Supporting Information

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SI Materials and Methods

Antibodies and DNA Constructs. Antibodies: (*i*) Mouse anti-GFP (Covance, 1:250); (*ii*) Rabbit anti-GFP (Abcam, 1:1,500); (*iii*) Mouse anti-HA (Covance, 1:1,000); (*iv*) Rabbit anti-mBDNF (custom made, 1:400); (*v*) chicken anti-proBDNF (Chemicon, 1:1,000); (*vi*) Rabbit anti-BDNF [N-20 Santa Cruz (Lot# J0807), 1:1,000]; (*vii*) Mouse anti-MAP2 (Sigma, 1:10,000); (*viii*) mouse anti-synapsin I (Stressgen, 1:500); (*ix*) Anti-rabbit (or mouse) Alexa Fluor 488 (Molecular probes, 1:1,500); (*x*) Anti-mouse (or rabbit) Alexa Fluor 633 (Molecular Probes, 1:1,500); (*xi*) anti-rabbit IgG-HRP (Pierce, 1:30,000); (*xii*) anti-chicken IgY-HRP (Promega,1:5,000). Unless indicated otherwise, all chemicals were from Sigma, and culture reagents from Invitrogen.

DNA sequence corresponding to the signal peptide followed by haemagglutinin (HA) tag, FLAG tag, and the pro-peptide of BDNF were synthesized with XbaI (5′) and NdeI (3′) ends and ligated upstream between XbaI-NdeI sites in the human BDNF-EGFP gene in pET21d(+). Subsequently, the entire ORF was PCR amplified using primers with sequence CACC at the 5′ and 3′ ends to clone into a lentiviral vector using the pLenti6/V5 directional topo-cloning kit (Invitrogen). Transcription in pLenti6/V5-D-TOPO vector is driven by the CMV minimal promoter without the enhancer. The plasmid was transfected by nucleofection [efficiency ≈50−60% when a control plasmid expressing GFP alone (pMax-GFP) is transfected] using the Amaxa rat neuron nucleofector kit following manufacturer's instructions. Based on the efficiency of transfection (50–60%) and the fold increase in BDNF expression from the construct (≈0.3-fold), we calculate a 0.54-fold increase in total BDNF increase when BDNF is expressed from this lentiviral construct. The rat tPA-Venus in the pEGFP-N1 vector was kindly provided by Dr. Wolfhard Almers (19).

Neuronal Culture and Transfection. Rat or mouse hippocampal neurons are prepared on embryonic day 18 or 16 of gestation, respectively (16). Briefly, hippocampi were dissected, trypsinized (0.05%) along with DNase I (0.1 μg/ml), washed in DMEM containing 10% serum, and dissociated. Transfections were done according to the manufacturer's protocol (Amaxa). Cells were plated on 18-mm glass cover slips at low density (50,000 cells) or in 6-well plate at high density (0.6×10^6 cells per well), coated overnight with PolyD-Lysine in 0.1M Borate Buffer pH 8.5 followed by Laminin I (ATCC) at 10 μg/ml. The cultures were maintained at 37 °C, 5% CO_2. The media was replaced with Neurobasal supplemented with B27 and 2-mM Glutamax I after 12 to 18 h, and then treated with 3-μM cytosine arabinoside after 48 h for 24 h. The cells were grown for at least 14 days and the medium was changed every 2 days.

Generation of mBDNF-Specific Antibody. B-peptide (HSDPARRC) (Fig. 2) corresponding to the amino terminus of the cleaved end of the mature BDNF conjugated to KLH through Cysteine at the C terminus was used for immunizing rabbits. The serum was affinity-purified using Sepharose conjugated to B-peptide. Following elution, the B-peptide antibody was depleted of antibodies that could bind to uncleaved proBDNF by passing through the second column conjugated to a peptide sequence (**CSMRVRR**HSDPARR) corresponding to the uncleaved sequence. The flow through of this column contained antibody that specifically recognized only mBDNF, not proBDNF.

Detection of BDNF Secretion. Following washes with neurobasal medium supplemented with B27, 2-mM Glutamax I, 0.1 mg/ml BSA, and 50 μg/ml protamine sulfate to block any nonspecific BDNF binding, neurons were incubated with TAC: 15 μM of Tetrodotoxin, 20 μM D-APV (D-2-amino5-phosphonovaleric acid), and 20 μM of CNQX (6-cyano-7-nitoquinoxaline-2, 3-dione) in neurobasal medium with 0.1-mg/ml BSA for 12 h or 15 min for constitutive secretion. For regulated secretion, cells were depolarized with 50 mM KCl in neurobasal medium containing 0.1 mg/ml BSA for 15 min or stimulated by field electrical stimulation. BDNF release from transfected neurons under each condition was measured from 3-ml medium (1 ml per well × 3 wells in a 12-well plate, with a plating density of 0.12×10^6 cells per well). Endogenous BDNF release was measured from 8 ml of medium collected from 10-cm dishes with a plating density of 10×10^6 cells. Protease inhibitor mixture (includes serine, cysteine, aspartate, metallo-proteases, and amino-peptidases) was added to the media immediately after stimulation, clarified by centrifugation (16,100 × *g*/15 min) to remove any cell debris, precleared with protein G Sepharose beads for 30 min at 4 °C, mixed with primary antibody for 2 h, and then pulled down with Protein G Sepharose beads (50 μl of 50% slurry) overnight. Following washes with Tris buffered saline containing 0.1% Tween 20 (TBST), BDNF in the immunoprecipitates was detected by Western blotting as described below.

Hippocampal Tissue/Neuronal Lysate Preparation for BDNF Detection. Hippocampi from wild type or BDNF–/– (p21) mice or cultured neurons was homogenized in RIPA buffer (50 mM Tris-HCl pH 7.2, 0.5 M NaCl, 1% SDS, 1% Triton X-100, 1% Deoxycholate, and 5 mM EDTA) containing protease inhibitors, sonicated, and extracted at 4 °C for 1 h in an end-to-end rotor. Lysates were clarified by centrifugation at 16,100 × *g* at 4 °C for 30 min and the protein content in the supernatant was estimated. Equal amount of proteins were then separated by electrophoresis using a Bis-Tris NuPAGE gel and transferred to PVDF membrane for Western blotting as described below.

Western Blotting. Immunoprecipitates from the culture medium or proteins from the neuronal/brain lysate was resolved on a Bis-Tris NuPAGE gels, transferred to 0.2 μm PVDF membrane, blocked with 5% BSA in TBST, and probed overnight with primary antibodies diluted in blocking buffer. Following 6 × 5 min washes with TBST, specific immunoreactive signals were visualized by chemiluminescent reaction using either anti-rabbit or mouse HRP secondary antibody and ECL-Plus kit (GE Healthcare). The signals were quantified using the National Insitutes of Health Image software, ImageJ Ver. 1.34n. The data for the same condition in multiple experiments were averaged and presented as mean ± SEM.

Statistical Analysis. For analysis of surface-staining experiments, the data from multiple dendrites of "n" neurons in the same condition in at least 3 independent experiments were averaged and presented as mean ± SEM. The results obtained from various cells under different experimental conditions were analyzed by appropriate statistical tests (two-tailed student's *t* test or ANOVA followed by posthoc analysis) as described in the figure legends using InStat3. Similarly, the data from Western blots were presented as mean ± SEM and analyzed using InStat3. The results were all normalized to total BDNF to faithfully measure changes after various stimulation paradigms from different cells.

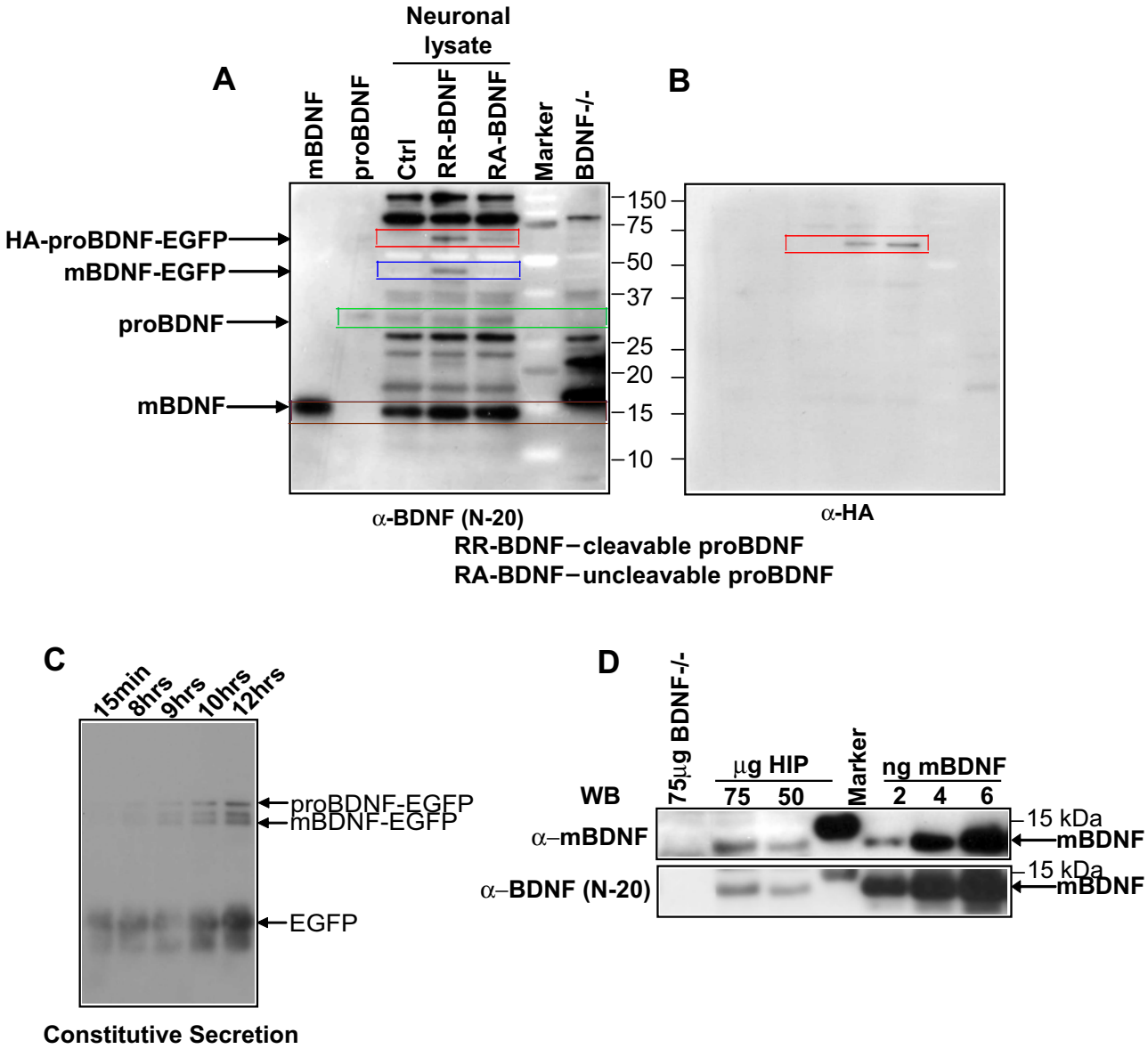

Fig. S1. (*A*) Western blot analysis of endogenous and exogenous proBDNF and mBDNF in lysates (15 μg per lane) from hippocampal neurons transfected with or without BDNF construct (shown in Fig. 1*A*). Recombinant proBDNF and mBDNF (10 ng each) was loaded as positive controls and BDNF–/– hippocampal tissue lysate (15 μg) was loaded as a negative control. Bands were visualized by a rabbit polyclonal ''pro/m'' antibody that detects both proBDNF and mBDNF (Santa-Cruz N-20 antibody). The identity of the epitope-tagged exogenous and the endogenous BDNF isoforms were confirmed by their (*i*) predicted molecular weights (in kDa: HA-proBDNF-EGFP:≈60; mBDNF-EGFP:≈45; proBDNF:≈30; and mBDNF:≈13); (*ii*) absence in BDNF knockout (–/–) brain lysates; (*iii*) detection with anti-HA (Fig. S1*B*) and anti-GFP antibodies (see Fig. 1*A*); (*iv*) absence in untransfected hippocampal neurons; and (*v*) absence of mBDNF-EGFP in neurons transfected with uncleavable proBDNF (RA-BDNF). (*B*) Blot in (*A*) was stripped and reprobed with anti-HA antibody to identify exogenously expressed HA-tagged proBDNF. (*C*) Time-course analysis of constitutive BDNF secretion. (*D*) Quantification of mBDNF in hippocampal lysate. Western blot was used to determine the endogenous mBDNF levels in hippocampal lysates from p21 wild-type mice. Lysates from BDNF–/– mice and different amounts of recombinant mBDNF were used as negative and positive controls, respectively. The blot was probed first with the B-antibody (*Top*), stripped, and reprobed with the Santa-Cruz antibody (N-20) (*Bottom*).

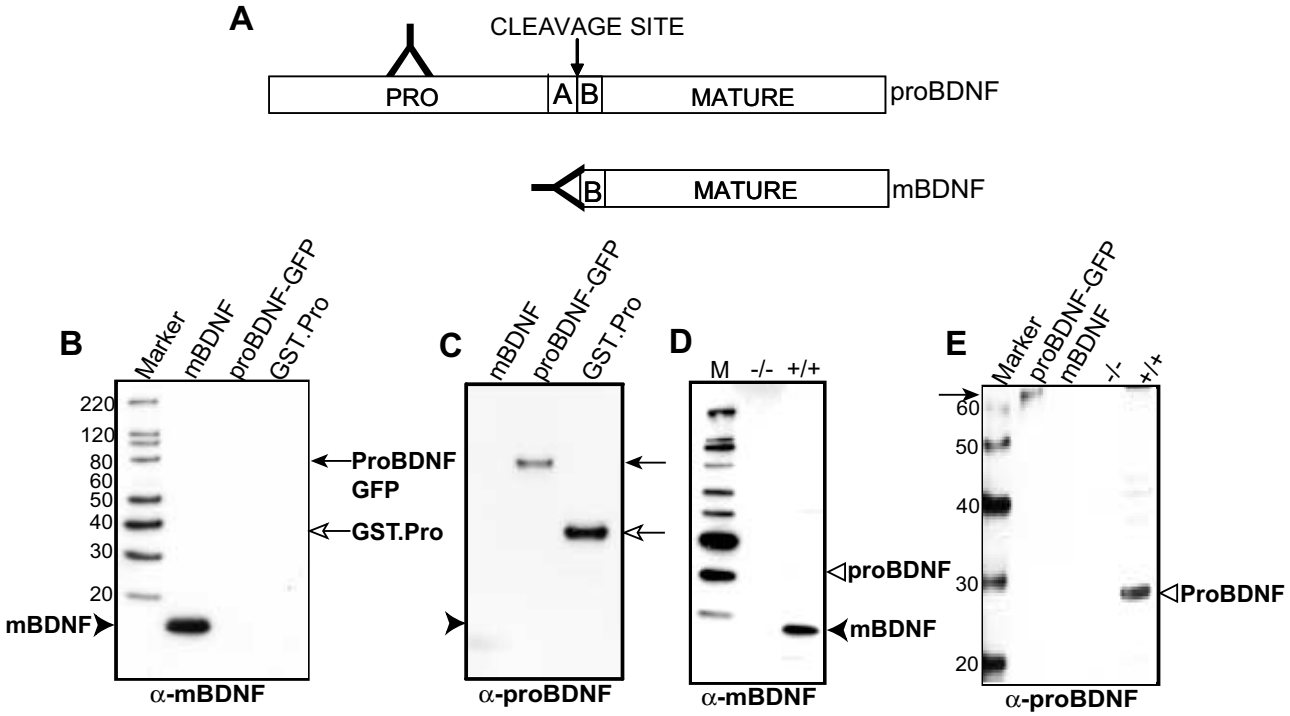

Fig. S2. Specificity of mBDNF antibody in Western blotting. (*A*) Schematic representation of proBDNF, mBDNF, and the epitopes to which the proBDNF and mBDNF antibodies are directed. The mBDNF-specific antibody was raised against the ''B'' peptide and depleted against the ''A+B'' peptide encompassing the cleavage sequence in proBDNF. (*B*) Detection of recombinant mBDNF by the anti-mBDNF antibody. Blot with equal amounts of purified recombinant proteins (10 ng of GST fused to prodomain of BDNF, proBDNF, and mBDNF) were probed with the mBDNF-specific antibody. (*C*) Detection of recombinant GST-Pro and proBDNF by the anti-proBDNF antibody. (*D*) Detection of mBDNF in hippocampal lysates by the anti-mBDNF antibody. mBDNF was detectable in hippocampal proteins derived from wild type, but not BDNF–/– mice. (*E*) Detection of proBDNF in hippocampal lysates by the chicken polyclonal antibody against pro-domain. Note that proBDNF was detectable in wild-type hippocampal lysates but not in BDNF–/– mice. Solid arrow, proBDNF-GFP; open arrow, GST-pro; filled arrowhead, mBDNF; open arrowhead, proBDNF.

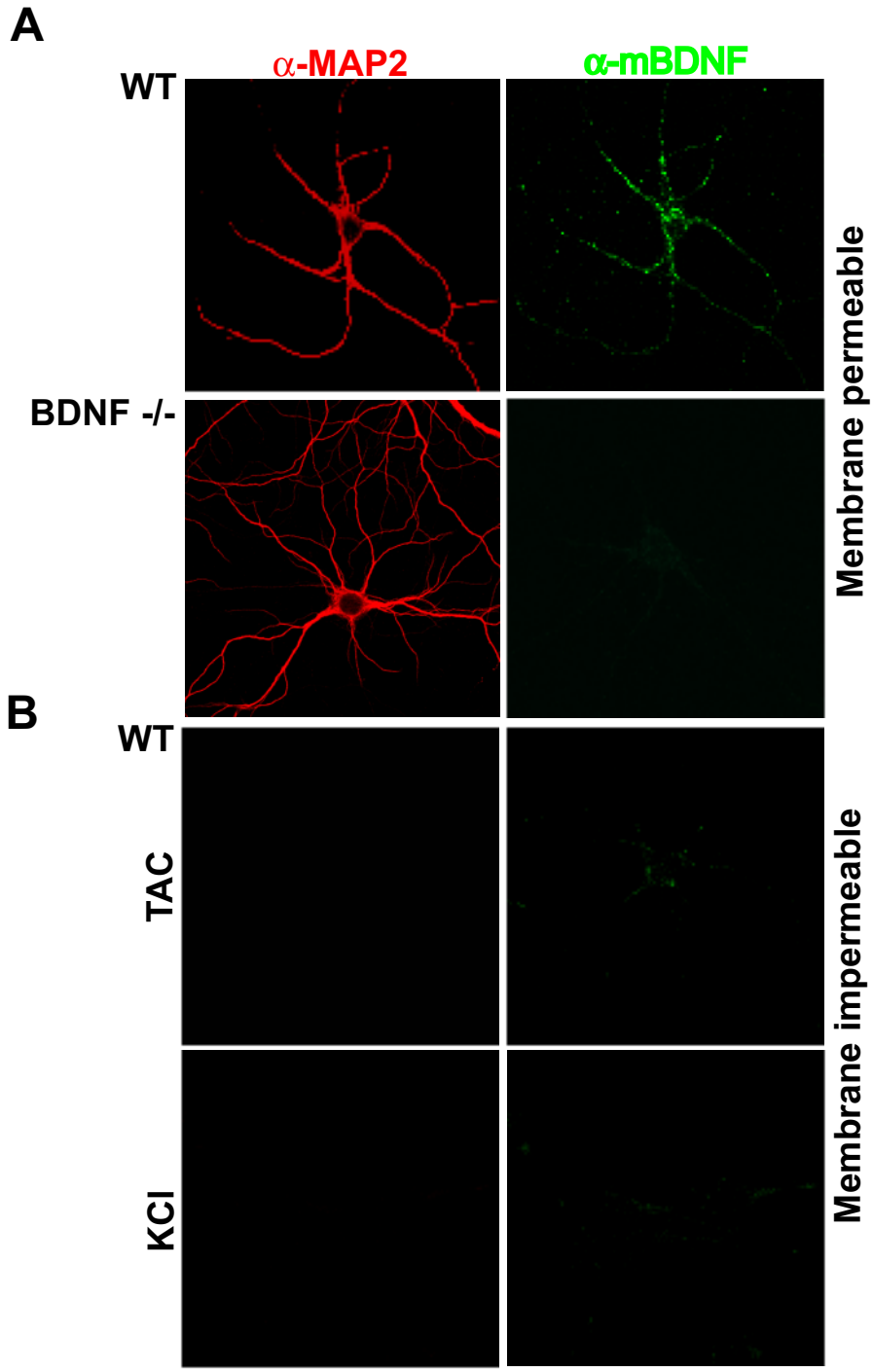

Fig. S3. (*A*) Specificity of mBDNF antibody in immunocytochemistry. Hippocampal neurons from WT (*Top*) or BDNF–/– mice (*Bottom*) were double-stained with the polyclonal anti-mBDNF antibody and a monoclonal anti-MAP2 antibody under membrane permeable conditions. (*B*) Surface staining of cultured hippocampal neurons in TAC and KCl under membrane impermeable conditions using the polyclonal anti-mBDNF antibody and a monoclonal anti-MAP2 antibody. Note that the staining for endogenously expressed mBDNF was almost negligible, and the antibody against intracellular protein MAP2 failed to stain, validating the method of surface staining.

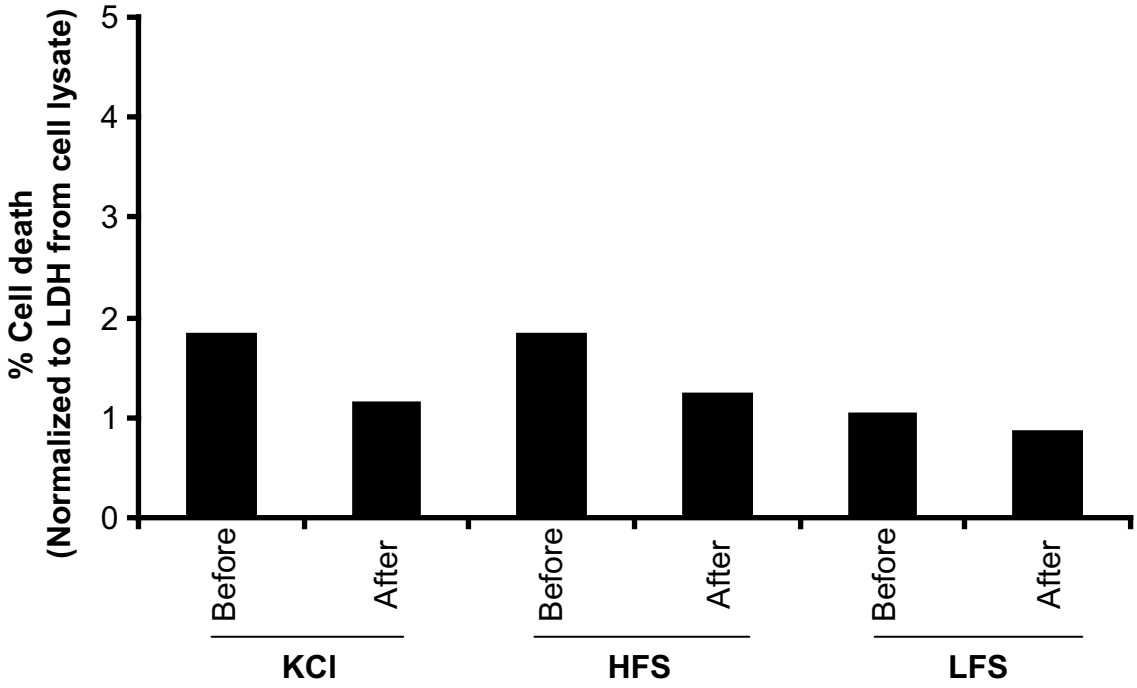

Fig. S4. Cytotoxicity assay. The cell viability of the neurons subjected to various stimulations (high K^+, HFS, LFS) was assessed for the presence of Lactate dehydrogenase (Promega, CytoTox 96 Non-Radioactive Cytotoxicity Assay) in the media collected from cultured neurons before and after stimulation. LDH activity from the media was normalized to LDH from neuronal lysates. Data presented is an average of 2 different experiments.

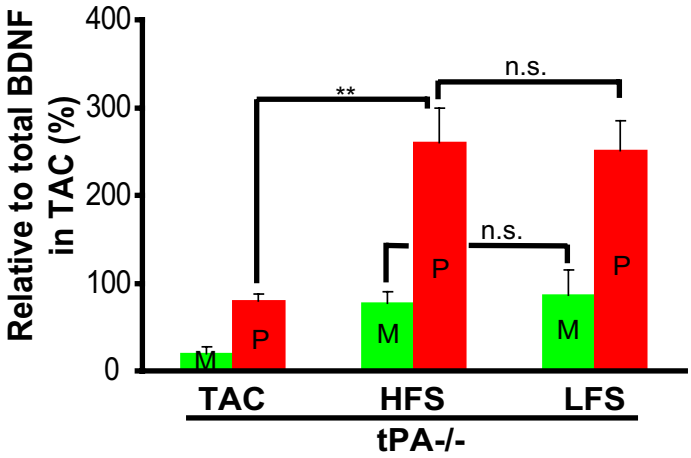

Fig. S5. Quantitative analysis of proBDNF and mBDNF surface immunostaining in tPA–/– neurons. TAC, 15 dendrites from 7 neurons; HFS, 31 dendrites from 5 neurons; LFS, 17 dendrites from 10 neurons; n.s., non-significant; **, $P < 0.001$, 1-way ANOVA.

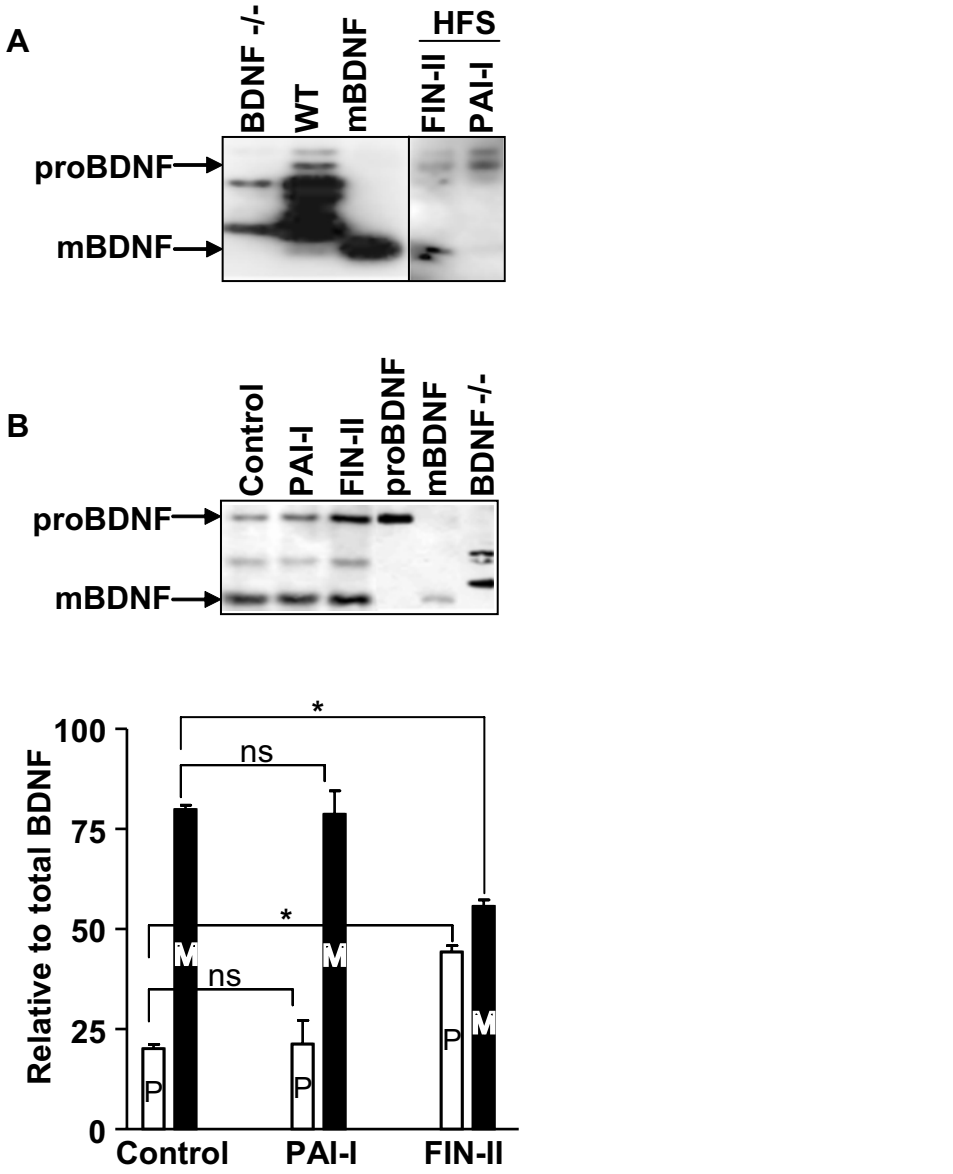

Fig. S6. Inhibition of proBDNF cleavage in hippocampal neurons by inhibitors of intracellular and extracellular proteases. Hippocampal neurons were treated with 1 μg/ml PAI-I, 13 μM FIN II. (*A*) Secretion of endogenous proBDNF and mBDNF induced by different stimulation paradigms. Positive (15 μg wild-type hippocampal lysate, 10 ng recombinant mBDNF) and negative (15 μg BDNF –/– hippocampal lysate) controls are included to identify proBDNF and mBDNF on the Western blot probed with ''pro/m'' antibody. (*B*) Analysis of intracellular BDNF following FIN-II and PAI-I treatments. (*Upper*) A representative Western blot with arrows indicating endogenous proBDNF ($\approx$30kDa) and mBDNF ($\approx$15kDa); 25 ng proBDNF; 5 ng mBDNF; 15 μg BDNF–/– lysate. (*Lower*) Quantification of the Western blots expressed as proBDNF (P) and mBDNF (M) relative to total BDNF ($n = 3$). n.s, non-significant; *, $P < 0.01$, 2-way ANOVA followed by Bonferroni posthoc analysis.

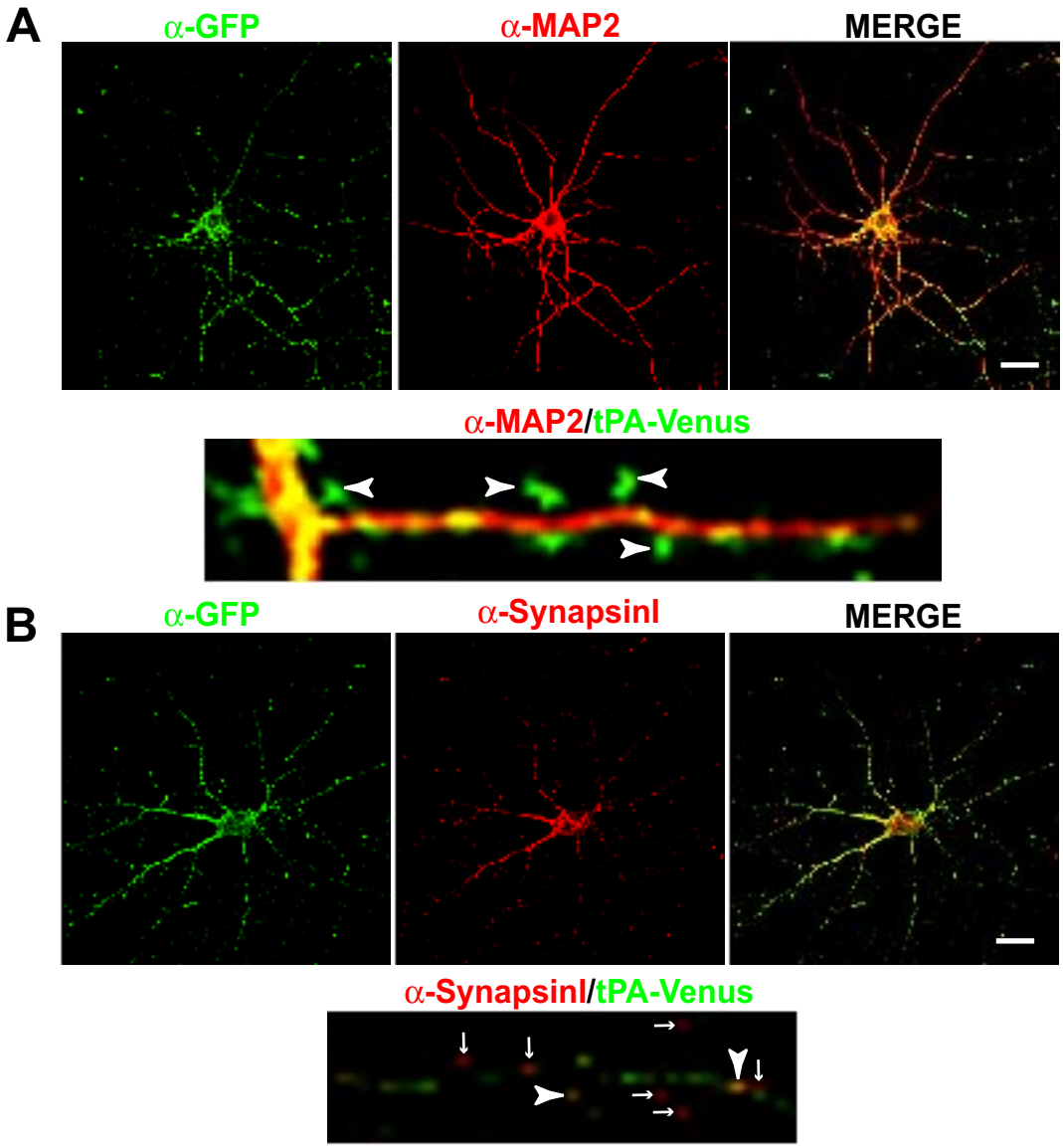

Fig. S7. Localization of tPA in dendritic spines and at synapses. Neurons transfected with tPA-Venus were stimulated with HFS for 15 min, double-stained with a polyclonal anti-GFP antibody (*green*) and a monoclonal antibody (*red*) against either MAP2 (*A*) or Synapsin I (*B*) under membrane-permeable conditions. (*A*) Localization of tPA in dendrites and spines in response to HFS. (*Lower*) Distribution of tPA in spines (*arrowheads*). (*B*) Localization of tPA at synapses. (*Lower*) Higher magnification of a dendritic region showing distribution of synapses (*red spots, arrows*, revealed by anti-Synapsin I antibody) and colocalization of tPA with Synapsin I (*yellow spots, arrowheads*).

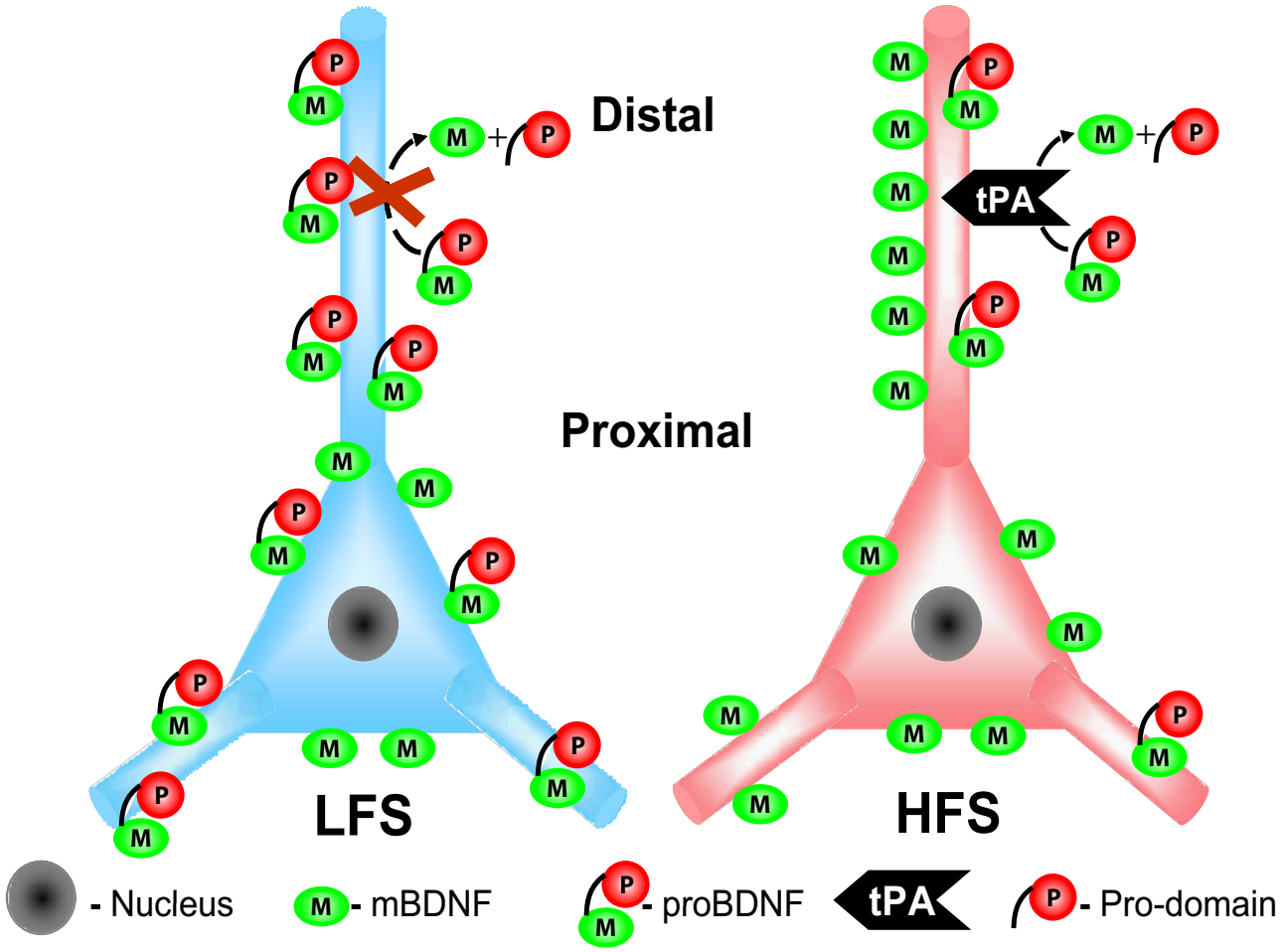

Fig. S8. Schematic representation of the extracellular conversion of proBDNF to mBDNF on the surface of a typical pyramidal neuron by tPA under HFS and LFS conditions.