Supporting Information

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SI Methods

Cell Culture and Transfection. All relevant aspects of the experimental procedures were approved by the Institutional Animal Care and Use Committee of Nara Institute of Science and Technology. Hippocampal neurons prepared from E18 rat embryos were seeded on glass coverslips (Matsunami) coated either sequentially with 100 μg/mL of polylysine (poly-D-lysine; Sigma) and 25 μg/mL of laminin (Wako Pure Chemical Industries) or with polylysine alone, and cultured in Neurobasal Medium (Thermo Fisher Scientific) containing B-27 supplement (Thermo Fisher Scientific) and 1 mM glutamine without a glia feeder layer as described (1). Preparation of microscale patterns of laminin on polylysine-coated coverslips is described in *Preparation of Microscale Patterns*. All experiments except for the measurement of forces were carried out on glass surfaces. Neurons were transfected with plasmid DNA using Nucleofector (Lonza) before plating. For the immunoblot analysis in Fig. S5*A*, we used rat cortical neurons, as the experiments required large numbers of neurons. These were prepared from E18 rat embryos using the same protocol as above. HEK293T cells were cultured in DMEM supplemented with 10% FBS, and transfected with vectors by the calcium phosphate method.

Preparation of Microscale Patterns of Laminin on Polylysine-Coated Coverslips.

Fabrication of PDMS stamp. To serve as masters for the subsequent fabrication of a polydimethylsiloxane (PDMS) stamp, micromolds with small hexagonal patterns were lithographically fabricated into an epoxy photoresist (SU-8; MicroChem) spin-coated on silicon substrate with 5.5 μm thickness. The PDMS stamp was formed by coating the micromold with silicone oil (Barrier Coat No. 6; ShinEtsu), pouring a layer of PDMS (KE-103; ShinEtsu) up to 5 mm thickness onto the master, applying a vacuum to remove entrapped air bubbles, curing at 60 °C for 1 h, cooling to room temperature, and carefully peeling off the mold (Fig. S1*A*). Actual sizes of the hexagonal patterns on PDMS stamps were measured using a 3D laser scanning confocal microscope (VK-X250; Keyence). The widths and heights of the hexagonal patterns were 14 μm and 5.5 μm, respectively.

Microscale patterning of laminin. Microscale patterns of laminin were formed on polylysine-coated coverslips by a wet-transfer process for laminin molecules using the PDMS stamp (Fig. 1*B*). First, PBS solution containing 25 μg/mL of laminin (Wako Pure Chemical Industries), 1 M sucrose, and 20 μg/mL of Texas Red-conjugated BSA (Thermo Fisher Scientific) was applied to the PDMS stamp with a micropipette to fill the dents of hexagonal lattice patterns at microscales. Excess solution on the PDMS surface was removed using a spin coater at 3,000 rpm for 15 s. The PDMS stamp was then attached to the polylysine-coated coverslip with the surfaces facing each other, pressed manually, and incubated at 37 °C overnight to transfer the laminin patterns onto the coverslip surface. Before seeding neurons, the coverslips were carefully separated from the PDMS stamp and washed with PBS.

RNAi. For RNAi experiments, we used a Block-iT Pol II miR RNAi expression kit (Invitrogen). The targeting sequences of shootin1a and cortactin miRNA were reported previously (2–4). The targeting sequence of L1-CAM miRNA #1, 5′-GTGGAGGAAGGAGAATCAGTA-3′, corresponds to nucleotides 439 to 459 in the coding region of rat L1-CAM; that of another miRNA (miRNA #2) was reported previously (3). As described previously (3), to ensure high-level expression of miRNA before neurite elongation, hippocampal neurons prepared from E18 rat embryo and transfected with the miRNA expression vector were plated on uncoated polystyrene plates. After a 20-h incubation to induce miRNA expression, the cells were collected, and then cultured on coverslips. Reduction of endogenous L1-CAM in neurons by L1-CAM miRNA #1 and #2 was confirmed using immunoblot analysis (Fig. S5*A*).

DNA Constructs. The cDNA encoding human L1-CAM was provided by Vance Lemmon (University of Miami Miller School of Medicine, Miami, FL). The RNAi refractory L1-CAM mutant was generated by joint-PCR using the flowing primers: 5′-GTCGAAGAAGGGGAGAGCGTGGTTCTGCCTTGCAACCCTCCC-3′, 5′-CACGCTCTCCCCTTCTTCGACCTCCACGGGCTTCACTGTCTC-3′, 5′-CTTCGAATTCCGGCGCCGGGAAAGATGGTC-3′, and 5′-GGAAGCGGTACCGCAGGTGGGGGCTGAG-3′. Eight mutations (underlined) in 5′- GTCGAAGAAGGGGAGAGCGTG were induced in the target sequence #1 of rat L1-CAM miRNA without changing the amino acid sequence. The ΔT273 mutant of human L1-CAM was generated using a QuikChange II site-directed mutagenesis kit (Stratagene) using the following primer: ΔT273, 5,-GAGGGCTTTCCCACGCCCATCAAATGGCTGCGCCCCAGTG. The cDNAs were subcloned into pEGFP (Clontech) and pFC14K HaloTag CMV Flexi (Promega) vectors. Localization of L1-CAM-WT and the ΔT273 mutant in the plasma membrane was confirmed by expressing them in HEK293T cells (Fig. S5 *B* and *C*).

Immunocytochemistry, Immunoblot, and Microscopy. Cultured neurons were fixed with 3.7% formaldehyde in Krebs buffer for 10 min at room temperature, and then treated for 15 min with 0.05% Triton X-100 in PBS on ice and 10% FBS in PBS for 1 h at room temperature. They were then stained with an anti-βIII-tubulin (BioLegend) or anti-myc (MBL) antibody, as described (2). Immunoblot was performed as described (2). Fluorescence and phase-contrast images of neurons were acquired using a fluorescence microscope (Axioplan2; Carl Zeiss) equipped with a plan-Neofluar 40× 0.75 NA or 63× oil 1.40 NA objective (Carl Zeiss), a charge-coupled device camera (AxioCam MRm; Carl Zeiss), and imaging software (Axiovision3; Carl Zeiss). Live-cell images of cultured hippocampal neurons were acquired at 37 °C using a fluorescence microscope (IX81; Olympus) equipped with an EM-CCD camera (Ixon DU888; Andor), using a plan-Fluar 20× 0.45 NA or 40× 0.60 NA objective (Olympus), and MetaMorph software. Fluorescent images of HEK293T cells were acquired using a confocal microscope (LSM700; ZEISS) equipped with an alpha Plan-Apochromat 100×/1.46 Oil DIC M27.

Analysis of Axons Located on Microscale Patterns of Laminin and Polylysine. For the analyses in Fig. 1 *C–F* and Figs. S1 *D* and *E* and S4*A*, the total lengths of axonal shafts that were located on laminin-coated areas were divided by the full lengths of the axonal shafts to obtain the percentages of axon length located on laminin (Fig. S1*C*).

Fluorescent Speckle Microscopy. Fluorescent speckle imaging and speckle tracking analysis of mRFP-actin and EGFP-shootin1a were performed as described previously (3). Fluorescent speckle imaging of L1-CAM-HaloTag was performed using neurons transfected with pFC14K-L1-CAM. Neurons were treated with HaloTag TMR ligand (Promega) at 1:1,500 dilution in the culture medium and incubated for 1 h at 37 °C. The ligand was then washed out with PBS, and the cells were incubated with culture medium for 30 min

at 37 °C. Before observation, the medium was replaced with L15 medium (Thermo Fisher Scientific) including B27 supplement and 1 mM glutamine. The fluorescent features of L1-CAM-HaloTag were observed using a TIRF microscope (IX81; Olympus) equipped with an EM-CCD camera (Ixon3; Andor), a complementary metal–oxide–semiconductor (CMOS) camera (ORCA Flash4.0LT; HAMAMATSU), a UAPON 100× 1.49 NA (Olympus), and MetaMorph software. For the grip and slip analysis, fluorescent signals of L1-CAM-HaloTag were monitored at 5-s intervals. L1-CAM puncta that were visible for at least 10 s (two intervals) were analyzed, and immobile ones were defined as L1-CAM in stop (grip) phase, while those that flowed retrogradely were defined as in flow (slip) phase.

Traction Force Microscopy. Traction force microscopy was performed as described (4, 5). Briefly, neurons were cultured on polyacrylamide gels with embedded 200-nm fluorescent microspheres (200 nm diameter; Invitrogen). Time-lapse imaging of fluorescent beads and growth cones was performed at 37 °C using a confocal microscope (LSM710; Carl Zeiss) equipped with a C-Apochromat 63×/1.2 W Corr objective. The growth cone area was determined by EGFP fluorescence or from differential interference contrast (DIC) images. Traction forces under the growth cones were monitored by visualizing force-induced deformation of the elastic substrate, which is reflected by displacement of the beads from their original positions, and expressed as vectors. The force vectors detected by the beads under individual growth cones were then averaged, and were expressed as vectors composed of magnitude and angle (θ) (Fig. 2*C*). To compare the forces under different conditions, the magnitude and angle (θ) of the force vectors of the individual growth cones were statistically analyzed and expressed as means ± SEM, separately. They were also analyzed by the unpaired Student's *t* test, To analyze the angle of the force in Fig. 2*C*, we also performed a χ^2 test.

Protein Preparation and in Vitro Binding Assay. To construct the vector which expresses FLAG-His-tagged L1-CAM-ECD (WT), a double-stranded DNA,

GGCGGCGGACTCAACGACATATTCGAGGCCCAGAA-
AATCGAGTGGCACGAAGGAGGTGGGCTGGAAGTG-
CTGTTTCAAGGGCCCGGAGGAGGCGATTACAAGGA-
TCATGATGGGGACTATAAGGATCACGACATTGACT-
ACAAGGATGATGACGACAAAGGCGGTGGCCATCACC-
ACCACCATCACCATCATCACCAC,

which encodes tandem multitags (BirA recognition site, HRV3C protease recognition site, 3xFLAG, and 10xHis tags) was synthesized (Eurofins Genomics). The DNA was then amplified by PCR with the primers

5′-TTAAGCGGCCGCTGGCGGCGGACTCAACGA-3′ and

5′-TTAAGAATTCTTATCAGTGGTGATGATGGTG-3′,

which contain NotI and EcoRI restriction enzyme sites, respectively. The amplicon was ligated into the pCAGGS-L1-CAM-Fc vector (3) to replace the Fc region with the tandem multitags. To construct a vector that expresses FLAG-His-tagged L1-CAM-ECD (ΔT273), the coding sequence of L1-CAM-ECD (ΔT273) was amplified with the primers

5′-TTAAGTCGACGCCACCATGGTCGTGGCGCT-3′ and

5′-TTAAGCGGCCGCAGGGAGCCTCACGCGGCC-3′.

Next, the amplicon was ligated into pCAGGS-L1-CAM-ECD (WT) FLAG-His vector to replace the L1-CAM-ECD (WT) region with L1-CAM-ECD (ΔT273).

FLAG-His-tagged L1-CAM-ECD (WT and ΔT273) were expressed in HEK293T cells. The supernatants of the culture media containing secreted L1-CAM-ECD proteins were applied to a Ni Sepharose excel column (GE Healthcare). After washing the column with wash buffer (20 mM Tris·HCl, pH 8.0, 300 mM NaCl, 20 mM imidazole), the proteins were eluted by elution buffer (20 mM Tris·HCl, pH 8.0, 300 mM NaCl, 200 mM imidazole) and dialyzed against HBS buffer (10 mM Hepes, pH 7.4, 150 mM NaCl) overnight at 4 °C. Purified laminin was obtained from Wako.

Purified laminin and purified FLAG-His-tagged L1-CAM-ECD (WT or ΔT273) were incubated overnight at 4 °C in reaction buffer (10 mM Tris·HCl, pH 8.0, 150 mM NaCl) and centrifuged for 15 min at 17,400 × *g* at 4 °C. The supernatants were incubated with Ni-NTA agarose beads (QIAGEN) for 2 h at 4 °C. The beads were washed three times with wash buffer (10 mM Tris·HCl, pH 8.0, 150 mM NaCl, 10 mM imidazole), and the bound proteins were eluted with elution buffer (20 mM Tris·HCl pH 8.0, 300 mM NaCl, 200 mM imidazole) for 2 h at 4 °C. The elution samples were analyzed by immunoblot. Laminin and L1-CAM-ECD (WT or ΔT273) were detected by anti-laminin (Sigma) and anti-FLAG (MBL) antibodies, respectively. As reported previously (6), purified laminin is composed of two bands at 220 kDa and 440 kDa (Fig. 4*F*).

Statistical Analysis. Significance was determined by the unpaired Student's *t* test and χ^2 test, using Excel 2011 (Microsoft) in most cases. For the multiple comparisons in Fig. 4 *B* and *C* and Fig. S3 *E* and *F*, we used one-way ANOVA with Schaffer's post hoc test.

1. Inagaki N, et al. (2001) CRMP-2 induces axons in cultured hippocampal neurons. *Nat Neurosci* 4:781–782.
2. Toriyama M, et al. (2006) Shootin1: A protein involved in the organization of an asymmetric signal for neuronal polarization. *J Cell Biol* 175:147–157.
3. Shimada T, et al. (2008) Shootin1 interacts with actin retrograde flow and L1-CAM to promote axon outgrowth. *J Cell Biol* 181:817–829.
4. Toriyama M, Kozawa S, Sakumura Y, Inagaki N (2013) Conversion of a signal into forces for axon outgrowth through Pak1-mediated shootin1 phosphorylation. *Curr Biol* 23:529–534.
5. Kubo Y, et al. (2015) Shootin1-cortactin interaction mediates signal-force transduction for axon outgrowth. *J Cell Biol* 210:663–676.
6. Timpl R, et al. (1979) Laminin–A glycoprotein from basement membranes. *J Biol Chem* 254:9933–9937.

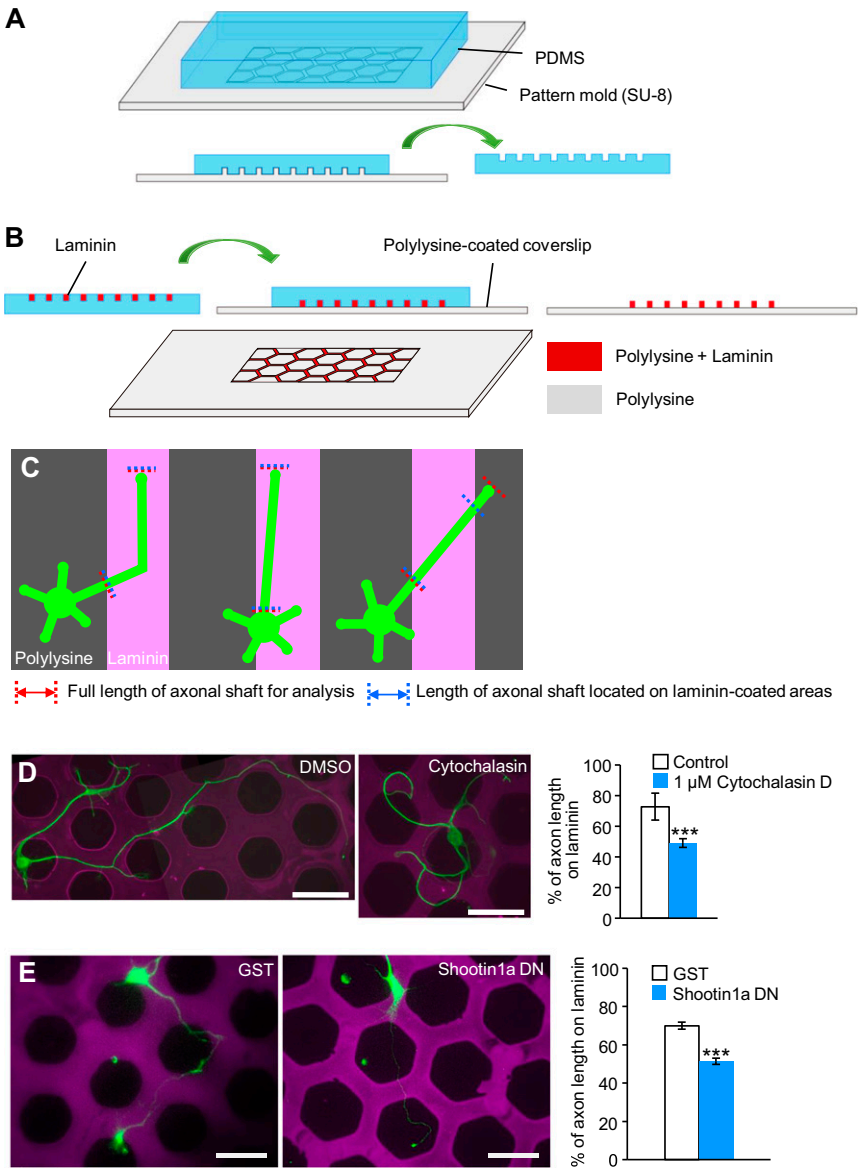


Fig. S1. Analysis of laminin-induced axonal haptotaxis. (*A* and *B*) Preparation of microscale patterns of laminin on polylysine-coated coverslips. For details, see *SI Methods*. (*C*) Analysis of axons located on laminin. To calculate percentage of axon length on laminin, the total lengths of axonal shafts located on laminin-coated areas (blue double arrow) were divided by the full lengths of the axonal shafts (red double arrow). (*D*) Hippocampal neurons treated with DMSO or 1 μM cytochalasin D were cultured on microscale patterns of laminin (pink) and polylysine (black) for 5 d and stained with an anti-βIII-tubulin antibody (green). The graph shows the percentage of axon length located on laminin ($n = 158$ neurons). (*E*) Neurons overexpressing myc-NES-GST (control) or myc-NES-shootin1 (261 to 377) (shootin1a DN) (1) were cultured on microscale patterns of laminin (pink) and polylysine (black) for 5 d and stained with an anti-myc antibody (green). The graph shows the percentage of axon length located on laminin ($n = 275$ neurons). Data represent means ± SEM; ***$P < 0.01$. (Scale bars: 50 μm.)

1. Kubo Y, et al. (2015) Shootin1-cortactin interaction mediates signal-force transduction for axon outgrowth. *J Cell Biol* 210:663–676.

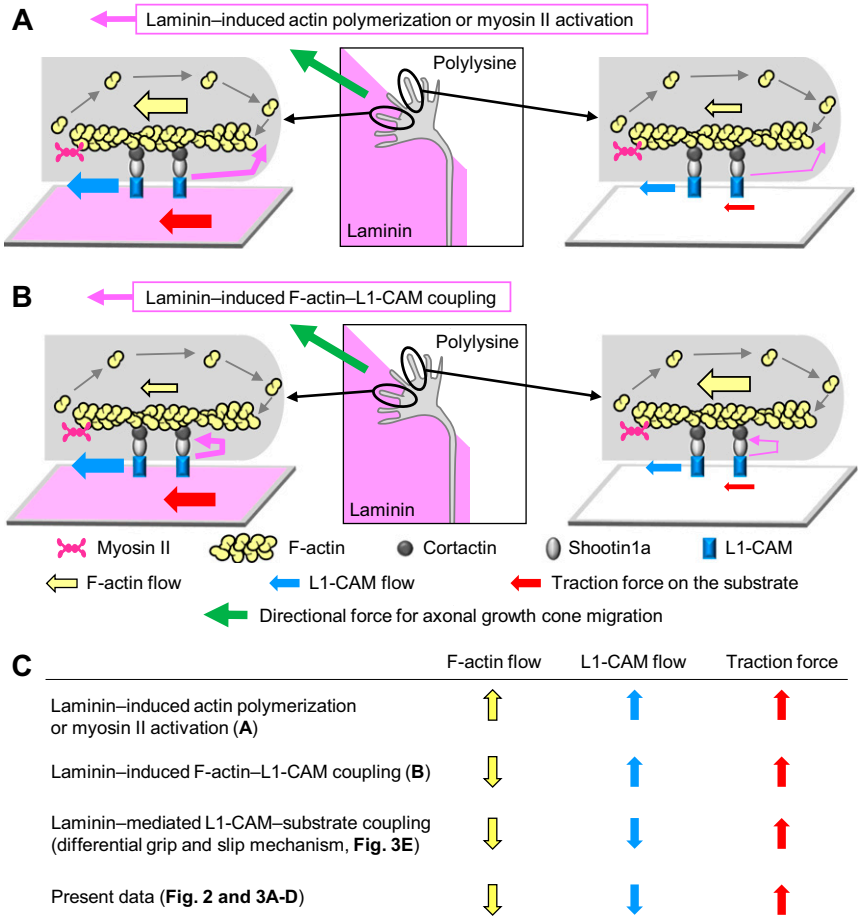


Fig. S2. Comparison of possible mechanisms for laminin-induced growth cone haptotaxis. (*A*) Laminin activates cell signaling, which leads to promotion of actin polymerization or myosin II activation. This, in turn, enhances F-actin retrograde flow (1, 2), force transmitted on L1-CAM, and traction force for growth cone migration. (*B*) Laminin activates cell signaling, which promotes mechanical coupling between F-actin flow and L1-CAM. This, in turn, decreases the F-actin flow rate, and enhances force transmitted on L1-CAM and traction force for growth cone migration (3, 4). (*C*) Comparison of the three possible mechanisms for laminin-induced growth cone haptotaxis with the experimental data. Note that the present data are consistent only with the differential grip and slip mechanism.

1. Medeiros NA, Burnette DT, Forscher P (2006) Myosin II functions in actin-bundle turnover in neuronal growth cones. *Nat Cell Biol* 8:215–226.
2. Craig EM, Van Goor D, Forscher P, Mogilner A (2012) Membrane tension, myosin force, and actin turnover maintain actin treadmill in the nerve growth cone. *Biophys J* 102:1503–1513.
3. Toriyama M, Kozawa S, Sakumura Y, Inagaki N (2013) Conversion of a signal into forces for axon outgrowth through Pak1-mediated shootin1 phosphorylation. *Curr Biol* 23:529–534.
4. Kubo Y, et al. (2015) Shootin1-cortactin interaction mediates signal-force transduction for axon outgrowth. *J Cell Biol* 210:663–676.

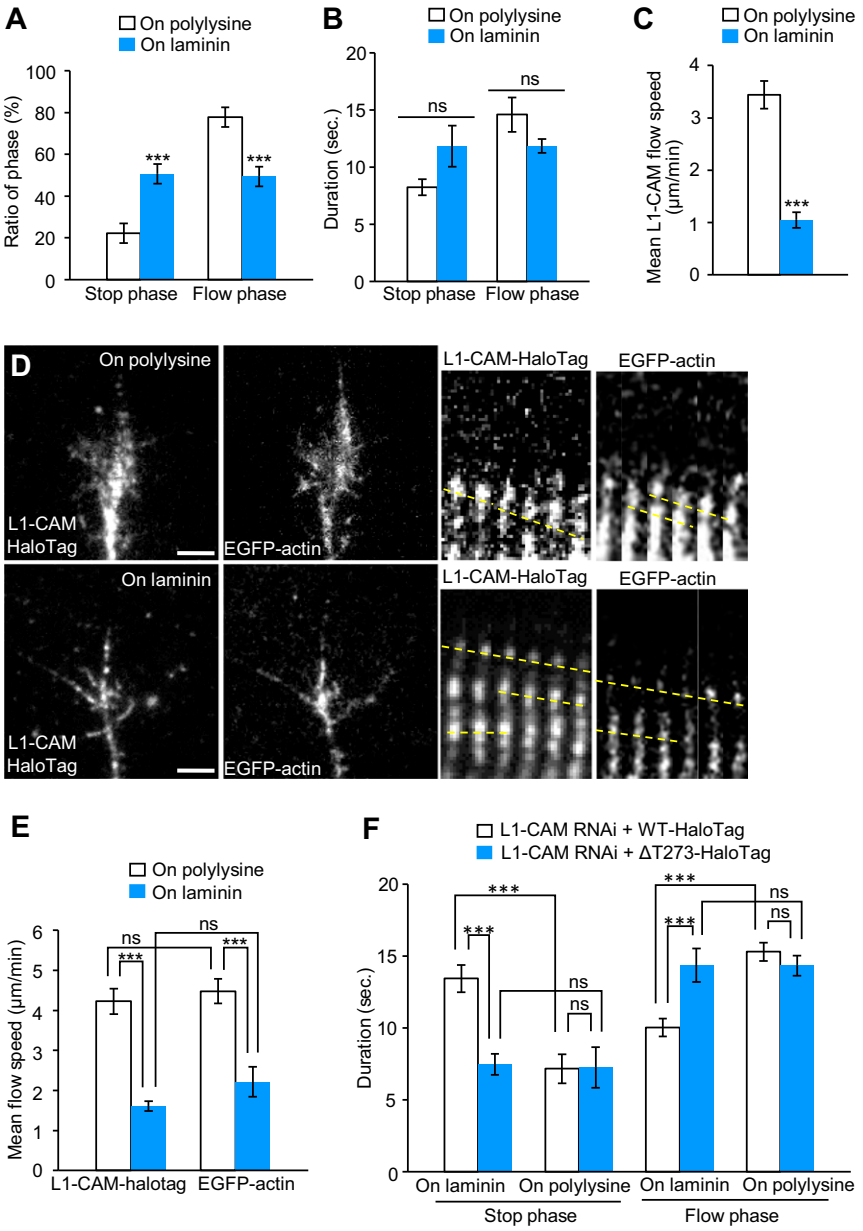


Fig. S3. Movements of L1-CAM in growth cone on polylysine and laminin. (*A*) Ratio and (*B*) duration of the stop and flow phases of L1-CAM-HaloTag in filopodia obtained from the kymograph analyses in Fig. 3*A* ($n = 503$ signals). (*C*) Retrograde flow speed of L1-CAM-HaloTag in filopodia obtained from the kymograph analyses in Fig. 3*A* ($n = 503$ signals). (*D*) (*Left*) Neurons coexpressing L1-CAM-HaloTag and EGFP-actin were cultured on polylysine or laminin, and fluorescent signals of these molecules in growth cones were analyzed. (*Right*) Kymographs of the fluorescent features of L1-CAM-HaloTag and EGFP-actin in filopodia are shown (L1-CAM and F-actin flows are indicated by dashed yellow lines) (Movies S6 and S7). (*E*) Retrograde flow rates obtained from the kymograph analyses in *D* ($n = 361$ signals). (*F*) Duration of the stop and flow phases of L1-CAM-HaloTag (WT or ΔT273) in filopodia obtained from the kymograph analyses in Fig. 4*A* ($n = 503$ signals). Data represent means ± SEM; ***$P < 0.01$; ns, nonsignificant. (Scale bars: 5 μm.)

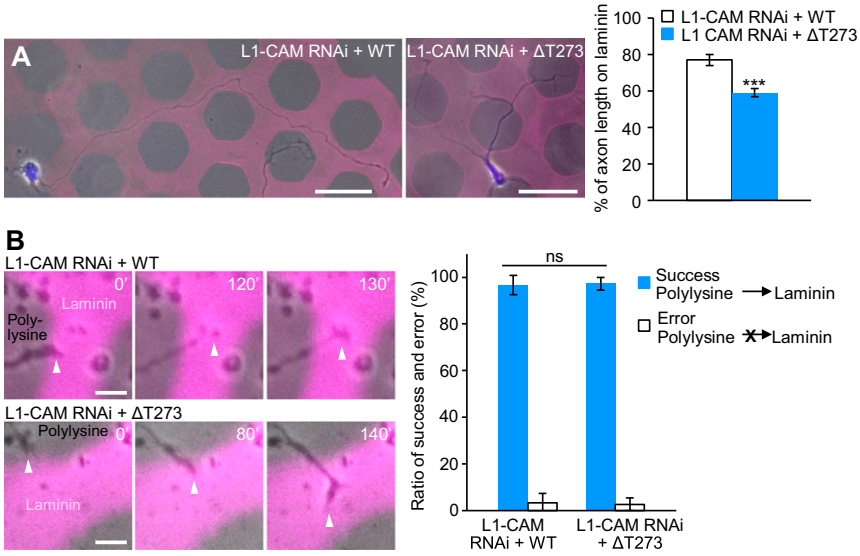


Fig. S4. Deletion of Thr273 in L1-CAM disrupts laminin-induced axonal haptotaxis. (*A*) Hippocampal neurons coexpressing L1-CAM miRNA and RNAi-refractory L1-CAM-EGFP (WT or ΔT273) were cultured on microscale patterns of laminin (pink) and polylysine (gray) for 3 d. Blue color is the signal of CFP. The graph shows the percentage of axon length located on laminin ($n = 127$ neurons). (*B*) (*Left*) Time-lapse images of growth cones (arrowheads) coexpressing L1-CAM miRNA and RNAi-refractory L1-CAM-EGFP (WT or ΔT273) cultured on microscale patterns of laminin (pink) and polylysine (gray). (*Right*) The graph shows the percentages of the growth cones that crossed the border from polylysine to laminin ($n = 53$ growth cones). Data in *B* represent means ± SEM; $***P < 0.01$; ns, nonsignificant. (Scale bars: 50 μm for *A* and 10 μm for *B*.)

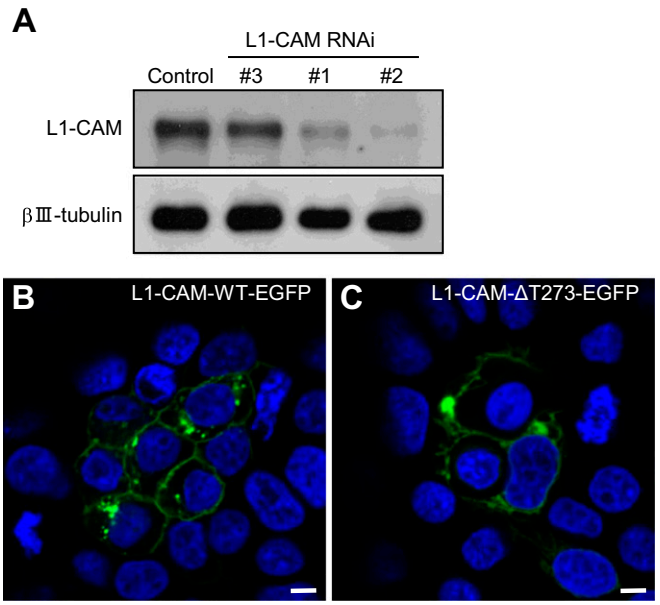
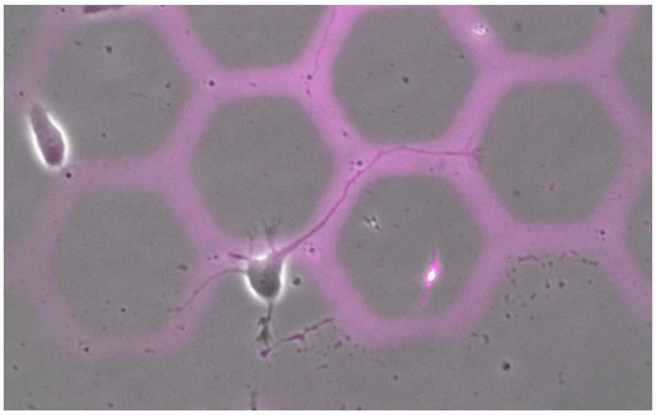
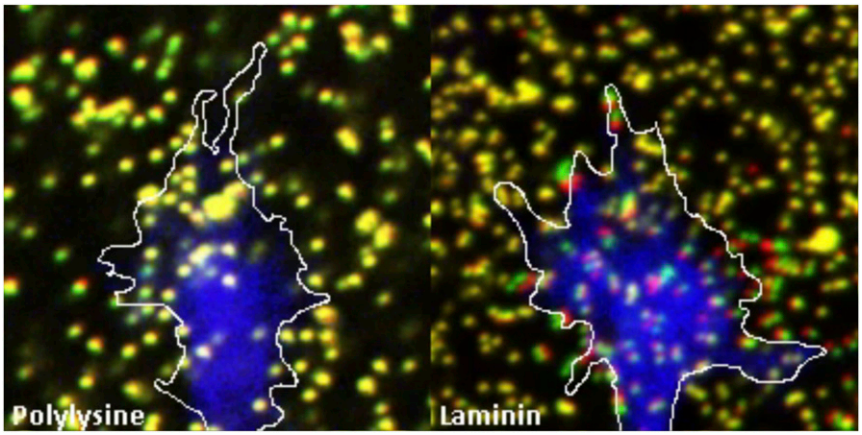


Fig. S5. Analyses of L1-CAM RNAi and L1-CAM-ΔT273 localization. (*A*) Immunoblot analysis of endogenous L1-CAM in cultured neurons (DIV2) transfected with control miRNA or miRNA against L1-CAM (#1, #2, or #3). Immunoblot with anti-βIII-tubulin antibody served as a loading control. The targeting sequence of L1-CAM miRNA #3 is 5′-CTCATACAGATTCCTGATGAA-3′. (*B* and *C*) Localizations of WT L1-CAM and the ΔT273 mutant in the plasma membrane. (*B*) L1-CAM-WT-EGFP and (*C*) L1-CAM-ΔT273-EGFP were expressed in HEK293T cells and observed by confocal microscopy (green). The blue color indicates DAPI staining. (Scale bars: 5 μm.)



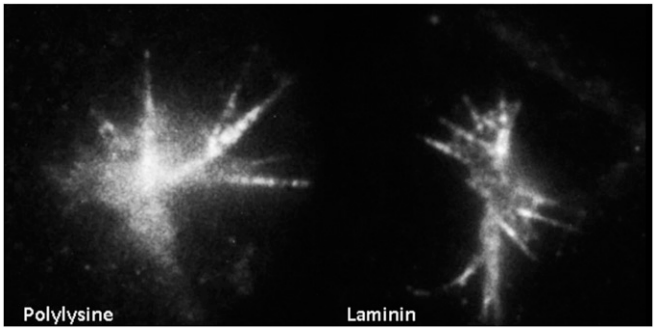
Movie S1. Growth cone migration on a microscale pattern of laminin. A time-lapse movie of a hippocampal neuron cultured on a microscale pattern of laminin (pink) and polylysine (gray) during DIV1-2 (Fig. 1*A*). Images of growth cone migration were captured using a fluorescence microscope (IX81; Olympus). Frames were taken every 30 min for 20 h.

Movie S1



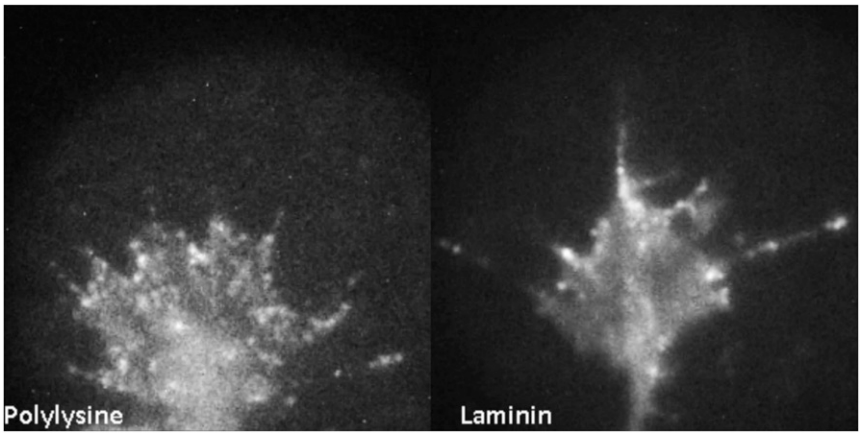
Movie S2. Laminin-induced promotion of traction force under axonal growth cones. Time-lapse movies of axonal growth cones of hippocampal neurons expressing EGFP (blue) and cultured on (*Left*) polylysine-coated or (*Right*) laminin-coated polyacrylamide gel with 200-nm fluorescent beads embedded (Fig. 2 *A* and *B*). Time-lapse imaging was performed using a confocal microscope (LSM710; Carl Zeiss). Frames were taken every 3 s for 147 s.

Movie S2



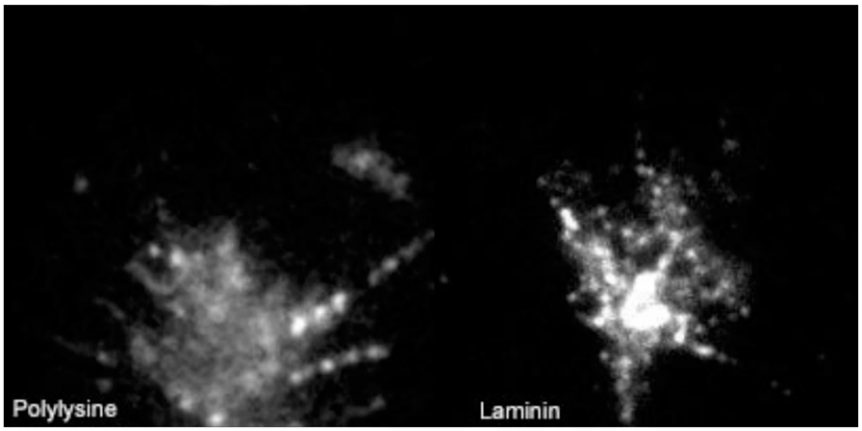
Movie S3. Movement of fluorescent features of mRFP-actin in the growth cones on polylysine and laminin. Time-lapse movies of mRFP-actin at axonal growth cones of hippocampal neurons cultured on (*Left*) polylysine or (*Right*) laminin (Fig. 2*D*). Images of mRFP-actin in the growth cones were captured using a fluorescence microscope (Axioplan2; Carl Zeiss). Frames were taken every 5 s for 145 s.

Movie S3



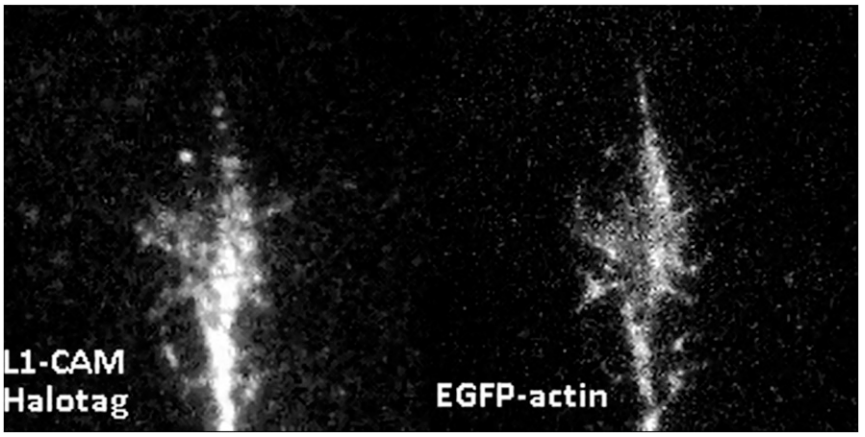
Movie S4. Movement of fluorescent features of EGFP-shootin1a in the growth cones on polylysine and laminin. Time-lapse movies of EGFP-shootin1a at axonal growth cones of hippocampal neurons cultured on (*Left*) polylysine or (*Right*) laminin (Fig. 2*E*). Images of EGFP-shootin1a in the growth cones were captured using a fluorescence microscope (Axioplan2; Carl Zeiss). Frames were taken every 5 s for 145 s.

Movie S4



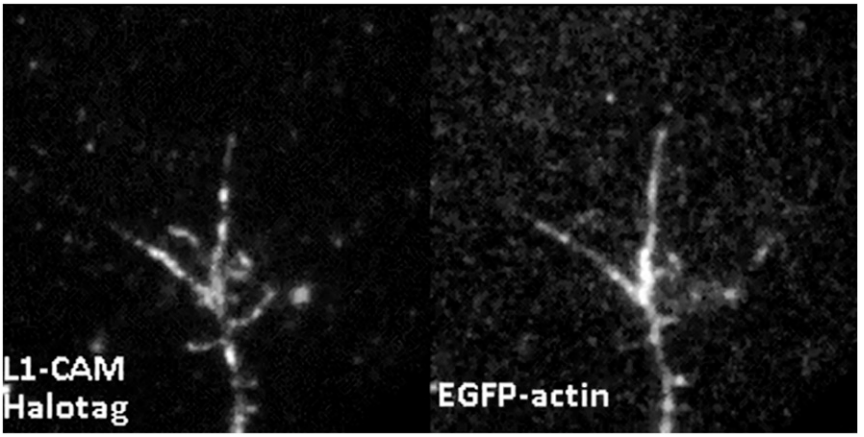
Movie S5. Movement of fluorescent features of L1-CAM-HaloTag in the growth cones on polylysine and laminin. Time-lapse movies of L1-CAM-HaloTag at axonal growth cones of hippocampal neurons cultured on (*Left*) polylysine or (*Right*) laminin (Fig. 3*A*). Images of L1-CAM-HaloTag in the growth cones were captured using a TIRF microscope (IX81; Olympus). Frames were taken every 5 s for 245 s.

Movie S5



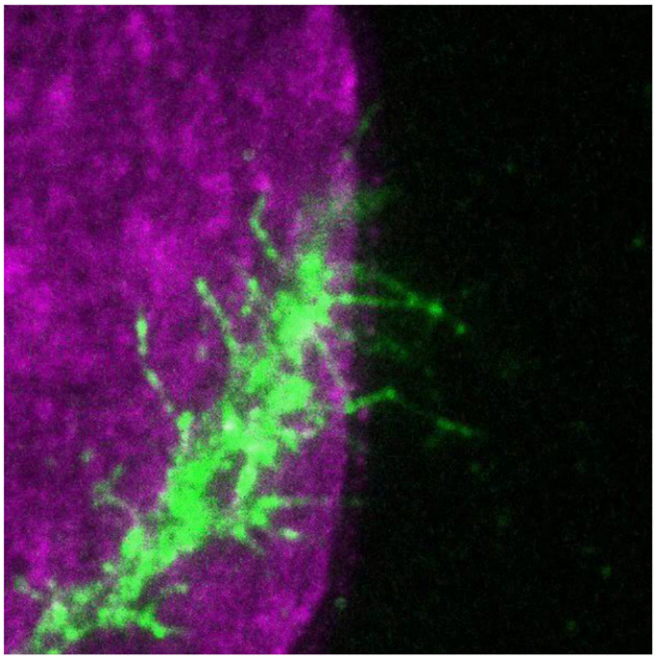
Movie S6. Time-lapse movies of fluorescent features of L1-CAM-HaloTag and EGFP-actin in the growth cones on polylysine. Neurons coexpressing L1-CAM-HaloTag and EGFP-actin were cultured on polylysine and fluorescent signals of these molecules in growth cones were analyzed (Fig. S3*D*). Images were captured using a TIRF microscope (IX81; Olympus). Frames were taken every 5 s for 245 s.

Movie S6



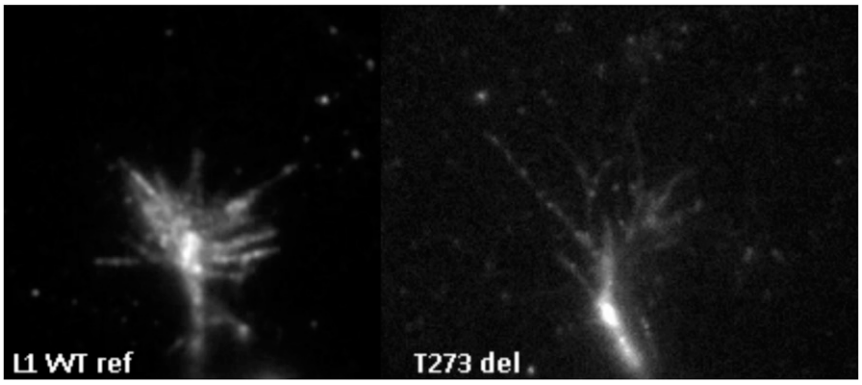
Movie S7. Time-lapse movies of fluorescent features of L1-CAM-HaloTag and EGFP-actin in the growth cones on laminin. Neurons coexpressing L1-CAM-HaloTag and EGFP-actin were cultured on laminin and fluorescent signals of these molecules in growth cones were analyzed (Fig. S3*D*). Images were captured using a TIRF microscope (IX81; Olympus). Frames were taken every 5 s for 245 s.

Movie S7



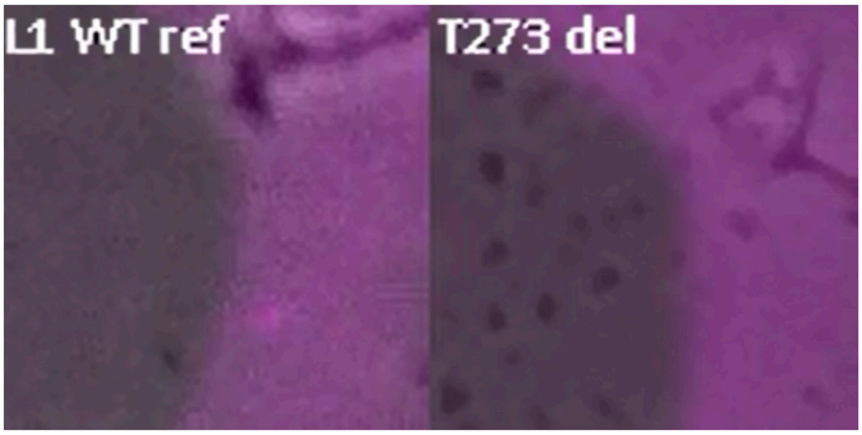
Movie S8. Movement of fluorescent features of L1-CAM-HaloTag in the growth cone on the border between laminin and polylysine. A time-lapse movie of L1-CAM-HaloTag at the axonal growth cone of hippocampal neurons located on the border between laminin (pink) and polylysine (black) (Fig. 3*B*). Images of L1-CAM-HaloTag (green) in the growth cone were captured using a TIRF microscope (IX81; Olympus). Frames were taken every 5 s for 245 s.

Movie S8



Movie S9. Movement of fluorescent features of L1-CAM-HaloTag and L1-CAM-ΔT273-HaloTag in the growth cones on laminin. Time-lapse movies showing fluorescent signals of (*Left*) L1-CAM-WT-HaloTag and (*Right*) L1-CAM-ΔT273-HaloTag at axonal growth cones of hippocampal neurons cultured on laminin (Fig. 4*A*). Images of L1-CAM-HaloTag in the growth cones were captured using a TIRF microscope (IX81; Olympus). Frames were taken every 5 s for 245 s.

Movie S9



Movie S10. Time-lapse movies of growth cones coexpressing L1-CAM miRNA and RNAi-refractory L1-CAM-EGFP (WT and ΔT273) cultured on microscale patterns of laminin (pink) and polylysine (gray) (Fig. 4*G*). Frames were taken every 10 min for 90 min.

Movie S10