Brain-derived Neurotrophic Factor Regulates Surface Expression of α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic Acid Receptors by Enhancing the N-Ethylmaleimide-sensitive Factor/GluR2 Interaction in Developing Neocortical Neurons*

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In hippocampal neurons, the exocytic process of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-type glutamate receptors is known to depend on activation of N-methyl-d-aspartate channels and its resultant Ca2⁺ influx from extracellular spaces. Here we found that brain-derived neurotrophic factor (BDNF) induced a rapid surface translocation of AMPA receptors in an activity-independent manner in developing neocortical neurons. The receptor translocation became evident within hours as monitored by [3H]AMPA binding and was resistant against ionotrophic glutamate receptor antagonists as evidenced with surface biotinylation assay. This process required intracellular Ca2⁺ and was inhibited by the blockers of conventional exocytosis, brefeldin A, botulinum toxin B, and N-ethylmaleimide. To explore the translocation mechanism of individual AMPA receptor subunits, we utilized the human embryonic kidney (HEK) 293 cells carrying the BDNF receptor TrkB. After the single transfection of GluR2 cDNA or GluR1 cDNA into HEK/TrkB cells, BDNF triggered the translocation of GluR2 but not that of GluR1. Subsequent mutation analysis of GluR2 carboxyl-terminal region indicated that the translocation of GluR2 subunit in HEK293 cells involved its N-ethylmaleimide-sensitive factor-binding domain but not its PDZ-interacting site. Following co-transfection of GluR1 and GluR2 cDNAs, solid phase cell sorting revealed that GluR1 subunits were also able to translocate to the cell surface in response to BDNF. An immunoprecipitation assay confirmed that BDNF stimulation can enhance the interaction of GluR2 with N-ethylmaleimide-sensitive factor. These results reveal a novel role of BDNF in regulating the surface expression of AMPA receptors through a GluR2-NSF interaction.

The neurotrophins, nerve growth factor, brain-derived neurotrophic factor (BDNF), neurotrophin-3, and neurotrophin-4/5, are implicated as important regulators of synaptic development and plasticity in both central and peripheral nervous systems. In particular, activity-dependent synthesis and release of neurotrophins are suggested to be key modulators of neuronal development and synaptic plasticity. BDNF can exert strong neurotrophic activities on immature brain neurons with a subchronic time scale of days. BDNF enhances the dendritic growth, induces synaptogenesis (1, 2), and induces various neurochemical phenotypes of developing excitatory and inhibitory neurons (3–6). The biological activity of neurotrophins on the induction of postsynaptic components and/or development of postsynaptic neurons needs to be evaluated, and their underlyling molecular mechanisms remain to be characterized in various types of neurons.

In addition to such chronic activity of BDNF for days, application of BDNF to cultured neurons enhances excitatory glutamatergic synaptic transmission with increased efficacy of glutamate release from the presynaptic neuron (7–10). Neurotrophins also acutely enhance postsynaptic responsiveness of N-methyl-D-aspartate (NMDA)-type glutamate receptors in mature synapses (11). In addition, endogenous neuronal BDNF is required for various forms of synaptic plasticity (12–14). However, the latest studies demonstrate that BDNF is released from nerve terminals in an activity-dependent manner and act on target neurons or postsynaptic sites (15, 16). Accordingly, anterograde neurotrophic functions of BDNF have now been established (17).

A large variety of molecules interacting with α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor subunits have been identified and characterized; SAP97 associates with the COOH termini of GluR1 subunits, whereas GRIP/ABP and Pick1 bind to those of GluR2 and GluR3 subunits (18–21). The intracellular domain of GluR1 protein associates with various signal transducers as well, such as G protein, and that of GluR2 with Lyn and N-ethylmaleimide-sensitive factor (NSF) (22–25). The interacting proteins carrying PDZ domains are suggested to cooperate to regulate the dynamics, targeting, and clustering of AMPA receptors at the postsynaptic membrane of the hippocampal neurons (19, 26–28). Similarly, the NSF-GluR2 interaction is also suggested to have some roles in the subcellular dynamics of AMPA receptors in such excitatory neurons. NSF might contribute to synaptoplasmic assault on synaptic plasticity.
aptic plasticity as evidenced by electrophysiological studies: Disruption of the interaction between GluR2 and NSF with a decay peptide influences hippocampal long term depression (29, 30). In contrast to the above information about AMPA receptors in hippocampal neurons, the characteristics of the AMPA receptor dynamics in other types of neurons are largely unknown.

We recently reported that chronic BDNF treatment increases and maintains total protein levels of AMPA receptor subunits in neocortical cultures without influencing their mRNA expression (4, 31). Consistent with this finding, there is impaired synaptic AMPA receptor expression in BDNF knockout mice (4). This process presumably depends on a post-translational mechanism and requires chronic stimulation of BDNF for days. In the present study, we found that BDNF triggers dynamic subcellular movement of AMPA receptors in cultured neocortical neurons. Using primary cultured neurons as well as the heterologous system of human embryonic kidney (HEK) cells, we explored the molecular mechanism underlying the rapid BDNF effects on surface translocation of AMPA receptors. We characterized this cellular phenomenon, paying attention to the difference between this BDNF-dependent process and the NMDA receptor-dependent plasticity reported previously. The potential contribution of the individual subunit regulation to the dynamics of the whole receptor complex is also examined and discussed.

EXPERIMENTAL PROCEDURES

Cell Culture—Whole cerebral neocortices of embryonic day 18 rats or neonatal rats were mechanically dissociated and plated onto poly-d-lysine-coated dishes at a low density (400–600 cells/mm²). Cortical neurons were grown in serum-free condition or in serum-containing medium as described previously (4, 31, 32). Purified human recombinant BDNF (50 ng/ml) was added 4–5 days after plating. HEK293 cells were transfected with a vector carrying the mouse TrkB cDNA in pBluescript (provided by Dr. S. Kozumi) with Ca²⁺-phosphate method. Cells were selected in medium containing 400 μg/ml Genetixnic, and the drug-resistant colonies were isolated and expanded (HEK-TrkB cells). HEK-TrkB cells were transiently transfected with cDNA for GluR1, GluR2, or its derivatives using the lipofection method, and their expression and subcellular dynamics were analyzed using Western blotting and immunostaining methods, respectively. Prior to the BDNF treatment, some cultures were pre-incubated with the reagents known to influence receptor internalization or endocytosis, brefeldin A (5 μg/ml; Wako Chemicals), nocodazole (1 μM; Sigma), calphostin C (1 μg/ml; Calbiochem), and 0.5 μM KN93 (Wako Chemicals) or with translation inhibitors (2 ng/ml cycloheximide Sigma) and 30 μM anisomycin (Sigma).

Surface Biotinylation and Precipitation—Neocortical cultures or HEK-TrkB cells were treated with 100 ng/ml BDNF for 30 min to 24 h and then incubated with 1 μg/ml sulfo-NHS-LC-biotin (Pierce) in phosphate-buffered saline containing 1 μM CaCl₂ and 1 μM MgCl₂ for 15 min on ice (33). Cell lysate was incubated with immobilized streptavidin-beaded agarose (Pierce) overnight at 4 °C. Biotinylation proteins were eluted with 2% sodium dodecyl sulfate (SDS) buffer at 95 °C and processed for Western blotting analysis.

Western Blotting—Cells were lysed in Laemmli sample loading buffer (10% glycerol, 2% SDS, 65 mM Tris). After centrifugation at 95 °C for 10,000 x g for 10 min, protein in supernatants was denatured at 95 °C in the presence of 0.5% 2-mercaptoethanol and protein samples (15 μg/lane) were separated by SDS-polyacrylamide gel electrophoresis (PAGE) with 1-mm-thick, 8% polyacrylamide slab gels. Protein was transferred to a nitrocellulose membrane by electrophoresis. Primary antibodies were diluted with blocking solution and incubated with the membrane at 4 °C overnight. Immunoreactivity was detected with goat anti-rabbit immunoglobulin conjugated to peroxidase (1:10,000) followed by chemiluminescence reaction combined with film exposure (ECL kit; Amershams Biosciences, Tokyo, Japan). The following primary antibodies were used: affinity-purified rabbit anti-GluR1 COOH terminus (Chemicon International, Temecula, CA), mouse monoclonal anti-GluR2 NH₂ terminus (Chemicon International), rabbit polyclonal anti-GluR2 antibody 397, Chemicon International) and the rabbit polyclonal antibody against the anti-GluR2/3 intracellular domain (made to a 50-amino acid peptide corresponding to the COOH terminus of GluR2 (4), rabbit antisera to NSF (a gift of Dr. M. Takahashi, 0.5 μg/ml), and mouse monoclonal pan-cadherin antibody (Sigma). The immunoreactivity of the bands was quantified by densitometric analysis.

[³H]AMPA Binding Assay—Cultured neurons or HEK-TrkB cells were rinsed with Tris buffer, pH 7.4, containing 100 μM sodium acetate, 2.5 mM CaCl₂, and 5 μl/iter glucose. [³H]AMPA (1,561 GBq/mmol, 37 MBq/ml, PerkinElmer Life Sciences) was adjusted to 50 nM with the Tris buffer and diluted with 0 to 800 nM cold AMPA (Sigma). Cells were incubated with these AMPA solutions on ice for 60 min as described previously (34). An excess amount of cold AMPA (100 μM) was applied to culture to estimate nonspecific [³H]AMPA binding to cells. After rapid washing with cold Tris buffer, cells were lysed with 0.5 N NaOH overnight at 4 °C. The bands was quantified by densitometric analysis.

Solid Phase Cell Sorting—Cell sorting was performed using a laser scanning cytometer (LSC2, Olympus). Co-transfected HEK293 cells in live culture were immunostained with anti-GluR1 NH₂-terminal antibodies (30 μg/ml) (35) followed by the FITC-conjugated secondary anti-rabbit immunoglobulin antibody (Jackson Laboratories, Bar Harbor, ME). After fixation, cells were incubated with an anti-GluR2 NH₂-terminal mouse monoclonal antibody (20 μg/ml), monoclonal antibody 397, Chemicon International) and the rabbit polyclonal antibody against the anti-GluR2/3 intracellular domain (10 μg/ml) (4). Total immunoreactivity for GluR1 and GluR2 was visualized with the Cy5-labeled secondary anti-rabbit immunoglobulin antibody (2.5 μg/
ml; Amersham Biosciences) and the R-phycocerythrin-labeled secondary anti-mouse immunoglobulin antibody (2.5 µg/ml; PharMingen, San Diego, CA), respectively. An argon laser (488 nm) was used to excite FITC and R-phycocerythrin, and a helium/neon laser (633 nm) was used for Cy-5.

Deletion Mutagenesis of GluR2—The COOH-terminal five amino acids (aa 858–862) and 20 amino acids (aa 843–862) of mouse GluR2 were deleted, using the pcDNA plasmid as a template. The deletion of the NSF binding region (aa 823–832; KRMVKAKNAQ) was performed with a site-directed mutagenesis kit (Clontech, Palo Alto, CA) according to the instructions from the manufacturer. Nucleotide deletion was confirmed by DNA sequencing, and the expression of the GluR2 mutant proteins was verified by Western blotting analysis.

Immunoprecipitation—HEK-TrkB cells were harvested in immunoprecipitation buffer containing 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5 mM ATP-S (Roche Molecular Biochemicals, Mannheim, Germany), 2 mM EDTA, 1 mM EGTA, and 1% sodium deoxycholate. Cell lysates were centrifuged at 10,000 × g for 10 min at 4 °C, and the supernatant was used for co-immunoprecipitation (36). Neocortices from young rats (postnatal day 3–4) were homogenized in immunoprecipitation buffer without deoxycholate, containing 5 µg/ml affinity-purified chicken anti-BDNF antibodies (4), and homogenization was performed on ice, using a Handy Sonic tissue grinder (Tomy Inc., Tokyo, Japan). The lysate was treated with 200 ng/ml BDNF for 30 min at 37 °C and centrifuged at 10,000 × g for 60 min at 4 °C, and the pellet was solubilized in immunoprecipitation buffer containing 1% sodium deoxycholate. All the cleared supernatants were pretreated with excess amounts of protein A-Sepharose and were then incubated with anti-GluR2 NH2-terminal antibodies (4 µg/ml; monoclonal antibody 397, Chemicon International) at 4 °C for 2 h. The immunocomplex was precipitated with protein A-Sepharose and was washed five times with immunoprecipitation buffer, boiled in 2× SDS buffer, and subjected to SDS-PAGE followed by Western blotting with anti-NSF antibody.

Statistical Analysis—Statistical analysis was performed using one-way analysis of variance followed by the Bonferroni test (for multiple factors). Alternatively, Student’s t test was applied to data with multiple variables and one factor. For data from cell sorting, the Mann-Whitney U test was applied. Results were obtained with multiple sister cultures or independent cultures and expressed as means ± S.E. (n = 3–6 cultures).

RESULTS

Effects of BDNF on Surface AMPA Receptors in Neocortical Neurons—To identify whether BDNF stimulation can influence functional AMPA receptor on the cell surface or not, [3H]AMPA binding assay was performed with cultured neocortical neurons grown for 5 days and then treated with or without BDNF (50 ng/ml). After a 30-min incubation with BDNF, various concentrations of [3H]AMPA were applied to cultures and the binding of the radioactive ligand to neurons was measured. The values of bound [3H]AMPA were subjected to the Scatchard plot analysis, and the total number (Bmax) and the dissociation constant (Kd) of the functional AMPA receptors were determined on the cell surface (Fig. 1A). The Bmax of the AMPA receptors on the cell surface increased from 3.88 ± 0.05 to 4.96 ± 0.27 pmol/mg (n = 4, p < 0.05). The dissociation constant of the AMPA receptors (Kd) increased from 192 ± 18 to 286 ± 26 nM. The time course of the maximum ligand binding activity of the AMPA receptors (Bmax) was assessed by stimulating neurons

| Reagent | Control | +BDNF |
|---------|---------|-------|
| A.      |         |       |
| Control | 100 ± 15| 171 ± 12**|
| 1 µM baclofen A | 168 ± 16** | 206 ± 10* |
| 5 µg/ml brefeldin A | 111 ± 15 | 136 ± 11 |
| 1 mM NEM | 102 ± 24 | 133 ± 17 |
| 100 mM botulinum toxin | 89 ± 6 | 101 ± 11 |
| B.      |         |       |
| Control | 100 ± 3 | 150 ± 6*** |
| 5 mM BAPTA | 84 ± 5* | 132 ± 5** |
| 0.1 mM BAPTA-AM | 85 ± 17 | 95 ± 4 |
| C.      |         |       |
| Control | 100 ± 2 | 153 ± 2*** |
| 1 µM calphostin C | 87 ± 2* | 96 ± 6 |
| 10 µM U73122 | 45 ± 1*** | 135 ± 8*** |
| 25 µM PD98059 | 99 ± 2 | 120 ± 2* |
| 0.5 µM KN93 | 58 ± 1** | 84 ± 5*** |

Fig. 2. BDNF triggers cell surface redistribution of AMPA receptors. A, surface GluR1 and GluR2/3 proteins of neurons, which were treated with or without BDNF for 24 h (n = 3 sister cultures), were labeled with a membrane-impermeable biotinylation reagent, sulfo-NHS-LC-biotin, and processed for Western blotting analysis with antibodies against GluR1 and GluR2/3. B. 10% of the total protein lysates prior to the streptavidin precipitation were subjected to Western blotting and are indicated as total protein. C, surface GluR1 and GluR2/3 proteins of neurons in culture (n = 4 sister cultures), which were pretreated with 10 µM CNQX and 50 µM AP-5, were measured as described above. Note that the difference between A and C was within an experimental variation. *, p < 0.05; **, p < 0.01.
with BDNF for various periods of time and then determined by exposing them to a submaximal concentration of $[^{3}H]$AMPA (250 nM; Fig. 1B). The AMPA binding to neocortical culture became evident after a 30-min incubation with BDNF and further increased until 24 h of incubation ($277 \pm 10\%$ of control, $n = 4$, $p < 0.0001$). These results reveal that BDNF triggers rapid subcellular redistribution of AMPA receptors, leading to the functional AMPA receptor increase on the cell surface.

**BDNF-triggered AMPA Receptor Translocation Reflects an Exocytotic Process Involving Intracellular Ca$^{2+}$**—To determine the mechanism of this rapid redistribution of AMPA receptors, several reagents known to influence cellular processes for exocytosis (brefeldin A, botulinum toxin B, and NEM) or endocytosis (bafilomycin A) were applied to primary cultures of neocortical neurons prior to BDNF treatment (7) (Table I, part A). After BDNF stimulation, the number of AMPA receptors on the cell surface was estimated by ligand-binding assay with $[^{3}H]$AMPA. Bafilomycin A, which blocks trans-Golgi transport to inhibit endocytosis, increased basal levels of surface AMPA receptors and BDNF further elevated their levels. In contrast, the exocytosis blockers, brefeldin A, botulinum toxin B, and NEM, inhibited or reduced the BDNF effects. The drug sensitivity suggests that the BDNF-triggered increase in AMPA receptors reflects an exocytotic process of the receptors.

Second, we assessed the involvement of Ca$^{2+}$ using its chelator, BAPTA, and its derivative. Intracellular Ca$^{2+}$ was trapped with cell-permeable BAPTA-AM, whereas extracellular Ca$^{2+}$ was chelated with nonpermeable BAPTA (Table I, part B). Under this condition, cultured neocortical neurons were incubated with BDNF for 3 h. BAPTA-AM inhibited the BDNF-triggered increase in $[^{3}H]$AMPA binding. In contrast, BAPTA had no significant effect. The above findings suggest that the surface translocation of AMPA receptors by BDNF involves the exocytotic pathway triggered by intracellular Ca$^{2+}$ (37).

BDNF is known to trigger intracellular signaling cascades of phospholipase C and mitogen-activated protein kinase. In addition, protein kinase C (PKC) and aCAMKII are often implicated in the subcellular dynamics of AMPA receptors (26–28). To explore intracellular signals leading to the surface AMPA receptor increase, cultured neurons were stimulated with BDNF in the presence of kinase inhibitors, U73122 (phospholipase C), PD98059 (mitogen-activated protein kinase), calphostin C (PKC), and KN93 (aCAMKII) (Table I, part C). Calphostin C almost abolished the BDNF-dependent increase in $[^{3}H]$AMPA binding. Although U73122 and KN93 themselves markedly reduced the basal $[^{3}H]$AMPA binding, BDNF treatment still markedly up-regulated the $[^{3}H]$AMPA binding in the presence of these inhibitors. This observation suggests that PKC kinase may involve the BDNF-dependent translocation of AMPA receptors. As the basal amount of $[^{3}H]$AMPA binding was modestly influenced by BAPTA and BAPTA-AM, as well as by the other kinase inhibitors, there might be an alternative signaling pathway that maintains the basal surface AMPA receptor levels.

**Differential Subcellular Redistribution of AMPA Receptor Subunits without Activity-dependent Processes**—To confirm the BDNF effects on subcellular distributions of individual AMPA receptor subunit proteins, cultured neocortical neurons were treated with or without BDNF. Following the treatment, proteins on cell surfaces were biotinylated, collected with streptavidin-coupled beads, and subjected to Western blotting. The biotinylated (cell surface-expressed) GluR1 and GluR2/3 proteins were markedly increased by BDNF treatment compared with nontreated cells (Fig. 2A). In parallel, AMPA receptor protein levels were also increased in total cellular lysates (Fig. 2B).

BDNF is known to trigger glutamate release from cultured neurons (32). Is the activation of glutamate receptors involved in the surface increase of AMPA receptors? To examine the contribution of endogenous glutamate to the BDNF effect, both NMDA-type and AMPA-type glutamate receptors were blocked by their inhibitors AP-5 and CNQX in culture, respectively (Fig. 2C). In the presence of these blockers, BDNF similarly increased the surface expression of the AMPA receptor subunits. These results suggest that BDNF significantly altered the levels of the AMPA receptors on neuronal surface, irrespective of excitatory synaptic activities.

**Reconstitution of the BDNF-triggered AMPA Receptor Translocation in HEK293 Cells**—To explore the above molecular mechanism for the surface translocation of AMPA receptors, we attempted to reconstitute AMPA receptor translocation in non-neuronal cells. This system presumably excluded the involvement of presynaptic elements or activity-dependent processes. HEK293 cells carrying TrkB receptors were established.
with permanent integration of human TrkB gene under the cytomegalovirus promoter. By transfecting cDNA for each AMPA receptor subunit into the HEK-TrkB cells, the influences of BDNF on the surface expression of each receptor subunit, GluR1 or GluR2, were evaluated by the cell surface biotinylation assay. BDNF triggered an increase in surface protein levels of GluR2 as determined after a 5-h incubation with BDNF (Fig. 3A). The up-regulation of surface expression was pronounced for GluR2 (392 ± 8% of control, n = 3, p < 0.01). In contrast, surface expression of GluR1 as well as that of cadherins was not significantly influenced (103 ± 15% and 106 ± 7% of control, respectively). This may eliminate the possibility that BDNF simply facilitates general turnover of intracellular membranes. The cell surface distribution of the active AMPA receptor subunits was ascertained by the ligand-binding assay using [3H]AMPA (Fig. 3B). When these cells were stimulated with BDNF, [3H]AMPA binding activity on the cell surface was markedly increased in the HEK-TrkB cells carrying GluR2 but not GluR1. These results indicate that the effects of BDNF on translocation specifically involve the GluR2 subunit in HEK-TrkB cells.

Contribution of Protein Synthesis to the Surface Expression of AMPA Receptors—We have found that BDNF can enhance translation rates to increase protein synthesis in cortical neurons (38). Thus, we assessed potential contribution of protein synthesis to the surface AMPA receptor increase both in cortical neurons and HEK293 cells. The total protein increase in GluR1 and GluR2/3 levels lagged behind the rapid surface increase in AMPA binding; a 5-h incubation with BDNF failed to alter GluR1 and GluR2/3 levels in total protein lysate from cortical culture (Fig. 4A), whereas the same treatment increased surface [3H]AMPA binding in cortical culture and surface AMPA receptor biotinylation on HEK-TrkB cells (see Figs. 1 and 3).

We also examined the effects of translation inhibitors, cycloheximide and anisomycin (Fig. 4B). The inhibition of new protein synthesis and supply significantly resulted in a decrease in basal AMPA binding activity (56.6 ± 5.1%, n = 4, p < 0.01 for cycloheximide; and 67.2 ± 6.4%, n = 4, p < 0.05 for anisomycin), suggesting the contribution of protein synthesis/supply to the maintenance of a basal surface receptor level. Even in the presence of the translation inhibitors, however, BDNF stimulation produced the same magnitude of the increase in AMPA binding activity as seen in control BDNF treatment; surface AMPA binding to cortical neurons was increased ~2-fold following a 3-h incubation with BDNF and the translation inhibitors. In addition, the AMPA binding activity of the BDNF- and anisomycin-treated culture was significantly higher than that of untreated control culture (135 ± 9.5%, n = 4, p < 0.05). In cultured HEK-TrkB cells, cycloheximide also failed to prevent the AMPA receptor translocation induced by BDNF. The surface biotinylation assay following transfection of GluR2 cDNA revealed that surface AMPA receptor levels were significantly increased by a 5-h incubation with BDNF, even in the presence of the translation inhibitor (38.7 ± 1.0% for cycloheximide alone and 74.7 ± 2.0% for cycloheximide plus BDNF, n = 3, p < 0.05), although the basal surface receptor level was reduced again in the absence of new protein supply during incubation with cycloheximide. These results indicate that the BDNF-triggered AMPA receptor translocation is independent from the BDNF-mediated translational regulation (38).

BDNF Regulation of GluR2 Insertion into the Plasma Membrane by Exocytosis—To compare the mechanism of this rapid redistribution of AMPA receptors in HEK293 cells with that in neurons, the same reagents that block exocytosis (brefeldin A and NEM) or exocytosis (bafilomycin A) were added to HEK-TrkB culture prior to BDNF application (Fig. 5). After a 3-h incubation with BDNF, cell surface-expressed GluR2 protein was examined on HEK-TrkB cells by surface biotinylation. Brefeldin and NEM reduced the basal level of the surface-expressed GluR2 protein and inhibited the BDNF-mediated cell surface redistribution as well. Bafilomycin A failed to in-

**Fig. 4. Interaction between the BDNF-mediated AMPA receptor increase and protein synthesis.** A, total GluR1 and GluR2/3 protein levels in cultured cortical neurons, which were incubated with or without BDNF for 5 h (n = 3 sister cultures), were determined by Western blotting analysis with antibodies against GluR1 and GluR2/3. Basal expression of GluR1 and GluR2/3 proteins was set to 100%. No significant change was observed. B, effects of translation inhibitors on the ligand binding activity of AMPA receptors were determined using 250 nM [3H]AMPA (see Fig. 1). Cultured cortical neurons were pretreated with cycloheximide (2.0 ng/ml) or anisomycin (30 µM) and then incubated with BDNF for 3 h. The presence of the translation inhibitors reduced protein synthesis to <1% as measured by [35S]methionine incorporation (data not shown) (38). C, the BDNF effects on surface GluR2 expression were determined in HEK-TrkB cells by biotinylation after a 5-h incubation with BDNF (50 ng/ml) plus cycloheximide (2 ng/ml) or BDNF alone. Prior to the BDNF treatment, an expression vector carrying GluR2 cDNA was transferred into HEK-TrkB cells. Statistical comparison was done in each group with and without BDNF (**, p < 0.01; ***, p < 0.001), between untreated control and anisomycin- or cycloheximide-treated culture (#, p < 0.05; ##, p < 0.01) or between untreated control and anisomycin/BDNF- or cycloheximide/BDNF-treated culture (+, p < 0.05).
Bafilomycin, a known inhibitor of the vacuolar H^+ ATPase, significantly reduced the basal surface expression of GluR2 protein, as determined by biotinylation assay. The exocytotic process (brefeldin A) and the endocytotic process (bafilomycin) were blocked by using their respective reagent. NEM, which blocked endocytosis, did not inhibit the BDNF effects, whereas Bafilomycin with bafilomycin (327/11006/40906) significantly inhibited the BDNF effects. The target protein of the chemical reagent NEM is known as NEM-sensitive factor (NSF), which is implicated in intracellular trafficking (39). The result indicates that BDNF accelerates the surface translocation of GluR2 subunits in non-neuronal cells through NSF-dependent exocytotic processes.

**BDNF Actions on GluR2 Carboxyl Termini**—Two regions have been identified in GluR2 COOH-terminal domain that interact with other proteins (40): the extreme COOH-terminal five amino acids of the PDZ binding region (18–21) and an NSF binding motif (24, 25, 36). The PDZ proteins interacting with GluR2 subunits include GRIP1, ABP/GRIP2, and Pick1 (41, 42). These interacting proteins are implicated in coordinating the targeting and clustering of AMPA receptors at the postsynaptic membrane. To assess whether BDNF acts on the GluR2 COOH-terminus interacting proteins, we generated GluR2 constructs containing a truncated PDZ binding domain (∆Δ and ∆20) and an NSF binding domain (∆NSF) to disrupt their interactions (Fig. 6A). Western blot analysis confirmed equal levels of GluR2 expression in each construct in HEK-TrkB cells. Endogenous NSF levels are shown as a control. C, 3 h after BDNF application, the cell surface redistribution of GluR2 protein was detectable by biotinylation assay in wild type GluR2 (*, p < 0.01; **, p < 0.005; n = 3 each).

The interaction of GluR2 with NSF in the above paradigm, immunoprecipitation with an anti-NH2-terminal GluR2 monoclonal antibody, was performed in cell lysates prepared from HEK-TrkB cells or from the neocortical tissue (Fig. 7). Increased association of GluR2 and NSF after BDNF treatment was observed in immunoprecipitates from both preparations. All these results indicate that BDNF regulates AMPA receptor redistribution by modulating the NSF-GluR2 interaction.

**Influences of BDNF on Subcellular Dynamics of the AMPA Receptor Complexes**—There are a variety of combinations of AMPA receptor subunits that can form the channel complexes (43). Accordingly, it raises the question as to how the heterooligomeric complexes of AMPA receptor subunits, such as GluR1/GluR2, behave in response to BDNF. We addressed this question using the double transfection protocol into HEK-TrkB cells with an aid of a three-channel laser scanning cytometer (i.e., solid-phase cell sorter) (Fig. 8). The lipofection protocol achieved 40–50% efficiency of gene transduction into HEK-TrkB cells.

The subcellular localization of GluR1 was monitored by its...
BDNF increases the association of GluR2 and NSF.

Neocortices from young rats (postnatal day 3–4) or cultured HEK-TrkB cells were homogenized in the immunoprecipitation buffer containing ATPγS and 1% sodium deoxycholate. Half of the cell lysate was stimulated with BDNF for 30 min, and the other half was not. Both were subjected to immunoprecipitation with the anti-GluR2 NH2-terminal antibody. A, immunoprecipitation assay was performed in cell lysate from HEK-TrkB cells. B, immunoprecipitation assay was similarly performed in tissue lysate from the neocortex. BDNF increased association of GluR2 and NSF in immunoprecipitates from both preparations ($n = 5$ each; **, $p < 0.01$).

**DISCUSSION**

In the present study, we found that BDNF is a potent regulator of AMPA receptor dynamics in developing neocortical neurons. The ligand binding assay, the cell surface biotinylation, and the cell sorting assay all indicate that AMPA receptor complexes carrying GluR2 subunits can be translocated from the cytoplasmic pool to the cell surface membranes in response to BDNF stimulation. The neurotrophin-dependent regulation of AMPA receptor redistribution occurred on a fairly rapid time scale of minutes to hours and preceded the cellular increase in the AMPA receptor content. The exocytotic process for AMPA receptors appears to depend on the interaction of GluR2 subunits with NSF and on intracellular Ca$^{2+}$ influx. The inhibitors for CaMKII as well as for translation failed to block this process. This contrasts well with the finding that activated CaMKII plays a central role in the AMPA receptor exocytosis responsible for hippocampal long term potentiation (27, 44–46). Thus, the BDNF-dependent process might form a basis for unique plasticity or development of AMPA receptor-carrying postsynaptic membranes of neocortical neurons.

Using HEK-TrkB cells, we further assessed the molecular contribution of each AMPA subunit and its domains to the subcellular translocation of the receptor complexes. In response to BDNF, wild-type GluR2 subunits were redistributed to the cell surface. Wild-type GluR1 as well as GluR2 mutants lacking the NSF binding domain, however, failed to translocate to the cell surface. Although the results from the HEK-TrkB cells agreed with those from neocortical neurons, we cannot deny the possibility that intracellular trafficking of AMPA receptor subunits or complexes may differ in real neurons (44–46).

Presynaptic and Postsynaptic Mechanisms of BDNF Activity—Neurotrophins have been extensively studied with respect to their developmental activity on growing presynaptic components and functions (47, 48). BDNF induces expression of presynaptic machinery and synthesis of neurotransmitters chronically to enhance presynaptic maturation (32). Recent biochemical and cellular localization studies of BDNF revealed that BDNF is stored in presynaptic vesicles and released toward postsynaptic neurons in an activity-dependent manner (15, 16). The evidence for its anterograde trophic activity ascertains the biological importance of the BDNF-mediated AMPA receptor regulation at developing postsynaptic areas. Vesicular dynamics at both pre- and post-synaptic sites presumably involve an NSF-dependent soluble NSF attachment protein receptor mechanism (39, 49). The surface translocation of AMPA receptor subunits followed various pharmacological features of typical exocytotic processes. The blockers of exocytosis, brefeldin A, botulinum toxin B, and NEM, all suppressed the BDNF effect. This process was slightly different from the conventional exocytotic process, as seen in neurotransmitter release. The exocytosis-like processes for the AMPA receptors had a different Ca$^{2+}$ requirement. The postsynaptic translocation of AMPA receptors involves an efflux of Ca$^{2+}$ from the intracellular stores, whereas presynaptic release of neurotransmitters requires a Ca$^{2+}$ influx from extracellular spaces. In agreement, BDNF is known to trigger an intracellular Ca$^{2+}$ influx, activating TrkB (50).

The enzyme blockers for PKC kinase prevented the surface translocation of AMPA receptors. It is consistent with the report that TrkB receptor directly activates this protein kinase (51). In particular, the activation of PKC is noteworthy because Matveeva et al. (52) demonstrated that PKC, which is activated by a calcium influx, phosphorylates NSF. A similar process might be recruited in the BDNF-dependent interaction between NSF and the AMPA receptor subunit. Subsequent studies should examine whether the activation of PKC kinase fol-
following BDNF stimulation is able to enhance the interaction between NSF and the AMPA receptor subunit.

Contribution of BDNF to AMPA Receptor Plasticity—Strength of the BDNF-dependent AMPA receptor expression in neocortical culture was influenced by cell density and culture age (31). Dense culture or long term culture resulted in a higher basal level of AMPA receptors and made the biochemical detection of the receptor translocation difficult (data not shown). Our serum-free neocortical culture consisted of various neuronal populations including pyramidal neurons and non-pyramidal neurons. Our preliminary studies suggest that the major population of neurons responding to BDNF was non-pyramidal neurons in the cortical culture. In this context, the ineffectiveness of the αCAMKII inhibitor on the BDNF-dependent AMPA receptor translocation is in reasonable agreement with the fact that αCAMKII is absent from neocortical non-pyramidal neurons (53). The specificity of the BDNF action on non-pyramidal neurons might explain the above effect of culture duration; the surface AMPA expression of pyramidal neurons, which constitutes more than 70% of cortical cells, masked the increase in non-pyramidal neurons, especially when the endogenous neuronal activity occurs in culture and facilitates surface translocation of AMPA receptors in pyramidal neurons in a αCAMKII-dependent manner. In agreement, we found significant influences of the αCAMKII inhibitor and the calcium chelator only on basal levels of the surface AMPA receptors in our mixed culture.

Exocytotic processes or periplasmic movement of AMPA receptor channels is implicated in synaptic plasticity of estab-

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2 M. Narisawa-Saito, Y. Iwakura, M. Kawamura, K. Araki, S. Kozaki, N. Takei, and H. Nawa, unpublished data.
lished brain circuits (44, 45). αCAMKII activation leading to long term potentiation is associated with postsynaptic translocation of AMPA receptors from intracellular pools in glutamatergic pyramidal neurons, involving the AMPA receptor subunit, GluR1 (27, 46). Alternatively, there might be a lateral movement of the receptors from persynaptic pool of the postsynaptic membrane (54). On the other hand, recent studies suggest that the AMPA receptor subunit, GluR2, is subjected to constitutive exocytosis or synaptic recycling in such excitatory neurons (29, 30, 49). Neuronal activity and the resultant influx of extracellular Ca2+ are known to trigger the GluR1-dependent receptor dynamics in hippocampal pyramidal neurons (46, 55). This process is suggested to involve the COOH terminus of the AMPA receptor subunit, GluR1, and presumably one of the postsynaptic density proteins carrying PDZ domain(s). In contrast, the constitutive exocytotic process of GluR2 subunits in hippocampal neurons requires its interaction with NSF (29, 30, 49). Together with our findings, therefore, individual neurons are subjected to different molecular mechanisms underlying the regulation of AMPA receptor dynamics. How individual types of brain neurons switch on and off the subcellular translocation of individual AMPA receptor subunits is an interesting question.

Differential Contribution of Neurotrophic Factors to Surface Expression of Transmitter Receptors—Among the neurotrophins, BDNF most potently increased AMPA receptor protein levels in young neocortical cultures even containing insulin (4). Platelet-derived growth factor and basic fibroblast growth factor appear to have a similar effect on the expression of the AMPA subunits, although the reacting neuronal population would be different (31). Our latest studies suggest that chronic application of BDNF for several days also up-regulates gene expression of various PDZ proteins to elevate total cellular levels of AMPA receptors (56). This process involves the interaction between AMPA receptors and their associating PDZ proteins. In agreement, the lack of the PDZ interacting COOH-terminal portion of GluR2 attenuated the BDNF-triggered surface translocation (see Fig. 6) and the following cellular increase in the GluR2 mutant protein.3 Presumably, such a stabilization process should follow the surface translocation of the receptors to maintain their functions there.

Many neurotrophic factors regulate neuronal differentiation and development. In contrast, those influencing postsynaptic structures and/or phenotypes have been limited. Neuregulins are important cytokines in both central and peripheral synapses. It will be interesting to elucidate whether these trans-synaptic factors distinctively regulate the dynamics of various transmitter receptors in neuronal subcellular compartments during development and how such regulation results in the characteristics of numerous postsynaptic sites in adults.

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