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Brain-derived Neurotrophic Factor Regulates the Expression and Synaptic Delivery of α-Amino-3-hydroxy-5-methyl-4-isoxazole Propionic Acid Receptor Subunits in Hippocampal Neurons*

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Margarida V. Caldeira[‡1], Carlos V. Melo[‡], Daniela B. Pereira[‡], Ricardo Carvalho[‡], Susana S. Correia[§], Donald S. Backos[§], Ana Luísa Carvalho[‡], José A. Esteban[§], and Carlos B. Duarte[‡2]

From the [‡]Center for Neuroscience and Cell Biology, Department of Zoology, University of Coimbra, 3004-517 Coimbra, Portugal and the [§]Department of Pharmacology, University of Michigan Medical School, Ann Arbor, Michigan 48109

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 tropomyosin-related kinase *in vitro* 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-amanitine 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 Ca^{2+}-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)[3] 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 Cγ (PLCγ), 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 recruit a presynaptic component (19). However, activation of post-synaptic TrkB receptors generates intracellular Ca^{2+} 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

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[1] A student of the Ph.D. Programme in Experimental Biology and Biomedicine, Centro de Neurociências e Biologia Celular de Coimbra, University of Coimbra.

[2] To whom correspondence should be addressed: Tel.: 351-239-480-209; Fax: 351-239-480-208; E-mail: cbduarte@ci.uc.pt.

[3] The abbreviations used are: BDNF, brain-derived neurotrophic factor; LTP, long term potentiation; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid; DIV, days *in vitro*; CaMKII, Ca^{2+}-calmodulin-dependent protein kinase II; GFP, green fluorescent protein; APV, DL-2-amino-5-phosphonovaleric acid; ANOVA, analysis of variance; PKC, protein kinase C; NMDA, *N*-methyl-D-aspartic acid; TrK, tropomyosin-related kinase.

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 I (5.36 mg/ml), in Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution (5.36 mM KCl, 0.44 mM KH_2PO_4, 137 mM NaCl, 4.16 mM $NaHCO_3$, 0.34 mM $Na_2HPO_4{\cdot}2H_2O$, 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), to stop trypsin activity, and transferred to Neurobasal medium (Invitrogen) supplemented with B27 supplement (1:50 dilution, Invitrogen), 25 μM glutamate, 0.5 mM glutamine, and 0.12 mg/ml gentamycin. The cells were dissociated in this solution and were then plated in 6-well plates (91.6×10^3 cells/cm^2), coated with poly-D-lysine (0.1 mg/ml). The cultures were maintained in a humidified incubator of 5% CO_2/95% air, at 37 °C, for 7 or 14 days. Cultures were stimulated with 100 ng/ml BDNF (kind gift from Regeneron, Tarrytown, NY) for the indicated periods of time. When appropriate, 200 nM K252a, 2.0 μM emetine, 2.0 μM anisomycin, 1.5 μM α-amanitine, 1.5 μM actinomycin D (Calbiochem), 5 μM chelerythrine (Sigma), or 10 μM KN-93 (Sigma) were added 30 min before stimulation, as indicated. Scavenging of endogenous extracellular BDNF was performed with TrkB-IgG (1 μg/ml, Sigma) for 24 h. Hippocampal slices were prepared from young rats (postnatal days 5 and 6) and cultured on semiporous membranes as previously described (25).

Preparation of Extracts—Hippocampal neurons were washed twice with ice-cold phosphate-buffered saline and once more with phosphate-buffered saline supplemented with 1 mM dithiothreitol and a mixture of protease inhibitors (0.1 mM phenylmethylsulfonyl fluoride, 1 μg/ml chymostatin, 1 μg/ml leupeptin, 1 μg/ml antipain, 1 μg/ml pepstatin, Sigma-Aldrich). The cells were then lysed with radioimmune precipitation assay buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 5 mM EGTA, 1% Triton, 0.5% deoxycholic acid, and 0.1% SDS at a final pH 7.5) supplemented with 50 mM NaF, 1.5 mM sodium orthovanadate, and the mixture of protease inhibitors. After centrifugation at 16,100 × *g* for 10 min, protein in the supernatants was quantified using the bicinchoninic acid method, and the samples were diluted with a 2× concentrated denaturating buffer (125 mM Tris, pH 6.8, 100 mM glycine, 4% SDS, 200 mM dithiothreitol, 40% glycerol, 3 mM sodium orthovanadate, and 0.01% bromphenol blue).

Extracts from treated hippocampal slices were prepared in homogenization buffer containing protease and phosphatase inhibitors (10 mM HEPES, 500 mM NaCl, 10 mM EDTA, 10 mM NaF, 1 μM microcystin, 0.1 mM phenylmethylsulfonyl fluoride, 2 μg/ml chymostatin, 2 μg/ml leupeptin, 2 μg/ml antipain, 2 μg/ml pepstatin, 10 μM sodium orthovanadate, and 1% Triton X-100). Samples were centrifuged at 16,100 × *g* for 4 min, and the protein present in the supernatant was quantified using the bicinchoninic acid method and denaturated with denaturating buffer at 95 °C for 5 min. The proteins of interest were then analyzed by Western blot.

Total RNA Isolation and Reverse Transcription for Real-time PCR—Total RNA from cultured hippocampal neurons was extracted with TRIzol reagent (Invitrogen), according to the instructions of the manufacturer. The full content of a 6-well cell cluster plate, with 870,000 cells/well (7 DIV), was collected for each experimental condition. For first strand cDNA synthesis, 3 μg of total RNA was reverse-transcribed with avian myeloblastosis (AMV) reverse transcriptase (Roche Applied Science) using random primer $p(dN)_6$ (3.2 μg), dNTPs (1 mM each), $MgCl_2$ (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 TCC GTG TTT GTT CG-3′ and RGR1R2937, 5′-TTC CTG TCT GCT CCA GTT AC-3′ for GluR1; RGR2F2522, 5′-GAA GCC TTG TGA CAC CAT GA-3′ and RGR2R3008, 5′-AGC CTT GCC TTG CTC CTC AT-3′ for GluR2; RGR3F2431, 5′-CAA AGG CTA TGG TGT GGC AA-3′ and RGR3R2927, 5′-ACA CCA GGG AGA GTG AAA TC-3′ for GluR3; S2288, 5′-TGG AGG GCG TGG CTC GTG TC-3′ and R2800, 5′-TTG GGG CAG TCA GGG GTA AG-3′ for GluR4. The primers used for the amplification of endogenous control gene 18 S 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 mM $MgCl_2$, 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

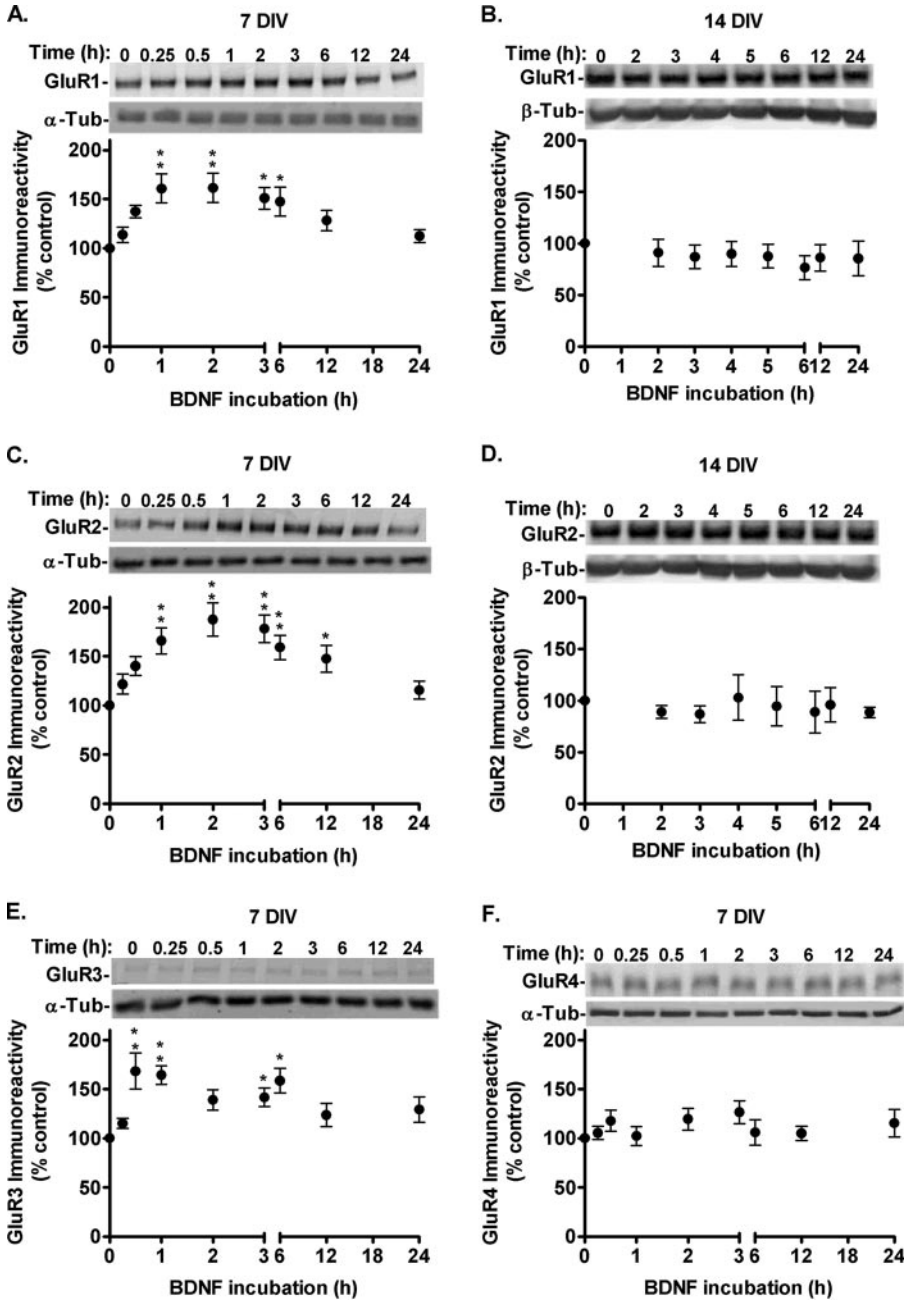

FIGURE 1. **BDNF up-regulates the protein levels of the GluR1, GluR2, and GluR3 AMPA receptor subunits in developing cultured hippocampal neurons.** 7 DIV (*A*, *C*, *E*, and *F*) or 14 DIV (*B* and *D*) cultured neurons were incubated with or without 100 ng/ml BDNF (15 min, 30 min, 1 h, 2 h, 3 h, 5 h, 6 h, 12 h, and 24 h) as indicated. Total GluR1 (*A*), GluR2 (*C*), GluR3 (*E*), and GluR4 (*F*) protein levels were determined by Western blot for 7 DIV, and GluR1 (*B*) and GluR2 (*D*) protein levels were determined for 14 DIV. Control (0 h) protein levels of AMPA receptor subunits were set to 100%. α-Tubulin or β-tubulin were used as loading controls. The results are the average ± S.E. of 5–9 independent experiments, performed in independent preparations. Statistical analysis was performed by one-way ANOVA, followed by the Dunnett test. *, $p < 0.05$; **, $p < 0.01$.

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 $\Delta\Delta C_P$ 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" (C_P) values given by the LightCycler system II software, for each target gene, were subtracted by the respective C_P value determined for the 18 S gene from the same sample and condition (ΔC_P). This allows normalizing changes in target gene expression. Afterward, the ΔC_P values were subtracted by the respective values of the control for the target gene giving $\Delta\Delta C_P$. The derivation to the value of $2^{-(\Delta\Delta C_P)}$ sets each control at the unity (or 100%), because $\Delta\Delta C_P$ (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 $CaCl_2$ and 1 mM $MgCl_2$, 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 denaturating buffer at 95 °C for 5 min (Fig. 7, *A*, *C*, and *D*) or at 65 °C for 15 min (Fig. 7*B*). 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 chemifluorescence (ECF) on the Storm 860 Gel and Blot Imaging System (Amersham Biosciences) or by ECL (Fig. 9*A*). The following

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 $CaCl_2$, 4 mM $MgCl_2$, 26 mM $NaHCO_3$, 1 mM NaH_2PO_4, 11 mM glucose, 0.1 mM picrotoxin, 0.1 mM DL-2-amino-5-phosphonovaleric acid (APV), and 2 μM 2-chloroadenosine (pH 7.4), gassed with 5% CO_2/95% O_2. Patch recording pipettes (4–7 MΩ) were filled with the 115 mM cesium methanesulfonate, 20 mM CsCl, 10 mM HEPES, 2.5 mM $MgCl_2$, 4 mM Na_2ATP, 0.4 mM Na_3GTP, 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 from 30 min to 2.5 h. When the effect of NMDA receptor activity on AMPA delivery to the synapse was studied, 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 blotting (Fig. 1). BDNF rapidly up-regulated GluR1-GluR3 protein levels, in a transient manner, with small effects observed already after 15 min of stimulation with the neurotrophin (Fig. 1, *A*, *C*, and *E*). In contrast, BDNF did not change GluR4 protein levels (Fig. 1*F*). The increases in GluR1, GluR2, and GluR3 had distinct kinetics, and the maximal effects were observed after incubation with BDNF for 1 h, 2 h, and 30 min, respectively. For longer periods of BDNF stimulation (12–24 h), the abundance of GluR1-GluR3 subunits decreased to values similar to the control. Because there was little GluR3 immunoreactivity with the antibody used, the effect of BDNF on this subunit was not further examined.

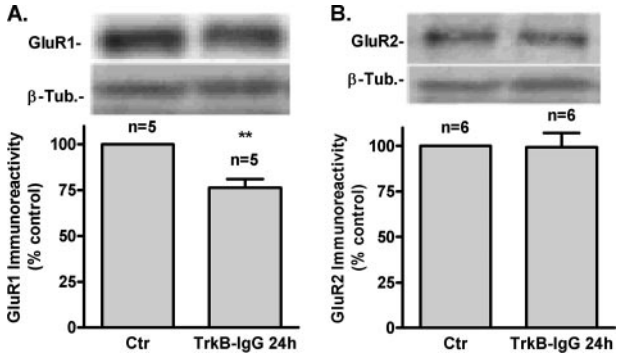

FIGURE 2. **Chelation of endogenous released BDNF with the fusion protein TrkB-IgG decreases the protein levels of GluR1 but was without effect on GluR2 protein levels.** 14 DIV neurons were incubated with or without 1 μg/ml TrkB-IgG for 24 h. Total GluR1 (*A*) and GluR2 (*B*) protein levels were measured 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 (Fig. 1, *B* and *D*). However, when the endogenous extracellular BDNF was chelated with the fusion protein TrkB-IgG for 24 h, the levels of GluR1 significantly decreased (Fig. 2*A*), whereas no changes were observed for the GluR2 subunit (Fig. 2*B*). These results indicate that endogenous BDNF regulates the GluR1 protein levels in 14 DIV cultures of hippocampal neurons. Activation of Trk 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).

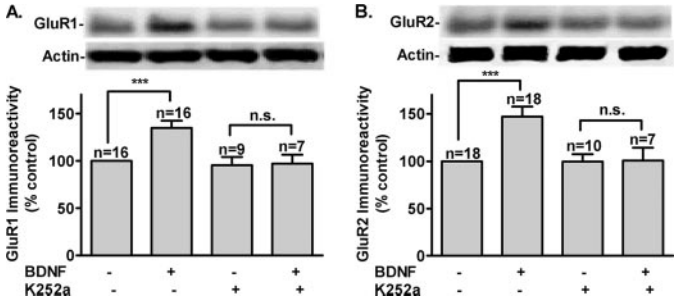

FIGURE 3. **Inhibition of Trk activity blocks the BDNF-induced up-regulation of GluR1 and GluR2 protein levels.** Neurons (7 DIV) were incubated with or without 100 ng/ml BDNF for 2 h, in the presence or in the absence of K252a (200 nM). When the inhibitor was present it was preincubated 30 min before stimulation with BDNF. Total GluR1 and GluR2 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 7–18 independent experiments, performed in independent preparations. Statistical analysis was performed by one-way ANOVA, followed by the Bonferroni test. ***, $p < 0.001$.

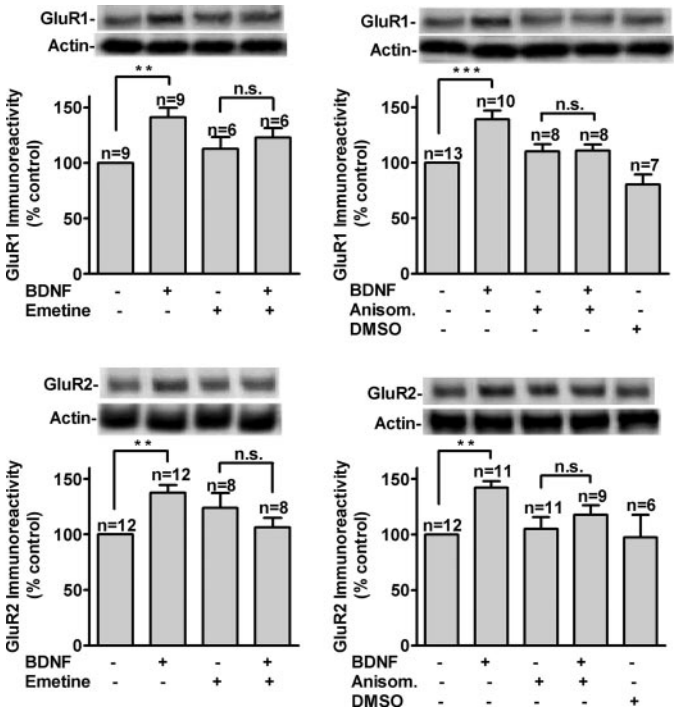

FIGURE 4. **Translation inhibitors impair the BDNF-induced up-regulation of GluR1 and GluR2 protein levels.** Neurons (7 DIV) were incubated with or without 100 ng/ml BDNF for 3 h, in the presence or in the absence of emetine (2.0 μM, *left panels*) or anisomycin (2.0 μM, *right panels*). When the inhibitors 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*, Me_2SO.

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) and actinomycin D (1.5 μM). Both transcription inhibitors blocked the effect of BDNF on GluR1 and GluR2 protein levels, but were without effect on the abundance of the receptor subunits in the absence of the neurotrophin (Fig. 5). In agreement with these observations, real-time PCR using the SYBR green assay showed that BDNF stimulation for 30 min increased significantly the mRNA levels for GluR1, and when the cells were incubated with the neurotrophin for 3 h a significant increase in the mRNA for GluR1 and GluR2 was also observed (Fig. 6). Taken together, the results indicate that BDNF regulates GluR1 and GluR2 AMPA receptor subunits at the transcription level. In contrast with the results obtained for GluR1 and GluR2, no effect of BDNF was observed on the mRNA levels for GluR3 and GluR4.

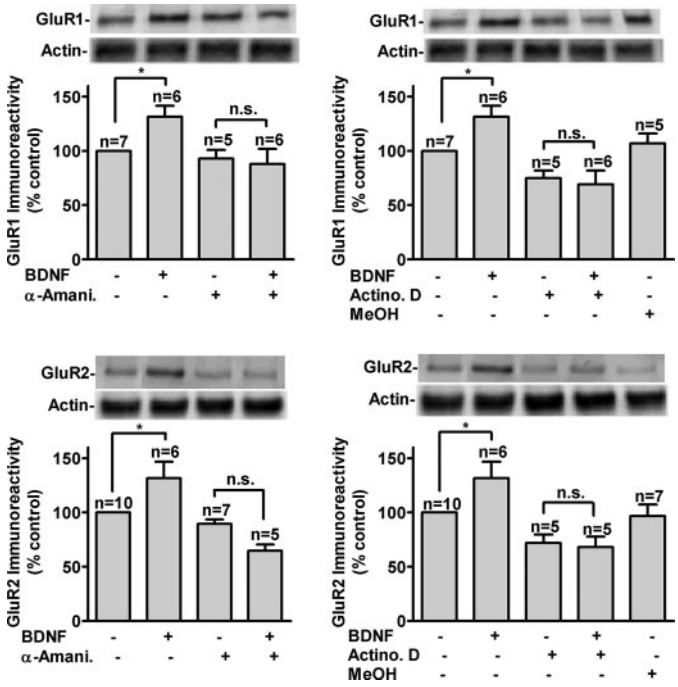

FIGURE 5. **Transcription inhibitors prevent the BDNF-induced up-regulation of the GluR1 and GluR2 protein levels.** Neurons (7 DIV) were incubated with or without 100 ng/ml BDNF for 3 h, in the presence or in the absence of α-amanitine (1.5 μM, *left panels*) or actinomycin D (1.5 μM, *right panels*). 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 5–10 independent experiments, performed in independent preparations. Statistical analysis was performed by one-way ANOVA, followed by the Bonferroni test. *, $p < 0.05$.

Effect of BDNF on GluR1 and GluR2 Protein Levels at the Plasma Membrane—Because a significant percentage of glutamate receptors expressed in neurons is intracellular (32) and, therefore, do not participate in the response to the neurotransmitter glutamate, we tested whether BDNF can affect the translocation of AMPA receptor subunits to the plasma membrane. To study the effect of BDNF on the cell surface distribution of the AMPA receptor subunits GluR1 and GluR2, 7 DIV cultured

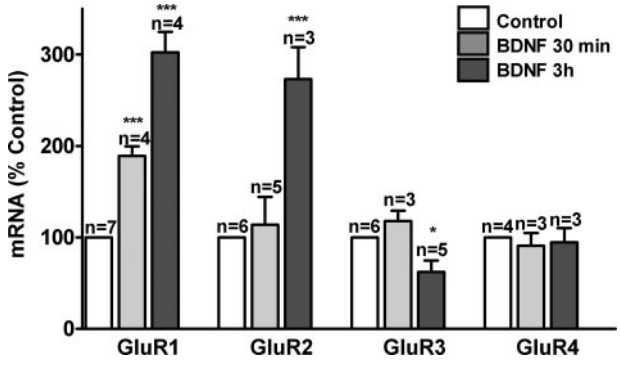

FIGURE 6. **BDNF increases the mRNA levels of the GluR1 and GluR2 AMPA receptor subunits.** The variation of GluR1 and GluR2 mRNA levels was assayed by SYBR Green real-time PCR of total RNA samples, converted to cDNA in reactions normalized to contain equal amounts of mRNA. The cells (7 DIV) were incubated in the presence or absence of 100 ng/ml BDNF, during 30 min (*gray columns*) or 3 h (*black columns*). The results are presented as mean percentage ± S.E. compared with the control (unstimulated), and normalized to the reference gene 18 S, and are the average ± S.E. of 3–7 independent experiments, performed in independent preparations. Statistical analysis was performed by one-way ANOVA, followed by the Dunnett test. *, $p < 0.05$; ***, $p < 0.001$.

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- or 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. 7*A*). The increase observed by BDNF treatment during 30 min was blocked by emetine (Fig. 7*B*), 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. 7*D*). 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. 7*C*).

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. 7*E*). 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. 7*F*, see also Fig. 1*A*). 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 μM) and KN-93 (10 μM) 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. 7*E*).

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. 8*B*), 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. 7*B* and 8*A*; compare *second* and *fourth columns* in Fig. 8*B*). BDNF had no significant effect on the rectification index of non-infected cells (Fig. 8*B*; 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 αCaMKII (*tCaMKII*, Fig. 7*B* and 8*A*; compare *fourth* and *seventh columns* in Fig. 8*B*), 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. 8*B*; 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 μM) for 30 min did not affect the increase in the rectification index induced by BDNF (Fig. 8*B*; 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. 7*E*). In contrast, no

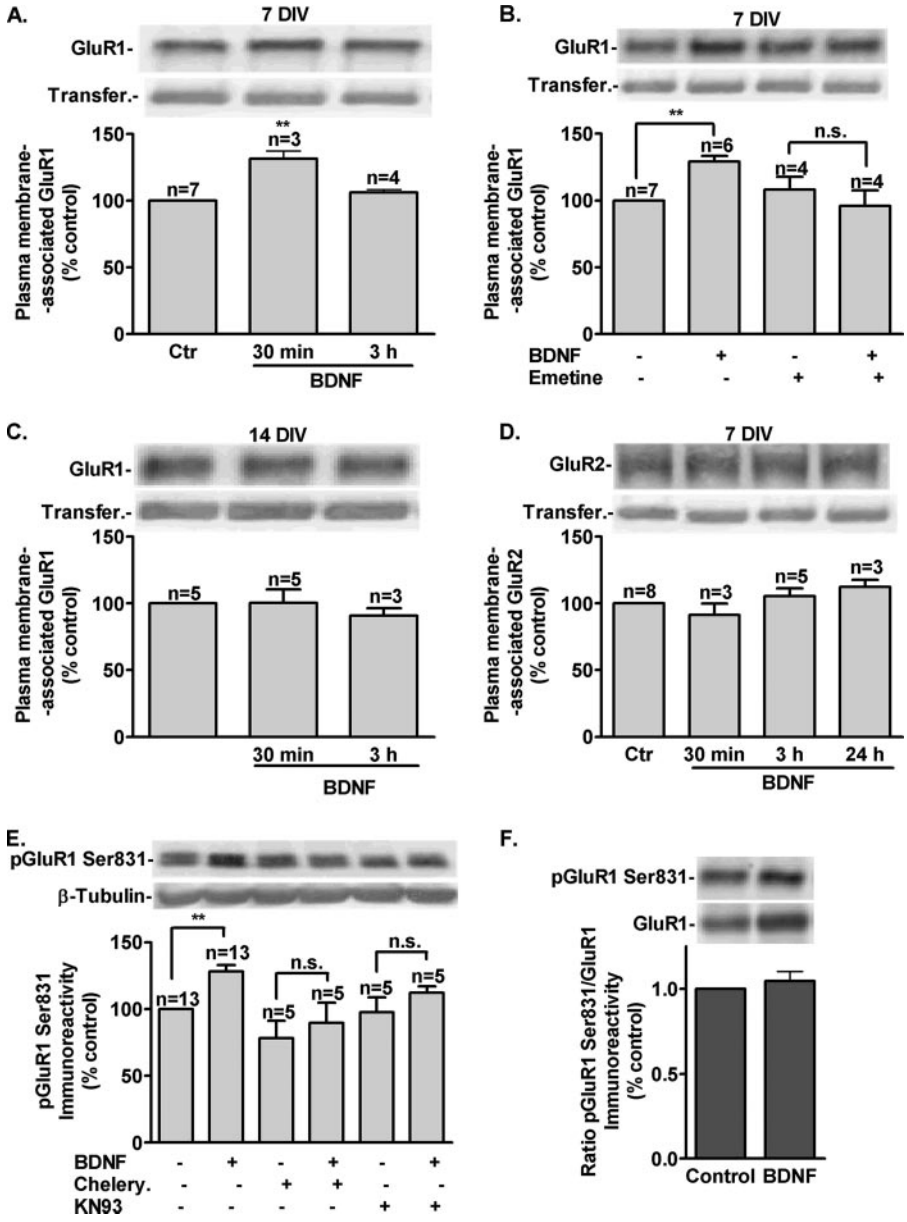

FIGURE 7. **BDNF increases the GluR1 subunit in the plasma membrane, but not the GluR2 subunit, in developing cultured hippocampal neurons.** 7 DIV (*A*, *B*, and *D*) or 14 DIV (*C*) cultured neurons were treated with or without 100 ng/ml BDNF (30 min, 3 h, and 24 h) as indicated. In *panel B*, 7 DIV neurons were preincubated or not with emetine (2.0 μM), for 30 min, before stimulation with BDNF, for 30 min, in the presence or in the absence of the inhibitor. Following treatment, cell surface proteins were labeled by biotinylation, followed by precipitation with streptavidin beads. The abundance of each subunit in the plasma membrane was then determined by Western blot. Control (unstimulated) levels of AMPA subunits protein was set to 100%. The transferrin receptor was used as loading control. The effect of BDNF on GluR1 phosphorylation on Ser-831 (pGuR1 Ser-831) is shown in *panel E*. 7 DIV cultured hippocampal neurons were incubated for 30 min with or without BDNF, in the presence or absence of chelerythrine (5 μM) or KN-93 (10 μM). The phosphorylation of GluR1 on Ser-831 was measured by Western blot, using a phosphospecific antibody. Control (unstimulated) phosphorylation of GluR1 was set to 100%. β-Tubulin was used as loading control. The total amount of GluR1 was determined after striping the membranes, and the ratio between pGluR1 Ser-831 and the total amount of GluR1 is plotted in *panel F*. The results are the average $\pm$ S.E. of 3–13 independent experiments, as indicated, performed in independent preparations. Statistical analysis was performed by one-way ANOVA, followed by the Dunnett test (*A*, *C*, and *D*) or Bonferroni test (*B* and *E*). **, $p < 0.01$.

change in GluR1 phosphorylation was detected for Ser-845 (Fig. 9*A*). 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. 9*A*). 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. 9*A*).

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

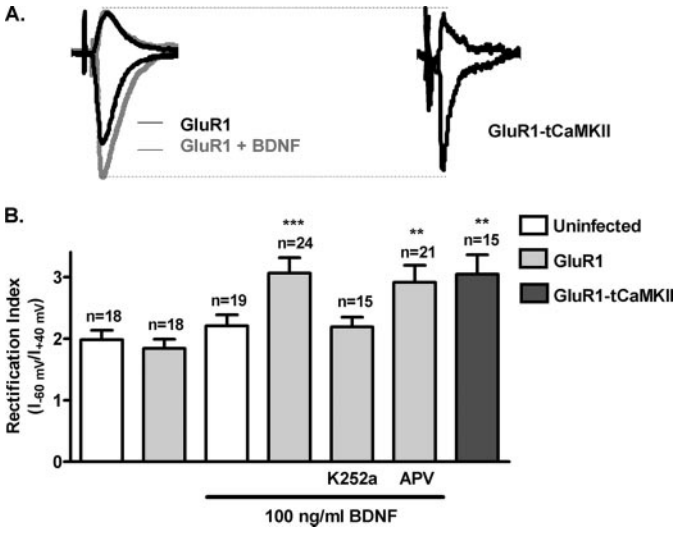

FIGURE 8. **BDNF increases the delivery of the AMPA receptors to the synapse.** AMPA receptor-mediated responses were recorded at −60 mV and +40 mV from CA1 neurons, and the rectification index was calculated as the ratio between the responses at these holding potentials ($I_{-60\,mV}/I_{+40\,mV}$). *A*, AMPA receptor-mediated responses at −60 mV and +40 mV in neurons infected with Sindbis virus expressing recombinant GluR1 or GluR1 plus the constitutive active αCaMKII tagged to GFP. GluR1-infected cells were incubated (*gray line*) or not (*black line*) with 100 ng/ml BDNF. *B*, rectification values were obtained from uninfected neurons, and from neurons infected with recombinant GluR1 or GluR1 plus the constitutive active αCaMKII (as indicated), tagged to GFP. Where indicated the cells were stimulated with 100 ng/ml BDNF for 30 min to 3 h. Because no difference was found between the results obtained for the various preincubation periods tested, the results were pooled together. The effect of 200 nM K252a and 0.1 mM APV on the BDNF-induced increase on the rectification index was also tested by perfusing the hippocampal slices with these compounds for 30 min prior to BDNF incubation and during the recordings. The results are the average ± S.E. of the indicated number of experiments. Statistical analysis was performed using the Student *t* test. **, $p < 0.01$; ***, $p < 0.001$.

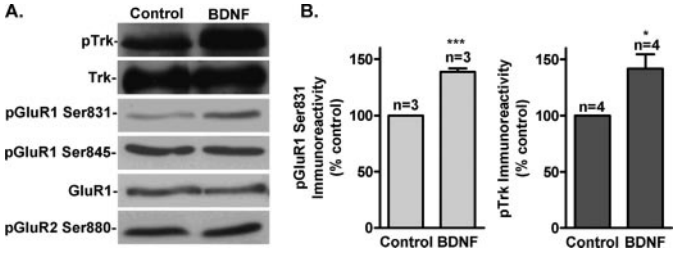

FIGURE 9. **BDNF induces the phosphorylation of GluR1 on Ser 831.** *A*, hippocampal slices were stimulated with 100 ng/ml BDNF for 30 min, and Trk, GluR1, and GluR2 phosphorylation was analyzed by Western blot, using phosphospecific antibodies. BDNF induces phosphorylation of Trk receptor, and GluR1 phosphorylation at Ser-831 but not at Ser-845. GluR2 phosphorylation at Ser-880 is not affected by BDNF (*A* and *B*). The phosphorylation of GluR1 on Ser-831 and the phosphorylation of Trk were normalized to GluR1 and Trk receptor protein levels, respectively. The results are the average ± S.E. of 3–4 independent experiments. Statistical analysis was performed using the Student *t* test. *, $p < 0.05$; ***, $p < 0.001$.

enhancing the translation activity (Fig. 7*B*). 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-associated receptors by interaction with protein(s) synthesized following Trk receptor stimulation. The acute effects of BDNF on the plasma membrane GluR1 protein levels may require continuous signaling activity by Trk receptors, because after 3-h stimulation with BDNF, when Trk receptors are desensitized to some extent (50), the receptor subunits found in the membrane were similar to the control. In contrast with the 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. 1*B*), and on the total amount of surface receptors (Fig. 7*C*), 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. 9*A*).

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. 8*B*). 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 naïve 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 Ca^{2+} 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

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 cultured 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 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.

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ADDITIONS AND CORRECTIONS

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THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 282, NO. 37, pp. 27556–27556, September 14, 2007

VOLUME 282 (2007) PAGES 14788–14796

RIP1-mediated AIP1 phosphorylation at a 14-3-3-binding site is critical for tumor necrosis factor-induced ASK1-JNK/p38 activation.

Haifeng Zhang, Yan Lin, Jiehui Li, Jordan S. Pober, and Wang Min

PAGE 14788:

The first author should be listed as Haifeng Zhang rather than Rong Zhang, whose name should be deleted.

VOLUME 282 (2007) PAGES 12619–12628

Brain-derived neurotrophic factor regulates the expression and synaptic delivery of α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor subunits in hippocampal neurons.

Margarida V. Caldeira, Carlos V. Melo, Daniela B. Pereira, Ricardo Carvalho, Susana S. Correia, Donald S. Backos, Ana Luísa Carvalho, José A. Esteban, and Carlos B. Duarte

PAGE 12619:

In the abstract, beginning with the third sentence, lines 6–13 should read: "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 *in vitro* cultured hippocampal neurons. The increase in GluR1 and GluR2 protein levels in developing cultures was impaired by K252a, a tropomyosin-related kinase (Trk) inhibitor, and by translation (emetine and anisomycin) and transcription (α-amanitine and actinomycin D) inhibitors."

VOLUME 282 (2007) PAGES 9105–9116

Dynamic assembly of TRPC1-STIM1-Orai1 ternary complex is involved in store-operated calcium influx. EVIDENCE FOR SIMILARITIES IN STORE-OPERATED AND CALCIUM RELEASE-ACTIVATED CALCIUM CHANNEL COMPONENTS.

Hwei Ling Ong, Kwong Tai Cheng, Xibao Liu, Bidhan C. Bandyopadhyay, Biman C. Paria, Jonathan Soboloff, Biswaranjan Pani, Yousang Gwack, Sonal Srikanth, Brij B. Singh, Donald L. Gill, and Indu S. Ambudkar

PAGE 9105:

The middle initial for Dr. Gill was inadvertently omitted. The correct author list is now shown above.

VOLUME 279 (2004) PAGES 55109–55116

Mixed macromolecular crowding accelerates the oxidative refolding of reduced, denatured lysozyme. IMPLICATIONS FOR PROTEIN FOLDING IN INTRACELLULAR ENVIRONMENTS.

Bing-Rui Zhou, Yi Liang, Fen Du, Zheng Zhou, and Jie Chen

PAGE 55111:

In Table I, the first two lines of the legend should read: "Dextran + BSA represents a mixed crowding agent containing 90 g/liter dextran 70 and 10 g/liter BSA. Ficoll + BSA represents a mixed crowding agent containing 90 g/liter Ficoll and 10 g/liter BSA."