Supporting Information

Chiaruttini et al. 10.1073/pnas.0902833106

SI Methods

Chimeric BDNF-GFP Constructs. Total RNA was extracted from whole rat brain using TriZol Reagent (Invitrogen). Since exon I is expressed in the hippocampus at low levels, RNA for exon 1 amplification was extracted from the whole brain of a rat treated for 3 h with kainic acid (12 mg/kg). After extraction, 1 μg total RNA was reverse-transcribed into cDNA with random primers (Roche) using 200 U SuperscriptIII Reverse transcriptase (Invitrogen) according to the manufacturer's instructions. PCR was performed in a total volume of 25 μL containing 1 μg cDNA, 10× Reaction Buffer, 500 mM KCl, 100 mM Tris-HCl, pH 9; 1.5 mM $MgCl_2$; 200 μM dNTPs mix; TaqDNA polymerase 0.04 units/reaction, all from Promega and specific forward and reverse primers 500 nM, (Celbio-Euroclone) to amplify the different BDNF isoforms. PCR protocol was 5 min at 95 °C; 35 cycles of the progression 1 min 95 °C, 1 min 55 °C, 2 min 72 °C. Unique forward primers were designed within each of the major *BDNF* exons and were paired with a common reverse primer at the end of the BDNF coding sequence (Exon 1: AATT*CTCGAG*GGTCTTC-CCGCCCTAGCCTGAC; exon 2: AATT*CTCGAG*CGGA-GCGTTTGGAGAGCCAGCC; exon 4: AATT*CTCGAG*TGAAG-GCGTGCGAGTATTACCTCC; exon 6: AATT*CTCGAG*TCG-CACGGTCCCCATTGCGCC; forward BDNF coding sequence GCG*CTCGAG*ATGA-CCATCCTTTTCCTTAC; reverse GTCA*CACGTG*TC-CCCTTTTAATG-GTCAGTG). The sequences in italics correspond to the *Xho*I (forward primers) and the *Sac*II (reverse) restriction sites for cloning into the pEGFPN-1 plasmid (Clontech). Expression of the BDNF-GFP chimaeras was tested in HEK-293T cells transfected with each BDNF-GFP construct. At 24 h after transfection, cells were washed in PBS, scraped into Laemli buffer, subjected to SDS/PAGE and blotted on nitrocellulose membrane (Schleicher & Schuell). Western blots were incubated at 4 °C overnight with the monoclonal anti-GFP antibody (1:1,000; Clontech) and 1 h at room temperature with secondary anti-mouse alkaline phosphatase-coupled antibody (DAKOCytomation) diluted 1:1,000. Membranes were developed at room temperature with 4-nitroblue tetrazolium 35 mg/mL (NBT; Labtek Eurobio) and 5-bromo-4-chloro-3-indolylphosphate 17.5 mg/mL (BCIP; Labtek Eurobio) in 100 mM Tris-HCl, pH 9.5, 50 mM $MgCl_2$, 100 mM NaCl.

Cell Cultures and Transfections. Primary hippocampal neurons were prepared from P2 rats as described before with slight modifications (1). Cells were plated on 2% Matrigel (BD Biosciences) coated coverslips in 24-well plates at a density of 4×10^5/mL cells per well and cultured for 8 days in a 5% CO_2-humidified incubator, in minimum essential medium with Earle's salts and Glutamax I (MEM, Life Technologies, Invitrogen) with 10% FBS (Gibco), 35 nM D-glucose (Lancaster), 14 mM Hepes (Sigma),1 mM vitamin B12 (Sigma), 0.36 mM d-Biotin (Sigma), 30 μg/mL insulin (Sigma), 100 μg/mL bovine transferring (Sigma), and antibiotics (Euroclone). The medium was changed every 2 days from the second day in culture onwards.

HEK-293T cells were used to analyze the expression of the BDNF-GFP chimeras because of their high level of transfection efficiency. Cells were cultured in MEM and Glutamax I with 10% FBS. The day before transfection, cells were plated at $1–3 \times 10^5$ cells per well in 24-well plates and 24 h after transfection they were analyzed by GFP autofluorescence or western blot analysis. HEK-293T cells and hippocampal neurons were transfected 1 day and 7 days after plating, respectively, with 1 μg GFP-chimaeric plasmid and 2 μL Lipofectamine 2000 solution (1 mg/mL) each diluted in 50 μL MEM without serum nor antibiotics. The Lipofectamine2000 and DNA mix was removed 24 h after transfection. Cells were then washed twice with PBS and then used for immunofluorescence, western blot, or nonradioactive in situ hybridization experiments.

RNAi Interference. To improve the likelihood that the RNAi approach was effective, we used RNAi "cocktails" generated by cleavage of relatively long (≈300 nt) double-stranded RNA produced from a segment of the target gene with a low level of homology to other genes. Target regions were identified in translin, CPEB1, and BDNF by consulting the NCBI database. To amplify these genes, PCR primers containing the T7 promoter were used: translin forward primer 5′-GCGTAATAC-GACTCACTATAGGGtgagcactggaggtttgtgc-3′; reverse primer 5′- GCGTAATACGACTCACTATAGGG cgtagcgtttcctcagg-gag-3′ Length of PCR Product = 324 bp. CPEB1 forward primer 5′-GCGTAATACGACTCACTATAGGGtctgacag-gctgggac-cgac-3′; reverse primer 5′-GCGTAATACGACTCACTAT-AGGGtgaagc-cactggtgtctgag-3′ Length of PCR Product = 267 bp. Scrambled control siRNA sequence 5′- AAGTAGTC-GAGTGCGTGGATT-3′. The target regions were amplified from whole rat brain and testis cDNA by PCR at 94 °C for 1 min, 55 °C for 1 min, 72 °C for 2 min (30 cycles), and 72 °C for 10 min. PCR products were gel-purified and used as template for in vitro transcription using T7 RNA polymerase and the Silencer siRNA mixture kit (Ambion). Following DNase and RNase A treatment for 1 h, double-stranded RNA was purified through spin columns (Promega) and digested with RNase III according to the manufacturer's instructions to yield siRNA mixtures. The mixtures of 12–30-bp double-stranded RNAs contain 5′-PO_4, 3′-OH, and 2-nucleotide 3′overhangs similar to siRNA produced in vivo. SK-N-BE cells were cotransfected with the plasmid pEGFP-N1-translin and siRNA for translin or the siRNA against CPEB1 as a control. The different assays were performed in 24 multiwell plates using 1 μg plasmid and 19 nM siRNA mixture with 2 μL Lipofectamine 2000 (Invitrogen) per well. Cells were fixed with PFA 4% 24 h after transfection. Counts of green cells was performed from three replicate assays. Statistical analysis of these data were performed with SigmaStat.

In addition to using RNAi cocktails, as described above, we tested the effects of defined siRNA oligo sequences targeting rat translin. The sense sequences for si690 and si744 are: 5′-acgacggcuuaaaguauga-3′ and 5′-ccauccgaggcuucaauaa-3′. Sense sequence for si744M is: 5′-**gcacccgacguu**ucaauaa-3′, which contains four mismatches with si744, shown in bold. An additional scrambled siRNA was also used as a control: 5′-aaguagucgagugcguggauu-3′. Each sense and antisense oligo was synthesized with a uu overhang at its 3′ end. Oligos were annealed before transfection. The effectiveness of the sequences used in si690, si744, and si744M to silence endogenous translin was confirmed by infecting rat cortical cultures with lentiviral constructs (plK0.1, generously provided by M. K. Meffert) containing the corresponding shRNA inserts and then analyzing translin and trax expression by immunoblotting in culture lysates harvested 6 days following infection. Infection efficiency was monitored by expression of mCherry generated by the virus.

Animal Treatment. Animals were treated according to the institutional guidelines in compliance with national and international laws (European Council Directive 86/609, Oja L 358,1, Decem-

ber 12, 1987; NIH Guide for the Care and Use of Laboratory Animals). Two-month-old Val/Val or Met/Met mice were pretreated with methylscopolamine (1 mg/kg, i.p.), 30 min before i.p. injection of 250 mg/kg pilocarpine in saline, or saline (controls). Mice were continuously monitored. Time of onset and severity of seizures were recorded. Mice injected with pilocarpine that did not show seizures were discarded. About 80% of mice developed class 5 seizures within 1 h after injection of pilocarpine.

In Situ Hybridization and Immunocytochemistry. Transfected hippocampal neurons, either in normal medium or after depolarization for 3 h with 10 mM KCl, were analyzed by in situ hybridization as described (1). Cells were fixed for 15 min at room temperature in 4% paraformaldehyde in PBS, washed in PBST, and permeabilized in absolute ethanol for 15 min at −20 °C. After rehydration, cells were hybridized with antisense or sense probes for GFP mRNA or BDNF CDS. The ORF of GFP or BDNF CDS were subcloned into pBluescript or pGEM vectors respectively and DIG- labeled probes were synthesized with the DIG-RNA labeling kit (La Roche Diagnostics) using linearized plasmids as templates, according to the manufacturer's instructions. Hybridization was followed by high stringency washes with 0.01× sodium saline citrate buffer containing 0.1% Tween-20 (SSCT) at 60 °C, then incubated with anti-digoxigenin alkaline phosphatase coupled antibody for 3 h diluted 1:1,000 and developed with NBT and BCIP for 40 min at room temperature. For immunocytochemistry, fixation and permeabilization of hippocampal neurons was performed as described above. After permeabilization, cells were incubated for 3 h at room temperature with a rabbit anti-MAP2 antibody (Roche) diluted 1:1,000 in 10% FCS in PBST. After washes in PBST, cells were incubated 1 h at room temperature with anti-rabbit-FITC coupled antibody (1:100 in 10% FCS in PBST; Dako).

For in situ hybridizations on free floating sections, mice were transcardially perfused with 4% paraformaldehyde under ketamine (100 mg/kg i.p.) anesthesia, and their brains removed and kept in 4% paraformaldehyde/20% sucrose [in PBS 1× (PO_4^{2-} 0.01 M; NaCl 0.15 M), pH 7.3] at 4 °C for at least 3 days before sectioning.

In situ hybridization was performed as described (2) on free-floating, 40-μm coronal sections cut at the level of dorsal hippocampus. Hybridization with BDNF CDS anti sense probe was performed at 55 °C followed by the same stringency washes done for hippocampal neurons. To obtain reproducible and comparable results and to avoid saturation of the reaction, alkaline phosphatase development was always performed for 5 h at room temperature. All in situ hybridizations on epileptic brain sections were conducted contemporary with brain sections from control animals.

Western Blots. Primary hippocampal neurons were transfected with si-translin and subsequently lysed in lysis buffer containing 150 mM NaCl, 50 mM Tris pH 7.4, 1%Nonidet P-40, and protease inhibitors. Non treated and si-translin treated samples were loaded on 12% polyacrilamide gel. Translin was detected with a rabbit polyclonal antibody [1:2,000 in 5% nonfat milk/PBS Tween 0.1% (PBST)] and α-tubulin with mouse monoclonal anti α-tubulin (1:20,000 in 5% nonfat milk PBST, Sigma). Anti rabbit- and anti mouse- alkaline phosphatase coupled secondary antibodies (Sigma) were used at 1:1,500 in 5% nonfat milk/PBST. Membranes were developed with NBT and BCIP as previously described. Densitometric analyses were performed with ImageJ software to quantify the extent of translin silencing. All data were normalized using α-tubulin as control.

Quantitative Imaging Analysis and Statistics. Nonradioactive in situ hybridization was analyzed by viewing stained cultures under bright-field illumination with a Nikon E800 microscope with a differential interference contrast-equipped lens (60× magnification). Images of stained neurons were acquired with a charge-coupled device (CCD) camera (Nikon ADX-1200) and digitalized with the image analysis program Image-ProPlus (Media Cybernetics). The function "trace" was used to measure, starting from the base of the dendrites, the MDDL as described (1). Dendrites were traced, in a conservative manner, up to the point at which the in situ labeling was no longer clearly distinguishable from the background. The background level obtained in sister cell cultures hybridized with the sense probes was used as a reference to set the threshold for specific labeling obtained with the antisense probes. Between 100 and 300 dendrites were measured for each construct (three independent experiments). The individual preparations were coded and analyzed in a blind manner. The MDDL data were normalized by dividing each single measurement by the mean dendritic length in sister cultures prepared at the same time to obtain a measure of the relative dendritic filling (%RDF) and were statistically analyzed with Kruskal–Wallis one-way ANOVA on ranks, followed by a multiple comparison procedure with Dunn's method.

Gel-Shift Competition Assay. Gel-shift competition experiments were performed using procedures described previously (3). In brief, freshly dissected rat cerebella or forebrain were homogenized in a solution containing 20 mM Hepes (pH 7.9), 400 mM NaCl, 20% glycerol, 1.5 mM $MgCl_2$, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM DTT, 1 μg/mL leupeptin, 5 μg/mL aprotinin, and 20 mM NaF, incubated on ice for 15 min, and then centrifuged for 15 min at 14,000 rpm in a microfuge. Aliquots of the supernatant were stored at −80 °C for later use. Prm-2 or BDNF probes were prepared by labeling synthetic RNA oligos with [γ-^{32}P] ATP by T4 polynucleotide kinase (New England Biolabs) according to the manufacturer's direction and purified with a Sephadex G-50 column (Amersham Pharmacia Biotech). For competition studies, 5 μg protein extract was incubated with unlabelled competitors in 12 mM Hepes (pH 7.9), 4 mM Tris-HCl (pH 7.9), 50 mM KCl, 50 mM NaCl, 1 mM EDTA, 1 mM DTT, 12% glycerol, and 2 μg poly(dI-dC) in 28-μL reaction volume for 15 min at room temperature and then 40,000 cpm of probe (2 μL) were added to the reaction for other 15 min of incubation. The mixture was then loaded onto a 4% native polyacrylamide gel. After electrophoresis, gels were dried and bands quantified following exposure in a phosphoimager cassette for 2–4 h at room temperature.

Immunoprecipitation and RT-PCR. Immunoprecipitation experiments were performed as described (4, 5) with minor modifications. Rat forebrains were harvested and homogenized in ice-cold buffer [10 mM Hepes (pH 7.4), 400 mM NaCl, 30 mM EDTA, and 0.5% Triton X-100] with 5 μg/mL aprotinin and 400 U/mL RNase inhibitor (Promega), then centrifuged at 14,000 rpm at 4 °C for 15 min. The supernatant was precleared and a 100-μL aliquot of precleared supernatant, called "offered" was saved for RNA extraction. Trax antibody was added to the precleared supernatant to immunoprecipitate the translin/trax complex. As a control of the specificity of the immunoprecipitation, we also included control samples in which trax antibody had been omitted or preincubated with the trax antigen peptide (10 μg/mL) for 1 h. RNA from the "offered" and immunoprecipitated samples were extracted with TRIzol reagent (Invitrogen). The first strand cDNA was generated by reverse transcription with random hexamers. Twenty-eight–cycle PCR was performed using 1 μL of the RT reaction to detect candidate transcripts and the products were analyzed by 1% agarose gel electrophoresis. The primer sequences used were: BDNF forward primer, 5′-CCATGAAAGAAGCAAACGT-3′, reverse primer, 5′-CTCCAGCAGAAAGAGCAGA-3′.

Secondary Structure Prediction and Modeling Methods. Folding predictions were made by using parameters at default settings and selection of the secondary structures was carried out using the following criteria: first the selected structures should be the most frequent; second the first and last nucleotides should fold separately; third the BDNF GFP-chimaeras configurations should contain domains very reminiscent to those found in the natural RNA sequences.

Modeling procedures were carried out with the GRID software (6). GRID is a computational procedure that determines energetically favorable binding sites on molecules of known structure, referred to as the 'target', by calculating the interaction energies between a small chemical probe (i.e., a chemical functional group) in all nodes of a three-dimensional grid, which spans the target molecule (6). This procedure generates molecular interaction fields (MIFs), which are visualized as isopotential surfaces. The corresponding MIFs were visualized to find the most relevant binding sites of translin. The same MIFs were used for the docking simulation by the GLUE module of GRID. First, the single nitrogen bases, with the sugar and phosphate groups substituted by a single methyl group, were used. Then the calculated conformations were used as the starting point for the construction of the whole nucleotides, with the support of the MOE program (MOE version 2004.03, distributed by Chemical Computing Group). All of the complexes were energy minimized using a three phases protocol (steepest descend, conjugated gradient and truncated Newton), with a termination gradient of 0.01 and MMFF94 force field. The minimization procedure was carried out by allowing the movement of the nucleotides and of those amino acid residues in direct contact with the nitrogen bases. The corresponding energies were used as a score for the selection of the conformers. The whole sequence of the RNA was incrementally constructed by linking sequentially all of the conformers previously calculated and by minimizing the energy of the system after each step. The molecular docking simulation was carried out using three different overlapping segments (right, middle and left) of 36 nt each from that sequence. The conformations and positions of each nucleotide, as calculated by the docking simulation, were then used as geometrical reference for the construction of the RNA sequence spanning the H and Y elements. In this process, attention was focused on the alignment of the sequence with the binding interface identified on each translin monomer. The central portion of the RNA was manually modeled separately starting from the secondary structure predicted by the mfold server (7) by using the MOE program and the AMBER94 force field. After having modeled the two termini of the RNA sequence, which are bound to translin, all of the possible orientations were tested in an attempt to connect the two termini with the central RNA portion previously constructed. The three portions were linked together on the basis of the sole conformation compatible with the different structural and conformational constraints and, finally, the energy of the whole system was minimized.

1. Tongiorgi E, Righi M, Cattaneo A (1997) Activity-dependent dendritic targeting of BDNF and TrkB mRNAs in hippocampal neurons. *J Neurosci* 17:9492–9505.
2. Tongiorgi E, et al. (2004) BDNF mRNA and protein are targeted to discrete dendritic laminae by event that trigger epileptogenesis. *J Neurosci* 24:6842–6852.
3. Li Z, Baraban J (2004) High affinity binding of the Translin/Trax complex to RNA does not require the presence of Y or H elements. *Brain Res Mol Brain Res* 120:123–129.
4. Brown V, et al. (2001) Microarray identification of FMRP-associated brain mRNAs and altered mRNA translational profiles in fragile X syndrome. *Cell* 107:477–487.
5. Finkenstadt PM, et al. (2000) Somatodendritic localization of Translin, a component of the Translin/Trax RNA binding complex. *J Neurochem* 75:1754–1762.
6. Goodford PJ (1985) A computational procedure for determining energetically favorable binding sites on biologically important macromolecules. *J Med Chem* 28:849–857.
7. Zuker M (2003) Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Res* 31:3406–3415.

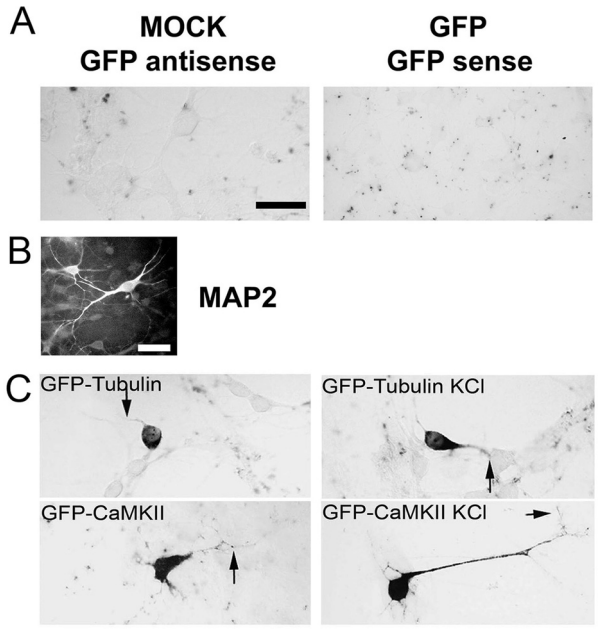

Fig. S1. Controls for in situ hybridization and MAP2 staining. (*A*) No signal was detected when in situ hybridization was carried out with anti-GFP antisense riboprobe on mock transfected neurons or with anti-GFP sense riboprobe on GFP transfected cultures. (*B*) The dendritic length used to calculate the RDF was obtained by measuring both apical and basal dendrites of neurons labeled by anti-MAP2 antibodies. (*C*) Representative neurons transfected with control GFP-tubulin or GFP-CaMKII chimeric transcripts that were left untreated (*Left*) or exposed to KCl (*Right*) and then analyzed by in situ hybridization for GFP mRNA. (Scale bars in *A* and *B*, 30 μm.)

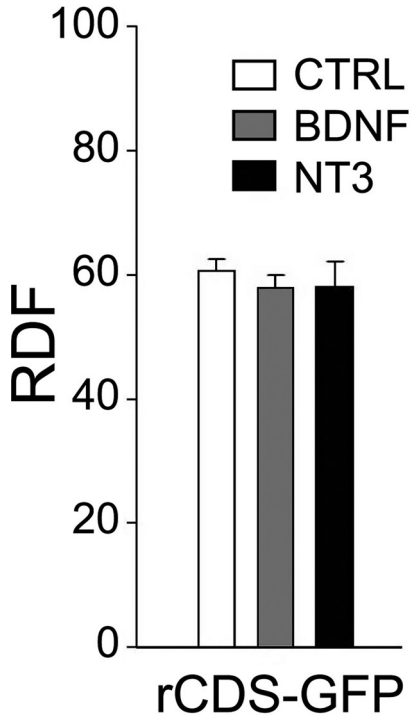

Fig. S2. No effect of neurotrophins on targeting of the rCDS-GFP construct. BDNF and NT3 (50 ng/mL) were applied to rat hippocampal cultures for 3 h.

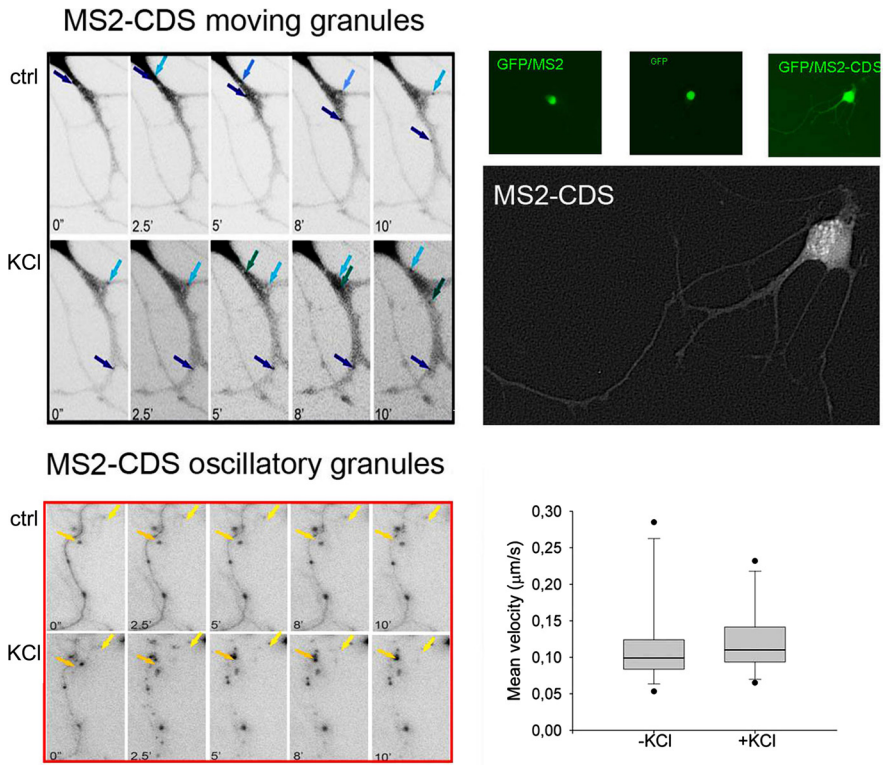

Fig. S3. Video time-lapse analysis of living neurons shows no effect of KCl treatment on dendritic trafficking of MS2-GFP-labeled rCDS RNA granules. The plasmid encoding the MS2-binding protein fused with GFP, or the GFP alone (GFP), has a nuclear localization (GFP/MS2), but diffuses in the soma and dendrites cytoplasm when co-transfected with a 6×MS2-BDNF CDS construct (GFP/MS2-CDS). MS2-BDNF CDS (MS-CDS) moving granules (75% of total granules) recorded over 10 min, move at the same speed as those under 10 mM KCl depolarization. Oscillatory granules (25% total granules) do not change behavior during 10 min depolarization with 10 mM KCl.

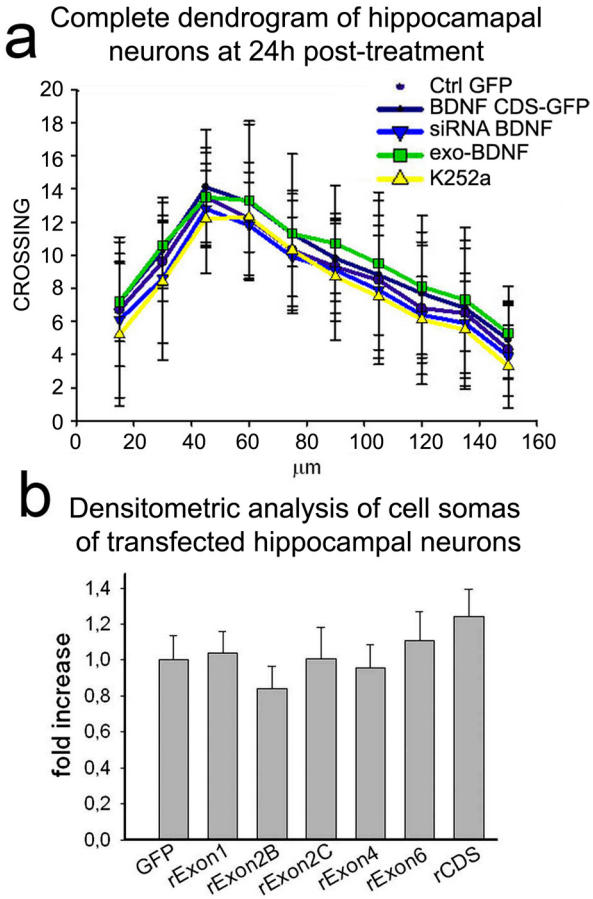

Fig. S4. (*a*) Sholl analysis of the effects of treatments that impact BDNF signaling on dendritic complexity of hippocampal neurons. Dendritic trees in hippocampal neurons at 8 DIV appears to be quite stable and not affected by 24 h exposure to treatments that modify BDNF expression or signaling, that is, BDNF overexpression using the CDS-GFP construct (BDNF CDS-GFP), siRNA targeting the CDS region (siRNA BDNF), recombinant BDNF (50 ng/mL; exo-BDNF) or the Trk inhibitor K252a (10 nM). (*b*) Densitometric analysis of chimaeric constructs in the cell bodies of transfected hippocampal neurons. No significant difference in expression levels between the different chimaeric constructs was found.

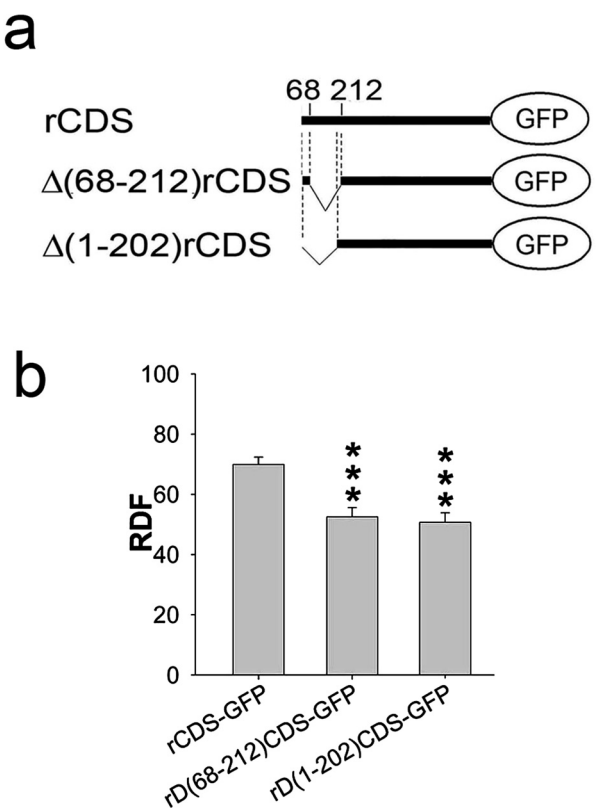

Fig. S5. Analysis of the effects on BDNF mRNA targeting of deletions in the region containing the putative translin binding site. (*a*) Schematic representation of rCDS-GFP chimaera, Δ(68–212)- and Δ(1–202)-rCDS-GFP deletion mutants lacking nucleotides 68–212 and 1–202, respectively. (*b*) Relative dendritic filling (RDF) values of rCDS-GFP, Δ(68–212)- and Δ(1–202)-rCDS-GFP chimaeras. The decrease in the RDF values was significant (***, $P < 0.001$) for both deletion mutants when compared to rCDS-GFP.

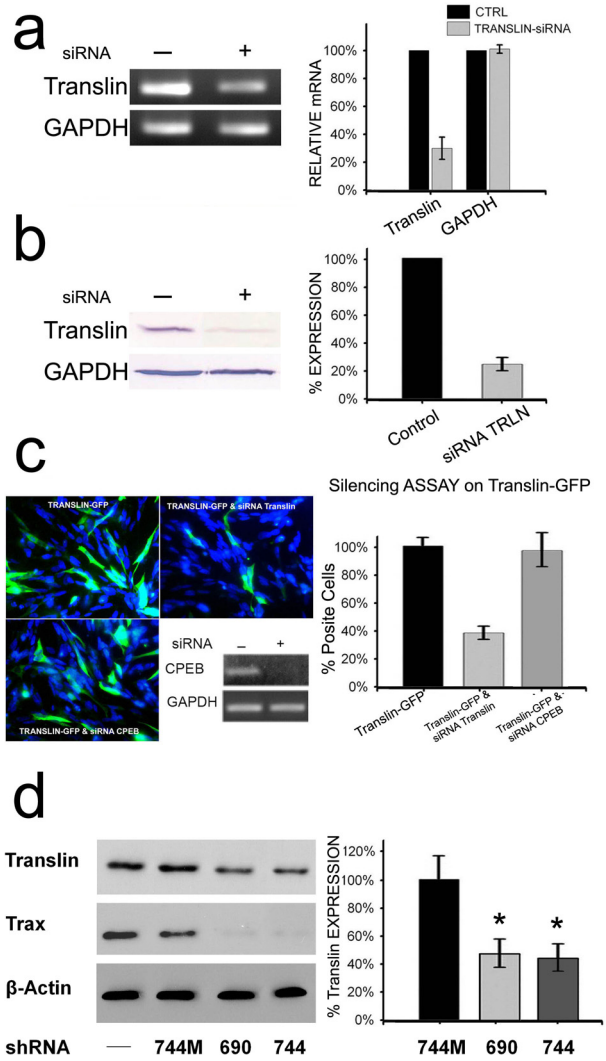

Fig. S6. Analysis of efficacy and specificity of siRNA- and shRNA-mediated translin knockdown. (*a*) Semiquantitive RT-PCR of hippocampal neuron cultures showed that siRNA to translin downregulated translin mRNA by 72% while the signal for the housekeeping gene GAPDH was unaffected. (*b*) Western blot analysis of hippocampal neurons transfected with si-translin showed a 73% down-regulation of translin protein. (*c*) Neuroblastoma SK-N-BE cells transfected with translin-GFP and si-translin showed 60% decrease in the number of translin positive transfected cells, while siRNA against CPEB1, which is highly effective at reducing CPEB1 mRNA levels as detected by RT-PCR, had no effect on the number of translin-positive cells ($n = 3$ cultures). (*d*) The ability of defined shRNA sequences to decrease endogenous expression of translin was tested in primary cortical cultures following infection with lentiviral constructs containing shRNA inserts. Immunoblots show that two sequences, called 690 and 744, reduce translin protein expression. Of note, deletion of translin also reduces trax protein levels. β-actin blot shown as a negative control. In contrast, the sequence generated by inserting multiple mismatches into 744, called 744M, did not decrease either translin or trax protein levels. For the values presented in the bar graph, the intensity of the translin bands was adjusted for changes in β-actin levels. Translin levels in cultures treated with either 690 or 744 were significantly reduced (*, $P < 0.05$; $n = 3$) compared to those found in either uninfected cultures or cultures infected with an ''empty'' virus containing no insert.

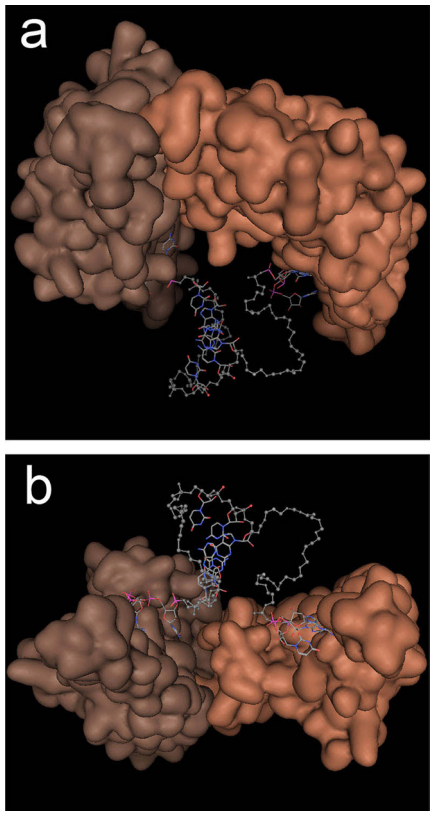

Fig. S7. Three-dimensional structure of the translin-hBDNF mRNA complex. (*a*) View from the top. (*b*) View from the bottom.