Two-tiered coupling between flowing actin and immobilized *N*-cadherin/catenin complexes in neuronal growth cones

Mikael Garcia^{a,b}, Cécile Leduc^c, Matthieu Lagardère^a, Amélie Argento^b, Jean-Baptiste Sibarita^a, and Olivier Thoumine^{a,1}

^aInterdisciplinary Institute for Neuroscience, UMR 5297, Centre National de la Recherche Scientifique (CNRS), University of Bordeaux, 33077 Bordeaux, France; ^bCYTOO SA, Minatec, 38040 Grenoble, France; and ^cCell Polarity, Migration, and Cancer Unit, CNRS, Unité de Recherche Associée 2582, Institut Pasteur, 75724 Paris Cedex 15, France

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Neuronal growth cones move forward by dynamically connecting actin-based motility to substrate adhesion, but the mechanisms at the individual molecular level remain unclear. We cultured primary neurons on *N*-cadherin-coated micropatterned substrates, and imaged adhesion and cytoskeletal proteins at the ventral surface of growth cones using single particle tracking combined to photoactivated localization microscopy (sptPALM). We demonstrate transient interactions in the second time scale between flowing actin filaments and immobilized *N*-cadherin/catenin complexes, translating into a local reduction of the actin retrograde flow. Normal actin flow on micropatterns was rescued by expression of a dominant negative *N*-cadherin construct competing for the coupling between actin and endogenous *N*-cadherin. Fluorescence recovery after photobleaching (FRAP) experiments confirmed the differential kinetics of actin and *N*-cadherin, and further revealed a 20% actin population confined at *N*-cadherin micropatterns, contributing to local actin accumulation. Computer simulations with relevant kinetic parameters modeled *N*-cadherin and actin turnover well, validating this mechanism. Such a combination of short- and long-lived interactions between the motile actin network and spatially restricted adhesive complexes represents a two-tiered clutch mechanism likely to sustain dynamic environment sensing and provide the force necessary for growth cone migration.

growth cone | actin flow | *N*-cadherin adhesion | micropatterned substrates | single-molecule tracking

Growth cones are motile structures at the extremity of axons responsible for path finding and neurite extension during nervous system development and repair. Growth cones translate extracellular signals into directional migration through a coordinated regulation of cytoskeleton, adhesion, and membrane processes (1). At the cytoskeletal level, motility is generated by polarized actin treadmilling, which, together with myosin contraction, generates a continuous retrograde actin flow from the periphery to the base of growth cones (2–7). At the membrane level, adhesion proteins form dynamic bonds with immobilized extracellular ligands, allowing step-by-step locomotion (8).

The molecular clutch model postulates that the mechanical coupling between ligand-bound transmembrane adhesion receptors and the actin flow allows traction forces to be transmitted to the substrate, resulting in local diminution of the retrograde flow and forward progression (9–11). Optical tweezers and flexible substrate experiments using microspheres coated with adhesion molecules revealed clutch-like mechanisms for integrins (12, 13), Ig cell adhesion molecules (14, 15), and cadherins (16, 17). However, the mechanism of clutch engagement at the individual molecular level remains elusive. For integrin-based adhesion, single-molecule tracking experiments suggested that talin and vinculin could switch between a state bound to flowing actin and a state bound to immobilized integrins (18, 19). For cadherin-based adhesion, biochemical experiments suggested that α -catenin could transit between being bound to actin or to the cadherin/ β -catenin complex (20, 21), but a direct visualization of such behavior is

lacking. In addition, vinculin, which can bind both actin and α -catenin, is recruited at cadherin-based intercellular junctions under mechanical force (22–24), but its dynamic behavior in growth cone migration is unknown.

By combining spatially controlled adhesion in growth cones with single-molecule tracking, fluorescence recovery after photobleaching (FRAP) experiments, and computer simulations, we report that *N*-cadherin/ α -catenin complexes, but not vinculin, are selectively trapped at *N*-cadherin micropatterns. In addition, 80% of actin molecules flow rearward, making transient pauses on the order of seconds with immobilized *N*-cadherin/ α -catenin complexes, whereas 20% of confined actin molecules contribute to local actin enrichment. This association of short- and long-lasting individual bonds underlies the differential coupling between the actin motile machinery and substrate adhesions supporting growth cone migration.

Results

***N*-Cadherin/Catenin Complex Is Diffusionally Trapped at *N*-Cadherin Micropatterns.** To induce *N*-cadherin adhesions at spatially controlled locations, we cultured primary hippocampal neurons on micropatterned substrates coated with recombinant *N*-cadherin-Fc (Ncad-Fc) as described (25) (Fig. 1A). Neurons developed faster on those substrates than on glass coverslips uniformly coated with polylysine (PLL) or micropatterned substrates coated with Fc (Fig. S1 A–D), in agreement with the growth-promoting activity of *N*-cadherin (26). Neurons extended numerous and long

Significance

Using state-of-the-art micropatterning technology and single-molecule imaging, we revisited a prevalent paradigm in cell locomotion, the molecular clutch, which states that the intracellular actin motile machinery is dynamically coupled to transmembrane adhesion, allowing cells to move forward by applying forces on the substrate. We combined primary neuronal cultures on micropatterned substrates to trigger selective micron-scale *N*-cadherin adhesions, with single-molecule mapping of actin, *N*-cadherin, and α -catenin at the ventral surface of growth cones using photoconvertible fluorescent proteins. We demonstrate that actin filaments transit on a time scale of seconds between a flowing state and a state bound to immobilized *N*-cadherin/catenin complexes. These transient connections represent the first direct evidence for an intrinsic molecular clutch mechanism underlying growth cone locomotion.

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¹To whom correspondence should be addressed. Email: olivier.thoumine@u-bordeaux.fr.

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Fig. 1. Membrane *N*-cadherin and α -catenin are selectively immobilized at *N*-cadherin micropatterns. (A) Neurons were cultured on micropatterned glass substrates comprising regularly spaced Ncad-Fc-coated dots (red) separated by a cytophobic agent (violet). (B) Side view of the molecular interactions at the *N*-cadherin/actin interface. (C and D) Epifluorescence images of growth cones expressing Ncad-GFP or α -catenin-GFP on Ncad-Fc patterns (red circles). (E and F, Left) Individual trajectories of *N*-cadherin-mEos2 (Ncad-mEos2) or α -catenin-mEos2 molecules (green) superimposed on Ncad-Fc patterns (red). (E and F, Right) In higher magnification images, confined trajectories with $D < 0.01 \mu\text{m}^2\text{s}^{-1}$ are white, whereas Brownian trajectories with $D > 0.01 \mu\text{m}^2\text{s}^{-1}$ are green. (Scale bars: C–F, 2 μm .) (G and H) Distributions of diffusion coefficients for Ncad-mEos2 and α -catenin-mEos2 molecules, respectively, at Ncad-Fc patterns or outside regions (between 1,222 and 4,062 trajectories analyzed). Gray-shaded areas represent confined molecules with $D < 0.01 \mu\text{m}^2\text{s}^{-1}$. (I) Corresponding fraction of confined trajectories. Data represent the mean $\pm$ SEM of confined fractions (*** $P < 0.0001$ by paired Student's *t* test). The number of growth cones examined is given in each column.

neurites as on substrates uniformly coated with Ncad-Fc (16), showing that the cytophobic layer between micropatterns did not alter axonal growth. A single growth cone typically spread over several Ncad-Fc-coated dots, allowing a paired comparison between *N*-cadherin adhesions and nonadhesive areas.

To visualize molecules engaged in adhesions at the ventral surface of growth cones (Fig. 1B), we electroporated neurons with *N*-cadherin, actin, α -catenin, or vinculin constructs fused to either GFP for conventional epifluorescence illumination or to the photoconvertible protein mEos2 (27) for observation of individual molecule behavior by single-particle tracking combined to photoactivated localization microscopy (sptPALM) (19, 28). We checked by immunocytochemistry that recombinant proteins were expressed at relatively low levels and incorporated well into endogenous protein pools (Fig. S1 E–G). *N*-cadherin GFP (Ncad-GFP) was strongly accumulated at Ncad-Fc-coated micropatterns (Fig. 1C and Table 1), indicating that membrane *N*-cadherin was engaged in homophilic adhesion. At the individual level, *N*-cadherin-mEos2 molecules displayed a variety

of behaviors, ranging from Brownian motion with a high diffusion coefficient ($D \geq 0.1 \mu\text{m}^2\text{s}^{-1}$), to mixed trajectories ($0.01 < D < 0.1 \mu\text{m}^2\text{s}^{-1}$), to complete immobilization ($D < 0.001 \mu\text{m}^2\text{s}^{-1}$) (Fig. 1 E and G, Fig. S2, and Movie S1). Trajectories with diffusion coefficients below $0.01 \mu\text{m}^2\text{s}^{-1}$ were called confined. The fraction of confined *N*-cadherin-mEos2 trajectories was significantly higher on Ncad-Fc micropatterns than on nonadhesive areas (42% vs. 25%, respectively) (Fig. 1 E and I), reflecting the formation of specific interactions with Ncad-Fc on the substrate. Similar to *N*-cadherin, α -catenin-GFP was strongly recruited at Ncad-Fc micropatterns (Fig. 1D and Table 1), and individual α -catenin-mEos2 molecules exhibited greater confinement at Ncad-Fc-coated micropatterns than on nonadhesive areas (Fig. 1 F, H, and I), revealing a preferential association with *N*-cadherin adhesions. The fact that we cannot resolve the motion of cytosolic molecules by sptPALM (19) and that the diffusion/trapping behaviors of *N*-cadherin-mEos2 and α -catenin-mEos2 were extremely similar strongly suggests that *N*-cadherin and α -catenin travel as a single complex in the membrane. In contrast, vinculin-GFP was not enriched at Ncad-Fc-coated micropatterns, and single vinculin-mEos2 molecules did not preferentially stop at these locations (Table 1 and Fig. S3 A–D), indicating a minor role of vinculin in the assembly of cadherin adhesions in hippocampal growth cones. Nevertheless, GFP- and mEos2-tagged vinculin, as well as endogenous vinculin, strongly accumulated at intercellular junctions between glial cells present in the cultures and at peripheral contacts between glial cells and Ncad-Fc-coated micropatterns (Fig. S3 F and G). These results demonstrate that tagged vinculin is functional and confirm that vinculin is selectively recruited at cadherin contacts under high mechanical constraint (22–24).

Mapping and Regulation of the Actin Flow in Growth Cones. In contrast to the diffusion/trapping behavior of *N*-cadherin and α -catenin, individual actin-mEos2 molecules displayed directed rearward motion at the periphery of growth cones and then became diffusive or confined in the intermediary and central regions (Fig. 2B and Movie S2). Three types of trajectories were distinguished (directed, Brownian, or confined) according to the shape of the mean squared displacement over time (parabolic, linear, or anomalous, respectively) (Fig. S4). Directed trajectories yielded an average velocity of $0.14 \mu\text{m}\text{s}^{-1}$, consistent with values obtained from actin-GFP kymographs in hippocampal neurons (11). The actin-capping drug cytochalasin D, the monomeric actin-sequestering drug latrunculin A, and the actin-stabilizing agent jasplakinolide all dramatically diminished the fraction of rearward-moving actin-mEos2 molecules (21%, 7%, and 2%, respectively) (Fig. S5), consistent with a major role of actin tread-milling in generating the flow (2, 3, 5). Myosin-light chain (MLC)-GFP was localized in the intermediary region of the growth cone, and MLC-Eos2 trajectories were mostly confined in this area (Fig. S6). Addition of the phosphatase inhibitor calyculin A to enhance myosin II contractility (29) induced a major retraction of the actin-GFP peripheral network in <5 min, an effect that was blocked by preincubation with the myosin II inhibitor blebbistatin. Finally, blebbistatin decreased the fraction of directed actin-mEos2 trajectories and reduced their velocity by 23%. Together, these results indicate that myosin II contributes to actin flow generation by pulling on actin filaments, consistent with previous studies (5, 30).

Engagement of *N*-Cadherin Adhesions Reduces Actin Flow. In contrast to *N*-cadherin and α -catenin, actin-GFP was not specifically enriched at Ncad-Fc-coated micropatterns under epifluorescence illumination (Fig. 2A and Table 1), suggesting that actin was weakly bound to the *N*-cadherin/ α -catenin complex in growth cones. However, rearward actin velocity dropped with increasing Ncad-Fc coating concentration (Fig. 2E) and negatively correlated with forward growth cone migration speed (Fig. 2F), revealing partial clutch engagement between *N*-cadherin adhesion and actin (6). Furthermore, individual actin-mEos2 molecules often slowed

Table 1. Enrichment of GFP tagged *N*-cadherin, α -catenin, actin, and vinculin at Ncad-Fc micropatterns

Transfected protein	Ncad-GFP	α -Catenin-GFP	Actin-GFP	Vinculin-GFP
Epifluorescence	1.35 $\pm$ 0.08 (19)	1.27 $\pm$ 0.05 (10)	1.04 $\pm$ 0.06 (15)	1.04 $\pm$ 0.07 (11)
TIRF	1.74 $\pm$ 0.09 (19)	1.51 $\pm$ 0.08 (9)	1.45 $\pm$ 0.05 (15)	1.04 $\pm$ 0.04 (9)

Fluorescence levels were normalized to control regions outside micropatterns, on the same growth cones. Values are presented as the mean $\pm$ SEM (number of growth cones examined).

down or stopped at Ncad-Fc-coated micropatterns (Fig. 2*B*), resulting in less directed (56% vs. 74%) and more confined (23% vs. 8%) trajectories at micropatterns compared with nonadhesive areas (Fig. 2*C*). The retrograde velocity of actin molecules displaying rearward motion was 20% lower on Ncad-Fc patterns than on cytophobic areas or on PLL-coated substrates (Fig. 2*D* and *E*). To address the molecular mechanisms underlying this effect, neurons were coelectroporated with actin-mEos2 and an *N*-cadherin construct deleted of the whole extracellular domain (Ncad- Δ E), which was thus unable to form homophilic adhesions. This mutant acts as a dominant negative construct by sequestering α - and β -catenins, thereby interfering with the binding of actin to endogenous *N*-cadherin (16, 29). Upon Ncad- Δ E expression, the velocity of actin-mEos2 molecules on Ncad-Fc micropatterns was rescued to the high levels outside micropatterns (Fig. 2*D*), suggesting that the reduction of actin velocity on Ncad-Fc micropatterns involves a connection through catenins. This experiment also demonstrates that the slowing down of actin filaments on Ncad-Fc micropatterns is not due to a nonspecific frictional effect attributable to molecular crowding at adhesion sites.

Transient Pauses of Actin Molecules on *N*-Cadherin Micropatterns. We then analyzed in detail actin-mEos2 trajectories, focusing on molecules spending part of their time inside a micropattern and the other part outside (Fig. 3*A* and *B*). Actin-mEos2 molecules entering a pattern showed a significant reduction in velocity (Fig. 3*A*), whereas molecules exiting a pattern displayed acceleration (Fig. 3*B*). Thus, individual rearward-moving actin filaments can rapidly reduce their speed by contacting *N*-cadherin adhesions, and these interactions are spatially restricted to Ncad-Fc micropatterns, suggesting that the actin network is loosely interconnected. Actin molecules within Ncad-Fc micropatterns often exhibited pauses around 1–2 s, which appeared less frequently outside patterns (Fig. 3*C*). The percentage of the trajectory duration spent in pauses was approximately fivefold higher for actin trajectories localized within Ncad-Fc patterns than for those actin trajectories localized outside (Fig. 3*D*). This significant increase in pause proportion was blocked by the expression of Ncad- Δ E (Fig. 3*D*), indicating that these pauses reflect specific labile interactions between actin filaments and *N*-cadherin adhesions, mediated by catenins.

Different Turnover of *N*-Cadherin and Actin at *N*-Cadherin Micropatterns. To assess whether *N*-cadherin and actin also exhibited differential dynamics at the ensemble level, we performed FRAP experiments on GFP-tagged *N*-cadherin, α -catenin, vinculin, and actin at Ncad-Fc micropatterns. We used total internal reflection fluorescence (TIRF) to isolate molecules close to the substrate ($\sim$ 100 nm) (Fig. 4*A*). TIRF illumination confirmed the robust accumulation of Ncad-GFP at Ncad-Fc micropatterns (175% enrichment), but also revealed significant enrichment of actin-GFP (145%) organized in various ways at or around the micropatterns (Fig. S5*C*). Phalloidin staining was very strong at Ncad-Fc micropatterns in TIRF (Fig. S5*D*), demonstrating the recruitment of endogenous F-actin. Addition of latrunculin A to growth cones migrating on Ncad-Fc-coated substrates decreased the number of confined actin-mEos2 trajectories compared with untreated growth cones (Fig. S5*F* and *H*), suggesting that G-actin was not selectively recruited at *N*-cadherin adhesions, consistent with assays using purified proteins (31). FRAP of

actin-GFP revealed a rapid 80% recovery in about 50 s, followed by a slower recovery to 100% in the remaining 150 s (Fig. 4*B* and *C*). These phases tightly correspond to the two populations of actin-mEos2 observed by sptPALM on Ncad-Fc-coated patterns (i.e., 80% of flowing molecules with directed or Brownian trajectories showing weak interactions with *N*-cadherin adhesions and 20% of confined molecules

Fig. 2. Actin flow is slowed down at *N*-cadherin micropatterns. (A) Growth cone expressing actin-GFP on Ncad-Fc-coated micropatterns (red circles) by epifluorescence. (B) Individual actin-mEos2 trajectories (green), with magnified views focusing on a micropattern or a neighboring nonadhesive area. Directed trajectories (exponent $\alpha > 1.4$) are shown in green, diffusive trajectories ($0.8 < \alpha < 1.2$) are shown in cyan, and confined trajectories ($\alpha < 0.8$) are shown in white. (Scale bars: A and B, 2 μ m; magnified views in B, 1 μ m.) (C) Repartition of the three types of behaviors (directed, Brownian, and confined) for trajectories outside (black) and inside (red) Ncad-Fc patterns (479 and 535 trajectories, respectively). Fractions were compared by paired *t* tests (**P* = 0.04; ****P* = 0.0045). (D) Velocity of actin-mEos2 molecules in growth cones from neurons electroporated with either actin-mEos2 alone or actin-mEos2 plus Ncad- Δ E and plated on Ncad-Fc-coated micropatterned substrates. Measurements were performed for trajectories exclusively inside or outside micropatterns. The mean $\pm$ SEM values of several growth cones are shown, and the number of growth cones examined is given in each column. Data were compared by paired *t* tests (****P* = 0.0003). n.s., not significant. (E) Retrograde velocity of actin-mEos2 molecules in growth cones migrating on substrates uniformly coated with increasing concentrations of Ncad-Fc. Data were compared with the control PLL coating condition by the Kruskal–Wallis test (***P* = 0.0062). (F) Negative correlation between actin-mEos2 velocity and growth cone forward velocity on substrates uniformly coated with Ncad-Fc (16). The numbers of growth cones examined are in the range of seven to 18 for actin-mEos2 velocity and 45–75 for growth cone migration speed.

Fig. 3. Transient pauses of actin filaments on *N*-cadherin micropatterns. (A and B) Examples of actin-mEos2 trajectories (green) entering and exiting Ncad-Fc patterns (red), respectively, and corresponding graphs showing displacement vs. time for the mean $\pm$ SEM of 23 entering and 31 exiting trajectories. Dashed lines that prolong the initial slopes are guides to the eye. (Scale bars: A and B, 1 μ m.) (C) Individual example showing the displacement of a molecule entering a region coated with Ncad-Fc. Note that the trajectory was fairly linear before entering the region and showed multiple pauses thereafter (arrows). (D) Median of pause durations, normalized by the total duration of each trajectory, for neurons expressing actin-mEos2 alone or actin-mEos2 plus Ncad-ΔE. Data are presented as the mean $\pm$ SEM, with the number of growth cones indicated in the columns. Regions within and outside patterns were compared by unpaired *t* tests (**P* < 0.05).

with long-lasting interactions) (Fig. 2 B and C). This second population likely contributes to actin enrichment at Ncad-Fc micropatterns. Ncad-GFP also recovered in two phases (Fig. 4 B and C): a rapid recovery within 20 s to $\sim$ 50%, roughly corresponding to the fraction of freely diffusing molecules identified in sptPALM, and a slower linear recovery reflecting molecules trapped by homophilic interactions at Ncad-Fc-coated micropatterns and slowly exchanging. The latter corresponds to the 45% fraction of confined *N*-cadherin molecules in sptPALM. α -Catenin-GFP behaved very similar to Ncad-GFP under FRAP (Fig. 4C), again indicating that *N*-cadherin and α -catenin were tightly associated. In contrast, vinculin-GFP recovered very fast (Fig. S3E), suggesting that it was not bound to Ncad-Fc patterns.

Model of Cadherin and Actin Dynamics. To integrate these experimental data into a single consistent framework allowing comparison between single-molecule recordings and ensemble measurements, we performed Monte Carlo simulations describing the motion of single *N*-cadherin and actin molecules within growth cones containing localized adherent zones (Fig. S7 A–C). *N*-cadherin molecules were considered to move in the plasma membrane with $D = 0.17 \mu\text{m}^2\text{s}^{-1}$, which was calculated from the mobile population in sptPALM, and were allowed to form transient interactions with Ncad-Fc immobilized at micropatterns, with a slow turnover rate (0.002 s^{-1}) in agreement with values reported in the literature (32, 33) (Fig. 5A and Movie S3). Superimposition of thousands of individual simulations, mimicking a fluorescence image, revealed strong enrichment of *N*-cadherin on Ncad-Fc-coated micropatterns compared with nonadhesive areas ($\sim$ 180%), as observed for Ncad-GFP with TIRF illumination (Fig. 5 C and F and Table 1). Furthermore, FRAP simulations showed recovery kinetics in close agreement with experimental data (Fig. 4C, Fig. S8A, and Movie S5), validating the coexistence of a rapidly diffusing (unbound) *N*-cadherin population and a fraction strongly bound to Ncad-Fc (Fig. S7D).

The actin model included fast free diffusion of monomers, slow rearward motion of actin filaments assembled at the tip,

intermittent coupling to localized *N*-cadherin adhesions, and disassembly at the base (Fig. 5B and Movie S4). Association and dissociation rates between actin and the α -catenin/*N*-cadherin complex were taken as the frequency of the pauses ($k_{\text{on}} = 0.2 \text{ s}^{-1}$) and the inverse of pause duration ($k_{\text{off}} = 0.8 \text{ s}^{-1}$), respectively. The average actin velocity measured for trajectories localized within micropatterns was consistent with experimental values ($0.11 \mu\text{m}\cdot\text{s}^{-1}$), whereas normal velocity ($0.14 \mu\text{m}\cdot\text{s}^{-1}$) was restored when k_{on} was set to zero, mimicking the effect of Ncad-ΔE (Fig. 5E). We further introduced a 20% actin population displaying durable interactions with *N*-cadherin adhesions ($k_{\text{off}} = 0.001 \text{ s}^{-1}$), allowing us to mimic the 145% actin enrichment at Ncad-Fc micropatterns observed with TIRF (Fig. 5 D and F and Table 1) and to simulate the slower phase of actin-GFP recovery observed in FRAP experiments (Fig. 4C, Fig. S8B, and Movie S6). The model also predicted regulation of the balance between G-actin and F-actin by latrunculin A and jasplakinolide in both sptPALM and FRAP paradigms (Figs. S7E and S8C).

Discussion

This study exploring the dynamic coupling between actin motility and *N*-cadherin adhesions in growth cones can be summarized by a two-step model (Fig. 6): (i) The membrane-diffusing *N*-cadherin/catenin complex is trapped at Ncad-Fc-coated micropatterns, and (ii) flowing actin filaments interact with this adhesive complex through a combination of short- and long-lived interactions.

At the adhesion level, diffusion and trapping of *N*-cadherin and α -catenin are reminiscent of the behavior of integrins and talin at focal contacts in fibroblasts (19). FRAP experiments allowed us to calculate an average dwell time of $\sim$ 500 s, which is compatible with previous measurements on Ncad-GFP accumulated at Ncad-Fc beads in growth cones (32) or at focal adhesion-like structures formed by myogenic cells on substrates coated with Ncad-Fc (33). Introducing this parameter in the simulations, we mimicked the experimentally observed $\sim$ 180%

Fig. 4. FRAP on actin-GFP, α -catenin-GFP, and Ncad-GFP accumulated at *N*-cadherin micropatterns. (A) Growth cones from neurons expressing Ncad-GFP or actin-GFP were observed by TIRF illumination, revealing significant enrichment of both proteins at Ncad-Fc patterns (circles). (Insets) Regions selected for photobleaching. (B) Ncad-GFP or actin-GFP signals were photobleached on two to three patterns per cone, with an $\sim$ 300-ms laser pulse, and the fluorescence recovery was recorded for 200 s (color coding). (Scale bars: A and B, 2 μ m.) (C) Graphs of fluorescence recovery over time for Ncad-GFP, α -catenin-GFP, and actin-GFP. Solid dots represent the average of three independent experiments [10–20 growth cones, 18–50 patterns per condition $\pm$ SEM (gray)], and plain curves are the average of three independent simulations.

phalloidin under TIRF illumination. This process revealing full clutch engagement (17) resembles the dynamic assembly of a flexible actin network occurring at restrained beads coated with *Aplysia* neural cell adhesion molecule or *N*-cadherin (14, 16, 41). The confined fraction may correspond to flowing actin filaments being stalled by interactions with a high local density of *N*-cadherin/ α -catenin complexes, as predicted theoretically (45). The various shapes of actin-GFP at Ncad-Fc patterns may reflect such a differential anchorage of flowing actin to *N*-cadherin adhesions. Alternatively, although G-actin sequestered by latrunculin A was not trapped at Ncad-Fc substrates, we cannot rule out a local nucleation of branched actin filaments (46, 47) at *N*-cadherin adhesions arising, for instance, from local depletion of homodimeric α -catenin competing with Arp2/3 for actin binding (20, 21). In any case, those strongly anchored actin filaments might bear the frictional force necessary for growth cones to move forward, whereas the more rapidly interacting filaments may allow dynamic sampling of the environment.

Methods

Experimental protocols are given in *SI Methods*. mEos2-tagged *N*-cadherin, α -catenin, vinculin, MLC, and actin were generated from GFP-tagged plasmids as described previously (16, 29, 32). Rat hippocampal neurons were dissected and electroporated with those plasmids before plating. Neurons

were cultured on coverslips homogeneously coated with PLL or Ncad-Fc, or on Ncad-Fc-coated micropatterned glass substrates (16, 25). Immunostaining with antibodies to *N*-cadherin, α -catenin, and actin or with phalloidin was performed as described (29). SptPALM experiments using a 405-nm photoactivation laser and a 561-nm excitation laser were performed as reported (19), with high acquisition frequency for mEos2-tagged *N*-cadherin, α -catenin, and vinculin (50 Hz) and a slow frame rate for actin-mEos2 and MLC-Eos2 (2 Hz) (40). Image stacks were analyzed using custom analysis programs. FRAP experiments on GFP-tagged *N*-cadherin, α -catenin, vinculin, and actin were performed using a focused 491-nm laser, and recordings were made by TIRF illumination. Computer simulations of *N*-cadherin and actin dynamics were based on previous frameworks (48).

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