Supporting Information

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SI Materials and Methods

Hippocampal Cell Cultures and Transfections. Primary hippocampal neurons were prepared from embryonic day 18 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 cells/mL per well and cultured in a 5% CO_2 humidified incubator in Neurobasal media (Invitrogen) supplemented with B27 (Invitrogen), 1 mM L-glutamine (Euroclone), and antibiotics (Euroclone). The medium was changed every 2 d from the second day in culture onward. Primary hippocampal neurons were transfected with the different BDNF constructs or siRNAs at 4 or 15 days in vitro (DIV) using Lipofectamine 2000 (Invitrogen) following manufacturer recommendations. Morphological studies were carried out in young neurons (DIV7) or mature neurons (DIV18) after fixation in 4% paraformaldehyde for 20 min at room temperature.

KCl Treatments. Hippocampal neurons were analyzed after control treatment (normal medium) or after depolarization for 3 h with 10 mM KCl as described (2). In brief, the cells were depolarized changing the normal medium to high-KCl medium (10 mM KCl, 1.8 mM $CaCl_2 \cdot 2H_2O$, 0.8 mM $MgSO_4 \cdot 7H_2O$, 101 mM NaCl, 26 mM $NaHCO_3$, 1 mM $NaH_2PO_4 \cdot 2H_2O$, 0.7% D-glucose, 15 mM Hepes, pH 7.4) and fixed using 4% paraformaldehyde (PFA) in PBS.

Cotransfection Control. To determine the efficiency of cotransfection used for morphological analysis, BDNF-GFP and red fluorescent protein (RFP) plasmids were cotransfected at a 20:1 ratio using Lipofectamine 2000 in DIV6 neurons and, 24 h posttransfection (DIV7), cells were fixed and RFP- and GFP-RFP-positive cell somata were counted. Almost all cells (97%) carrying RFP were also transfected with GFP.

RNA Interference. RNAi pools were generated from each transcript of BDNF by PCR amplification of ~200–300 nt-long segments with low levels of homology to other genes and high scores as targets for silencing as evaluated with the software "siRNA Target finder" (Ambion). Primers (Table S1) contained the T7-polymerase sequence at their 5′ end (Proligo). To obtain siRNA pools (average length 22 bp), double-stranded RNA was synthesized using T7-RNA polymerase and the mixture kit (Ambion) and were digested with RNase III (Ambion). For a control, we used the GAPDH siRNA duplex (Ambion), which has no effect on neuronal morphology (3) and survival (4). Efficiency of siRNAs was analyzed by semiquantitative PCR for each transcript using ImageJ software (http://rsb.info.nih.gov; National Institutes of Health; NIH), normalizing with GAPDH.

Riboprobe Preparation. The GFP gene fragment was excised from the commercial vector pEGFP-N1 using the available restriction sites BamHI and NotI (restriction enzymes from New England BioLabs) and incorporated into pBLKS II vector (Fermentas). BDNF transcript-specific probes were previously described (5). Antisense digoxigenin-labeled RNA probes were synthesized with the DIG-RNA Labeling Kit (Roche Diagnostics) according to the manufacturer's instructions.

In Situ Hybridization. Nonradioactive in situ hybridization on hippocampal neurons in culture was performed as described (2, 6). In situ hybridization with antisense RNA probe for GFP was carried out overnight (at least 16 h) in multiwell plates at 55 °C. Posthybridization washes were 2× saline sodium citrate, 0.1% Tween 20 (SSCT), 50% deionized formamide at 55 °C for 30 min, 20 min in 2× SSCT, and twice in 0.05% SSCT at 60 °C for 30 min.

Immunocytofluorescence. Immunostaining of cultured hippocampal neurons was performed with the following procedure. Cells were fixed in 4% PFA in PBS for 20 min and washed in PBS. After a preliminary permeabilization with PBS, 0.1% Triton (PBST) for 1 h, cells were incubated overnight at 4 °C with anti-TrkB polyclonal antibodies (Sigma-Aldrich) diluted 1:200 or with rabbit anti-phospho-TrkB polyclonal antibodies (M.V.C. laboratory) diluted 1:250 in 5% normal goat serum in PBST. After washes in PBST, cells were incubated 1 h at room temperature with secondary goat anti-rabbit IgG antibodies Alexa 488 (1:250; Invitrogen) for TrkB and Alexa 568 (1:250; Invitrogen) in 5% normal goat serum in PBST. Subsequently, cells were washed in PBST and mounted in Mowiol antifade mountant (Sigma). Fluorescence was analyzed with a Nikon C1Si confocal microscope. For MAP2 and GFP staining, the cells were incubated for 3 h at room temperature with a rabbit anti-MAP2 antibody (1:500; Sigma-Aldrich) and anti-GFP (1:1,000; Clontech) diluted in 10% FCS in PBST. After washes in PBST, cells were incubated 1 h at room temperature with secondary goat anti-mouse IgG Alexa 488 (Invitrogen) and secondary goat anti-rabbit IgG Alexa 568 (Invitrogen).

Quantitative Imaging Analysis and Statistics. For experiments on local translation, neurons were transfected with the different BDNF transcripts and, using glass pipettes mounted on a single-arm micromanipulator (Nikon), apical dendrites were severed from the cell soma. Individual cultures were used for each neuron tested. Time-lapse images of BDNF-GFP in the soma and severed dendrites were acquired for 1 h with a Nikon TE300 microscope (40× magnification) and CCD camera (Sensicam qe; PCO) in 10 mM high-KCl medium at room temperature. To block protein synthesis, neurons were pretreated with 1 μM (280 ng/mL) of cycloheximide (Sigma-Aldrich) 20 min prior to and then during KCl depolarization. Changes in BDNF-GFP fluorescence are expressed as fluorescence variations on basal fluorescence at the beginning of the experiment. In particular, $\Delta F/F$ was evaluated and normalized on a background of unstimulated cultures. All quantitative measures were performed using the program ImageJ (NIH), and statistical analyses were performed using SigmaStat software (SYSTAT Software Inc.). Data are expressed as the mean of 7 (exon 1 and exon 2C) to 10 (exon 4 and exon 6) dendrites or somata for each condition. Statistical analyses on GFP fluorescence variations were verified using a two-way ANOVA on the entire range of time points; however, we report in the graphs only the results given by a point-by-point statistically significant difference among treatments (Kruskal–Wallis one-way ANOVA), $^*P < 0.05$; $^{**}P < 0.01$.

GFP fluorescence, immunocytochemistry staining, and nonradioactive in situ hybridization were analyzed with a Nikon E800 microscope (60× magnification) and CCD camera (Nikon; ADX-1200). The function "trace" of the image analysis program Image-ProPlus (Media Cybernetics) was used to measure, starting from the base of the dendrites, the maximal distance of dendritic labeling (MDDL), as described (2). 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 set as threshold. Around 50 dendrites were measured for each

construct (three independent experiments). Individual preparations were coded and analyzed in a blind manner, and neurons were randomly sampled from each culture, discarding bipolar neurons. MAP2 staining allowed us to calculate the total length of apical dendrites and to calculate the relative dendritic filling of different constructs. All measures were performed using the "straighten" plug-in of ImageJ (NIH). High-magnification pictures of dendrites were collected acquiring z-series of 15–25 optical sections at 0.5 μm with a 63× oil objective on a confocal system (Bio-Rad; MRC-1024) on a BX50WI Olympus microscope. Dendrites were counted from 15 μm up to 135 μm from the cell soma with 15-μm intervals. The number of primary and secondary dendrites was obtained from the tracing data collected. Quantitative analysis was performed using the program ImageJ from the NIH using the "Neuromorpho" package for the semiautomated tracing of neurons and the "NeuronJ" and "Sholl analysis" plug-ins for data collections. Data were statistically analyzed with Kruskal–Wallis one-way ANOVA on ranks, followed by a multiple comparison procedure with Dunn's method. Sholl analysis was performed on at least 50 neurons per culture (three cultures in total) from an experimenter blind to the treatment. Statistical analyses on dendrograms were verified using a two-way ANOVA on the entire range of data points; however, we report in the graphs only the results given by a point-by-point statistically significant difference among treatments (Kruskal–Wallis one-way ANOVA), $^{*}P < 0.05$; $^{**}P < 0.01$.

Semiquantitative RT-PCR. Total RNA was extracted from hippocampal neurons in culture at DIV7 (72 h posttransfection with siRNA) using TRIzol reagent (Invitrogen). Total RNA (1 μg) was reverse-transcribed into cDNA using 200 U SuperScriptIII reverse transcriptase (Invitrogen) and random hexanucleotides. PCR was performed in a 25-μL volume containing 1 μg cDNA, 10× reaction buffer, 500 mM KCl, 100 mM Tris·HCl (pH 9), 1.5 mM $MgCl_2$, 200 μM dNTP mix, 0.04 U Taq DNA polymerase (all from Invitrogen), and specific forward and reverse primers (MWG) for the different BDNF isoforms. Twenty-eight-cycle PCR was performed using 1 μL of the reverse-transcription reaction to detect candidate transcripts, and the products were analyzed by 1% agarose gel electrophoresis. The PCR program and primer sequences used are reported in Table S2. Quantification analysis was performed using the densitometry protocol of ImageJ software (NIH) and normalizing each band to the internal controls. The conditions used for RT-PCR are provided in Table S3.

1. Aibel L, Martin-Zanca D, Perez P, Chao MV (1998) Functional expression of TrkA receptors in hippocampal neurons. *J Neurosci Res* 54:424e431.
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3. Omi K, Tokunaga K, Hohjoh H (2004) Long-lasting RNAi activity in mammalian neurons. *FEBS Lett* 558:89−95.
4. Baj G, Tongiorgi E (2009) BDNF splice variants from the second promoter cluster support cell survival of differentiated neuroblastoma upon cytotoxic stress. *J Cell Sci* 122:36−43.
5. Chiaruttini C, Sonego M, Baj G, Simonato M, Tongiorgi E (2008) BDNF mRNA splice variants display activity-dependent targeting to distinct hippocampal laminae. *Mol Cell Neurosci* 37:11−19.
6. Tongiorgi E, Righi M, Cattaneo A (1998) A non-radioactive in situ hybridization method that does not require RNAse-free conditions. *J Neurosci Methods* 85:129−139.

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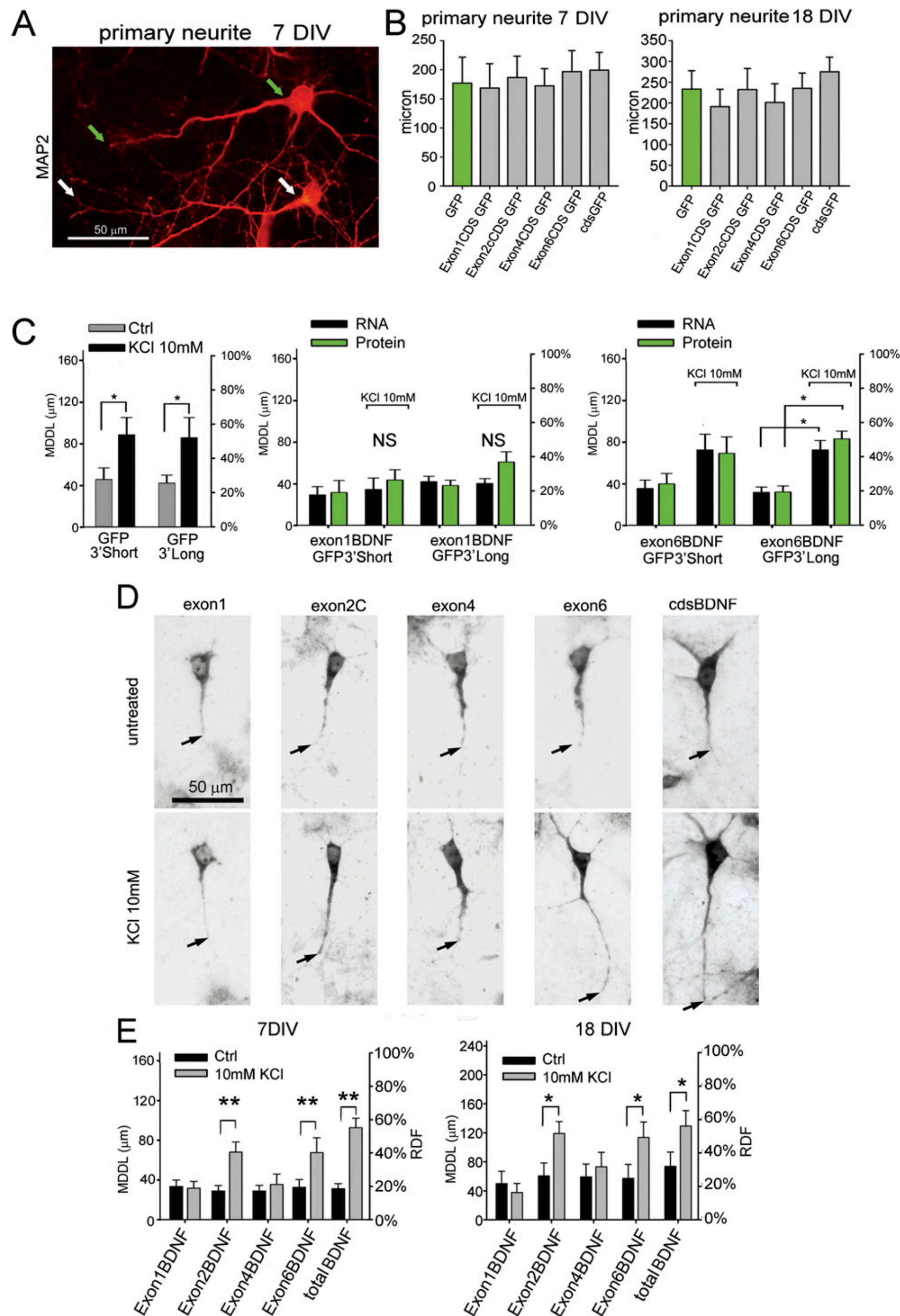

Fig. S1. (*A*) Example of MAP2 staining of 7-d-old hippocampal neurons used for quantification of apical dendrite length. White (start) and green (end) arrows show apical dendrites. (*B*) Quantification of the average maximal length reached by apical dendrites at 7- and 18-d-old neurons transfected with BDNF-GFP constructs. No significant variations were recorded. (*C*) Quantification of MDDL and relative dendritic filling (RDF) of GFP-3′UTR short and GFP-3′UTR long mRNA (*Left*) and full-length BDNF transcripts encoding for exon1 BDNF-GFP-3′UTR short or long (*Central*) and for exon6 BDNF-GFP-3′UTR short or long (*Right*). Histogram bars show the maximal distance reached by BDNF-GFP mRNAs (black bars) and BDNF-GFP protein (green bars) in primary dendrites of 7-d-old hippocampal neurons under control conditions or after 3 h stimulation with 10 mM KCl (n = 25–35 neurons per bar). (*D*) In situ hybridization on endogenous BDNF transcripts under control conditions and after a 3 h-long treatment with 10 mM KCl depolarizing solution in 7-d-old cultures. (*E*) Quantification of MDDL and RDF of endogenous transcripts under control conditions or after 3 h stimulation with 10 mM KCl in 7- and 18-d-old hippocampal neurons (n = 50 dendrites per bar; error whiskers indicate SE; $*P < 0.05$; $**P < 0.01$; NS, not significant; Student's t test).

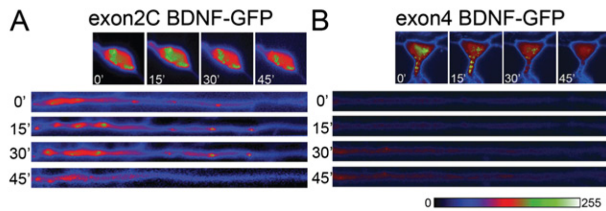

Fig. S2. Video time-lapse analysis of exon 2C (*A*) and exon 4 BDNF (*B*). Translation living in the soma and in dendrites that were mechanically separated and then stimulated with 10 mM KCl. Exon2C BDNF-GFP and exon4 BDNF-GFP translation at the indicated times following KCl treatment in the soma and in dendrites severed from the cell body. Note that in the absence of the soma, there is no newly synthesized exon4 BDNF-GFP in dendrites, whereas dendritic synthesis of exon2C BDNF-GFP is evident. Images of dendrites show a straightened 150 μm-long segment blunted from the soma. Proximal stump is on the left.

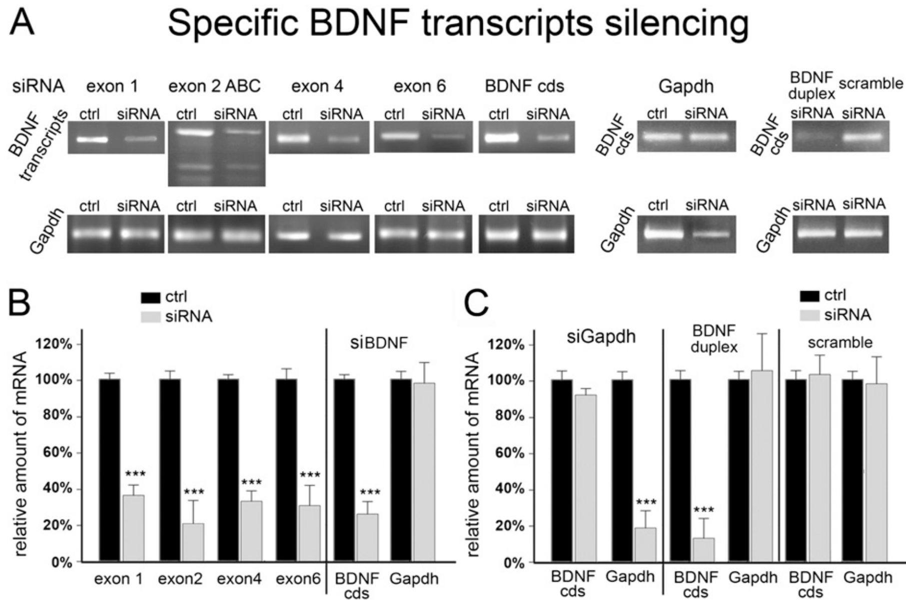

Fig. S3. (*A*) Representative PCR amplifications showing the efficacy of silencing RNA pools against BDNF exons 1, 2ABC, 4, and 6, transcripts, or total BDNF (coding sequence; cds) in hippocampal neurons and control siRNA pool against GAPDH, siRNA duplex against BDNF, and scramble siRNA. (*B*) Single BDNF isoform quantification after silencing, normalized on GAPDH mRNA used as an internal control, showed reductions ranging between 60 and 80% ($n = 3$; $***P < 0.001$). The siRNA pool against total BDNF (siBDNF) was able to produce a 73% reduction of cdsBDNF but did not affect GAPDH mRNA levels. (*C*) Densitometric quantification after silencing with siRNA against GAPDH showing 81% reduction of GAPDH mRNA ($n = 3$; $***P < 0.001$). Under the same condition, no variation of total BDNF mRNA was detectable. An siRNA duplex against BDNF produced 87% reduction of total BDNF but not of GAPDH ($n = 3$; $***P < 0.001$). Scramble siRNA used at the same concentration and for the same time (72 h) did not affect GAPDH or BDNF mRNAs.

Table S1. Primers used to generate dsRNA template for siRNA production

Exon1T7 fw	5′-GCGTAATACGACTCACTATAGGGTGGGGGTACTCTGAAACTCC-3′
Exon1T7 rev	5′-GCGTAATACGACTCACTATAGGGCAACTCTCATCCACCTTGGC-3′
Exon2T7 fw	5′-GCGTAATACGACTCACTATAGGGGTTTGGTCCCCTCATTGAGC-3′
Exon2T7 rev	5′-GCGTAATACGACTCACTATAGGGTCTTTGCGGCTTACACCACC-3′
Exon4T7 fw	5′-GCGTAATACGACTCACTATAGGGTGAAATCTCCCAGTCTCTGC-3′
Exon4T7 rev	5′-GCGTAATACGACTCACTATAGGGAGTCTTTGGTGGCCGATATG-3′
Exon6T7 fw	5′-GCGTAATACGACTCACTATAGGGCGGCTTGGAGAAGGAAAC-3′
Exon6T7 rev	5′-GCGTAATACGACTCACTATAGGGGAGTCACATTGTTGTCACGC-3′
BDNFcdsT7 fw	5′-GCGTAATACGACTCACTATAGGGGGTCACAGTCCTGGAGAAAGTC-3′
BDNFcdsT7 rev	5′-GCGTAATACGACTCACTATAGGGCCCCTTTTAATGGTCAGTGTAC-3′

The underlined nucleotides correspond to the T7 promoter sequence. Fw, forward; rev, reverse.

Table S2. Primers used for semiquantitative PCR

Exon1 fw	5′-TCTTCCCGCCCTAGCCTGAC-3′
Exon2abc fw	5′-TTCAGCACCTTGGACAGAGC-3′
Exon4 fw	5′-AGGCGTGCGAGTATTACCTCC-3′
Exon6 fw	5′-TCGCACGGTCCCCATTGCGCC-3′
BDNFcds fw	5′-GATGACCATCCTTTTCCTTAC-3′
Common BDNF rev	5′-attcacgctctccagagtcc-3′
GAPDH fw	5′-TGGAGTCTACTGGCGTCTT-3′
GAPDH rev	5′-TGTCATATTTCTCGTGGTTCA-3′

Table S3. PCR program: 94 °C 4 min, 94 °C 1 min, 55 °C 1 min (28 cycles), 72 °C 1 min, 72 °C 10 min

Reverse transcribed cDNA total cells (template)		1 μL
Buffer 10×		2.5 μL
$MgCl_2$	25 mM	1.5 μL
dNTPs	10 mM	0.5 μL
Taq (Promega)	5 U/mL	0.125 μL
H_2O		16.875 μL
Oligo forward	10 mM	1.25 μL
Oligo reverse	10 mM	1.25 μL
		Volume total 25 μL