# Protein Synthesis-dependent and -independent Regulation of Hippocampal Synapses by Brain-derived Neurotrophic Factor\*

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as long as the recordings can be maintained (1). Similar short-

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A fundamental difference between short-term and long-term forms of synaptic plasticity is the dependence on transcription and translation of new genes. Using organotypic cultures of hippocampal slices, we have investigated whether the modulation of synapses by brain-derived neurotrophic factor (BDNF) also requires protein synthesis. Long-term treatment of hippocampal slice cultures with BDNF increased the number of docked vesicles, but not that of reserve pool vesicles, at CA1 excitatory synapses. BDNF also increased the levels of the vesicle proteins synaptophysin, synaptobrevin, and synaptotagmin, without affecting the presynaptic membrane proteins syntaxin and SNAP-25, or the vesicle-binding protein synapsin-I. The increase in synaptophysin and synaptobrevin expression was moderate (2fold) and occurred within 6 h after BDNF application. In contrast, synaptotagmin expression took 24 h to reach maximum levels (5-fold). The delayed increase in synaptotagmin was blocked by protein synthesis inhibitors, while the early increase in synaptophysin and synaptobrevin was not. Moreover, the BDNF-induced increase of synaptotagmin was blocked by inhibiting the cAMP/ protein kinase A (PKA) pathway. However, BDNF did not activate PKA, and application of a PKA activator did not mimic the BDNF effect. Taken together, these results suggest a novel, protein synthesis-dependent form of BDNF modulation that requires cAMP gating.

As the cellular basis for learning and memory, two forms of synaptic plasticity have been subjects of intensive study: shortterm changes in synaptic strength within minutes and hours, and long-term modulation of the structure and function of synapses over the course of days. In the mammalian hippocampus, a single high-frequency stimulation of afferent fibers elicits an early phase of long-term potentiation (E-LTP)<sup>1</sup> lasting for 2–3 h, while repeated (3–4 times) high-frequency stimulation results in long-lasting, late phase LTP (L-LTP) that lasts

and long-term forms of synaptic plasticity have been observed in the sea slug Aplysia (2) and the fruit fly Drosophila (3, 4). A single application of the neuromodulator serotonin facilitates synaptic transmission at these synapses for a few hours. In contrast, 4-5 repeated applications of serotonin elicit a longterm facilitation that lasts for days. These forms of short- and long-term facilitation of synaptic transmission are thought to underlie short- and long-term sensitization, a simple form of learning and memory (2). Similarly, brief training of the fruit fly Drosophila results in short-term memory, while repeated, spaced training leads to long-term memory formation (3, 4). A unique feature sets long-term modulation apart from shortterm plasticity: its dependence on protein synthesis (1-3). It has been proposed that repeated stimulation leads to a cAMPdependent, sustained phosphorylation of the transcription factor CREB (cAMP response element binding protein), which in turn triggers the expression of several genes responsible for long-term structural and functional changes at synapses (2, 3, 5). Serotonin has been identified as the neuromodulator that mediates both short- and long-term synaptic facilitation in Aplysia (2). Recent studies suggest that neurotrophins, originally defined as a family of trophic factors for neuronal survival and differentiation, may serve as a new class of neuromodulators that regulate synaptic transmission, synapse development, and plasticity in vertebrates (6). Two major effects have been described at the neuromuscular synapses: (i) acute potentiation of transmitter release; (ii) long-term regulation of synapse development. Acute application of BDNF or neurotrophin-3 (NT-3) rapidly enhances synaptic transmission at the neuromuscular junction (7). The acute effect of neurotrophins is due strictly to an enhancement of transmitter release probability at presynaptic sites (7, 8). Moreover, the protein synthesis inhibitor anisomycin or cycloheximide does not prevent the effects of neurotrophins, suggesting that acute potentiation of synaptic transmission by neurotrophins is completely independent of protein synthesis (8, 9). In the long-term mode, treatment with BDNF or NT-3 (2-3 days) results in a sustained increase in quantal size as well as a more reliable impulseevoked synaptic transmission (10). Moreover, BDNF or NT-3 enhances the expression of synaptic vesicle proteins such as synaptophysin and synapsin-I, and increases the number of synaptic varicosities in the presynaptic site (10).

Acute neurotrophic modulation of synaptic transmission and plasticity has also been observed in the central nervous system (CNS). For example, application of BDNF or NT-3 to cultured hippocampal or cortical neurons rapidly enhances neuronal activity and synaptic transmission (11-13). Moreover, substantial evidence indicates that BDNF acutely modulates E-LTP in the hippocampus (14-16). Application of exogenous BDNF fa-

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<sup>&</sup>lt;sup>1</sup> The abbreviations used are: E-LTP, early phase long-term potentiation; L-LTP, late phase long-term potentiation; BDNF, brain-derived neurotrophic factor; NT-3, neurotrophin-3; CNS, central nervous system; PKA, protein kinase A.

cilitates tetanus-induced LTP at the CA1 synapses in neonatal hippocampal slices, in which the endogenous BDNF levels are low (15). In contrast, inhibition of endogenous BDNF activity, either by the BDNF scavenger TrkB-IgG, or by BDNF gene knockout, reduces the magnitude of tetanus-induced LTP in adult hippocampus, in which the endogenous BDNF levels are high (14-17). Acute modulation of hippocampal LTP by BDNF may result from the BDNF-induced increase in the synaptic responses to high-frequency stimulation (15, 18). This effect requires activation of mitogen-activated protein kinase and phosphatidylinositol 3-kinase, and is insensitive to inhibitors of new protein synthesis (19). Analysis of the hippocampal synapses in BDNF knockout mice reveals three major deficits: (i) a pronounced impairment in the synaptic responses to high frequency stimulation that correlates with a reduction in LTP; (ii) a selective decrease in synaptobrevin and synaptophysin in synaptosomes; and (iii) a marked reduction in the number of docked synaptic vesicles, without affecting the total number of vesicles (20). The defects in LTP and synaptic responses to high frequency stimulation, as well as the reduction in the two vesicle proteins, can be rescued by treatment of the knockout slices with BDNF for a few hours (16, 20). Moreover, application of BDNF to cortical synaptosomes elicits a mitogen-activated protein kinase-dependent phosphorylation of synapsin-I, leading to an increase in availability of synaptic vesicles for release (21). These results suggest that acute modulation of CNS synapses by BDNF is achieved by protein synthesis-independent modifications of existing presynaptic proteins.

Neurotrophins are also involved in long-term modulation of CNS synapses. Substantial evidence suggests their role in the development of ocular dominance columns in cat and rodents in vivo (22, 23). Long-term treatment of slices derived from the visual cortex with neurotrophins elicits profound effects on dendritic growth (24). In dissociated cultures of hippocampal or cortical neurons, chronic application of BDNF results in complex effects on synaptic transmission (25-29). However, the molecular mechanisms underlying long-term modulation of synaptic transmission in the CNS by BDNF are essentially unexplored. Using organotypic slice cultures as a model system, the present study shows that long-term BDNF treatment increases the number of synaptic vesicles docked at active zones of excitatory CA1 synapses. The expression of specific synaptic vesicle proteins synaptophysin, synaptobrevin, and synaptotagmin hippocampal slices is also enhanced by longterm treatment with BDNF. The delayed increase in synaptotagmin levels is prevented by inhibiting either protein synthesis, while the early increases in synaptophysin and synaptobrevin are not. Moreover, the effect of BDNF on synaptotagmin could be blocked by the inhibitor of cAMP-signaling cascade, but the activation of the cascade does not mimic the BDNF effect. These results suggest that BDNF exerts its actions on hippocampal synapses by two different mechanisms, a rapid protein synthesis-independent pathway, and a delayed cAMP- and protein synthesis-dependent cascade reminiscent of the late phase of LTP.

### EXPERIMENTAL PROCEDURES

Hippocampal Slice Cultures—Hippocampal slices (400  $\mu$ m thick) were prepared from postnatal day 7 (P7) rats. The slices were cultured on Unlaminated Hydrophilic Fluoropore membranes (Millipore) placed on the inserts of 6-well plates (Fisher) in a 37 °C, 5% CO<sub>2</sub>, 99% humidity incubator. The slices were maintained for 5 days in horse serum-containing medium (50% minimal essential medium with Earle's salts, 25% HBSS, 10 mM HEPES, 0.5% GlutaMax II, glucose, pH 7.2, 25% horse serum), then for 3 days in Dulbecco's modified Eagle's medium N1 biotin-containing medium plus 3% serum, and finally for 1 day in serum-free Dulbecco's modified Eagle's medium N1 biotin-containing medium was then replaced with the serum-free medium containing 6 nM BDNF (kindly provided by Regeneron Pharmaceuti-

cals), and the cultures were treated with BDNF for 3–72 h. For some experiments, drugs such as PKA inhibitor  $R_{\rm p}$ -cAMPs or activator  $S_{\rm p}$ -cAMPs (100  $\mu$ M; Biolog Life Science Institute), or the protein synthesis inhibitor anisomycin (20  $\mu$ M; Sigma) were added to the medium. Slices were pretreated with drugs for 30 min before the BDNF treatment was initiated. All medium reagents were purchased from Life Technologies, Inc.

Quantitative Electron Microscopy-Three independent pairs of cultures (control and BDNF-treated), each contained multiple slices, were used for electron microscopy (EM) analysis. In each pair of cultures, sister slices were used for Western blot analysis of synaptotagmin to confirm the effectiveness of BDNF. The remaining sister slices from the pair of cultures were fixed and processed for EM using standard procedures (30). Briefly, slices with the attached filter membrane were transferred to 35-mm plastic culture dishes and fixed with 2.5% glutaraldehyde in 0.15 M sodium cacodylate (pH 7.3), post-fixed in 1% osmium tetroxide in 0.1 M sodium cacodylate, stained en bloc with 1% uranyl acetate, dehydrated, and flat embedded in Epon (24 h at 60 °C). After curing, the Epon disc containing the slices was separated from the plastic dish. Semi-thin sections  $(0.5 \ \mu m)$  were cut, stained with toluidine blue, and used to locate and trim the CA1 region. Ultra-thin sections were cut at  ${\sim}80$  nm, collected on grids, stained with uranyl acetate and lead citrate, and then examined in a JEOL-100CX transmission electron microscope operated at 80 kV. Asymmetric spine synapses within CA1 stratum radiatum were photographed at a final magnification of  $\times$  33,000. The EM negatives were then scanned at 300 dots per inch, converted to positive images, and saved as digital images. The area of the presynaptic terminals and the length of the active zones were measured using NIH Image directly from the digital images.

Treatment with BDNF in general did not significantly affect the size of CA1 synapses. Preliminary results indicated that treatment of the slice cultures with BDNF for 5-9 days increased active zone length by only  $\sim 11\%$ <sup>2</sup> However, larger objects within the tissue block have a higher probability of being sectioned, and thus observed in the sections. To avoid the bias of selecting the larger synapses during sampling in single thin sections, only synapses having approximately equal area of presynaptic terminals and length of active zones were chosen for analysis. This approach also permitted the normalization of the vesicle number per length of active zone (in microns) and presynaptic terminal area (in squared microns), allowing unequivocal assessment of vesicle distributions within presynaptic terminals without the confounding effect of variable synapse size. A total of 84 synapses from three pairs of slices (control n = 43 synapses, N = 3; BDNF n = 41 synapses, N = 3) fitted the above criteria were used for analysis. Corrections for remaining biases, such as tissue shrinkage and section thickness, were not performed because it is expected that those biases would affect both treatment groups equally. The diameter of small, clear and round synaptic vesicles are fairly homogeneous of  $\sim$ 50 nm, smaller than the thickness of ultra-thin sections. Thus, most vesicles can be observed within the thickness of the sections. We counted only the small, clear and round vesicles with at least one-half the circumference of their total plasma membrane clearly visible (30). Docked vesicles were defined as those located within one vesicle diameter (~50 nm) from presynaptic membrane (30), while reserve pool vesicles included the remaining set of vesicles within the presynaptic terminal profile. All analyses were performed in a blinded fashion; the observers acquiring the images and performing the measurements were not aware of the treatment groups.

Western Blot Analysis-Slice cultures were harvested and homogenized in lysis buffer (20 mM HEPES, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mm sodium orthovanadate, 50 mm NaF, 1 mm phenylmethylsulfonyl fluoride, 1% Nonidet P-40, 0.1% SDS, 1% deoxycholic acid, 10% glycerol, 10  $\mu$ g/ml leupeptin, and 1  $\mu$ g/ml aprotinin, pH 7.4) using a Dounce tissue grinder at 4 °C. The homogenates were incubated on ice for 30 min. Insoluble materials were removed by high-speed centrifugation for 20 min (12,000  $\times$  g). The supernatants were carefully taken into clean tubes, and protein concentrations of the supernatants were determined by the Bio-Rad protein assay. Equal amounts of protein (3)  $\mu g$  per lane) in sample buffer (1% SDS, 10% glycerol, 100 mm Tris, 0.05% biphenyl blue, 5% 2-mercaptoethanol) were denatured, separated on a 4-20% Tris glycine gel. The proteins on the gels were transferred to an Immobilon P membrane (Millipore), and the membranes were incubated with blocking buffer (0.2 mM Tris, 137 mM NaCl, 5% (w/v) nonfat milk, and 0.1% Tween 20) for 1 h, and then probed at 4 °C overnight with the following monoclonal antibodies from Chemicon: actin (1:20,000), synaptophysin (1:2,000), synapsin I (1:5,000), syntaxin

<sup>2</sup> W. J. Tyler and L. Pozzo-Miller, unpublished observations.

FIG. 1. BDNF selectively increases the number of docked vesicles per active zone at CA1 excitatory spine synapses in hippocampal slice cultures. Neonatal hippocampal slices were treated with BDNF for 48 h and the slices were harvested for quantitative EM analysis. A, representative electron micrographs of excitatory spine synapses from CA1 stratum radiatum of control (left) and BDNFtreated (right) hippocampal slice cultures. The arrowheads mark the length of postsynaptic density, and the arrows point to representative docked vesicles included in the quantitative analyses. B, quantitative analyses of synaptic vesicle density at excitatory spine synapses from CA1 stratum radiatum. The histograms plot the number of docked vesicles (DV)normalized per active zone length  $(DV/\mu m \text{ of active zone, } left)$  and the number of reserve pool vesicles (RV) normalized per terminal area  $(RV/\mu m^2 \text{ of termi-}$ nal, right). Data are presented as mean  $\pm$ S.E. \*: p < 0.05, Student's t test. Control, n = 43 synapses; BDNF, n = 41 synapses; N = 3 slices in each condition.



(1:10,000), SNAP-25 (1:500), neuron-specific enolase (1:1,000); or the following antibodies from Dr. M. Takahashi synaptotagmin (monoclonal, 1:10,000), or antibody for synaptobrevin (polyclonal, 1:20,000). In most cases, two membranes were used. One membrane was cut to three pieces and reacted with synaptotagmin (65 kDa), synaptophysin (38 kDa), and SNAP-25 (25 kDa) antibodies, while the other membrane was cut to three pieces and reacted with synapsin I (85 kDa), actin (43 kDa), syntaxin (35 kDa), and synaptobrevin (18 kDa) antibodies. The membranes were rinsed with washing buffer (0.1% Tween 20, 0.2 mm Tris, 137 mM NaCl) and incubated with secondary antibody (1:10,000) for 1 h at room temperature, followed by chemiluminescent detection (ECL; Pierce).

In each experiment with multiple experiment conditions (control or drug-treatments), 3–5 slices for each condition were pooled and harvested. Protein samples from the same experimental conditions were run on an SDS gel in triplicate (n = 3). The blot was scanned and the intensities of the bands were quantified using NIH Image software. Signals from experimental conditions were normalized to the average signals in the control condition in the same blot. The same experiments (with multiple experiment conditions) were repeated 5–10 times (N = 5-10), and mean  $\pm$  S.E. are presented.

Immunocytochemistry-Slice cultures were fixed by 4% paraformaldehyde in phosphate-buffered saline, blocked and permeabilized with 10% horse serum, 2% bovine serum albumin, and 0.1% Triton X-100, all in phosphate-buffered saline. The cultures were incubated at 4 °C overnight in one of the following primary antibodies: anti-synaptophysin and anti-synaptobrevin (1:250, both polyclonal from Santa Cruz), and anti-synaptotagmin (1:10,000, monoclonal from Dr. Takahashi). After incubation with biotinylated secondary antibodies (1:250, Santa Cruz), the cultures were treated with avidin-fluorescein isothiocyanate or avidin-Thy3 (Vector Labs), mounted with Vectashield and imaged with a confocal microscope. Low magnification images were taken to include as much as the CA1 region as possible, mostly with a  $\times 10$  objective, and exported to NIH Image. For synaptophysin and synaptobrevin stainings, all the pixels were selected and their intensity was plotted in a frequency distribution histogram. Appropriate controls lacking primary antibodies were performed for each antibody used.

Protein Kinase A Assay—Slices were cultured in the same way as described above. On the day of PKA assay, the cultures were incubated with nothing (control), forskolin (20  $\mu$ M, positive control), or BDNF (4 nM) for 10 min at 37 °C and harvested in the following PKA lysis buffer: 50 mM Tris (pH 6.8), 1 mM EDTA, 0.1% Triton X-100, 0.1 mM dithio-threitol, protease inhibitor mixture (Calbiochem), and phosphatase inhibitor mixture I (Sigma). The lysates were homogenized using a Dounce glass homogenizer with pestle "B" and centrifuged at 16,000 × g for 10 min at 4 °C. Protein concentrations of the supernatants were determined by Bio-Rad protein assay. PKA assay (31) were performed at 30 °C for 30 min in a final volume of 100  $\mu$ l containing 20 mM Na citrate (pH 7.2), 5  $\mu$ g of PKA substrate peptide (PGRQRRHTLPANE-

FRC), 15 mM MgCl<sub>2</sub>, 0.2 mM ATP, 2  $\mu$ Ci of [ $\gamma$ -<sup>32</sup>P]ATP (6  $\mu$ Ci/nmol, Amersham Pharmacia Biotech), and either 5  $\mu$ g of cytosolic extracts of the slice cultures or 1–30 units of recombinant catalytic subunit of PKA enzyme (Promega). After incubation 5- $\mu$ l aliquots were spotted onto P81 Whatmman filter papers and the filers were washed 5  $\times$  20 min in 75 mM phosphoric acid. The samples were air-dried and the radioactivity counted. A standard curve using different amounts of recombinant PKA was constructed and the PKA activities in slices treated with or without forskolin or BDNF were calculated using the standard curve.

*Statistics*—All parameters were analyzed using Student's *t* test, or ANOVA followed by Scheffé's procedure for multiple comparisons as *post-hoc* analysis; all data shown is presented as mean  $\pm$  S.E. of the mean (S.E.).

#### RESULTS

BDNF Increases the Number of Docked Synaptic Vesicles-Organotypic hippocampal slice cultures prepared from P7 rats were used to determine the structural changes at the hippocampal synapses induced by long-term treatment with BDNF. After 9 days in vitro, the hippocampal slice cultures were treated with 6 nm BDNF for 48 h, before harvesting for quantitative electron microscopy (EM) analysis. The slices were fixed, stained, and embedded. The Epon blocks were trimmed, and the CA1 region identified. Excitatory synapses on CA1 dendritic spines were identified by the following criteria: (i) presynaptic profiles containing small (~50 nm diameter), round and clear synaptic vesicles; (ii) prominent electron-dense postsynaptic densities, characteristic of type-I asymmetric synaptic junctions; and (iii) postsynaptic dendritic spines, identified by their lack of microtubules and mitochondria, continuity with a dendritic shaft, and overall size and shape. Synaptic junctions fulfilling the above criteria are well established to represent glutamatergic excitatory synapses (32). To avoid the bias of selecting the larger synapses during sampling in single thin sections, only synapses having approximately equal area of presynaptic terminals and length of active zones were chosen for analysis. From three independent pairs of slices, we analyzed a total of 84 synapses (control n = 43; BDNF n = 41) that fitted the above criteria. On average, the terminal areas were 0.229  $\pm$  0.009  $\mu m^2$  in control and 0.233  $\pm$  0.009  $\mu m^2$  in BDNF-treated slices, and the active zone lengths (L) were 0.251  $\pm$  0.006  $\mu$ m in control and 0.264  $\pm$  0.006  $\mu$ m in BDNF-treated slices.

Small, round and clear synaptic vesicles were clearly ob-

served in the CA1 spine synapses. The distribution of synaptic vesicles was measured in two distinct and mutually exclusive pools within presynaptic terminals at CA1 excitatory synapses. Docked vesicles are defined as those in close apposition to, or within 50 nm from the presynaptic active zone (33). Reserve pool vesicles are identified as those synaptic vesicles within the presynaptic terminals, but outside of the docked vesicle region (20). Long-term BDNF treatment significantly increased the number of docked vesicles per unit length of active zone at these excitatory spine synapses in CA1 stratum radiatum (Fig. 1A). The number of docked vesicles per micron active zone (DV/ $\mu$ m) increased from 14.7  $\pm$  0.7 in control, to 17.3  $\pm$  0.8 after BDNF treatment (p = 0.0139; Fig. 1B). In contrast, there were no differences in the number of reserve pool vesicles per unit terminal area between control  $(304.4 \pm 7.3 \text{ reserve vesicle})$  $\mu$ m<sup>2</sup> of presynaptic terminal area) and BDNF-treated slices  $(311.9 \pm 9.2 \text{ reverse vesicle}/\mu\text{m}^2; p = 0.5269; \text{Fig. 1}B)$ . From the length of the active zone (L) and the number of docked vesicle counted in the two-dimensional electron micrographs, we estimated the total number of docked vesicles per active zone (TDV = 0.907  $\times$   $(DV/\mu m)^2$   $\times$  L^2 (20). TDV was 14.1 in control and 21.0 in BDNF-treated slices, a 50% increase. These results suggest that BDNF selectively increases the number of docked vesicles without affecting the reserve pool vesicles at excitatory CA3-CA1 synapses in hippocampal slices.

BDNF Differentially Regulates the Expression Levels of Specific Synaptic Vesicle Proteins-To determine whether longterm treatment with BDNF also elicits biochemical changes in the hippocampal synapses, we performed Western blot analysis. After 9 days in vitro, the hippocampal slice cultures were treated with 6 nm BDNF for 48 h. In each experiment, samples from the same experimental conditions were run in triplicate (n = 3), and multiple proteins were simultaneously measured on the same blots. The same experiment was repeated 5-10 times (N = 5-10), using samples from independent culture preparations. As shown in Fig. 2, long-term (48 h) treatment with BDNF elicited a significant increase in the levels of several synaptic vesicle proteins. When all data from BDNFtreated group were normalized to those from control group, we found that levels of synaptophysin and synaptobrevin, two integral membrane proteins of the synaptic vesicle membrane, increased by 2-fold in slices treated with BDNF (Fig. 2B). A 5-fold increase was observed in the level of synaptotagmin, a vesicle membrane protein proposed to be the  $Ca^{2+}$  sensor for vesicle fusion (Fig. 2B) (34). The levels of the cytoskeletal protein actin and of the neuron-specific marker neuron-specific enolase did not show any significant changes after BDNF treatment (Fig. 2B), suggesting that there was no general increase in cellular proteins.

The increase in synaptotagmin, synaptobrevin, and synaptophysin were relatively specific. The levels of syntaxin and SNAP-25, two proteins located on presynaptic membrane, did not change after BDNF treatment (Fig. 2B), suggesting that the total number of synapses was not significantly altered. Synapsin-I, a non-integral membrane protein tightly associated with synaptic vesicles, remained unchanged in BDNFtreated slices (Fig. 2B). Furthermore, the increase in synaptotagmin was far more pronounced (5-fold) than that of synaptobrevin and synaptophysin (2-fold). These results are consistent with the idea that BDNF differentially increases the amount of certain proteins per vesicle rather than elevating the total number of synaptic vesicles per synapse (see below).

The effects of BDNF on synaptic protein expression were further confirmed by immunocytochemistry, using antibodies against aforementioned synaptic proteins. Control and BDNFtreated slices from sister cultures were processed side by side



FIG. 2. Long-term effects of BDNF on the levels of synaptic proteins in cultured hippocampal slices. The slice cultures were prepared in the same way as described in the legend to Fig. 1, and harvested for quantitative Western blot analysis. A, representative Western blots showing specific bands of synaptic proteins. B, summary of all results. In each experiment, 3–5 slices for each condition (control or BDNF-treated) were pooled and harvested. Protein samples from the control or BDNF-treated groups were run on an SDS gel in triplicates (n = 3). The blot was scanned and the intensities of the bands were quantified using NIH Image software. Signals from experimental conditions were normalized to the average signals in the control condition in the same blot. The same experiments were repeated 8 times using independent samples (N = 8). \*, p < 0.05, ANOVA followed by post-hoc tests. In all remaining figures, quantitation of Western blots was done in the same way, based on 5–10 independent experiments (N = 5-10), and mean  $\pm$  S.E. are presented.

for immunofluorescence staining, and images were acquired by a confocal microscope using exactly the same conditions. As shown in Fig. 3, A and B, the BDNF-treated slices exhibited brighter immunofluorescence for synaptobrevin and synaptophysin, as compared with untreated controls. The increase in the immunofluorescence appears to be widespread in the hippocampus, rather than limited to the CA1 area. Although the immunofluorescence studies were not quantitative, it was quite clear that the BDNF-induced increase in synaptotagmin expression was much more pronounced (Fig. 4), as compared with that in synaptobrevin and synaptophysin expression (Fig. 3). The increase in synaptotagmin was observed throughout hippocampus, but most obviously in CA1 and dentate areas (Fig. 4).

To reveal the kinetics of the long-term BDNF effects, we examined the time course of the changes in various synaptic proteins by treating the slice cultures with or without BDNF at different time points (6, 12, 24, 48, and 72 h) before harvesting. This procedure allowed all slices to be cultured for the same length of time while being exposed to BDNF for different durations. The expression levels of synaptophysin and synaptobrevin increased within 6 h of BDNF treatment, an effect that lasted for at least 72 h (Fig. 5). In contrast, the increase in

FIG. 3. Confocal images of immunofluorescence staining of hippocampal slice cultures, using specific antibodies against synaptobrevin and synaptophysin. The control and BDNFtreated slices were processed and imaged identically. A, examples of immunostaining of synaptophysin and synaptobrevin in control and BDNF-treated slices. B, mean fluorescence intensity plots are shown with the x axis corresponding to fluorescence intensity in arbitrary units and the y axis corresponding to the mean number of pixels at each intensity level.



synaptotagmin occurred more slowly, reaching its maximum levels by 48 h (Fig. 5). The difference in the kinetics of these protein changes suggests that the mechanism by which BDNF regulates synaptotagmin may be different from that used to modulate synaptobrevin and synaptophysin.

BDNF Modulation of Synaptotagmin Requires Protein Synthesis and cAMP-Several forms of long-term synaptic plasticity require new protein synthesis (1-3). To determine whether the increase in synaptic protein levels after long-term BDNF treatment also depends on new protein synthesis, we investigated the effect of the protein synthesis inhibitor anisomycin on the BDNF-induced increase in synaptic proteins. Cultures were treated with BDNF, anisomycin, or a combination of BDNF and anisomycin for 12 h, since the increase in synaptotagmin was evident during this period (Fig. 5). Anisomycin (20 μM) was added 30 min prior to BDNF treatment. Pretreatment with anisomycin blocked the BDNF-induced increase in synaptotagmin expression (Fig. 6A). Similar results were obtained when another protein synthesis inhibitor cycloheximide was used (data not shown). We also examined the effect of anisomycin on BDNF modulation of synaptophysin and synaptobrevin. In most cases, we determined the levels of synaptophysin and synaptobrevin after 6-12 h of BDNF/anisomvcin treatment because the maximal effect of BDNF was already achieved during this short period (Fig. 5). Surprisingly, the effects of BDNF on the levels of synaptophysin or synaptobrevin were not altered by the pretreatment with anisomycin (Fig. 6B). Treatment with an isomycin alone for the same duration of time did not decrease the levels of any of three synaptic proteins measured, implying a slow turnover of these proteins (Fig. 6, *A* and *B*). Furthermore, the levels of actin and neuronspecific enolase were not significantly affected by the anisomycin treatment, suggesting that anisomycin treatment for 6-12h was not overtly toxic (data not shown).

Protein synthesis-dependent long-term synaptic plasticity often involves a cAMP/PKA-dependent signaling pathway (2, 3, 5). Therefore, we tested whether the BDNF-induced increase in synaptic proteins requires activation of this pathway, using a potent inhibitor for the cAMP/PKA pathway,  $R_{\rm p}$ -cAMPs (100  $\mu$ M). Cultures were treated with BDNF, R<sub>p</sub>-cAMPs alone, or a combination of BDNF and  $R_{\rm p}$ -cAMPs for 12 h. Treatment with  $R_{\rm p}$ -cAMPs alone had no effect on any of the proteins examined (Fig. 7, A and B). Treatment with BDNF markedly increased the levels of synaptotagmin, but this effect was specifically blocked by  $R_{\rm p}$ -cAMPs (Fig. 6B). In contrast, the increase of synaptophysin (Fig. 7B) or synaptobrevin (data not shown) was not affected by  $R_{\rm p}$ -cAMPs. Neither BDNF,  $R_{\rm p}$ -cAMPs, nor the two together had any effect on the levels of syntaxin (Fig. 7B) or actin (data not shown). Thus, the effect of BDNF on synaptotagmin involves activation of a cAMP/PKA pathway, making it distinct from the effects of BDNF on synaptophysin and synaptobrevin.

The BDNF-induced increase in synaptotagmin expression could be mediated by the cAMP/PKA pathway. Alternatively, cAMP could serve as a "gate" that allows BDNF to achieve this effect (35–37). If the effect of BDNF were mediated by cAMP, one would predict that: 1) cAMP/PKA pathway should be activated by BDNF; and 2) cAMP analogues should mimic the BDNF effect. However, BDNF was unable to activate PKA in the cultured hippocampal slices. PKA activity did not increase,



FIG. 4. Confocal images of immunofluorescence staining of hippocampal slice cultures, using specific antibodies against synaptotagmin. The control and BDNF-treated slices were processed and imaged identically.

but slightly decreased, upon application of BDNF (Fig. 7*C*). Moreover, enhancement of endogenous PKA activity by  $S_{\rm p}$ -cAMPs (100  $\mu$ M), a potent activator of PKA, did not alter the expression of synaptotagmin, synaptophysin, or synaptobrevin in the hippocampal slices. The levels of these proteins in slice cultures treated with  $S_{\rm p}$ -cAMPs for 12 (not shown) or 24 (Fig. 7*D*) h were the same as those in control cultures. These results, together with the finding that the BDNF-induced increase in synaptotagmin expression could be blocked by  $R_{\rm p}$ -cAMPs, strongly suggest that cAMP is not a downstream effector in the signaling cascade activated by BDNF, but instead acts in a permissive capacity for the BDNF effect.

## DISCUSSION

While significant progress has been made in studying the acute effects of neurotrophins, the molecular mechanisms underlying long-term modulation of synaptic transmission in the CNS by neurotrophins are much less well understood. In dissociated cultures, long-term treatment with BDNF increases the AMPA-receptor-mediated synaptic currents in hippocampal neurons (29), but inhibits the increase of AMPA currents



FIG. 5. Time course of the BDNF effect on the expression of **specific synaptic proteins.** Cultured hippocampal slices were treated with BDNF and harvested for quantitative Western blot analysis at different time points. *Top*, example of Western blot showing the BDNF-induced change of synaptotagmin level over time. *Bottom*, summary of results showing time courses of all synaptic proteins investigated. The number of experiments is indicated in appearances.

induced by activity blockade in cortical neurons (27). Moreover, chronic application of BDNF or NT-3 may or may not increase the number of synaptic connections depending on the age of embryonic brain used for cultures (26, 28). These discrepancies may be due largely to the heterogeneity of the culture systems, which lack the appropriate afferent and efferent synaptic connections observed in vivo. To avoid problems associated with dissociated cultures, we investigated the molecular mechanism(s) mediating long-term BDNF modulation of hippocampal synapses using organotypic slice cultures, which maintain a pattern of synaptic connectivity quite similar to that of the hippocampal formation in vivo. We have made two interesting observations. First, we found that BDNF exerts a long-term modulatory effect on the expression levels of several proteins on synaptic vesicles, as well as changes in the distribution of synaptic vesicles within presynaptic terminals at excitatory spine synapses. Second, we showed that the BDNF-induced increase in synaptotagmin is mediated through a protein synthesis and cAMP-dependent mechanism, an effect reminiscent of long-term facilitation in Aplysia sensory-motor synapses, and of L-LTP. Thus, long-term modulation by BDNF may share mechanisms similar to other types of long-term synaptic modifications.

In the brain of awake, living animals, acute and long-term actions of BDNF on synapses may have different physiological consequences. Acute application of neurotrophins to neuromuscular or central synapses has been shown to cause an instant but transient modulation of the efficacy of synaptic transmission (7, 8, 11–13). These short-term effects are mediated by rapid changes in intracellular  $Ca^{2+}$  and protein phosphorylation, rather than new protein synthesis (8, 9, 38, 39). It is conceivable that activity dependent secretion of neurotrophins may play an important role in this type of modulation. On the other hand, BDNF mRNA and protein are highly expressed in the brain, particularly in the hippocampus (40, 41), suggesting that CNS synapses are constantly exposed to BDNF under physiological conditions *in vivo*. Thus, BDNF may play a long-



FIG. 6. **BDNF modulation of synaptotagmin, but not that of synaptophysin or synaptobrevin, requires protein synthesis.** *A*, *top*: representative Western blots showing the effect of BDNF and anisomycin on the levels of synaptotagmin in cultured hippocampal slices. *Bottom*, summary of results showing that anisomycin blocks the BDNF-induced increase in synaptotagmin (N = 7). *B*, *top*: representative Western blots showing the effect of BDNF and anisomycin on the levels of synaptobrevin. *Bottom*, summary of results showing that anisomycin on the levels of synaptophysin and synaptobrevin. *Bottom*, summary of results showing that anisomycin (N = 9) or synaptophysin (N = 8).

term modulatory role in the development and/or function of hippocampal synapses under physiological conditions, in addition to its acute effects on synaptic transmission and plasticity. It has been shown that repeated high-frequency afferent stimulation elicits a marked increase in BDNF mRNA in the hippocampus (42). Time course studies indicate that the activitydependent increase in BDNF gene expression occurs within 3-5 h, correlating very well with the occurrence of protein synthesis and cAMP-dependent L-LTP. BDNF knockout mice exhibit impairments in L-LTP, in addition to defects in E-LTP (43). Application of TrkB-IgG, a scavenger for BDNF and NT-4, 30 min after tetanic stimulation to hippocampal slices reverses previously established LTP (17). All these studies raise the possibility that activity-dependent expression of BDNF plays a role in L-LTP. The mechanisms by which BDNF modulates hippocampal L-LTP are unknown. Long-term treatment of cortical slices with BDNF regulates dendritic growth of cortical pyramidal neurons (24). We demonstrated here that chronic exposure of hippocampal slices to BDNF increases the number of vesicles docked at active zones of CA1 synapses. Furthermore, BDNF increases the expression of synaptotagmin in a protein synthesis and cAMP-dependent manner. It is tempting to speculate that these changes contribute to the BDNF modulation of L-LTP.

Takei *et al.* (25) reported that treatment of newly dissociated cultures of cortical neurons with BDNF in serum-containing conditions for 5 days appears to increase all synaptic proteins,

suggesting an increase in synapse number. The present paper has made a number of conceptual and technical advances. First, we studied the long-term effects of BDNF on hippocampal synapses in slices, which resemble more closely the hippocampus in vivo in terms of synaptic circuits. Second, our experiments were done in better controlled conditions. We examined the effects of BDNF in serum-free conditions, avoiding the potential interactions between BDNF and other unknown serum factors. Our slices were derived from postnatal day 7 (P7) hippocampus, right around the time of synapse formation. We also treated slices for much shorter periods (maximum 2 days). These measures made it less likely that we were looking at the survival effect of BDNF. Third, we showed that BDNF selectively enhances the expression of synaptophysin, synaptobrevin, and synaptotagmin, without affecting other synaptic proteins. Thus, we demonstrated specific changes in the properties of synapses, rather than general increase in synapse number. Finally and most importantly, our study revealed mechanistic differences between the short-term and long-term synaptic effects of BDNF: dependence on protein synthesis and cAMP. We believe that this represents a novel and significant conceptual advance.

We have previously shown a selective reduction in the amount of synaptophysin and synaptobrevin in hippocampal synaptosomes prepared from BDNF knockout mice (20). Moreover, the reduction in the levels of synaptobrevin and synaptophysin was reversed after incubation with BDNF for a few hours, suggesting an acute, rather than a long-term effect of BDNF. Using hippocampal slice cultures, we now studied acute as well as long-term effects of BDNF on the expression of synaptic proteins in the hippocampus. We found that the levels of synaptobrevin and synaptophysin increase within a few hours after BDNF application, and this increase is sustained as long as BDNF is present in the cultures. Interestingly, the increase in synaptobrevin and synaptophysin cannot be blocked by the protein synthesis inhibitor anisomycin. We do not know how the increase in synaptobrevin and synaptophysin could occur without protein synthesis, but it is possible that BDNF could alter the processing, post-translational modification, or re-distribution into different pools/complexes of the two proteins, leading to a better detection by the antibodies (44). Further experiments are required to distinguish these possibilities. The most interesting finding in the present study is the BDNF-induced increase in synaptotagmin, a multifunctional protein on the synaptic vesicles (34). We demonstrated here that long-term treatment of cultured hippocampal slices with BDNF elicits an increase in synaptotagmin in ways quite distinct from the BDNF-induced increase in synaptobrevin and synaptophysin. First, the increase in synaptotagmin requires a considerably longer time of BDNF exposure (12-24 h), and is much more pronounced. Second, this increase is dependent on protein synthesis, and on a cAMP-signaling pathway. The present results, together with other studies discussed above, suggests that BDNF has two modes of actions on hippocampal synapses: an acute effect due at least in part to rapid protein phosphorylation, and a long-term effect mediated by a protein synthesis and cAMP-dependent mechanism.

There are two potential mechanisms that cAMP pathway could be involved in neurotrophin signaling. One is that a particular neurotrophin activates the cAMP/PKA pathway, which in turn mediates its neurotrophic effects. BDNF does not seem to activate the cAMP/PKA pathway in a number of systems tested (45–47). In the present study, we found that BDNF does not activate PKA in hippocampal neurons in slice cultures. The second mechanism is the "cAMP gating." This mechanism was first proposed to explain the regulatory role of cAMP



FIG. 7. BDNF modulation of synaptotagmin requires activation of a cAMP-signaling pathway. A, representative examples of gels showing that the effect of BDNF on synaptotagmin, but not synaptophysin, is blocked by  $R_{\rm v}$ -cAMPs. Slice cultures were treated with BDNF, the PKA inhibitor  $R_p$ -cAMPs, or the two together for 12–24 h and then harvested for Western blot analysis. *B*, quantification of the levels of synaptotagmin (N = 9), synaptophysin (N = 8), and syntaxin (N = 10) after treatment with  $R_p$ -cAMPs and/or BDNF. The data were normalized to control slices. C, BDNF does not enhance PKA activity. Slice cultures were treated with or without BDNF or forskolin (Forsk, positive control) for 10 min and harvested for PKA assay. PKA activities are expressed as unit per µg of cytosolic proteins extracted from the slices, based on a standard curved generated using the recombinant catalytic subunit of PKA. N = 6 in all conditions. D, lack of effect of  $S_{v}$ -cAMPs on the expression of synaptotagmin (black bar, N = 5), synaptophysin (white bar, N = 6), or synaptobrevin (gray bar, N = 5). Slices were treated with or without  $S_{\rm p}$ -cAMPs, and all data were normalized to control slices.

during LTP induction (35, 36). It has been reported that cAMP gating is also involved in BDNF regulation of the survival of retinal ganglion cells and release of neurotransmitters at the neuromuscular junction (37, 48). In this mechanism, cAMP is not a downstream effector in the signaling cascade activated by a neurotrophin, but instead acts in a permissive capacity. Experimentally, the cAMP gating theory predicts the following: 1) cAMP/PKA pathway is not activated directly by BDNF; 2) cAMP analogues cannot mimic the BDNF effects; 3) the effects of BDNF can be blocked by inhibition of cAMP/PKA pathway. We found that all three are true for the enhancement of synaptotagmin expression by BDNF. Thus, our data supports the cAMP gating theory for BDNF-induced synaptic modulation in the hippocampus.

Whether and how changes in synaptic vesicle proteins contribute to the BDNF modulation of vesicle docking are interesting questions for further investigation. Synaptophysin is tightly associated with synaptobrevin (49, 50), but there is little evidence for its involvement in vesicle docking. Rather, recent studies in the squid giant synapse have implicated synaptophysin in rapid clathrin-independent vesicle endocytosis at the active zone (51). The role of synaptobrevin in vesicle docking has been controversial. Biochemical experiments indicate that the v-SNARE synaptobrevin binds to the t-SNARE proteins syntaxin and SNAP25 to form the SNARE core complex (20 S), suggesting that synaptobrevin is a required component for vesicle docking (52, 53). However, cleavage of synaptobrevin in squid giant synapse or Drosophila neuromuscular junction by tetanus toxin caused a slight increase, rather than a decrease, in the numbers of docked vesicles due to an accumulation of vesicles after fusion is blocked by the toxin (54, 55). Synaptotagmin is a Ca<sup>2+</sup> sensor known to play a key role in vesicle fusion (34). It has also been implicated in endocytosis and recycling of synaptic vesicles (56, 57). Moreover, morphologically docked vesicles are reduced in the synaptotagmin mutant of Drosophila (58). A recent study suggests the involvement of C terminus of synaptotagmin in vesicle docking (59). In the present study, we demonstrated that long-term exposure of hippocampal slices to BDNF increases the number of vesicles docked at the active zone, as well as the level of synaptotagmin. These experiments support the notion that synaptotagmin plays a role in vesicle docking. It is also possible that both synaptobrevin and synaptotagmin participate in vesicle docking, but the former is involved in the initiation of docking complex, while the later may be required for maintenance or stabilization of vesicles docked at the active zone.

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