Suzuki et al. http://www.jcb.org/cgi/doi/10.1083/jcb.200404106

# Supplemental materials and methods

## Antibodies and chemicals

Antibodies: anti-Grb2, anti–TrkB-T1, anti-EGFR, anti–phospho-Erks and anti-Erks were purchased from Santa Cruz Biotechnology Inc.; anti-TrkB, anti-Fyn, anti-Shc, anti–PI3-K p85 subunit, and anti–caveolin-2 were purchased from BD Transduction Laboratories; anti–phospho-Trk (Y490), anti–phospho-Akt, and anti-Akt were purchased from Cell Signaling; anti–transferrin receptor was purchased from Zymed Laboratories; anti-PLCγ and anti-PSD95 were purchased from Upstate Biotechnology; anti-synaptophysin was purchased from Boehringer; anti-MAP2 was purchased from Sigma-Aldrich; anti-$p75^{NTR}$ was purchased from Promega; anti-GFAP was purchased from DakoCytomation; anti-NSE was purchased from Polysciences Inc. Reagents: MCD, MCD-cholesterol complex, and filipin were purchased from Sigma-Aldrich; DAPI was purchased from Molecular probes; NT-4 was purchased from R&D Systems. Wistar ST rats were purchased from NIPPON SLC.

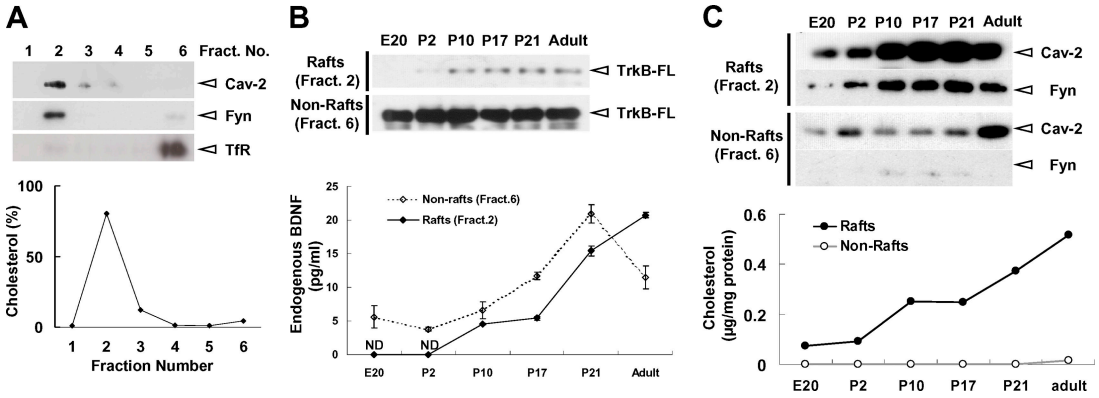


Figure S1. **Localization of TrkB and BDNF in lipid rafts during cortical development.** (A) Lipid raft preparation. Cultured neurons were homogenized in ice-cold lysis buffer containing Triton X-100 and centrifuged in discontinuous 5–35% sucrose gradients. (Top) Six fractions (from top to bottom) were separated on SDS-PAGE, and immunoblotted for the indicated proteins. (Bottom) Graph shows the percentage of cholesterol content in each fraction, revealing a peak concentration of cholesterol in the raft fraction (fraction 2). Note that lipid raft marker proteins caveolin-2 and Fyn are enriched in fraction 2 where cholesterol concentration is the highest while a nonraft marker protein transferrin receptor is concentrated in fraction 6. (B) Increase of BDNF and TrkB-FL in lipid rafts during cortical development. Lipid raft fraction was prepared from tissues of cerebral cortex as described in Materials and methods. (Top) TrkB-FL expression in rafts and nonrafts during cortical development. Lipid rafts (fraction 2) and nonrafts (fraction 6) from cerebral cortex of indicated ages were immunoblotted with a TrkB antibody. At E20 and P2, TrkB-FL was detected in nonraft fraction, but was virtually absent in lipid raft fraction (TrkB in rafts/ TrkB outside rafts: 0%) while after P10 TrkB-FL was in both fractions (TrkB in rafts/ TrkB outside rafts: 14.3 ± 3.1%). (Bottom) BDNF expression in lipid rafts and nonrafts during cortical development. The amounts of BDNF in fractions 2 and 6 were determined using BDNF ELISA. $n = 3$ independent preparations. ND, not detected. (C) Incre ase of caveolin-2 and Fyn (top) and cholesterol content (bottom) in lipid rafts during cortical development. Western blotting was performed for the indicated proteins. Graph represents cholesterol content relative to protein content. $n = 3$ independent preparations. Error bars represent SEM.

## Primary cultures

Primary cultures were according to the method of Suzuki et al. (2002). The cells were cultured in medium (5/5/DME) consisting of 5% FBS (Cell Culture Technologies), 5% heat-inactivated horse serum (GIBCO BRL) and 90% Dulbecco's minimum essential medium (DME; GIBCO BRL), at final cell counts of $2 \times 10^7$ cells per 100-mm polyethyleneimine-coated plate. After culturing for 4 d, the medium was changed to serum-containing 5/5/DME with 1 μM cytosine arabinoside (Sigma-Aldrich), and the cells were cultured for further 4 d. The medium was replaced with serum-free DME and cells were cultured overnight to perform the assays described in Results.

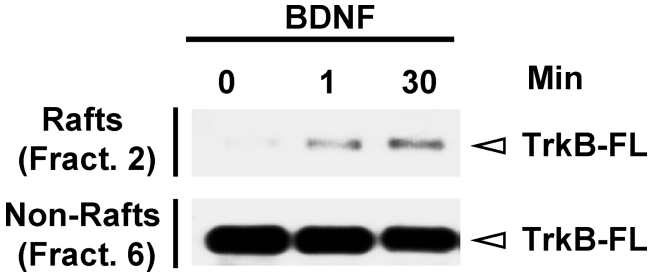


Figure S2. **Association of TrkB-FL with lipid rafts 1 min after BDNF stimulation.** Cultured cortical neurons were treated with or without 200 ng/ml BDNF for 1 and 30 min, and lysed for lipid raft collection. Western blot analysis was done with TrkB antibody. Similar results were obtained from two separate experiments.

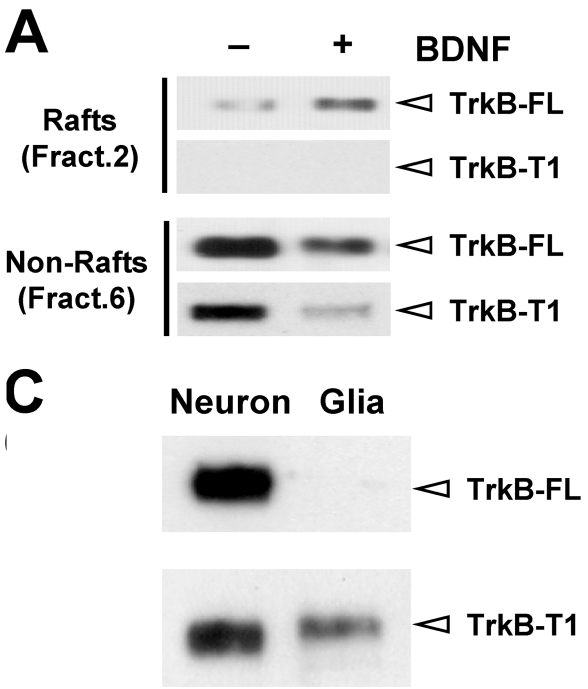


Figure S3. **TrkB-T1 expression in rafts and nonrafts and NT-4-induced recruitment of TrkB-FL into lipid rafts.** (A) TrkB-FL, but not TrkB-T1, is recruited into rafts by BDNF stimulation. Cultured cortical neurons were treated with or without 200 ng/ml BDNF for 30 min. Fraction 2 and 6 were immunoblotted for the indicated proteins. (B) TrkB-FL translocation into rafts is induced by NT-4 (200 ng/ml, 30 min), another ligand that activates TrkB tyrosine kinase. Cultured neurons were stimulated with either 200 ng/ml BDNF or 200 ng/ml NT-4 for 30 min. Raft and nonraft fractions were immunoblotted using TrkB antibody and phospho-Trk antibody. (C) Cultured astrocytes expressed TrkB-T1, but not TrkB-FL. Cortical neuron cultures (Neuron) and astrocyte cultures (Glia) were prepared as described in supplemental Materials and methods. Immunoblots were performed for total lysates using antibodies specific for TrkB-T1 and TrkB-FL. Similar results were obtained from at least two separate experiments.

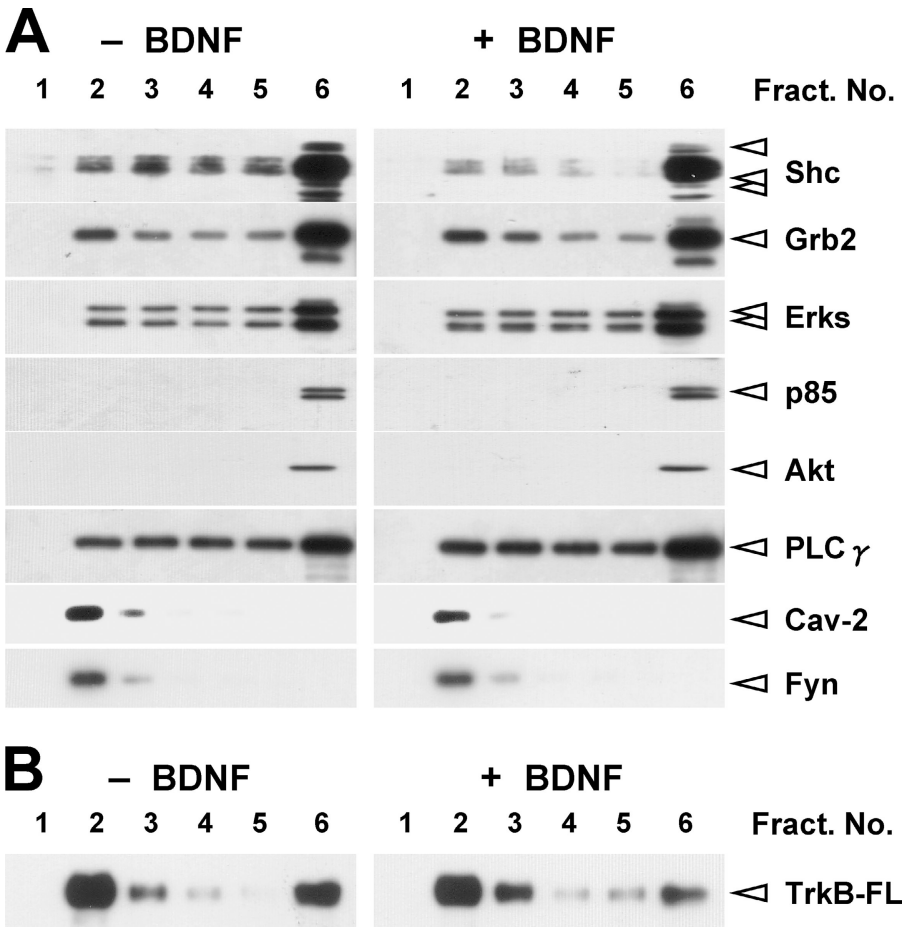


Figure S4. **Association of signaling molecules and TrkB with lipid rafts.** Cultured cortical neurons were treated with or without 200 ng/ml BDNF for 30 min. For lipid raft preparation, a less stringent detergent 1% Triton X-165 was used. Immunoblots were performed using antibodies specific for the indicated proteins. (A) BDNF does not induce the translocation of signaling components downstream of TrkB-FL. (B) A larger amount of TrkB-FL is associated with rafts in naïve cells. Note that under this condition, BDNF induced minimal translocation of TrkB-FL from nonrafts to rafts. Similar results were obtained from at least two separate experiments.

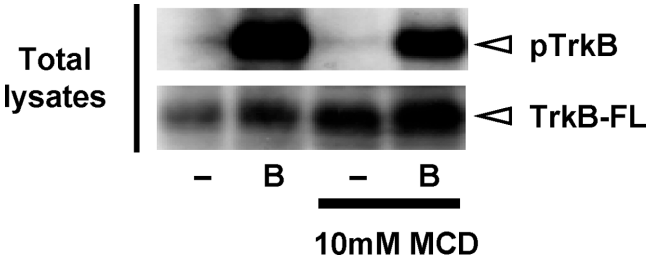


Figure S5. **Treatment with 10 mM MCD led to a partial decrease in BDNF-induced activation of TrkB-FL.** Neurons were pretreated with or without 10 mM MCD for 10 min, followed by incubation with or without 200 ng/ml BDNF for 30 min. Total lysates were prepared as described previously (Suzuki et al., 2002) and immunoblotted for the indicated proteins. Similar results were obtained from two separate experiments

### Generation of adenovirus

For the generation of adenovirus, the cDNAs of nuclear localization signal-possessing LacZ and rat $p75^{NTR}$ were subcloned into the multiple cloning site of the adenovirus vector pAxCAwt under the control of the CAG promoter (Niwa et al. 1991; Kanegae et al. 1995). The recombinant adenovirus expressing $p75^{NTR}$ or lacZ only was generated, amplified, purified, as described previously (Araki et al.,2000).

### Quantitative analyses of dendritic spines and synapse number

For these experiments, the neurons with large cell body (10–17 μm-diam) were selected. Fluorescent images were acquired by a confocal unit (RTS2000; Bio-Rad Laboratories) with 2-s exposure time with a 64 × 1.4 NA objective (Nikon). For colocalization assay, optical 4 sections were sequentially collected along the z axis at 0.2-μm intervals, and the reconstruction images were created using MetaMorph software (Universal Imaging Co., USA). The diameter of dendrites and number of dendritic spines were quantified according to the previous report (Murphy and Segal, 1996). Cultured hippocampal neurons (21–27 days in vitro) were infected with Sindbis virus expressing GFP as described previously (Egan et al., 2003). After 24–36 h, cells were treated with or without 2 mM MCD for 30 min and fixed. Individual spines were identified as 1–3 μm protrusions from the dendrite, having a distinct spine head. Averaged spine densities per 10 μm dendrite were determined. The number of synaptic puncta labeled by both anti-synaptophysin and anti–PSD-95 was counted in 50 μm dendrite, and statistically analyzed (Carroll et al., 1999). The entire experiments were performed in a blind fashion. Fields of stained cells were chosen randomly.

### BDNF ELISA

To determine the concentration of BDNF, we used BDNF $E_{max}$ Immunoassay System (Promega).

## References

Araki, T., M. Yamada, H. Ohnishi, S.I. Sano, and H. Hatanaka. 2000. BIT/SHPS-1 enhances brain-derived neurotrophic factor-promoted neuronal survival in cultured cerebral cortical neurons. *J. Neurochem.* 75:1502–1510.

Carroll, R.C., D.V. Lissin, M.V. Zastrow, R.A. Nicoll, and R.C. Malenka. 1999. Rapid redistribution of glutamate receptors contributes to long-term depression in hippocampal cultures. *Nat. Neurosci.* 2:454–460.

Egan, M.F., M. Kojima, J.H. Callicott, T.E. Goldberg, B.S. Kolachana, A. Bertolino, E. Zaitsev, B. Gold, D. Goldman, M. Dean, et al. 2003. The BDNF val66met polymorphism affects activity-dependent secretion of BDNF and human memory and hippocampal function. *Cell.* 112:257–269.

Kanegae, Y., G. Lee, Y. Sato, M. Tanaka, M. Nakai, T. Sakaki, S. Sugano, and I. Saito. 1995. Efficient gene activation in mammalian cells by using recombinant adenovirus expressing site-specific Cre recombinase. *Nucleic Acids Res.* 23:3816–3821.

Murphy, D.D., and M. Segal. 1996. Regulation of dendritic spine density in cultured rat hippocampal neurons by steroid hormones. *J. Neurosci.* 16:4059–4068.

Niwa, H., K. Yamamura, and J. Miyazaki. 1991. Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene.* 108:193–199.

Suzuki, S., M. Mizutani, K. Suzuki, M. Yamada, M. Kojima, H. Hatanaka, and S. Koizumi. 2002. Brain-derived neurotrophic factor promotes interaction of the Nck2 adaptor protein with the TrkB tyrosine kinase receptor. *Biochem. Biophys. Res. Commun.* 294:1087–1092.