N-Cadherin Regulates Ingrowth and Laminar Targeting of Thalamocortical Axons

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Thalamocortical axons are precisely targeted to cortical layer IV, but the identity of specific molecules that govern the establishment of laminar specificity in the thalamocortical projection has been elusive. In this study, we test the role of N-cadherin, a homophilic cell adhesion molecule, in laminar targeting of thalamocortical axons using cocultured thalamic and cortical slice explants exposed to N-cadherin function-blocking antibodies or inhibitory peptides. In untreated cocultures, labeled thalamocortical axons normally grow to and stop in layer IV, forming terminal-like arbors. In the N-cadherin-blocked cocultures, thalamic axons reach layer IV by growing through deep layers at the same rate as those in the untreated cocultures, but instead of terminating in layer IV, they continue growing uninterruptedly through layer IV and extend into supragranular layers to reach the outermost cortical edge, where some form terminal-like arbors in this aberrant laminar position. In cocultures in which the cortical slice is taken at an earlier maturational stage, one that corresponds to a time when thalamic axons are normally growing through deep layers before the emergence of layer IV from the cortical plate, thalamic axon ingrowth through deep layers is significantly attenuated by N-cadherin blocking reagents. These data indicate that N-cadherin has multifaceted roles in establishing the thalamocortical projection, governing aspects of both thalamic axon ingrowth and laminar targeting by acting as a layer IV stop signal, which progressively change in parallel with the maturational state of the cortex.

Key words: **synaptogenesis; axon outgrowth; cell adhesion molecules; stop signal; axon targeting; ventrobasal nucleus; barrel cortex; thalamocortical**

Introduction

The termination of thalamic axons in the cerebral cortex is highly ordered in both radial (laminar) and tangential dimensions (Jones, 1998). Such precise axon targeting is the basis for topographic mapping of sensory surfaces onto target layer IV that is fundamental to normal information processing (Harris et al., 2001). Characterizing mechanisms that enable precision in thalamic axon targeting is critical for understanding how thalamocortical function arises and is broadly applicable to understanding how circuit specificity is achieved generally in the CNS.

In the end-stages of thalamocortical innervation, mostly unbranched thalamic axons grow radially through deep layers V and VI, recognize their target layer IV by stopping growth, and elaborate terminal arbors (Senft and Woolsey, 1991; Agmon et al., 1993; Kageyama and Robertson, 1993; Catalano et al., 1996). Thalamic axon ingrowth appears to be regulated by the maturational state of the cortex via differential expression of several types of molecules, including those of the extracellular matrix (Bicknese et al., 1994; Emerling and Lander, 1994, 1996; Miller et al., 1995), Ig superfamily members such as LAMP (limbic system-associated membrane protein) (Mann et al., 1998), ephrins (Gao et al., 1998), and other unidentified membrane-bound molecules (Gotz et al., 1992; Tuttle et al., 1995; Yamamoto et al., 2000a). Other molecules have been identified that selectively regulate arborization of thalamic and other types of axons (Cohen-Cory and Fraser, 1995; Inoue and Sanes, 1997; Borrell et al., 1999; Castellani and Bolz, 1999; Wang et al., 1999; Boylan et al., 2000; Vanderhaeghen et al., 2000; Yamamoto et al., 2000b; Yates et al., 2001; Mann et al., 2002; Ozdinler and Erzurumlu, 2002; Rebsam et al., 2002). Different molecular cues appear to regulate axon arbor formation and growth-arrestin layer IV because these events can occur independently (Yamamoto et al., 1997).

In contrast to these advances, the molecules that signal thalamic axon growth-arrest (stopping) in layer IV are unknown. Evidence suggests that layer IV cells furnish a cell-surface-bound molecule specifically recognized by thalamic axons (O'Leary et al., 1994; Molnár and Blakemore, 1995; Bolz et al., 1996; Yamamoto, 2002). Recent studies show that N-cadherin, one of a family of transmembrane glycoproteins that mediates Ca^{2+}-dependent, predominantly homophilic adhesion (Geiger and Ayalon, 1992), plays an important role in retinal cell axon outgrowth and laminar targeting in the developing visual system of flies, frogs, and chicks (Riehl et al., 1996; Inoue and Sanes, 1997; Lee et al., 2001). This raises the possibility that cadherins function similarly in establishing the mammalian thalamocortical projection. Compelling support for this hypothesis comes from studies of cadherin localization during development of the thalamocortical innervation of rat somatosensory (barrel) cortex (Huntley and Benson, 1999; Gil et al., 2002). N-cadherin is expressed by neurons in layer IV and by those of the thalamic ventrobasal (VB) nucleus. The N-cadherin protein is rapidly upregulated in layer IV contemporaneously with the arrival of thalamic axons and progressively accumulates at thalamocortical synapses during

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subsequent formation of the characteristic whisker-map. N-cadherin may therefore function as a stop-cue by virtue of homophilic recognition between ingrowing thalamic axons and their target layer IV neurons.

Here, we test this hypothesis by using N-cadherin function-blocking antibodies and peptides in organotypic thalamic and cortical explant cocultures. Organotypic cocultures are ideal for molecular perturbation studies because cortical lamination is preserved and laminar targeting by thalamic axons is remarkably precise and similar to that *in vivo* (Gotz and Bolz, 1992; Yamamoto et al., 1992; Molnár and Blakemore, 1999). Our results indicate that N-cadherin regulates thalamic axon ingrowth through deep layers and functions as a layer IV target-layer recognition (stop) signal.

Materials and Methods

Animals. All thalamic and cortical slice explants were taken from Sprague Dawley rats. The first appearance of the vaginal plug was denoted embryonic day (E) 0; the first 24 hr after birth was designated postnatal day (P) 1. The care and treatment of all animals were in strict accordance with guidelines established by the National Institutes of Health and protocols approved by Mount Sinai's Institutional Animal Care and Use Committee.

Coculture preparation. Cocultures of thalamic and cortical slice explants were prepared under sterile conditions. The numbers of explants prepared and the particular experimental conditions to which they were subjected are given in Table 1. For all coculture experiments, thalamic explants were obtained from E16 embryos (Molnár et al., 1998). Fetuses were removed by Caesarian section from timed-pregnant mothers that were asphyxiated by regulated delivery of CO_2. Brains were rapidly removed, immersed in chilled Gey's balanced salt solution (BSS; Invitrogen, Carlsbad, CA), and hemisected. Under microscope guidance, an explant of the presumptive VB nucleus was isolated using landmarks determined previously from pilot experiments in which the position of the VB analage was identified in E16 brains by visualizing the thalamic (VB nucleus) terminations of the medial lemniscus labeled by placing crystals of the carbocyanine dye 1,1′-dioctadecyl-3,3,3′-tetramethylindo-carbocyanine perchlorate (DiI; Molecular Probes, Eugene, OR) into the dorsal column nuclei. Thalamic explants were placed onto collagen-coated membranes (pore size, 0.4 μm; Corning Costar Transwell inserts; Corning, Corning, NY) and placed into Petri dishes containing 3.5 ml of serum-based media. The composition of the culture media (Gähwiler, 1981) included 50 ml basal Eagle medium with Earle's salts, 25 ml Earle's balanced salt solution, 25 ml heat-inactivated horse serum, 1 ml 50% glucose, and 0.5 ml 200 mM L-glutamine (all reagents from Invitrogen). Thalamic explants were incubated for 2–3 hr in a humidified 37°C incubator with a continuous flow of 5% CO_2 before the cortical explants were added.

Cortical slice explants were obtained from postnatal rat pups aged either P1 or P6 (see Results for the rationale for these two representative ages). Pups were immobilized by hypothermia and decapitated, and the brains were rapidly isolated and immersed in chilled Gey's BSS. After removal of the pia mater, brains were then sliced in the frontal plane on a vibratome at a setting of 300 μm (for P1 brains) or 400 μm (for P6 brains). Explants were prepared from the dorsolateral aspect of the slices through the primary somatosensory cortex (S1) by making radial pial-to-white-matter cuts and removing most of the underlying white matter. Each cortical slice explant was then placed onto a collagen insert and paired with a single thalamic explant by positioning the cortical slice with its ventral-most (white matter) edge apposed to the thalamic piece, separated by ~0.5 mm. The cocultures were held stationary in the incubator for 24 hr and then transferred to a rocking platform for the remainder of the culture period. Media was replaced every 2–4 d, and a mixture of anti-mitotic inhibitors (10^{-6} M of cytosine arabinoside, uridine, and fluorodeoxyuridine (Sigma, St. Louis, MO) was added on day 2 or 4 for 24 hr. Cocultures were maintained for variable periods ranging from 1 to 10 d *in vitro* (DIV), depending on the experimental conditions (see Table 1). At removal, cocultures were fixed in 4% paraformaldehyde. The cocultures listed in Table 1 were all deemed healthy by several criteria including the appearance of laminar architecture, morphology of cortical neurons (either back-labeled by thalamic DiI injection or transfected with green fluorescent protein; see below), and the ability of thalamic axons to grow through the slices, which never occurs in unhealthy slices. Thus, all cocultures listed in Table 1 were included in this study. An additional 11 cocultures became contaminated and were immediately discarded.

Carbocyanine dye labeling of thalamocortical axons. Thalamocortical axons were visualized in paraformaldehyde-fixed cocultures by labeling with the carbocyanine dye DiI. DiI was dissolved in dimethylformamide, to produce a 0.5% solution, and pressure-injected through a glass micropipette attached to a Picospritzer. Under microscope guidance, multiple closely spaced injections were placed around the perimeter and interior of the thalamic explant to maximize thalamic axon labeling. The cocultures were then counterstained with 4′, 6′-diamidino-2-phenylindole (DAPI; Sigma) to reveal laminar architecture, placed into 0.1 M PBS containing 0.2% sodium azide, and incubated at 37°C in the dark for 2–4 weeks.

N-cadherin function-blocking experiments. Two different types of function-blocking reagents (synthetic inhibitory peptides and N-cadherin-blocking antibodies) were applied to cocultures in separate experiments to perturb N-cadherin function (see Table 1). Both types of blocking reagents are directed against portions of the distalmost N-cadherin ectodomain (EC1) region, thought to be critical for cadherin–cadherin adhesive binding *in trans* (Nose et al., 1990; Shapiro et al., 1995). A 16 mer synthetic peptide (HLRAHAVDINGNQVEN) containing the conserved histadine–alanine–valine (HAV) cadherin recognition sequence, present within the EC1 presumptive binding domain of all type I classic cadherins, was generated with flanking amino acids corresponding to a mouse N-cadherin sequence (Miyatani et al., 1989). It is likely that the HAV peptide is specific for N-cadherin because previous studies have shown that the flanking sequence confers cadherin-type specificity (Noe et al., 1999; Williams et al., 2000). A second scrambled (SCR) peptide (ARLQHDVNANVHEING) was applied to cocultures as a control. HAV peptides have been shown to inhibit a variety of cadherin-based functions, including neurite outgrowth from sensory and hippocampal neurons (Blaschuk et al., 1990; Doherty et al., 1991), and they block hippocampal long-term potentiation (Tang et al., 1998). Peptides were first added to the media at the time cortical explants were positioned adjacent to the thalamic piece and used at a final concentration of 200 μg/ml (Doherty et al., 1991). Peptides were replenished each time the media was changed.

An N-cadherin-blocking antibody (1260; generous gift from Dr. David Colman, Montreal Neurological Institute, Montreal, Quebec, Canada) was raised against an N-cadherin EC1 fusion protein. The function-blocking attributes and specificity of this antibody have been detailed previously (Bozdagi et al., 2000). This antibody recognizes N-cadherin in Western blot analysis of rat brain hippocampal tissue in which labeling can be competed out with purified N-cadherin EC1 protein but not BSA. The antibody blocks adhesive aggregation of N-cadherin-transfected L-cells in a standard adhesion assay and blocks the late phase of hippocampal long-term potentiation. Recently, it has also been used to perturb junction formation between Schwann cells and axon-Schwann cell alignment (Wanner and Wood, 2002). As an additional measure of the specificity of the function-blocking antibody under the culturing conditions imposed by our experiments, immunoblotting of cultured and acutely dissected somatosensory cortical tissue was performed (see Fig. 5). Cultures from P1 and P6 pups were prepared as described above ($n = 9$ cultures per each age; maintained 5 DIV). After the culture period, samples were prepared from the cortical explants as well as from acutely dissected somatosensory cortex taken from P1 and P6 pups ($n = 3$ pups for each age). Samples were lysed in 5% SDS, adjusted to equal protein concentrations (20 μg total protein per condition), and separated by 7.5% SDS-PAGE. Samples were then transferred to nitrocellulose and probed overnight with N-cadherin antibody 1260. Immunoblots were developed by alkaline phosphatase colorimetric reaction. For the function-blocking experiments, N-cadherin antibody or

Table 1. Experimental conditions of the thalamic–cortical cocultures used in this study

Age[a]	DIV	Treatment/experiment	Number of slices	Number of axon endpoints counted per slice (mean ± SEM)
P1	5	None/immunocytochemistry	5	
P1	5	None/*in situ* hybridization	6	
P1	8	GFP transfection/Nissl	3	
P1	5	Immunoblot	9	
P1	5–10	None/DiI labeling	40	223 ± 22
P1	5 or 10	SCR peptide/DiI labeling	14	197 ± 20
P1	5 or 10	HAV peptide/DiI labeling	16	215 ± 18
P1	5 or 10	N-cadherin IgG/DiI labeling	12	189 ± 24
P1	5 or 10	Preimmune IgG/DiI labeling	14	210 ± 17
P6	5	None/immunocytochemistry	6	
P6	5	None/*in situ* hybridization	5	
P6	8	GFP transfection/Nissl	4	
P6	5	Immunoblot	9	
P6	5–10	None/DiI labeling	37	242 ± 17
P6	5 or 10	SCR peptide/DiI labeling	17	203 ± 31
P6	5 or 10	HAV peptide/DiI labeling	12	251 ± 18
P6	5 or 10	N-cadherin IgG/DiI labeling	10	199 ± 17
P6	5 or 10	Preimmune IgG/DiI labeling	11	214 ± 18
P6	1	None or N-cadherin IgG/DiI labeling	3 each condition	157 ± 22
P6	2	None or N-cadherin IgG/DiI labeling	3 each condition	198 ± 17
P6	3	None or N-cadherin IgG/DiI labeling	3 each condition	176 ± 18
P6	4	None or N-cadherin IgG/DiI labeling	3 each condition	166 ± 15

[a]Age at time of plating. All cortical slices were paired with E16 thalamus.

preimmune serum were first added to the media at the time cortical explants were positioned adjacent to the thalamic piece and used at a final dilution of 1:100. Antibody or preimmune serum was replenished each time the media was changed. At the end of the designated culture period (see Table 1), cocultures were fixed and counterstained with DAPI, and thalamocortical axons were labeled with DiI as described above.

Microscopy and quantitative analysis. The extent to which DiI-labeled thalamocortical axons grew into the cortical slices under the various control and blocking conditions was analyzed quantitatively using a computer-interfaced Axiophot Photomicroscope and Neurolucida morphometry software (MicroBrightField, Colchester, VT). Laminar boundaries and the outermost (pial) edge of the cortical slices were first traced using a 20× objective under fluorescent illumination appropriate for visualizing the DAPI-stained cytoarchitecture. Next, DiI-labeled axons were visualized using the appropriate fluorescent filter and analyzed in successive microscope fields that, in sum, systematically tiled the entire cortical slice spanning the pial surface to the white-matter edge. In each microscope field, all terminal endings (end-points) within that field were marked. Terminal endings were considered for this analysis to be the end of a fiber (Palmer et al., 2001). Software was then used to calculate the shortest distance between each axon end-point and the circumferential arc representing the pial edge, providing for each axon end-point a measure of its shortest distance from the cortical surface. Mean values of axon end-point distances from the cortical surface and total number of end-points counted were compiled for each coculture and compared with similarly treated cocultures using Student's *t* tests (within-group comparisons; level of significance, $p < 0.05$). Significant differences between the different treatment groups in axon end-point distance from the cortical surface, and numbers of axon end-points, were evaluated using ANOVA and a *post hoc* Scheffé's test (across-group comparisons; level of significance, $p < 0.05$). Schematic maps of DiI-labeled thalamocortical axons were generated using Neurolucida software by tracing labeled axons from their position of entry into the cortical slice at the white-matter side to their terminal ending. Images of the DiI-labeling patterns were acquired by capturing single optical sections using a Zeiss LSM 410 confocal microscope (Zeiss, Thornwood, NY). The images were imported into Adobe Photoshop (Adobe Systems, San Jose, CA) where minimal adjustments in contrast and brightness were made. Final figure layout and graphics were completed using QuarkXpress 4.1 (Quark, Denver, CO). To ensure that DiI-labeled axons grew within the cortical slice rather than on its surface, a representative series of DiI-labeled cocultures from all treatment groups was additionally analyzed by confocal microscopy. Cortical slices were optically sectioned in the *z*-axis plane from surface to surface (i.e., from that closest to the air to that closest to the collagen membrane) in 5 μm increments to generate a collapsed projection that was viewed orthogonally. In all cases, DiI-labeled axons were present throughout the full depth of the tissue slices (data not shown).

Immunocytochemistry. Procedures, reagents, and confocal microscopy were used as detailed previously (Huntley and Benson, 1999; Bozdagi et al., 2000; Gil et al., 2002). Briefly, cocultures were immersed in blocking solution (3% bovine serum albumin and 5% normal serum) and then exposed to a guinea-pig N-cadherin polyclonal antibody (1273; generous gift from Dr. David Colman, Montreal Neurological Institute) that was raised against a portion of the EC1 ectodomain of N-cadherin. After overnight incubation, antibody binding was visualized by subsequent incubation in biotinylated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA) followed by streptavidin-conjugated Alexa 488 (Molecular Probes, Eugene, OR). For double-labeling experiments, cocultures were exposed to a mixture of N-cadherin antibody and a mouse synaptophysin antibody (1:10; Boehringer Mannheim, Carlsbad, CA). Binding of one primary antibody was visualized as described above, whereas the other was visualized using species-appropriate secondary antibodies directly conjugated to Cy3 or Cy5 (Jackson ImmunoResearch). Control experiments consisted of omitting the primary antibody or replacing it with preimmune serum. Single optical sections were acquired from the confocal microscope using a 100× objective. Emission spectra were clearly separated as determined by comparing data collected from two channels simultaneously with a dichroic beam splitter and with those obtained sequentially with one laser line.

In situ *hybridization histochemistry.* A radioactively labeled (^{35}S) antisense N-cadherin cRNA probe was used to identify the distribution of N-cadherin mRNAs in the cocultures. The construction and specificity of the N-cadherin cRNA probe and the hybridization histochemical methods used to visualize probe hybridization were described previously in detail (Huntley and Benson, 1999; Gil et al., 2002). Control cocultures were hybridized with the sense-strand probe.

Green fluorescent protein transfection. A Bio-Rad (Hercules, CA) Helios gene gun was used to transfect neurons in the cocultures with a green fluorescent protein (GFP)-containing plasmid (pEGFP-N1; Clontech, Palo Alto, CA) via particle-mediated gene transfer. Gold particles (1.0 μm; Bio-Rad) were coated with DNA according to procedures supplied

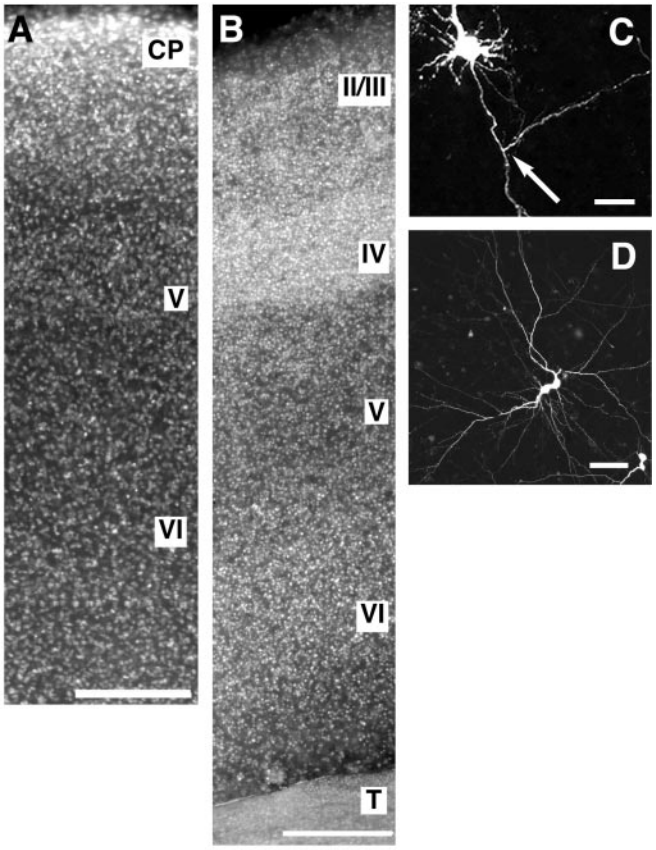

Figure 1. Lamination and neuronal morphology in normal (untreated) cocultures. *A*, *B*, Fluorescent DAPI-staining of cortical slices taken from P1 animal (*A*) or P6 animal (*B*) cocultured with E16 thalamus for 8 DIV showing largely normal cortical lamination. *C*, *D*, GFP-transfected neurons in layer III of P6 cortical slice (*C*) or E16 thalamus (*D*) from cocultures maintained 8 DIV. Layer III pyramidal neuron (*C*) has a labeled axon that gives rise to a horizontally oriented collateral (*arrow*); thalamic cell (*D*) displays multipolar morphology typical of relay neurons. *CP*, Cortical plate; *T*, thalamus. Orientation of all images: pial-most cortical surface is toward the *top*. Scale bars: *A*, 250 μm; *B*, 200 μm; *C*, *D*, 50 μm.

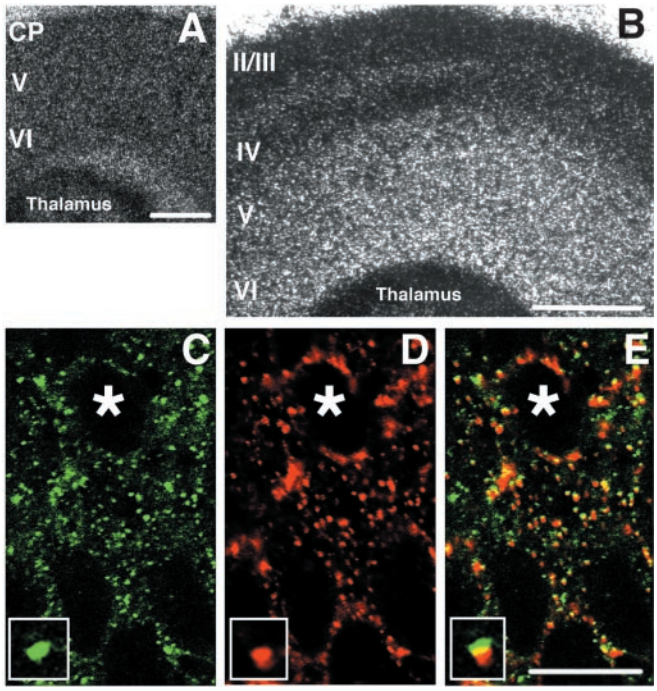

Figure 2. N-cadherin mRNA and protein distribution in normal cocultures. *A*, *B*, Film autoradiograms showing N-cadherin mRNA probe hybridization in P1 cortical slice (*A*) or P6 cortical slice (*B*) from cocultures maintained 5 DIV. Laminar patterns of N-cadherin mRNA probe hybridization under culture conditions are similar to those in acutely fixed slices at comparable ages. *C–E*, Immunofluorescent localization of N-cadherin (*C*) or synaptophysin (*D*) in P6 cortical slice from coculture maintained 5 DIV. Many N-cadherin puncta codistribute with synaptophysin puncta indicating synaptic localization, as shown by the *overlay* (*E*) and at higher power in the *insets*, where *yellow* indicates regions of codistribution of the two markers. The *large black holes* are unlabeled cell somata, one of which is demarcated by the *asterisks*. Orientation of all images: pial-most cortical surface is toward the *top*. Scale bars: *A*, *B*, 500 μm; *C–E*, 10 μm.

by Bio-Rad. Cocultures were removed from the incubator (see Table 1), transfected, and then returned to the incubator for 24 hr before fixation.

Results

A total of 242 cocultured thalamic and somatosensory cortical slice explants were used in this study (Table 1). In all cases, thalamic explants were taken from E16 embryos, an age when thalamic axons are extending readily *in vivo* (Catalano et al., 1996; Molnár et al., 1998). Cortical slice explants were taken from P1 or P6 pups. Our rationale for these two ages was to test the role of N-cadherin adhesion in thalamic axon innervation at two representative stages: an early stage (P1) when thalamocortical axons are normally growing through deep layers just before the emergence of layer IV from the dense cortical plate (~P2), and a later stage (P6) when layer IV is present.

Lamination and cellular morphology in normal organotypic cocultures

Lamination in the cocultured cortical slice explants was evident but less distinct in comparison with acute slices (Fig. 1*A*,*B*). In the P1 cortical slices, a dense band of cells abutting the pial-most surface of the slice presumably corresponds to the cortical plate, whereas deeper presumptive layers V and VI were discernable (Fig. 1*A*). In the P6 cortical slices, presumptive layer IV was evident as a prominent band of more densely packed cells, whereas supragranular and infragranular layers were also apparent (Fig. 1*B*). These features of laminar architecture are similar to those described previously (Gotz and Bolz, 1992; Yamamoto et al., 1992; Molnár and Blakemore, 1999).

Cellular morphology in both the cortical and thalamic slice explants appeared normal and healthy after culturing for 5–10 DIV. In the cortical slices, GFP-transfected pyramidal neurons were found throughout all layers of the slice, and all were properly oriented with a pial-directed apical dendrite and numerous basilar dendrites (Fig. 1*C*). Labeled axons all emerged from the base of the soma and descended toward the white matter side of the slices, often emitting horizontally oriented collaterals (Fig. 1*C*, *arrow*). In the thalamic explants, GFP-transfected neurons displayed a range of somal sizes and had a multipolar morphology characteristic of thalamocortical relay neurons (Fig. 1*D*).

Expression and synaptic localization of *N*-cadherin in normal organotypic cocultures

In situ hybridization histochemistry revealed that *N*-cadherin mRNAs were abundantly expressed in the cortical and thalamic explants after 5–10 DIV (Fig. 2*A*,*B*). No hybridization was evident in the cultures treated with the sense-strand probe (data not shown). In the P1 explants, hybridization signal of relatively homogeneous intensity extended from the cortical plate through layer VI (Fig. 2*A*), a pattern comparable with that observed in paraformaldehyde-fixed P1 slices through barrel cortex (Obst-Pernberg et al., 2001; Gil et al., 2002). In the cocultured P6 slices,

laminar variations in the intensity of the hybridization signal, specifically superficial and middle-layer bands of greater signal intensity, were evident (Fig. 2*B*). The middle-layer band corresponded to layer IV, a general pattern similar to that observed in paraformaldehyde-fixed P6 tissue slices (Huntley and Benson, 1999; Gil et al., 2002). In contrast, the intensity of probe hybridization in layer VI, although evident in the P6 cortical explant, appeared less in comparison with that in layer VI of acute slices. It is unknown whether this is attributed to a lower mRNA expression level in layer VI *in vitro* or reflects differences in packing density of cells as a result of culturing.

N-cadherin immunolabeling in the cocultured cortical explants revealed numerous puncta distributed throughout all of the layers of the slice in both P1 and P6 cortical explants (Fig. 2*C*). Double-immunofluorescent labeling with synaptophysin (Fig. 2*D*), a synaptic vesicle protein used as a marker of presynaptic boutons, demonstrated that most of the N-cadherin labeling is codistributed with that for synaptophysin, indicating that N-cadherin is largely localized to the synaptic junctional complex (Fig. 2*E*). However, some N-cadherin puncta were not synaptophysin co-positive, presumably representing nonsynaptic puncta adherens. Together, these data indicate that patterns of N-cadherin mRNA expression and synaptic distribution are largely similar after 5–10 DIV to those in acutely fixed tissue slices and are not overtly altered by culturing conditions.

Normal pattern of thalamic axon innervation of P1 cortical slice explants

After 5–10 DIV, cocultures were fixed, and small multiple injections of DiI were placed into the thalamic explants to label the thalamic axons within the cortical slices. Figure 3 shows the normal pattern of thalamic axon ingrowth into P1 cortical slices after 5 DIV, a pattern which is similar to that observed in cocultures maintained *in vitro* for longer periods (≤10 DIV). In untreated (control) cocultures, DiI-labeled thalamic axons grew robustly into the cortical slices. Axons were oriented predominantly radially and were mostly unbranched (Fig. 3*A*,*B*). Many of these were fasciculated (Fig. 3*C*, *arrow*), whereas numerous other single axons were studded along their trajectories with small varicosities that looked like en passant boutons. Because at this early stage the cortical slices lack a layer IV, the bulk of the axons grew readily through layers VI and V, terminating close to the pial-most surface of the cortical slice (Fig. 3*A*,*B*). At their terminal ends, many axons were tipped with growth cones, even after 10 DIV (Fig. 3*C*, *inset*), whereas others ended in what appeared to be a spray of terminal boutons (Fig. 3*D*). In some cases, axons penetrated the cortical plate. These general characteristics of normal thalamic axon ingrowth and termination in the P1 cortical explants are identical to those described previously (Yamamoto et al., 1992; Molnár and Blakemore, 1999).

Blocking N-cadherin in P1 cortex alters thalamic axon ingrowth through deep layers

We exposed otherwise identically prepared cocultures to one of two different classes of N-cadherin-blocking reagents. In one set of coculture experiments (Table 1), we introduced at the time of plating an HAV-blocking peptide or a scrambled control peptide (see Materials and Methods for details of the peptides). Exposure to HAV peptide significantly attenuated thalamic axon ingrowth through deep layers after 5 DIV (Fig. 4*A*,*E*,*F*), with the majority of the axons terminating on average ~500 μm deep to the cortical surface in layers V and VI (Fig. 4*A*, *arrows*, *E*,*F*). This effect could not be attributed to differences across conditions in the mean numbers of axon end-points counted (Table 1) ($p > 0.1$) and was apparent in cocultures maintained for up to 10 DIV, indicating that at least within this doubled timeframe, axons were not simply proceeding to the pial surface at a slower pace. Moreover, there appeared to be fewer fasiculated bundles of axons, and rather than a mostly radial orientation typical of the untreated cocultures, the trajectory of many axons was oblique or horizontal (Fig. 4*E*). However, many of these axons retained the periodic varicosities typical of en passant boutons. Additionally, in deep layers, there were occasional dense collections of labeled processes that were clumped together in a roughly circular pattern (Fig. 4*B*, *arrowheads*, *inset*), something that was never observed in the untreated cocultures. In contrast with these abnormal features, a small variable number of axons in the HAV-treated cocultures did reach the pial-most surface after 5 DIV or longer (Fig. 4*A*, *arrowheads*, *E*), and many of these were studded with en passant boutons similar to those in the untreated cocultures. It is unlikely that the HAV peptide effects on axon ingrowth were caused by some nonspecific toxic effect or gross changes in neuronal integrity. Lamination appeared normal, and a number of deep-layer pyramidal cells were fortuitously back-labeled by the DiI thalamic injections (Fig. 4*B*, *arrow*), indicating that cortical neurons were capable of sending an axonal projection to the thalamic explant in the presence of HAV. Additionally, although N-cadherin has been implicated in dendritic outgrowth (Esch et al., 2000), the dendritic morphology of the back-labeled pyramidal cells appeared grossly normal (Fig. 4*B*, *arrow*).

In contrast to the dramatic effects on ingrowth evident in the HAV cocultures, those exposed to the scrambled peptide ap-

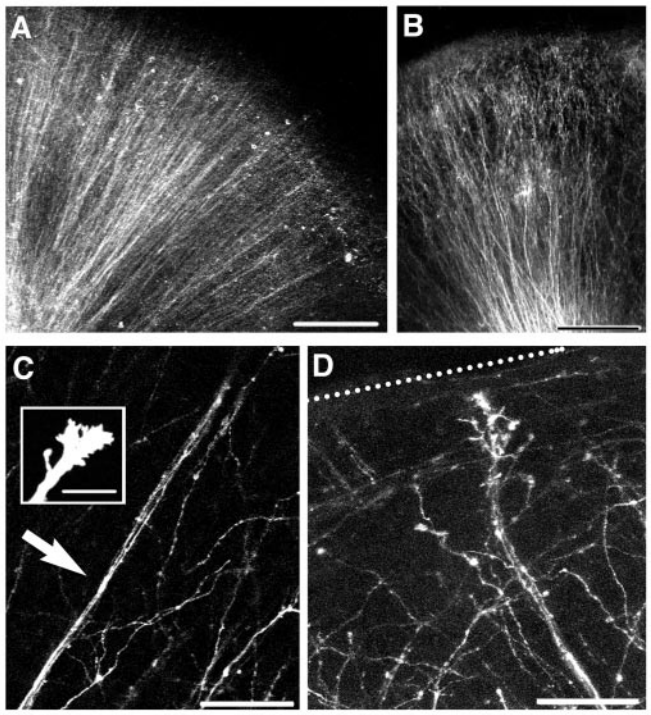

Figure 3. Normal patterns of thalamocortical axon ingrowth in P1 cortical slices. *A*, *B*, DiI-labeled axons grow mostly unbranched and radially oriented through deep layers, extending close to the pial-most surface. *C*, Many such axons are fasciculated (*arrow*); other single axons display periodic varicosities along their length that look like en passant boutons, and growth cones are prominent (*inset*). *D*, Many of those that end close to the pial-most surface possess terminal-like boutons. The *dotted line* delineates the pial-edge of cortical slice. All images are taken from untreated cocultures maintained for 5 DIV. Orientation of all images: pial-most cortical surface is toward the *top*. Scale bars: *A*, *B*, 250 μm; *C*, *D*, 50 μm; *C*, *inset*, 10 μm.

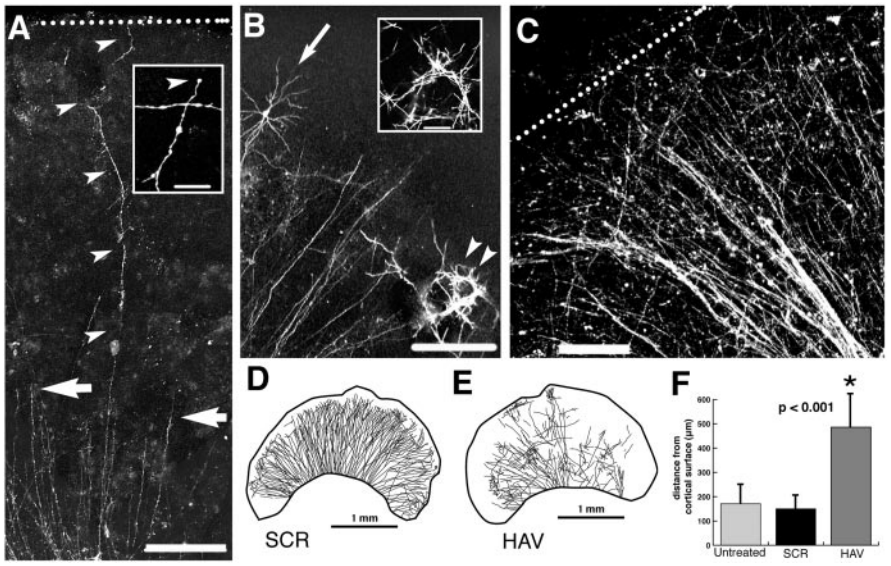

Figure 4. Thalamic axon ingrowth through deep layers is disrupted by N-cadherin-blocking peptide. *A–E*, Confocal images (*A–C*) or maps (*D*, *E*) of DiI-labeled thalamocortical axons in P1 cortical slices from cocultures exposed to HAV-peptide (*A*, *B*, *E*) or from those exposed to a scrambled (*SCR*) control peptide (*C*, *D*). The majority of DiI-labeled thalamocortical axons in the HAV cocultures terminate abnormally in deep layers (*A*, *arrows*), and many have oblique or horizontal trajectories (*E*). This altered pattern is significantly different from untreated cocultures or those treated with the SCR peptide (*F*, *asterisk*) (means + SEM). Some axons do extend to the pial surface (*A*, *arrowheads*, *E*). Axons in the HAV-treated cocultures appear grossly normal, ending in growth cones (*A*, *inset*, *arrowhead*) and possessing en passant-like boutons along their length. Neuronal morphology also appears normal, and cortical cells remain capable of sending an axon to the thalamus (*B*, *arrow*) (backfilled corticothalamic cell is shown). However, there were occasional dense whorls of labeled processes in deep layers (*B*, *arrowheads*, *inset*,) that were never observed in untreated cocultures. The pattern and extent of thalamocortical axon ingrowth in cocultures treated with the SCR peptide (*C*, *D*) are indistinguishable from untreated cocultures (*F*). All images are taken from cocultures maintained for 5 DIV. Orientation of all images: pial-most cortical surface is toward the *top*. *Dotted lines* (*A*, *C*) delineate pial-most surface. Thalamic explants are not shown in maps (*D*, *E*) for clarity. Scale bars: *A*, 125 μm; *A*, *inset*, 10 μm; *B*, 250 μm; *B*, *inset*, 10 μm; *C*, 100 μm.

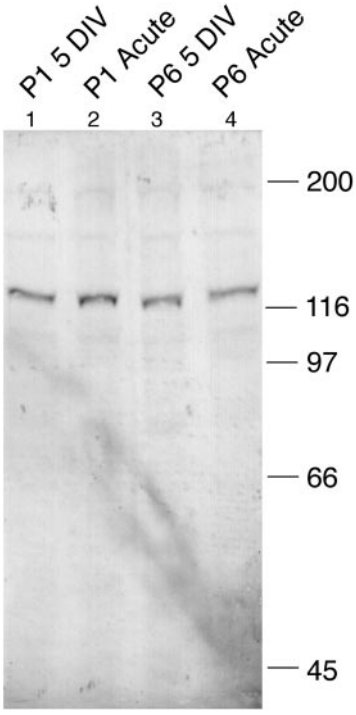

Figure 5. Function-blocking antibody 1260 is specific for N-cadherin and does not cross-react with other proteins. Immunoblots of tissue samples from P1 or P6 cultures maintained 5 DIV (*lanes 1*, *3*) or from P1 or P6 acutely dissected S1 cortex (*lanes 2*, *4*). Under all conditions, only a single band of the expected size of N-cadherin (~127 kDa) is observed. Size standards are shown on the *right*.

peared indistinguishable from the untreated cocultures (Fig. 4*C*,*D*), with no qualitative or quantitative differences in the extent to which thalamic axons grew into the cortical slices (Fig. 4*F*).

To corroborate the effects observed with HAV peptides, we exposed a second set of cocultures to a function-blocking N-cadherin antibody raised against the EC1 binding domain. This antibody is shown by Western blot analysis to be specific for N-cadherin and without cross-reactivity in tissue samples from either P1 or P6 cultures maintained 5 DIV as well as from acutely dissected P1 or P6 somatosensory cortex (Fig. 5) (see Materials and Methods for additional details of specificity and function-blocking attributes). In cultures exposed to N-cadherin IgG for 5 or 10 DIV (Table 1), thalamic axon ingrowth was significantly attenuated, with the majority of the axon terminations restricted to deeper layers ~500 μm from the pial-most surface (Fig. 6*A*,*D*). This pattern was identical to that seen in the HAV cultures and could not be attributed to differences across conditions in the mean number of axon end-points counted (Table 1) ($p > 0.1$). Also similar to the HAV cultures, a small number of axons did grow to the cortical surface (Fig. 6*B*, *arrowheads*). Many axons were tipped with growth cones (Fig. 6*A*, *inset*) and also appeared to grow more obliquely or horizontally than the mostly radially oriented axons of the untreated cocultures (Fig. 6*A*, *arrow*). However, in contrast to the HAV cocultures, there were no circularly oriented clumps of labeled processes such as those shown in Figure 4*B*. The reason for the apparent discrepancy between the two types of blocking reagents in this particular aspect of perturbed ingrowth is unclear, but it is possible that the HAV peptide affects other molecules that possess an HAV motif, or the peptide and the antibodies differentially affect N-cadherin-dependent intracellular-signaling mechanisms required for growth or fasciculation (Bixby and Jhabvala, 1990). Thalamic axon ingrowth in cocultures exposed to pre-immune serum was qualitatively (Fig. 6*C*) and quantitatively (Fig. 6*D*) identical to untreated cocultures. Together, these data indicate that thalamic axons require N-cadherin to navigate through deep layers of somatosensory cortex at early stages of innervation when they are normally growing through deep layers toward the cortical plate.

N-cadherin acts as a target-layer recognition (stop) signal at later stages

Our next step was to examine the effects of disrupting N-cadherin at a later stage of thalamocortical innervation (P6) when the target layer IV is present. In untreated (control) P6 cocultures maintained 5–10 DIV, DiI-labeled thalamic afferents grew mostly unbranched and radially oriented through deep layers but now, at this stage, ended abruptly in a broad middle-layer band that corresponded to presumptive layer IV, ~400–500 μm deep to the cortical surface (Figs. 7*A*, *arrow*, 8*A*). Here, many axons branched and formed terminal-like arbors (Fig. 7*A*, *inset*) and were studded with en passant and terminal boutons. Such lamina-specific termination patterns are identical to cocultured thalamic and corti-

cal explants described previously (Bolz et al., 1992; Yamamoto et al., 1997; Molnár and Blakemore, 1999; Palmer et al., 2001).

The pattern of thalamic axon ingrowth and termination in P6 cocultures treated with N-cadherin-blocking reagents was strikingly altered in comparison with untreated control cocultures (Figs. 7, 8). In those treated with HAV peptide, most thalamic afferents grew readily to, and through, presumptive layer IV, virtually reaching the outermost cortical edge (Fig. 7*B*), a highly significant deviation from the normal pattern of middle layer-specific termination (Fig. 7*E*). This enhanced growth pattern was already evident by 5 DIV and was similar to that found in cocultures maintained for 10 DIV. As described previously, these patterns could not be attributed to differences across conditions in numbers of end-points counted (Table 1) ($p > 0.1$). Most of the axons traversed the cortical slices radially oriented and were unbranched until they reached within 100–200 μm from the pial surface (Fig. 7*B*). Here, axons branched (Fig. 7*B*, *arrowheads*), with some forming terminal-like arbors and others ending in growth cones (Fig. 7*B*, *arrow*). Unlike the P1 HAV cocultures, there were no abnormal whorls of labeled processes found in any layers of the P6 cortical slice cocultures. In contrast with the apparent elimination of layer IV-specific targeting as a consequence of HAV treatment, thalamic axon ingrowth and termination in cocultures treated with the scrambled peptide appeared qualitatively (Fig. 7*C*,*D*) and quantitatively (Fig. 7*E*) indistinguishable from untreated control cocultures.

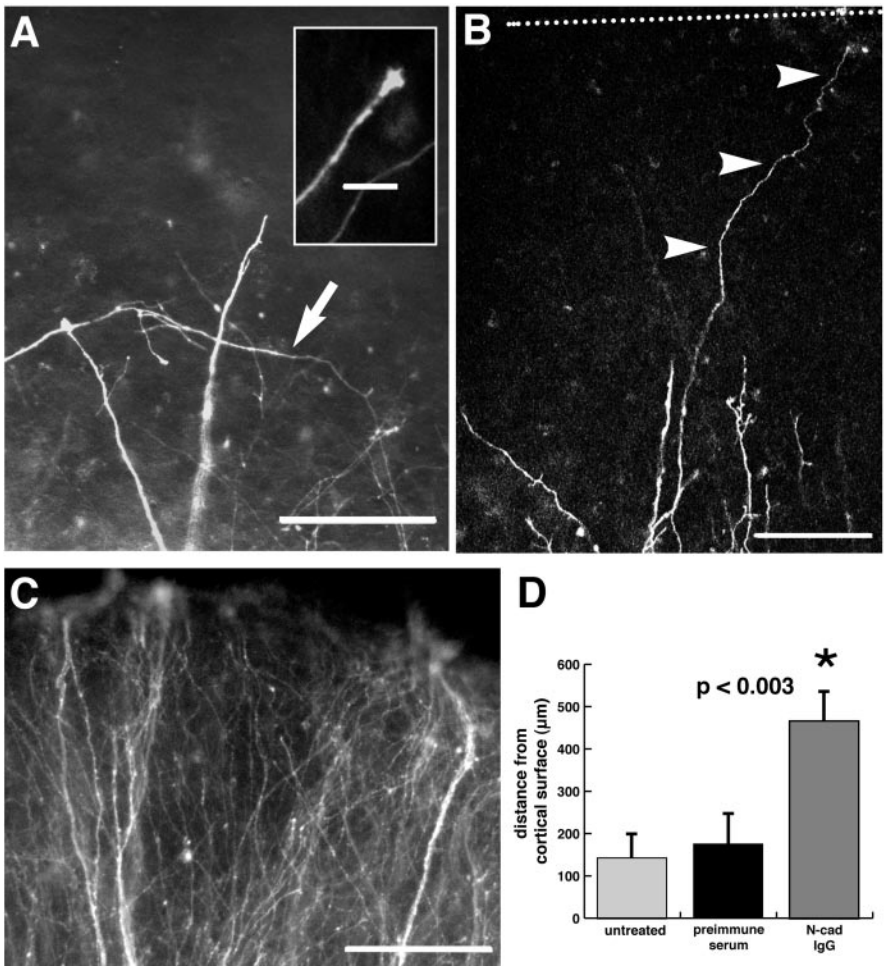

Figure 6. Thalamic axon ingrowth through deep layers is altered by N-cadherin function-blocking antibody. *A–C*, Confocal images of DiI-labeled thalamocortical axons in P1 cortical slices from cocultures exposed to blocking antibody (*A*, *B*) or preimmune serum (*C*). In the antibody-treated cocultures, most of the thalamic axons terminate in deep layers (*A*, *B*), and many have oblique or horizontal trajectories (*A*, *arrow*). This altered pattern is significantly different in comparison with untreated cocultures (*D*, *asterisk*) (means + SEM). Axons otherwise appear grossly normal, some with growth cones (*A*, *inset*) and en passant-like boutons. Some axons in the antibody-treated cocultures do extend to the pial-most surface (*B*, *arrowheads*). Ingrowth in the cocultures exposed to preimmune serum is indistinguishable from untreated cocultures (*C*, *D*). All images are taken from cocultures maintained for 5 DIV. Orientation of all images: pial-most cortical surface is toward the *top*. *Dotted line* (*B*) delineates pial-most surface. Scale bars: *A–D*, 100 μm; *A*, *inset*, 10 μm.

The dramatic effects on thalamic axon laminar targeting observed with HAV peptide treatment were mimicked by treatment of a separate series of P6 cocultures with the function-blocking N-cadherin antibody (Fig. 8). At 5 DIV, labeled thalamic afferents in the antibody-treated cocultures had grown largely unbranched through the middle-layer region, in which untreated cocultures normally terminate (Fig. 8*A*), to mostly reach the pial-most edge of the cortical slice (Fig. 8*B*,*F*). As with the HAV cocultures, many such axons branched and turned to extend parallel to the cortical surface (Fig. 8*C*, *arrowheads*), sometimes ending in terminal-like arbors or growth cones (Fig. 8*C*, *inset*). In contrast, laminar specificity in thalamic axon terminations in cocultures treated with preimmune serum was indistinguishable from untreated cocultures (Fig. 8*D–F*). These patterns could not be attributed to differences across conditions in numbers of end-points counted (Table 1) ($p > 0.1$). Together, these data indicate that N-cadherin functions as a layer IV stop-cue by which thalamic axons recognize and terminate in layer IV. This result is similar to one attributed to N-cadherin in establishing laminar specificity of developing retinal ganglion cell projections in flies and chicks (Inoue and Sanes, 1997; Lee et al., 2001).

Rate of thalamic axon growth through deep layers not affected by blocking reagents

The exuberant thalamocortical axon growth beyond their normal target layer that was observed with N-cadherin-blocking reagents in the P6 cortical slices was already evident by 5 DIV, raising the possibility that they might be acting to increase the rate of axon growth through deep layers or speed up a normally much slower progression of growth to the cortical surface. To investigate these possibilities, we prepared sister cocultures (one set treated with N-cadherin IgG, the other an untreated control set) and fixed and DiI-labeled thalamic axons in paired sets every 24 hr up to 5 DIV (Fig. 9, Table 1). Here, we found that the rate of thalamic axon ingrowth was identical over the first 3 DIV, with the front of thalamic axons in each of the coculture conditions reaching presumptive layer IV at the same time. Thereafter, those in the untreated control cocultures remained in layer IV, forming terminal branches as described above, whereas those in the N-cadherin IgG-treated cocultures continued growing, reaching the vicinity

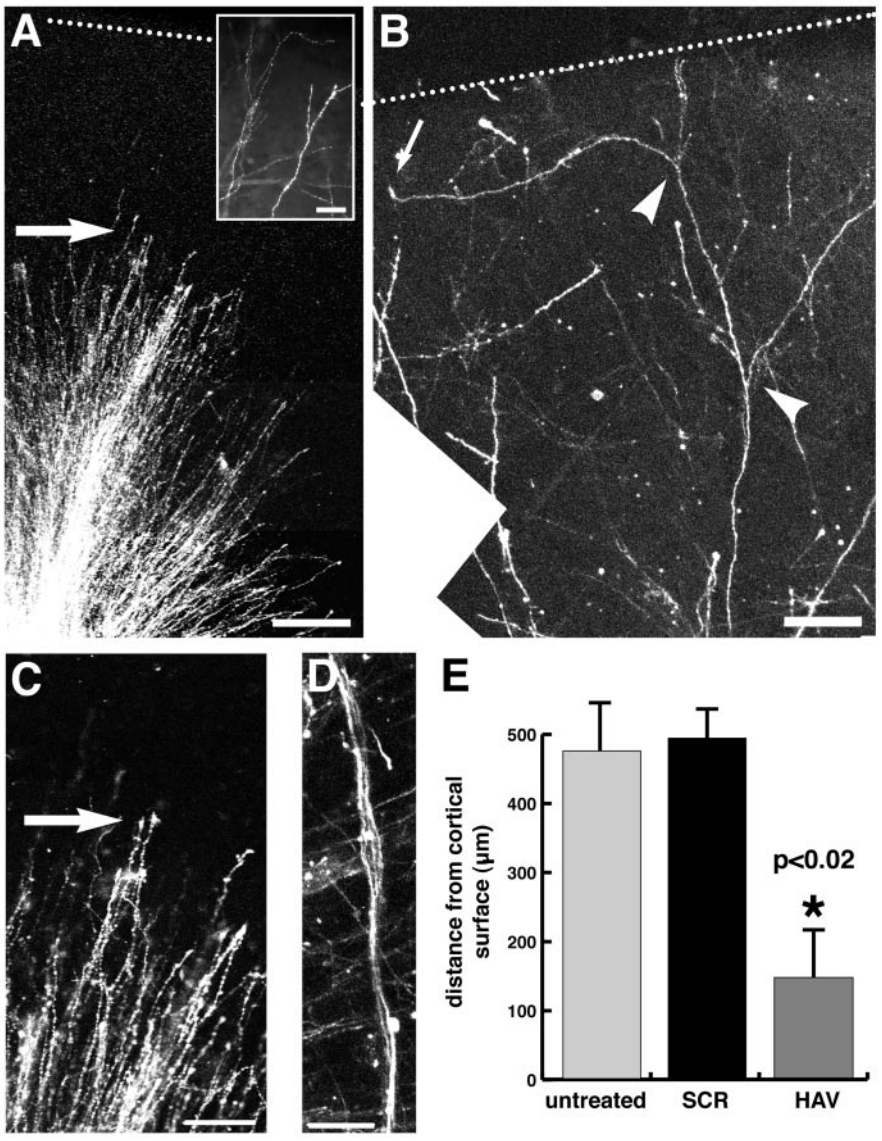

Figure 7. Thalamic axon targeting to layer IV is disrupted by N-cadherin-blocking peptide. *A–D*, Confocal images of DiI-labeled thalamic axons in P6 cortical slices from untreated control cocultures (*A*), those treated with HAV-peptide (*B*), or those treated with scrambled (*SCR*) peptide (*C*, *D*). At this stage (P6), the bulk of the thalamic axons normally stops in a broad middle-layer band corresponding to layer IV (*A*, *arrow*), in which many axons form terminal-like arbors that are studded with boutons (*A*, *inset*). In contrast, thalamic axons in the HAV-treated cocultures grow through layer IV to reach the pial-most surface of the cortical slice (*B*), in which many branch (*B*, *arrowheads*) and extend parallel and subjacent to the cortical edge (*B*, *arrow*). Laminar targeting is thus significantly altered by the N-cadherin-blocking peptide (*E*, *asterisk*) (means + SEM). Thalamic axons in the cocultures treated with the SCR peptide terminate in layer IV as normal (*C*, arrow); some fasciculated axon bundles are found in deeper layers (*D*). All images are taken from cocultures maintained for 5 DIV. Orientation of all images: pial-most cortical surface is toward the *top*. *Dotted lines* (*A*, *B*) delineate the pial-most surface. Scale bars: *A*, 250 μm; *A*, *inset*, 50 μm; *B*, 50 μm; *C*, 100 μm; *D*, 25 μm.

of the pial-most surface by ~P4. Additionally, in a separate set of experiments, we compared the ingrowth of thalamic axons in untreated control cultures maintained for twice as long (10 DIV) with untreated cocultures maintained for 5 DIV and found no significant differences between these two time periods ($p > 0.5$), suggesting that thalamic axons do not normally continue to grow slowly toward the cortical surface, which is consistent with previous reports (Yamamoto et al., 1997; Molnár and Blakemore, 1999). Together, these data indicate that blocking *N*-cadherin function in the P6 slices has no effect on the rate of growth into the cortex and the time at which axons reach layer IV but abolishes a stop-cue that allows them to continue growing to the superficial-most cortical edge.

Discussion

We show here that N-cadherin functions as a layer IV stop signal for ingrowing thalamocortical axons, using organotypic thalamic and cortical explant cocultures in combination with N-cadherin function-blocking antibodies and peptides. Additionally, the characteristically radial-ordered progressive thalamic axon ingrowth through deep layers is significantly altered by N-cadherin-blocking reagents at earlier stages of postnatal cortical maturation, when thalamic axons are normally growing through deep layers before the emergence of layer IV. These data indicate that N-cadherin has multifaceted roles in establishing the thalamocortical projection, governing aspects of thalamic axon ingrowth and laminar targeting, roles that progressively change in parallel with the maturational state of the cortex.

Role as a layer IV stop signal

The association of a stop signal with layer IV cells is supported by two principal observations. First, ingrowing thalamic axons stop in layer IV regardless of whether they enter from the pial or white matter sides (Caviness and Frost, 1983; Yamamoto et al., 1989, 1992, 1997; Bolz et al., 1992; Molnár and Blakemore, 1999; Palmer et al., 2001). Second, cytotoxic deletion of layer IV cells alters patterns of thalamocortical axon termination (Jones et al., 1982; Yurkewicz et al., 1984; Woo and Finlay, 1996; Noctor et al., 2001; Palmer et al., 2001). The results of this study indicate that a specific N-cadherin-mediated interaction between thalamic axons and layer IV cells is required for thalamic axon recognition of their terminal target layer. Because N-cadherin is expressed throughout all cortical layers (Gil et al., 2002; present results), the question is how do thalamic axons respond by stopping when encountering N-cadherin in layer IV in the apparent absence of such a response when encountering N-cadherin in other layers.

One possibility is that N-cadherin-mediated recognition between thalamic axons and layer IV cells is selectively enhanced in comparison with cells in other layers. This could be based simply on differential levels of N-cadherin expression. For example, cells expressing high levels of one cadherin type will sort out from those expressing low levels of the same cadherin type (Steinberg and Takeichi, 1994). Similarly, in *Drosophila*, changing the relative levels of expression of muscle fasciclin II leads to dramatic effects on target muscle recognition by motoneuron axons (Davis et al., 1997). It is also possible that on initial contact between the thalamic axon and layer IV target cell, the dynamics of N-cadherin recruitment to, and adhesive strength at, the junc-

tional interfaces are enhanced. In N-cadherin-expressing cell lines, initial N-cadherin-mediated contact on lamellipodia surfaces increases N-cadherin recruitment to the adhesion complex and alters the dynamics of cadherin anchoring to the actin cytoskeleton (Lambert et al., 2002). This idea is consistent with the rapid accumulation of cadherins at thalamocortical synapses in layer IV observed on the arrival of thalamic axons (Huntley and Benson, 1999; Gil et al., 2002). Cadherins are also bi-directional-signaling molecules (Lambert et al., 2000) the adhesive properties of which can be dynamically modulated by a number of convergent signaling pathways (Gumbiner, 2000). Potential loci for such regulation include reversible tyrosine phosphorylation of the cadherin–catenin complex (Daniel and Reynolds, 1997), cadherin–catenin interactions with the actin cytoskeleton through the small GTPases Rac, Rho, and Cdc-42 (Kaibuchi et al., 1999), or conversion from a weakly adhesive monomeric form to a strongly adhesive strand-dimeric form (Shapiro et al., 1995; Brieher et al., 1996; Colman, 1997; Ozawa and Kemler, 1998; Tamura et al., 1998; Yap et al., 1998). The subsequent onset of thalamocortical synaptic activity may also augment adhesive strength at the thalamocortical synapse. Synaptic depolarization of hippocampal neurons modifies the molecular configuration of N-cadherin to a state that represents heightened adhesive force (Bozdagi et al., 2000; Tanaka et al., 2000) and promotes redistribution of β-catenin into dendritic spines, in which its association with cadherins is enhanced (Murase et al., 2002).

A second possibility is that multiple cadherins interact combinatorially to produce the relevant stop signal. Although cadherin interactions are predominantly homophilic (Geiger and Ayalon, 1992), N- and R-cadherin can form functional *cis*-heterodimers and codistribute at synaptic sites (Shan et al., 2000). Although a number of classic cadherin mRNAs are expressed in overlapping laminar patterns in barrel cortex and the VB thalamus (Suzuki et al., 1997; Obst-Pernberg et al., 2001; Gil et al., 2002), the identity of those that might combine functionally at the membrane interface between thalamic axons and layer IV cells remains to be determined.

A third possibility is that the N-cadherin stop function in layer IV is aided by collaborative actions of other types of molecules. Neurocan, a chondroitin sulfate proteoglycan enriched in supragranular layers of somatosensory cortex (Watanabe et al., 1995), can bind to a cell-surface glycosyltransferase, which is tightly associated with N-cadherin (Balsamo and Lilien, 1990), resulting in a coordinated loss of both N-cadherin and β-1 integrin-mediated outgrowth (Li et al., 2000). It is possible the N-cadherin-blocking reagents interfere with the formation of the neurocan–N-cadherin complex, resulting in unimpeded growth through superficial layers. Abnormal thalamocortical axon extension into superficial layers has also been observed in TrkB-deficient mice (Vitalis et al., 2002). The similarity in the abnormal radial exten-

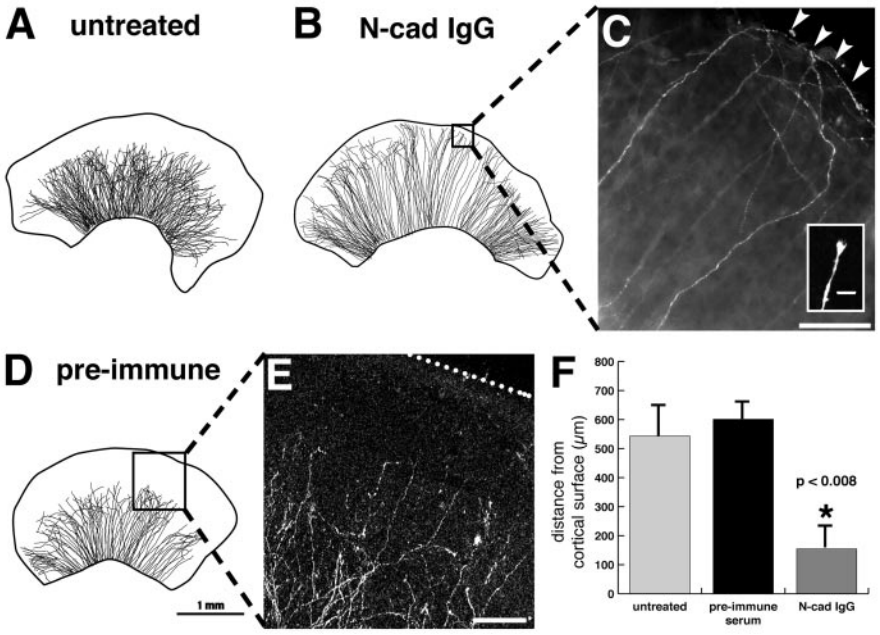

Figure 8. Thalamic axon targeting to layer IV is disrupted by N-cadherin function-blocking antibody. *A–E*, Maps (*A*, *B*, *D*) and confocal images (*C*, *E*) showing ingrowth and termination patterns of DiI-labeled thalamic axons in normal (*untreated*) P6 cortical slices (*A*), slices exposed to N-cadherin function-blocking antibody (*B*, *C*), or slices exposed to preimmune serum (*D*, *E*). When N-cadherin is blocked, thalamic axons bypass their normal target layer IV and extend through supragranular layers, terminating close to the pial edge (*B*, *boxed region* shown at higher power in *C*). Many of these abnormally positioned axons branch and extend underneath the cortical surface (*C*, *arrowheads*); many contain varicosities, and some end in growth cones (*C*, *inset*). This termination pattern is significantly different from that seen in untreated cocultures (*F*, *asterisk*) (means + SEM). Thalamic axon ingrowth and termination in cocultures exposed to preimmune serum are similar to those in untreated control cocultures (*D*, *boxed region* shown at higher power in *E*). All images are taken from cocultures maintained for 5 DIV. Thalamic explants are not shown in maps (*A*, *B*, *D*) for clarity. Orientation of all images: pial-most cortical surface is toward the *top*. *Dotted line* (*E*) delineates pial-most surface. Scale bars: (in *D*), *A*, *B*, *D*, 1 mm; *C*, 100 μm; *C*, *inset*, 10 μm; *E*, 250 μm.

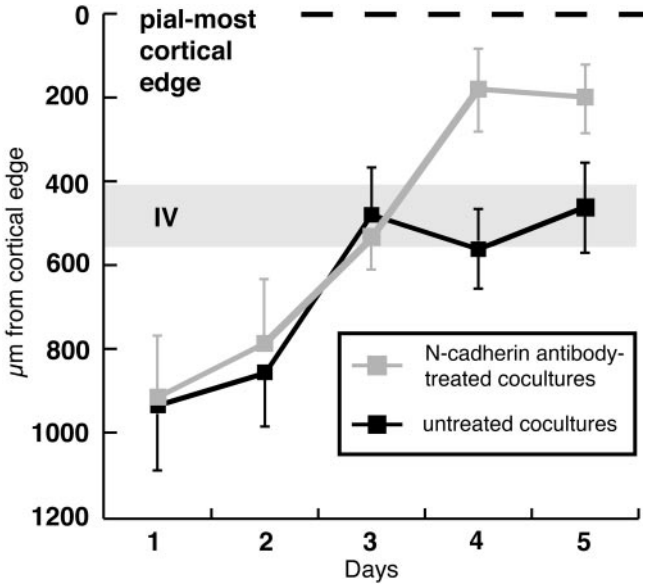

Figure 9. Rate of thalamic axon growth through P6 cortical slices is not affected by blocking N-cadherin. The graph shows a comparison in the extent of thalamic axon growth through the cortical layers between untreated control cocultures (*black lines*) and those exposed to the N-cadherin function-blocking antibody (*gray lines*) measured at 24 hr intervals. Note that the rate of ingrowth through deep layers in the two conditions is identical, with both sets of axons reaching layer IV at the same time (day 3). Over the next 1–2 d, those in the antibody-treated cocultures continue growing through superficial layers to terminate close to the pial edge (*dotted line*). The position of layer IV (*gray bar*) was determined by DAPI-staining. Data are means + SEM.

sion of thalamic axons raises the possibility that cross-talk between neurotrophin signaling and cadherin function may be important for regulating thalamic axon growth through superficial layers. Studies in PC12 cells suggest that neurotrophin signaling can regulate cadherin-adhesive function by modulating tyrosine phosphorylation of β-catenin (Kypta et al., 1996).

Many of the thalamic axons that extended into the superficial layers in the treated cocultures formed terminal-like arbors, despite the lack of a layer IV stop signal. These data are consistent with the prevailing notion that cues that regulate thalamic axon growth-arrest within layer IV and those that regulate terminal arbor formation can be separable molecular mechanisms (Yamamoto, 2002) and indicate that the appropriate branch-formation cues must be present in supragranular layers. Determining whether functional synapses formed on these or other axons in the treated cocultures was beyond this study, although en passant and terminal bouton-like varicosities along the thalamic axons remained readily identifiable in the treated cocultures. Any effects on finer aspects of synaptic function and morphology, such as altered dendritic spine morphology (Togashi et al., 2002), await future studies.

Role in thalamic axon ingrowth

Ventrobasal thalamic axons reach S1 cortex by ~E17 in rats and grow radially through deep layers before the emergence of layer IV at ~P2 (Schlaggar and O'Leary, 1994; Catalano et al., 1996). Our data indicate that N-cadherin is required for orderly and progressive thalamic axon growth through the deep layers at these earliest stages of cortical maturation but is otherwise not required as a general permissive factor because thalamic axons readily entered the cortex. It is known that N-cadherin potently stimulates axonal outgrowth when neurons are grown on N-cadherin-expressing non-neuronal cells or on purified N-cadherin substrates (Matsunaga et al., 1988; Tomaselli et al., 1988; Bixby and Zhang, 1990; Doherty et al., 1991; Lemmon et al., 1992; Williams et al., 1994; Burden-Gulley et al., 1995). Our findings are consistent with this but are more physiologically relevant, akin to the role of N-cadherin in retinal ganglion cell outgrowth *in vivo* (Riehl et al., 1996; Inoue and Sanes, 1997; Lee et al., 2001). Because thalamic axons *in vivo* form protobarrel clusters in deep layers during this stage (before invading layer IV) (Agmon et al., 1993), N-cadherin may be required for this aspect of targeting. This is supported by our observation of abnormally oblique or horizontal axon trajectories in the treated P1 cocultures. Cadherins have also been implicated in axon fasciculation (Drazba and Lemmon, 1990; Iwai et al., 1997; Honig et al., 1998; Lee et al., 2001; Treubert-Zimmermann et al., 2002), consistent with our observation of fewer bundles of fasciculated axons in the treated P1 cortical slices.

Mechanistically, the ingrowth-attenuating effects of the blocking reagents likely reflect interference with an adhesive linkage between thalamic axons and both cortical neurons and neuroglia, because both cell types express N-cadherin (Obst-Pernberg et al., 2001; Gil et al., 2002). The reagents also likely perturb N-cadherin-dependent intracellular signal cascades critical for outgrowth (Utton et al., 2001). Such signaling affects extracellular signal-regulated kinase activation (Perron and Bixby, 1999) and can regulate the phosphorylation state of the cadherin–catenin complex (Balsamo et al., 1998; Burden-Gulley and Brady-Kalnay, 1999; Pathre et al., 2001). N-cadherin-stimulated outgrowth also requires activation of the FGF receptor (Williams et al., 1994, 2001). Because thalamic neurons express FGF receptor mRNAs (Lotto et al., 1997), the blocking reagents could interfere with an interaction between N-cadherin and the FGF receptor within the thalamic axon membrane.

One of the striking findings of this study was the absence of any blocking effects on the rate of axon ingrowth through deep layers in the P6 cortical slices. Such loss of responsiveness by thalamic neurons is not likely to reflect changes in N-cadherin expression, because this remains largely constant throughout the period examined (see Results) (Huntley and Benson, 1999; Gil et al., 2002). It is also unlikely that the different effects of the blocking reagents at the two stages could be attributed to developmental upregulation of other molecules to which the reagents cross-react because the immunoblot analysis (Fig. 5) shows that only N-cadherin is recognized by the function-blocking antibody. It is thought that the cortex controls thalamic axon ingrowth in an age-dependent manner by the specific upregulation of growth-promoting molecules by cortical cells (Gotz et al., 1992; Emerling and Lander, 1994; Tuttle et al., 1995; Molnár and Blakemore, 1999). Our findings suggest an additional mechanism: a developmental switch in N-cadherin function that is temporally regulated by the cortex (because the thalamic explants were always taken at the same age). A developmentally regulated switch in responsiveness to N-cadherin has also been described for axon outgrowth from hippocampal neurons (Doherty et al., 1992), although it remains to be determined what the mechanisms are of such a switch in function.

In summary, our results provide evidence that N-cadherin has fundamentally critical roles in establishing the mammalian thalamocortical projection, functioning as a layer IV stop signal and regulating orderly and progressive axon growth through deep layers.

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