

BDNF-induced recruitment of TrkB receptor into neuronal lipid rafts: roles in synaptic modulation

Shingo Suzuki,^{1,2,3} Tadahiro Numakawa,⁴ Kazuhiro Shimazu,⁶ Hisatsugu Koshimizu,¹ Tomoko Hara,¹ Hiroshi Hatanaka,² Lin Mei,⁵ Bai Lu,⁶ and Masami Kojima^{1,3}

¹Research Institute for Cell Engineering, National Institute of Advanced Industrial Science and Technology (AIST), Ikeda, Osaka, 563-8577, Japan

²Institute for Protein Research, Osaka University, Suita, Osaka, 565-0871, Japan

³Solution Oriented Research for Science and Technology, Japan Science and Technology Agency, Kawaguchi, Saitama, 332-0012, Japan

⁴National Institute of Neuroscience, National Center of Neurology and Psychiatry, Kodaira, Tokyo, 187-8502, Japan

⁵Institute of Molecular Medicine and Genetics, Medical College of Georgia, Augusta, GA 30912

⁶Section on Neural Development and Plasticity, NICHD, National Institutes of Health, Bethesda, MD 20892

Brain-derived neurotrophic factor (BDNF) plays an important role in synaptic plasticity but the underlying signaling mechanisms remain unknown. Here, we show that BDNF rapidly recruits full-length TrkB (TrkB-FL) receptor into cholesterol-rich lipid rafts from nonraft regions of neuronal plasma membranes. Translocation of TrkB-FL was blocked by Trk inhibitors, suggesting a role of TrkB tyrosine kinase in the translocation. Disruption of lipid rafts by depleting cholesterol from cell surface blocked

the ligand-induced translocation. Moreover, disruption of lipid rafts prevented potentiating effects of BDNF on transmitter release in cultured neurons and synaptic response to tetanus in hippocampal slices. In contrast, lipid rafts are not required for BDNF regulation of neuronal survival. Thus, ligand-induced TrkB translocation into lipid rafts may represent a signaling mechanism selective for synaptic modulation by BDNF in the central nervous system.

Introduction

In the central nervous system (CNS), brain-derived neurotrophic factor (BDNF) not only promotes neuronal survival and differentiation, but also regulates synaptic transmission and plasticity. Pharmacological studies demonstrate BDNF enhances the survival of cortical neurons in culture (Ghosh et al., 1994). On the other hand, substantial experiments suggest that a major function of BDNF in the CNS is to regulate synaptic transmission and plasticity (Lu, 2003). In cultured hippocampal or cortical neurons, application of BDNF elicits a rapid potentiation of excitatory synaptic transmission primarily by enhancing presynaptic transmitter release (Lessmann, 1998; Takei et al., 1998). In slices, BDNF facilitates hippocampal long-term potentiation (LTP) and enhances synaptic response to LTP-inducing tetanus (Figurov et al., 1996; Patterson et al., 1996). Both *in vitro* and *in vivo* studies demonstrate that BDNF in-

duces complex effects on dendritic arborization of pyramidal neurons (McAllister et al., 1995).

Despite rapid progress in this area, the molecular mechanisms remain ill defined (Lu, 2003). All the functions of BDNF are mediated by TrkB, a receptor tyrosine kinase (RTK; Kaplan and Miller, 2000). Binding of BDNF rapidly activates its tyrosine kinase, which in turn triggers multiple intracellular signaling pathways. Downstream pathways include MAPK, phosphatidylinositol 3-kinase (PI3-K) and PLC γ . A critical yet poorly understood issue is how signals from this receptor are transduced to mediate diverse biological functions in CNS neurons.

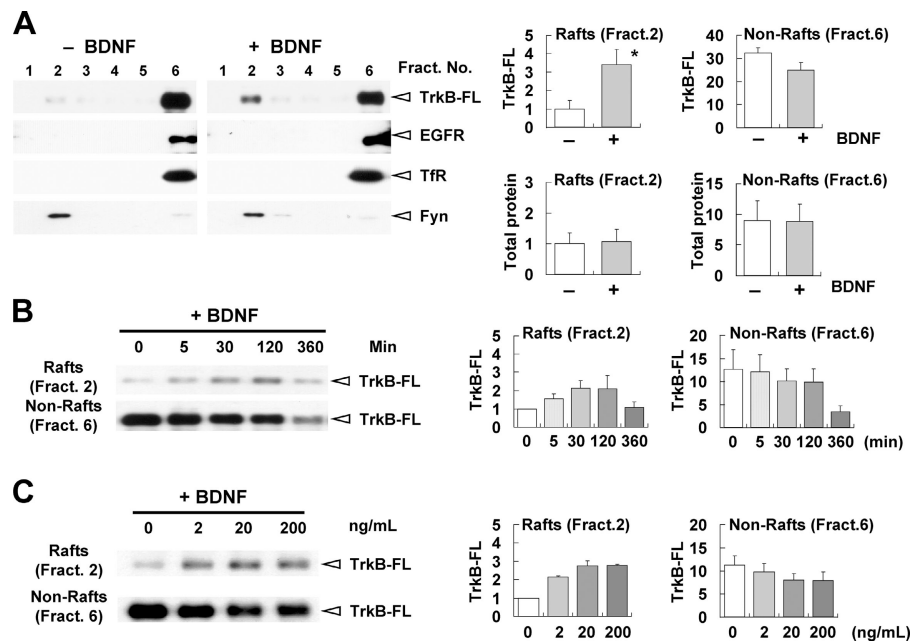
One idea for specific signal-function coupling is that different signaling pathways may be transduced in different subcellular compartments. More specifically, it has been proposed that cholesterol/sphingolipid-rich microdomains called lipid rafts make a specialized signaling platform in the plasma membrane, and therefore can transduce signals different from those in the nonraft membrane (Simons and Toomre, 2000; Anderson and Jacobson, 2002). Because both lipid components are resistant to solubilization with nonionic detergents, lipid rafts can be biochemically isolated as detergent-resistant membrane fractions. Raft fractions prepared from brain tissues are enriched in proteins that carry lipid modifications such as

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Correspondence to Masami Kojima: m-kojima@aist.go.jp; or Bai Lu: bailu@mail.nih.gov

Abbreviations used in this paper: 4-AP, 4-amino pyridine; BDNF, brain-derived neurotrophic factor; CNS, central nervous system; GDNF, glial cell line-derived neurotrophic factor; GPI, glycosylphosphatidylinositol; HFS, high frequency stimulation; KRH, Krebs'-Ringer's-Henseleit; LTP, long-term potentiation; MCD, methyl- β -cyclodextrin; PI3-K, phosphatidylinositol 3-kinase; RTK, receptor tyrosine kinase; TrkB-FL, full-length TrkB.

Figure 1. BDNF-induced translocation of TrkB-FL into lipid rafts in cultured cortical neurons. (A) Effect of BDNF on the distribution of membrane proteins in lipid raft and nonraft fractions. Cultured neurons were treated with or without 200 ng/ml BDNF for 30 min. An example is shown on the left. Note that TrkB-FL, but not EGFR or TrkR, was recruited into fraction 2 after BDNF stimulation. The quantification of TrkB-FL and total proteins in fractions 2 and 6 is shown on the right. The value of densitometry unit was determined for each TrkB-FL band, and normalized to that of “-BDNF” in fraction 2. (B) Time course of BDNF-induced recruitment of TrkB-FL into rafts. Neurons were treated with 200 ng/ml BDNF for the indicated times. (C) Dose-dependent translocation of TrkB-FL into rafts. Neurons were treated with the indicated concentration of BDNF for 30 min. *Indicates significantly different from “-BDNF” in fraction 2; *t* test; *P* < 0.05. *n* = 6 preparations from five independent experiments (A), 4 preparations from three independent experiments (B), and 3 preparations from two independent experiments (C). Error bars in this and all other figures represent SEM.



glycosylphosphatidylinositol (GPI)-anchored proteins, as well as palmitylated or myristoylated proteins such as Src-family kinases and trimeric or small G proteins, suggesting a crucial role of lipid rafts in signal transduction in the CNS (Paratcha and Ibanez, 2002). Recently, lipid rafts have been shown to serve as organizing platforms for chemotropic guidance of nerve growth cones (Guirland et al., 2004). Transmembrane RTKs, including EGF receptor (Mineo et al., 1999) and FGF receptor (Citores et al., 1999) are associated with rafts. The localization of certain signaling molecules in the rafts allows them to interact with each other more efficiently, and prevents them from interacting with the proteins outside rafts (Simons and Toomre, 2000). Thus, entering and exiting lipid rafts of RTKs represent a unique mechanism that transduces differential signals at the subcellular levels. In the present study, we used brain tissues, slices and dissociated cultures to examine whether TrkB receptor is localized in lipid rafts of the plasma membrane, and if so, how the localization is regulated and what the functional roles are. Our results reveal a BDNF-induced TrkB translocation into the lipid rafts, and such translocation is important for BDNF-induced synaptic modulation in CNS neurons.

Results

BDNF-induced translocation of TrkB into lipid rafts

Lipid raft fraction was prepared from tissues or primary cultures of cerebral cortex according to the method of Kawabuchi et al. (2000; Fig. S1A, available at <http://www.jcb.org/cgi/content/full/jcb.200404106/DC1>). We first examined whether full-length TrkB (TrkB-FL) and its ligand BDNF were localized in lipid rafts at different stages of cortical development. Both proteins exhibit a gradual increase in lipid rafts after birth (Fig. S1 B). The components of the lipid rafts, such as cholesterol and raft marker proteins caveolin-2 and Fyn, also increased in rafts

during postnatal development (Fig. S1 C), raising the possibility that the raft localization of TrkB and BDNF may depend on the developmental expression of these components in rafts. The parallel increase of TrkB and BDNF in lipid rafts also suggests that BDNF may regulate the localization of TrkB in lipid rafts.

To directly test whether BDNF recruits TrkB into lipid rafts, we prepared the rafts in cultured cortical neurons treated with BDNF. In the cortical cultures used here, $93.3 \pm 2.4\%$ and $3.8 \pm 0.7\%$ are NSE-positive neurons and GFAP-positive astrocytes, respectively (*n* = 6 independent experiments). As shown in Fig. 1 A, application of BDNF induced an increase in TrkB-FL in the raft fraction. There was a low amount of TrkB-FL in lipid rafts in naïve neurons, suggesting that in naïve cells TrkB-FL may be associated with rafts with a low affinity. In cultures stimulated with 200 ng/ml BDNF for 30 min, the amount of TrkB-FL was markedly increased in rafts (TrkB-FL_{BDNF-treated}/TrkB-FL_{control}: 3.4 ± 0.8 -fold, *P* < 0.03) but decreased slightly in nonrafts ($23.1 \pm 6.4\%$). Total proteins in both regions were not changed by the BDNF treatment (Fig. 1 A). When expressed as TrkB-FL/total protein, BDNF increased TrkB-FL in rafts by 3.63 ± 0.73 -fold, indicating that BDNF selectively increases the amount of TrkB-FL, but not protein concentration, in rafts.

A time course study demonstrated that the partition of TrkB-FL into rafts was significant at 5 min, peaked at 30–120 min after BDNF application, and returned to control levels within 6 h (Fig. 1 B). The translocation was observed 1 min after BDNF addition (Fig. S2, available at <http://www.jcb.org/cgi/content/full/jcb.200404106/DC1>). On the other hand, TrkB-FL in nonraft fraction exhibited a gradual decrease after BDNF exposure (Fig. 1 B). BDNF-induced recruitment of TrkB-FL into rafts was dose dependent (Fig. 1 C). To confirm that BDNF selectively translocated TrkB-FL into lipid rafts, we examined whether BDNF stimulation also affects the distribution of other receptors in the rafts and nonraft membrane frac-

tions. A 30-min treatment with BDNF did not change the distribution of TrkR, a nontyrosine kinase receptor, or EGF receptor, a tyrosine kinase receptor (Fig. 1 A, left). Together, these data suggest that BDNF rapidly and selectively induces the translocation of TrkB-FL from nonrafts into lipid rafts.

TrkB translocation: dependence on its tyrosine kinase activity

Binding of BDNF to TrkB-FL induces the autophosphorylation of its tyrosine residues on the intracellular kinase domain, leading to its activation (Kaplan and Miller, 2000). To test whether TrkB recruited into rafts is activated, we performed Western blot analysis using anti-phospho-Trk antibody (pY490; Binder et al., 1999). In neurons treated with BDNF for 30 min, a substantial amount of TrkB-FL recruited into rafts was tyrosine phosphorylated (Fig. 2 A, top). In BDNF-treated cultures, TrkB-FL tyrosine phosphorylation relative to total TrkB-FL protein was not significantly different between in rafts and in nonrafts (Fig. 2 A, bottom; $P > 0.64$). To investigate whether activation of TrkB tyrosine kinase was required for the translocation of TrkB-FL into lipid rafts, we performed the following experiments. First, we treated cortical neurons with the Trk kinase inhibitors, K252a or AG879 for 3 h before BDNF stimulation (Fig. 2 B). K252a (100 nM), which reduced BDNF-dependent TrkB tyrosine phosphorylation by $68.8 \pm 12.2\%$ in rafts and by $49.4 \pm 7.0\%$ in the nonrafts ($n = 3$ independent experiments), inhibited BDNF-induced TrkB-FL translocation (Fig. 2 B). BDNF-dependent recruitment of TrkB-FL into rafts was also blocked by another Trk kinase inhibitor AG879 (10 μ M). Second, because TrkB activation could be induced by a 1-min exposure to BDNF (Takei et al., 1998), we tested whether this short-term stimulation would allow recruitment and partition of TrkB-FL in rafts. Cultured cortical neurons were stimulated with BDNF for 1 min, followed by incubation with medium containing no BDNF for 5–360 min. As shown in Fig. 2 C, a 1-min stimulation with BDNF was sufficient to recruit TrkB-FL into rafts. The amount of TrkB-FL continued to increase after BDNF was washed out, suggesting that once activated TrkB-FL can move into lipid rafts. Third, TrkB-T1, a truncated form of the TrkB receptor lacking tyrosine kinase domain, did not appear to be partitioned into rafts by BDNF (Fig. S3A, available at <http://www.jcb.org/cgi/content/full/jcb.200404106/DC1>). Finally, NT-4 (200 ng/ml, 30 min), another ligand that activates TrkB tyrosine kinase, recruited TrkB-FL into rafts (Fig. S3 B). Together, these results suggest that BDNF-induced translocation of TrkB into rafts requires its tyrosine kinase activity. Because cultured astrocytes expressed TrkB-T1, but not TrkB-FL (Fig. S3 C), the TrkB-FL translocation is likely to occur in neurons only.

Regulation of BDNF-induced translocation of TrkB into rafts by p75^{NTR}

How is BDNF-dependent translocation of TrkB-FL into rafts regulated? Given that the pan-neurotrophin receptor, p75^{NTR} associates with lipid rafts (Higuchi et al., 2003) and forms a heterodimer with Trk family receptors (Hempstead, 2002),

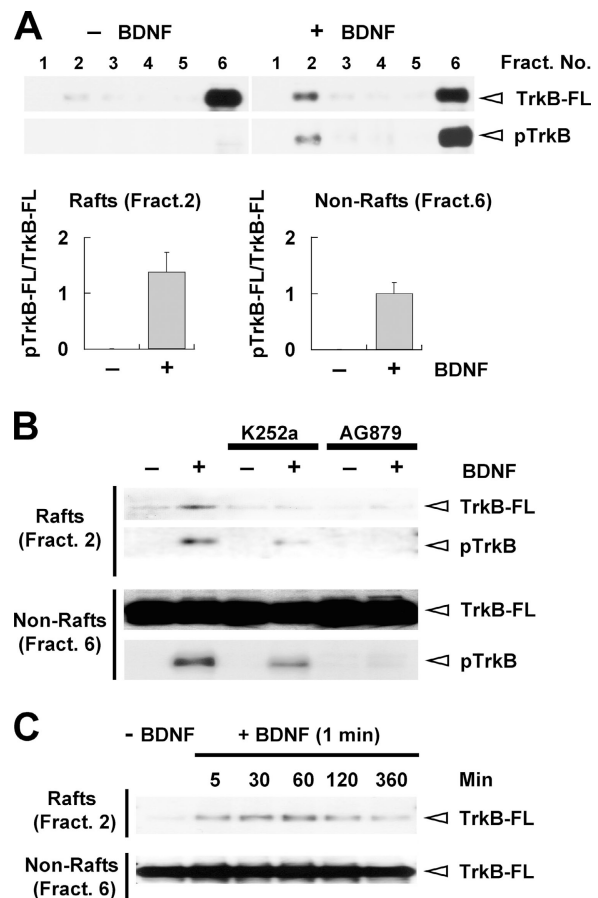


Figure 2. Requirement of TrkB activation for BDNF-induced TrkB translocation into lipid rafts. Immunoblots were performed using anti-TrkB and anti-phospho-Trk antibodies. (A) TrkB-FL recruited into rafts is tyrosine phosphorylated. Cortical neurons were treated with (200 ng/ml) or without BDNF for 30 min. (Top) Sample blots. (Bottom) Quantification. pTrkB-FL/total TrkB-FL was quantified in fractions 2 and 6, and shown as relative to that of "+BDNF" in fraction 6. $n = 5$ preparations from five independent experiments. (B) Inhibition of TrkB activation prevents TrkB-FL translocation into lipid rafts. Neurons were treated with 100 nM K252a or 10 μ M AG879 for 3 h, followed by a 30-min stimulation with 200 ng/ml BDNF. (C) Effect of a brief exposure to BDNF on TrkB-FL translocation. Cortical neurons were treated with 200 ng/ml BDNF for only 1 min followed by incubating with medium containing no BDNF for the indicated times. Note that once activated, TrkB-FL continued to be partitioned into rafts in the absence of BDNF.

p75^{NTR} may facilitate BDNF-dependent association of TrkB with rafts. To test this, we introduced p75^{NTR} or lacZ into cortical neurons using adenovirus-mediated gene transfer. In cortical neurons expressing lacZ, p75^{NTR} was not detectable both in raft fraction and nonraft fraction (Fig. 3, lacZ). Infection of cortical neurons with p75^{NTR} adenovirus resulted in the expression of the 75-kD p75^{NTR} (Fig. 3, p75). The p75^{NTR} did not interfere with basal association of TrkB with rafts or TrkB phosphorylation outside rafts. Interestingly, the expression of exogenous p75^{NTR} in cortical neurons markedly inhibited BDNF-dependent translocation of TrkB-FL into lipid rafts (Fig. 3, TrkB-FL_{rafts}). Consequently, phosphorylated TrkB-FL was not detected in the rafts (Fig. 3, pTrkB_{rafts}). Moreover, application of BDNF did not increase the amount of p75^{NTR} in the rafts (Fig. 3, p75_{rafts}). Together, these data suggest that a lipid raft-associated

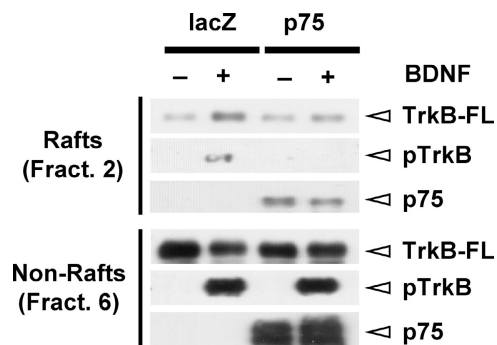


Figure 3. Inhibition of BDNF-induced TrkB translocation by p75^{NTR}. Cultured cortical neurons were infected with adenovirus expressing p75^{NTR} or lacZ as described in Materials and methods. Cells were treated without or with 200 ng/ml BDNF for 30 min. The fractions of rafts and nonrafts were immunoblotted for the indicated proteins. (Top) BDNF-induced translocation and activation of TrkB-FL is inhibited by p75^{NTR} expression. (Bottom) The location and activation of TrkB-FL in nonrafts are not influenced by p75^{NTR} expression. BDNF did not affect the distribution of p75^{NTR} in rafts and nonrafts. Similar results were obtained from two separate experiments.

pan-neurotrophin receptor p75^{NTR} has an inhibitory role in BDNF-dependent translocation of TrkB-FL into rafts.

Distribution of signaling molecules downstream of TrkB-FL in lipid rafts and nonrafts

Phosphorylated tyrosine residues on the TrkB intracellular domain form docking sites for the signaling proteins Shc, Grb2, and PLC γ , leading to activation of MAPK (Erks), PLC γ , and PI3-K pathways (Kaplan and Miller, 2000). We tested whether TrkB-FL, upon BDNF stimulation, carries these signaling molecules into lipid rafts during its translocation. In naïve neurons, Erks extended throughout the gradient (fractions 2–5), whereas Shc, Grb2, p85 subunit of PI3-K, Akt, and PLC γ were primarily localized in the bottom, nonraft fraction (Fig. 4 A, left). BDNF application did not increase the amount of any of these proteins in fraction 2 (Fig. 4 A, right), suggesting that TrkB-FL does not carry its associated signaling molecules into lipid rafts during its translocation. When a milder detergent Triton X-165 was used to prepare rafts (Fig. S4 A, available at <http://www.jcb.org/cgi/content/full/jcb.200404106/DC1>), many of them (Shc, Grb2, Erks, and PLC γ) were extended throughout the gradient whereas p85 subunit of PI3-K and Akt appeared to be still in nonraft fraction. It was notable that BDNF did not stimulate the translocation of these signaling molecules into rafts (Fig. S4 A). Parallel to these, BDNF-induced translocation of TrkB-FL (Fig. 4 B, left) was accompanied by a significant increase in the phosphorylation of Erks (Figs. 4 B, middle and Fig. 4 D), but not that of Akt (Fig. 4 B, right and Fig. 4 D), in lipid rafts. In nonraft regions, however, both Erks and Akt were activated by BDNF application (Fig. 4, C and D). Thus, although TrkB-FL does not move with its associated proteins during translocation, the translocation of TrkB-FL itself into rafts may be a key event in forming TrkB signaling complex, including TrkB-FL and its associated proteins, in rafts, leading to preferential activation of Erks over Akt in neuronal lipid rafts.

Requirement of lipid rafts for short-term synaptic modulation by BDNF

What is the functional role of BDNF-induced TrkB translocation into lipid rafts? Because the translocation was observed 1 min after BDNF application (Fig. S2), we reasoned that this process may be involved in the short-term actions of BDNF (Lu, 2003). BDNF has been shown to rapidly enhance transmitter release in cultured cortical neurons (Matsumoto et al., 2001). Therefore, we tested whether disruption of lipid rafts would interfere with BDNF modulation of depolarization-evoked transmitter release at CNS synapses. Methyl- β -cyclodextrin (MCD) binds and depletes cholesterol from the plasma membrane, and thereby disrupts lipid rafts (Simons and Toomre, 2000). A 10-min treatment with MCD (2 mM) removed membrane cholesterol by $33 \pm 2\%$ (91 ± 2 ng/well in control cultures, 61.2 ± 2 ng/well in MCD-treated cultures, $n = 4$ independent experiments), but did not affect the amount of caveolin-2 and Fyn in lipid rafts (Fig. 5 A, right). Treatment of the cultured cortical neurons with 2 mM MCD significantly attenuated the BDNF-induced recruitment of TrkB-FL into the rafts (Fig. 5 A, left and middle). Consequently, the amount of phosphorylated TrkB was also reduced in the rafts. Importantly, TrkB phosphorylation in the nonraft fraction was not affected. Thus, cholesterol may play a role in TrkB translocation into lipid rafts.

We reported previously that in cultured cortical neurons, pretreatment with BDNF enhanced glutamate release evoked by 4-amino pyridine (4-AP), a K⁺ channel blocker frequently used in biochemical assays of evoked neurotransmitter release (Matsumoto et al., 2001). As shown in Fig. 5 B, pretreatment with BDNF for 30 min had no effect on the content of glutamate in the medium (white bars in BDNF-pretreated cultures), but significantly enhanced 4-AP-induced glutamate release (hatched bars in BDNF-pretreated cultures). A 10-min treatment with 2 mM MCD before BDNF exposure, however, abolished BDNF enhancement of 4-AP-evoked glutamate release. The neurons pretreated with 2mM MCD alone displayed 4-AP-evoked glutamate release, which was comparable to that in control neurons, indicating that cholesterol depletion itself does not impair the exocytosis machinery. Long-term treatment with raft depleting agents such as fumonisins B₁ and mevastatin has been reported to lead to instability of AMPA receptors and loss of dendritic spines and synapses (Hering et al., 2003). In contrast, dendritic parameters (Murphy and Segal, 1996) were not affected by short-term treatment (30 min) with 2 mM MCD. The dendritic diameter was about the same in control cultures and MCD-treated cultures (1.19 ± 0.04 μ m in control cells and 1.18 ± 0.04 μ m in MCD-treated cells, $n = 415$ segments). Likewise, MCD treatment did not change spine density (spine number per 10 μ m dendritic segment; 2.47 ± 0.11 in control cells, 2.36 ± 0.10 in cells treated with 2 mM MCD, $n = 945$). Furthermore, immunocytochemistry using antibodies against the presynaptic marker synaptophysin and postsynaptic marker PSD95 indicated that short-term MCD treatment did not alter the number or the distribution of synapses (Fig. 5 C). These data support the notion that MCD selectively prevents the residing of activated TrkB in lipid rafts, leading to impairments of BDNF modulation of glutamate release.

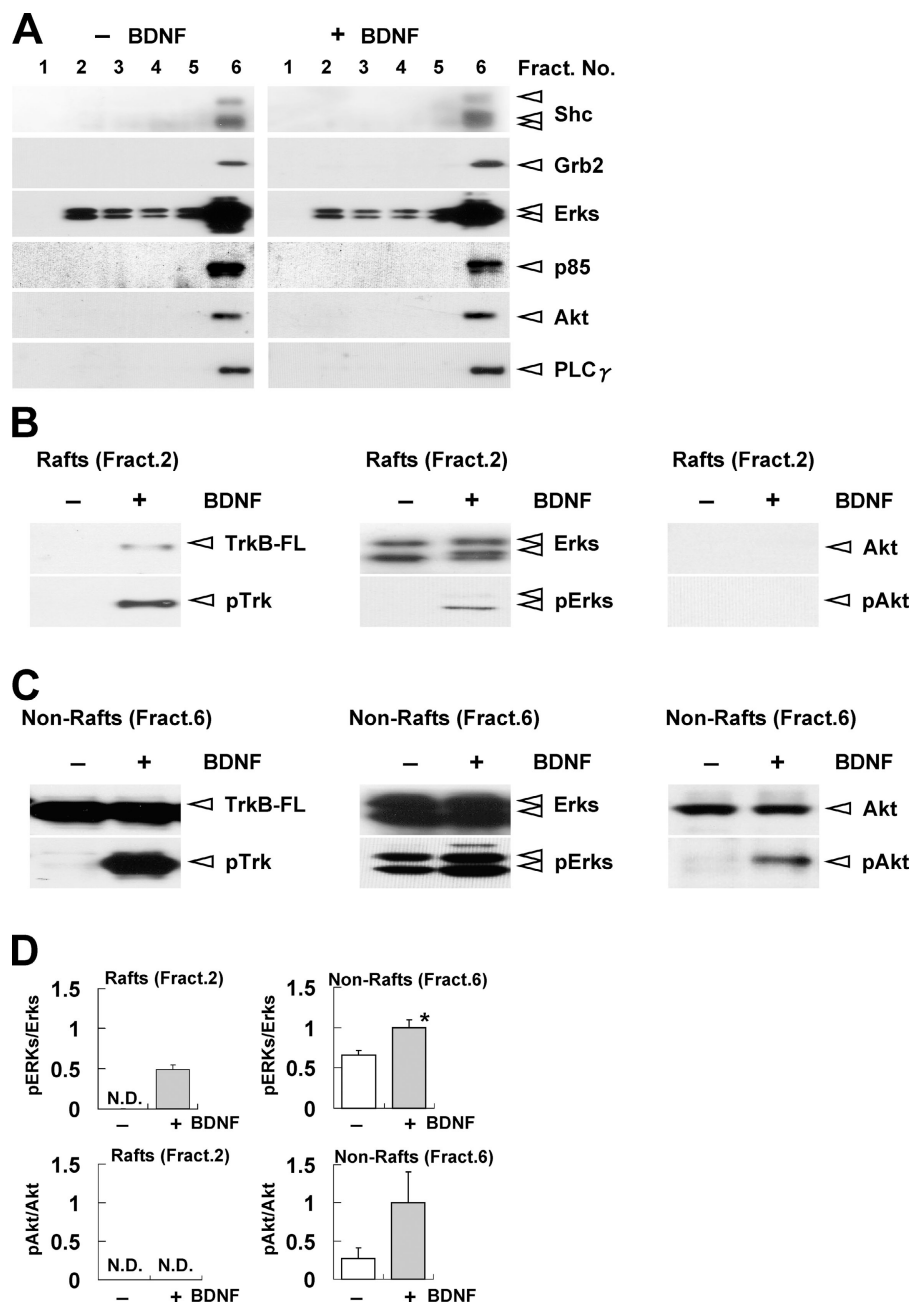


Figure 4. Differential activation of Erks and Ark in lipid rafts upon BDNF-induced TrkB-FL translocation. Cultured cortical neurons were treated with or without BDNF (200 ng/ml) for 30 min. (A) Western blot analysis of the distribution of major signaling molecules downstream of TrkB before and after BDNF stimulation. Similar results were obtained from two separate experiments. (B and C) Differential activation of Erks and Akt in lipid rafts and nonrafts after a 30-min treatment with BDNF. Fractions 2 and 6 were immunoblotted. (D) Quantitative analysis of BDNF effect on the activation of Erks and Akt in rafts and nonrafts. For each protein, phosphorylation relative to total amount was quantified in fractions 2 and 6, and shown as relative to that of "+BDNF" in fraction 6. *Indicates significantly different from "-BDNF"; *t* test; *P* < 0.05. *n* = 4 independent experiments. In A–C, immunoblots were performed using antibodies specific for indicated proteins and phospho-proteins.

The biochemical assay described above measures both synaptic and nonsynaptic glutamate release. To determine whether lipid rafts are important for BDNF modulation of neurotransmitter release at synapses, we measured synaptic exocytosis in cultured hippocampal neurons using a style membrane dye FM1-43 (Ryan et al., 1993). A second depolarization of these FM dye-loaded neurons by high K^+ solution (see Materials and methods) resulted in a rapid destaining of the FM dye-labeled spots, reflecting transmitter release at the synapses (Fig. 6, A and B). Pre-treatment with BDNF for 30 min enhanced depolarization-induced FM1-43 destaining (Fig. 6 B). A 10-min treatment with MCD (2 mM) before BDNF application prevented the enhancement effect of BDNF (Fig. 6 C). The neurons pretreated with MCD alone exhibited FM1-43 destain-

ing similar to that in control neurons (Fig. 6 C), indicating that neither uptake nor destaining of FM 1-43 dye was affected by MCD pretreatment.

Similar to the effect of BDNF in hippocampal cultures, BDNF enhanced depolarization-induced FM dye destaining, and MCD attenuated the BDNF effect, in cultured cortical neurons (Fig. 6 D). Several experiments were performed to ensure that the MCD effect was due specifically to removal of cholesterol and disruption of lipid rafts, rather than to a nonselective, pharmacological effect such as removal of proteins from the plasma membrane. First, we pretreated the cultures with 50 ng/ml filipin, a cholesterol sequestration agent with a structure distinct from that of MCD (Simons and Toomre, 2000; Ma et al., 2003). We found that, although FM 1-43 uptake appeared to be

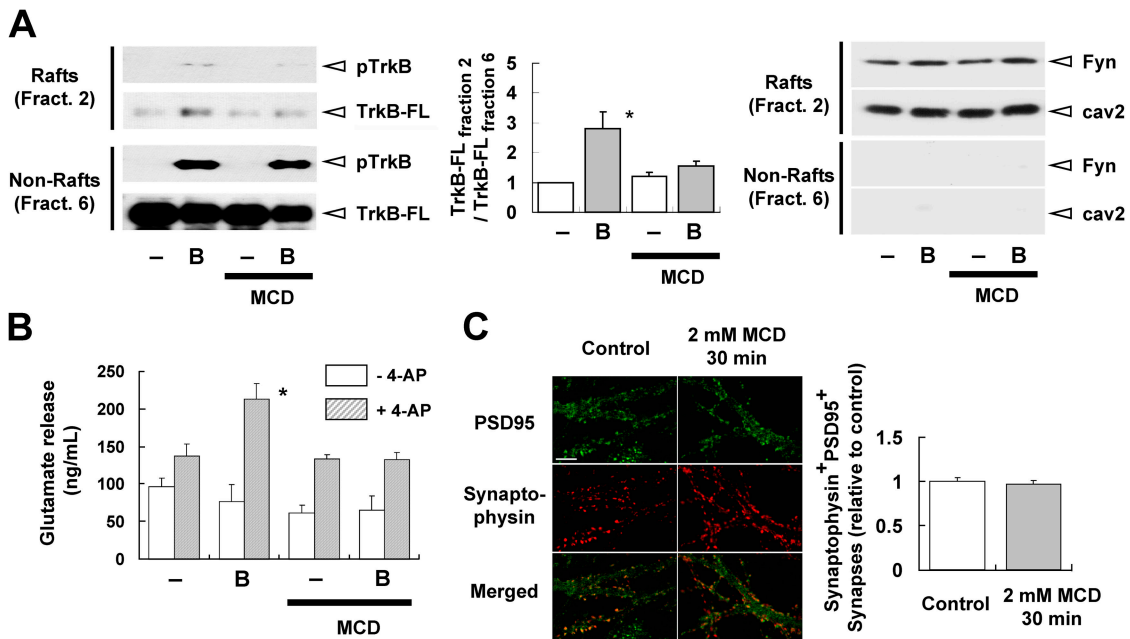


Figure 5. Inhibition of BDNF-induced TrkB-FL translocation and evoked glutamate release by lipid raft disruption. (A) Effect of MCD on BDNF-induced TrkB translocation. Cortical neurons were treated with or without MCD (2 mM) for 10 min. (Left) Raft (fraction 2) and nonraft fractions (fraction 6) were immunoblotted for the indicated proteins. Note that MCD blocked BDNF-induced TrkB phosphorylation in rafts and translocation to rafts. (Middle) TrkB-FL_{fraction 2}/TrkB-FL_{fraction 6} was quantified and shown as relative to “-BDNF” in control cultures ($n = 4$ preparations from three independent experiments). *Indicates significantly different from “-BDNF” in control cultures; t test; $P < 0.01$. (Right) Treatment with 2 mM MCD for 10 min did not influence the amount of caveolin-2 and Fyn in lipid raft fraction. (B) Effect of MCD on BDNF enhancement of 4-AP-evoked glutamate release from cortical neurons. Neurons were pretreated with or without 2 mM MCD for 10 min, followed by incubation with or without BDNF for 30 min. After washing four times, neurons were stimulated with 1 mM 4-AP. *Indicates significantly higher than all other 4-AP-treated groups; ANOVA test; $P < 0.05$; $n = 4$ cultures. There was no statistically significant difference among all white bars (ANOVA test, $P > 0.48$). Similar results were obtained from three independent experiments. (C) Effect of short-term treatment with MCD on the number and distribution of synapses. (Left) Immunocytochemistry for the indicated proteins was done after a 30-min incubation with or without 2 mM MCD. (Right) The number of synapses (doubly labeled by anti-PSD-95 and anti-synaptophysin antibodies) was determined and shown as relative to that of “Control” ($n = 90$ dendritic segments with 50- μ m length from three independent coverslips and experiments). Note that there is no significant difference in distribution and number of the immunopositive synapses between control and MCD-treated cultures. Bar, 5 μ m.

slightly lower, filipin, like MCD, completely reversed the effect of BDNF on depolarization-induced FM dye destaining (Fig. 6 E). Neurons pretreated with filipin alone exhibited FM dye destaining similar to that in control neurons (Fig. 6 E). Second, if the MCD effect were due to a nonspecific removal of membrane protein, addition of MCD-cholesterol complex should not rescue the deficits induced by MCD. We treated the cultures with MCD-cholesterol complex (1 mg/ml MCD balanced with 40 μ g/ml cholesterol) for 10 min (Thiele et al., 2000). Remarkably, this treatment did not affect the potentiating effect of BDNF on depolarization-induced destaining of FM 1-43 (Fig. 6 D). We therefore conclude that lipid rafts are important for the modulation of neurotransmitter release and synaptic exocytosis by BDNF.

One of the major functions of BDNF in the intact hippocampal synaptic circuits is to attenuate synaptic fatigue induced by a train of high frequency stimulation (HFS; or tetanus, 100 Hz, 1 s; Figuero et al., 1996). We next examined the role of lipid rafts in this form of synaptic modulation. In neonatal hippocampal slices (P12-13) in which the level of endogenous BDNF is low, application of tetanus resulted in pronounced synaptic fatigue at Schaffer collateral-CA1 synapses (Fig. 7 A). Consistent with our previous reports (Figuero et al., 1996), treatment with exogenous BDNF (2 nM) for 1–2 h sig-

nificantly attenuated the synaptic fatigue (Fig. 7 B). However, pretreatment with MCD for 30 min completely abolished the attenuating effect of BDNF on HFS-induced synaptic fatigue. Quantitative analysis indicated that treatment with BDNF markedly increased the rate constant (τ) for synaptic fatigue and disruption of lipid rafts with MCD completely prevented such an increase (Fig. 7 C). It is important to note that treatment with MCD for 3 h had no effect on synaptic responses to HFS (Fig. 7, B and C), nor did MCD affect basal synaptic transmission or tetanus induced LTP (Ma et al., 2003). These results suggest that short-term exposure to MCD per se does not affect the number of readily releasable vesicles in the presynaptic terminals or the number or properties of AMPA or NMDA receptors on the postsynaptic density.

Requirement of lipid rafts for long-term regulation of dendritic growth, but not neuronal survival, by BDNF

To test whether lipid rafts are also involved in long-term effects of BDNF, we treated cultures with cholesterol synthesis inhibitors, mevastatin or pravastatin, which effectively deplete rafts from cortical neurons over days (Hering et al., 2003). The cholesterol level was significantly reduced in mevastatin- or pravastatin-treated cortical neurons (Fig. 8 A). We first exam-

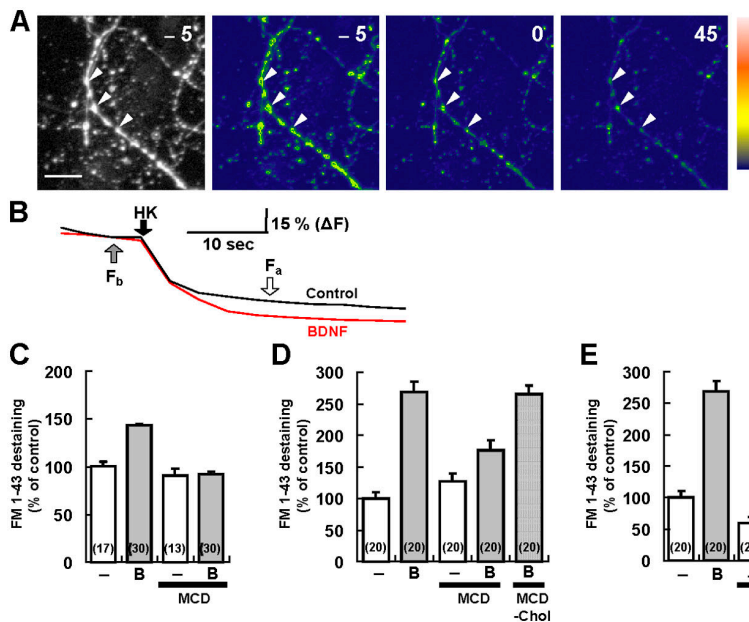


Figure 6. Attenuation of BDNF enhancement of synaptic exocytosis by lipid raft disruption. Recycling synaptic vesicles in cultured hippocampal or cortical neurons were labeled by FM1-43, and exocytosis was induced by a perfusion of 50 mM KCl containing KRH buffer (high K⁺ solution). (A) Pseudo-colored images showing high K⁺ solution-dependent reduction in FM1-43 intensity. FM 1-43 images were captured at the indicated times (s) and baseline intensity was captured at 5 s before depolarization. The monochromatic image shows the neuron 5 min before stimulation. Arrowheads indicate representative spots with a gradual reduction of FM dye labeling after depolarization. Bar, 10 μ m. (B) Representative recordings of FM 1-43 destaining. Neurons were incubated with or without BDNF for 30 min, followed by a 1-min exposure to high K⁺ solution to load FM dye. After three washes for 5 min, FM dye was destained with high K⁺ solution (black arrow). FM 1-43 intensities at 4 s before (gray arrow) and at 15 s after (white arrow) depolarization are defined as F_b and F_a, respectively. ΔF (%) = (F_b - F_a)/F_b \times 100. (C-E) Summary of FM dye destaining experiments using hippocampal neurons (C) and cortical neurons (D and E). Cells were pretreated with MCD, MCD-cholesterol complex (MCD-Chol), or filipin for 10 min, followed by BDNF incubation for 30 min and stimulation with high K⁺ solution. Data were collected at 4 s before and at 15 s after stimulation with high K⁺ solution. The number associated with

each column represents the number of spots recorded for each condition. A 1-min incubation with 5 mM KCl did not elicit any significant change in the intensity of FM 1-43 (not depicted). In all experiments similar results were obtained from at least two independent experiments.

ined the effect of these lipid raft disrupters on BDNF regulation of neuronal survival, using the WST-1 assay, which quantitatively determines cell viability based on the cleavage activity of a soluble tetrazolium salt WST-1 by mitochondrial dehydrogenases, and by counting the number of MAP2-positive neurons in culture. 3-d treatment with BDNF significantly enhanced the survival of cortical neurons in serum-free medium (Fig. 8, B and C, left; Yamada et al., 2001). Both mevastatin and pravastatin, however, failed to block this effect of BDNF (Fig. 8, B and C, middle and right), indicating that lipid rafts are not required for the regulatory effect of BDNF on cortical neuron survival.

BDNF has also been shown to be a potential regulator for dendritic growth of CNS neurons (McAllister et al., 1995). We next examined whether lipid rafts are important for BDNF regulation of dendritic growth. 3-d exposure of BDNF led to robust growth of MAP2-positive primary dendrites (Fig. 8 D, BDNF). Quantitative analysis revealed that BDNF increased the number of primary dendrites by >50% ($P < 0.001$). This effect of BDNF, however, was effectively blocked by mevastatin, whereas treatment with mevastatin alone had no effect (Fig. 8 D). Thus, while required for short-term modulation of synaptic transmission and plasticity by BDNF, lipid rafts may mediate some, but not all long-term effects of BDNF in CNS neurons.

Discussion

One of the conceptually difficult issues in neurotrophin research is how a single neurotrophin could elicit different effects under different conditions on the same neurons, or on certain parts of the same neurons (Bibel and Barde, 2000). How could, for example, BDNF regulate synaptic transmission without altering the survival of neurons of adult cortex? One way to

ensure signaling specificity is to induce BDNF signaling in specific subcellular compartments. In the present study, we demonstrated that BDNF stimulation induced the translocation of activated TrkB-FL from nonrafts to lipid rafts in cortical neurons. We further showed that attenuation of such translocation by depleting cholesterol in the lipid rafts prevented the effects of BDNF on synaptic transmission and plasticity, but not neuronal survival. These results reveal a new mechanism for the signaling specificity of BDNF, and suggest a critical role of lipid rafts in the synaptic function of BDNF in CNS neurons.

BDNF-induced translocation of activated TrkB into lipid rafts

One of the key findings in the present study is that TrkB translocation to rafts depends on its tyrosine kinase activity. This notion is supported by a number of observations: (a) inhibition of the Trk kinase activity by K252a or AG879 significantly inhibited BDNF-induced translocation of TrkB-FL; (b) NT-4, which activates the TrkB RTK, also induced TrkB-FL translocation; (c) 1-min exposure of BDNF, which activates TrkB, was sufficient to partition TrkB-FL into rafts. Thus, TrkB activation has to occur before the translocation; and (d) TrkB-T1, the truncated form of TrkB lacking the tyrosine kinase domain, was not recruited into rafts by BDNF. This last result, however, has to be interpreted with caution. It is also possible that the intracellular domain of the TrkB receptor may contain motifs that interact with other proteins important for the delivery of TrkB to the lipid rafts.

Another important observation is that removing of cholesterol, a major component in the lipid rafts, with 2 mM MCD inhibited TrkB signaling, leading to impairments in synaptic transmission and plasticity. The most straightforward interpretation is that MCD disrupted BDNF-induced translocation of TrkB-FL into rafts, and consequently impaired TrkB signaling

in rafts. Alternatively, TrkB may need to interact with cholesterol to become fully functional, and therefore depletion of cholesterol directly affects TrkB signaling, rather than TrkB translocation. Consistent with this idea, BDNF failed to induce TrkB phosphorylation on the cell surface in cultured striatal neurons from Niemann-Pick type C mice (NPC^{-/-}), which have abnormal cholesterol metabolism (Henderson et al., 2000). The fact that there was a small amount of TrkB in lipid rafts in naïve cells (Fig. 1 A) also supports the idea that TrkB interacts with rafts, perhaps with a low affinity. However, several observations argue against this idea. First, in cultured striatal neurons from NPC^{-/-}, the content of free cholesterol was normal, suggesting that the cholesterol-rich microdomains on cell surface are probably normal. Thus, the dysfunction of TrkB on cell surface in NPC^{-/-} is probably due to reasons other than lack of cholesterol in the plasma membrane. Second, we demonstrated that treatment with 2 mM MCD, which removed membrane cholesterol by 33%, interfered with TrkB recruitment into rafts, but not TrkB activation outside rafts (Fig. 5 A). The basal level of TrkB localization in rafts in resting cells was normal as well. Finally, treatment with 10 mM MCD, which removed >95% cholesterol, did not diminish TrkB kinase activity (Fig. S5, available at <http://www.jcb.org/cgi/content/full/jcb.200404106/DC1>). These data suggest that cholesterol is involved in the recruitment of TrkB into lipid rafts, but not directly in TrkB function.

A third possible mechanism for TrkB translocation is that BDNF signaling involves an interaction between TrkB and protein X, which could be a protein embedded in cholesterol-rich membranes. Cholesterol depletion could in principle lead to structural changes in the protein X (Simons and Toomre, 2000; Munro, 2003; Glebov and Nichols, 2004), and therefore disturb its interaction with TrkB. Although the nature of protein X remains unknown, we tested whether p75^{NTR}, which is primarily associated with rafts (Higuchi et al., 2003) and is capable of binding to Trk receptors (Hempstead, 2002), facilitates BDNF-dependent translocation of TrkB into rafts. Interestingly, expression of exogenous p75^{NTR} in cortical neurons inhibited TrkB translocation into lipid rafts, as well as the level of TrkB phosphorylation in the rafts, but not the basal association of TrkB with rafts or TrkB phosphorylation outside rafts (Fig. 3). These data are opposite to the above prediction and suggest that p75^{NTR} has an inhibitory function in TrkB translocation and lipid raft-mediated BDNF/TrkB signaling. Thus, although it is unlikely that BDNF-induced TrkB translocation into lipid rafts is mediated by p75^{NTR} through its interaction with TrkB, our experiments could not rule out the possibility that a protein X is an intermediate for BDNF-induced TrkB translocation into rafts.

The dynamic behavior of TrkB into lipid rafts is most analogous to that of two tyrosine kinase receptors, c-Ret for glial cell line-derived neurotrophic factor (GDNF; Tansey et al., 2000; Paratcha et al., 2001) and ErbB4 for neuregulin (Ma et al., 2003). c-Ret is recruited into lipid rafts upon GDNF stimulation by two distinct mechanisms. In cells expressing c-Ret and GPI-anchored protein GFR α 1 (GDNF family receptor α 1), which is primarily associated with lipid rafts, the binding

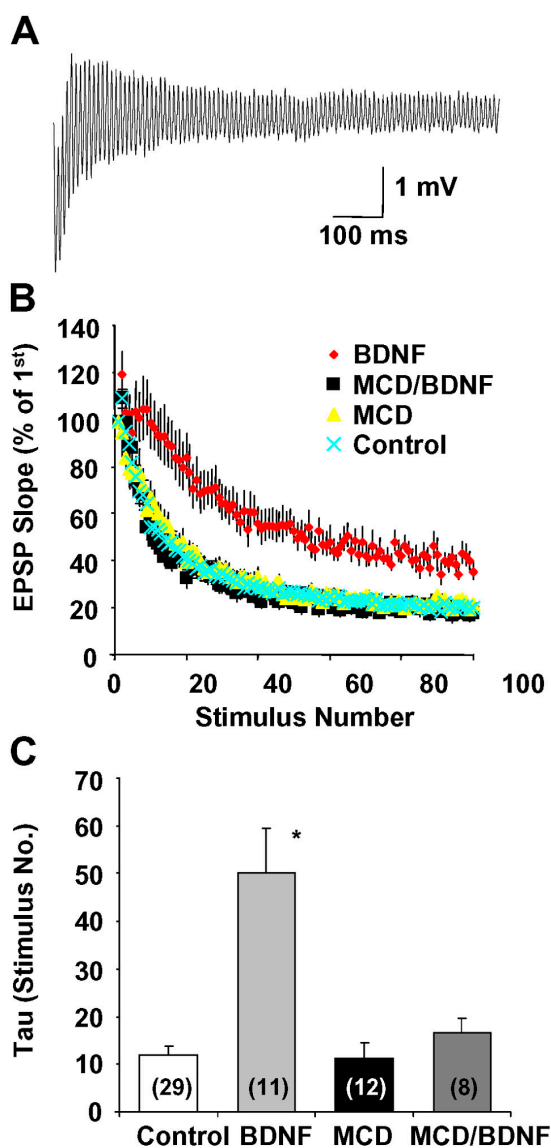


Figure 7. Effect of MCD on BDNF modulation of synaptic fatigue at hippocampal CA1 synapses. Neonatal hippocampal slices were pretreated with MCD (2 mM) for 30 min before treatment with BDNF (2 nM) for 1–2 h. A train of HFS (100 Hz, 1 s) was applied to Shaffer collaterals and field EPSPs were recorded from stratum radiatum. The slopes of EPSPs during the entire recording were normalized to the first EPSP slope in each recording. (A) An example of EPSPs elicited by HFS recorded from a BDNF-treated slice. (B) Effect of MCD on synaptic depression induced by HFS. Normalized EPSP slopes in each condition were averaged and plotted against the number of stimulus during HFS. (C) Summary of the MCD effect on the rate of synaptic depression. The plot for each recording was fitted with a single exponential curve, and rate constant (τ) for each condition was averaged and presented. *Indicates significantly higher than all other groups; ANOVA followed by post hoc tests; $P < 0.001$. The number associated with each column represents the number of slices used for each condition.

of GDNF to GFR α 1 results in a transient recruitment of c-Ret into the compartment. Unlike TrkB translocation, this movement is independent of the kinase activity. In cells lacking GPI-anchored GFR α 1, however, coapplication of GDNF and soluble GFR α 1 allows stabilization of c-Ret in rafts, and this effect is tyrosine kinase-activity dependent. These mechanisms lead to the association of c-Ret with different adaptor

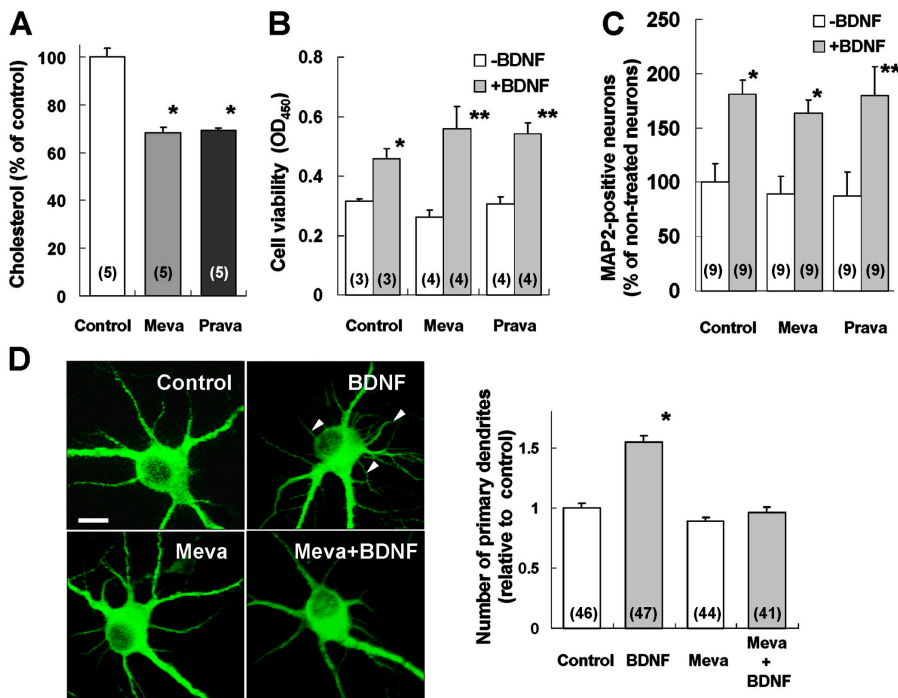


Figure 8. Effects of raft depletion on BDNF regulation of dendritic growth and cell survival. Cultured cortical neurons were treated with or without 10 μ M mevastatin (Meva) or 10 μ M pravastatin (Prava) in the absence or presence of 200 ng/ml BDNF. Membrane cholesterol content, cell survival, or the number of primary dendrites was measured 3 d later. (A) Effect of mevastatin or pravastatin on membrane cholesterol levels. The data are expressed as the percentage of control (nontreated cultures). *Indicates significantly lower than control; $P < 0.001$ (*t* test). $n = 5$ cultures in each group. (B) Effect of mevastatin or pravastatin on cell viability. Viability of cortical neurons was measured by the WST-1 colorimetric assay, and expressed as OD₄₅₀. *t* test; * $P < 0.05$, ** $P < 0.01$. (C) Effect of mevastatin or pravastatin on the number of MAP2-positive neurons. Data are expressed as the percentage of control (nontreated cultures). * $P < 0.01$, ** $P < 0.05$. In both B and C, the number associated with each column represents the number of cultures used in a single experiment, and three independent experiments were performed. No difference was found between the "+BDNF" groups, nor between the "-BDNF" groups (ANOVA test). (D) The effect of mevastatin on BDNF modulation of dendritic growth. (Left) Representative images of MAP2-stained neurons.

After 3-d incubation with BDNF and/or Meva, cells were stained with anti-MAP2 antibody. Note that the number of primary dendrites (arrowheads) is increased by BDNF treatment, and that the effect is completely blocked by mevastatin. Bar, 10 μ m. (Right) The number of primary dendrites was quantified and normalized to that of the control. *Indicates significantly higher than all other groups; ANOVA followed by post hoc test; $P < 0.00002$. The number associated with each column indicates the number of neurons measured in a single experiment, and three independent experiments were performed.

proteins inside rafts and elicit neuronal survival and differentiation. Similar to TrkB-FL, ErbB4 is recruited into lipid rafts by neuregulin. However, it is unclear whether ErbB4 translocation depends on its kinase activity. Although neuregulin translocates the whole receptor complex, including ErbB4 and its adaptor proteins, such as Grb2 and Shc, into rafts, BDNF recruited TrkB alone into lipid rafts, without carrying its associated proteins Shc, Grb2, and PLC γ (Fig. 4 A and Fig. S4 A). However, we cannot completely rule out the possibility that the movement of these associated proteins into rafts was under the detection limit of our immunoblotting analysis. Given that Shc, Grb2, Erks, and PLC γ were present in lipid rafts in an experiment using a milder detergent Triton X-165 (Fig. S4 A), it is possible that the movement of TrkB-FL into rafts allows TrkB to interact with signaling molecules inside the rafts, a step crucial in transmitting lipid raft-mediated BDNF/TrkB signal transduction.

The raft localization of detergent insoluble proteins including RTKs depends on cell type and detergent stringency (Pike, 2003; Schuck et al., 2003). For example, when a less stringent detergent (Triton X-165) was used, a larger amount of TrkB-FL was associated with rafts in naïve cells, and BDNF-induced movement of TrkB-FL from nonrafts to rafts became less apparent (Fig. S4 B). In PC12 cells, both TrkA and p75^{NTR} are constitutively localized in caveolae/lipid rafts (Bilderback et al., 1999; Huang et al., 1999) and NGF binding does not alter the partition of TrkA and p75^{NTR} in lipid rafts (Huang et al., 1999). This may be due to the specific cell types they are expressed or their association with lipid raft-anchored proteins.

Role of BDNF-dependent TrkB translocation into lipid raft in synaptic modulation

The present study has revealed the importance of TrkB translocation into rafts in short-term modulation of synaptic transmission and plasticity by BDNF. In contrast, lipid rafts are not required for BDNF regulation of cortical neuron survival. In neurons, lipid rafts are preferentially distributed in synaptic membranes (Ma et al., 2003). In cerebral cortex *in vivo*, TrkB-FL is present in lipid rafts (Fig. S1 B). Moreover, the endogenous BDNF appears to be enriched in lipid raft fractions in the adult cortex (Fig. S1 B), stored in vesicles in synaptosomes (Fawcett et al., 1997), and secreted at synapses in an activity-dependent manner (Hartmann et al., 2001; Kojima et al., 2001). Thus, the translocation of TrkB into lipid rafts may imply a BDNF-induced recruitment of TrkB into the synapses.

Because BDNF elicits the synaptic effects through presynaptic mechanisms (Gottschalk et al., 1998; Lessmann, 1998), activated TrkB may need to be translocated to the rafts in the presynaptic terminals to interact with its target molecules. The specific target molecules mediating the presynaptic effects of BDNF, however, remain to be fully identified. In purified nerve terminals (synaptosomes) from cerebral cortices, application of BDNF facilitates evoked glutamate release and increases the MAPK-dependent phosphorylation of synapsin I, a synaptic vesicle-associated protein implicated in the control of transmitter release (Jovanovic et al., 2000), suggesting a critical role in the TrkB/MAPK/synapsin signaling cascade. BDNF also rapidly induces MAPK phosphorylation in these slices and inhibition of MAPK signaling blocks BDNF modulation of

HFS response (Gottschalk et al., 1999). In the present study, when TrkB was translocated into lipid rafts, Erks appeared to be activated in the rafts (Fig. 4 B). Assuming that TrkB-FL is rapidly translocated into the rafts in synaptic membranes, activation of Erks in rafts may lead to the phosphorylation of pre-synaptic molecules (e.g., synapsin, synaptobrevin, or synaptophysin) necessary to mediate BDNF effects at CNS synapses.

Materials and methods

Primary cultures

Dissociated cultures of cortical and hippocampal neurons were prepared from embryonic day 20 and cultured. The details were described in On-line supplemental material.

Preparation of lipid rafts and Western blot analysis

Lipid rafts were prepared according to the method of Kawabuchi et al. (2000). Cultured neurons (3.5×10^5 cells/cm²) were rinsed with PBS and quickly lysed in 0.5 ml ice-cold lysis buffer (50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.15 M NaCl, 10 mM NaF, 1 mM Na₃VO₄, 1% Triton X-100, 1 mM PMSF, 10 mM Na₂P₂O₇, 100 μM phenylarsine oxide) and incubated at 4°C for 30 min. For a separate experiment (Fig. S4), 1% Triton X-165 was used. The lysates were mixed with an equal volume of 100% (wt/vol) sucrose in buffer A (50 mM Tris-HCl, pH 7.4, 5 mM NaCl, 1 mM Na₃VO₄, 1 mM PMSF, 100 μM phenylarsine oxide). The mixture was transferred to a centrifuge tube, and 8 ml of 35% (wt/vol) sucrose in buffer A and 3.5 ml of 5% (wt/vol) sucrose in buffer A were overlaid sequentially. After centrifugation at 2×10^5 g for 13 h at 4°C, six fractions were collected from the top of the gradient (the first fraction, 2.5 ml; other fractions, 2.0 ml). To measure cholesterol, 50 μl of each fraction was analyzed with the cholesterol assay kit (Wako). To isolate lipid rafts from cortex, rats of different ages were decapitated and cortex were removed quickly. This procedure was strictly in accord with the protocols approved by the Institutional Animal Care and Use Committee of AIST. Western blot analysis was performed as described previously (Guirland et al., 2004). To determine the concentration of protein, we used BCA protein assay kit (Pierce Chemical Co.).

Recombinant adenovirus

The rat p75^{NTR} cDNA was supplied by E. Shooter (Stanford University, Stanford, CA). The adenovirus vector pAxCawt was kindly provided by I. Saito (University of Tokyo, Tokyo, Japan). For adenovirus generation, see Online supplemental material. The recombinant adenovirus was used at a multiplicity of infection of 5. After 6 d in culture, cells were infected with adenovirus for 24 h followed by a 24-h incubation in serum-free DME to assay.

Glutamate release

For this assay, cultured neurons (6×10^5 cells/cm²) were prepared as described previously (Matsumoto et al., 2001). After 5 h incubation in serum-free medium, the neurons were washed four times with Krebs-Ringer's-Henseleit (KRH) buffer containing 130 mM NaCl, 5 mM KCl, 1.2 mM NaH₂PO₄, 1.8 mM CaCl₂, 10 mM glucose, and 25 mM Hepes, pH 7.4, and pretreated with or without MCD for 10 min followed by BDNF incubation for 30 min. To induce glutamate release, 1 mM 4-AP was applied (Matsumoto et al., 2001). The glutamate released was measured using Amplex red glutamic acid oxidase kit (Molecular Probes).

Exocytosis assay using FM dye destaining

Dissociated neurons (5×10^4 cells/cm²) were cultured on polyethyleneimine-coated glasses (Matsumami; Numakawa et al., 2002). After washing three times with KRH buffer, neurons were pretreated with or without MCD, MCD-cholesterol complex or filipin for 10 min followed by BDNF incubation for 30 min. FM dye (2 mM; Molecular Probes) was loaded by incubating the cells with the high K⁺ solution (KRH buffer containing 56 mM KCl and 79 mM NaCl) for 1 min at 37°C. After washing the cells with KRH buffer, fluorescence images of FM-labeled spots were taken every second using fluorescence microscope (model IX70; Olympus) equipped with a Cool SNAP HQ CCD camera (Roper Scientific) and a 20 × 0.8 NA objective (Olympus). The baseline intensity was at 4 s before the stimulation with high K⁺ solution. To induce FM dye destaining, neurons were depolarized with high K⁺ solution. FM-labeled spots/50-μm dendrite were analyzed using a quantification menu of the MetaMorph software (Universal Imaging Co.).

Slice preparation and electrophysiology

Recording of hippocampal slices, prepared from neonatal rat (P12-13), was described previously (Figurov et al., 1996). Field EPSPs were evoked in CA1 stratum radiatum by stimulating Schaeffer-commissurals and were recorded with ACSF-filled glass pipettes (<5 MΩ). Only slices exhibiting EPSPs of 2–3 mV in amplitude without superimposed population spikes were used. Stimulus intensity was adjusted to evoked EPSPs of ~1.3 mV. A train of HFS (100 Hz, 1 s) was used to induce synaptic depression. All experimental data were collected at least 10 min after stable EPSPs were achieved. Slices were treated with MCD (2 mM) or BDNF (2 nM) or them together for 2 h before synaptic responses to HFS were tested.

Assays of cell survival and dendritic growth

Cortical neurons (5×10^4 cells/cm²) were cultured in Neurobasal medium containing B27 supplement (GIBCO BRL), 0.5 mM glutamine, for 3 d, and treated with indicated agents in Neurobasal medium containing no B27 supplement for 3 d. Cell survival was quantitated by WST-1 assay, which determines cell viability based on the cleavage activity of a soluble tetrazolium salt WST-1 by mitochondrial dehydrogenases. In brief, cultured cortical neurons were incubated with a tetrazolium salt WST-1 (Roche) for 30 min and viable cells were then determined by a microplate reader at 450 nm with 650 nm as a reference wavelength. Alternatively, the number of MAP2-positive neurons was counted (Yamada et al., 2001). To identify primary dendrites from each neuron, cells were stained with anti-MAP2 antibody, followed by DAPI staining. After taking images of MAP2-positive neurons with cell body of diameter (16.9 ± 0.5 μm diam, $n = 32$ cells from four independent coverslips), the number of the primary dendrites was counted.

Online supplemental material

Fig. S1 shows localization of TrkB and BDNF in lipid rafts during cortical development. Fig. S2 shows association of TrkB-FL with lipid rafts 1 min after BDNF stimulation. Fig. S3 shows TrkB-T1 expression in rafts and non-rafts and NT-4-induced recruitment of TrkB-FL into lipid rafts. Fig. S4 shows association of signaling molecules and TrkB with lipid rafts. Fig. S5 shows that treatment with 10 mM MCD lead to a partial decrease in BDNF-induced activation of TrkB-FL. Further comments on the data reported can be found in the legends. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.200404106/DC1>.

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