was made between primary dendrites or secondary branches. For the analysis of INL cells, only cells that could be clearly identified as belonging to the INL were used. The different regions of the brain were distinguished by the shape of the ventricle in cross-section, and the presence or absence of the hypothalamus, roofplate, and notochord.

Cultures

Transfected neurons were identified with fluorescence optics and neurite length was measured on captured phase images using NIH Image. For each cell, the length of the longest neurite was recorded. Only neurons that were growing directly on the substrate, isolated from cell aggregates, were included in the data analysis.

Acknowledgments

The authors thank Lisa McNeill for invaluable technical assistance. We thank Sarah McFarlane, Bill Harris, Chris Kintner, and Barbara Ranscht for insightful discussions of the results and Emilie Marcus, Barbara Lom, Wes Chang, Chi-Bin Chien, Terri Worley, and Andreas Walz for helpful comments on the manuscript. Andrew Benedict helped with the Image and Excel analysis; Andreas Walz helped with figure preparation. We are grateful to Chris Kintner for the gifts of the N-cadherin constructs and antibody, to Dave Turner for the CS2+ expression vector, and to J. Willoughby for help with Western blots. This work was supported by National Institutes of Health grant RO1 NS23780, the March of Dimes, and a Pew Scholars Award (C. E. H.), National Institutes of Health grant EY06658 (G. B. G.), and a National Research Service Award from the National Cancer Institute 1F32CA66313-01 (R. B.).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *"advertisement"* in accordance with 18 USC Section 1734 solely to indicate this fact.

Received July 2, 1996; revised September 20, 1996.

References

Aberle, H., Butz, S., Stappert, J., Weissig, H., Kemler, R., and Hoschuetzky, H. (1994). Assembly of the cadherin–catenin complex in vitro with recombinant proteins. J. Cell Sci. *107*, 3655–3663.

Akitaya, T., and Bronner-Fraser, M. (1992). Expression of cell adhesion molecules during initiation and cessation of neural crest cell migration. Dev. Dyn. *194*, 12–20.

Barami, K., Kirschenbaum, B., Lemmon, V., and Goldman, S.A. (1994). N-cadherin and Ng-CAM/8D9 are involved serially in the migration of newly generated neurons into the adult songbird brain. Neuron *13*, 567–582.

Bixby, J.L., and Harris, W.A. (1991). Molecular mechanisms of axon growth and guidance. Annu. Rev. Cell Biol. *7*, 117–159.

Bixby, J.L., and Zhang, R. (1990). Purified N-cadherin is a potent substrate for the rapid induction of neurite outgrowth. J. Cell Biol. *110*, 1253–1260.

Bixby, J.L., Grunwald, G.B., and Bookman, R.J. (1994). Ca^{2+} influx and neurite growth in response to purified N-cadherin and laminin. J. Cell Biol. *127*, 1461–1475.

Boller, K., Vestweber, D., and Kemler, R. (1985). Cell-adhesion molecule uvomorulin is localized in the intermediate junctions of adult intestinal epithelial cells. J. Cell Biol. *100*, 327–332.

Chng, S.K., and Straznicky, C. (1992). The generation and changing retinal distribution of dis-placed amacrine cells in Bufo marinus from metamorphosis to adult. Anat. Embryol. *186*, 175–181.

Cornel, E., and Holt, C.E. (1992). Precocious pathfinding: retinal axons can navigate in an axonless brain. Neuron *9*, 1001–1011.

Detrick, R.J., Dickey, D., and Kintner, C.R. (1990). The effects of N-cadherin misexpression on morphogenesis in Xenopus embryos. Neuron *4*, 493–506.

de Wet, J., Wood, K.V., DeLuca, M., Helinski, D.R., and Subramani, S. (1987). Firefly luciferase gene: structure and expression in mammalian cells. Mol. Cell. Biol. *7*, 725–737.

Drazba, J., and Lemmon, V. (1990). The role of cell adhesion molecules in neurite outgrowth on Muller cells. Dev. Biol. *138*, 82–93.

Dufour, S., Saint, J.J., Broders, F., Wedlich, D., and Thiery, J.P. (1994). Differential perturbations in the morphogenesis of anterior structures induced by overexpression of truncated XB- and N-cadherins in Xenopus embryos. J. Cell Biol. *127*, 521–535.

Dunlop, S.A., and Beazley, L.D. (1984). A morphometric study of the retinal ganglion cell layer and optic nerve from metamorphosis in Xenopus laevis. Vision Res. *24*, 417–427.

Evan, G.I., Lewis, G.K., Ramsay, G., and Bishop, J.M. (1985). Isolation of monoclonal antibodies specific for human c-myc proto-oncogene product. Mol. Cell. Biol. *5*, 3610–3616.

Fujimori, T., and Takeichi, M. (1993). Disruption of epithelial cell–cell adhesion by exogenous expression of a mutated nonfunctional N-cadherin. Mol. Biol. Cell *4*, 37–47.

Gimlich, R.L., and Gerhart, J.C. (1984). Early cellular interactions promote embryonic axis formation in Xenopus laevis. Dev. Biol. *104*, 117–130.

Grant, P., and Rubin, E. (1980). Ontogeny of the retina and optic nerve of *Xenopus laevis*. J. Comp. Neurol. *189*, 671–698.

Harris, W.A., and Messersmith, S.L. (1992). Two cellular inductions involved in photoreceptor determination in the Xenopus retina. Neuron *9*, 357–372.

Hatta, K., Takagi, S., Fujisawa, H., and Takeichi, M. (1987). Spatial and temporal expression pattern of N-cadherin cell adhesion molecules correlated with morphogenetic processes of chicken embryos. Dev. Biol. *120*, 215–227.

Hermiston, M.L., and Gordon, J.I. (1995). Inflammatory bowel disease and adenomas in mice expressing a dominant negative N-cadherin. Science *270*, 1203–1207.

Holt, C.E. (1989). A single-cell analysis of early retinal ganglion cell differentiation in Xenopus: from soma to axon tip. J. Neurosci. *9*, 3123–3145.

Holt, C.E., Bertsch, T.W., Ellis, H.M., and Harris, W.A. (1988). Cellular determination in the Xenopus retina is independent of lineage and birthdate. Neuron *1*, 15–26.

Holt, C.E., Garlick, N., and Cornel, E. (1990). Lipofection of cDNAs in the embryonic vertebrate central nervous system. Neuron *4,* 203–214.

Holt, C.E., Lemaire, P., and Gurdon, J.B. (1994). Cadherin-mediated cell interactions are necessary for the activation of MyoD in Xenopus mesoderm. Proc. Natl. Acad. Sci. USA *91*, 10844–10848.

Holtfreter, J. (1943). Properties and functions of the surface coat in amphibian embryos. J. Exp. Zool. *93*, 251–323.

Inuzuka, H., Miyatani, S., and Takeichi, M. (1991). R-cadherin: a novel Ca(2+)-dependent cell–cell adhesion molecule expressed in the retina. Neuron *7*, 69–79.

Kintner, C. (1992). Regulation of embryonic cell adhesion by the cadherin cytoplasmic domain. Cell *69*, 225–236.

Kljavin, I.J., Lagenaur, C., Bixby, J.L., and Reh, T.A. (1994). Cell adhesion molecules regulating neurite growth from amacrine and rod photoreceptor cells. J. Neurosci. *14*, 5035–5049.

Lemmon, V., Burden, S.M., Payne, H.R., Elmslie, G.J., and Hlavin, M.L. (1992). Neurite growth on different substrates: permissive versus instructive influences and the role of adhesive strength. J. Neurosci. *12*, 818–826.

Levine, E., Lee, C.H., Kintner, C., and Gumbiner, B.M. (1994). Selective disruption of E-cadherin function in early Xenopus embryos by a dominant negative mutant. Development *120*, 901–909.

Lilienbaum, A.L., Reszka, A.A., Horwitz, A.F., and Holt, C.E. (1995). Chimeric integrins ex-pressed in retinal ganglion cells impair process outgrowth in vivo. Mol. Cell. Neurosci. *6*, 139–152.

Matsunaga, M., Hatta, K., Nagafuchi, A., and Takeichi, M. (1988a). Guidance of optic nerve fibres by N-cadherin adhesion molecules. Nature *334*, 62–64.

Matsunaga, M., Hatta, K., and Takeichi, M. (1988b). Role of N-cadherin cell adhesion molecules in the histogenesis of neural retina. Neuron *1*, 289–295.

Muller, H.A., Kuhl, M., Finnemann, S., Schneider, S., van der Poel, S.Z., Hausen, P., and Wedlich, D. (1994). Xenopus cadherins: the maternal pool comprises distinguishable members of the family. Mech. Dev. *47*, 213–223.

Murphy, E.C., Napolitano, E.W., and Reichardt, L.F. (1994). The expression of B-cadherin during embryonic chick development. Dev. Biol. *161*, 107–125.

Nagafuchi, A., Ishihara, S., and Tsukita, S. (1994). The roles of catenins in the cadherin-mediated cell adhesion: functional analysis of E-cadherin-α catenin fusion molecules. J. Cell Biol. *127*, 235–245.

Neugebauer, K.M., Tomaselli, K.J., Lilien, J., and Reichardt, L.F. (1988). N-cadherin, NCAM, and integrins promote retinal neurite outgrowth on astrocytes in vitro. J. Cell Biol. *107*, 1177–1187.

Nieuwkoop, P.D., and Faber, J. (1967). Normal Table of *Xenopus laevis* (Amsterdam, North-Holland: Daudin).

Paradies, N.E., and Grunwald, G.B. (1993). Purification and characterization of NCAD90, a soluble endogenous form of N-cadherin, which is generated by proteolysis during retinal development and retains adhesive and neurite-promoting function. J. Neurosci. Res. *36*, 33–45.

Payne, H.R., Burden, S.M., and Lemmon, V. (1992). Modulation of growth cone morphology by substrate-bound adhesion molecules. Cell Motil. Cytoskel. *21*, 65–73.

Redies, C., and Takeichi, M. (1993). N- and R-cadherin expression in the optic nerve of the chicken embryo. Glia *8*, 161–171.

Redies, C., Inuzuka, H., and Takeichi, M. (1992). Restricted expression of N- and R-cadherin on neurites of the developing chicken CNS. J. Neurosci. *12*, 3525–3534.

Redies, C., Engelhart, K., and Takeichi, M. (1993). Differential expression of N- and R-cadherin in functional neuronal systems and other structures of the developing chicken brain. J. Comp. Neurol. *333*, 398–416.

Reichardt, L.F., Bixby, J.L., Hall, D.E., Ignatius, M.J., Neugebauer, K.M., Tomaselli, K.J. (1989). Integrins and cell adhesion molecules: neuronal receptors that regulate axon growth on extracellular matrices and cell surfaces. Dev. Neurosci. *11*, 332–334.

Reszka, A.A., Hayashi, Y., and Horwitz, A.F. (1992). Identification of amino acid sequences in the integrin β1 cytoplasmic domain implicated in cytoskeletal association. J. Cell Biol. *117*, 1321–1330.

Simonneau, L., Broders, F., and Thiery, J.P. (1992). N-cadherin transcripts in Xenopus laevis from early tailbud to tadpole. Dev. Dyn. *194*, 247–260.

Stier, H., and Schlosshauer, B. (1995). Axonal guidance in the chicken retina. Development *121*, 1443–1454.

Suzuki, S., Sano, K., and Tanihara, H. (1991). Diversity of the cadherin family: evidence for eight new cadherins in nervous tissue. Cell Regul. *2*, 261–270.