THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 286, NO. 6, pp. 4357–4363, February 11, 2011

RNA Polymerase 1-driven Transcription as a Mediator of BDNF-induced Neurite Outgrowth*[S]

Received for publication, July 29, 2010, and in revised form, November 12, 2010 Published, JBC Papers in Press, November 23, 2010, DOI 10.1074/jbc.M110.170134

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Neurite outgrowth is essential for development of the nervous system. Neurotrophins including BDNF are among extracellular signals that regulate neurite outgrowth. The ERK1/2 pathway contributes to intracellular signaling networks transducing the pro-neuritic effects of BDNF. In the nucleolus, RNA polymerase-1 (Pol1)-mediated transcription regulates ribosomal biogenesis, enabling cellular protein synthesis and growth. Hence, we tested the possibility that Pol1 is an effector for pro-neuritic signals such as BDNF. We report that Pol1-mediated nucleolar transcription was increased by BDNF in an ERK1/2-dependent manner in rat forebrain neurons. Conversely, in cultured hippocampal neurons, knockdown of a Pol1 coactivator, transcription initiation factor 1A (TIF1A), attenuated BDNF- or ERK1/2-induced neurite outgrowth. Also, upon overexpression, a constitutively active mutant of TIF1A strongly promoted neurite outgrowth, including increases in total neurite length and branching. Finally, overexpression of wild-type TIF1A enhanced the pro-neuritic effects of ERK1/2 activation. These observations indicate that the Pol1-mediated nucleolar transcription regulates neurite outgrowth and serves as a major pro-neuritic effector of the BDNF-activated ERK1/2 pathway. Thus, development of the nervous system appears critically dependent on the nucleolus.

Neurite outgrowth and maturation are critical for development of the nervous system determining neuronal connectivity. Neurite outgrowth/maturation is stimulated by extracellular signals, including neurotrophins and electrical activity. In forebrain neurons, the neurotrophin BDNF and/or neuronal electrical activity stimulates morphogenesis of the postsynaptic neurites (dendrites) by activation of several signaling pathways, including calcium/calmodulin-dependent protein kinase (CaMK)[2] I/II/IV, ERK1/2 (extracellular signal-regulated kinase-1/2), and PI3K (phosphatidylinositol 3-kinase)/mTOR (mammalian target of rapamycin). Rapid regulation of cytoskeletal dynamics and/or long-term changes in gene expression programs have been implicated as pro-neuritic effector mechanisms for these signaling mediators (1–3). The CaMK/ERK-regulated transcription factor cAMP response element-binding protein (CREB) is critical for neuritogenesis stimulated by electrical activity (4–6). Although BDNF has been recognized as one of the most important drivers of neurite outgrowth (1, 2), the BDNF-activated pro-neuritic transcription factors, as well as their target genes, remain to be identified.

The nucleolus is a structure within the nucleus that contains hundreds of clustered repeats of 45 S rRNA genes (rDNA) whose primary 45 S transcript is rapidly processed, producing 5.8, 18, and 28 S rRNAs (7, 8). Transcription of rDNA is mediated by RNA polymerase-1 (Pol1), initiating the nucleolus-based process of ribosomal biogenesis. Nucleolar transcription is tightly regulated to adjust ribosomal production to cellular needs. For instance, growth factors stimulate Pol1 by the ERK1/2 pathway-mediated phosphorylation of the Pol1-specific coactivator transcription initiation factor 1A (TIF1A) (9). Conversely, growth of cycling cells is critically dependent on Pol1 (8, 9).

Morphological studies of the developing nervous system revealed positive correlations between the size/activity of neuronal nucleoli and neuron growth (10, 11). Conversely, increases in nucleolar size and Pol1 activity have been observed during regenerative axonal growth of adult motor neurons (12, 13). Although these observations suggest that the nucleolus plays a role in neuronal morphogenesis, direct testing of such a possibility has not been yet reported. Thus, we investigated whether nucleolar transcription in developing rat forebrain contributes to BDNF-mediated neurite outgrowth.

EXPERIMENTAL PROCEDURES

Materials—The following reagents were obtained from commercial sources: rabbit anti-GFP polyclonal antibody (MBL International, Woburn, MA), mouse anti-β-gal monoclonal antibody (Promega, Madison, WI), mouse anti-B23 monoclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), rabbit anti-MAP2 polyclonal antibody (Cell Signaling Technology, Inc., Danvers, MA), Oregon Green® 6-carboxamido-(6-azidohexanyl) triethylammonium salt (Oregon Green® azide, Invitrogen), BDNF (Alomone Labs, Jeru-

* This work was supported, in whole or in part, by National Institutes of Health Grants NS047341 and RR015576 (to M. H.). This work was also supported by National Science Foundation Grant IOS1021860 (to M. H.), the Commonwealth of Kentucky Challenge for Excellence and Norton Healthcare (to M. H. and T. H.), and the University of Louisville School of Medicine Summer Research Student Program (to M. N. Y.).

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental "Experimental Procedures" and Fig. 1.

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[2] The abbreviations used are: CaMK, calcium/calmodulin-dependent protein kinase; CREB, cAMP response element-binding protein; Pol1, RNA polymerase-1; TIF1A, transcription initiation factor 1A; ca, constitutively active; DIV, day *in vitro*; 5-EU, 5-ethynyl uridine; shTIF1A, TIF1A shRNA.

salem, Israel), and TrkB-Fc peptide (R&D Systems, Minneapolis, MN). All other reagents were purchased from Sigma, VWR (West Chester, PA), and EMD (Darmstadt, Germany).

Plasmids—The following plasmids have been described previously: pEF1αLacZ (14), CMV-p53-DD (dominant-negative mutant of p53) (15), pCEP4-HA-δN4-MKK1-S218E/S222/D (constitutively active form of MKK1 (mitogen-activated protein kinase kinase-1)) (16), pGFP-TIF1Awt (17), pSUPER-based shRNA constructs targeting GFP and TIF1A (18), and human rDNA promoter-driven luciferase reporter construct (19). pmaxGFP expression vector was purchased from Lonza (Walkersville, MD). A pSUPER-based shRNA targeting the *Renilla* luciferase sequence 5′-caaaggaaacggatgataa-3′ was generated as described previously (18). An expression vector for the constitutively active mutant form of TIF1A (S633D/S649D; TIF1Aca) was obtained by site-directed mutagenesis of the pGFP-TIF1Awt plasmid.

Cell Culture and Transfections—Neurons from the cerebral cortex or hippocampus were prepared from newborn Sprague-Dawley rats at postnatal day 0 as we have described previously (20). Cortical neurons were grown in Eagle's basal medium supplemented with 10% heat-inactivated bovine calf serum (HyClone brand from Thermo Scientific, Waltham, MA), 35 mM glucose, 1 mM L-glutamine, 100 units/ml penicillin, and 0.1 mg/ml streptomycin. Cytosine arabinoside (2.5 μM) was added to cortical cultures on the 2nd day after seeding (day *in vitro* (DIV) 2) to inhibit the proliferation of non-neuronal cells. Cells were used for experiments on DIV6–7 unless indicated otherwise. Transient transfections with Lipofectamine 2000 (Invitrogen) were performed on DIV4 as described previously (20). Electroporations of freshly dissociated neurons were conducted using a rat neuron Nucleofection reagent kit (Lonza). Hippocampal neurons were grown in Neurobasal medium/B27 supplement (Invitrogen) containing 1 mM L-glutamine, 100 units/ml penicillin, and 0.1 mg/ml streptomycin and transfected on DIV6 using Lipofectamine 2000.

Intracerebroventricular Injections—Sprague-Dawley rats received an injection at postnatal day 7 based on a previously described method (21) and according to Institutional Animal Care and Use Committee and National Institutes of Health guidelines. Injections of 5 μl of PBS and 0.1% BSA with and without 3 μg of BDNF were made with a Hamilton needle inserted into the left lateral ventricle at the following coordinates: 1.5 mm rostral and 1.5 mm lateral to lambda (incorrectly named bregma in Ref. 19),[3] 2 mm deep from the skull surface. Lambda was readily identified by the underlying venous sinuses, which were visualized by shining light from two focal sources placed on either side of the head.[3] The consistency of intraventricular delivery was confirmed in a pilot experiment in which methylene blue solution was injected into euthanized postnatal day 7 pups using the outlined coordinates.

RNA Isolation and Quantitative RT-PCR—TRIzol RNA extraction, random-primed cDNA synthesis, and pre-rRNA/18 S rRNA quantitative RT-PCR were performed as reported previously (18).

Reporter Gene Assay—Luciferase and β-gal activities were assayed as described previously (14). Transcriptional activity was determined as luciferase activity normalized to β-gal activity.

In Situ Run-on Assay—The RNA precursor 5-ethynyl uridine (5-EU; Berry & Associates, Inc., Dexter, MI) was diluted in diethyl pyrocarbonate-treated water. To label nascent RNA, glass coverslip-cultured cells were incubated with 1 mM 5-EU for 1 h (37 °C, 5% CO_2), followed by fixation with 4% paraformaldehyde. Co-immunofluorescence for the nucleolar marker B23 and the neuronal marker MAP2 was performed according to standard protocols. Following incubations with the secondary antibodies, the 5-EU-labeled nascent RNA was detected using the previously described "click" chemistry methodology (22). The click buffer (1 M Tris-HCl (pH 8.5), 100 mM $CuSO_4$, 0.5 M ascorbic acid, and 5 mM Oregon Green® azide in diethyl pyrocarbonate-treated water) was applied for 30 min, followed by a wash with PBS and mounting of the coverslips onto slides. Z-stacked images were captured with a Zeiss Axio Observer inverted microscope and AxioVision software using green (5-EU), blue (MAP2), and red (B23) channels. In MAP2-positive cells, the integrated brightness density of 5-EU was calculated in the B23-positive nucleoli using NIH ImageJ. For each cell, the nucleolar signal was normalized against the 5-EU staining in the whole nucleus. For each experiment, at least 25 neurons were analyzed.

Image Acquisition and Morphometric Analysis—Image acquisition and analysis were performed according to previously published methodologies (23) with some modifications (for details, see supplemental "Experimental Procedures").

Statistical Analysis—Statistical analysis of the data was performed using analysis of variance, followed by post hoc Fisher least significant difference comparisons.

RESULTS

First, we determined whether BDNF stimulates nucleolar transcription in developing forebrain neurons. We used the ratio between the levels of the unstable 45 S rRNA primary transcript and its relatively more stable processing product, the 18 S rRNA, as an indicator of nucleolar transcription (18). In cultured cortical neurons from newborn rats, 10 ng/ml BDNF increased the 45/18 S ratio (Fig. 1*A*). Similar effects were also seen in ipsilateral cortices or hippocampi of postnatal day 7 rat pups that received unilateral injections of 3 μg of BDNF into the lateral ventricle 4 h earlier (Fig. 1*B*). As cultured rat cortical neurons have been shown to produce endogenous BDNF (24, 25), we determined whether their nucleolar transcription is sensitive to the decoy BDNF receptor TrkB-Fc. The 45/18 S ratios were reduced by TrkB-Fc (Fig. 1*C*), indicating that endogenous BDNF/TrkB signaling drives nucleolar transcription. Using a construct consisting of a luciferase reporter gene under the control of the rDNA promoter, we observed increased activity of the latter upon cortical neuron exposure to BDNF (Fig. 1*D*).

Although we have previously shown that at least 80% of cells in newborn rat cortical cultures are neurons, the BDNF

[3] D. M. Holtzman, personal communication.

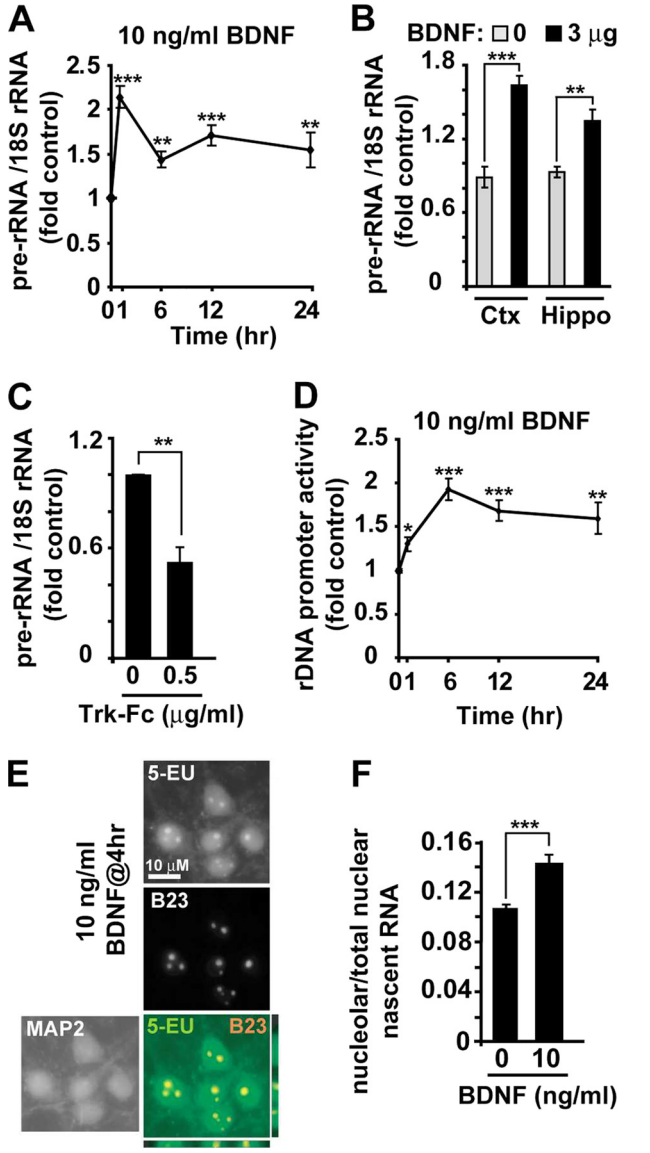

FIGURE 1. **BDNF activates nucleolar transcription.** *A* and *B*, BDNF stimulation increased the 45 S pre-rRNA/18 S rRNA ratio. In *A*, DIV6 cortical neuron cultures were used; averages of three independent experiments are shown. In *B*, postnatal day 7 rat pups (four animals/condition) received BDNF injections into the left lateral ventricle; after 4 h, ipsilateral cortices and hippocampi were dissected and analyzed. *C*, blocking endogenous BDNF signaling with the soluble TrkB-Fc peptide reduced 45 S pre-rRNA levels. DIV6 cortical neurons were treated with 0.5 μg of TrkB-Fc or IgG control for 6 h; averages of three independent experiments are depicted. *D*, BDNF activated the rDNA promoter. DIV4 cortical neurons were cotransfected with the rDNA promoter-luciferase reporter construct and the pEF1αLacZ plasmid (0.2 + 0.2 μg of plasmid DNAs/5 $\times$ 10^5 neurons, respectively). Two days later, cells were treated with BDNF as indicated. The activity of the rDNA promoter was determined by the activity ratio of luciferase to β-gal. Data represent four sister cultures from three independent experiments. *E* and *F*, increased nascent RNA levels in neuronal nucleoli after BDNF stimulation of cortical cultures. At DIV6, BDNF was added to the cells as indicated, followed by a 1-h incubation with the RNA precursor 5-EU (1 mM). After fixation and co-immunofluorescence for the neuronal marker MAP2 and the nucleolar marker B23, nascent RNA was visualized using click chemistry (see "Experimental Procedures" for more details). In MAP2-positive neurons, BDNF increased the ratio between nucleolar and nuclear nascent RNA levels. The nucleolar compartment was defined by B23 immunofluorescence. In *F*, data are the means of at least 75 randomly selected individual neurons/condition from three independent experiments. In all graphs, *error bars* indicate S.E. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

response of the nucleolar transcription may also come from the remaining glia (26). The glial origin of the BDNF effects on the overexpressed rDNA-reporter construct is unlikely, as in rat cortical cultures, virtually all successfully transfected cells were identified as MAP2-positive neurons (data not shown). To verify neuronal contribution to the BDNF-mediated transcriptional activation of endogenous rDNA, *in situ* run-on assay was performed with an RNA precursor, 5-EU, followed by co-immunofluorescence for the nucleolar marker B23 and the neuronal marker MAP2 (Fig. 1*E*). Increased content of nascent RNA was detected in cortical neuron nucleoli in response to a 4-h BDNF treatment (Fig. 1*F*). The increase in the ratio between nucleolar and total nuclear nascent RNA levels also suggests that the BDNF-mediated stimulation of nucleolar transcription is not due to enhancement of overall transcriptional activity but instead is Pol1-specific (Fig. 1*F*). Altogether, these results demonstrate that nucleolar transcription is elevated in developing forebrain neurons upon exposure to their major neurite growth-promoting signal, BDNF.

To test whether nucleolar transcription is required for BDNF-stimulated neuritic morphogenesis, we blocked Pol1 activity using the shRNA against TIF1A (shTIF1A). This reagent was previously validated and found to induce apoptosis of cultured neurons, as Pol1 is required for degradation of the pro-apoptotic transcription factor p53 (18). To avoid apoptotic interferences with morphogenesis, we cotransfected shTIF1A with an expression vector for the dominant-negative mutant form of p53. Our prior work has demonstrated its anti-apoptotic potential in shTIF1A-expressing neurons (18). The GFP expression vector was also included to provide a marker for identification of transfected cell bodies and neurites. In hippocampal neurons that received a control shRNA targeting *Renilla* luciferase, the 24-h BDNF treatment increased total neurite length, neurite branching, and cell body volume but not the number of primary neurites (Fig. 2). The shTIF1A markedly reduced the morphogenic effects of BDNF (Fig. 2), indicating that nucleolar transcription is required for somatoneuritic growth and neurite branching in response to neurotrophic stimulation.

It is noteworthy that the anti-morphogenic effects of shTIF1A were not accompanied by reductions in general protein synthesis (supplemental Fig. 1). Such findings are consistent with the relatively long half-life time of neuronal ribosomes estimated to be at least 8 days (27). Therefore, a 2-day expression of shTIF1A such as in the studies presented in Fig. 2 will unlikely cause ribosomal depletion and affect general protein synthesis.

As the ERK1/2 pathway is the major mediator of both neurotrophin-induced neuritic morphogenesis and Pol1 activation by growth factors (2, 9), we evaluated its contribution to BDNF effects on nucleolar transcription. In cultured cortical neurons, the ERK1/2 pathway inhibitor U0126 blocked BDNF-induced increases of the 45/18 S ratio and rDNA promoter activity (Fig. 3, *A* and *B*). Conversely, overexpression of a constitutively active mutant form of the ERK1/2 activator MKK1 (MKK1ca) stimulated the rDNA promoter (Fig. 3*C*). Hence, we investigated the effects of shTIF1A on the morphogenic activity of MKK1ca. After a 48-h coexpression of

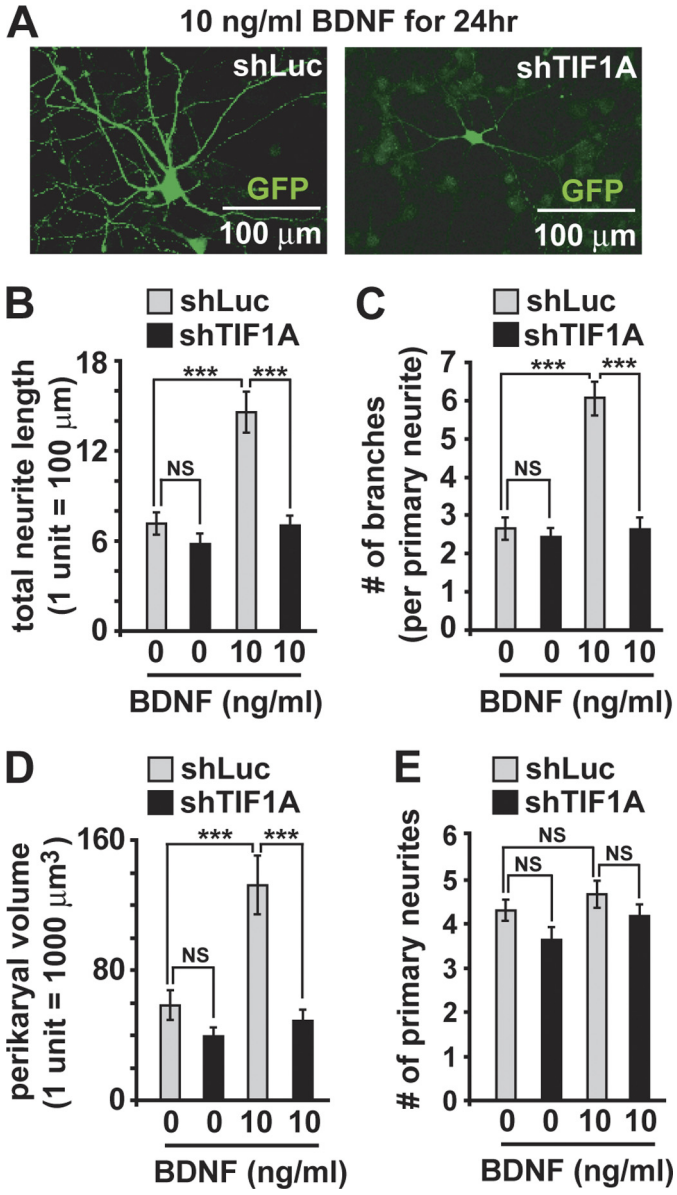

FIGURE 2. **Pol1-driven transcription is required for BDNF-induced morphogenesis.** DIV6 hippocampal neurons were cotransfected with expression plasmids for GFP (pmaxGFP) and shTIF1A or control *Renilla* luciferase shRNA (*shLuc*; 0.1 + 0.6 μg of plasmid DNAs/2 $\times$ 10^5 neurons, respectively). The expression plasmid for the dominant-negative mutant of p53 (CMV-p53-DD; 0.2 μg of DNA/2 $\times$ 10^5 cells) was added to all transfections to prevent apoptotic consequences of blocking neuronal Pol1 (18). The next day, neurons were treated with BDNF for 24 h. Transfected neurons were visualized by GFP immunostaining. *A*, representative images of transfected neurons. *B–D*, BDNF-induced increases in total neurite length, neurite branching, and perikaryal volume, respectively, were reduced by shTIF1A. *E*, the number of primary neurites was unaffected with or without BDNF. Data are means $\pm$ S.E. of at least 45 randomly selected individual neurons/condition from three independent experiments. ***, $p < 0.001$; *NS* (not significant), $p > 0.05$.

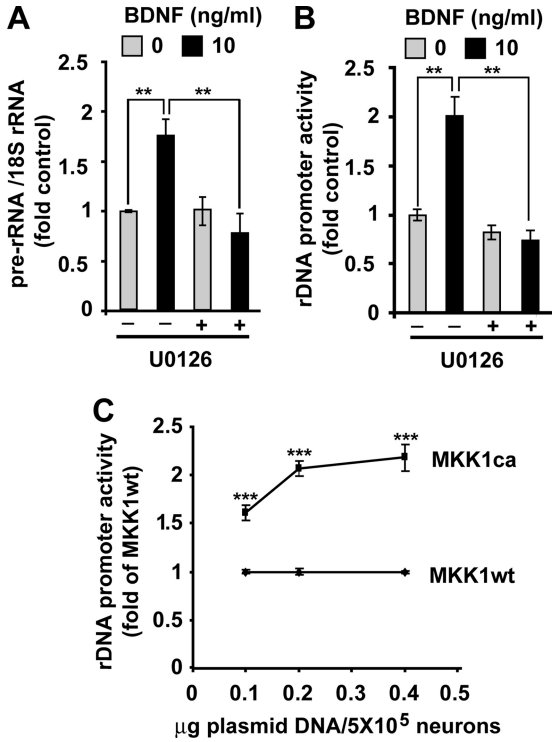

FIGURE 3. **BDNF regulates Pol1-driven transcription through the ERK1/2 signaling pathway.** *A*, pharmacological inhibition of the ERK1/2 signaling pathway attenuated the BDNF-induced increase in 45 S pre-rRNA levels. DIV6 cortical neurons were treated with BDNF and the MKK1/2 inhibitor U0126 (50 μM) or its vehicle control (0.2% Me_2SO). After 8 h, cells were lysed, and 45 S pre-rRNA/18 S rRNA ratios were determined by quantitative RT-PCR. Our previously published studies validated the effectiveness and specificity of 50 μM U0126 as an ERK1/2 pathway inhibitor in cultured cortical neurons (46). *B*, pharmacological inhibition of the ERK1/2 signaling pathway attenuated BDNF-induced activation of the rDNA promoter. DIV4 cortical neurons were cotransfected with the β-gal expression plasmid (pEF1αLacZ) and the rDNA promoter-luciferase construct (0.2 + 0.2 μg of plasmid DNAs/5 $\times$ 10^5 cells, respectively). Two days after transfection, neurons were stimulated as described for *A*. *C*, selective activation of the ERK1/2 signaling pathway with MKK1ca was sufficient to stimulate the rDNA promoter. DIV4 cortical neurons were transfected as described for *B*. In addition, MKK1ca or MKK1wt was added to the transfection mixtures as indicated. At 48 h post-transfection, cells were washed twice with serum-free medium and then placed in serum-free medium containing the NMDA receptor antagonist MK801 (1 μM). This treatment was used to reduce the basal levels of the ERK1/2 pathway activity. Four hours later, cells were lysed, and rDNA promoter activity was determined. In *A–C*, data from three independent experiments are presented; four sister cultures from each experiment were analyzed in *B* and *C*. *Error bars* are S.E. **, $p < 0.01$; ***, $p < 0.001$.

MKK1ca and *Renilla* luciferase shRNA (control shRNA), neurites of hippocampal neurons increased in length and branching (Fig. 4, *A–C*). In addition, MKK1ca stimulated perikaryal growth without effects on the number of primary neurites (Fig. 4, *D* and *E*). These morphogenic responses to MKK1ca were attenuated by shTIF1A (Fig. 4, *A–D*). These results suggest that neuronal nucleolar transcription is regulated by ERK1/2 and that such regulation is required for ERK1/2-mediated neuronal morphogenesis.

To determine whether selective activation of Pol1 is sufficient to stimulate neuronal morphogenesis, hippocampal neurons were transfected with a constitutively active mutant form of TIF1A that was generated by substituting the ERK pathway-regulated serines 633 and 649 with aspartic acid residues (TIF1Aca). As shown previously in cycling cells (9), also in neurons, TIF1Aca activated the rDNA gene promoter (Fig. 5*A*). Overexpression of TIF1Aca, but not TIF1Awt, stimulated neuronal morphogenesis by increasing total neurite length, neurite branching, and cell body volume (Fig. 5, *B–E*). TIF1Aca did not affect the number of primary neurites (Fig. 5*F*). The morphogenic effects of TIF1Aca were similar or greater than those of BDNF (Fig. 2). In addition, the morpho-

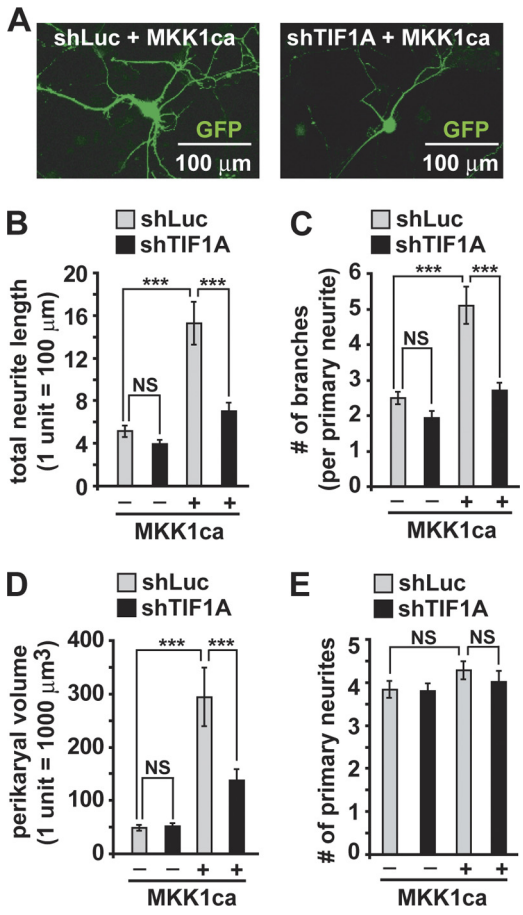

FIGURE 4. **Pol1-driven transcription is required for the morphogenic effects of the ERK1/2 signaling pathway.** DIV6 hippocampal neurons were cotransfected with expression plasmids for dominant-negative p53, GFP, MKK1ca, and shTIF1A or control *Renilla* luciferase shRNA (*shLuc*; 0.2 + 0.1 + 0.4 + 0.6 μg of plasmid DNAs/2 × 10^5 neurons, respectively). The empty expression vector pCEP4 was used as a negative control for MKK1ca. After 48 h, cells were fixed. GFP-positive neurons were analyzed morphometrically. *A*, representative images of transfected neurons. *B–D*, MKK1ca-induced increases in total neurite length, neurite branching, and perikaryal volume, respectively, were reduced by shTIF1A. *E*, the number of primary neurites was unaffected by shTIF1A or MKK1ca. Data are means ± S.E. of at least 45 randomly selected individual neurons/condition from three independent experiments. ***, $p < 0.001$; *NS*, $p > 0.05$.

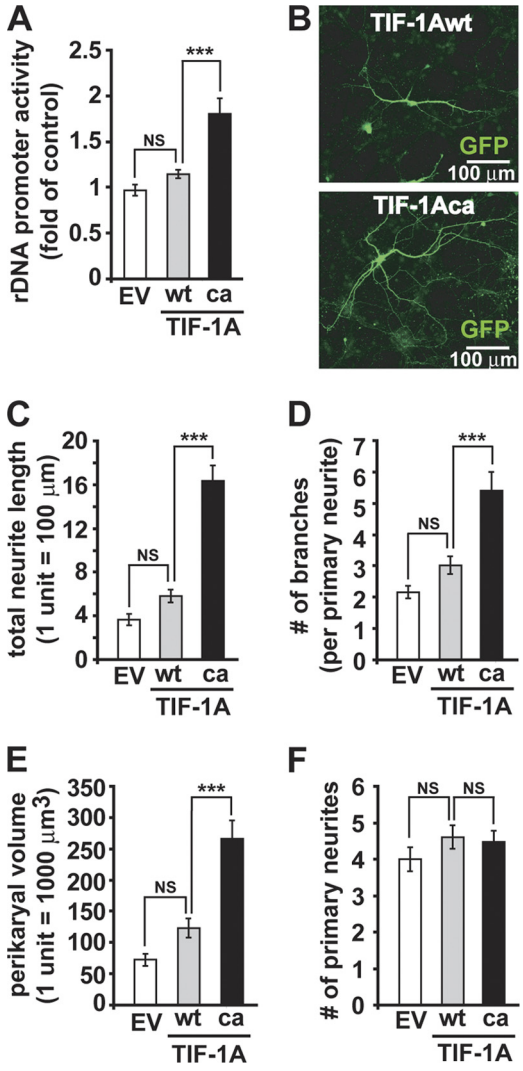

FIGURE 5. **Selective activation of Pol1-driven transcription induces neuronal morphogenesis.** *A*, the constitutively active mutant form of TIF1A that contains phosphomimetic substitutions of the ERK1/2-regulated sites (S633D/S649D; TIF1Aca) increased rDNA promoter activity. DIV4 cortical neurons were cotransfected with the rDNA promoter-luciferase construct together with expression plasmids for β-gal (pEF1αLacZ) and TIF1Aca (0.2 + 0.2 + 0.6 μg of plasmid DNAs/5 × 10^5 neurons, respectively). TIF1Awt or empty expression vector (*EV*) served as a control for TIF1Aca. The activity of the rDNA promoter was determined 48 h post-transfection; means ± S.E. of four sister cultures from three independent experiments are presented. *B–F*, DIV6 hippocampal neurons were cotransfected with expression plasmids for enhanced GFP and TIF1Aca or its controls (0.02 + 0.6 μg of plasmid DNAs/2 × 10^5 neurons, respectively). Fixation and analysis were as performed as described for Fig. 3. *B*, representative images of transfected neurons. TIF1Aca increased total neurite length (*C*), neurite branching (*D*), and perikaryal volume (*E*) but not the number of primary neurites (*F*). Data are means ± S.E. of at least 45 randomly selected individual neurons/condition from three independent experiments. ***, $p < 0.001$; *NS*, $p > 0.05$.

genic potency of TIF1Aca was similar to that of the ERK1/2 activator MKK1ca (Fig. 4). Therefore, our results indicate that selective activation of nucleolar transcription is sufficient to stimulate somatoneuritic morphogenesis. Because TIF1Aca contained mutations that mimicked regulation by the ERK1/2 pathway, such sufficiency further supports the notion that Pol1-driven transcription is the major morphogenic effector for the ERK1/2 signaling unit.

To test this possibility further, we investigated whether overexpression of TIF1Awt enhances the effects of MKK1ca on hippocampal neuron morphogenesis. To avoid saturation of ERK1/2-mediated morphogenic activity, we used a lower plasmid dose of MKK1ca than that used in the experiments presented in Fig. 4. As expected, the plasmid dose reduction from 0.4 to 0.1 μg of plasmid DNA/2 × 10^5 cells reduced the morphogenic effectiveness of MKK1ca (Fig. 6 *versus* Fig. 4). Combining MKK1ca and TIF1Awt resulted in enhanced stimulation of neurite outgrowth, neurite branching, and cell body growth (Fig. 6, *A–C*). Conversely, the number of primary neurites was unaffected (Fig. 6*D*). These results suggest that TIF1A is the ERK-sensitive regulator of the pro-morphogenic Pol1.

DISCUSSION

We have demonstrated that Pol1 activity increased in an ERK1/2-dependent manner in BDNF-stimulated neurons from developing rat forebrain. In addition, inactivation of Pol1 reduced BDNF- or ERK1/2-induced neuritic morphogenesis. Conversely, robust neuritic morphogenesis followed

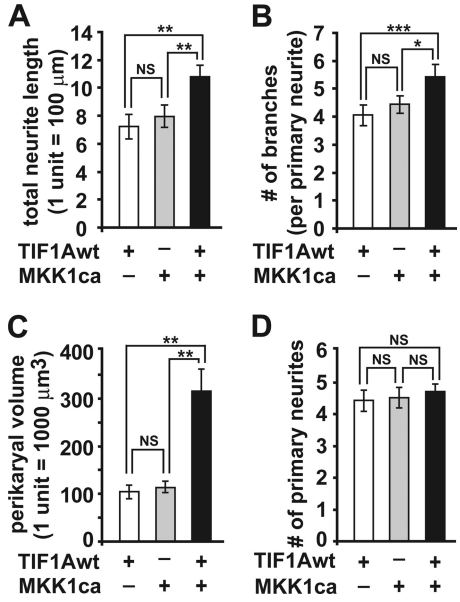

FIGURE 6. **Enhanced morphogenic effects upon coexpression of MKK1ca and TIF1Awt.** DIV6 hippocampal neurons were cotransfected with expression plasmids for GFP, TIF1Awt, and/or MKK1ca (0.02 + 0.3 + 0.1 μg of plasmid DNAs/2 × 10^5 neurons, respectively) as indicated. In addition, empty cloning vectors were used as negative controls for the TIF1Awt and MKK1ca constructs. To avoid saturation of the morphogenic response induced by MKK1ca, its dose was 4-fold lower than in Fig. 3. Cell fixation and analysis were as performed as described for Fig. 3. Coexpression of TIF1A and MKK1ca increased total neurite length (*A*), neurite branching (*B*), and perikaryal volume (*C*) but not the number of primary neurites (*D*). Data are means ± S.E. of at least 45 randomly selected individual neurons/condition from three independent experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; *NS*, $p > 0.05$.

overexpression of TIF1Aca that contained mutations mimicking ERK1/2-regulated phosphorylations. Finally, overexpression of the ERK1/2-regulated Pol1 coactivator TIF1A enhanced neurite development in response to activation of ERK1/2. Therefore, our results identified nucleolar transcription as a pro-neuritic effector of the BDNF-activated ERK1/2 signaling pathway, uncovering the critical involvement of the nucleolus in neuronal morphogenesis.

Neurites include presynaptic axons and postsynaptic dendrites. They differ in function and morphology (28). As in hippocampal pyramidal neurons, the dendrites greatly outnumber the axons, pro-neuritic effects of Pol1 manipulations are mostly, if not exclusively, in dendrites. We also observed that all treatments that modified neuritic morphogenesis had parallel effects on cell soma growth. The dendrites and the cell soma share many features and are distinct from the axons (28). Hence, the morphogenic effects presented in this study likely concern the somatodendritic compartment. However, Pol1 contribution to axonal development is also possible and will be a subject of our future research. Future studies will also determine whether Pol1 regulates the essential maturation steps of dendrite development, including spine formation and synaptogenesis.

We studied the morphogenic effects of nucleolar manipulations in fixed hippocampal pyramidal neurons after their prior labeling by the low efficiency transfection of a GFP expression plasmid. Therefore, a possibility exists that the reported effects are due, at least in part, to differential GFP transfection/expression efficiency in neuronal subpopulations with distinct morphological features. Time-lapse microscopy studies of living neurons could exclude such an artifact. Unfortunately, in our hands, prolonged time-lapse fluorescence imaging of the transfected neurons was associated with cytotoxicity, preventing us from using such an approach. However, the relative homogeneity of cultured pyramidal neurons from rat hippocampi reduces the likelihood of misinterpreting morphological diversity as a differential growth response (29, 30). Indeed, due to cell-to-cell consistency of morphogenic responses, the rat hippocampal pyramidal neurons have become one of the favorite systems to study brain neuron morphogenesis in culture (30).

Our results support the notion that neuronal nucleolar transcription is regulated by neurotrophins acting via the high affinity Trk receptors and the ERK1/2 signaling pathway. Moreover, neuronal Pol1 regulation by ERK1/2 is mediated, at least in part, by TIF1A. In non-neuronal cells, TIF1A appears to rapidly shuttle between the nucleolus and the nucleus/cytosol (31). Therefore, one can consider the possibility that TIF1A undergoes ERK1/2-dependent phosphorylations in non-nucleolar locations and then transiently enters the nucleolus to stimulate Pol1. Besides regulating TIF1A, ERK1/2 may stimulate nucleolar transcription by phosphorylation of the upstream binding factor or increased abundance of the TATA-binding protein (32, 33). Such mechanisms may also contribute to ERK1/2-mediated regulation of neuronal Pol1.

Pol1 might also be stimulated by pro-morphogenic PI3K/mTOR signaling, which also targets TIF1A (34, 35). In addition, neuronal activity, whose morphogenic effects are mediated by CaMKI/II/IV (3–5), appears to increase nucleolar transcription (36). The relative contribution of the PI3K/mTOR or CaMK pathway to the morphogenic regulation of neuronal nucleoli remains to be determined.

Besides TIF1A, only a few other extracellular signal-regulated transcription factors are sufficient to induce neuritic morphogenesis. The most robust effects resembling those of TIF1Aca were reported on dendrite development in *Drosophila* motor neurons that overexpressed the AP1 (activating protein-1) transcription factors c-Fos and c-Jun (37). Likewise, overexpression of the p50 and p65 components of NF-κB stimulates neurite outgrowth and branching in cultured mouse nodose ganglion neurons (38). In cultured rat hippocampal neurons, increased dendrite length follows transfection of a constitutively active mutant of CREB (5). As both c-Fos and CREB are well established targets for the neurotrophin-activated ERK1/2 pathway (39, 40), it is tempting to speculate that at least part of their morphogenic activity may be mediated by regulation of Pol1-driven transcription. Indeed, there is a growing list of transcription factors that, besides having previously recognized roles in modulating RNA polymerase-2 activity, also regulate nucleolar transcription (8).

At present, a mechanism underlying the morphogenic effects of nucleolar transcription is unknown. Interestingly, inhibition of Pol1 reduced BDNF-induced neurite growth but not general protein synthesis, suggesting that changes in the total translational capacity do not explain the nucleolar tran-

scription requirement for morphogenesis. Such a notion is consistent with the relatively long half-life time of neuronal ribosomes estimated to be at least 8 days (27). In contrast, disruption of ribosomal biogenesis may prevent supply of newly generated ribosomes to the growing neurites. Reductions in the neurite ribosomal component could limit morphogenesis by reducing local protein synthesis. In addition, as the previously unrecognized diversity of eukaryotic ribosomes becomes evident (41), it is conceivable that, upon morphogenic stimulation, pro-morphogenic ribosomal species are rapidly produced to initiate the neurite outgrowth response. Indeed, knockdowns of various ribosomal proteins in developing zebrafish embryos result in distinct neurodevelopmental defects, including disrupted brain growth (42). Besides ribosomal biogenesis, there are also other emerging functions of the nucleolus, including sequestration/inactivation of transcription factors such as p53 and NF-κB (43, 44). As the nucleolar structure is regulated by rDNA transcription, such "non-classical" nucleolar activities may also contribute to the morphogenic effects of Pol1. The pro-neuritic mechanisms of nucleolar transcription will be a subject of our future studies.

Taken together, our results identified the nucleolus as a critical contributor to neuronal morphogenesis. We have demonstrated that stimulation of nucleolar transcription in developing forebrain neurons is the major morphogenic effector mechanism of the ERK1/2 pathway. As the nucleolar activation occurs in regenerating neurons (12, 13), the morphogenic activity of the nucleolus may also play a role in neuroregeneration. Finally, in severe depression of developmental origin, hippocampal atrophy correlates with reduced nucleolar transcription (45). Therefore, the pro-morphogenic activity of the nucleolus may be disrupted in neurodevelopmental disorders.

Acknowledgments—We thank Drs. Ingrid Grummt and Tom Misteli for providing reagents. We are also grateful to Dr. David Holtzman for advice on the intraventricular injections and Drs. Jeffrey Twiss and Jacek Jaworski for critical reading of this manuscript.

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