Brain-derived Neurotrophic Factor-Tropomyosin-related Kinase B Signaling Contributes to Activity-dependent Changes in Synaptic Proteins*

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The ability of synapses to undergo changes in structure and function in response to alterations of neuronal activity is an essential property of neural circuits. One way that this is achieved is through global changes in the molecular composition of the synapse; however, it is not clear how these changes are coupled to the dynamics of neuronal activity. Here we found that, in cultured rat cortical neurons, bidirectional changes of neuronal activity led to corresponding alterations in the expression of brain-derived neurotrophic factor (BDNF) and phosphorylation of its receptor tropomyosin-related kinase B (TrkB), as well as in the level of synaptic proteins. Exogenous BDNF reversed changes in synaptic proteins induced by chronic activity blockade, while inhibiting Trk kinase activity or depleting endogenous BDNF abolished the concentration changes induced by chronic activity elevation. Both tetrodotoxin and bicuculline had significant, but opposite, effects on synaptic protein ubiquitination in a time-dependent manner. Furthermore, exogenous BDNF was sufficient to increase ubiquitination of synaptic proteins, whereas scavenging endogenous BDNF or inhibiting Trk kinase activity prevented the ubiquitination of synaptic proteins induced by chronic elevation of neuronal activity. Inhibiting the proteasome or blocking protein polyubiquitination mimicked the effect of tetrodotoxin on the levels of synaptic proteins and canceled the effects of BDNF. Our study indicates that BDNF-TrkB signaling acts upstream of the ubiquitin proteasome system, linking neuronal activity to protein turnover at the synapse.

Synaptic remodeling is critical for brain development and neural plasticity (1–3). During neural circuit maturation and in response to alterations in neuronal activity, synapses undergo remarkable cytoarchitectural and functional changes (1, 4–6). Such plasticity is associated with changes to the molecular composition of synapses (1). In dissociated neuron cultures, for example, chronic increases or reductions in neuronal activity cause dramatic changes in the expression of a large number of proteins at the synapse. These changes are at least in part determined by the rate of protein degradation by the ubiquitin proteasome system (UPS)2 (4). It remains to be determined, however, how modulation of neural activity translates into changes in the rate of protein degradation.

Previous studies have shown that the expression of brain-derived neurotrophic factor (BDNF) in cortical neurons and its subsequent secretion are regulated by neuronal activity in vivo and in vitro (7–9). As BDNF is known to exert many modulatory actions on neuronal and synaptic functions (10–13), we hypothesized that BDNF signaling serves to link neuronal activity to proteasome-dependent synapse remodeling. In this study, we show that BDNF expression and TrkB activation in cultures of cortical neurons are modulated by chronic alteration of activity with tetrodotoxin (TTX) or bicuculline. Application of exogenous BDNF prevents TTX-induced changes in synaptic proteins and is sufficient to mimic the effects of bicuculline on the expression of a battery of synaptic proteins. On the other hand, inhibiting TrkB or scavenging endogenous BDNF blocks bicuculline-induced changes and mimics the effect of TTX on synaptic protein concentrations. We provide evidence to suggest that BDNF-TrkB signaling modulates synaptic protein levels by regulating the activity of the ubiquitin-proteasome system. Changes in synaptic protein concentrations by BDNF or bicuculline correlate with elevated global ubiquitination. Furthermore, blocking UPS activity with pharmacological agents or dominant negative ubiquitin causes the same profile of effects as blocking activity of BDNF signaling. Together these results demonstrate how BDNF signaling couples neuronal activity to UPS activity, thereby controlling the concentration of specific synaptic targets.

**EXPERIMENTAL PROCEDURES**

**Antibodies**—Antibodies used in this study included monoclonal antibodies against NR1 (BD Biosciences), PSD-95

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2 The abbreviations used are: UPS, ubiquitin proteasome system; BDNF, brain-derived neurotrophic factor; TTX, tetrodotoxin; TORC1, transducer of regulated CREB activity 1; DN-TORC1, dominant negative TORC1; DN-Ub, dominant negative form of ubiquitin; ERK, extracellular signal-regulated kinase; Trk, tropomyosin-related kinase; AKAP 79/150, A-kinase anchor protein 79/150; DIV, days in vitro; GFP, green fluorescent protein; EGFP, enhanced GFP; RT-PCR, reverse transcription PCR; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CREB, cAMP-response element-binding protein; SFV, Semliki Forest virus.
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—Crude synaptosome purification—Crude synaptosome fractions of cultured cortical neurons were purified as described previously (16) with minor modifications. Briefly, cells were scraped in 500 μl of 4 mM Hepes buffer containing 0.32 mM sucrose and a mixture of protease inhibitors and homogenized for 25–30 strokes with a glass homogenizer. Whole cell lysates were then centrifuged at 800 × g for 10 min at 4 °C to pellet nuclei. The supernatant was centrifuged again at 10,000 × g for 15 min at 4 °C to obtain a crude synaptosomal fraction. Synaptosome purity was then assayed by Western blot with antibodies against the postsynaptic marker PSD-95, presynaptic marker synapsin-1, and nuclear marker CREB.

Immunoblot Analysis of Synaptosome Proteins—Protein samples (10–20 μg) were resolved by SDS-PAGE and transferred to polyvinylidene fluoride membranes. Proteins were detected by immunoblot using indicated antibodies. Horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibodies (1:5,000; Amersham Biosciences) were used as secondary antibodies. Immunoreactive bands were visualized with ECL-plus (Amersham Biosciences). Band intensities were measured with an Imaging Densitometer (Bio-Rad Model GS-700) and quantified with Image Quant 5.2. Each experiment was repeated at least three times with lysates from separate neuron preparations.

Reverse Transcription PCR—Total RNA was extracted from cultured neurons using TRIzol (Invitrogen). mRNA was reverse transcribed using oligo(dT) primers and Moloney murine leukemia virus transcriptase (Invitrogen). RT-PCR was performed using a Promega kit (Madison, WI). PCR primer sets used in the study were designed to span intron/exon junctions to avoid genomic DNA contamination. The BDNF primer set was: 5'-TCACTCAGTTTACCCAGG-3', 5'-CCTCCATAGACATGTTTGC-3'; GAPDH primer set: 5'-TGTCTGTGAGCTCTAC-TGG-3', 5'-CAGCATAAACGTTGAGG-3'. PCR products were evaluated by gel electrophoresis. Quantification of band intensities was performed with PhosphorImage software (GE Healthcare), and the relative amount of message was normalized to the internal control GAPDH. Each experiment was repeated at least three times with RNA from separate preparations.

DNA Constructs—CDNA encoding dominant negative ubiquitin (DN-Ub, K48R) was amplified by PCR to generate 5'-Xhol and 3'-NotI sites flanking the 5' and 3'-ends and inserted into the Semliki Forest virus vector pSFV(pd)-EGFP (17) with Xhol and NotI to produce pSFV(pd)-DN-Ub-EGFP constructs. A modified enhanced GFP (EGFP) was designed by fusing an ubiquitination signal sequence (CL1 degron, ACKNWFSSLS-HFVIIHL) to the C terminus of EGFP (GFPU). GFPU or EGFP was then inserted directly into the pSFV(pd) vector (a generous gift from Kenneth Lundstrom, Switzerland). The fidelity of constructs was verified by DNA sequencing.

Virus Packaging and Infection—Packaging of recombinant virions capable of high infection rates in neurons was performed as described previously (17). Cultured cortical neurons (DIV 10) were infected with pSFV(pd)-EGFP, pSFV(pd)-GFPU, or pSFV(pd)-DN-Ub-EGFP, and expression levels were assessed by the intensity of EGFP fluorescence.

Immunoprecipitation—Cultured cortical neurons (DIV 12) were dissolved with radioimmune precipitation buffer solution containing 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 5 mM EDTA, 20 mM Hepes, and protease inhibitor mixture. Total protein concentrations were quantified with the protein concentrate assay kit from Bio-Rad. Protein A/G-agarose beads (Roche Applied Science or Santa Cruz Biotechnology) were washed with radioimmune precipitation buffer three times and incubated with 2 μl of anti-ubiquitin antibodies (Calbiochem) for 2 h at 4 °C. After incubation, agarose/antibody complexes were washed thoroughly three times with radioimmune precip-
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initiation buffer before incubating for 4 h at 4 °C with 500 μg of protein from treated or untreated neurons. Agarose-protein complexes were washed three times and dissolved in 2× SDS-PAGE buffer. Protein complexes were dissolved and denatured by boiling at 100 °C for 5 min and then analyzed by Western blotting.

*Statistical Analysis*—All statistical analyses were performed using software of SPSS 13.0 (Cary, NC). Statistical comparisons were performed using one-way analysis of variance followed by a Bonferroni correction factor for multiple comparisons. *p* values of <0.05 were considered statistically significant.

**RESULTS**

**Chronic Activity Modulation Alters BDNF Expression and TrkB Phosphorylation**—To test our hypothesis that BDNF couples neuronal activity to synaptic remodeling, we first assessed the level of BDNF mRNA in cultured cortical neurons in response to increases and decreases of network activity. After 10 days *in vitro*, we added the Na⁺ channel blocker TTX (2 μM) or the γ-aminobutyric acid, type A (GABA_A) receptor antagonist bicculline (40 μM) to the medium and measured BDNF mRNA levels by semiquantitative RT-PCR 8 h later. Suppressing neuronal activity with TTX decreased the level of BDNF mRNA by 42.9%, whereas elevating neuronal activity by blocking GABAergic transmission increased BDNF mRNA ~3-fold (Fig. 1A). To characterize the time course of activity-induced changes in BDNF mRNA expression, cultures were treated with TTX or bicculline for time periods ranging from 0 to 48 h. The level of BDNF was first observed to diminish after 4 h of activity blockade and continued to decline over the next 9 h to roughly half of the base-line level, remaining low with little change for the final 36 h (Fig. 1B). Treating cultures with bicculline caused a rapid increase in the level of BDNF mRNA that peaked by 4 h and remained elevated to the 24-h time point before gradually declining toward the base-line level by 48 h (Fig. 1B). These results show that bidirectional changes in synaptic activity are accompanied by corresponding changes in BDNF expression.

BDNF is a well established target of cAMP-response element-mediated transcription in neurons (18–20). Our group and others have recently reported that neuronal activity-dependent nuclear accumulation of TORC1 (transducer of regulated CREB activity) is required for cAMP-response element-dependent gene transcription (21, 22). We thus reasoned that the effects of TTX and bicculline on BDNF expression might be due to effects on the subcellular localization of TORC1. To investigate this, we stained cultured hippocampal neurons with anti-TORC1 after treatment with TTX or bicculline. In untreated neurons, TORC1 is primarily localized to the cytoplasm with only low levels detected in the nucleus (Fig. 1C, top panel) (21). Treating cultures for 8 h with bicculline dramatically increased the proportion of TORC1 protein in the nucleus, whereas TTX had the opposite effect (Fig. 1C).

TORC1 translocates to the nucleus in the dephosphorylated state (23); accordingly, we observed an increase in the level of phosphorylated TORC1 after treatment with TTX, as assessed by Western blotting (Fig. 1D). To determine whether TORC1 nuclear translocation is required for triggering activity-dependent increases in BDNF expression, we treated cultures with cyclopamine A (1 μg/ml) and FK506 (1 μg/ml), inhibitors of the TORC1 phosphatase calcineurin (24). Co-treatment of cortical neurons with both cyclopamine A and FK506 reduced bicculline-induced BDNF mRNA expression by 58% but had no effect on basal BDNF expression (Fig. 1E). TORC1 binds to CREB upon entering the nucleus, triggering the expression of cAMP-response element-containing target genes (25), and...
CREB target gene transcription can be blocked by dominant negative TORC1 (DN-TORC1) (24). We expressed DN-TORC1 in cultured cortical neurons using a Semliki Forest virus gene-delivering system on DIV10 and examined BDNF mRNA expression on DIV12 immediately after 8 h of treatment with bicuculline. DN-TORC1 completely abolished bicuculline-induced BDNF expression without affecting TORC1 nuclear translocation (Fig. 1F and data not shown). Taken together, these results suggest that the differential effects of TTX and bicuculline on BDNF expression are determined by the activity-dependent dynamics of TORC1 phosphorylation and subcellular localization.

Many neuronal actions of BDNF are exerted by its high affinity receptor TrkB. Interaction of BDNF with TrkB leads to activation of downstream signaling pathways by triggering TrkB dimerization and cross-phosphorylation at multiple intracellular sites (26). Activity-dependent changes in BDNF transcriptional levels are expected to modulate BDNF protein expression and secretion, thus leading to alterations of TrkB activation. Using an antibody specific to phosphorylated Trk receptors (phospho-Trk), we indeed found that treating cortical neuron cultures with TTX for 12 h decreased Trk phosphorylation, whereas 12 h of treatment with bicuculline increased the level of phosphorylation (Fig. 2A). Following the time course of changes to Trk phosphorylation under conditions of increased or decreased activity (Fig. 2B), we found that the dynamics of Trk phosphorylation closely resemble those of BDNF transcription after modulating neuronal activity, although the onset of changes to BDNF transcription occurred more rapidly (Fig. 1B versus Fig. 2B). Incubating cultures with a secreted form of TrkB, TrkB-IgG (10 μg/ml), to scavenge secreted BDNF or the tyrosine kinase inhibitor K252a (100 nM) to inhibit Trk kinase activity reduced bicuculline-induced TrkB phosphorylation (Fig. 2C). Furthermore, application of TrkB-IgG prevented TrkB phosphorylation induced by exogenous BDNF (25 ng/ml) but had no effect on nerve growth factor-induced TrkA phosphorylation in PC12 cells, confirming the efficacy and specificity of TrkB-IgG in scavenging BDNF (data not shown). Together, these results indicate that BDNF expression and subsequent TrkB activation are sensitive to the level of synaptic activity.

**BDNF-TrkB Signaling Is Required for Activity-dependent Changes in Synaptic Proteins**—To test whether BDNF-TrkB signaling cascades participate in activity-dependent changes in the expression of protein constituents at the synapse, we modified the activity of cultured cortical neurons and assayed the expression of 17 synaptic proteins in purified synaptosomes. We first blotted for the postsynaptic marker PSD95 and presynaptic marker synapsin 1 and the nuclear marker CREB to assess the purity of synaptosomal fractions. Both PSD95 and synapsin 1 were highly enriched in the synaptosomal fraction relative to whole cell lysate, whereas CREB was only observed in whole cell lysate (data not shown). As revealed by quantitative immunoblot analysis, chronic blockade of neuronal activity by TTX for 48 h resulted in striking changes to the expression of many of the assayed neurotransmitter receptors, synaptic scaffolding proteins, and signaling molecules (Fig. 3A), consistent with a previous report (4). After normalizing band intensities and calculating the mean -fold increase relative to mean level in synaptosomes of untreated controls, changes in the abundance of synaptic proteins could be separated into three distinct populations: those that significantly increased (NR2B, NR1, AKAP 79/150, PKA-cat, PKA-RIIβ, and spinophilin), significantly decreased (NR2A, PSD95, myosin Va, PP1, SOS1/2, and CaMKIIα), or remained unchanged (nNOS, SAP97, PKCα, tubulin, and actin) in response to TTX (Fig. 3B). To determine whether the changes were direct effects of decreased BDNF expression we co-treated cultures with TTX and BDNF (25 ng/ml) for 48 h. Strikingly, chronic application of BDNF reversed all TTX-induced changes to synaptic protein expression without exception (Fig. 3). To test the specificity of BDNF in suppressing the effect of TTX treatment, we co-treated cultures with TTX and nerve growth factor for 48 h and found that nerve growth factor had no obvious effect on TTX-induced changes to synaptic protein levels (data not shown).

Elevation of neuronal activity by chronic treatment with bircuculline for 48 h led to changes in the abundance of these three categories of synaptic proteins that were the mirror opposite of those observed after TTX treatment (Fig. 3). Proteins that increased in the presence of TTX decreased in the presence
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A Western blot analysis of 17 proteins present in synaptosomal fractions isolated from cortical neurons treated for 48 h with TTX, BDNF (25 ng/ml), TTX and BDNF (TTX+BDNF), bicuculline (Bic), TrkB-IgG, bicuculline and K252a (Bic+K252a), or bicuculline and TrkB-IgG (Bic+TrkB-IgG). B, quantification of immunoblot band intensities normalized to levels in untreated controls, presented as means±S.E. (n=3–8 for each protein and treatment). *p<0.05.

of bicuculline and vice versa, whereas those unaffected by TTX were not affected by bicuculline either. The effects were again dependent on BDNF-TrkB signaling because K252a or TrkB-IgG completely abolished bicuculline-induced alterations to synaptic protein levels (Fig. 3). From these results, it follows that if neuronal activity-induced changes in levels of synaptic proteins were wholly determined by modulation of BDNF-TrkB signaling, then manipulating BDNF-TrkB signaling alone should be sufficient to produce changes similar to activity modulation. Indeed, treating neuronal cultures with TrkB-IgG alone for 48 h faithfully mimicked the effect of TTX on synaptic protein concentrations, whereas treating cultures with BDNF alone for 48 h produced similar effects, although less robust, to those of bicuculline treatment (Fig. 3). Taken together, these findings indicate the essential requirement of BDNF-TrkB signaling in regulating changes to the concentration of proteins at the synapse in response to neuronal activity.

BDNF-TrkB Signaling Mediates Activity-dependent Ubiquitination of Synaptic Proteins—To distinguish whether activity-induced changes in synaptic protein levels reflect changes in the rate of protein synthesis, degradation, or both, we assayed mRNA expression of NR2A, NR2B, and spinophilin. TTX or bicuculline for 48 h did not affect NR2B mRNA levels, whereas NR2A mRNA levels changed in the same direction as NR2A protein levels, and spinophilin mRNA levels changed in the opposite direction as spinophilin protein levels in response to TTX and bicuculline (data not shown). These results indicate that BDNF-mediated changes to synaptic protein concentrations are not merely the result of altered gene expression and that activity has complex, gene-specific effects on transcriptional regulation.

Previous studies have shown that activity-induced modulation of synaptic composition is regulated by protein turnover rates that are controlled by ubiquitin conjugation and enzymatic activity of the proteasome (4). To characterize the dynamics of synaptic protein ubiquitination during prolonged modulation of neuronal activity, we measured global ubiquitination in the synaptic fraction of cultured cortical neurons in the presence of TTX or bicuculline over a 48-h time course. As shown in Fig. 4, A and B, TTX and bicuculline each had significant, but opposite, time-dependent effects on global synaptosomal protein ubiquitination. TTX-treated neurons began to show a decrease in ubiquitination in the synaptosomal fraction by 8 h and a continued decline until the 24-h mark when ubiquitin content leveled off (Fig. 4, A and B). Ubiquitination levels increased in the synaptosomal fraction of bicuculline-treated neurons by 4 h and continued to increase for the full 48 h. Chronic TTX treatment led to a 44.2% decrease, whereas bicuculline treatment led to a 52.1% increase, in the level of ubiquitination of synaptic proteins at the 48-h time point (Fig. 4C). These time courses match well with those of activity-dependent BDNF expression (Fig. 1B) and Trk activation (Fig. 2B). To assess the significance of this correlation, we incubated cultures in TTX and BDNF or bicuculline and TrkB-IgG or K252a. Co-treatment with BDNF completely reversed the TTX-induced reduction in ubiquitin levels, and both TrkB-IgG and K252a prevented bicuculline-induced ubiquitination (Fig. 4C). Furthermore, application of BDNF alone significantly increased ubiquitination of synaptic proteins and this effect could be prevented by co-application of K252a, whereas application of TrkB-IgG alone decreased the level of ubiquitination in the synaptosomal fraction (Fig. 5, A and B). These results suggest that BDNF signaling is both necessary and sufficient for regulating activity-dependent global synaptic ubiquitination.

An earlier study surveying substrates of ubiquitin ligation in the postsynaptic compartment identified only three proteins, Shank, GKAP, and AKAP 79/150, of a total of more than 30 assayed proteins (4). Consistent with those findings, AKAP 79/150 could be immunoprecipitated from cortical neuron cultures with anti-polyubiquitin antibody, showing that AKAP 79/150 is ubiquitinated under control conditions. We next confirmed the observation that the level of ubiquitinated AKAP 79/150 decreases after treatment with TTX and increases after treatment with bicuculline (Fig. 4D). Coincubation with BDNF, however, reversed the effect of TTX on AKAP 79/150 ubiquitination, and K252a or TrkB-IgG not only abolished bicuculline-induced enhancement but also resulted in marked reduction in ubiquitin-bound AKAP 79/150 relative to untreated cultures (Fig. 4D). Similarly, TrkB-IgG treatment alone decreased the level of ubiquitin-conjugated AKAP 79/150 whereas BDNF
alone increased ubiquitination (Fig. 5C). Inhibiting mitogen-activated protein kinase signaling, one of three signaling cascades downstream of BDNF-TrkB signaling, with PD98059 (20 \( \mu \)M) completely blocked the effect of BDNF on AKAP 79/150 ubiquitination (Fig. 5D). Taken together, these findings reveal that activity-induced changes in BDNF-TrkB signaling trigger gene-specific modifications of ubiquitination levels at the synapse.

**BDNF Alters Protein Ubiquitination but Not Proteasome Activity**—The elevated levels of ubiquitination we observed in response to BDNF signaling could be attributable to inhibition of proteasome activity, increased ubiquitin ligase activity, or both. To distinguish between these possibilities, we first followed an established in vitro assay (27) to measure the enzymatic kinetics of cortical neuron proteasomes following BDNF treatment. We found that BDNF did not affect the rate of degradation of the reporter substrate Suc-LLVY-MCA relative to untreated controls, suggesting that the enzymatic activity of 26 S proteasomes isolated from cultured cells was not affected by BDNF (data not shown).

To monitor proteasome activity in situ, we infected cultured cortical neurons by SFV-mediated gene delivery with a modified GFP construct containing a ubiquitination signal sequence in the C terminus (GFPu) (28–30) or unmodified GFP. Western blot analysis of ubiquitination levels in lysates from cortical neurons treated with BDNF, BDNF and K252a, or TrkB-IgG for 2 h were analyzed by anti-Ub Western blotting. B, quantification of ubiquitination after treatment as in A; mean levels are presented relative to control (\( \pm S.E., *p < 0.05; **p < 0.01, n = 6 \text{/group} \)). C, ubiquitination of AKAP 79/150 in cultured cortical neurons treated with BDNF for 2 h. Cell lysates were immunoprecipitated with anti-polyubiquitin and blotted with anti-AKAP 79/150 antibody. D, GFP fluorescence in cortical neurons infected with pSFV(pd)-EGFP or pSFV(pd)-GFPu and treated with MG132 (10 \( \mu \)M) or U0126 (20 \( \mu \)M) or control solution. Cell lysates were immunoprecipitated with anti-Ub and blotted with7 anti-AKAP 79/150. E and F, BDNF has no effect on proteasome activity. E, GFP fluorescence in cortical neurons infected with pSFV(pd)-GFPu (see “Experimental Procedures”) and treated with MG132 (10 \( \mu \)M) for 6 h or BDNF (100 ng/ml) for 2 h (scale bar, 50 \( \mu \)m). F, representative anti-GFP immunoblots of cell lysates from cortical neurons infected with pSFV(pd)-EGFP or pSFV(pd)-GFPu and treated as in E. Note that the molecular mass of GFPu is slightly higher than EGF due to the addition of the ubiquitination signal sequence at the C terminus. G, quantification of GFP and GFPu expression in Western blot. Data represent mean \( \pm S.E. \) relative to untreated controls (*, p < 0.05; **, p < 0.001, n = 2 blots/group).
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or band intensity (Fig. 5, E–G). GFPu, on the other hand, was expressed only at low levels in untreated cortical neuron cultures but was markedly increased in neurons treated with MG132 (Fig. 5, E and F), consistent with the effects of MG132 on GFPu expression in cultured cardiac myocytes (30). We then treated GFPu-expressing neurons with 100 ng/ml BDNF. Relative to untreated controls, no change in GFPu expression after 2 h of treatment with BDNF was observed (Fig. 5, E and F). Thus, increased global synaptic protein ubiquitination induced by BDNF is most likely due to enhancement of ubiquitin conjugation, rather than a reduction of proteasome activity.

BDNF-TrkB Affects Synaptic Protein Expression Upstream of the UPS—Previous studies have demonstrated that UPS-dependent protein degradation in response to neuronal activity leads to changes in the level of synaptic proteins (4, 31). To determine whether BDNF regulates UPS-mediated changes in the expression of specific synaptic proteins, we compared the effects of UPS blockade with those of BDNF inhibition on individual synaptic proteins. Treatment of cortical neurons with MG132 mimicked the effect of synaptic blockade by TTX on the levels of synaptic proteins and thus had the mirror opposite effect of bicineulline (Fig. 6, A and B), consistent with previous results (4). Application of another proteasome inhibitor, lactacystine (10 μM), had the same effect as MG132 (data not shown). Ubiquitin-proteasome-dependent protein degradation requires the formation of polyubiquitin chains at specific amino acid residues of substrate polypeptides, which directs the modified protein into proteasome apparatus. To specifically block polyubiquitination, we expressed a dominant negative form of ubiquitin, K48R, in cultured cortical neurons by SFV-mediated gene delivery and then measured synaptic protein concentrations 24 h later. Overexpression of DN-Ub had similar effects as MG132 and lactacystine on the level of synaptic proteins (Fig. 6, A and B).

Because both pharmacological inhibition of proteasome activity and blockade of polyubiquitination mimicked TTX-induced changes in the level of synaptic proteins (Fig. 6B) and BDNF reversed the effects of TTX (Fig. 3), we asked whether BDNF could also reverse the effects of UPS inhibition. Co-treatment of neuronal cultures with MG132 and BDNF resulted in a similar profile of expression as treatment with MG132 alone (Fig. 6B), demonstrating that increasing BDNF-TrkB signaling cannot reverse the effects of UPS inhibition. To verify that BDNF signaling is unaffected under conditions of proteasome inhibition, we monitored BDNF-induced phosphorylation of Trk, ERK1/2, Akt, and phospholipase C-γ in the presence of MG132 and found no difference relative to control cultures (Fig. 6C). Together these findings indicate that BDNF signaling acts upstream of the UPS in mediating activity-dependent regulation of synaptic proteins.

DISCUSSION

Changes in neuronal activity are accompanied by complex changes in the molecular makeup of the synapse (1, 4). We have demonstrated that chronic changes in network activity of cultured cortical neurons alter the expression of an array of proteins at the synapse and that regulated BDNF signaling is critically involved in orchestrating these complex, gene-specific effects. Activity-dependent changes in BDNF expression and subsequent TrkB activation correlate with increased global and gene-specific ubiquitination levels of synaptic proteins, and blocking proteasome activity mimics the effects of reduced network activity or reduced BDNF signaling. Together these results show that the dynamics of activity-dependent changes in the composition of synapse are controlled by BDNF signaling, which mediates its effects by regulating ubiquitination.

Studies have shown that the degree of BDNF expression and release are determined by the degree of neuronal activity both in vivo and in vitro (7–9, 32). Increasing network activity of cultured cortical neurons with the γ-aminobutyric acid, type A receptor antagonist bicuculline led to nuclear accumulation of TORC1, whereas decreasing network activity with the Na+ channel blocker TTX decreased the proportion of TORC1 in the nucleus. In the nucleus, TORC1 binds to CREB and acti-
vates CREB-mediated transcription. Accordingly, blocking TORC1-CREB interaction with DN-TORC1 was sufficient to block activity-dependent increases in the transcription of BDNF (21, 22), a well-established CREB target gene (18–20). Changes in BDNF expression and secretion lead to changes in TrkB activation, and these changes are thought to be important events in regulating activity-dependent changes at the synapse (10–13). We found that reducing BDNF signaling blocked the effect of chronic increases in activity and mimicked the effect of chronically reducing network activity. In contrast, increased BDNF completely reversed the effects of reduced network activity and mimicked the effects of increased network activity, although the magnitude of change affected by BDNF treatment was less profound than that of bicuculline treatment. The lesser potency of exogenous BDNF relative to bicuculline may reflect a less dramatic activation of TrkB, as it has been found that prolonged exposure of cultured cortical neurons to BDNF induces down-regulation of TrkB mRNA and protein and tyrosine phosphorylation levels, as well as decreases in phospholipase C-γ phosphorylation (33). Alternatively, the less robust effect of BDNF relative to bicuculline suggests that other signaling cascades downstream of network activity may also regulate synaptic content. Together, these results show that the level of BDNF signaling is determined by activity and that BDNF signaling, in turn, is necessary and possibly sufficient for regulating activity-dependent changes in synaptic protein concentrations.

Ubiquitin and proteasomes are both present in the synaptic fraction of adult brains and, more specifically, have been detected in hippocampal dendrites, retinal growth cones, and presynaptic fractions (2, 34–36). UPS-dependent protein degradation is required for neuronal development, synaptic plasticity, and activity-dependent changes in the steady state concentration of synaptic receptors and kinases (4, 31, 37, 38). Modulating the activity of the ubiquitin-proteasome pathway with pharmacological agents leads to acute and long-term changes in synaptic function (2, 31, 34). Furthermore, UPS dysfunctions have been implicated in the pathogenesis of several neurological disorders, including Alzheimer disease, Parkinson disease, Huntington disease, and Angelman syndrome (39). Thus, activity-dependent ubiquitination and degradation of synaptic proteins may provide an important regulatory mechanism for synapse remodeling (4). We demonstrated that exogenous BDNF prevented activity blockade-associated decreases in synaptic protein ubiquitin conjugation and scavenging BDNF with TrkB-IgG or inhibiting TrkB activity with K252a mimicked the effect of reduced activity. Also, BDNF alone was sufficient to increase ubiquitin conjugation in a manner reminiscent of increased activity, whereas TrkB-IgG and K252a blocked the effects of bicuculline. Blocking proteasome activity with pharmacological inhibitors (MG132 or lactacystin) led to a profile of up-regulation and down-regulation of proteins in the synaptosome strikingly similar to that observed after chronically suppressing network activity with TTX (4). Furthermore, treating neurons with BDNF in the presence of MG132 did not reverse the effects of MG132 alone, revealing that UPS activity is downstream of activity-dependent BDNF signaling. These findings point to BDNF as a regulator of the UPS in activity-dependent ubiquitin conjugation and regulation of synaptic proteins.

The functions of ubiquitination are not limited to protein degradation. Recent studies have shown that protein ubiquitination is involved in regulating protein activity, intracellular trafficking, and subcellular localization, as well as modulating signaling (40). The outcome of ubiquitination depends on the orientation of ubiquitin molecules in polyubiquitin chains. For example, a chain of covalent bonds at the conserved lysine residue Lys-63 on ubiquitin tags receptor proteins for endocytosis (40) and is required for nerve growth factor-induced TrkA internalization and activation of intracellular signaling (40, 41). In contrast, polyubiquitination at Lys-48 is critical for UPS-mediated degradation (42). Thus, changes in synaptic proteins correlated with increased global ubiquitination could be due either to their degradation or to other cellular processes. We overexpressed a dominant negative ubiquitin (K48R) that selectively interferes with proteasome-dependent degradation to discriminate the two possibilities. We found that overexpression of DN-UbK48R mimicked the effects of proteasome inhibitors on the pattern of synaptic proteins. Together, these findings suggest that proteasome-dependent degradation is the primary mechanism for activity-dependent changes in synaptic proteins.

BDNF and TrkB play important roles in neuronal development, synaptic plasticity, and brain diseases (10–13). Conditional knockout of TrkB in mice results in impairment of long-term potentiation (43) and blockade of epileptogenesis in the kindling model of epilepsy (44). Human patients with TrkB mutations exhibit developmental delays and memory retardation (45). The UPS is also involved in many activity-dependent cellular functions and neurobiological diseases (39). We conclude that BDNF-TrkB signaling couples neuronal activity to synaptic protein ubiquitination and predict that BDNF-regulated control of the UPS is an important molecular mechanism underlying activity-dependent changes in neuronal plasticity.

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