Regulation of N-Cadherin Dynamics at Neuronal Contacts by Ligand Binding and Cytoskeletal Coupling

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N-cadherin plays a key role in axonal outgrowth and synaptogenesis, but how neurons initiate and remodel N-cadherin-based adhesions remains unclear. We addressed this issue with a semiartificial system consisting of N-cadherin coated microspheres adhering to cultured neurons transfected for N-cadherin-GFP. Using optical tweezers, we show that growth cones are particularly reactive to N-cadherin coated microspheres, which they capture in a few seconds and drag rearward. Such strong coupling requires an intact connection between N-cadherin receptors and catenins. As they move to the basis of growth cones, microspheres slow down while gradually accumulating N-cadherin-GFP, demonstrating a clear delay between bead coupling to the actin flow and receptor recruitment. Using FRAP and photoactivation, N-cadherin receptors at bead-to-cell contacts were found to continuously recycle, consistently with a model of ligand-receptor reaction not limited by membrane diffusion. The use of N-cadherin-GFP receptors truncated or mutated in specific cytoplasmic regions show that N-cadherin turnover is exquisitely regulated by catenin partners. Turnover rates are considerably lower than those obtained previously in single molecule studies, demonstrating an active regulation of cadherin bond kinetics in intact cells. Finally, spontaneous neuronal contacts enriched in N-cadherin exhibited similar turnover rates, suggesting that such dynamics of N-cadherin may represent an intrinsic mechanism underlying the plasticity of neuronal adhesions.

INTRODUCTION

Cadherins form a large family of adhesion molecules involved in cell-cell recognition and tissue morphogenesis (Yap *et al.*, 1997). N-cadherin is expressed predominantly in the nervous system and participates in the development and functional organization of the adult neural tissue. N-cadherin is implicated in neurite outgrowth, dendritic arborization, axon guidance, and in the early stages of synaptogenesis (Benson and Tanaka, 1998; Nakai and Kamiguchi, 2002; Yu and Malenka, 2003). Later in development, N-cadherin localizes at synapses (Beesley *et al.*, 1995; Uchida *et al.*, 1996), where it not only plays an adhesive role but also participates to the regulation of synaptic function and plasticity (Bozdagi *et al.*, 2000; Tanaka *et al.*, 2000; Murase *et al.*, 2002; Togashi *et al.*, 2002; Okamura *et al.*, 2004).

Cadherins are single-pass transmembrane proteins forming homophilic calcium-dependent bonds by transassociations of their extracellular domains (Pertz *et al.*, 1999; Boggon *et al.*, 2002). Cadherin ectodomains are also able to form lateral oligomers (Iino *et al.*, 2001; Troyanovsky *et al.*, 2003), resulting in complex adhesive structures (He *et al.*, 2003). On the intracellular side, the cadherin cytoplasmic tail can cou-

ple to actin via the adaptor proteins α - and β -catenin (Yap *et al.*, 1998). Such mechanical coupling could represent the molecular basis for the strengthening of intercellular contacts (Adams *et al.*, 1998; Vasioukhin *et al.*, 2000; Chu *et al.*, 2004). Recent findings indicate that, in addition to their role as adhesive moieties, cadherins also behave as signaling receptors (Yap and Kovacs, 2003). In particular, cadherin ligation has been shown to activate Rho family GTPases known to affect actin assembly (Noren *et al.*, 2001; Kovacs *et al.*, 2002). Conversely, these enzymes together with the catenin complex participate to the regulation of cadherin adhesiveness. For example, a dominant negative form of Rac inhibits the extension of cadherin-dependent contact zones (Ehrlich *et al.*, 2002; Gavard *et al.*, 2004) as well as the rapid linkage of N-cadherin to the actively moving actin network in lamellipodia (Lambert *et al.*, 2002).

Although the molecular components involved in the formation of cadherin contacts are beginning to be characterized, the issue of how cells control the strength of such adhesive zones and remodel them remains unclear. It is often difficult in these processes to distinguish the respective effects of ligand-receptor binding, receptor clustering, and receptor coupling to the cytoskeleton. To answer these questions, it is necessary to investigate the dynamics of formation and renewal of cadherin-mediated adhesive contacts. Biophysical approaches using purified fragments of cadherin extracellular domains and techniques such as atomic force microscopy (Sivasankar *et al.*, 1999), laminar flow chamber (Perret *et al.*, 2002), or single molecule fluorescence detection (Baumgartner *et al.*, 2003) have shed light on the kinetics and strength of the cadherin homophilic interactions at the individual molecular level. They showed that the lifetime of a single cadherin-cadherin bond, irrespective of the cadherin subtype, is about 1 s. It is intriguing that cadherin interactions, apparently so labile at the individual level, can sup-

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Abbreviations used: DIV, days in vitro; CytD, cytochalasin D; FRAP, fluorescence recovery after photobleaching; Ncad-Fc, N-cadherin fused to Fc; Ncad-GFP, N-cadherin fused to GFP; Noc, nocodazole; PAGFP, photoactivatable GFP.

port long-term adhesion between cells. Clearly, it seems important to extend such measurements to living cells, in which an active regulation of cadherin adhesiveness can take place.

To probe the dynamics of N-cadherin accumulation and turnover at neuronal adhesion sites, we essentially used microspheres coated with purified N-cadherin interacting with primary cultures of neurons transfected with N-cadherin fused to GFP. This biomimetic system allowed a precise control of the type and density of ligand molecules presented to the cells and of the time and duration of the interaction, which is not possible in natural contacts. Furthermore, it has the advantage over purely artificial systems that one can probe the dynamics of wild-type or mutated receptors in a living cellular environment. Using a series of optical microscopy techniques, we characterized the kinetics of recruitment of N-cadherin receptors, their anchoring to the actin cytoskeleton, and the turnover of N-cadherin bonds at equilibrium within both semiartificial and spontaneous contacts. Our results demonstrate that the formation and stabilization of N-cadherin bonds in neurons is critically regulated by interactions with catenins.

MATERIALS AND METHODS

Plasmid Constructs

The construction of chicken N-cadherin fused to GFP (*Ncad-GFP*) was described earlier (Gavard *et al.*, 2004). To construct N-cadherin fused to photoactivatable GFP (*Ncad-PAGFP*), we replaced the 740-base pair *Bam*HI-*Not*I GFP-encoding region of pNcadGFP by the one of pPA-GFP (Patterson and Lippincott-Schwartz, 2002; a gift of J. Lippincott-Schwartz). The pNcadGFP plasmid was digested by *Not*I, then by *Bam*HI in conditions allowing a partial digestion, and the 6793-base pair fragment corresponding to molecules deleted from GFP cDNA were purified and ligated with the *Not*I-*Bam*HI pPA-GFP fragment. The plasmid encoding the N-cadherin sequence deleted in the β -catenin binding region and fused at its C-terminal end in frame with the GFP cDNA (*Ncad Δ β cat-GFP*) was obtained by ligation of a 2633-base pair *Sac*I-*Sac*I fragment of pNA 18–53 (Matsuzaki *et al.*, 1990) into the *Sac*I site of pEGFP N2 (Clontech, Palo Alto, CA). To construct the expression plasmid for N-cadherin deleted of its entire cytoplasmic domain and fused with GFP (*Ncad Δ cyto-GFP*), a 2265 base pairs *Sac*I-*Sac*I fragment encoding the ectodomain and the transmembrane region of chicken N-cadherin was inserted into the *Sac*I site of pEGFP N1 (Clontech). Mouse Ncad-GFP was a gift of C. Gauthier-Rouvière (Causseret *et al.*, 2005). Mouse N-cadherin-YFP with triple alanine mutation in the juxtamembrane domain, known to abolish binding to p120^{cas} (*NcadAAA-YFP*), was a gift of K. G. Green (Chen *et al.*, 2003). NrCAM-GFP was a gift of J. Falk and C. Faivre-Sarrailh (Falk *et al.*, 2004).

Protein Expression and Cell Extracts

The mouse sarcoma cell line S180, the mouse L cell line and the stable N-cadherin expressing S180Ncad cell line (Matsuzaki *et al.*, 1990) were cultured at 37°C in 5–7.5% CO₂ in DMEM medium containing 10% fetal calf serum (FCS). About 5 million cells were briefly trypsinized and resuspended in 400 μ l DMEM, 10% FCS, 50 mM HEPES pH 7.4. After addition of 40 μ g plasmid, cells were electroporated at 280 V and 1500 μ F for 50 ms (Easyject, Equibio, Ashford, United Kingdom) and seeded in DMEM with 10% FCS and placed back in the incubator. Two days later, cells were lysed in Laemmli, scraped, and sonicated to disrupt high-molecular-weight DNA molecules. For Western Blot analysis, equivalent amounts of total protein were separated on 7.5% homemade polyacrylamide-SDS gels or NuPAGE 4–12% (Invitrogen, Carlsbad, CA) in reducing conditions, transferred to 0.45- μ m nitrocellulose membranes, and immunoblotted as previously described (Lambert *et al.*, 2000) with either monoclonal anti-N-cadherin (1/2000, Transduction Laboratories, Lexington, KY), monoclonal anti-GFP (1/2000, Roche, Indianapolis, IN), or polyclonal anti- β -catenin (1/5000, Sigma, St. Louis, MO) antibodies. After extensive washing, membranes were probed with either HRP-conjugated anti-mouse or anti-rabbit immunoglobulin (Ig) antibodies (1/5000, Dako, Carpinteria, CA) and developed using the ECL method (Amersham, Indianapolis, IN). For Figure 1D, this step was performed using IRDye 800CW-conjugated antibodies (1/5000, Rockland, Gilbertsville, PA) and signal detection was performed at 800 nm (excitation wavelength: 774 nm) with the Odyssey Infrared Imaging System (Li-Cor Biotechnology, Lincoln, NE).

Microsphere Coating

The fusion protein between mouse Fc and the extracellular domain of chick N-cadherin (*Ncad-Fc*) was produced as described earlier (Lambert *et al.*, 2000).

Protein concentration about 200 μ g/ml was measured by a protein assay (Bio-Rad, Hercules, CA) using rabbit anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA) as a standard. Latex microspheres (4 μ m sulfate, Interfacial Dynamics, Eugene, OR) were coated with goat anti-mouse Fc antibodies (Jackson ImmunoResearch) using 20 μ g antibody for 20 μ l of the 8% solids bead stock solution (overnight incubation at 4°C in borate buffer 0.2 M, pH 8.5). Beads were rinsed in borate buffer containing 0.3% globulin-free bovine serum albumin (BSA; Sigma) and then incubated with 2 μ g Ncad-Fc or equimolar amounts of monoclonal anti-N-cadherin (clone GC4, Sigma) or mouse Fc (Jackson ImmunoResearch) for 3 h at room temperature, rinsed, and resuspended in 100 μ l of buffer. Coated beads were kept on ice and used within 8 h. To estimate the density of ligands on microspheres, the proteins coupled to the beads were solubilized in sample buffer and run on a polyacrylamide gel, together with known amounts of Ncad-Fc. The gel was processed for immunoblotting using GC4 as a primary antibody and HRP goat anti-mouse IgG (Dako) as a secondary antibody and then revealed by ECL. The band intensity was compared with the standards, yielding a surface density around 300 Ncad-Fc dimers/ μ m² on the beads.

Neuronal Culture and Transfection

Hippocampal neurons from E18 Sprague-Dawley rat embryos were seeded on 15-mm polylysine-coated glass coverslips at a density of 10,000 cells per cm² and cultured on a layer of glial cells in Neurobasal medium supplemented with B27 (Invitrogen; Hemar *et al.*, 1997). Neurons were transfected 1 or 2 d after plating, using a phosphate calcium method with 30 μ g DNA for five coverslips (Xia *et al.*, 1996) and processed 24 h later. Cells were placed in 1-ml culture medium supplemented with 1% BSA, 20 mM HEPES, and 10 μ l of the bead solution, left at 37°C for 0.5 h and then rinsed three times in warm medium (except for optical tweezers experiments where cells were observed immediately). For live experiments, coverslips were mounted in a homemade aluminum chamber sealed with vacuum grease. GC4 antibody (1:50 from 10 mg/ml ascites fluid), EGTA (4 mM), cytochalasin D (1 μ M), nocodazole (3 μ M), or dimethylsulfoxide (DMSO; 1:2000) were added at this stage when indicated. For the characterization of spontaneous neuronal contacts, cells at 6 d in vitro (DIV) were transfected with Ncad-GFP using a lipofection kit (Effectene, Qiagen, Chatsworth, CA), and observed 24 h later.

Optical Tweezers and Epifluorescence

The optical tweezers setup consists of an inverted microscope (Olympus IX 70, Lake Success, NY) fed through its epifluorescence port by a Nd:YAG laser beam (Compass 1064 nm, Coherent, Palo Alto, CA) expanded by a 10 $\times$ telescope. Fluorescence illumination comes from a Xenon lamp deposited at 90° and reflected by a 750-nm cutoff dichroic mirror (Omega Optical, Brattleboro, VT). Both the infrared beam and the GFP excitation light (filter 480/20 nm, Chroma Technology, Brattleboro, VT) are reflected inside the microscope by a dual dichroic mirror (Chroma Technology) and focused by a 100 $\times$ /1.40 NA oil objective. The laser power was 100 mW at the back of the objective. A set of two lenses in a near afocal configuration, with one lens on a three-dimensional (3D) translator were used for fine centering of the beam. Digital images were acquired using a cooled CCD camera (HQ Cool Snap, Roper Scientific, Tucson, AZ) driven by the Metamorph software (Universal Imaging, West Chester, PA). The temperature was maintained at 37°C with an air blower (World Precision Instruments, Sarasota, FL) and an objective heater (Biopetechs, Butler, PA). Using a motorized stage (MarzHauser, Wetzlar, Germany), microbeads were captured and maintained on neuronal growth cones for 10 s. In short-term experiments, the microsphere trajectory was monitored for 2 min by 10-Hz image acquisition in DIC illumination mode. The coordinates of the microspheres were tracked automatically through the Metamorph software, and the mean squared displacement over time was computed using a homemade algorithm (Serge *et al.*, 2002). In longer term experiments, images were taken every 10 s, alternating between bright field and fluorescence illumination (emission filter 525/50 nm) using shutters (Uniblitz, Vincent Associates, Rochester, NY). To estimate the force that could be applied on microspheres by optical tweezers, the stage was moved at increasing velocities until a trapped microsphere escaped. The maximal trap force was taken as Stokes' drag on the sphere ($6\pi\eta av$), where a is the radius of the microsphere (2 μ m), η the viscosity of the medium (10^{-3} kg·m⁻¹s⁻¹), and v the critical velocity for escape (about 150 μ m/s), leading to a value of 6 pN.

Fluorescence Recovery after Photobleaching

The same microscope was used for photobleaching experiments, except that the long-pass dichroic mirror was replaced by a 70/30 beam splitter (Chroma Technology), allowing illumination with the 488- and 514-nm lines of an Argon laser (Innova 300, Coherent) together with that of the Xenon lamp. The laser beam was also expanded 10 times to fill the back aperture of the objective, and the movable lens was adjusted to yield a diffraction-limited spot on the focal plane. The laser power was 2.5 mW at the back of the objective, either with GFP or YFP filter sets. Both wide-field and laser illuminations were controlled by shutters. A region of interest on a neuron expressing Ncad-GFP was brought to the position of the laser spot and the fluorescence recovery after photobleaching (FRAP) sequence was started: five reference images were acquired first and then the sample was bleached by the

Table 1. Kinetic parameters obtained from recruitment experiments

Transfection	Bead coating	Receptor/ligand density (%)	Surface/total (%)	Plateau value $1+C_{\infty}/R$	k (h^{-1})	$k_{\text{on}}R$ (h^{-1})	k_{off} (h^{-1})	n
Ncad-GFP								
WT	GC4	3.4 ± 0.4	31 ± 4	2.5 ± 0.2	13 ± 3	3	10	9
WT	Ncad-Fc	3.4 ± 0.4	31 ± 4	2.0 ± 0.1	12 ± 3	3	9	9
$\Delta\beta\text{cat}$	Ncad-Fc	2.1 ± 0.4	50 ± 9	1.8 ± 0.4	13 ± 5	1	12	5
Δcyto	Ncad-Fc	3.9 ± 0.3	43 ± 3	1.8 ± 0.3	7 ± 1	1	6	8
AAA	Ncad-Fc	ND	ND	2.1 ± 0.2	15 ± 4	ND	ND	7

ND, not determined. The surface levels of murine NcadAAA-YFP could not be quantitated because this construct is not recognized by the GC4 antibody, which is chicken specific. However, the facts that Ncad-Fc coated microspheres bind more to neurons transfected for Ncad-AAA-YFP and that Ncad-AAA-YFP is recruited around beads and enriched at glial contacts indicate that it is expressed at the plasma membrane like the other constructs.

laser for 0.3 s, and fluorescence recovery was recorded for 12 min. Images were acquired in full-field illumination with exposure times of 50–200 ms. The whole sequence was driven by a journal written in the Metamorph software. Three FRAP sequences were run per coverslip, bringing the experiment duration to ~45 min. Using Ncad-GFP-positive cells fixed with paraformaldehyde, we measured the diameter of the bleached area ($2r = 4 \mu\text{m}$) and the photobleaching due to the illumination sequence itself, which was <5%.

Photoactivation of Fluorescence

Cells transfected for Ncad-PAGFP and incubated with Ncad-Fc beads were mounted on a confocal microscope (Leica TCS, Deerfield, IL) equipped with a pulsed laser (Mira 900, Coherent) set at 800 nm, whose power was controlled by an electro-optic modulator (Linco Photonics, Milford, MA). The sample was scanned at 800 Hz by the 488-nm laser line and the fluorescence between 500 and 600 nm was collected by a photomultiplier, using a $60\times/1.3$ objective and a pinhole open to three times the Airy disk ($180 \mu\text{m}$). To select cells expressing Ncad-PAGFP, we superimposed illumination with the 800-nm biphoton light at low power (~3 mW at the front of the objective), until a cell lighted up (only the cell body, where most of the fluorescence is concentrated was activated during this process). Alternatively, neurons were cotransfected with DsRed, allowing the identification of Ncad-PAGFP-positive cells. Then, a photoactivation sequence was run using the FRAP module on the Leica software: a few reference images were acquired and then an area of $6 \times 6 \mu\text{m}$ around a bead was illuminated with the 800-nm biphoton laser at high power (30 mW) for one scan of 600 ms, and the fluorescence decay was recorded for 10 min with no further photoactivation. Measurements were also made on cell bodies for comparison.

Immunocytochemistry

Cells were fixed for 10 min in warm paraformaldehyde (4% in phosphate-buffered saline [PBS]), and remaining active sites were saturated with 50 mM NH_4Cl in PBS for 5 min. For staining of intracellular epitopes, cells were permeabilized with 0.1% Triton X-100 in PBS for 5 min, and nonspecific binding sites were blocked with 1% BSA in PBS for 30 min. Cells were incubated in PBS-BSA with 1:400 rabbit anti-GFP (Molecular Probes, Eugene, OR), 1:100 anti-N-cadherin (Transduction Laboratories), 1:400 anti- β -catenin (Sigma), or 1:500 anti- β -tubulin (Sigma) for 2 h, rinsed extensively, and incubated with 1:800 Alexa⁵⁶⁸-conjugated goat anti-rabbit antibody (2 mg/ml, Molecular Probes) for 1 h. For F-actin labeling, cells were incubated with 1:500 Alexa⁴⁸⁸-phalloidin (Molecular Probes) in place of the secondary antibody. After rinsing, coverslips were mounted with Moviol and observed under the Olympus microscope with a $100\times$ objective and appropriate filter sets (Chroma). For surface staining, neurons transfected for Ncad-GFP were incubated in $50 \mu\text{l}$ culture medium containing 1% BSA and $5 \mu\text{l}$ GC4 antibody for 20 min at room temperature, then rinsed, and processed as above without the permeabilization step. To estimate the surface versus total density of exogenous N-cadherin receptors, Ncad-GFP transfected cells were labeled using GC4 as a primary antibody under permeabilized or nonpermeabilized conditions, respectively, and the fluorescent signals on neurites and growth cones were compared with those of latex microspheres coated with known amounts of Ncad-Fc and stained identically.

Model of Diffusion-Reaction for Membrane N-Cadherin

Transient Phase: Recruitment of N-Cadherin Receptors. The interaction between Ncad-Fc ligands on the microspheres and Ncad-GFP receptors on the cell surface was treated as a first-order chemical reaction, with association rate k_{on} ($\mu\text{m}^2 \text{s}^{-1}$) and dissociation rate k_{off} (s^{-1}), in agreement with measure-

ments on individual bonds (Perret *et al.*, 2002). The formation of a ligand-receptor bond depends on the diffusion of free receptors to the vicinity of ligand molecules, followed by the reaction step itself (Bell, 1978). Here, we assumed that the process was reaction-limited, so that the density of homophilic bonds at the microsphere-to-cell interface, $C(t)$, was given by $dC/dt = k_{\text{on}}(L - C)R - k_{\text{off}}C$, where L is the ligand density on the microspheres and R is the receptor density on cell surface (both in μm^{-2}). In the binding term, we assumed that the pool of surface receptors was sufficiently large so as to neglect receptor depletion, consistent with the fact that there was no decrease in fluorescence over time in areas outside bead-to-cell contacts. Given the initial condition of no bond and after introducing the pooled parameters $K = k_{\text{off}}/k_{\text{on}}R$, $k = k_{\text{on}}R + k_{\text{off}}$, and $C_{\infty} = L/(1 + K)$, the solution of this differential equation is: $C(t) = C_{\infty}[1 - \exp(-kt)]$. The fluorescence level around beads that were placed on growth cones at time zero using optical tweezers was normalized by a control level on regions containing no-bead, and the data were fit by the expression $1 + C(t)/(R\phi)$, where ϕ is the ratio between total and surface receptors. The measurements of L/R and ϕ allowed us to compute the intrinsic rates k_{on} and k_{off} (Table 1).

Dynamic Equilibrium: Continuous Exchange of Receptors. At steady state ($dC/dt = 0$), the bond density reaches the value C_{∞} , and there is a continuous exchange between free and bound receptors at a rate $k_{\text{off}}C_{\infty}$. FRAP experiments in areas outside bead contacts were interpreted using a 1D model of unrestricted diffusion with infinite boundaries, consistent with the geometry of neurites and with the fact that the bleached zone was small compared with the total neuronal surface area. Accordingly, the recovered fluorescence intensity was fit by the expression $1 - \text{erf}[1/2(Dt/r^2)^{1/2}]$, where erf is the error function, t is the time and $r = 2 \mu\text{m}$ the radius of the area bleached at time zero (Crank, 1975). Because it takes on average a time $\tau = d^2/2D = 20 \text{ s}$ to diffuse across the bead diameter d , whereas the accumulation of fluorescence under beads occurs in several minutes, our hypothesis that diffusion was much faster than reaction seems valid. At bead contacts, the recovery of fluorescence should then occur in two steps: a fast one due to diffusion of unbound receptors and a slower phase due to replacement of bound receptors by unbleached Ncad-GFP molecules. The diffusion term was fit as above by the error function. To obtain the reaction term, we assumed that the rate at which bleached receptors leave the contact was proportional to the density of bleached receptors at time t , as in a Poisson process. The recovered intensity due to ligand-receptor exchange was then $(C_{\infty} - R)[1 - \exp(-k_{\text{off}}t)]$. Normalized fluorescence data were fit by the sum of these two expressions, yielding the two parameters $k_{\text{diff}} = D/r^2$ and k_{off} .

RESULTS

Biochemical and Functional Analysis of N-Cadherin Fusion Molecules

To study the dynamics of N-cadherin and its regulation by the actin cytoskeleton, we generated a series of plasmids encoding GFP tagged N-cadherin molecules (Figure 1A). Plasmids encoding full-length N-cadherin fused at its C-terminal with conventional GFP (Ncad-GFP) or a photoactivatable variant of GFP (Patterson and Lippincott-Schwartz, 2002; Ncad-PAGFP) were transfected in S180 cells, and protein expression was analyzed by Western blotting. A monoclonal antibody (mAb) against N-cadherin revealed a double band around 150 kDa for both Ncad-GFP and Ncad-PAGFP

Figure 1. Expression and biochemical characterization of N-cadherin-GFP constructs. (A) Schematic diagram of the various N-cadherin-GFP constructs. Full-length cDNA encoding chicken N-cadherin was either fused in frame to conventional GFP or a photoactivatable form (PAGFP) in pEGFP N₂. NcadΔβcat-GFP presents a carboxyl-terminal deletion of 35 amino acid residues (nt 2666–2736) and NcadΔcyto-GFP a carboxyl-terminal deletion of 158 amino acid residues (nt 2265–2736) corresponding to the whole intracytoplasmic domain. NcadAAA-YFP encodes the full-length mouse N-cadherin fused to YFP where residues 780–782 (GGG) have been mutated in AAA, abrogating p120 binding (Chen *et al.*, 2003). Ext, extracellular domain; TM, transmembrane domain; cyto, cytoplasmic domain; GFP, green fluorescent protein; PAGFP, Photoactivatable GFP. (B) Western blot of protein extracts from S180 cells transfected with empty vector (mock), Ncad-GFP, or Ncad-PAGFP, or from the NcadS180 cell line (Ncad), revealed with a mAb to N-cadherin or a polyclonal antibody to β-catenin. (C) Western blot of protein extracts from cells expressing Ncad-GFP, NcadΔcat-GFP, and NcadΔcyto-GFP stained with monoclonal anti-GFP or anti-β-catenin antibodies. (D) Western blot of protein extracts from cells expressing empty vector (mock), Ncad-GFP, or Ncad-AAA-YFP stained as in B.

(Figure 1B). The upper band likely corresponded to the immature form of the N-cadherin fusion protein, including leader peptide and propeptide sequences. The size of the mature form (lower band) agreed with that of full-length chicken N-cadherin (127 kDa) augmented by 25 kDa, the expected size of the GFP tag. Although β-catenin was barely expressed in S180 cells that do not express any cadherin (Papkoff, 1997; Ozawa and Kemler, 1998; Lambert *et al.*, 2000), the transfection of either untagged N-cadherin, Ncad-GFP, or Ncad-PAGFP strongly increased β-catenin levels (Figure 1B). Therefore, despite the presence of GFP and PAGFP sequences close to their β-catenin binding site, N-cadherin GFP/PAGFP fusion proteins conserve the ability to interact with and stabilize β-catenin.

Mutants deleted either in the β-catenin binding site (NcadΔβcat-GFP) or in the whole cytoplasmic domain (NcadΔcyto-GFP), or mutated in the p120^{ctn} interacting region (NcadAAA-YFP; Chen *et al.*, 2003) were used to investigate the respective roles of N-cadherin intracytoplasmic

regions (Figure 1A). Western blots analysis after transient expression of these mutants into S180 or L cells showed that each fusion protein migrates as a double band with expected molecular weights (Figures 1, C and D). Moreover, in contrast to NcadAAA-YFP, which recruited β-catenin as much as wild-type Ncad-GFP, neither NcadΔβcat-GFP nor NcadΔcyto-GFP were able to stabilize β-catenin (Figure 1C), which was expected because both mutants lack the β-catenin interacting domain.

N-Cadherin-GFP Proteins Are Expressed at the Neuronal Surface and Present in Growth Cones

We transfected these Ncad-GFP constructs in rat hippocampal neurons at 2–3 DIV and compared their distribution with that of native N-cadherin. All constructs were of chicken origin (except NcadAAA-YFP of mouse origin), allowing in subsequent experiments the use of a chicken-specific antibody directed against the N-cadherin ectodomain (clone GC4) and were used as surrogates of endogenous rat N-cadherin. Both endogenous and transfected N-cadherin were recognized by another mAb directed against the cytoplasmic tail of mouse N-cadherin (immunogen 638–655 of the mature protein that bears 100% sequence homology between rat, mouse, and chicken). In agreement with such homology, the staining intensities for cells transfected for chicken or mouse Ncad-GFP were very similar (Figure 2A). This allowed us to quantify the relative expression levels of Ncad-GFP versus native N-cadherin, which we found to be moderately overexpressed with a ratio of 1.9 ± 0.7 (mean $\pm$ SEM, $n = 15$ cells).

The protein products of all constructs showed distributions similar to that of endogenous N-cadherin, with a strong perinuclear staining as well as membrane and vesicular localization in neurites and growth cones (Benson and Tanaka, 1998; Figure 2A). Furthermore, the shape and viability of neurons did not seem affected by the transfection of Ncad-GFP constructs at these expression levels. All Ncad-GFP proteins were present at the cell surface (Figure 2B), as shown by live labeling with the GC4 antibody, which recognizes selectively the EC1 extracellular region of chicken N-cadherin (Harrison *et al.*, 2005). A quantification of receptor density was carried out by comparing the fluorescent signals of cells transfected with the various forms of Ncad-GFP and labeled with GC4 antibody, to those on beads coated with known amounts of Ncad-Fc (Figure 2C). By comparing nonpermeabilized versus permeabilized cells, we could compute the ratio between surface and total Ncad-GFP, which fell around 30–50% (Table 1).

N-Cadherin-coated Microspheres Adhere Specifically to N-Cadherin-expressing Neurons

We used a recombinant dimeric N-cadherin-Fc fusion molecule, hereafter abbreviated Ncad-Fc, whose functional properties were described previously (Lambert *et al.*, 2000). Microspheres coated with Ncad-Fc adhered stably to the surface of untransfected cells (Figure 3, A and E). Such adhesion was specific of N-cadherin homophilic interaction because beads coated with Fc fragment, or Ncad-Fc-coated beads in the presence of EGTA (4 mM) or of a function blocking antibody (GC4, dilution 1:50) did not bind to neurons (Figure 3, B and E). Neither treatment was found to particularly affect cell morphology or motility (unpublished data). Neurons transfected with either Ncad-GFP, NcadAAA-YFP, NcadΔβcat-GFP, or NcadΔcyto-GFP bound about two times more Ncad-Fc-coated microspheres than untransfected cells or cells transfected with GFP alone (Figure 3E). Moreover, microspheres coated with the GC4 anti-

Figure 2. Distribution of N-cadherin fusion proteins in cultured neurons. (A) Distribution of endogenous and exogenous N-cadherin in neurons. Neurons transfected for Ncad-GFP (left panel) were immunofluorescently stained with an antibody directed against an intracellular epitope of N-cadherin (center panel). The Ncad-GFP expressing cell is stained more intensely than its untransfected neighbor, with an almost perfect colocalization (right panel), showing that the antibody recognizes the exogenous protein. The staining intensity measured on neurites and growth cones was 760 ± 80 grey levels for cells expressing chicken Ncad-GFP (mean $\pm$ SEM, $n = 10$), 770 ± 70 for cells expressing mouse Ncad-GFP ($n = 8$), and 260 ± 50 for nontransfected cells ($n = 13$), after subtraction of nonspecific staining (Fc used in place of N-cadherin antibody). (B) Live cells transfected for Ncad-GFP (left) were incubated with an antibody recognizing the extracellular domain of chicken N-cadherin (clone GC4), followed by fixation and immunofluorescent detection (center). Note that the Ncad-GFP-expressing cell shows specific surface labeling, with a good degree of colocalization with the GFP signal (right). Bar, 20 μ m. (C) Microspheres coated with various amounts of Ncad-Fc (here 4 and 12 μ g/ml) were fixed and stained with GC4 antibody followed by a fluorescent secondary antibody. (D and E) Neurons transfected for Ncad-GFP were fixed only (D) or fixed and permeabilized (E) and then revealed identically. Growth cones from such cells are shown for the Ncad-GFP signal and GC4 staining. Images were taken at the same illumination and camera exposure and color coded. Bar, 10 μ m.

body adhered only to cells expressing the chicken Ncad-GFP sequence and not to untransfected cells or GFP-expressing cells (Figure 3, C–E). Taken together, these data show that hippocampal neurons express endogenous N-cadherin, which binds Ncad-Fc ligands on microspheres and that transfection for Ncad-GFP increases the number of functional receptors at the cell surface. Furthermore, we detected strong immunofluorescent signals around Ncad-Fc-coated beads for endogenous N-cadherin, α -catenin (unpublished data), β -catenin, and F-actin (see Figure 5A), all usual markers of cadherin-dependent adhesions (Lambert *et al.*, 2000, 2002).

On Ligation, N-Cadherin Receptors at Growth Cones Rapidly Couple to the Actin Cytoskeleton

We next examined the early steps of N-cadherin dependent contact formation by placing Ncad-Fc- or GC4-coated microspheres on neuronal growth cones with optical tweezers. We chose growth cones exhibiting active lamellipodial and filopodial movements. After maintaining a bead in contact for 10 s, the bead trajectory was followed for 2 min (Figure 4A). Ncad-Fc-coated microspheres displayed a characteristic behavior: 1) they adhered quickly and firmly to the cell surface and could not be subsequently displaced by the optical trap, whose maximal force was around 6 pN; 2) they exhibited a low lateral diffusion coefficient and moved backward, with velocities in the range of 1.5–4 μ m/min (Figure 4, B and E). These observations suggested that microspheres were coupled to the continuous retrograde actin flow underlying growth cone motility (Suter *et al.*, 1998). Indeed, an inhibitor of actin polymerization (cytochalasin D) which dramatically affected the distribution of F-actin and reduced the motility of growth cones (unpublished data), blocked the retrograde motion of the beads, whereas an inhibitor of microtubule polymerization (nocodazole), which efficiently depolymerized microtubules (unpublished data), did not affect bead velocity (Figure 4, B–E). Coupling to the actin

flow was independent of bead size, because microspheres of 1 or 4 μ m diameter had an equal probability of moving rearward ($\sim 90\%$ of the trials) and showed similar velocities (3.2 ± 0.7 μ m/min, $n = 7$ for 1- μ m beads vs. 2.7 ± 0.3 μ m/min, $n = 13$ for 4- μ m beads). In the presence of blocking antibodies or EGTA, Ncad-Fc-coated microspheres interacted loosely with the cell surface and often detached, as did control microspheres coated with Fc alone, further demonstrating the specificity of the cadherin-cadherin interaction (unpublished data). Moreover, Ncad-Fc-coated beads placed on neurites showed random motion with a high diffusion coefficient (Figure 4, B and C), indicating that the robust coupling was specific to growth cones. Microspheres coated with GC4 antibodies also moved rearward on the growth cones of Ncad-GFP-transfected cells, suggesting that cross-linking of receptors alone was sufficient to induce such coupling to the actin flow (Figure 4, B–E).

To assess if the connection to the actin flow was mediated by molecular interactions between N-cadherin and catenins, we transfected cells with either NcadAAA-YFP, Ncad Δ cat-GFP, or Ncad Δ cyto-GFP. Transfection per se did not affect the motile activity of growth cones. We reasoned that these mutant molecules should compete with endogenous N-cadherin receptors for ligand sites on microspheres and thus reduce the coupling because of their inability to bind cytoplasmic partners. Indeed, the velocity of Ncad-Fc-coated beads was reduced for all three mutants (40% inhibition for Ncad Δ cyto-GFP, 55% for NcadAAA-YFP, and 16% for Ncad Δ cat-GFP), compared with cells transfected with wild-type Ncad-GFP (Figure 4, C–E), suggesting that interactions with catenins are critical for the coupling of ligated receptors to the actin flow.

N-Cadherin Receptors Slowly Accumulate around Ncad-Fc-coated Microspheres

We then asked if the coupling between microspheres and the actin cytoskeleton was related to the recruitment of N-cad-

Figure 3. Adhesion of N-cadherin microspheres to neurons. Neurons untransfected or transfected for the various Ncad-GFP constructs (WT, AAA, $\Delta\beta\text{cat}$, or Δcyto) or GFP alone were incubated for 0.5 h with beads coated with various ligands (mouse Fc, Ncad-Fc, or GC4 antibody), then fixed, and observed. Representative examples are shown. (A and B) Untransfected neurons with Ncad-Fc beads (A) or Fc beads (B). Phase contrast; bar, 20 μm . (C and D) Neurons transfected for Ncad-GFP and incubated with beads coated with GC4 antibody. Note that beads bind only to the Ncad-GFP-expressing cell (left) and not to a nontransfected counterpart (right). DIC image (C) and GFP fluorescence (D); bar, 20 μm . (E) The number of beads bound per cell in each condition is expressed as mean $\pm$ SEM, with the number of cells examined in italic.

herin receptors by ligands exposed on microspheres. We thus monitored the fluorescence distribution of Ncad-GFP during 15 min after placing Ncad-Fc- or GC4-coated microspheres on neuronal growth cones by optical tweezers and in parallel measured the microsphere movement. Transfected cells with moderate levels of fluorescence and showing active growth cone motion were selected. We observed a progressive accumulation of fluorescence signal around

beads, usually reaching a plateau in ~ 15 min (Figure 5B). In few cases, fluorescent packets came directly to the proximity of the beads, possibly corresponding to N-cadherin-rich vesicles or clusters, but most of the time a monotonous increase in fluorescence around beads was observed, supporting the idea that N-cadherin accumulates around beads via ligand-receptor binding. All beads did not recruit N-cadherin to the same extent (Figure 5A), showing that this effect was specific and not resulting from optical artifacts.

In the same time as they accumulated Ncad-GFP signal, microspheres still moved rearward on growth cones, but with a velocity that decreased in a few minutes, independently of bead coating and mutations in the receptor (Figure 5D). Such tendency of microspheres to progressively slow down was already apparent as a downward deflection of the displacement curves at short term (Figure 4C). Therefore, bead coupling to the retrograde flow and recruitment of receptors occur on two separate time scales.

Fluorescence levels at bead-to-cell contacts were quantified and the data were fit by an equation derived from first-order chemical kinetics (Figure 5C), giving the intrinsic reaction rates of the adhesive interactions and the equilibrium values (Table 1). The accumulation of Ncad-GFP was higher for GC4- than for Ncad-Fc-coated beads, owing to the different affinities of GC4 and Ncad-Fc for Ncad-GFP receptors (Table 1) and also to a competition between endogenous N-cadherin and exogenous Ncad-GFP in the case of Ncad-Fc ligands (the GC4 antibody recognizes only Ncad-GFP). The truncated molecule Ncad Δcyto -GFP reached equilibrium later than its wild-type counterpart, reflecting slower kinetics both in the association and the dissociation steps (Figure 5C; Table 1). In contrast, the NcadAAA-YFP mutant reached equilibrium faster than both Ncad-GFP and Ncad Δcyto -GFP, indicating a higher association rate and/or lower dissociation rate, although we could not distinguish between these possibilities (Figure 5C; Table 1). Finally, Ncad $\Delta\beta\text{cat}$ -GFP was characterized by a lower association rate and a higher dissociation rate than the wild-type molecule (Figure 5C; Table 1). These data thus indicated subtle differences in the dynamic behavior of the different receptor constructs. However, this approach required the quantification of several parameters (ligand and receptor densities, surface vs. total receptor expression, and kinetic rates), which had a high variance. We therefore performed more direct measurements of receptor turnover in areas of bead contact at equilibrium, using a photobleaching technique.

FRAP Experiments Reveal the Slow Turnover of N-Cadherin Receptors at Cell-Microsphere Contacts

We photobleached bead-to-cell contacts exhibiting Ncad-GFP recruitment, with the idea that if bleached receptors could dissociate from their ligands on the bead and be replaced by unbleached receptors, the fluorescent signal should increase over time and reveal such exchange. To first evaluate the diffusion properties of Ncad-GFP receptors, we carried out photobleaching experiments on control regions of neurites and growth cones (Figure 6A). The fluorescence recovery was well described by a model of unbiased diffusion (Figure 6C), indicating that most N-cadherin receptors were freely mobile with a diffusion coefficient of 0.1 $\mu\text{m}^2/\text{s}$. This value is slightly higher than that reported for E-cadherin using single particle tracking (Sako *et al.*, 1998) and can be attributed to differences in the measurement methods (Kucik *et al.*, 1999) or to a fraction of intracellular Ncad-GFP, which diffuses more quickly.

For photobleaching experiments performed on bead-to-cell contacts, the fluorescent level around microspheres was

Figure 4. Movement of N-cadherin coated microspheres on growth cones. (A) Ncad-Fc or GC4 coated microspheres were placed on growth cones or neurites for 10 s using optical tweezers, and their subsequent motion was followed over time. (B) Representative examples of 2-min trajectories (grey traces), the beads being shown at time zero; bar, 5 μ m. (B) Untransfected neurons (left). (B) neurons transfected for Ncad-GFP (right, top and bottom) or Ncad Δ cyto-GFP (center). Insets, the corresponding fluorescence images taken after tracking; note a slight accumulation of signal around beads (arrows). (C) Individual examples of the distance traveled by microspheres in the radial axis of the growth cone, for various conditions. (D) The mean squared displacement calculated from the trajectories shown in B was plotted versus time (t), and fit with the equation V^2t^2 (plain curves), where V is the mean rearward velocity of the bead. (E) Mean velocity of Ncad-Fc- or GC4-coated beads measured for the different conditions (mean $\pm$ SEM, number of experiments in italic). * $p < 0.05$; *** $p < 0.01$; **** $p < 0.005$ by Student's *t* test.

again normalized by the control level on the same neurite or growth cone, to better focus on the fraction of bound receptors within the contact. The ratio of accumulation was about 2.3 before bleach (Figure 6C), corresponding well to the steady state value measured from recruitment experiments (Figure 5C; Table 1). The fluorescence ratio recovered in two steps (Figure 6C): 1) a rapid phase during the first 2 min and then a slower phase reaching a value of ~ 1.2 in 12 min. In its initial phase, the recovery curve was superimposable to that obtained from control experiments on neurites or growth cones (no bead), suggesting that the gain of fluorescence was due to the diffusion of unbound receptors (Figure 6C). However, the fluorescent ratio around beads finally reached higher levels than on control areas, suggesting that a fraction of the fluorescence on the beads corresponding to trapped receptors recovered with a slow turnover. The data were well described by a diffusion-reaction model (Figure 6C), allowing the characterization of the turnover rate of ligand-receptor bonds $k_{off} = 2.3 \pm 0.4 \text{ h}^{-1}$ ($n = 31$), which was in the same range as that obtained by quantifying the kinetics of Ncad-GFP recruitment (Table 1). Such a value means that the whole population of receptors trapped beneath a microsphere exchanges within 45 min (30% in 12 min). This turnover was insensitive to bead size, because FRAP experiments on Ncad-GFP receptors using 1- μ m microspheres coated with Ncad-Fc gave a similar turnover rate ($k_{off} = 3.6 \pm 1.8 \text{ h}^{-1}$, $n = 7$). Thereafter, we used 4- μ m beads, which provide a higher signal-to-noise ratio in fluorescence.

The Exchange Regime Is Characteristic of the N-Cadherin Homophilic Interaction

To further validate the hypothesis that fluorescence recovery originates from turnover of N-cadherin homophilic bonds at the bead-to-cell interface, we carried out a series of control experiments. We first blocked N-cadherin homophilic adhesion by adding 4 mM EGTA at the beginning of the FRAP experiment and observed a disappearance of the slow recovery regime (Figure 6C). This came in agreement with the hypothesis that bonds which dissociate cannot be replaced by new bonds, because free receptors and free ligands are unable to bind again. At higher concentrations of EGTA (10 mM), we observed a progressive disappearance of Ncad-GFP signal at N-cadherin contacts (unpublished data). In addition, when the GC4 antibody was used in place of Ncad-Fc as a ligand on the microspheres, fluorescence recovery was significantly inhibited (Figures 6D). This was likely due to the fact that the antibody-antigen bond is more stable than the natural N-cadherin/N-cadherin bond, such that bleached receptors stay longer in the contact. As the reverse control, we studied the mobility of NrCAM, a member of the IgCAM family not reported to interact with cadherins (Falk *et al.*, 2004), at contacts with Ncad-Fc microspheres. Transfected NrCAM-GFP receptors were expressed at the neuronal surface, as revealed by live staining with anti-GFP (unpublished data) and, as expected, showed little accumulation around Ncad-Fc-coated beads (baseline, Figure 6D). More precisely, in neurons cotransfected for Ncad-DsRed (Lambert *et al.*, unpublished results) and NrCAM-GFP, the enrichment factor around Ncad-Fc or GC4

Figure 5. Kinetics of N-cadherin accumulation around N-cadherin-coated microspheres. (A) Untransfected neurons incubated with Ncad-Fc-coated beads were stained for endogenous N-cadherin, β -catenin, or F-actin. Bar, 5 μ m. (B–D) Neurons were transfected for various Ncad-GFP constructs, and Ncad-Fc- or GC4-coated beads were placed on growth cones at time zero with optical tweezers. (B) Representative time sequence of Ncad-GFP accumulation around a Ncad-Fc-coated bead. Note a slight retrograde motion of the bead in this time period. (C) The fluorescence level around microspheres was normalized by the level on adjacent regions of the same growth cone, forcing the time zero point to a ratio of 1, and the average data for 5–10 cells in each condition is plotted over time. SEs, about 3–12% of the mean, are omitted for clarity. The different conditions represent Ncad-Fc beads on Ncad-GFP ($\square$), Ncad Δ cyto-GFP ($\diamond$), Ncad Δ β cat-GFP (Δ), or NcadAAA-YFP ($\times$) expressing neurons, or GC4-coated beads on Ncad-GFP-expressing neurons ($\circ$). The data were fit with a first-order kinetics equation, yielding the parameters $k = k_{\text{on}}R + k_{\text{off}}$ and the plateau value C_{∞} . (D) The instantaneous bead velocity dropped to zero in a few minutes, for all conditions.

microspheres was 2.6 ± 0.2 for the Ncad-DsRed signal versus 1.3 ± 0.1 in the NrCAM-GFP channel, as measured on the same beads ($n = 33$, $p < 0.0001$). This experiment dem-

onstrated the specificity of fluorescence accumulation around microspheres and ruled out potential optical artifacts, owing for example to membrane wrapping of micro-

Figure 6. Steady exchange of N-cadherin/N-cadherin bonds at cell-microsphere contacts. (A and B) Time sequences of typical FRAP experiments. Neurons transfected for Ncad-GFP were either untreated (A) or incubated with Ncad-Fc-coated beads for 0.5 h, leading to fluorescence accumulation around beads (B). A brief laser pulse was imposed at time zero to photobleach a small area at the center of the image (arrow), and the fluorescence level in this area was monitored for 12 min thereafter. In B, the laser spot was centered precisely at a bead location. Bar, 10 μ m. (C) The fluorescence level around beads normalized by the level on adjacent areas with no bead is plotted over time ($\square$). Recall that the normalized prebleach level on the beads is at 2.3, and so recovery curves can very well go above 1. The data were fit by a model of diffusion-reaction (plain curve), giving the average parameters k_{diff} and k_{off} . In the presence of EGTA (Δ), the fluorescence recovers as for control experiments obtained for Ncad-GFP-positive cells not incubated with beads ($\circ$, neurite). Those latter data were fit with a model of unrestrained 1D diffusion (plain curve). (D) FRAP experiments were carried out on contacts between Ncad-Fc-coated beads and cells transfected with NrCAM-GFP (Δ) or, conversely, between beads coated with GC4 antibody and cells transfected with Ncad-GFP ($\circ$). Data of more than 10 cells for each condition are expressed as mean $\pm$ SEM.

spheres within partial phagocytosis (Lambert *et al.*, 2000). The recovery of fluorescence for NrCAM-GFP was analogous to that on control neurites with an absence of long-term recovery, showing that the exchange regime was indeed a specific feature of Ncad-GFP receptors (Figure 6D). Finally, to rule out possible photodamage, we bleached the whole Ncad-GFP signal of several neurons using the laser then incubated cells with Ncad-Fc-coated beads, and stained the receptors with anti-GFP. Beads were found to recruit immunoreactive GFP (unpublished data), indicating that photo-bleached Ncad-GFP receptors were still functional.

Photoactivation Experiments Confirm that N-Cadherin Receptors Slowly Leave Adhesive Contacts

To show unambiguously that N-cadherin receptors could leave the adhesive contact and diffuse away, we carried out experiments using neurons transfected with Ncad-PAGFP. The fluorescence was briefly activated in areas of contact with Ncad-Fc-coated beads, and the fluorescence level was monitored over time (Figure 7A). A significant decay of fluorescence was observed in areas of bead contacts, which was not due to photobleaching because control experiments on cell bodies showed no decrease of fluorescence under the same conditions (Figure 7B). The curves were not the exact mirror images of FRAP experiments, in that they lacked the fast initial phase that was expected from the presence of free receptors. This was somewhat expected because photoactivation was carried out using a confocal microscope in bi-photon mode, so that Ncad-PAGFP was excited in a narrow zone and fast diffusion of out-of-focus components was not detected. Indeed, FRAP experiments carried out with a confocal microscope also showed a reduction of the fast regime fraction (unpublished data). Furthermore, because of the low efficiency of the photoactivation process, we were selecting beads with a high Ncad-GFP signal, where the density of bound receptors was much higher than that of free receptors. Accordingly, we found it quite difficult to photo-activate Ncad-PAGFP on neurites or growth cones alone. Nevertheless, the fit with a monoexponential decay gave a turnover rate of $5.7 \pm 0.9 \text{ h}^{-1}$ ($n = 9$) in relative agreement with that obtained from FRAP experiments.

N-Cadherin Turnover Is Controlled by Its Connection to the Actin Cytoskeleton

Because the linkage between cadherin and actin is implicated in the strength of adhesion (Ozawa and Kemler, 1998; Yap *et al.*, 1998) and the transport of N-cadherin to adhesive sites is associated with the microtubular motor kinesin (Chen *et al.*, 2003), we tested if the turnover of N-cadherin adhesions was regulated by the cytoskeleton. In the presence of cytochalasin D or nocodazole, the binding of Ncad-Fc beads to neurons and the recruitment of Ncad-GFP around beads was similar to that of DMSO-treated cells (unpublished data). In nocodazole-treated cells, the recovery of fluorescence of Ncad-GFP on Ncad-Fc beads after photobleaching was slightly (but not significantly) lower than that of DMSO treated cells (Figure 8, B and D), indicating that an active transport of N-cadherin receptors along microtubules is not a requisite for the recycling of N-cadherin adhesions in our conditions. In contrast, the exchange regime was abolished in the presence of cytochalasin D (Figure 8, A and D), indicating that an intact connection to actin is essential for N-cadherin turnover.

To more specifically probe the molecular interactions involved in this process, we carried out FRAP experiments on mature contacts between Ncad-Fc-coated beads and neurons transfected for the mutated receptors Ncad Δ cyto-GFP,

Figure 7. Dissociation kinetics of N-cadherin receptors from bead-to-cell contacts. Neurons transfected for Ncad-PAGFP were incubated with Ncad-Fc-coated beads for 0.5 h. The fluorescence at Ncad-Fc-coated beads was activated at time zero using a brief pulse of 800-nm biphoton light, on a $8 \times 8\text{-}\mu\text{m}$ area (white square). (A) Time sequence of a typical experiment. (B) The fluorescence level in the selected area was normalized by its level at time zero (right after activation) and plotted over time for an average of 10 cells ($\square$). We observed a decline in signal after initial activation, which was fit by a monoexponentially decreasing function $\exp(-k_{\text{off}} t)$. Control measurements on cell bodies ($\circ$) showed no photobleaching in the same time frame. Data are expressed as mean $\pm$ SEM.

Ncad $\Delta\beta$ cat-GFP, or NcadAAA-YFP. The initial phase of the recovery curve were indistinguishable for all constructs (Figure 8, B and C), indicating that the diffusion properties of the truncated molecules were similar to those of the wild type. However, the second phase of the fluorescence recovery was markedly different. For the constructs Ncad Δ cyto-GFP and NcadAAA-YFP, which were as enriched as Ncad-GFP at bead contacts (ratio bead/neurite about 2), the

Figure 8. Regulation of N-cadherin turnover by cytoskeletal partners. Photobleaching experiments were carried out at sites of contacts between Ncad-Fc-coated beads and neurons transfected for various Ncad-GFP constructs. (A) Neurons transfected for wild-type Ncad-GFP were treated with cytochalasin D (Cyt D), nocodazole (Noc), or the carrier DMSO alone at the beginning of the experiment. (B and C) Neurons were transfected for Ncad-GFP, Ncad Δ cyto-GFP, Ncad Δ β cat-GFP, or NcadAAA-YFP and untreated thereafter. Data are expressed as mean $\pm$ SEM and fit with the diffusion-reaction model (plain curves). (D) Summary graph of the turnover rates measured by FRAP for all conditions. Turnover rates were compared two by two using Student's *t*-tests: Cytochalasin versus DMSO; EGTA versus untreated, Ncad Δ cyto-GFP, Ncad Δ β cat-GFP, NcadAAA-YFP, and NrCAM-GFP versus Ncad-GFP, all for Ncad-Fc beads; GC4 bead versus Ncad-Fc bead, both for Ncad-GFP transfected cells. Significance levels: **p* < 0.05; ***p* < 0.025; ****p* < 0.01; *****p* < 0.0025.

exchange regime was abolished (Figure 8, B and C) and characterized by significantly smaller k_{off} values (Figure 8D). In contrast, Ncad Δ β cat-GFP receptors were recruited less than Ncad-GFP receptors at bead contacts (ratio bead/neurite 1.7) and recycled more rapidly, as revealed by a higher k_{off} (Figure 8D). These data validated the measurements obtained from recruitment experiments (Table 1) and demonstrated that the turnover of N-cadherin receptors at N-cadherin contacts is finely regulated by catenin partners.

Bond Exchange Occurs at Similar Rates in Natural Neuronal and Glial Contacts

Finally, to show that microsphere adhesion mimicked natural contacts quite well, we carried out similar FRAP experiments on spontaneous neuronal and glial contacts also showing Ncad-GFP accumulation (Figure 9, A and B). The ratio between the fluorescence level in the contacts and that on glial lamellipodia or adjacent regions on neurites was also about 2 (Figure 9C), as reported in other studies (Chen *et al.*, 2003). Strikingly, the recovery curve obtained for these contacts followed closely that obtained for contacts between Ncad-Fc-coated microspheres and neurons (Figure 9C), with very similar exchange rates (Figure 8D).

DISCUSSION

Because of its importance in the contexts of growth cone targeting and synaptogenesis, we focused on the dynamic regulation of early N-cadherin-dependent neuronal contacts. Using Ncad-Fc-coated microspheres binding to cultured neurons, we show that the establishment of N-cadherin-dependent contacts occurs in several successive phases: first a rapid coupling of a few N-cadherin receptors to the actin flow in growth cones, followed by a progressive recruitment of more receptors around ligand-coated microspheres and then by a slow steady state turnover of bonds controlled by the connection to the actin cytoskeleton. A summary diagram of the effect of the various mutated re-

ceptors on ligand and cytoskeleton interactions is shown in Figure 10.

Using an optical trap to impose brief contacts between microspheres and the cell surface, we found that the growth cones of hippocampal neurons were particularly reactive to Ncad-Fc beads, which escaped the optical trap within seconds and moved in a retrograde manner. In contrast, microspheres coated with fibronectin did not adhere well and rarely coupled to the retrograde flow in the same neurons (unpublished data), as already reported for DRG neurons (Schmidt *et al.*, 1995). Ncad-Fc thus behaves as a natural ligand for growth cones, which are enriched in endogenous counterreceptors. In agreement with our previous observations that Ncad-Fc-coated beads placed on the lamellipodia of N-cadherin-expressing cells start a retrograde motion with a latency time that varies inversely with ligand density (Lambert *et al.*, 2002), we observed here that larger beads coated at maximal ligand density were immediately dragged to the rear of the growth cone. Thus, the number of ligand-receptor bonds between the microsphere and the cell surface is probably sufficient to trigger immediate connection of a few receptors to the cytoskeleton and allows the microsphere to escape a moderate trapping force (Thoumine and Meister, 2000).

The expression of NcadAAA-YFP, Ncad Δ β cat-GFP, or Ncad Δ cyto-GFP mutants all diminished the rearward motion of Ncad-Fc-coated beads on growth cones, albeit to varying degrees. Although we cannot totally exclude the possibility that the expression of mutated receptors may affect actin motility itself, the fact that NcadAAA-YFP and Ncad Δ cyto-GFP were more potent inhibitors than Ncad Δ β cat-GFP suggests that the juxtamembrane domain, rather than the β -catenin binding region, is implicated in the coupling of N-cadherin to the actin flow. This may involve a direct physical linkage, for example, via p120, which has recently been shown to bind cortactin (Martinez *et al.*, 2003), a protein that interacts with filamentous actin and is closely associated with cadherin contacts (El Sayegh *et al.*, 2004;

Figure 9. Turnover of N-cadherin receptors in neuronal and glial contacts. Primary hippocampal cultures, which contain both glial cells and neurons, were transfected with Ncad-GFP. (A) In glial cells at 3 DIV, Ncad-GFP is enriched at cell-cell contacts where it localizes in a linear manner. (B) In 7 DIV neurons, there is partial accumulation of Ncad-GFP when neurites cross one another. Bar, 10 μ m. (A and B) Time sequences of typical FRAP experiments carried out on such contacts. Arrows indicate the position of the laser spot. (C) Recovery of fluorescence over time, normalized by the level at regions outside the contact. Data are expressed as mean $\pm$ SEM and fit with the diffusion-reaction model (plain curves). Control experiments were performed on glial lamellipodia and neurites, and data were fit by the diffusion equation alone (plain curve).

Helwani *et al.*, 2004). It may also involve signaling molecules such as the nonreceptor kinase FER and its downstream target cortactin, which regulates the actin nucleating complex Arp 2/3, or a Rac1 pathway (El Sayegh *et al.*, 2005; Kovacs *et al.*, 2002; Lambert *et al.*, 2002). Alternatively, the ability of mutant molecules to compete with endogenous N-cadherin for the coupling to the actin flow also depends on their ligand-binding affinity, which varies among mutants and is particularly low for Ncad $\Delta\beta$ cat-GFP (see below). Beads coated with GC4 antibody on cells transfected for Ncad-GFP moved rearward as fast as beads coated with Ncad-Fc, suggesting that the lateral clustering of N-cadherin receptors is alone responsible for the efficient coupling to the actin flow. It is not excluded that the antibody, whose epitope lies close to the ligand-binding site, also activates the receptor (Harrison *et al.*, 2005). The clustering effect was also observed for L1-myc receptors bound to anti-myc-coated

beads (Gil *et al.*, 2003) and at variance with the behavior of integrins, whose coupling to the actin flow specifically requires ligand binding (Choquet *et al.*, 1997). Altogether, these data show that neuronal N-cadherin mobilization in the growth cone is strongly coupled to the anchoring of this receptor to rearward moving actin, an event that was never observed along neurite shafts. This process may be important for both axonal elongation and neuronal contact formation requiring extensive actin cytoskeleton reorganization.

When monitoring the fluorescence distribution around microspheres, both Ncad-Fc- and GC4-coated beads recruited Ncad-GFP in a time course of several minutes. This process was much slower than the rapid coupling to the actin flow, suggesting that the retrograde motion of beads required only a limited number of bonds not detectable from the GFP signal at early time points. Conversely, the decrease of microsphere velocity on growth cones may be related to

Figure 10. Schematic view of the molecular interactions implicated in cadherin bond dynamics and coupling to the actin cytoskeleton. The various Ncad-GFP receptors as well as endogenous N-cadherin are shown, together with their Ncad-Fc ligands and main cytoskeletal partners (p120, α -catenin, β -catenin, cortactin, and actin). The effect of the mutated and truncated forms of Ncad-GFP on the kinetic rates and on the coupling to the actin flow are shown by plus, equal, or minus signs. *cis*-interactions between mutated receptors and endogenous N-cadherin may take place through the ectodomain. The dimeric receptor and ligand forms are omitted for clarity.

the accumulation of receptors beneath the beads, tending to increase lateral friction, possibly through connection to immobile components of the cytoskeleton. Likewise, in cells transfected with full-length E-cadherin, anti-E-cadherin-coated beads can be displaced over very limited distances by an optical trap due to strong connection of the receptor to the cytoskeleton (Sako *et al.*, 1998). At equilibrium, the recruitment of N-cadherin-GFP beneath Ncad-Fc beads remained moderate (ratio bead/neurite about 2.3), suggesting that it may be limited either by the availability of N-cadherin receptors or the affinity of N-cadherin bonds. The fast and unrestrained mobility of unbound N-cadherin receptors measured by FRAP on growth cones and neurites indicated that recruitment was not limited by receptor diffusion, but rather by the reaction itself, in agreement with a recent study showing a weak effect of lateral mobility on the rate of IgCAM recruitment at neuronal contacts (Thoumine *et al.*, 2005). Indeed, the kinetic analysis showed that the recruitment of N-cadherin around beads corresponded to a dynamic equilibrium with continuous bond association and dissociation, which we explored in more detail using photobleaching experiments at bead-to-cell contacts.

After previous FRAP studies on E-cadherin (Adams *et al.*, 1998), VE-cadherin (Delanoe-Ayari *et al.*, 2004), N-cadherin (Causeret *et al.*, 2005), or NCAM receptors (Jacobson *et al.*, 1997) at cell-cell contacts, we initially carried out a conventional analysis, normalizing all the recovery curves by their initial values. When we did so, the fluorescence intensity in contact areas reached a plateau about 50% of the initial value (in agreement with the reports cited above), revealing the presence of an immobile fraction of receptors. This suggested that the various receptors were trapped almost irreversibly by long-lasting interactions with their counterreceptors. However, a careful analysis based on diffusion and trapping, longer observations, and normalization against initial fluorescent intensities in control areas where N-cadherin remained unbound made possible the identification of a slow turnover of receptors. The recovery curves were strikingly similar for bead-cell contacts, where ligands are immobilized, and for cell-cell contacts, where receptors are freely moving within the two apposed cell membranes. In that case, bonds can a priori slide as individual pairs, although in practice they may be stabilized by interactions with cytoplasmic partners (Sako *et al.*, 1998; Nishimura *et al.*, 2003). Because similar recovery curves were obtained for these two situations, it seems again that diffusion is not a limiting step in the renewal of N-cadherin adhesions. Recycling of cadherins through endo-exocytosis has been documented (Chen *et al.*, 2003), but involves a relatively small fraction of membrane receptors, i.e., only 15% in the case of E-cadherin under basal conditions (Le *et al.*, 1999). Furthermore, depolymerization of microtubules did not significantly affect the recovery of fluorescence, so it is unlikely that the receptor turnover observed here comes from trafficking events from and to intracellular compartments, but rather involves molecules already present at the cell surface.

Analogous photobleaching experiments on contacts between TAG-1-coated beads and NrCAM-GFP-expressing cells showed an absence of exchange regime (Falk *et al.*, 2004), suggesting that the linkage between NrCAM and TAG-1 is rather stable. Similarly, the recovery of Ncad-GFP signal around GC4-coated beads was very slow, giving a lifetime of several hours typical of an antibody-antigen bond (Pierres *et al.*, 1998). Furthermore, FRAP experiments carried out on Ncad-GFP-rich adhesion sites formed between myogenic cells and a flat substrate coated with Ncad-Fc (Gavard *et al.*, 2004) gave turnover rates similar to those obtained for

contacts between Ncad-Fc beads and neurons, showing that the particular geometry of microspheres does not perturb the measurements (Lambert *et al.*, unpublished results). Thus, the data allowed to unravel the existence of long-lasting N-cadherin adhesive bonds compatible with the maintenance and the plasticity of cellular contacts needed during initiation and maturation of contacts between growth cones and neighboring neuronal or glial cell surfaces.

The lifetime of about 1 h for the N-cadherin bond obtained here is much longer than the values reported earlier for other cadherins in single molecule studies, i.e., on the order of 1 s (Perret *et al.*, 2002; Baumgartner *et al.*, 2003). Several differences can explain this apparent discrepancy. First, in laminar flow chamber studies, the authors used EC1-EC2 fragments grafted to microspheres or flat surfaces. The other domains EC3–5 might affect the adhesive capabilities, as shown by the unbinding force profile obtained by AFM (Sivasankar *et al.*, 1999). Recent measurements on entire extracellular domains using the bioforce probe apparatus suggest indeed that E-cadherin bonds can show a hierarchy of lifetimes, according to the EC regions involved (Perret *et al.*, 2004). Second, by using microspheres coated with a high density of ligands in dimeric configuration, lateral associations between N-cadherin receptors may be promoted. This should affect bond formation, because *cis*-interactions are a prerequisite for the formation of adhesive dimers (Chitaev and Troyanovsky, 1998). Third, in experiments using purely artificial systems, the transmembrane and cytoplasmic domains of the cadherin molecules are lacking, which also affect the affinity of the homophilic bond, as shown here.

Indeed, the use of the Ncad Δ cyto-GFP construct caused a clear disappearance of the exchange regime. The fully truncated receptor thus becomes locked in an almost irreversible binding state and can no longer dissociate and leave space for free receptors. This difficulty to dissociate from its ligand may explain the slower kinetics of Ncad Δ cyto-GFP recruitment around Ncad-Fc-coated beads, compared with the wild-type molecule (Figure 5; Table 1). In fact, the characteristic rate constant associated with fluorescence accumulation around beads is $k = k_{on}R + k_{off}$, which becomes small as k_{off} decreases. Furthermore, because Ncad Δ cyto-GFP stably bound to Ncad-Fc ligands on the beads, it behaved as an efficient competitor for ligand binding and thus reduced coupling between endogenous N-cadherin and the actin flow. In contrast, the turnover rate of the Ncad Δ bcat-GFP-truncated protein within N-cadherin contacts was twice as fast as that of the wild-type receptor, indicating that β -catenin binding or the N-cadherin cytoplasmic tail itself intrinsically slow down N-cadherin dynamics. Because the plateau value $C_{\infty} = L/(1 + k_{off}/k_{on}R)$ decreases with increasing k_{off} , the high turnover of Ncad Δ bcat-GFP is associated with a lower recruitment around Ncad-Fc-coated beads at steady state (ratio bead/neurite of 1.7). It also makes this Ncad Δ bcat-GFP mutant a poor competitor for ligand binding, and that may explain its weak effect on the coupling of Ncad-Fc-coated beads to the actin flow. By truncating further the whole intracellular domain of N-cadherin, turnover was abolished, supporting the concept that the membrane proximal region where p120^{ctn} binds (Ohkubo and Ozawa, 1999) is involved instead in accelerating ligand-receptor dissociation. Indeed, the mutated receptor NcadAAA-YFP, which cannot bind p120^{ctn} (Chen *et al.*, 2003), exhibited a much reduced turnover rate. Because the recruitment of this mutant around Ncad-Fc-coated beads was fast (Figure 5C), it means that the interaction between NcadAAA-YFP and its N-cadherin ligand has a high association rate and a low

dissociation rate. The underlying mechanism is unclear, but may be a positive action of p120^{ctn} on the exchange of cadherin receptors from adhesive to lateral dimers, which would tend to decrease adhesiveness (Ozawa and Kemler, 1998). Alternatively, because p120^{ctn} is implicated in the transport of N-cadherin toward the plasma membrane (Chen *et al.*, 2003) and turnover of E-cadherin at the cell surface (Davis *et al.*, 2003), it is possible that the loss of exchange at adhesive contacts observed for NcadAAA-YFP is also due to reduced trafficking events. Another possibility is that Ncad-AAA-YFP could be trapped via a stronger interaction with the cytoskeleton, resulting in reduced turnover.

Finally, an important question is how such dynamics of individual cadherin bonds can affect the overall durability and strength of adhesive zones. High bond kinetics (as for the Ncad $\Delta\beta$ cat-GFP mutant) should reduce adhesiveness, whereas reduced bond kinetics (as for the Ncad Δ cyto-GFP or NcadAAA-YFP mutants) should increase it. Indeed, E-cadherin mutants deleted of their β -catenin interaction domain cannot mediate cell aggregation, whereas fully truncated E-cadherin promotes cell aggregation to a similar extent as its wild-type counterpart and allows cells to resist high-shear forces (Ozawa and Kemler, 1998). At longer term, cadherin adhesions develop stronger forces, involving the connection of cadherin receptors to actin (Ozawa and Kemler, 1998) and signaling through Rac and cdc42 GTPases (Chu *et al.*, 2004). Despite such robustness, mature cadherin adhesive zones retain some plasticity, as shown here. This feature may be essential in the remodeling of neuronal connections, with strong implications in the control of synaptic transmission. For example, it has been shown that KCl-induced depolarization of hippocampal slices leads to β -catenin relocation and increased association to N-cadherin (Murase *et al.*, 2002) as well as dimerization of N-cadherin (Tanaka *et al.*, 2000), influencing synaptic size and strength. Furthermore, a mutation in δ -catenin, a close relative of p120 implicated in dendritic branching, correlates with mental retardation in humans as well as severe cognition defects and abnormal hippocampal synaptic plasticity in mutant mice (Israel *et al.*, 2004). Although the physiological responses to such modulation of the cadherin-catenin complex are far from being understood, our results constitute a substratum to further understand the underlying molecular and cellular mechanisms.

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