Brain-derived neurotrophic factor (BDNF) plays an important role in synaptic plasticity in the hippocampus, but the mechanisms involved are not fully understood. The neurotrophin couples synaptic activation to changes in gene expression underlying long term potentiation and short term plasticity. Here we show that BDNF acutely up-regulates GluR1, GluR2, and GluR3 α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor subunits in 7-day cultured hippocampal neurons. The increase in GluR1 and GluR2 protein levels in developing cultures was impaired by K252a, a Trk inhibitor, and by translation (emetine and anisomycin) and transcription (α-amanitin and actinomycin D) inhibitors. Accordingly, BDNF increased the mRNA levels for GluR1 and GluR2 subunits. Biotinylation studies showed that stimulation with BDNF for 30 min selectively increased the amount of GluR1 associated with the plasma membrane, and this effect was abrogated by emetine. Under the same conditions, BDNF induced GluR1 phosphorylation on Ser-831 through activation of protein kinase C and Ca2+-calmodulin-dependent protein kinase II. Chelation of endogenous extracellular BDNF with TrkB-IgG selectively decreased GluR1 protein levels in 14-day in vitro cultures of hippocampal neurons. Moreover, BDNF promoted synaptic delivery of homomeric GluR1 AMPA receptors in cultured organotypic slices, by a mechanism independent of NMDA receptor activation. Taken together, the results indicate that BDNF up-regulates the protein levels of AMPA receptor subunits in hippocampal neurons and induces the delivery of AMPA receptors to the synapse.

Neurotrophins are essential for the development of the vertebrate nervous system, modulate synaptic function, and play an important role in synaptic plasticity (1, 2). Brain-derived neurotrophic factor (BDNF) has been implicated in activity-dependent synaptic plasticity, particularly in long term potentiation (LTP) induced by high frequency stimulation. Accordingly, LTP is impaired in the hippocampal CA1 region of animals deficient in BDNF, but it can be rescued by supplying the neurotrophin (3–5). Chelation of endogenous BDNF also prevents the induction of LTP by theta burst stimulation and reduces late phase LTP induced by high frequency stimulation (6, 7). Furthermore, the late phase LTP induced by tetanic stimulation was not observed in slices from BDNF knock-out mice and was also abrogated when TrkB receptors were blocked (8). Taken together, the available evidences point to a direct role of BDNF in the early and late phases of LTP.

Binding of BDNF to TrkB receptors is followed by activation of intracellular signaling pathways, including the Ras/extracellular signal-regulated protein kinase, phospholipase Cβ (PLCβ), phosphatidylinositol-3-kinase/Akt, and Src pathways (9–11). TrkB receptors are located on axon terminals and in the post-synaptic density of glutamatergic synapses (12–14), but whether the effects of BDNF on synaptic plasticity are mediated by pre- and/or post-synaptic receptors is not fully elucidated.

BDNF was originally shown to induce a long lasting potentiation of excitatory synaptic transmission in the hippocampal CA1 region, acting at a presynaptic site (15) (for conflicting results see Refs. 4, 6, 16–18), and subsequent studies showed that BDNF was selectively required for those forms of LTP that concentration transients in dentate granule cells, which induce LTP when paired with weak synaptic stimulation (20). The enhancement of synaptic transmission by BDNF observed in the dentate gyrus in vivo (21) may also be due, at least in part, to the activation of post-synaptic receptors (22, 23). Nevertheless, a post-synaptic role of BDNF in synaptic plasticity, and the potential mechanisms involved, remain controversial.

The number, composition, and location of α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors...
Modulation of AMPA Receptors by BDNF

in neurons, together with receptor phosphorylation, are critically important factors in determining the neuronal response to glutamate, and play an important role in the mechanisms of synaptic plasticity (24). AMPA receptors are formed by the association of GluR1-GluR4 subunits, and their delivery to the synapse is tightly controlled by the intracellular signaling activity. In this work, we characterized the effect of BDNF on the abundance of AMPA receptors in cultured hippocampal neurons and on their cellular distribution. Furthermore, we investigated the effect of BDNF on the synaptic delivery of GluR1-containing AMPA receptors in CA1 hippocampal neurons, which could account for the postsynaptic effects of the neurotrophin in the early phase of LTP.

EXPERIMENTAL PROCEDURES

Hippocampal Cultures—Primary cultures of rat hippocampal neurons were prepared from the hippocampi of E18–E19 Wistar rat embryos, after treatment with trypsin (0.06%, 15 min, and 37 °C, Invitrogen) and deoxyribonuclease 1 (5.36 mg/ml), in Ca2+/Mg2+-free Hanks’ balanced salt solution (5.36 mM KCl, 0.44 mM KH2PO4, 137 mM NaCl, 4.16 mM NaHCO3, 0.34 mM Na2HPO4, 2H2O, 5 mM glucose, 1 mM sodium pyruvate, 10 mM HEPES, and 0.001% phenol red). The hippocampi were then washed with Hanks’ balanced salt solution containing 10% fetal bovine serum (BioWhittaker), 2.0 μM chelerythrine (Sigma), or 10 μM KN-93 (Sigma) were added 30 min before stimulation, as indicated. Scavenging of endogenous extracellular BDNF was performed using random primer r(dN)6 (3.2 μg), dNTPs (1 mM each), MgCl2 (25 mM), RNase inhibitor (50 units) and gelatin (0.01 μg/μl) in reaction buffer (10 mM Tris, 50 mM KCl, pH 8.3) in a total volume of 40 μl. The reaction was performed at 25 °C for 10 min, followed by 60 min at 42 °C, for primer annealing to the RNA template and cDNA synthesis, respectively. The reverse transcriptase was then denatured during 5 min at 99 °C, and the sample was cooled to 4 °C for 5 min and finally stored at −80 °C until further use.

Real-time PCR—Real-Time PCR analysis of gene expression was performed using the LightCycler System II (Roche Applied Science). The PCR reactions were performed using LightCycler FastStart DNA Master SYBR Green I (26) in 20-μl capillaries. The primers used for amplification of genes encoding AMPA receptor subunits were, respectively, RGR1F2271, 5′-GAA CCA CTC TGG TTT TTG CG-3′ and RGR1R2937, 5′-TTC CTG TCT GCT CCA GTC AC-3′ for GluR1; RGR2F2522, 5′-GAA GCC TTT TGG TGA CAT GA-3′ and RGR2R3008, 5′-AGC CTT GCC TGG TCG CTC AT-3′ for GluR2; RGR3F2431, 5′-CAA AGG TTA TGG TGT GGC AA-3′ and RGR3R2927, 5′-ACA CCA GGG AGA GTG AAA TC-3′ for GluR3; S2288, 5′-TGC AGG AGG CTC GGT TGG TC-3′ and S2800, 5′-TTG GGG CAC TTA GGG TCA AG-3′ for GluR4. The primers used for the amplification of endogenous control gene 18S rRNA were those included in the Applied Biosystems TaqMan Ribosomal RNA Control Reagents Kit (Porto, Portugal). Each primer of a pair was added to the reaction mixture (10 μl) at a final concentration of 0.8 μM with 3 μM MgCl2, in addition to the “Hot Start” LightCycler Fast Start DNA Master SYBR Green I mix (1×) and 1.2 μl of cDNA sample. Thermal cycling was initiated with activation of the FastStart TaqDNA polymerase by denaturation during 10 min at 95 °C followed by 45 cycles of a 30-s melting step at 95 °C, a 5-s annealing step at 58 °C, and a 25-s elongation step at 72 °C. All temperature transition rates used were at 20 °C/s. After amplification for 45 cycles, at least 10 cycles beyond the beginning of the linear phase of amplification, samples were subjected to a melting
curve analysis according to the instructions of the manufacturer to confirm the absence of unspecific amplification products and primer-dimers. In all experiments, samples containing no template were included as negative controls.

**mRNA Quantitative Analysis**—The mRNA levels of the constitutively expressed housekeeping gene encoding 18 S ribosomal RNA were used as a control, in all experiments. The relative changes in the mRNA levels of glutamate receptor subunits in cultured hippocampal neurons were determined using the ΔCₚ method. Accordingly, for each experimental condition (unstimulated neurons and neurons treated with 100 ng/ml BDNF for 30 min or 3 h) the “crossing point” (Cₚ) values given by the LightCycler system II software, for each target gene, were subtracted by the respective Cₚ value determined for the 18 S gene from the same sample and condition (ΔCₚ). This allows normalizing changes in target gene expression. Afterward, the ΔCₚ values were subtracted by the respective values of the control for the target gene giving ΔΔCₚ. The derivation to the value of 2⁻¹(ΔΔCₚ) sets each control at the unity (or 100%), because ΔΔCₚ (control) = 0, and the stimuli conditions used were at a percentage relative to the control.

**Surface Biotinylation and Precipitation**—Hippocampal cell cultures were treated or not with 100 ng/ml BDNF and then incubated with 1 mg/ml EZ-Link™ Sulfo-NHS-SS-biotin (Pierce) in ice-cold phosphate-buffered saline containing 1 mM CaCl₂ and 1 mM MgCl₂, for 30 min (27). The non-bound biotin was removed by washing the cells with phosphate-buffered saline containing 100 mM glycine. Cell lysates were obtained as described above and were incubated with UltraLink Plus™ immobilized streptavidin or UltraLink™ immobilized NeutrAvidin™ plus beads (Pierce), for 2 h at 4 °C, under constant agitation. Non-biotinylated proteins were removed by centrifugation at 2,500 g for 3 min, and the beads were washed three times with radioimmune precipitation assay buffer. Biotinylated proteins were then eluted with denaturing buffer at 95 °C for 5 min (Fig. 7, A, C, and D) or at 65 °C for 15 min (Fig. 7B). Samples were then processed for Western blotting analysis.

**Western Blotting**—Protein samples were separated by SDS-PAGE, in 6% polyacrylamide gels, transferred to polyvinylidene membranes (Bio-Rad), and immunoblotted. Blots were incubated with primary antibodies (overnight at 4 °C), washed, and exposed to alkaline phosphatase-conjugated secondary antibodies (1:20,000 dilution; 1 h at room temperature). Alkaline phosphatase activity was visualized by enhanced chemiluminescence (ECF) on the Storm 860 Gel and Blot Imaging System (Amersham Biosciences) or by ECL (Fig. 9A). The following

![Image](https://via.placeholder.com/150)
primary antibodies were used: anti-GluR1 (1:1,500, Upstate, Waltham, MA), anti-GluR2 (1:600, Chemicon International, Temecula, CA), anti-GluR3 (1:200, Zymed Laboratories Inc., San Francisco, CA), anti-GluR4 (1:200, Chemicon International), anti-pGluR1 Ser-831 (1:1,000, Chemicon International or 1:1,500, Tocris, UK), anti-pGluR1 Ser-845 (1:1,000, Chemicon International), anti-pGluR2 Ser-880 (1:1,000, Chemicon International), anti-pTrk (1:1,000, Cell Signaling, Beverly, MA), and anti- TrkB (clone 47, 1:1,000, BD Biosciences). Anti-α-tubulin (1:1,000, Zymed Laboratories Inc.), anti-β-tubulin I (1:150,000, Sigma), anti-actin (1:20,000, Chemicon), and anti-transferrin receptor (1:3,000, Zymed Laboratories Inc.) antibodies were used as loading controls.

Electrophysiology—After 3–5 days in culture, the organotypic cultures of rat hippocampal slices, prepared as previously described (25), were infected (36 h) with the Sindbis virus expressing recombinant GluR1 or GluR1 plus the constitutive active αCaMKII (as indicated), tagged to GFP. Voltage clamp whole cell recordings were obtained from infected and uninfected CA1 pyramidal neurons, under visual guidance using fluorescence and transmitted light illumination. The recording chamber was perfused with 119 mM NaCl, 2.5 mM KCl, 4 mM CaCl2, 4 mM MgCl2, 26 mM NaHCO3, 1 mM Na2PO4, 11 mM glucose, 0.1 mM picrotoxin, 0.1 mM DL-2-aminoglycoside (APV), and 2 μM 2-chloroadenosine (pH 7.4), gassed with 5% CO2/95% O2. Patch recording pipettes (4–7 MΩ) were filled with the 115 mM cesium methanesulfonate, 20 mM CsCl, 10 mM HEPES, 2.5 mM MgCl2, 4 mM Na2ATP, 0.4 mM Na3GTP, 10 mM sodium phosphocreatine, 0.6 mM EGTA, and 0.1 mM spermine (pH 7.25). Whole cell recordings were carried out with a MultiClamp 700A amplifier (Axon Instruments, Union City, CA). Synaptic responses were evoked with two bipolar electrodes with single voltage pulses (duration of 0.3 ms, up to 20 V). The stimulating electrodes were placed over Schaffer collateral fibers between 200 and 300 μm from the recorded cells. Synaptic AMPA receptor-mediated responses were collected at −60 mV and +40 mV with glass electrodes placed in CA1 stratum radiatum and averaged over 50–60 trials; their ratio was used as an index of rectification. All electrophysiological data were collected with pCLAMP software (Axon Instruments). When the effect of K252a (200 nM) was tested, the drug was added to the perfusion medium 30 min prior to incubation with 100 ng/ml BDNF. Incubation with BDNF was performed for 30 min to 2.5 h. When the effect of NMDA receptor activity on AMPA delivery to the synapse was further examined, NMDA receptor-mediated responses were blocked pharmacologically using 0.1 mM APV.

Statistical Analysis—Statistical analysis was performed using one-way ANOVA followed by the Dunnett test or Bonferroni test, or using the Student’s t test, as indicated in the figure captions.

RESULTS

Effect of BDNF on the Total Protein Levels of AMPA Receptor Subunits—To determine whether acute stimulation with BDNF affects the abundance of glutamate receptor subunits, 7 DIV cultured hippocampal neurons were incubated with or without 100 ng/ml BDNF, for various periods of time (15 min to 24 h). The AMPA receptor subunit (GluR1, GluR2, GluR3, and GluR4) protein levels were determined by Western blot. Control protein levels of AMPA receptor subunits were set to 100%. β-Tubulin was used as loading control. The results are the average ± S.E. of 5–6 independent experiments, performed in independent preparations. Statistical analysis was performed by one-way ANOVA, followed by the Dunnett test.* p < 0.05.

In contrast with the results obtained using 7 DIV hippocampal neurons, BDNF did not affect GluR1 and GluR2 protein levels in neurons cultured for 14 DIV, for incubation periods with the neurotrophin of 2–24 h. However, when the endogenous extracellular BDNF was chelated with the fusion protein TrkB-IgG for 24 h, the levels of GluR1 significantly decreased (Fig. 2A). These results indicate that endogenous BDNF regulates the GluR1 protein levels in 14 DIV cultures of hippocampal neurons. Activation of TrkB neurotrophin receptors was required for up-regulation of GluR1 and GluR2 AMPA receptor subunits by BDNF in 7 DIV cultures, because no effect of the neurotrophin was observed when the experiments were conducted in the presence of K252a (200 nM), an inhibitor of this family of receptors (Fig. 3).

To test whether the effect of BDNF was due to an increase in protein synthesis, we used two translation inhibitors, emetine and anisomycin. Hippocampal neurons were stimulated with BDNF for 3 h, in the presence or in the absence of the translation inhibitors. Emetine (2 μM) and anisomycin (2 μM) abrogated the effect of BDNF on GluR1 and GluR2 subunits (Fig. 4). None of the protein synthesis inhibitors reduced the GluR1 or GluR2 protein levels under control conditions, in agreement with the long half-life of AMPA receptor subunits (28–30).
Taken together, our findings indicate that the effect of BDNF on AMPA receptor subunits is mediated by activation of TrkB receptor and is due to an up-regulation of protein synthesis instead of a reduction in protein degradation.

**BDNF Up-regulates AMPA Receptor Subunits by Promoting Transcription Activity**—BDNF may stimulate either transcription of genes (22) or stimulate protein synthesis by activating translation cascades (31). Therefore, to test for the role of transcription in the up-regulation of glutamate receptor subunits by BDNF we used two different transcription inhibitors, α-amanitine (1.5 μM, left panels) or actinomycin D (1.5 μM, right panels). When the inhibitors were used the cells were preincubated with the compounds for 30 min before stimulation with BDNF. Total GluR1 (top panels) and GluR2 (bottom panels) protein levels were measured by Western blot. Control (unstimulated) protein levels of AMPA receptor subunits were set to 100%. Actin was used as loading control. The results are the average ± S.E. of 6–12 independent experiments, performed in independent preparations. Statistical analysis was performed by one-way ANOVA, followed by the Bonferroni test. *p < 0.05.

**FIGURE 4.** Translation inhibitors impair the BDNF-induced up-regulation of GluR1 and GluR2 protein levels. Neurons (7 DIV) were incubated with or without 100 ng/ml BDNF for 3 h, in the presence or in the absence of emetine (2.0 μM, left panels) or anisomycin (2.0 μM, right panels). When the inhibitors were used the cells were preincubated with the compounds for 30 min before stimulation with BDNF. Total GluR1 (top panels) and GluR2 (bottom panels) protein levels were measured by Western blot. Control (unstimulated) protein levels of AMPA receptor subunits were set to 100%. Actin was used as loading control. The results are the average ± S.E. of 6–12 independent experiments, performed in independent preparations. Statistical analysis was performed by one-way ANOVA, followed by the Bonferroni test. *p < 0.01; **p < 0.001. DMSO, Me2SO.

**FIGURE 5.** Transcription inhibitors prevent the BDNF-induced up-regulation of the GluR1 and GluR2 protein levels. Neurons (7 DIV) were incubated with or without 100 ng/ml BDNF for 3 h, in the presence or in the absence of α-amanitine (1.5 μM, left panels) or actinomycin D (1.5 μM, right panels). When the inhibitors were used the cells were preincubated with the compounds for 30 min before stimulation with BDNF. Total GluR1 (top panels) and GluR2 (bottom panels) protein levels were measured by Western blot. Control (unstimulated) protein levels of AMPA receptor subunits were set to 100%. Actin was used as loading control. The results are the average ± S.E. of 6–10 independent experiments, performed in independent preparations. Statistical analysis was performed by one-way ANOVA, followed by the Bonferroni test. *p < 0.05.
hippocampal neurons were treated with or without 100 ng/ml BDNF (30 min, 3 h, and 24 h). Following treatment, proteins on the cell surface were biotinylated, collected with streptavidin-coated NeutrAvidin-coupled beads, and subjected to Western blotting (Fig. 7). GluR1 protein associated with the plasma membrane was markedly increased by BDNF treatment during 30 min when compared with non-treated cells. However, incubation with BDNF for 3 h was without effect (Fig. 7A). The increase observed by BDNF treatment during 30 min was blocked by emetine (Fig. 7B), indicating that the BDNF-induced up-regulation in the amount of plasma membrane-associated GluR1 requires de novo protein synthesis. In contrast, BDNF did not affect significantly the GluR2 protein levels at the plasma membrane (Fig. 7D). These results indicate that BDNF has a differential effect on the traffic of AMPA receptor subunits in developing cultured hippocampal neurons. Also, the distinct time courses for the increase in surface and total GluR1 expression suggest that the effects of BDNF on total GluR1 content and receptor trafficking may be mediated by separate signaling pathways. In contrast with the results obtained in developing neurons, BDNF did not affect the surface expression of GluR1 in 14 DIV cultured hippocampal neurons (Fig. 7C).

The delivery of GluR1 subunits to the synapse is regulated by phosphorylation in the C-terminal region. GluR1 is phosphorylated on Ser-831 during LTP, although this phosphorylation is not sufficient to induce synaptic delivery of AMPA receptors (24, 26, 33, 34). Stimulation of cultured hippocampal neurons (7 DIV) with BDNF (100 ng/ml) increased the phosphorylation of GluR1 on Ser-831, as determined by Western blot using a phosphospecific antibody (Fig. 7E). However, because the total amount of GluR1 present in the cells was also increased upon stimulation with the neurotrophin, the percentage of total protein that was phosphorylated was not significantly changed (Fig. 7F, see also Fig. 1A). To better understand which signaling pathways may be involved in GluR1 phosphorylation following Trk receptor activation, and because Ser-831 is a phosphorylation site for protein kinase C (PKC) and Ca\(^{2+}\)-calmodulin-dependent protein kinase II (CaMKII) (24, 26), the effect of the kinase inhibitors chelerythrine (5 \(\mu\)M) and KN-93 (10 \(\mu\)M) was tested. Inhibition of PKC and CaMKII with chelerythrine and KN-93, respectively, inhibited GluR1 phosphorylation on Ser-831, suggesting that both pathways contribute to the effect of BDNF (Fig. 7E).

**Effect of BDNF on Synaptic Delivery of AMPA Receptors**—We next examined the role of BDNF in the trafficking of AMPA receptors into the synapse, in CA1 neurons of rat hippocampal organotypic slices expressing GluR1-GFP. Overexpression of GluR1-GFP, with a Sindbis virus expression system, leads to the formation of homomeric AMPA receptors containing the GluR1 subunit (33). These GluR2-lacking receptors are inwardly rectifying (35–37), and therefore their recruitment to the synapse increases the inward currents at \(-60\) mV, with no effect on the outward currents. Hence, synaptic delivery of GluR1-containing AMPA receptors to the synapse can be monitored as an increase in the ratio between the currents at \(-60\) mV versus the currents at +40 mV (rectification index) (33). As expected, overexpression of GluR1 in CA1 hippocampal neurons did not increase the rectification index of synaptic responses (Fig. 8, A and B; compare first and second columns in Fig. 8B), confirming that GluR1 is not spontaneously delivered into synapses (33). In marked contrast, the rectification index of homomeric GluR1-expressing neurons was significantly increased upon incubation with BDNF (100 ng/ml) for 30 min to 3 h (Figs. 7B and 8A; compare second and fourth columns in Fig. 8B). BDNF had no significant effect on the rectification index of non-infected cells (Fig. 8B; compare first and third columns). The effect of BDNF was similar to that observed in cells where GluR1 was coexpressed with a constitutively active form of aCaMKII (tCaMKII, Fig. 7B and 8A; compare fourth and seventh columns in Fig. 8B), which is known to induce synaptic delivery of GluR1-containing AMPA receptors (33, 38). Inhibition of the Trk receptors with K252a (200 nM) completely prevented synaptic delivery of AMPA receptors induced by BDNF (Fig. 8B; compare fourth and fifth columns). Taken together, the results indicate that BDNF induces a very efficient delivery of GluR1 homomeric receptors into synapse and that this effect is mediated by binding of BDNF to Trk receptors.

Tetanic stimulation was shown to induce a rapid delivery of GFP-GluR1-containing AMPA receptors into dendritic spines and cause receptor clustering in dendrites by a mechanism requiring activation of NMDA receptors (39). To determine whether synaptic delivery of GFP-GluR1-containing AMPA receptors induced by BDNF requires activation of NMDA receptors, experiments were performed in the presence of the NMDA receptor antagonist APV. Preincubation of the slices with APV (100 \(\mu\)M) for 30 min did not affect the increase in the rectification index induced by BDNF (Fig. 8B; compare fourth and sixth columns).

Stimulation of hippocampal slices with BDNF (100 ng/ml) for 30-min increased GluR1 phosphorylation on Ser-831 (Fig. 9, A and B), in agreement with the results obtained in monolayer cultures of hippocampal neurons (Fig. 7E). In contrast, no
change in GluR1 phosphorylation was detected for Ser-845 (Fig. 9A). The activation of Trk receptors by BDNF was confirmed by Western blot, using an antibody that recognizes phosphotyrosine 490 in TrkA, a residue conserved in the other Trk receptors. Stimulation of hippocampal slices with BDNF for 30 min significantly increased Trk (presumably TrkB) phosphorylation. Control experiments showed that the total GluR1 and Trk protein levels did not change significantly in the slices under the experimental conditions used (Fig. 9A). The effect of BDNF on GluR1 phosphorylation was specific, because no phosphorylation of GluR2 on the PKC phosphorylation site (Ser-880 (24)) was observed (Fig. 9A).

DISCUSSION

It is well known that BDNF plays an important role in synaptic plasticity (1, 40, 41), particularly in LTP induced by high frequency stimulation (42–44), which in late phase requires transcription activation and protein synthesis (45). Accordingly, LTP is impaired in the hippocampal CA3-CA1 region of bdnf null mutant and forebrain-specific trkB knock-out mice (3, 4, 46, 47). In the latter case, a compromised learning ability was also shown (46). However, the mechanisms whereby BDNF contributes to LTP are not fully understood. In this study, we show that Trk receptor activation (presumably TrkB) by BDNF rapidly up-regulates GluR1 and GluR2 protein levels in cultured hippocampal neurons, by increasing transcription activity. Although BDNF also up-regulated GluR3 protein levels, no effect on the mRNA for this receptor subunit was observed. The neurotrophin also induced phosphorylation of GluR1 on Ser-831, most likely by activating PKC and CaMKII, and promoted synaptic delivery of GluR1-containing AMPA receptors in the CA1 region of the hippocampus.

Short incubation periods with BDNF (30 min) up-regulated GluR1 and GluR2 protein levels to about the same extent in cultured hippocampal neurons (Fig. 1, A and C) but selectively increased the amount of GluR1 subunits associated with the plasma membrane (Fig. 7). These findings indicate that the delivery of GluR1 and GluR2 subunits to the membrane is differentially regulated, as shown for synaptic delivery of the AMPA receptor subunits in the adult hippocampus (48, 49). Furthermore, we found that the BDNF acutely increases GluR1 subunit associated with the plasma membrane by
results obtained in 7 DIV cultures, when synaptogenesis is particularly active (51), addition of BDNF had no effect on GluR1 and GluR2 protein levels in hippocampal neurons cultured for 14 DIV (Fig. 1, B and D). In these cultures, chelation of endogenous extracellular BDNF with the fusion protein chimera TrkB-IgG selectively decreased the GluR1 levels, indicating that BDNF has a tonic effect on GluR1 protein levels (Fig. 2). These results suggest that the lack of effect of BDNF on total GluR1 protein levels in 14 DIV hippocampal cultures (Fig. 1B), and on the total amount of surface receptors (Fig. 7C), may be due to the activity of endogenous BDNF, which controls to some extent the abundance of GluR1, thereby precluding an effect of exogenous addition of the neurotrophin. This may also explain the results showing no effect of BDNF on total GluR1 protein levels in the organotypic slices (Fig. 9A).

Electrophysiology studies have also shown that BDNF induces synaptic delivery of GluR1-containing AMPA receptors in CA1 neurons of hippocampal organotypic slices expressing GluR1-GFP, by a mechanism independent of NMDA receptor activity (Fig. 8B). This contrasts with the role of NMDA receptors in synaptic delivery of GluR1-containing AMPA receptors following tetanic stimulation (39). The synaptic delivery of GluR1 induced by BDNF was sensitive to the Trk inhibitor K252a and was associated with the phosphorylation of the protein in Ser-831, the CaMKII and PKC phosphorylation site (24). Interestingly, GluR1 phosphorylation in Ser-831 was also observed upon induction of LTP in naive synapses (34). In contrast, BDNF did not affect GluR1 phosphorylation in Ser-845, which is preferentially phosphorylated upon high frequency stimulation of previously depressed synapses (34). Because GluR1 phosphorylation in Ser-831 is not sufficient to induce synaptic delivery of AMPA receptors (33), the effect of BDNF may also involve GluR1 phosphorylation on Ser-818. Phosphorylation of this site is significantly increased during hippocampal LTP, playing an important role in synaptic incorporation of GluR1 (52). Alternatively, BDNF may induce the phosphorylation of a regulatory protein, leading to the release of a retention interaction and allowing the incorporation of GluR1 subunits into the synapse. Phosphorylation of GluR1 on Ser-831 may also allow the interaction with a protein that anchors the receptor at the plasma membrane.

The effect of BDNF on synaptic delivery of GluR1 was similar to that observed in cells where a constitutively active form of αCaMKII was coexpressed, suggesting that analogous signaling mechanisms may be involved. In fact, treatment of cultured hippocampal neurons also increased the phosphorylation of GluR1 on Ser-831, and this effect was abolished by inhibition of PKC and CaMKII, suggesting that both pathways are involved in the BDNF-induced GluR1 phosphorylation and possibly on the delivery of AMPA receptors to the synapse. In agreement with the role of PKC and CaMKII in BDNF-induced GluR1 phosphorylation, activation of TrkB receptors stimulates the phospholipase Cγ pathway, giving rise to diacylglycerol, which activates PKC, and inositol-1,4,5-trisphosphate, which mobilizes Ca2+ from intracellular stores (10, 11). Interestingly, this signaling pathway is involved in the synaptic changes resulting from local application of BDNF to the *Xenopus laevis* optic tectum, which cause rapid modifications of synaptic inputs at

enhancing the translation activity (Fig. 7B). This may be due to the delivery of newly synthesized receptors to the plasma membrane and/or to an increased stability of the plasma membrane-associate receptors by interaction with protein(s) synthesized following TrkB receptor stimulation. The acute effects of BDNF on the plasma membrane GluR1 protein levels may require continuous signalling activity by TrkB receptors, because after 3-h stimulation with BDNF, when TrkB receptors are desensitized to some extent (50), the receptor subunits found in the membrane were similar to the control. In contrast with the
the dendrites of retinal ganglion cells by up-regulating AMPA receptors at the retinal ganglion cells (53). Furthermore, recruitment of phospholipase Cγ by active TrkB receptors was shown to play a role in hippocampal LTP (54).

Although activation of NMDA receptors induces a rapid delivery of GluR1-containing AMPA receptors to the synapse, and their clustering in dendrites (39), this does not account for the effect of BDNF, because we found no effect of the NMDA receptor antagonist APV on BDNF-induced delivery of GluR1 subunits to the synapse. Interestingly, inhibition of NMDA receptors was also shown not to affect BDNF-induced LTP at medial perforant path to granule cell synapses in the rat dentate gyrus, in contrast with the high frequency stimulation-induced LTP, which depends on the activation of NMDA receptors (22).

In addition to the rapid effects on delivery of GluR1 subunits to the synapse in hippocampal CA1 neurons, and to the plasma membrane in developing cultured hippocampal neurons, BDNF also rapidly up-regulated GluR1, GluR2, and GluR3 protein levels in the latter preparation. This effect was transient, most likely due to the desensitization of the Trk receptors, followed by a decrease in intracellular signaling activity (50, 55). The up-regulation in AMPA receptor subunits induced by BDNF was due to an increase in transcription activity, as demonstrated by the effect of transcription inhibitors, followed by synthesis of the receptor subunits. The role of transcription activity on the effect of BDNF on the protein levels of the GluR1 and GluR2 subunits is not surprising, because BDNF is known to regulate transcription of several genes (44, 56) and to modulate transcription during the late phase LTP (22). In agreement with the present findings, chronic treatment of cerebrocortical neurons with BDNF also increased GluR1 and GluR2/3 protein levels, but no effect on the mRNA for the receptor subunits was found under these conditions (57, 58). However, in this case, the sustained stimulation of neurotrophin receptors from early in development may have caused changes in cell phenotype.

The signaling mechanisms involved in the rapid increase in the transcription of GluR1 and GluR2 in hippocampal neurons exposed to BDNF remain to be identified. Previous studies have shown GluR1 protein levels may be regulated by the serum- and glucocorticoid-inducible kinase 3, one of the downstream targets of phosphatidylinositol 3-kinase (59), and by the tyrosine kinase Fyn, which also regulates GluR2/3 (9). Studies in a heterologous system showed that the expression of GluR2 can be up-regulated by BDNF, through a mechanism involving a neuron-restrictive silencer element present within the GluR2 promoter (60). These results are in agreement with our own results and contrast with the effects observed in cerebrocortical neurons chronically exposed to BDNF, where no change in the mRNA for GluR2 was observed (57).

In addition to the effect of BDNF on the mRNA levels for GluR1 and GluR2, which accounted for the up-regulation in the AMPA receptor subunits in cultured hippocampal neurons, a recent study showed that BDNF also increased GluR1 protein levels in synaptoneurosomes (61). This effect, mediated by a mammalian target of rapamycin-phosphatidylinositol 3-kinase-dependent pathway, may contribute to increase locally the amount GluR1 after high frequency stimulation. Delivery of these receptors to the synapse may contribute to LTP. Chronic stimulation of neocortical neuronal cultures with BDNF also increased the abundance of the AMPA receptor-interacting proteins SAP97, GRIP1, and PICK1 (62). Under the same conditions there was an increase in the interaction between GluR1 and SAP97, as well as between GluR2 and GRIP1, which was suggested to play a role in the up-regulation of AMPA receptors by BDNF (62). It remains to be determined whether BDNF induces a local synthesis and increase in the protein levels of these AMPA receptor-interacting proteins, which may contribute to stabilize the receptors at the synapse.

In conclusion, our results strongly suggest that BDNF plays a direct role in the early phase of synaptic plasticity by triggering the delivery of GluR1 subunits to the synapse. Furthermore, BDNF rapidly up-regulated AMPA receptor subunits in developing hippocampal neurons and induced an overall increase in the number of receptors associated with the membrane during a period of active synaptogenesis.

Acknowledgments—We thank Dr. Gina Marrão (Faculty of Medicine, University of Coimbra) for kindly providing us the use of the Light-Cycler System II, and Regeneron for the kind gift of BDNF.

REFERENCES

1. Poo, M. M. (2001) Nat. Rev. Neurosci. 2, 24–32
2. Vicario-Abejon, C., Owens, D., McKay, R., and Segal, M. (2002) Nat. Rev. Neurosci. 3, 965–974
3. Korte, M., Carroll, P., Wolf, E., Brem, G., Thoenen, H., and Bonhoeffer, T. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 8856–8860
4. Patterson, S. L., Abel, T., Deuel, T. A. S., Martin, K. C., Rose, J. C., and Kandel, E. R. (1996) Neuron 16, 1137–1145
5. Korte, M., Griesbeck, O., Gravel, C., Carroll, P., Staiger, V., Thoenen, H., and Bonhoeffer, T. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 12547–12552
6. Figueroa, A., Pozzo-Miller, L. D., Olafsson, P., Wang, T., and Lu, B. (1996) Neuron 31, 706–709
7. Kang, H., Welcher, A. A., Shelton, D., and Schuman, E. M. (1997) Neuron 19, 653–664
8. Korte, M., Kang, H., Bonhoeffer, T., and Schuman, E. (1998) Neuropharmacology 37, 553–559
9. Narisawa-Saito, M., Silva, A. J., Yamaguchi, T., Hayashi, T., Yamamoto, T., and Nawa, H. (1999b) Proc. Natl. Acad. Sci. U. S. A. 96, 2461–2466
10. Kaplan, D. R., and Miller, F. D. (2000) Curr. Opin. Neurobiol. 10, 381–391
11. Huang, E. J., and Reichardt, L. F. (2003) Annu. Rev. Biochem. 72, 609–642
12. Drake, C. T., Milner, T. A., and Patterson, S. L. (1999) J. Neurosci. 19, 8009–8026
13. Aoki, C., Wu, K., Elste, A., Len, G., Lin, S., McAluliffe, G., and Black, I. B. (2000) J. Neurosci. Res. 59, 454–463
14. Pereira, D. B., Rebola, N., Rodrigues, R. J., Cunha, R. A., Carvalho, A. P., and Duarte, C. B. (2006) J. Neurosci. Res. 83, 822–844
15. Kang, H., and Schuman, E. M. (1995) Science 267, 1658–1662
16. Tanaka, T., Saito, H., and Matsuki, N. (1997) J. Neurosci. 17, 2959–2966
17. Gottschalk, W., Pozzo-Miller, L. D., Figueroa, A., and Lu, B. (1998) J. Neurosci. 18, 6830–6839
18. Freking, M., Malenka, R. C., and Nicol, R. A. (1998) J. Neurophysiol. 80, 3383–3386
19. Zakharenko, S. S., Patterson, S. L., Dragatsis, I., Zeitlin, S. O., Siegelbaum, S. A., Kandel, E. R., and Morozov, A. (2003) Neuron 39, 975–990
20. Kovalchuk, Y., Hanse, E., Kafitz, K. W., and Konnerth, A. (2002) Science 295, 1729–1734
21. Messaoudi, E., Bardsen, K., Srebro, B., and Bramham, C. R. (1998) J. Neurophysiol. 79, 496–499
22. Messaoudi, E., Ying, S. W., Kanhema, T., Croll, S. D., and Bramham, C. R. (2002) J. Neurosci. 22, 7453–7461
Modulation of AMPA Receptors by BDNF

23. Ying, S. W., Futter, M., Rosenblum, K., Webber, M. J., Hunt, S. P., Bliss, T. V., and Bramham, C. R. (2002) J. Neurosci. 22, 1532–1540
24. Gomes, A. R., Correia, S. S., Carvalho, A. L., and Duarte, C. B. (2003) Neurochem. Res. 28, 1459–1473
25. Gahwiler, B. H., Capogna, M., Debanne, D., McKinney, R. A., and Thompson, S. M. (1997) Trends Neurosci. 20, 471–477
26. Roche, K. W., O’Brien, R. J., Mammen, A. L., Bernhardt, J., and Huganir, R. L. (1996) Neuron 16, 1179–1188
27. Gomes, A. R., Cunha, P., Nuriya, M., Faro, C. J., Huganir, R. L., Pires, E. V., Carvalho, A. L., and Duarte, C. B. (2004) J. Neurochem. 90, 673–682
28. O’Brien, R. J., Kamboj, P., Shen, A., Chalk, R., and Thompson, S. M. (1992) Trends Neurosci. 15, 471–477
29. Archibald, K., Perry, M. J., Molnar, E., and Henley, J. M. (1998) J. Biol. Chem. 273, 2262–2267
30. Huh, K. H., and Wenthold, R. J. (1999) J. Biol. Chem. 274, 151–157
31. Takei, N., Kawamura, M., Hara, K., Yonezawa, K., and Nawa, H. (2001) J. Biol. Chem. 276, 42818–42825
32. Hall, R. A., and Soderling, T. R. (1997) Neuroscience 78, 361–371
33. Hayashi, Y., Shi, S. H., Esteban, J. A., Piccini, A., Poncer, J. C., and Malinow, R. (2000) Science 287, 2262–2267
34. Lee, H. K., Barbarosie, M., Kameyama, K., Bear, M. F., and Huganir, R. L. (2000) Nature 405, 955–959
35. Boulter, J., Hollmann, M., O’Shea-Greenfield, A., Hartley, M., Deneris, E., Maron, C., and Heinemann, S. (1990) Science 249, 1033–1037
36. Hollmann, M., Hartley, M., and Heinemann, S. (1990) Science 252, 851–853
37. Verdoorn, T. A., Burnashev, N., Monyer, H., Seeburg, P. H., and Sakmann, B. (1991) Science 252, 1715–1718
38. Poncer, J. C., Esteban, J. A., and Malinow, R. (2002) J. Neurosci. 22, 4406–4411
39. Shi, S. H., Hayashi, Y., Petralia, R. S., Zaman, S. H., Wenthold, R. J., Svoboda, K., and Malinow, R. (1999) Science 284, 1811–1816
40. Lu, B. (2003) Learn. Mem. 10, 86–98
41. Alder, J., Thakker-Varia, S., Crozier, R. A., Shaheen, A., Plummer, M. R., and Black, I. B. (2005) J. Neurosci. 25, 3080–3085
42. Chen, G., Kalbeck, R., Barde, Y. A., Bonhoeffer, T., and Kossel, A. (1999) J. Neurovirol. 19, 7983–7990
43. Schuman, E. M. (1999) Cur. Opin. Neurobiol. 9, 105–109
44. Ernfors, P., and Bramham, C. R. (2003) Trends Neurosci. 26, 171–173
45. Nguyen, P. V., and Kandel, E. R. (1996) J. Neurosci. 16, 3189–3198
46. Minichiello, L., Korte, M., Wolfer, D., Kuhn, R., Unsicker, K., Cestari, V., Rossi-Arnaud, C., Lipp, H. P., Bonhoeffer, T., and Klein, R. (1999) Neuron 24, 401–414
47. Xu, B., Gottschalk, W., Chow, A., Wilson, R. I., Schnell, E., Zang, K., Wang, D., Nicoll, R. A., Lu, B., and Reichhardt, L. F. (2000) J. Neurosci. 20, 6888–6897
48. Shi, S., Hayashi, Y., Esteban, J. A., and Malinow, R. (2001) Cell 105, 331–343
49. Passafaro, M., Piech, V., and Sheng, M. (2001) Nat. Neurosci. 4, 917–926
50. Almeida, R. D., Manadas, B. J., Melo, C. V., Gomes, J. R., Mendes, C. S., Grãos, M. M., Carvalho, R. F., Carvalho, A. P., and Duarte, C. B. (2005) Cell Death Differ. 12, 1329–1343
51. Fletcher, T. L., Cameron, P., De Camilli, P., and Banker, G. (1991) J. Neurosci. 11, 1617–1626
52. Boehm, J., Kang, M. G., Johnson, R. C., Esteban, J., Huganir, R. L., and Malinow, R. (2006) Neuron 51, 213–225
53. Du, J. L., and Poo, M. M. (2004) Nature 429, 878–883
54. Minichiello, L., Calella, A. M., Medina, D. L., Bonhoeffer, T., Klein, R., and Korte, M. (2002) Neuron 36, 121–137
55. Sommerfeld, M. T., Schweigreiter, R., Barde, Y. A., and Hoppe, E. (2000) J. Biol. Chem. 275, 8982–8990
56. Groth, R. D., and Mermelstein, P. G. (2003) J. Neurosci. 23, 8125–8134
57. Narisawa-Saito, M., Carnahan, J., Araki, K., Yamaguchi, T., and Nawa, H. (1999) Neuroscience 88, 1009–1014
58. Nakanishi, T., Yanagawa, Y., Obata, K., Narisawa-Saito, M., Namba, H., Otsu, Y., Takei, N., and Nawa, H. (2003) Mol. Cell Neurosci. 24, 340–356
59. Strutz-Seebohm, N., Seebohm, G., Mack, A. F., Wagner, H. J., Just, L., Skutella, T., Lang, U. E., Henke, G., Striegel, M., Hollmann, M., Rouach, N., Nicoll, R. A., McCormick, J. A., Wang, J., Pearce, D., and Lang, F. (2005) J. Physiol. 565, 381–390
60. Bren, S., Messer, C., Okado, H., Hartley, M., Heinemann, S. F., and Nestler, E. J. (2000) Eur. J. Neurosci. 12, 1525–1533
61. Schratt, G. M., Nigh, E. A., Stoy, G., Higa, T., and Reichardt, L. F. (2004) J. Neurosci. 24, 7366–7377
62. Jourdi, H., Iwakura, Y., Narisawa-Saito, M., Ibaraki, K., Xiong, H., Watanabe, M., Hayashi, Y., Takei, N., and Nawa, H. (2003) Dev. Biol. 263, 216–230