Molecular Interactions

Optical Tweezers and Fluorescence Recovery After Photo-Bleaching to Measure Molecular Interactions at the Cell Surface

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Abstract—This paper describes applications of two optical microscopy techniques, namely laser tweezers and fluorescence recovery after photo-bleaching (FRAP), to the measurement of ligand/receptor/cytoskeleton interactions. These methods are used in combination with ligand-coated microspheres, binding to specific membrane receptors at the dorsal cell surface. A first application exploits the possibility to impose piconewton forces on microspheres. At low ligand density, one can identify the rupture of individual bonds between ligand/receptor complexes and the motile actin cytoskeleton. The second application uses control of the initial contact time by optical tweezers, together with time lapse imaging of GFP-tagged receptor accumulation around the microspheres. Quantification of fluorescence enrichment together with a simple chemical reaction model allows characterization of global ligand/receptor on-rates. These contacts eventually reach steady-state corresponding to continuous formation and dissociation of ligand/receptor bonds. FRAP is then used to probe the equilibrium dynamics of this system, by photo-bleaching GFP-tagged receptors recruited at ligand-coated beads. The recovery curves are fitted by a diffusion/reaction model, to yield turnover rates between adhesion proteins. To illustrate the potential of these techniques, we take examples from our previously published data on neuronal adhesion proteins involved in growth cone locomotion, in particular N-cadherin and immunoglobulin cell adhesion molecules.

Keywords—Optical microscopy, Neuronal growth cone, Actin, N-cadherin, Immunoglobulin cell adhesion molecules, L1, Ligand/receptor interactions.

INTRODUCTION: THE BIOLOGICAL CONTEXT OF GROWTH CONE MIGRATION

Growth cones are motile structures at the extremity of axons responsible for pathfinding decisions and neurite extension during nervous system development and repair. In this process, transient contacts are made between growth cones and the extracellular matrix or surrounding cells, as well as more stable contacts between axons that will make nerve fibers (i.e. fasciculation). These contacts are mediated by specific adhesion receptors expressed at the cell membrane, including immunoglobulin cell adhesion molecules (IgCAMs) and cadherins. These proteins are expressed with precise developmental profiles in the brain and serve many different functions. For example, IgCAMs such as L1 and NrCAM are involved in axon fasciculation and outgrowth, whereas N-cadherins are involved cell positioning, axon outgrowth, dendritic branching, synaptogenesis, and synaptic plasticity.

In terms of molecular structure, N-cadherins are single-pass transmembrane proteins forming homophilic calcium-dependent bonds by trans-associations of their extracellular domains (Fig. 1). Cadherin ectodomains are also able to form lateral oligomers, resulting in complex adhesive structures. On the intracellular side, the cadherin cytoplasmic tail can couple in a dynamic fashion to actin filaments via the adaptor proteins \(\alpha\)-catenin and \(\beta\)-catenin. IgCAMs are transmembrane proteins with an ectodomain formed of several FnIII and Ig-like repeats. IgCAMs share a conserved cytoplasmic tail mediating interactions with ankyrin, ezrin-radixin-moesin members, PDZ domain proteins, and, exclusively for the neuronal form of L1, with the clathrin adaptor protein AP-2. Thus, adhesions formed by both IgCAMs and cadherins can make a transmembrane anchor to the highly dynamic actin cytoskeleton, which allows for force transmission and enables neuronal growth cones to move forward.
The formation and maintenance of cell-cell contacts intimately relies on the individual kinetic properties of the molecular bonds between CAMs, but also on more complex processes such as clustering with cis-interacting partners, coupling to the cytoskeleton, and intracellular targets which can modulate ligand/receptor adhesiveness. Indeed, both proteins are linked to specific signaling pathways, e.g. tyrosine phosphorylation of the L1 tail regulating endocytosis,9,24 and ankyrin binding,17,18 and Rac-1 regulating the anchorage of N-cadherin to the actin flow.27 Hence, it is important to study the behavior of populations of adhesion receptors in natural cell-cell contacts. Here, we provide examples of how optical microscopy techniques (optical tweezers, time lapse fluorescence imaging, and photo-bleaching) can be used to probe the association and dissociation rates between assemblies of adhesion molecules in living neurons (Table 1).

METHODS

All materials, methods, equipment and manufacturers were described in length in previous papers,2,12,16,46,47 and are not reproduced here for the sake of room. Briefly, DNA plasmids coding for GFP- or RFP-tagged receptors were transfected into 2–3 DIV primary rat hippocampal neurons using calcium phosphate precipitation, and neurons were observed 24–48 h later. Sulfate latex microspheres were coated with a secondary anti-Fc antibody, then with primary anti-GFP, or purified N-cadherin-Fc, TAG-1-Fc, or L1-Fc proteins. Beads were either placed on growth cones with optical tweezers for 2 s to 2 min, or incubated for 30 min on neurons, then processed for FRAP. The microscope set-up combining FRAP, optical tweezers, and time lapse imaging is described in Fig. 2.

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<tr>
<th>Technique</th>
<th>Advantages</th>
<th>Limitations</th>
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<tr>
<td>Ligand-coated microspheres</td>
<td>Good orientation and flexibility of the molecules through Fc-fusion</td>
<td>No translational diffusion of the ligands.</td>
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<td>of the molecules through Fc-fusion</td>
<td>However, techniques exist to coat microspheres with lipid bilayers and freely diffusible</td>
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<td>protein and anti-Fc fragment antibody</td>
<td>GPl-anchored proteins11</td>
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<td>Essentially probes cell surface events</td>
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<td>Signal contaminated by intracellular receptors.</td>
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<td>However, pH-sensitive GFP allows identification of surface-associated receptors</td>
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<td>GFP-tagged receptors</td>
<td>Highly specific labeling</td>
<td>Detected forces are typically smaller than 50 pN, i.e. not accessible to all ligand/receptor interactions</td>
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<td>Receptors lie in their native membrane environment</td>
<td>Accumulation of GFP tagged receptors at beads yield fairly noisy curves which do not allow easy discrimination between various chemical models</td>
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<td>Possibility to express mutated receptors</td>
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<td>Optical tweezers</td>
<td>Impose and measure forces after proper calibration</td>
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<td>Exquisitely good at low forces, e.g. motor proteins or receptor/cytoskeleton bonds involved in the clutch mechanism</td>
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<td>Precise control of initial encounter time</td>
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<td>FRAP</td>
<td>Fairly quick ensemble measurements</td>
<td>Local photo-damage to test</td>
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<td>Easy to implement on a standard confocal microscope</td>
<td>Yields only global rates, involving multiple ligand/receptor pairs</td>
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FIGURE 1. Schematic diagram showing the structure and intracellular binding partners of cadherin and immunoglobulin cell adhesion molecules. EC extracellular module. The EC1 domain is the one that primarily mediates homophilic adhesion. There are 3 calcium ions localized between each EC subdomains, which stabilize the structure of the cadherin ectodomain, and provide calcium dependent adhesion. Ig immunoglobulin domain; FnIII fibronectin type III module. L1 has been shown to spontaneously adopt a horseshoe conformation, corresponding to a kink between Ig domains 2 and 3.

TABLE 1. Advantages and limitations of the various approaches.
FIGURE 2. Description of the optical tweezers/FRAP set-up. The optical tweezers/FRAP set-up consists of an inverted microscope (Olympus IX 70) fed through its epifluorescence port by an Argon laser (Innova 1064 nm, 2.5 W, Coherent) or an Argon laser (Innova 300, 8 W, Coherent), both expanded by 8x telescopes (one lens of 25 mm focal length followed by another lens of 200 mm focal length). Merging of the two laser lines is achieved by a long-pass dichroic mirror with 750 nm cut-off (Omega optical) which transmits the 1064 nm line and reflects the 488/514 nm lines (position 0). Fluorescence illumination comes from a 100 W Xenon lamp placed in position 1, optical tweezers through a second long-pass dichroic mirror (position 2) hit inside the microscope a dual dichroic mirror which reflects blue light below 500 nm and infrared light above 750 nm (Chroma). GFP emission light is then collected using a 525/25 nm filter in position 3. In a low-pass filter which does not transmit above 750 nm is placed in position 4 for attenuating infrared laser reflection. For FRAP, a 70/30 beam splitter (Chroma) is used instead in position 1 together with a standard cube for GFP illumination (Chroma) within the microscope. Both lasers are focused through a 100×/1.40 NA oil objective. Laser powers are 100 and 2.5 mW at the back of the objective for optical tweezers and FRAP, respectively. A set of two lenses in a near afocal configuration with one lens on a 3D translator are used for fine centering of the beam. The focal length (200 mm) is chosen so that it matches the spacing between the second lens and the back aperture of the objective. In that configuration, the 2D movement of the lens translates into a 2D movement in the specimen plane, and the z position is adjusted to yield a diffraction-limited spot on the focal plane. Transmitted light, epifluorescence, and laser illuminations are all controlled independently by shutters (Uniblitz, Vincent Associates). Temperature is maintained at 37°C with an air blower (World Precision Instruments) and an objective heater (Biotechs). Digital images are acquired using a cooled CCD camera (HQ Cool Snap, Roper Scientific) driven by the Meta morph software (Universal Imaging). For optical tweezers experiments, microspheres are brought to the optical trap using a motorized stage (MarzHauser), and captured microspheres are placed on the dorsal surface of growth cones, for variable time periods (up to 2 min). The position of the microsphere is monitored by transmitted light, and the distribution of GFP-tagged receptors is followed in the fluorescence channel. For FRAP experiments, a region of interest on a neuron expressing GFP-tagged adhesion receptors is brought into the position of the laser spot. The sample is bleached by the laser for 0.1–0.3 s, and fluorescence recovery is recorded for typically 10 min. Images are analyzed using Metamorph.

LIGAND/RECEPTOR ON RATES MEASURED BY OPTICAL TWEEZERS AND FLUORESCENCE MICROSCOPY

Optical tweezers consist of a near infrared laser beam focused by a high numerical aperture microscope objective. The convergent rays of light behave as a three-dimensional trap for dielectric particles such as latex or glass microspheres, and can also be used to capture and manipulate bacteria or even whole eukaryotic cells. Optical trapping can be super-imposed to fluorescence imaging using appropriate optical components (Fig. 2). Latex microspheres can be coated with specific antibodies or ligands, such as the outer domains of adhesion proteins fused to the constant fragment of human IgG and produced as recombinant proteins. These constructs, coated onto a layer of anti-Fc antibodies grafted on the microspheres, allow a proper orientation and functionality of the adhesive moieties. Such bio-mimetic system allows a precise control of the type of ligand molecules presented to the cell and of the timing and duration of the interaction, which is not possible for natural contacts that form at arbitrary moments and where different adhesion proteins can co-exist in unknown stoichiometries. One can also achieve a reasonable estimate of the ligand coating density using immunofluorescence staining against a scale of purified proteins, although one is never sure of the actual number of functional ligands on the microspheres, and whether some molecules are hidden within the latex matrix. Microspheres are brought into contact with the region of interest, e.g. the dorsal surface of a neuronal growth cone (Figs. 3a and 3b). In these experiments, the optical trap is used essentially to control the time zero of the adhesive interaction. The position of the microsphere is then tracked in the transmitted light channel, and the redistribution of GFP-tagged molecules transacted into the neuron is followed in the epifluorescence channel (Fig. 3c). One can thus observe a progressive accumulation of adhesion receptors at microsphere contacts, and quantify its kinetics, starting from the initial contact (Fig. 3d).

The interaction between a receptor \( R \) expressed at the cell surface and a ligand \( L \) grafted on the microsphere, to form the molecular complex \( R \cdot L \) is described by a simple chemical reaction, hypothesizing first order kinetics with association rate \( k_{on} (\mu m^2/s) \) and dissociation rate \( k_{off} (s^{-1}) \): 

\[
R + L \leftrightarrow R \cdot L
\]

Assuming that diffusion of receptors in the plasma membrane is fast and not the dominant limitation, the surface density of receptor–ligand complexes \( (R \cdot L) \) at
the microsphere-to-cell interface, noted $[C]$, is then given by:

$$\frac{d[C]}{dt} = k_{\text{on}}[R][L] - k_{\text{off}}[C]$$  \hspace{1cm} (1)$$

where $[R]$ and $[L]$ are the free receptor and ligand densities at the cell and bead surfaces (in units of #/μm$^2$). The pool of surface receptors is sufficiently large when compared to the bead area so we can neglect receptor depletion and set $[R] = [R]_t - [C] = [R]_t$, where $[R]_t$ is the total (uniform) receptor density at the cell surface. Conversely, because of a limited supply of ligands at the bead surface, we hypothesize ligand depletion, written as $[L] = [L]_t - [C]$, where $[L]_t$ is the total ligand coating density on the microspheres. Given the initial condition of no bond, the solution of this differential equation is:

$$[C(t)] = [C]_\infty[1 - \exp(-kt)]$$  \hspace{1cm} (2)$$

with two pooled parameters $k = k_{\text{on}}[R]_t + k_{\text{off}}$ which represents an overall rate constant, and $[C]_\infty = [L]_t / (1 + k_{\text{off}}/[R]_t)$ which is the equilibrium bond density. In the case of antibody-antigen interactions which are very stable, we can set $k_{\text{off}} = 0$, and the steady state corresponds to saturation of ligand sites on the microspheres. The fluorescence level around beads is normalized by a control level on a nearby region, allowing reduction of potential photo-bleaching effects, and the data are fitted by the expression $1 + [C(t)]/[R]_t$ (Fig. 3d). The measurements of $[L]_t$ and $[R]_t$ by independent biochemistry and immuno-cytochemistry allow us to compute the intrinsic rates $k_{\text{on}}$ and $k_{\text{off}}$. 

FIGURE 3. Diffusion/trapping of IgCAMs at biomimetic contacts. (a) Illustration of the experiment. Cells are transfected with IgCAMs bearing an extracellular GFP tag and beads coated with anti-GFP antibodies are brought into contact with the growth cone surface by optical tweezers. (b) Membrane IgCAMs diffusing into the vicinity of a bead get captured almost irreversibly by the antibody. (c) Example of recruitment of NrCAM-GFP by an anti-GFP coated bead (4 μm diameter) on a growth cone. The left panel shows images taken in transmitted light, and the right panel shows the epifluorescence channel. The red trace is the trajectory of the bead. Note that rearward motion of the bead with the actin flow precedes accumulation of NrCAM-GFP at the bead contact. Time is indicated in minutes. (d) Representative examples of IgCAM recruitment kinetics. The fluorescence at the bead contact is normalized by that of a control region on the same growth cone. The average of 15 traces is indicated in red circles, together with a mono exponential fit (Eq. 2).
We present here some examples of data obtained by this method in a test experiment based on GFP/anti-GFP recognition (Fig. 3). In this particular study, we questioned whether the regulated interactions of IgCAMs to a static actin cytoskeleton in growth cones, e.g. through ankyrin and Ezrin-Radixin-Moesin proteins, could affect the rate of adhesion formation by reducing the diffusiveness of IgCAMs on the cell surface. Using various NrCAM-GFP receptors truncated in extracellular or cytoplasmic domains and followed by single nanoparticle tracking, we showed that the lateral mobility of a receptor is inversely correlated to its size. This is likely due to several processes, including interaction with cytoskeletal partners, formation of clusters in the membrane plane, and steric hindrance with the glycocalyx. These differences in lateral mobility translated into substantial effects on the rates of receptor accumulation at anti-GFP coated microspheres. For example, the NrCAM transmembrane domain fused with GFP diffuses five times more rapidly than the full length L1 molecule, and shows a two-fold higher on-rate. This agrees with the basic concept that adhesion is composed of two steps: (1) receptor diffusion to the vicinity of its ligand; and (2) receptor–ligand interaction per se. However, the effect of diffusion is rather modest and our initial hypothesis that receptor recruitment is mostly reaction-limited stays valid. We have also studied more physiological adhesions between adhesive ligands and their corresponding receptors, i.e. N-cadherin and L1, where the reaction step is even slower.

MEASURING THE LIFETIME OF ADHESIVE BONDS AT NEURONAL CONTACTS BY FLUORESCENCE RECOVERY AFTER PHOTO-BLEACHING

After initial bead-cell contact and the progressive recruitment of adhesion proteins as described in the previous paragraph, the contacts reach a dynamic equilibrium. This steady-state is characterized by a bond density \( C_{\infty} \) and a continuous exchange between free and bound receptors at a rate \( k_{\text{off}} [C_{\infty}] \). We have previously studied phenomena related to its size. This is likely due to several processes, including interaction with cytoskeletal partners, formation of clusters in the membrane plane, and steric hindrance with the glycocalyx. These differences in lateral mobility translated into substantial effects on the rates of receptor accumulation at anti-GFP coated microspheres. For example, the NrCAM transmembrane domain fused with GFP diffuses five times more rapidly than the full length L1 molecule, and shows a two-fold higher on-rate. This agrees with the basic concept that adhesion is composed of two steps: (1) receptor diffusion to the vicinity of its ligand; and (2) receptor–ligand interaction per se. However, the effect of diffusion is rather modest and our initial hypothesis that receptor recruitment is mostly reaction-limited stays valid. We have also studied more physiological adhesions between adhesive ligands and their corresponding receptors, i.e. N-cadherin and L1, where the reaction step is even slower.

This model nicely fits the data for N-cadherin and L1 homophilic interactions and allows extracting the respective \( k_{\text{off}} \) values (Fig. 4c). The diffusion coefficient of unbound receptors can be obtained as a second parameter but it is more variable and less informative. Control experiments with antibody-coated beads yield much reduced turnover rates, in agreement with the stability of antigen-antibody bonds (Fig. 4d). From a biological perspective, this approach allowed us to demonstrate that the turnover rate of N-cadherin homophilic adhesion is regulated by interactions with the actin cytoskeleton via catenin partners. Overall, the lifetime of adhesion receptors in neuronal contacts involving many molecules, in the order of several minutes to hours, are much lower than those found at the individual molecule level. This is also the case for integrins trapped in focal contacts, suggesting that adhesion receptors in native clusters are stabilized by mechanisms other than the ligand-receptor transmembrane adhesion per se. Several differences can explain this apparent discrepancy. First, in experiments with purely artificial systems, the transmembrane and cytoplasmic domains of the receptor molecules are lacking. However, binding of the intracellular tail of adhesion receptors to specific protein partners, including signalling molecules, may affect ligand/receptor affinity by an inside out mechanism, as demonstrated for integrins and cadherins. Second, by using microspheres coated with a high density of ligands in dimeric configuration, lateral associations between several receptors may be promoted, which may affect turnover rate by an avidity effect.

MEASURING THE SLIPPAGE OR REINFORCEMENT OF N-CADHERIN-CYTOSKELETON BONDS USING OPTICAL TWEEZERS AND MICROMANIPULATION

Growth cone intrinsic motility relies on a dynamic regulation of the actin network, with polymerization occurring at the leading edge, depolymerization in the
central region, and myosin motors pulling on lamellipodial actin filaments (Fig. 5a). This results altogether in a continuous retrograde flow of actin. An important question is how growth cone progression on adhesive substrates is coupled to actin motility. A prevalent hypothesis, the “molecular clutch” mechanism, is that the mechanical coupling between ligand-bound 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. We tested this model for N-cadherin, which is known to stimulate axon outgrowth but whose mode of action remains largely unknown. We measured the velocity of growth cones migrating on N-cadherin coated glass, and in parallel experiments, placed N-cadherin coated microspheres on the dorsal surface of growth cones using optical tweezers. Microspheres escaped the optical trap in a few seconds and showed rearward movement with minimal lateral diffusion (Fig. 5b). By varying ligand density and expressing mutated N-cadherin receptors or inhibitory RNAs to perturb binding to catenins, we showed a strong correlation between the physical coupling of ligand-bound N-cadherin receptors to the retrograde actin flow and growth cone velocity. These data support a direct transmission of actin-based traction forces to N-cadherin adhesions, through catenin partners, driving growth cone advance and neurite extension.

The difference in velocity between growth cone translocation (about 1 μm/min) and the actin flow rate (about 5 μm/min) implies some slippage between the moving actin network and N-cadherin receptors engaged in adhesive interactions at the basal surface. Indeed, using beads coated with intermediate N-cadherin densities, we recorded breaking events in which beads started to move rearward then snapped back into the trap centre (Fig. 5b). By multiplying the traveled distance by the trap stiffness previously calibrated from thermal fluctuations (Fig. 6), we computed the corresponding forces, which were found to be very small (main peak at 1.2 pN). Because beads coated with

FIGURE 4. Measuring the turnover rate of adhesive interactions using fluorescence recovery after photo-bleaching. (a) Schematic diagram of the molecular interactions and diffusion/reaction processes at the bead contact. Bleached GFP molecules are shown in black. (b) N-cadherin-GFP molecules on the growth cone surface accumulate at microspheres coated with N-cadherin-Fc, until reaching equilibrium. The bead diameter is 4 μm. At time zero, the GFP signal is photo-bleached with an argon laser focused by a high numerical aperture objective (red arrow), and fluorescence recovery is followed for several minutes. Images are in false color to better visualize intensity changes, with corresponding LUT. (c) The N-cadherin-GFP fluorescence signal is normalized by that on a control neurite region without bead, revealing a fraction of free receptors (0–100%) and a fraction of receptors bound to the beads (above 100%). The rapid recovery (2 min) corresponds to the diffusion of free receptors, and the long-term regime to the slower turnover of N-cadherin homophilic bonds. (d) Despite a similar fraction of bound receptors, beads coated with antibodies to N-cadherin display a very limited exchange regime.
FIGURE 5. Molecular clutch mechanism between N-cadherin adhesions and the actin flow in the growth cones of hippocampal neurons. (a) Schematic diagram of the molecular interactions, showing homophilic adhesion between N-cadherin ligands grafted to the beads and endogenous N-cadherin receptors on the cell surface, together with the coupling between liganded N-cadherin receptors and the retrograde flow of cross-linked actin filaments, which occurs through catenin partners. (b) On the growth cone surface, 1 μm microspheres coated with high density of N-cadherin easily escape the force (spring) imposed by an optical trap (red cone of light), and move rearward (blue trace); beads coated with lower N-cadherin density display individual breaking events (yellow trace), whereby they start to move rearward for about 0.5–1 μm, then quickly snap back into the trap center (yellow line). The baseline indicates the equilibrium trapping position, and deviation from this reference shows the distance traveled by the bead vs. time. (c) Inside the growth cone, GFP-tagged actin (shown here in false color scale) accumulates at bead contacts when microspheres are restrained by a micro-needle (blue profile), mimicking a stiff substrate. (d) DIC image showing a 4 μm microsphere on a growth cone and a microneedle placed transversally. (e) Corresponding fluorescence image showing actin accumulation at the bead contact restrained by the microneedle.
a small number of N-cadherin ligands are likely to attach to very few receptors,\textsuperscript{45} the retrapping events were interpreted as the breaking of a small number of molecular bonds. Since the optical trap is not powerful enough to break homophilic bonds between cadherin molecules, in the order of 30–60 pN,\textsuperscript{39} and because beads remain attached to the growth cone surface when the trap is stopped, rupture is unlikely to occur between N-cadherin ligands and receptors. Instead, the 1 pN retrapping events most likely reflect the rupture of single molecular bonds between the N-cadherin-catenin complex and the rearward moving actin network, which occurs when few N-cadherin receptors are engaged. In contrast, at high N-cadherin coating density, when large numbers of receptors are recruited, we could impose high forces on N-cadherin adhesions (about 1 nN) using a micro-needle placed downstream of the bead (Figs. 5c and 5d). This mimicked a stiff substrate and locally stopped the actin flow. We then observed dramatic actin-GFP accumulation at restrained bead contacts, suggesting an active cellular response to this mechanical stimulus (Figs. 5c and 5e). Growth cones could follow the imposed tension at a speed of about 1 μm/min, suggesting that such mechanism of stiff substrate recognition (durotaxis), as reported for fibroblasts,\textsuperscript{32} is actually driving N-cadherin mediated growth cone locomotion.

INVOLVEMENT OF INTERNAL TRAFFICKING AND LIPID RAFTS IN THE REGULATION OF Ig-CAM ADHESIONS

IgCAMs have the property to interact closely with dynamic membrane components, that can regulate their availability at the cell surface and functional properties. Indeed, both L1 and NrCAM were found by biochemistry and cholesterol depletion experiments to be associated with lipid rafts.\textsuperscript{16,36} Using beads coated with TAG-1 (a natural ligand for NrCAM) together with optical tweezers, single particle tracking and FRAP, we showed that anchoring of NrCAM with the retrograde actin flow can be triggered by adhesive contacts via cooperative processes including interactions with the cytoplasmic tail, formation of cis-complex via the FnIII repeats, and lipid raft aggregation.\textsuperscript{16} In addition, by interacting with the clathrin adaptor AP-2 through a specific YRSL motif in its
intracellular tail, L1 was shown to actively recycle within growth cones. L1 undergoes endocytosis mainly in the central domain (Fig. 7b), travels forward through recycling endosomes probably attached to microtubule motors (Fig. 7c), and is exocytosed preferentially at the periphery of the growth cone (Fig. 7d). This mechanism generates a density gradient of L1 molecules which helps growth cones to progress forward. Indeed, we showed that L1-GFP accumulated faster at microspheres coated with L1-Fc proteins than at antibody coated beads, indicating either faster reactivity and/or additional processes. The use of an L1-GFP construct in which the N-terminal GFP could be rapidly cleaved off by thrombin allowed the identification of newly exocytosed L1-GFP molecules at the growth cone periphery (Fig. 7b). L1-GFP still accumulated at L1-Fc microspheres after thrombin treatment, indicating that local exocytosis of L1-rich vesicles at the growth cone participates in enhancing the formation of L1 homophilic contacts. These effects were specifically triggered by L1–L1 adhesion since they were not observed for beads coated with antibodies, and because the exocytosis rate of L1 was much higher than in the absence of beads. Rapid saturation in the level of L1 molecules at L1-Fc microspheres was interpreted by the fact that L1–L1 bonds can also undergo rapid dissociation (non-zero $k_{\text{off}}$), so the steady-state regime is achieved faster than with antibodies ($k_{\text{off}} = 0$). This was confirmed by FRAP experiments on L1-GFP receptors accumulated at L1-Fc coated microspheres, which showed that L1 homophilic adhesions recycle 3 times faster than N-cadherin homophilic bonds. L1-GFP molecules truncated in the intracellular tail as well as NrCAM
missing the clathrin adaptor binding sequence, showed both little internalization and reduced turnover rates, indicating conversely a role of endocytosis in the recycling of mature L1 contacts at the base of the growth cone (Fig. 7a). Thus, unlike for other molecules such as NrCAM or N-cadherin, diffusion/trapping and exo/endocytosis events cooperate to allow the fast renewal of L1 adhesions.

CONCLUSION

These optical based techniques have been used to resolve in fine detail the dynamics of adhesion proteins NrCAM, L1, and N-cadherin within the growth cones of hippocampal neurons. In particular, they revealed how ligand/receptor binding and unbinding events are controlled by lateral membrane diffusion, connection to the actin cytoskeleton, and intracellular pathways such as endocytosis and exocytosis. The next step is to understand how all these physical interactions are integrated in time and space to allow for migration and guidance of growth cones in vivo.14

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REFERENCES


