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Suppression of the Basic Transcription Element-Binding Protein in Brain Neuronal Cultures Inhibits Thyroid Hormone-Induced Neurite Branching

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The molecular mechanisms underlying the effect of thyroid hormone (T_3) on neurite outgrowth are unknown. We recently identified the small GC-box binding protein BTEB (basic transcription element-binding protein) as a T_3-regulated gene in the developing rat brain. BTEB mRNAs are rapidly (by 1 h) up-regulated by T_3 in primary rat embryonic neuronal cultures. Antisense oligodeoxynucleotides (ODNs), added to the cultures, reduced by 60% the level of BTEB mRNA. Addition of BTEB antisense ODNs to the cultures, before the onset of neurite polarity, had no effect on neurite elaboration but significantly decreased, in a dose-dependent manner, the effect of T_3 on neurite branching. We then examined the effects of antisense ODNs on a thyroid hormone target neuronal population, *i.e.* the acetylcholinesterase-positive neurons after the onset of neurite polarity. Exposure to BTEB antisense ODNs completely abolished the effects of T_3 on neurite branching and on the elaboration of neuritic filopodia-like structures in acetylcholinesterase cells. By contrast, antisense ODNs did not alter the effect of T_3 on neurite length. Our results show that titration of BTEB levels by T_3 regulates the degree of neurite branching and that the T_3-induced neurite elongation and the T_3-induced neurite branching are regulated by distinct mechanisms. (*Endocrinology* 143: 2242–2249, 2002)

THYROID HORMONE (T_3) plays an essential role in development of the central nervous system. Thyroid hormone deficiency during neonatal and early postnatal life results in irreversible mental retardation, a condition known as cretinism (1). One of the major and best documented actions of T_3 on the developing brain is its effect on neurite outgrowth (Refs. 2–8; for review, see Ref. 1). However, the molecular mechanisms underlying this action remain unknown. The actions of T_3 are mediated by ligand-dependent transcription factors (9, 10). The binding of T_3 to its receptors regulates the expression of a limited number of genes that code for a set of specific proteins. Several T_3-regulated genes have been identified in the developing brain, but none have been shown to be directly involved in the effect of T_3 on neurite outgrowth (11–15).

There is evidence that shows that the expression and subsequent accumulation of brain microtubule-associated proteins (MAPs) are critical steps in the regulation of neurite outgrowth (for reviews, see Refs. 16–18), but T_3 does not seem to regulate expression of MAPs in the developing brain (16, 19). Recently, we identified the small GC-box binding transcription factor, basic transcription element-binding protein (BTEB), as a T_3-up-regulated gene in the developing rat brain. We also showed that overexpression of BTEB in N-2a cells induced neurites outgrowth (20). In the present study, we blocked BTEB gene expression in primary rat embryonic neuronal cultures using antisense oligonucleotides (ODNs) to determine whether BTEB is involved in the T_3-induced neurite outgrowth observed *in vivo*. We analyzed the consequences of exposure to antisense ODNs on the effects of T_3 on a specific neuronal population, the acetylcholinesterase (AChE)-positive neurons (AChE cells), which are known to be responsive to T_3 (6, 7). We found that inhibition of BTEB gene expression completely blocked the effect of T_3 on neurite branching, but not neurite elongation, in AChE cells. Titration of BTEB levels by T_3 regulates the degree of neurite branching. BTEB is the first T_3-regulated gene identified, thus far, to be implicated in the T_3-induced neurite branching signaling pathway.

Materials and Methods

Phosphorothioate oligodeoxynucleotide (ODN) synthesis

All ODNs were synthesized on a 380B synthesizer (PE Applied Biosystems, Foster City, CA) and purified over an NAP5 column (Amersham Pharmacia Biotech, Baie d'Urfé, Québec, Canada). The sequence of the ODNs used in this study were as follows: antisense 5′GGCCGCGGACATGGTGC3′ corresponding to bases 506–523 of the rat BTEB mRNA (21) and a scrambled sequence corresponding to the control non-sense 5′GATCGCGCGCATGACGC3′. These sequences were chosen using Oligo 4.0 software (National Bioscience, Inc., Plymouth, MN). Furthermore, searches of the sequence data bases showed that these sequences were not present in any other known sequence. ODNs were diluted in PBS to a concentration of 1 mM and stored in aliquots at −20 C.

Cell culture

Dissociated cultures of cerebral hemisphere neurons, prepared from embryonic E-16 rat embryos, were dissociated and plated onto gelatin/liter-polylysine-coated coverslips at a uniform density of 150,000 or 25,000 cells (for tubulin experiments)/15-mm diameter wells, as previously described (7). Cells were grown in serum-free medium, in a 37 C incubator, with 5% CO2.

Abbreviations: AchE, Acetylcholinesterase; BTEB, basic transcription element-binding protein; DTT, dithiothreitol; GST, glutathione-*S*-transferase; MAP, microtubule-associated protein; MTT, 3-(4,5-dimethylthiazole-2-yl) 2,5-diphenyl tetrazolium bromide; ODN, oligodeoxynucleotide.

3-(4,5-dimethylthiazole-2-yl) 2,5-diphenyl tetrazolium bromide (MTT) assay

The potential toxicity of ODNs was determined using an MTT assay (22). Cells treated or not with ODNs were incubated with MTT (250 μg/ml) for 3 h at 37 C, and reduction was measured by colorimetric detection (540 nm) of the blue insoluble formazan product. This assay provides an estimate of the number of functioning mitochondria present in the cells; *i.e.* the quantity of formazan product is directly proportional to the number of metabolically active cells in the culture.

AChE and tubulin staining

For AchE staining, cells were fixed with 3% paraformaldehyde and incubated for 1 h with substrate solution containing 72 mM acetylthiocholine, 10 mM potassium ferricyanide, 60 mM cupric sulfate, and 100 mM sodium citrate in 50 mM Tris-HCl (pH 7.6), followed by a second incubation with 0.04% 3,3′-diaminobenzidine, 0.3% nickel ammonium sulfate, and 0.003% H_2O_2, as described (7).

For immunofluorescent detection of tubulin, cells were fixed with 3% paraformaldehyde for 10 min, washed with PBS, and permeabilized with 0.1% Triton X-100 for 10 min. The cells were incubated for 1 h with the β-tubulin antibody (1:50; Roche Diagnostics, Laval, Québec, Canada), washed with PBS, and incubated with secondary antibody for 45 min (antimouse IgG-rhodamine; 1:50; Roche Diagnostics). Stained cells were mounted on a glass slide with PBS-glycerol 50% and stored at 4 C until morphometric analysis.

RNA extraction, RT-PCR, and Northern blot analysis

Total RNA was isolated from cells with Trizol reagent (Life Technologies, Inc., Grand Island, NY) and treated with deoxyribonuclease (Promega Corp., Madison, WI), following the manufacturer's instructions. Two micrograms of RNA were reverse transcribed into cDNA using a reaction mixture of 200 U Moloney murine leukemia virus reverse transcriptase (Promega, Mont-Royal, Québec, Canada), 1× RT reaction buffer, 10 mM of each deoxynucleotide triphosphate, 30 U RNAguard (Amersham Pharmacia Biotech), and 50 pmol of each sequence specific-primer. cDNA was synthesized at 37 C for 1 h. Subsequently, one fourth of the RT reaction was used as a template for PCR analyses. We previously showed that c-*jun* mRNA levels were unaffected by T_3 in primary neuronal cultures (Puymirat, J., personal communication). We therefore used c-*jun* mRNAs as our internal control in the RT-PCR assay. Oligonucleotide primer sequences were as follows: c-*jun*, F-5′GCTCCGAGGAACCGCTGCT3′ and R-5′TCACGTTCTTGGGGCACAAG3′; BTEB F-5′GAACCGGCTCAGGAGGAGGG3′ and R-5′GTCGCAGTCGCTCGGCGTCC3′. Standard PCR reaction mixture conditions, containing 200 μM deoxynucleotide triphosphates, 1.0 U *Taq* DNA polymerase (QIAGEN, Chatsworth, CA), 1× PCR reaction buffer, and 50 pmol of each primer set were used. Cycle characteristics for these primers were 94 C for 10 sec, 55 C for 30 sec, and 72 C for 30 sec. The PCR amplification products were resolved on a 1% agarose gel and stained with ethidium bromide. Peak areas associated with DNA bands were determined using the AlphaImager scan (Alpha Innovatech Corp., San Leandro, CA). PCR amplification gave products of 610- and 405-bp for c-*jun* and BTEB, respectively.

Western blot analysis

Cerebral hemisphere cultures, treated or not with 30 nM T_3 in the absence or presence of ODNs (1.5 μM), were homogenized in 1 ml buffer A [250 mM sucrose; 20 mM Tris HCl, pH 7.8; 1. 1 mM $MgCl_2$; 0.1% Triton X-100; 1 mM phenylmethylsulfonylfluoride; and a mix of protease inhibitors (Roche Diagnostics)] for 10 min at 4 C. Nuclei were removed by centrifugation, 5 min at 5,000 rpm, washed 3 times in the same buffer. Nuclear proteins were then extracted in a lysis buffer B [Buffer A, 5 mM dithiothreitol (DTT), 20% glycerol, and 400 mM KCl] for 20 min at 4 C. The samples were centrifuged at 16,000 × *g* for 5 min, and supernatant was diluted in Laemmli sample buffer. Samples were stored at −20 C until used. Fifty micrograms of nuclear proteins were separated by 10% SDS-polyacrylamide gels, transferred to polyvinylidene difluoride membrane, and probed with a affinity-purified anti-BTEB antibody (1/1000) in TBS containing 0.1% Tween. Detection was performed with a horseradish peroxidase-coupled antirabbit antibody (1:10,000; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) and ECL Western blotting detection reagents (Amersham Pharmacia Biotech). Western blots were normalized using a monoclonal antibody (2B3G8) prepared against an unknown 55-kDa nuclear protein. This nuclear protein is constitutively expressed in various tissues including neural cells, and its expression is independent of thyroid hormones (Puymirat, J., personal communication).

BTEB antibody production and affinity purification

The IgG fraction of a rabbit polyclonal antiserum, raised against a glutathione-*S*-transferase (GST)-*Xenopus* BTEB fusion protein (GST-xBTEB; Hoopfer, E. D., and R. J. Denver, unpublished), was further purified using an affinity column made with a GST-xBTEB-DNA-binding domain (GST-xBTEB[DBD]) fusion protein. A subtractive approach was used, where the column flow-through was retained, in which antibodies directed against the GST fusion tag and the highly conserved DBD (*i.e.* conserved among Sp family members) had been removed. Thus, the final IgG fraction used (the column flow-through) contained antibodies directed only against the N-terminal region of BTEB. Because the frog and rodent share sequence similarity in the N-terminal region, we reasoned that the anti-xBTEB IgGs would recognize a number of epitopes on the rodent BTEB protein.

GST-xBTEB[DBD] affinity column purification. The affinity column was prepared using the Affi-Gel 10 support (Bio-Rad Laboratories, Inc., Hercules, CA), following the manufacturer's protocol by coupling 2 mg GST-xBTEB[DBD] (2 mg/ml in 0.01 M 4-morpholinepropanesulfonic acid, pH 7.0) to 1 ml washed support (50% vol/vol bead suspension) for 4 h at 4 C. The support was incubated with 1 bed volume of 1 M ethanolamine HCl (pH 8.0) for 1 h at 4 C, transferred to a 10-ml Poly-Prep disposable column (Bio-Rad Laboratories, Inc.), and washed with 10 bed volumes of PBS (pH 7.0). The coupling efficiency was determined by comparing the OD_{280} values of the ligand solution before and after coupling. The IgG fraction of the anti-xBTEB serum was passed through the GST-xBTEB[DBD] affinity column equilibrated with 0.01 M Tris (pH 8.0), 0.15 M NaCl. The flow-through, which contained antibodies to the N-terminal region of xBTEB, was collected and reapplied to the affinity column twice. The specificity of the resultant IgGs obtained in the column flow-through (anti-xBTEB N-terminal region) was verified by Western blotting (*i.e.* this IgG fraction reacted strongly with the full-length xBTEB fusion protein but did not recognize GST-xBTEB[DBD] or GST alone; data not shown).

Morphometric analysis

Neurite outgrowth was estimated as described previously (2, 6). Several randomly chosen fields within the cultures were photographed in either a phase-contrast light microscope (for AChE neurons) or an epifluorescent microscope (for tubulin-positive neurons). Only neurons that were outside aggregates were analyzed. The number of neurites on each cell was counted, and cell length and point-branching were measured. The length of neurites was estimated by the index of neurite length. The index of neurite length was determined as x-fold cell diameter.

EMSA

Cell extracts were prepared, following methods described by Ranjan *et al.* (23). Cells were resuspended in a 5-fold packed cell volume of lysis buffer (0.4 M KCl; 20 mM HEPES, pH 7.8; 20% glycerol; 2 mM DTT; 0.5% IGEPAL CA-630; 75 U/ml aprotinin; 1 μg/ml leupeptin; 1 μg/ml pepstatin A). Cells were lysed by three cycles of freeze-thawing, and the lysate was clarified by centrifugation at 10,000 × *g* at 4 C. Protein content of the extract was determined using the Pierce Chemical Co. (Rockford, IL) protein assay.

For EMSA, a synthetic ODN corresponding to the sequence of the basic transcription element (21) was prepared: 5′gatcGAGAAGGAGGCGTGGCCAACCTCTTCCTCCGCACCGGTTGctag.

The 5′ and 3′ strands of the synthetic BTE ODN were annealed in a buffer containing 10 mM Tris (pH 7.5), 500 mM NaCl, 10 mM EDTA. Annealing was performed at 70 C for 5 min, followed by 37 C for 30 min.

The double-stranded BTE was radiolabeled by Klenow fill-in with [α-^{32}P]deoxy-CTP for 15 min at 30 C. Unincorporated [α-^{32}P]deoxy-CTP was removed by Sephadex G50 spin column chromatography. For EMSA, 15 μg cellular protein was combined with 20,000 cpm ^{32}P-labeled BTE in a buffer containing 1.4 μg poly(deoxyinosine-deoxycytidine), 20 mM HEPES (pH 7.8), 1 mM DTT, 0.1% IGEPAL CA-630, 50 mM KCl, and 20% glycerol and incubated at room temperature for 40 min. Unlabeled BTE or cytoplasmic actin cDNA were added to some reactions as specific and nonspecific competitors, respectively. Protein-DNA complexes were resolved on a 6% polyacrylamide, 0.25× Tris-borate EDTA minigel; the gel was dried and analyzed by phosphorimaging (Bio-Rad Laboratories, Inc.).

Statistical analysis

The data were expressed as mean ± SE. Results were analyzed by unpaired *t* test or one-way ANOVA on untransformed data.

Results

T_3 regulates BTEB gene expression in primary neuronal cultures

The time-course analysis of the effect of T_3 on BTEB mRNA in 7-d-old primary neuronal cultures is shown in Fig. 1. The levels of BTEB mRNAs were low, but detectable, in cultures grown in the absence of T_3, by Northern blot analysis. There was a significant (3.6-fold) increase in BTEB mRNA after 1 h of treatment with 30 nM T_3 ($P < 0.03$), with the maximal effect occurring after 12 h of treatment (5-fold the levels observed in untreated cells, $P < 0.001$). No further increase in BTEB mRNAs was observed after longer treatment with T_3.

Effect of antisense oligonucleotides on BTEB gene expression

BTEB antisense ODNs were added to the media of cultured neurons, 1 h before the addition of 30 nM T_3. The effects of antisense exposure on the levels of BTEB mRNA was determined by RT-PCR, 24 h later (Fig. 2). No significant reduction was observed with 0.5 μM antisense ODNs ($P = 0.28$). At the 1-μM concentration, antisense ODNs significantly decreased (by 33%) the levels of BTEB mRNA ($P < 0.03$), with maximal inhibition (60%) occurring at 1.5 μM ($P < 0.001$). No further reduction of BTEB mRNA was observed with higher doses of antisense (data not shown). No significant decrease in the levels of BTEB mRNA was observed with control non-sense ODNs (see Fig. 4). Based on these dose-response results, all experiments were performed with ODNs at a 1.5-μM concentration. To confirm that 1.5 μM antisense ODNs induce BTEB mRNA degradation, Northern blot analysis was performed with RNAs prepared from either 30 nM T_3 or 30 nM T_3 + 1.5 μM antisense ODN-treated cultures for 24 h. As shown in Fig. 2C, the levels of BTEB mRNAs were decreased by antisense ODNs, and a smear was clearly observed below the BTEB band, which most likely corresponds to BTEB mRNA degradation.

Western blot analysis was performed to confirm the decreased levels in BTEB protein by antisense ODNs. T_3 treatment of the cultures increased, by 2.4-fold, the levels of BTEB protein (Fig. 2D, lane 1 *vs.* lane 4). Antisense ODNs decreased, by 60–80%, the levels of BTEB protein in T_3-treated cultures, depending of the experiments. No effect of control non-sense ODNs was observed on the levels of BTEB protein (Fig. 2D, lane 3).

EMSA analysis was performed to determine whether antisense ODNs may affect the expression of other members of the Sp family proteins. EMSA detected several bands corresponding to Sp1 and Sp3, and the intensities of these bands were unaffected by any of the treatments (data not shown).

Potential effects of antisense or control non-sense ODNs on cell survival were determined using the MTT assay. Addition of 1.5 μM antisense or control non-sense ODNs had no effect on cell survival (0.377 ± 0.02, 0.42 ± 0.02, and 0.43 ± 0.005 optic density per well in control cells and cells treated with 1.5 μM antisense or control non-sense ODNs, respectively).

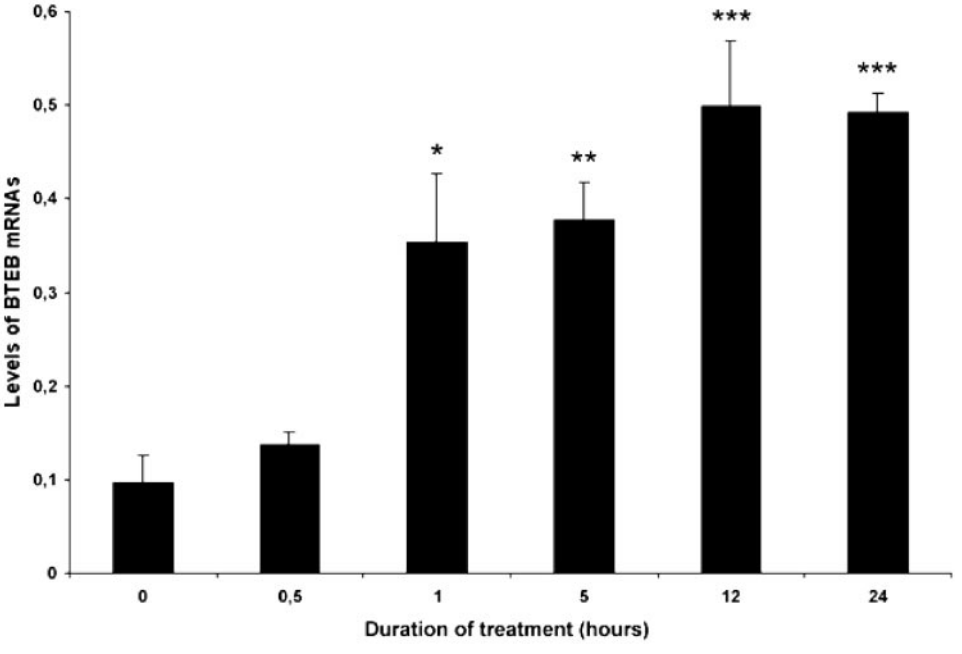

FIG. 1. T_3 up-regulates BTEB mRNA in primary rat embryonic neurons. Northern blot analysis of total RNA (20 μg), isolated from 7-d-old cultures of embryonic E-16 cerebral hemisphere neurons grown in the absence or presence of T_3 (30 nM) for the specified times. Normalization was realized by probing blots with cyclophilin cDNA. Quantitation of the signals was done by densitometry. The data represent the mean ± SEM of three independent experiments. *, **, and ***, Significantly different from control, by *t* test, at $P < 0.03$, $P < 0.01$, and $P < 0.001$, respectively.

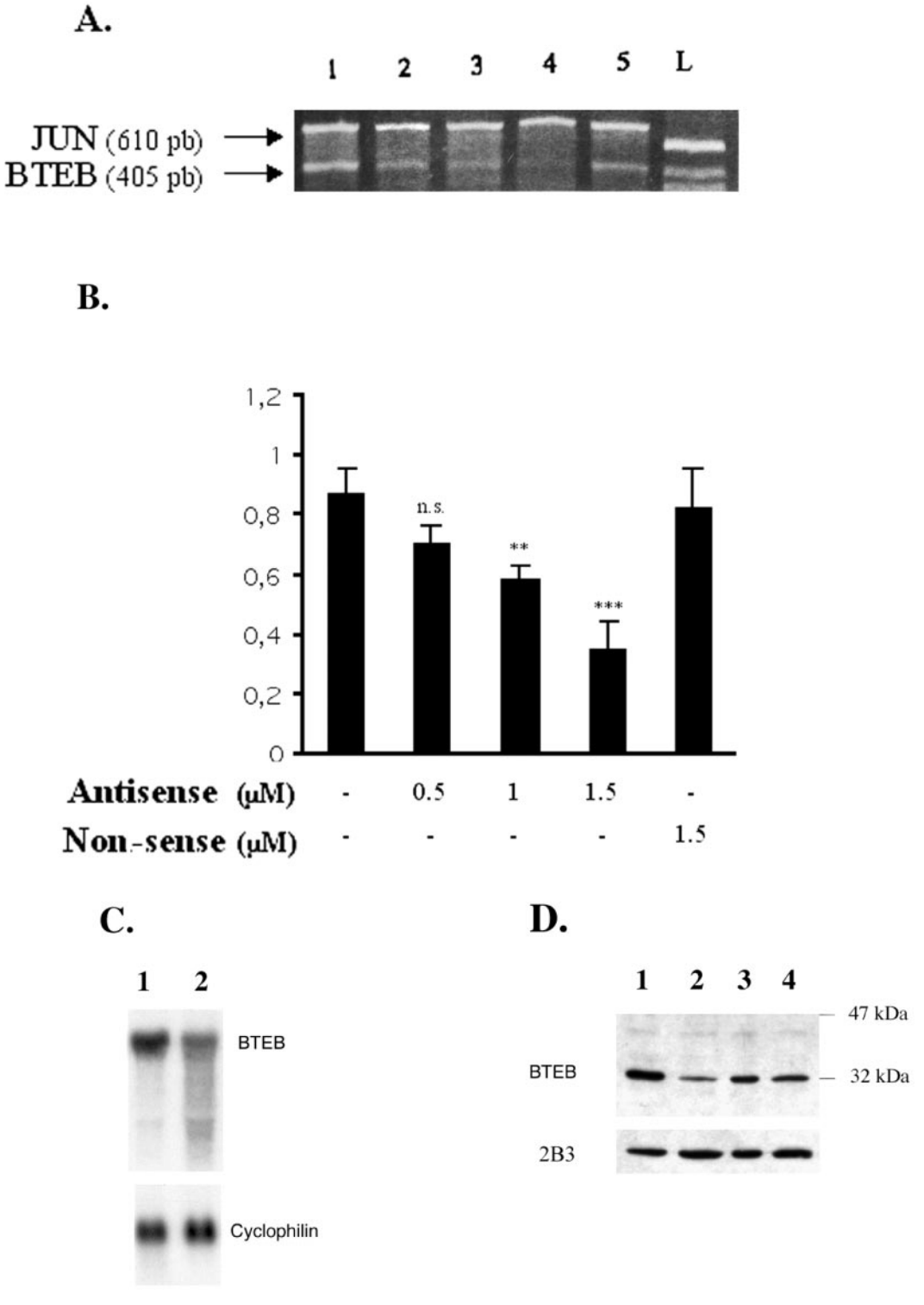

FIG. 2. Effect of BTEB antisense ODNs on BTEB mRNA in primary neuronal cultures. Cells were grown, in the absence of T_3, for 7 d. At d 5, different concentrations of ODNs (0.5, 1, and 1.5 μM for antisense ODNs; 1.5 μM for control non-sense ODNs) were added to the media, 24 h before treatment of the cells with 30 nM T_3. Total RNA was prepared, 24 h later, and analyzed by RT-PCR, as described in *Materials and Methods*. A, Ethidium bromide-stained agarose gel. Lanes 1–5 correspond to treatments shown under graph B. L, Ladder; JUN, Represents the RT-PCR 610pb band of c-Jun mRNA. B, Peak areas associated with DNA bands were quantified by densitometry, and the ratio of BTEB/c-*Jun* was calculated. Results are expressed as the mean ± SEM of three different experiments. ** and ***, Significantly different from T_3-treated cells, by ANOVA, at $P < 0.03$ and 0.001, respectively. n.s., Not significant. C, Northern blot of total RNA, prepared from 30 nM T_3 (lane 1)-, or 30 nM T_3 + 1.5 μM antisense ODNs (lane 2)-treated cells, for 24 h. D, Western blot, prepared from cultures treated or not with 30 nM T_3 for 24 h, in the presence or in the absence of antisense- or control non-sense ODNs (1.5 μM) and blotted with the anti-BTEB antibody. Lane1, T_3-treated cultures; lane 2, T_3 + 1.5 μM antisense ODNs; lane 3, T_3 + 1.5 μM control non-sense ODNs; lane 4, untreated cultures.

Effects of BTEB antisense oligonucleotides on T_3-induced neurite outgrowth

Under our culture conditions, initiation of neurite outgrowth occurs during the first 3 d of culture, whereas elongation takes place after 3 d (see Ref. 26). To determine whether T_3 influences the elaboration of neurites, we studied its effect on neurite outgrowth during the first 24 h of culture. Cells stained with the antitubulin antibody were examined. Treatment of the cultures, with 30 nM T_3 during the first 24 h, did not affect the number of neurites per neuron nor the index of neurite length but significantly increased the number of branchpoints per neuron (Fig. 3, compare B with A). The results were quantified and are presented in Table 1. BTEB antisense ODNs were added to the media of cerebral hemisphere neurons, in the absence or presence of T_3 during the first 24 h, to determine the effects of antisense exposure on neurite polarity and on T_3-induced neurite branching. As shown in Table 1, antisense ODNs (1.5 μM) completely abolished the T_3-induced increase in the number of branchpoints per neuron (Fig. 3C). This effect of antisense was dose-dependent (Table 1). There was no significant effect of antisense ODNs on the elaboration of neurites nor on the index length of neurites (Table 1). No effect of control non-sense or antisense ODNs was observed on neurite outgrowth in cell grown in the absence of T_3 (data not shown). No effect was observed with 1.5 μM control non-sense ODNs, and neurons were indistinguishable from those grown without ODNs in the presence of T_3 (Fig. 3, compare B with D; Table 1).

In further experiments, we examined the effect of T_3, after the onset of neurite polarity, to determine its effects on cells that had already formed neurites. Because the degree of neurite arborization visualized by the antitubulin antibody in T_3-treated cells was too dense to permit morphometric

TABLE 1. Effects of BTEB ODNs on T_3-induced neurite outgrowth in rat embryonic cerebral hemisphere neurons after culture for 24 h

	Neurite per neuron	Index of neurite length	Number of branchpoints per neuron
No treatment	1.9 ± 0.08	4.7 ± 0.2	1.30 ± 0.15
T_3 (30 nM)	2.0 ± 0.1	4.5 ± 0.2	2.14 ± 0.13[a]
T_3 + non-sense			
1.5 μM	2.0 ± 0.05	4.8 ± 0.1	1.95 ± 0.11
T_3 + antisense			
0.5 μM			1.75 ± 0.1[b]
1.0 μM			1.53 ± 0.1[c]
1.5 μM	1.9 ± 0.04	4.8 ± 0.1	1.32 ± 0.1[c]

Cells were cultured with or without T_3 (30 nM) and in the presence or absence of ODNs for 24 h. A total of 300 cells were analysed for each group. Each value represents the mean ± SEM.

[a] Significantly different from untreated cells, $P < 0.001$.

[b] Significantly different from T_3-treated cells, $P < 0.001$.

[c] Significantly different from T_3-treated cells, $P < 0.01$.

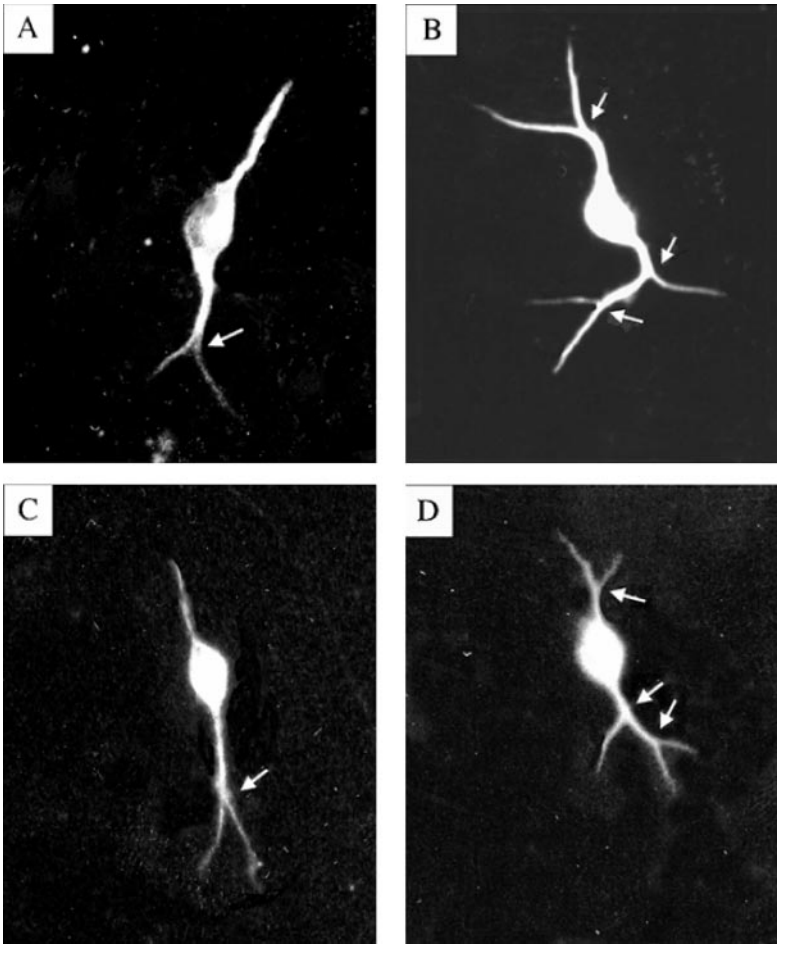

FIG. 3. Inhibition of neurite branching by BTEB antisense ODNs after 24 h in culture. T_3 and ODNs were added to the media, 1 h after plating, and morphometric analysis was performed 24 h later. Cells were labeled with a monoclonal antitubulin antibody. Three hundred neurons were analyzed per condition. Control culture grown in the absence of T_3 (A) or T_3-treated cells for 24 h (B). C, Antisense ODN-treated neurons grown in the presence of T_3; D, control non-sense ODN-treated neurons grown in the presence of T_3. Branchpoints are indicated by an *arrow*.

measurements, we chose to study a specific neuronal population, *i.e.* the AChE-positive neurons. These cells have been shown to be a target nerve cell population for T_3 (6, 7). In cells treated with T_3, the hormone was added to the media on d 6, and morphometric analysis was performed on d 7. Treatment of the cultures with 30 nM T_3 had no significant effect on the number of neurites per AChE cell (2.8 ± 1.1 and 3.1 ± 1.1 neurites per cell for control and T_3-treated cells, respectively; $P = 0.12$) but significantly increased both the index of neurite length (4.7 ± 0.23- and 6.0 ± 0.2-fold cell diameter for control and T_3-treated cells, respectively; $P < 0.001$) and neurite branching (Fig. 4, compare A with D). The effects on neurite branching were quantified and are presented in Fig. 4, A and B. In culture grown in the absence of T_3, 70% of the cells have less than three branchpoints, whereas this percentage falls to 45% in T_3-treated cells. Furthermore, short filopodia-like processes were observed, extending from neurites in T_3-treated cells (Fig. 4A). We then examined the effect of BTEB antisense ODNs on T_3-induced neurite outgrowth in AChE cells. Oligonucleotides (1.5 μM) were added to the cultures at d 5, 24 h before T_3, and morphometric analyses were performed at d 7. Addition of BTEB antisense ODNs to the media of cultures treated with T_3 completely abolished T_3-induced neurite branching in AChE cells but had no significant effect on the T_3-induced neurite elongation (Fig. 4, compare B with A). The index of neurite length was 6.0 ± 0.2- and 6.2 ± 0.24-fold cell diameter for T_3-treated cells and cells treated with both T_3 and antisense ODNs, respectively. The effects of antisense ODNs on neurite branching were quantified and are presented in Fig. 5D. In cells treated with T_3 and antisense ODNs, 65% of the cells have less than three branchpoints, which is similar to that observed in untreated cells (Fig. 5D). In cultures grown in the absence of T_3, antisense ODNs had no significant effect on the number of neurites per AChE cell (2.2 ± 0.9 and 2.3 ± 0.9 for antisense and control non-sense ODN-treated cultures, respectively; $P = 0.7$), on the index of neurite length (4.7 ± 0.23- and 4.0 ± 0.27-fold cell diameter for non-sense and antisense ODN-treated cells) nor on the number of branchpoints per neuron (Fig. 5C).

Culture in the presence of control non-sense ODNs did not affect the index of neurite length (5.4 ± 0.27- *vs.* 4.7 ± 0.23-fold cell diameter for ODN-treated and untreated cells, respectively) or neurite branching in AChE cells grown in the absence (Fig. 4, compare F with D; and Fig. 5C) or the presence of T_3 (index of neurite length, 6.0 ± 0.2- *vs.* 6.6 ± 0.23-fold cell diameter for ODN-treated cells and untreated cells, respectively; Fig. 4, compare C with A; and Fig. 5D).

Discussion

Our findings support the hypothesis that the T_3-regulated protein BTEB, which we previously showed increases neurite outgrowth in a neuroblastoma cell line (20), influences neurite growth in primary embryonic neurons. The effect of thyroid hormones on neurite outgrowth is well documented (Refs. 2–8; for review, see Ref. 1). However, whether T_3 affects the number of neurites per neuron, and/or the length and/or the branching of neurites is not well known. It has been shown that decrease of the T_3 level does not alter the number of primary neurites (3, 8). Our data show that T_3 did not affect the number of neurites per neuron but increased both the length and branching of neurites. These results are in good agreement with our previous data showing that treatment of primary neuronal cultures with T_3 does not affect the number of neurites per neuron but increases neu-

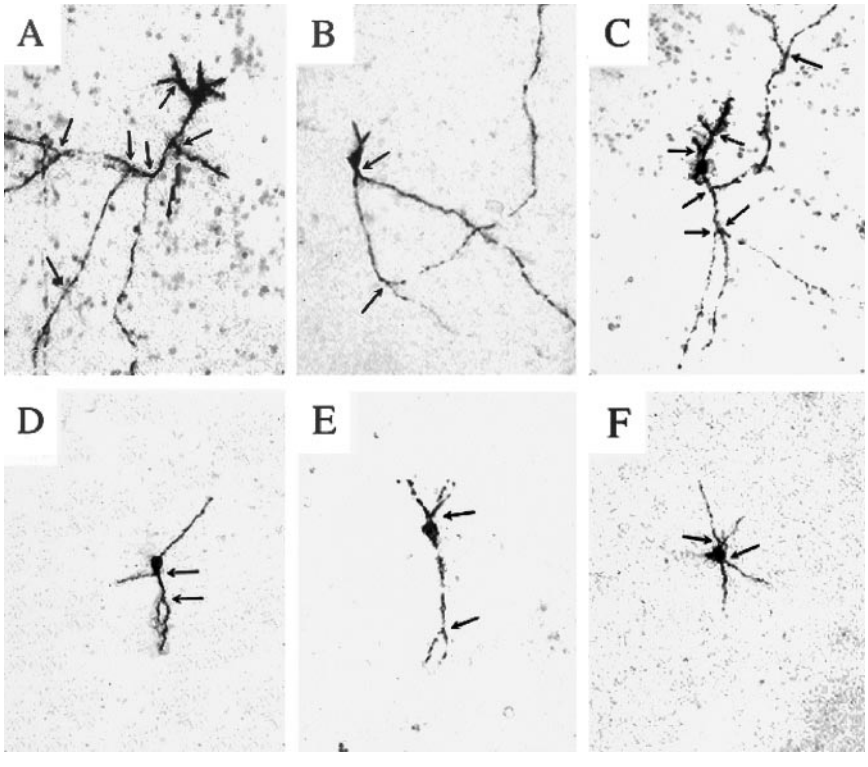

FIG. 4. Inhibition of AChE-neurite outgrowth by antisense ODNs after 7 d in culture. Cells were grown for 6 d in the absence of T_3. At d 6, T_3 (30 nM) was added to the culture for 24 h, and cell analysis was performed at d 7. In experiments with ODNs, control non-sense or antisense ODNs were added to the culture at d 5. AChE staining in cells grown in the absence (D) or in presence (A) of T_3 after 7 d *in vitro*; antisense ODN-treated neurons grown in the absence (E) or presence (B) of T_3; control non-sense ODN-treated neurons grown in the absence (F) or presence (C) of T_3.

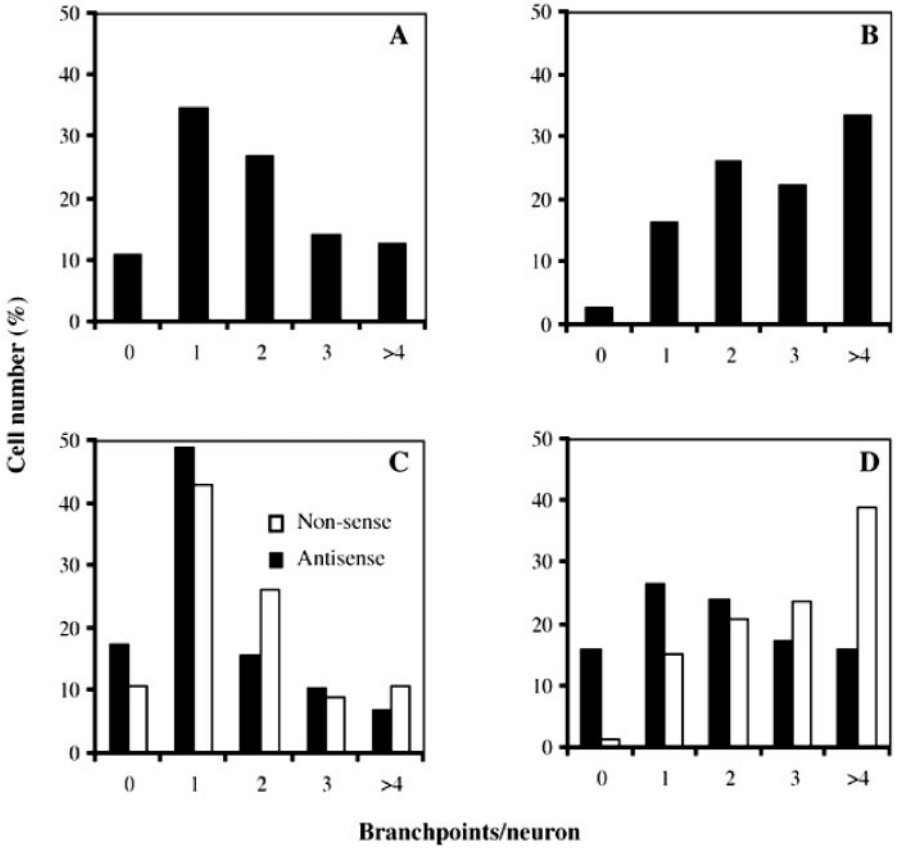

FIG. 5. Effects of BTEB antisense ODNs on T_3-induced neurite outgrowth in AchE-positive cells from rat embryonic cerebral hemisphere cultures. T_3 (30 nM) was added to 6-d-old cultures that have been treated with 1.5 μM ODNs for 24 h. Morphometric analysis was performed 24 h later. Results are expressed as the percent of cells having 0, 1, 2, 3, and more than 4 neurite branchpoints. Cultures grown in the absence (A) or presence (B) of T_3. C, Cultures grown in the absence of T_3 and treated with either antisense or control sense ODNs. D, Cultures grown in the presence of T_3 and treated with either antisense or control sense ODNs. Statistical analysis was performed by using chi-square *P* value. The distribution of the number of branchpoints per neuron is significantly different for: T_3-treated *vs.* untreated cells ($P < 0.003$); antisense ODN-treated cultures grown in the presence of T_3 *vs.* T_3-treated cultures ($P = 0.006$) or control non-sense-treated T_3 cultures ($P = 0.0006$). No significant difference was observed for: control non-sense-treated cultures grown in the absence or presence of T_3 *vs.* cells grown in the absence ($P = 0.85$) or presence of T_3 ($P = 0.85$); antisense-treated cells grown in the absence of T_3 *vs.* cells grown in the absence of T_3 without ($P = 0.25$) or with ($P = 0.5$) control non-sense ODNs.

rite density (including both neurite length and neurite branching) (2, 8).

Despite the fact that the effect of T_3 on neurite outgrowth has been known for several years, the genes that mediate this effect have not been identified. We previously identified the small GC box-binding transcription factor BTEB as a T_3-regulated gene in N-2aTRβ1 and in the developing brain (20). To determine the functions of BTEB in the developing brain, we blocked the expression of BTEB by antisense ODNs in primary neuronal cultures. We show that the effects of T_3 on neurite branching are completely abolished by BTEB antisense ODNs, whereas BTEB antisense ODNs had no effect on the T_3-induced increase in neurite length. Surprisingly, we found no significant effect of BTEB antisense ODNs on neurite branching in cultures grown in the absence of T_3, although BTEB mRNAs are expressed in these cells, albeit at a very low level. This lack of effect may be explained by the difficulties with quantifying a small effect on outgrowth by the methodology that we used. Although the effects of ODNs are relatively weak, they are statistically significant. The small effects of ODNs might be explained by the fact that the effect of T_3 on neurite outgrowth is weak after 24 h of treatment and requires several days of treatment to become pronounced (6). Several criteria support the conclusion that this effect of antisense is specific to BTEB. The decreased level in BTEB mRNAs is associated with a decreased level in BTEB protein. In contrast, neither antisense nor control sense ODNs affected the levels of c-*jun* mRNA (see Fig. 2), Sp1 protein, or Sp3 protein (determined by EMSA; data not shown). The fact that the levels of Sp1 and Sp3 proteins were unaltered in antisense ODN-treated cells argues against the effect on neurite branching being mediated by other Sp family proteins. Control non-sense ODN-treated neurons were indistinguishable from those cultured in the absence of ODNs. In addition, a potential cytotoxic effect of the ODNs was excluded by the MTT test. Taken together, our findings support the hypothesis that: 1) BTEB is involved in the neurite branching signaling pathway activated by T_3 and; and 2) T_3-induced neurite elongation and branching are controlled by different mechanisms. This is the first demonstration that the T_3-induced increase in neurite length and arborization are regulated by distinct mechanisms. These results differ, however, from those previously reported in neuro-2a cells that overexpress BTEB (20). In these cells, the index of neurite outgrowth length was found to be increased by BTEB overexpression. This discrepancy may be explained by the fact that MAPs (including tau), which regulate the assembly and stability of microtubules and therefore neurite outgrowth, differ between neuro-2a cells and primary neurons (24). It has been hypothesized that neurite outgrowth results from changes in the cytoskeleton. The assembly and stability of microtubules are regulated by MAPs, including tau (found predominantly in axons) and MAP2 (found predominantly in dendrites) (for reviews, see Refs. 17 and 18). There is now evidence that the initial establishment of neurites depends, in part, on MAP2, whereas further neurite elongation depends, in part, on tau and microtubule stabilization (25–28). MAP1 was found as a prominent component of microtubule proteins in neuro-2a cells, whereas MAP2 was found the major component of microtubule proteins in neurons (24).

This may explain the differences observed between neuro-2a cells and primary neurons.

Recent data obtained in our laboratory indicate that T_3 does not affect the levels of MAP2 and tau proteins in primary neurons, suggesting that the T_3-induced neurite outgrowth is not mediated by the changes in the levels of MAPs by T_3. This is in good agreement with other reports that show that the processing, rather than the expression, of MAPs is impaired in the hypothyroid brain (for review, see Ref. 19). The phosphorylation state of MAPs modulates their interaction with microtubules (17), and Diez-Guerra and Avila (29) recently showed that MAP2 phosphorylation plays a part in dendrite arborization in cultured hippocampal neurons. Furthermore, phosphorylation of MAPs modulates both dendrite branching and axon branching but with differences in sensitivity to phosphorylation and/or dephosphorylation by specific kinases and phosphatases (30). It is therefore possible that T_3 up-regulates BTEB gene expression, which, in turn, modulates the phosphorylation and/or dephosphorylation of MAPs and/or tau by specific kinases and phosphatases. In conclusion, our results suggest that the immediate early transcription factor BTEB is one of the first intermediates in the T_3-induced signaling pathway leading to neurite branching in the developing brain.

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