Supporting Information

Staquicini *et al.* 10.1073/pnas.0813286106

SI Text

Reagents. The following antibodies were used: anti-netrin-4 antibodies from Santa Cruz Biotechnology (C-15) and R&D Systems; anti-netrin-1, anti-DCC, anti-UNC5H1, anti-UNC5H2, anti-integrin subunits α6 and β1, and anti-laminin from R&D Systems; anti-laminin γ1 chain from AbCam; anti-integrin subunits α6, α1, α2, α3, α7, β1, and β4 from Santa Cruz; anti-phospho p44/42 from Cell Signaling; anti-α6β1 integrin from Chemicon; anti-β-actin from Novus Biologicals; anti-BrdU from Chemicon; and nonimmune IgG from Jackson Immunoresearch Laboratories. Anti-bacteriophage was purchased from Sigma. Secondary horseradish peroxidase (HRP)-conjugated anti-rabbit and anti-goat IgG, Cy-3-conjugated anti-rabbit IgG and anti-goat IgG, and FITC-conjugated anti-rat IgG were from Jackson Immunoresearch Laboratories. Recombinant netrin-1 and netrin-4, recombinant mouse DCC-Fc-chimera, rat UNC5H1, and UNC5H2-Fc-chimera were purchased from R&D Systems; mouse laminin was purchased from Chemicon; and fibronectin was from GIBCO. UO126 was purchased from Calbiochem.

Peptide Affinity Chromatography and Phage Binding Assays. Total protein extracts from NSCs were solubilized in extraction buffer (PBS) containing 1 mM $CaCl_2$, 1 mM $MgCl_2$, 50 mM octyl glucoside, and a mixture of protease inhibitors (PI) (Roche). Extracted proteins were loaded onto an affinity chromatography column (EDC/DADPA immobilization resin, Pierce) previously conjugated with CGLPYSSVC peptide. The proteins were allowed to bind for 30 min at room temperature (RT). The column was washed extensively and eluted first with 2 mM of unrelated peptide (CARAC), second with 2 mM of the targeted peptide (CGLPYSSVC), and finally with extraction buffer containing 2.5 mM EDTA. Fractions of 0.5 ml were collected, and those with positive OD (280 nm) were dialyzed and prepared for phage binding assays.

Phage Display Library Screening. We used a random phage library displaying the insert CX_7C (C, cysteine; X, any residue) for selection of peptides binding to the NSCs (clone C17.2) as described (1). A total of 10^6 NSCs were detached in PBS containing 2 mM EDTA, washed once in PBS, and resuspended in DMEM containing 2% BSA plus 10^9 transducing units (TU) of unselected phage library for 1 h on ice. Cells and phage were gently transferred to the top of a nonmiscible organic lower phase [dibutyl phthalate: cyclohexane, 9:1 (vol:vol)] and centrifuged at 10,000 $\times$ *g* for 10 min. Bound phage in the organic lower phase were recovered from the cell pellet after centrifugation by bacterial host infection with 200 μL of *Escherichia coli* K91 Kan growing in log phase. For the phage binding assay, phage displaying the selected peptide sequences were incubated with cells as described above. Colonies were counted after overnight (ON) incubation at 37 °C.

Two-Dimensional Electrophoresis. Proteins from NSC lysates were treated with a 2D Clean-up Kit (Amersham Biosciences). The resulting pellet was solubilized in a rehydration buffer [8 M urea, 4% CHAPS, 10 mM DTT, and 0.2% Bio-lytes (pI 3–10)] (BioRad). Proteins were loaded onto either 7 cm linear pH 4.7−5.6 or pH 3.0–7.0 IPG (immobilized pH gradient) strips (BioRad). Focusing of the first dimension took place on a Protean IEF Cell (BioRad) according to specification. The second-dimension separation was performed on a 4−15% SDS polyacrylamide gel (BioRad). The gel was probed by immunoblotting with the anti-netrin-4 antibodies. The spot of interest was excised from a Coomassie blue-stained gel and submitted for Mass Spectroscopy analysis (ProtTech). Two distinct peptides were identified: EAQLALGNAAADATEAK and VTDLDNEVNGMLR.

Proliferation, Adhesion, and Migration Assays. Cell proliferation was assessed by measuring the capacity of cells to metabolize the tetrazolium salt WST-1 to formazan (Roche). Biologically active concentrations (50 ng/ml of protein and 50 μM of peptides) were determined after testing a total of 5 different doses, ranging from 10 to 1000 ng/mL of protein and from 10 to 1000 μM of peptide. The cells were starved for 4 h; serum-free medium was removed and replaced by low-serum medium (1% FCS) containing 50 ng/mL of netrin-4, 50 ng/mL of netrin-1, 50 μM of CGLPYSSVC peptide, or 50 μM CARAC (negative control). Cells were cultured in the presence of proteins or peptides for 48−72 h at 37 °C. Treatment with UO126 was performed 30 min before cell activation.

For cell adhesion, proteins were coated ON at 4 °C onto 24-well plates at a concentration of 10 μg/mL. Wells were blocked and cell adhesion was carried out in DMEM containing 0.1% FCS, 1 mM $MgCl_2$, and 1 mM $CaCl_2$. Cells (10^5/well) were added to the wells and incubated for 1 h at 37 °C followed by washes. Total cell number was estimated on the basis of the conversion of salt WST-1 to formazan and measurement at 450 nm. Inhibition of cell adhesion to netrin-4 by specific antibodies was performed in 96-well plates in the presence or absence of antibodies (1 μg/mL), under the same experimental conditions. Cell migration was performed in modified Boyden chambers (BD BioCoat) with membranes of 8 μm pore size.

Bromodeoxyuridine (BrdU) Labeling and Flow Cytometry Analysis. NSCs were synchronized ON in serum-free medium (DMEM) containing 0.2% BSA and stimulated with proteins or peptides as indicated. Proliferating C17.2 NSCs were labeled by administration of BrdU (Sigma–Aldrich) at 10 μM for 60 min. NSCs were collected by trypsinization, washed in ice-cold PBS, and fixed in 100% ethanol for 30 min on ice. DNA denaturation was achieved by incubation of fixed cells in 2N HCL containing 0.05% Triton X-100 for 30 min at RT. Residual acid was neutralized with 0.1 M sodium borate, pH 8.5. Samples were further incubated with anti-BrdU monoclonal antibody followed by FITC conjugated secondary antibody. Total DNA content was measured with TO-PRO-3 iodide (Invitrogen). Flow cytometry analysis was performed in a FACSCanto II System and with FACSDiva software.

Cell Internalization Assay. Cells were grown on tissue chamber slides (Lab-Tek II Chamber Slide System, Nalge Nunc International Corp.) and incubated with 10^9 TU of the CGLPYSSVC-phage or insertless control phage. After extensive washes, cells were incubated with the primary anti-bacteriophage antibody, washed with PBS, incubated with Cy3-conjugated anti-rabbit secondary antibody, and visualized under a fluorescence microscope.

RNA Isolation and Quantitative Real-Time PCR. Two sets of total RNA were independently isolated from NSCs and differentiated NSCs with the RNeasy Mini Kit (Qiagen). First-strand cDNA synthesis was carried out with the ImProm-II Reverse Transcription System (Promega). Quantitative real-time PCR analysis was performed in

a 7500 Fast Real-Time PCR System instrument (Applied Biosystems). Probes were as follows: Mm00439445_m1 for LAMA1, Mm00550083_m1 for LAMA2, Mm01254801_m1 for LAMA3, Mn01193659_m1 for LAMA4, Mm01222049_g1 for LAMA5, Mm00801853_m1 for LAMB1, Mm00493080_m1 for LAMB2, Mm00493108_m1 for LAMB3, Mm00711808_m1 for LAMC1, Mm00500494_m1 for LAMC2, and Mm01324510_m1 for LAMC3. The gene expression ratio was normalized to that of 18S.

Phage Binding Assays. DCC, UNC5H1, UNC5H2, and BSA were immobilized on the wells of 96-well microtiter plates ON at 4 °C. Wells were washed twice with PBS, blocked with PBS containing 3% BSA for 1 h at RT, and incubated with 10^9 TU of netrin-4-like phage or insertless phage. After 1 h at RT, wells were washed with PBS, and bound phage were recovered by infection of host bacteria. The same procedure was adopted to analyze phage binding to immobilized netrin-4. Phage binding inhibition was performed in the presence of increasing concentrations of anti-netrin-4 antibody. Irrelevant IgG was used as a negative control.

Solution Binding Assays. All assays were performed using recombinant proteins and receptor-Fc chimeras in binding buffer [20 mM Hepes (pH 6.8), 150 mM KOAc, 2 mM $Mg(OAc)_2$, 2 mM DTT, 0.1% Tween 20] (2). For each experiment receptor-Fc chimeras were incubated with Protein-G coupled beads (0.6 μg of receptor-Fc chimera per 10 μL of packed beads) in 500 μL of binding buffer for 30 min at RT. The beads were collected by centrifugation at 5,000 rpm for 30 s and were washed 4 times by resuspension in 500 μL of binding buffer, followed by centrifugation. Washed collected beads were resuspended in 20 μL of binding buffer, and the recombinant protein (0.2, 0.5, and 1 μg) was added for a total final volume of 40 μL. The beads were gently shaken for 45 min at RT. At the end of the incubation time, beads were collected by centrifugation, and unbound proteins in the supernatant were collected by removal of 28 μL from the meniscus (unbound fraction). After washing twice with 500 μL of binding buffer, the beads were resuspended in 20 μL of buffer. All samples were processed by addition of 10 μL of 4× sample buffer containing β-mercaptoethanol and by heating at 95 °C for 10 min. Proteins in one half were resolved by SDS/PAGE and stained with Coomassie blue or were transferred to a nitrocellulose membrane and analyzed by immunoblotting.

NSC Differentiation. For NSC differentiation in vitro, cells at <33% confluence were treated with 10 μM of mitomycin-C for at least 2 weeks. The media were changed every 3 days for 3 weeks.

Primary Progenitors Culture. We used a standard procedure to isolate primary cells from the OB and RMS of adult mouse brain (3). Briefly, brain regions of interest were isolated, dissected, sliced, and digested with papain (30 min, 30 °C). Using a siliconized 9-inch Pasteur pipette with a fire-polished tip, tissue was triturated and cell suspension was carefully applied to the top of an OptiPrep (Sigma) density gradient (3) and centrifuged at 1,900 rpm for 15 min at RT. Fractions enriched for neuron progenitors were collected, washed, and plated at the desired concentration in low-adhesion 24-well plates in medium containing NeurobasalA, B27, 0.5 mM GIn, 10 μg/mL gentamycin, 5 ng/mL mouse FGF2, and 5 ng/mL mouse PDGFbb.

Neurosphere Assay. Freshly isolated neuronal progenitors proliferated in culture for 7 days in the presence of mitogens (mouse FGF2 and mouse PDGFbb) (4). Spheres were harvested, redissociated into single cells, and replated at a density of 2.5 cells/μL into 24-well low-adhesion plates in media containing mitogens and test reagents.

Immunofluorescence-Based Cell Staining. NSCs were cultured in 8-well chamber slides (Lab-Teck II, Nalge Nunc International) for 72 h and were placed in serum-free medium for 4 h before treatment with netrin-4 (50 ng/mL), netrin-1 (50 ng/mL), or the peptides CGLPYSSVC or CARAC (50 μM), unless otherwise specified. After 15 min of activation, cells were washed once with PBS, fixed in PBS containing 4% paraformaldehyde (PFA) for 5 min at RT, and permeabilized with PBS containing 0.2% Triton X-100 for 5 min at RT. Next, cells were quenched in fresh TBS containing 0.1% sodium borohydride for 5 min and blocked for 1 h with PBS containing 10% goat serum, 1% BSA, and 0.02% NaN_3. After washing with PBS, cells were incubated ON at 4 °C with specific antibodies in PBS containing 1% BSA. Cells were washed 3 times with PBS and incubated with appropriate fluorescently labeled secondary antibody in PBS containing 1% BSA for 45 min at RT. Finally, slips were washed 3 times with PBS, dried, and mounted with DAPI-containing Vectashield Mounting Medium (Vector Laboratories).

Immunofluorescence Staining of Frozen Tissue Sections. Immunofluorescence was performed on cryostat sections (20 μm thick) of adult BALB/cByJ mouse brain (4 months old). Tissue sections were ON with specific antibodies diluted in PBS containing appropriate normal sera and detergent. Anti-netrin-4 antibody purchased from Santa Cruz (C-15) showed no cross-reactivity with laminin γ1 chain and netrin-1 on immunoblots [supporting information (*SI*) Fig. S3*B*] and was therefore used for immunostaining. Confocal images were obtained with a Zeiss Axiovert 100M microscope and Carl Zeiss AIM software.

ELISA. Five micrograms of total proteins were coated in microtiter wells (ON at 4 °C), blocked with PBS containing 1% BSA for 1 h, and incubated with specific antibodies. HRP-conjugated antibodies were used as secondary reagents.

Immunoblotting. Total proteins from eluted fractions or crude cell lysate were resolved on a SDS/PAGE gel (BioRad), transferred to nitrocellulose membranes (BioRad), incubated with primary antibody, washed, incubated with HRP-conjugated secondary antibody, and developed with the enhanced chemiluminescence (ECL) reagent (BioRad). For detection of phosphorylated ERK1/2, total proteins were extracted in lysis buffer (20 mM Tris-HCl, 100 mM NaCl, 0.5% Nonidet P-40) containing 1 mM Na_3VO_4, 50 mM NaF, and PI.

Co-Immunoprecipitation Assays. Cells were lysed with Nonidet P-40 lysis buffer plus PI for 30 min at 4 °C and centrifuged for 10 min (14,000 rpm, 4 °C). The supernatant containing soluble proteins was precleared with protein-A beads (Amersham Bioscience) previously coupled to normal rabbit serum. Pellet-containing beads were discarded, and the remaining proteins were incubated with specific antibodies (ON at 4 °C). The next day, 50 μL of protein-A beads were added to the samples and incubated for 2 h at 4 °C. After centrifugation, pellets containing beads were washed with Nonidet P-40 high-salt lysis buffer and once with PBS.

Allen Brain Atlas Analysis. Expression profiles of the selected genes were evaluated in the brains of 8-week-old male adult C57BL/6J mice with the high-throughput in situ hybridization platform developed by the Allen Institute for Brain Science (Allen Brain Atlas) (5). Briefly, for each evaluated gene, in situ hybridization (ISH) signals were produced after specific antisense digoxigenin-labeled probes were hybridized to cellular (sense) transcripts, followed by incubation with HRP-conjugated anti-digoxigenin antibody. Colorimetric data were captured from sets of slides and were transformed into 3-dimensional data with the Brain Explorer application (Allen Institute for Brain Science, Version 1.0, July 28, 2006).

Peptide Modeling. We applied Molecular Mechanics Force Field Methods (MM+ of CHARMM, Hyperchem 7.5 from Hypercube Inc.); force field components were bond, angle, torsion, nonbonded, electrostatic, and hydrogen bonded for geometry optimization (6). We used molecular dynamics simulations (simulation temperature, 300 K) for determination of the structure (7) of netrin-4 protein and its peptide mimic.

1. Giordano RJ, Cardó-Vila M, Lahdenranta J, Pasqualini R, Arap W (2001) Biopanning and rapid analysis of selective interactive ligands. *Nat Med* 7:1249–1253.
2. Rexach M, Blobel G (1995) Protein import into nuclei: association and dissociation reactions involving transport substrate, transport factors, and nucleoporins. *Cell* 83:683–692.
3. Brewer GJ, Torricelli JR (2007) Isolation and culture of adult neurons and neurospheres. *Nat Protoc* 2:1490–1498.
4. Ferrón SR, *et al.* (2007) A combined ex/in vivo assay to detect effects of exogenously added factors in neural stem cells. *Nat Protoc* 2:849–859.
5. Lein ES, *et al.* (2007) Genome-wide atlas of gene expression in the adult mouse brain. *Nature* 445:168–176.
6. Weiner SJK, *et al.* (1984) A new force field for molecular mechanical simulation of nucleic acids and proteins. *J Am Chem Soc* 106:765–784.
7. Brooks BRB, *et al.* (1983) CHARMM: a program for macromolecular energy, minimization, and dynamics calculations. *J Comput Chem* 4:187–217.

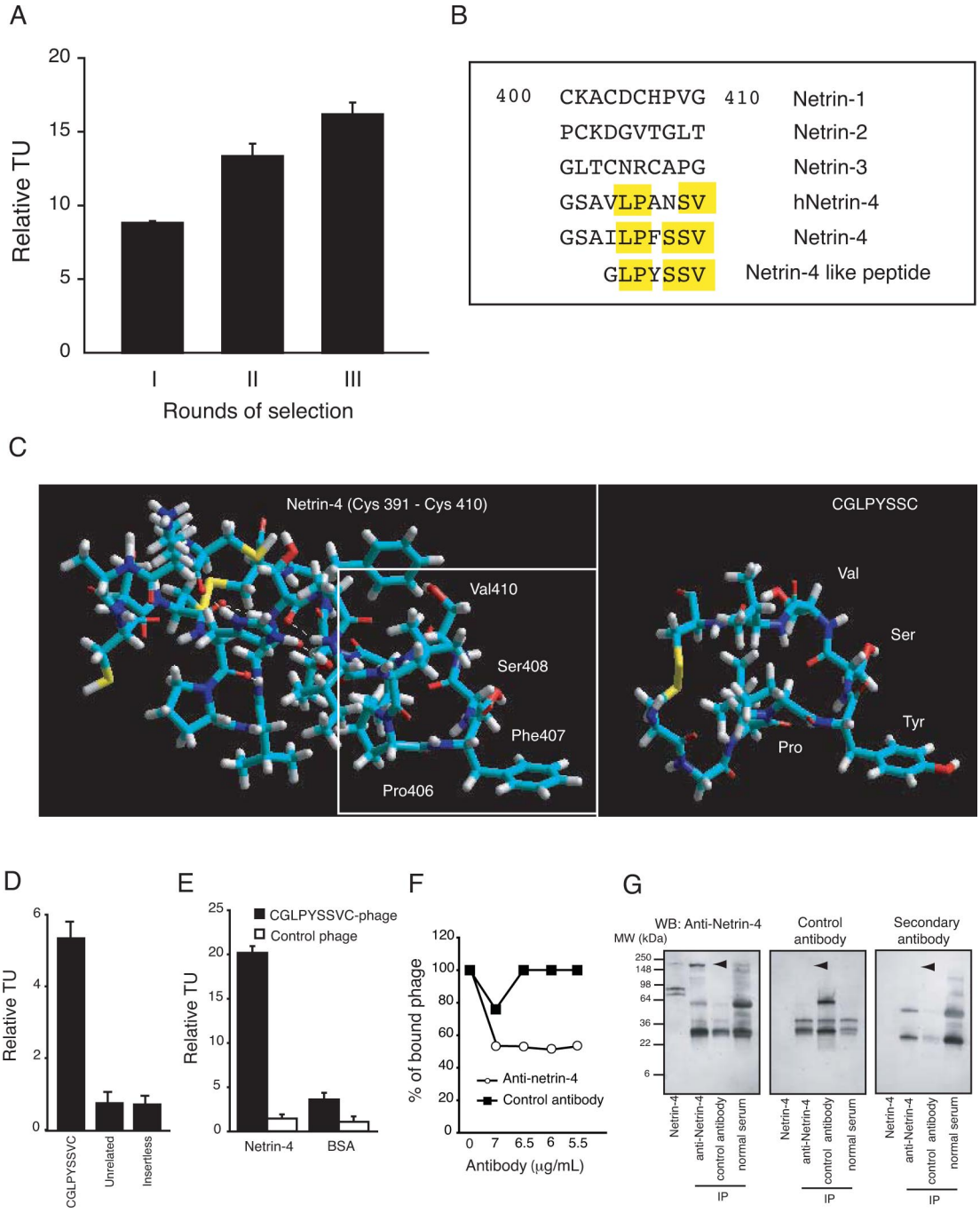

Fig. S1. Phage display screening on clonal mouse C17.2 neural stem cells. (*A*) A 3-fold phage binding enrichment was obtained after 3 rounds of selection. (*B*) Sequence alignment of CGLPYSSVC peptide and netrins. Similarities among residues are depicted in yellow. (*C*) Three-dimensional model of the Cys-391−Cys-410 motif of netrin-4 and the peptide CGLPYSSVC. Striking similarities were observed between the peptide and netrin-4 structures. After geometry and molecular dynamics optimization, the position and orientation of several corresponding amino acids are nearly identical, such as Pro-406, Ser-408, and Val-410. (*D*) Binding of individual phage clones to NSCs. A phage clone displaying the peptide sequence CGLPYSSVC bound preferentially to NSC relative to control insertless phage (≈7.5-fold). Experiments were performed 3 times with similar results; a representative binding experiment is shown. Bars are mean ± SD. (*E*) Phage binding to netrin-4 recombinant protein. Microtiter wells were coated ON with netrin-4 and incubated with of phage for 1 h. After several washes, bound phage were recovered by bacterial infection. Insertless phage (fd-tet) and BSA were used as negative controls. The experiment was performed 3 times with similar results. (*F*) Inhibition of CGLPYSSVC-phage to netrin-4 by anti-netrin-4 antibody. (*G*) Immunoprecipitation followed by immunoblotting shows that NSCs produce only the 220-kDa protein (arrowhead) but not netrin-4. The high molecular weight protein present in the eluted fractions is specifically immunoprecipitated by the anti-netrin-4 antibody (*Left*) but not by control antibodies (*Middle* and *Right*).

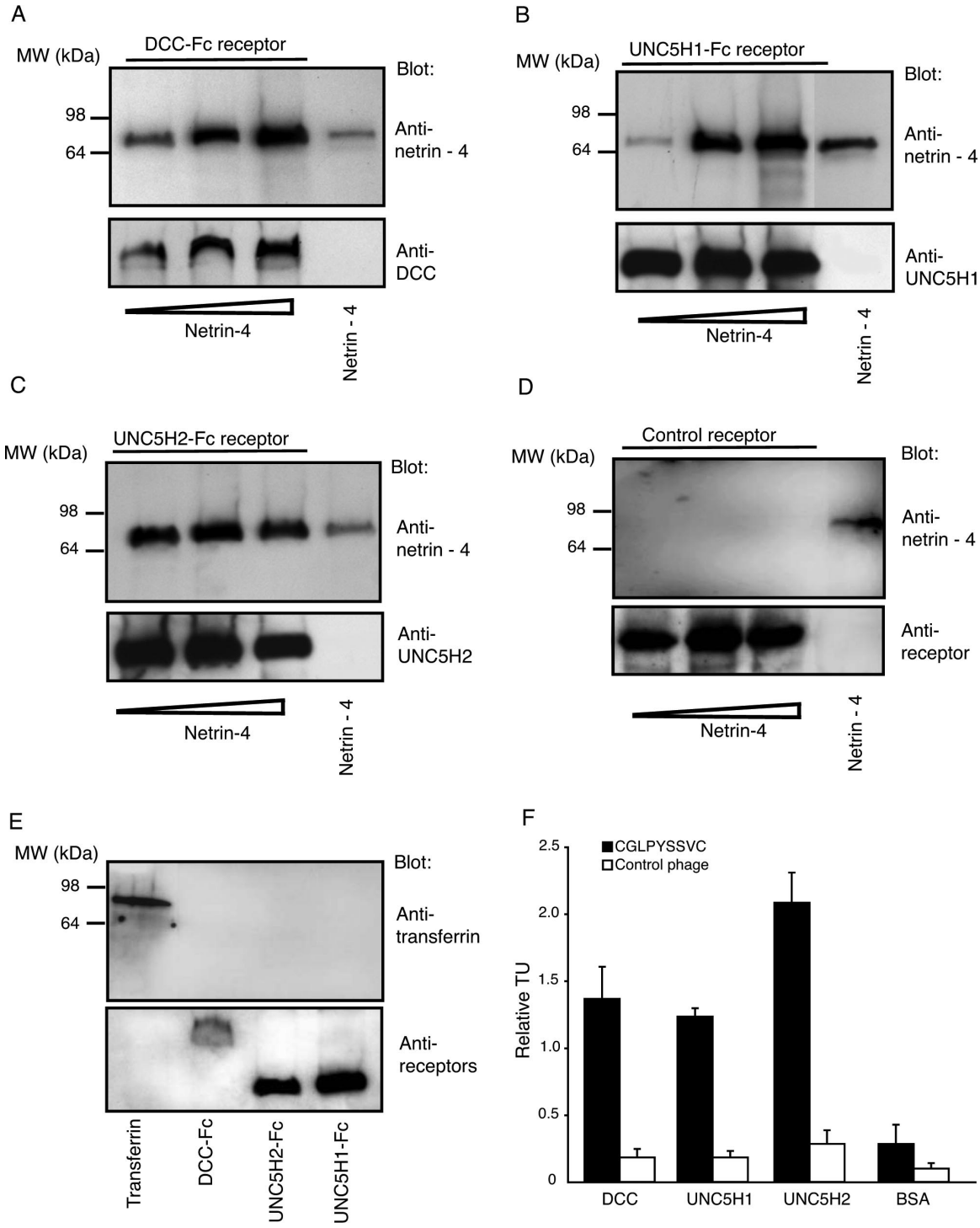

Fig. S2. Netrin-4 binds specifically to the extracellular domain of the receptors DCC and UNC5. (*A–C*) Fc-chimera receptors (DCC, UNC5H1, and UNC5H2) and control receptor were immobilized on protein-G beads and incubated with recombinant netrin-4 (0.2, 0.5, or 1 μg). Proteins bound to the receptors were separated by SDS–polyacrylamide gel electrophoresis and were analyzed by immunoblotting with anti-netrin-4 antibody. Unrelated receptor (*D*) and protein (*E*) were used as negative controls. (*F*) Phage binding to candidate receptors DCC, UNC5H1, and UNC5H2. Microtiter wells were coated ON with 0.25 μg of each receptor and incubated with phage for 1 h. After several washes, bound phage were recovered by bacterial infection. Insertless phage (fd-tet) and BSA were used as negative controls.

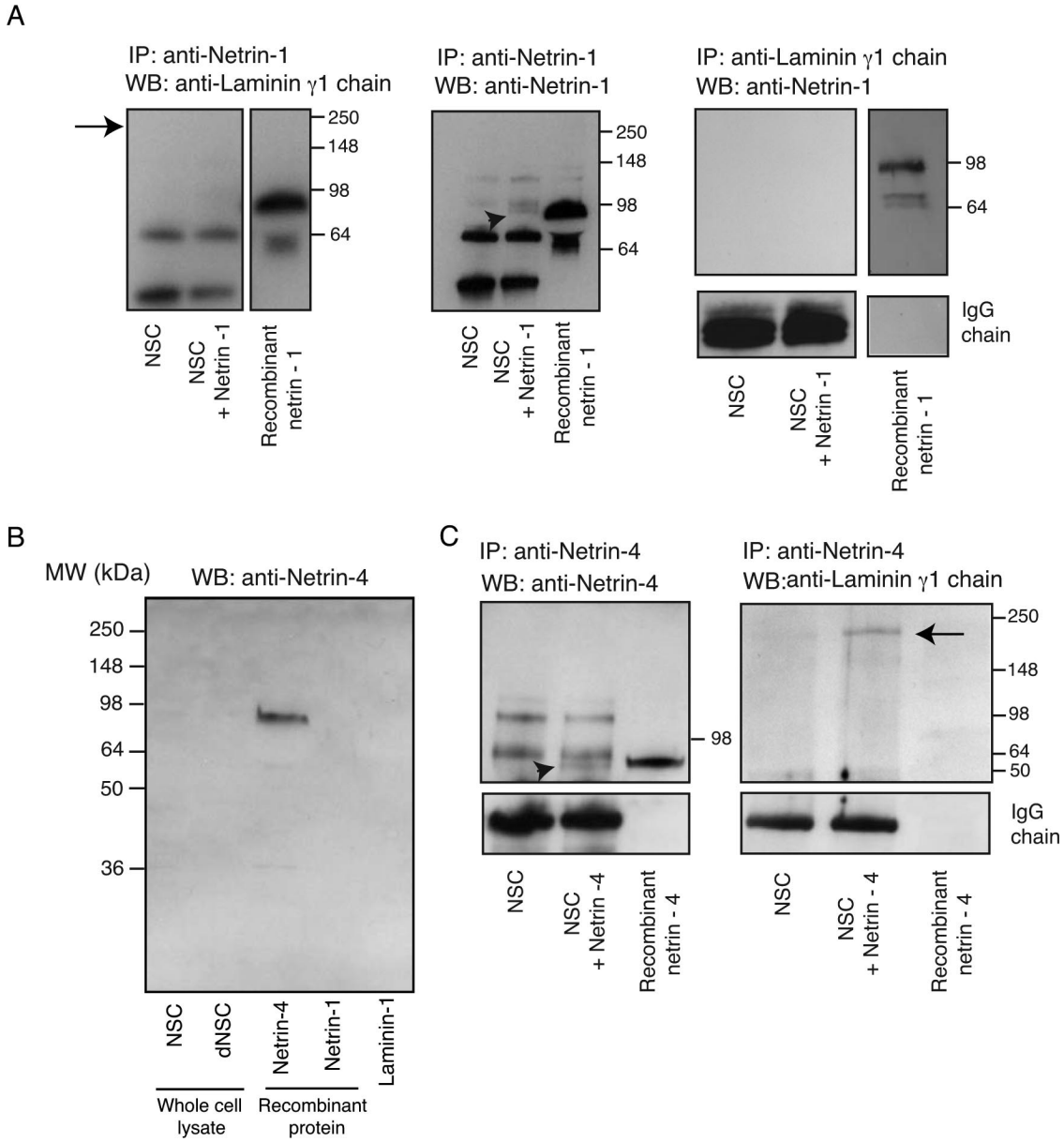

Fig. S3. Netrin-4 binds to laminin γ1 chain. (*A*) Ligand-directed co-immunoprecipitation. Immunoprecipitation with anti-netrin-1 was used as a control for binding specificity. No interaction is observed between netrin-1 and laminin γ1 chain. Arrows point to the expected position of laminin γ1 chain (*Left*) and netrin-1 (*Middle*). (*B*) Anti-netrin-4 antibody (C-15) recognizes only netrin-4 in immunoblotting analysis of total cell lysates and recombinant proteins. (*C*) Co-immunoprecipitation with anti-netrin-4 antibody. Immunoprecipitation of total NSCs lysate with anti-netrin-4 antibody coprecipitates the laminin γ1 chain (≈220 kDa, arrow, *Right*) only when netrin-4 is added to the cells.

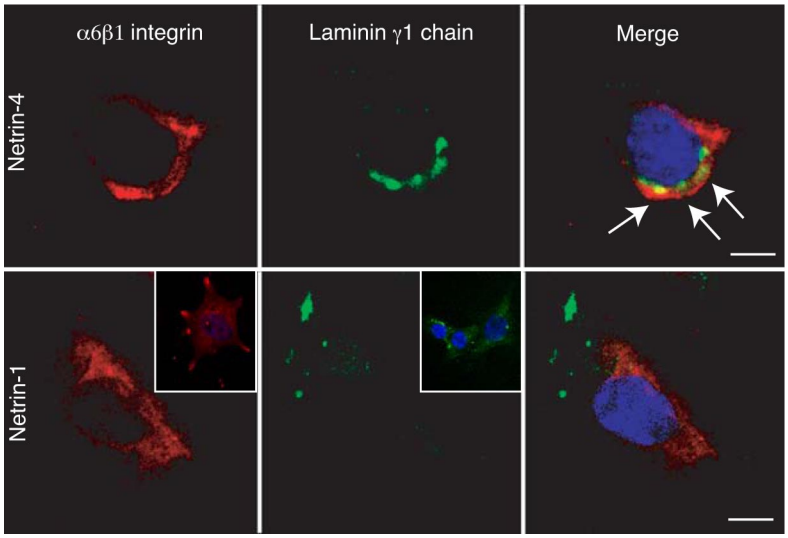

Fig. S4. Laminin γ1 chain and α6β1 integrin colocalize in NSCs stimulated with netrin-4 (*Top*) but not netrin-1 (*Bottom*). NSCs were cultured in serum-free media for 4 h and were subsequently stimulated with 50 ng/mL of netrin-4 or netrin-1 for 15 min. Cells were washed, fixed, and examined by immunofluorescence. Arrows indicate points of colocalization of α6β1 integrin and laminin γ1 chain. (Magnification, ×20.) *Insets* show localization of α6β1 integrin and laminin γ1 chain before treatment.

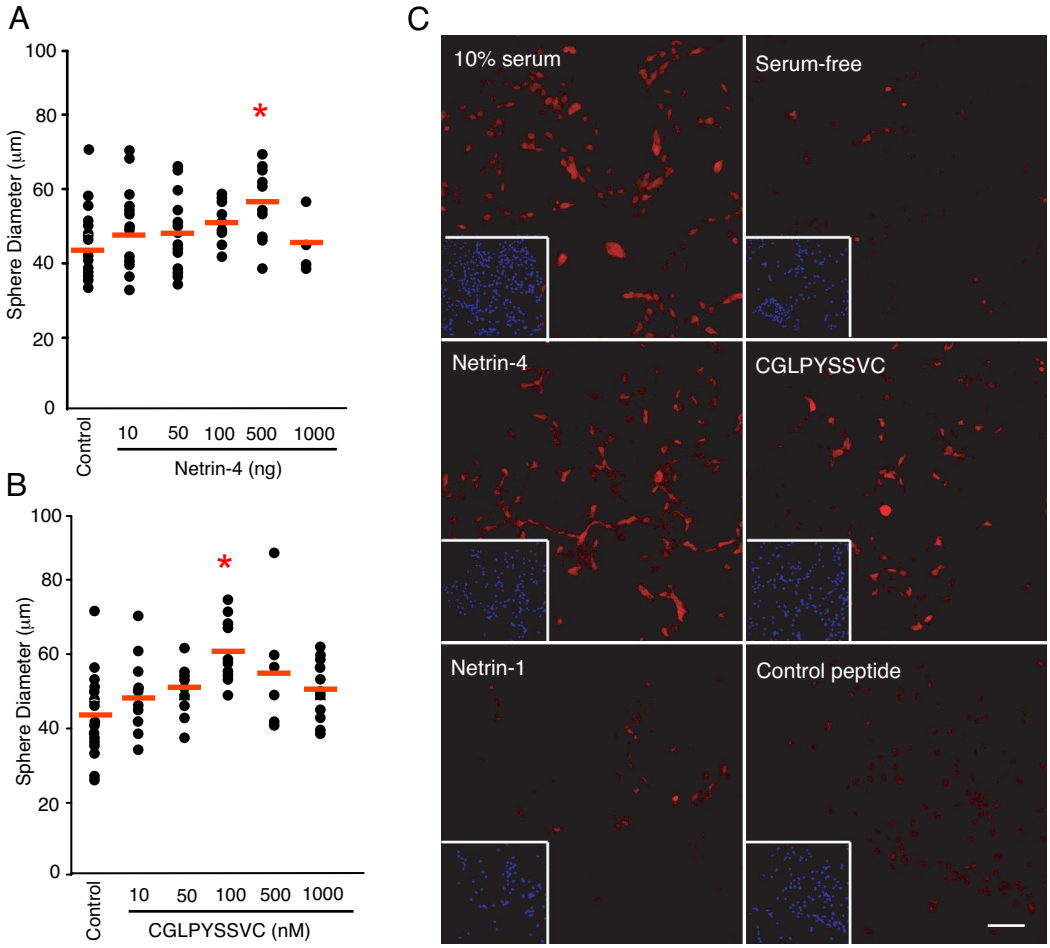

Fig. S5. Netrin-4 and the CGLPYSSVC peptide promote proliferation of neural progenitors. (*A* and *B*) Neurosphere assay. Freshly isolated neuronal progenitors were plated at a density of 2.5 cells/μL into 24-well low-adhesion plates in media containing mitogens, recombinant netrin-4 (*A*), and CGLPYSSVC peptide (*B*). The diameter of the spheres was measured after 6 days. *, $P < 0.001$. (*C*) Immunostaining of ERK1 and ERK2 phosphorylated proteins in different experimental conditions as indicated. (Magnification, $\times$10.)

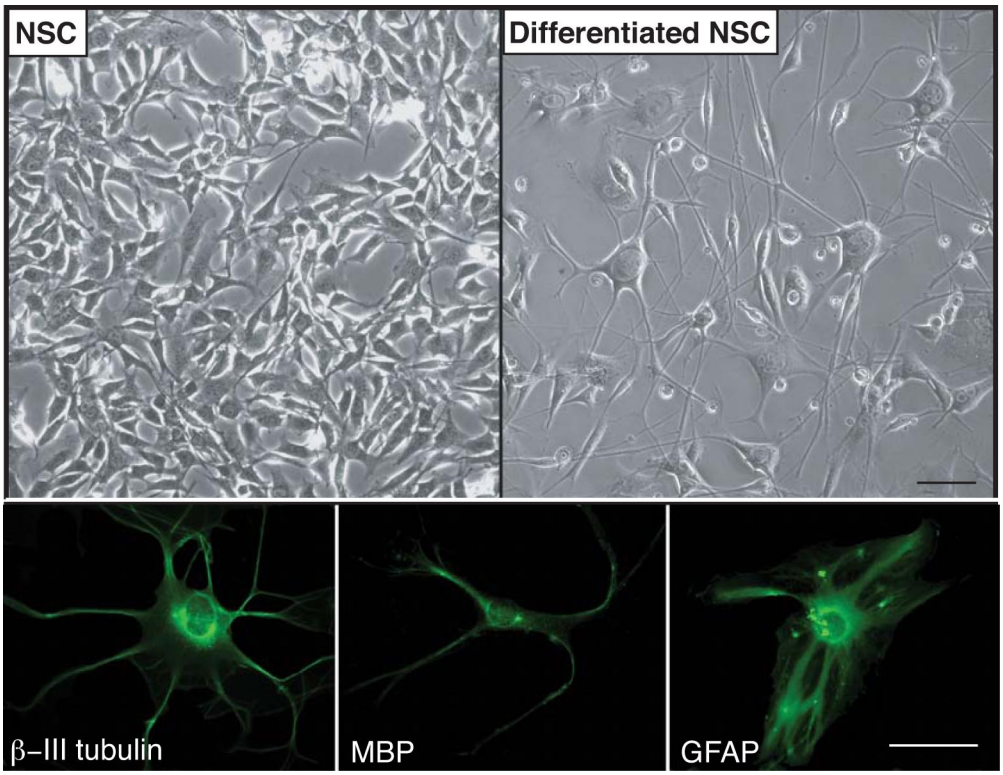

Fig. S6. Representative images of NSCs and differentiated NSCs. (Magnification: bright field, ×10.) Cell differentiation was confirmed by expression of β-III tubulin (neurons), myelin basic protein (MBP) (oligodendrocytes), and GFAP (astrocytes). (Magnification, ×20.)

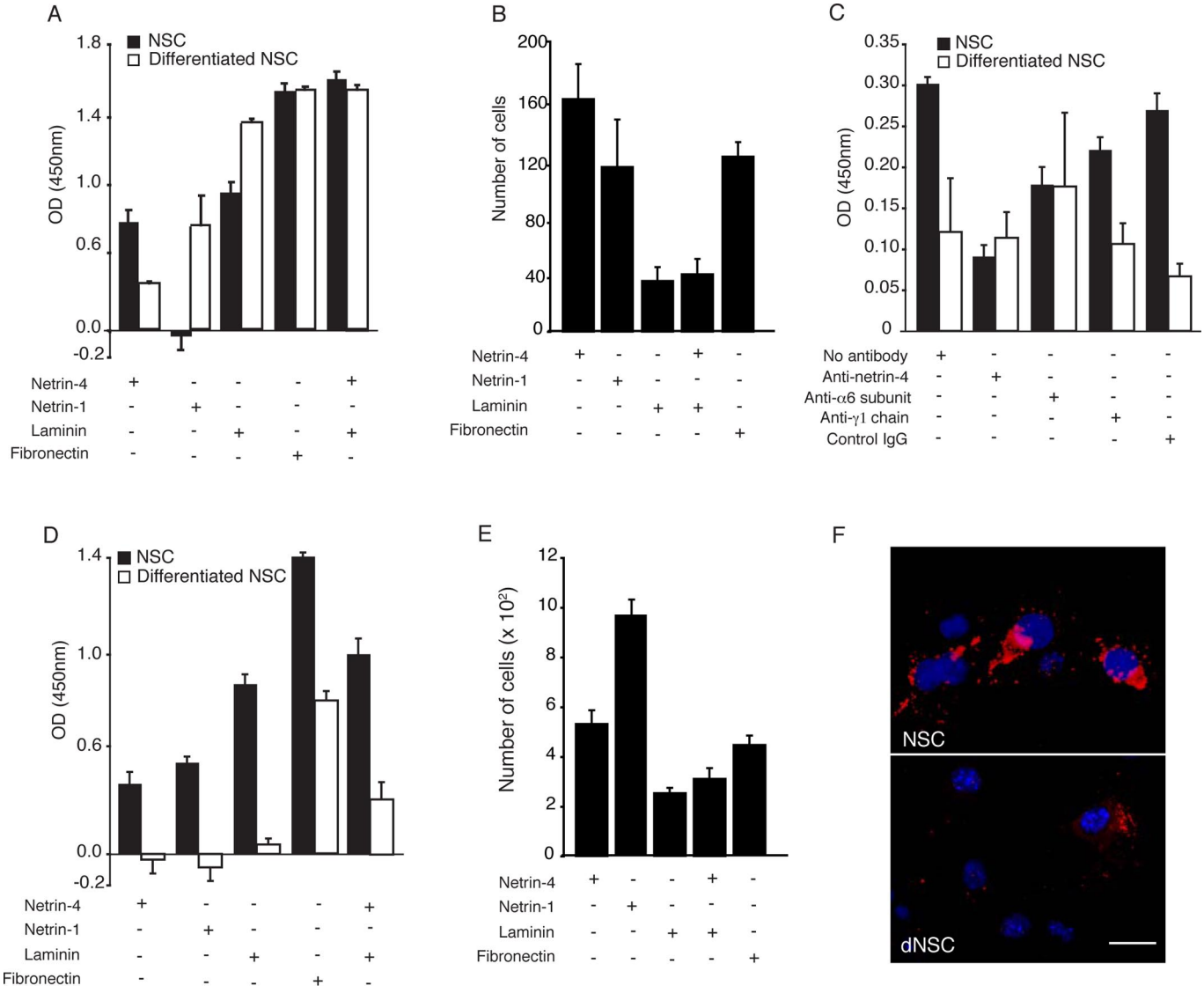

Fig. S7. (*A*) Adhesion of proliferating and differentiated C17.2 NSCs to netrin-4, netrin-1, laminin, and fibronectin. (*B*) Adhesion of primary neural progenitors to netrin-4. Cells were cultured for 7 days in appropriate media, and spheres were dissociated into single cells and assayed for their ability to attach to immobilized proteins as indicated. Netrin-1, laminin, and fibronectin were used as control proteins. (*C*) Cell adhesion to netrin-4 is specifically inhibited by anti-netrin-4, anti-α6 integrin subunit, and anti-laminin γ1 chain antibodies. A control antibody had no detectable effects in cell adhesion. (*D*) Migration of C17.2 NSCs and differentiated C17.2 NSCs toward netrin-4, netrin-1, laminin, and fibronectin in Boyden chambers. (*E*) Migration of primary neuronal progenitors toward netrin-4 in Boyden chambers. Again, netrin-1, laminin, and fibronectin were used as control proteins. (*F*) Decrease in phage internalization was observed after cell differentiation. (Magnification, ×20.)

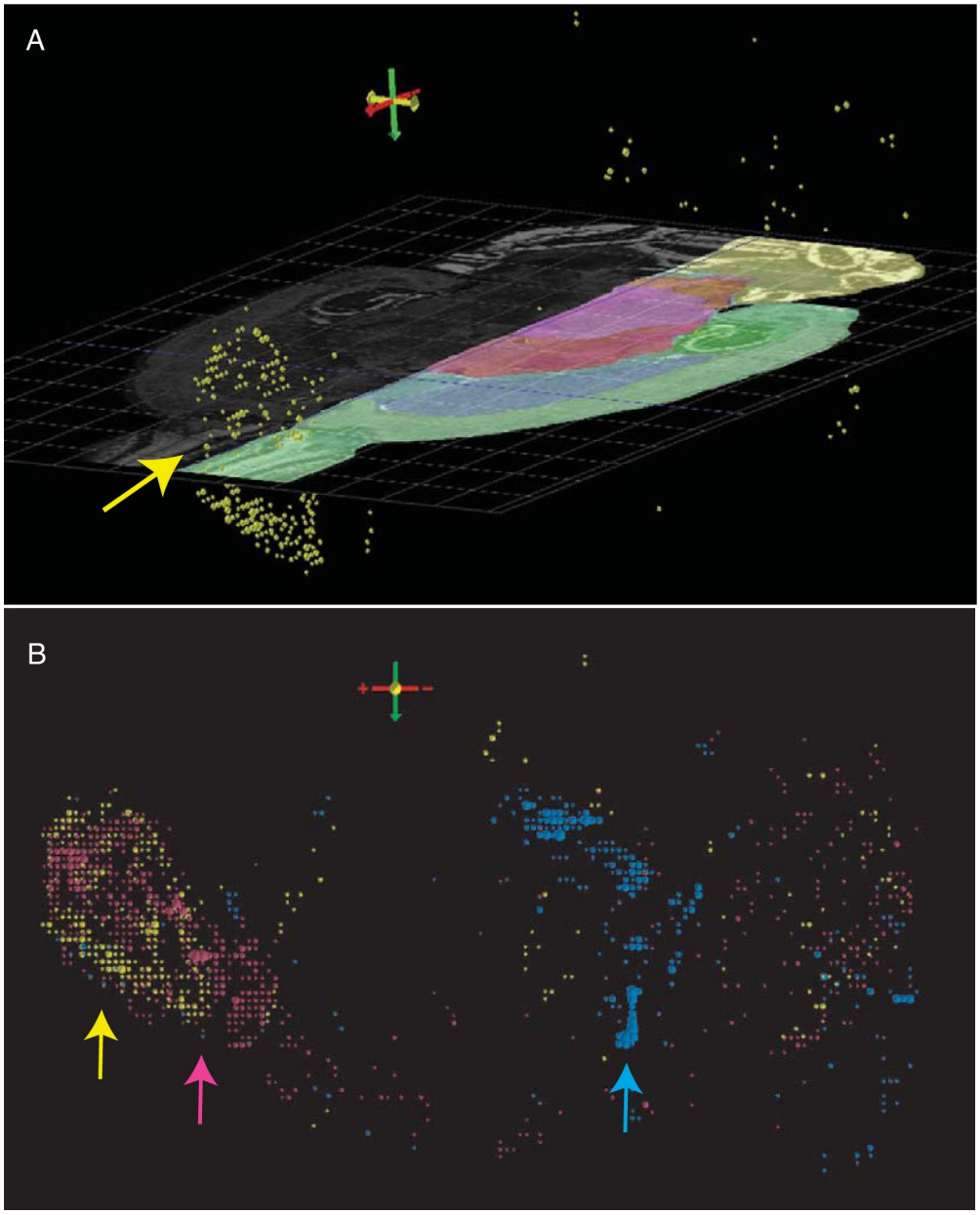

Fig. S8. Expression of netrin-4 and its receptors in the adult normal brain. (*A* and *B*) Digital images of a horizontal section (*A*) and a sagittal section (*B*) of adult mouse brain derived from the database provided in the Allen Brain Atlas (www.brainatlas.org). mRNA expression of netrin-4 was observed mainly in the OB (*A*). We also observed colocalization of netrin-4 (yellow) and α6 integrin subunit (pink) mRNAs in the same region (*B*). mRNA expression of the laminin γ1 chain is shown in blue. Spheres represent gene expression in different brain regions (determined by quadrants of 100 μm^3). The size of the spheres is proportional to the number of expressing cells detected in each quadrant. The compass is shown at the bregma position.

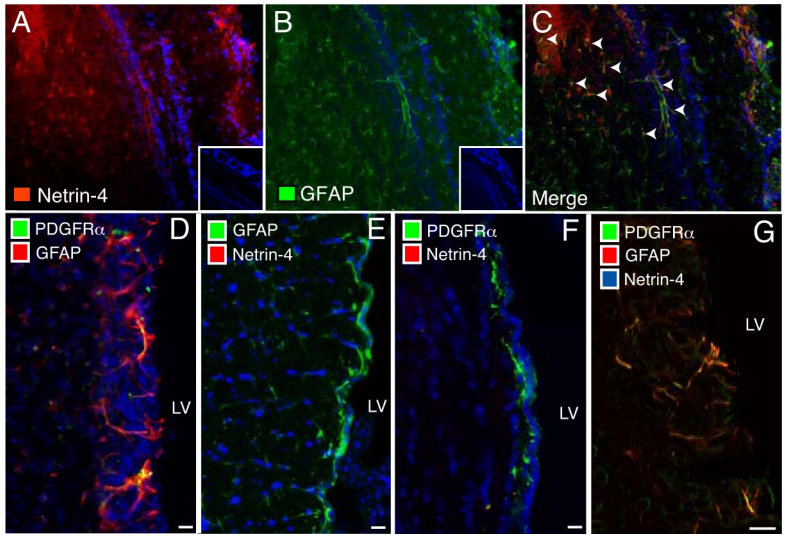

Fig. S9. Netrin-4 is expressed by a subset of astrocytes near and within the olfactory bulb (OB), but is not expressed by neural stem cells residing in the SVZ on the edge of the lateral ventricle. (*A*–C) Many cells in the OB are stained for netrin-4 (*A*) and GFAP (*B*). (C) Merged image, with arrowheads pointing to many astrocytes positively stained for netrin-4. *Insets* show tissue staining with secondary antibodies only (donkey anti-goat Cy3 and donkey anti-rabbit FITC). (*D*–*G*) Expression of netrin-4 in the subventricular zone of adult mouse brain. (*D*) PDGFRα^+/GFAP$^+$ cells were located in the subventricular zone of the lateral ventricle of mouse brain. (*E*–*G*) We used confocal microscopy to demonstrate that neither GFAP$^+$ cells (*E*) nor PDGFRα^+ cells (*F*) express netrin-4. (*G*) Triple staining of brain sections with anti-PDGFRα^+ (green), anti-GFAP (red), and anti-netrin-4 (blue) antibodies confirmed that cells of the subventricular zone of adult mice do not produce netrin-4. (Magnifications: *C*−*E*, ×40; *F*, ×60.)