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# Effect of thymosin β15 on the branching of developing neurons

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**Abstract**

The thymosin βs (Tβs) are polypeptide regulators of actin dynamics that are critical for the growth and branching of neurites in developing neurons. We found that mRNAs for Tβ4, Tβ10, and Tβ15 were highly expressed in the developing rat brain during neuritogenesis, supporting a role for the Tβs in this process. Overexpression of the Tβs increased the number of neurite branches per neuron in cultured hippocampal and cerebral cortex neurons, and Tβ15 had the greatest effect. Actin binding activity appears to be essential for the branch-promoting activity of Tβs because two mutants of Tβ15 lacking monomeric actin binding activity failed to stimulate branch formation. We also found that transfection of siRNA against Tβ15 reduced branching. Taken together, these data suggest that the three Tβs, and especially Tβ15, stimulate neurite branching during brain development.


*Keywords:* Thymosin β; Neuritogenesis; Branching; Actin dynamics; Actin binding protein

Neuritogenesis is essential in development to allow neurons to make several thousand different connections with their partners in different brain regions [1–3]. Neuronal development is initiated upon activation of membrane receptors by extracellular cues [4], and this triggers intracellular cascades some of which alter the organization of the actin cytoskeleton [5]. As a result, the original rounded shape of the cells is broken down giving rise to buds, which become neurites and are later transformed into axons and dendrites [1].

The motility of growth cones, the main determinant of neurite elongation and branching [6–8], is thus largely dependent on the reorganization of the actin cytoskeleton [9], and actin dynamics are believed to be critical for neurite growth, branching, and pathfinding [7,10]. Branching occurs via changes of actin and microtubule structure in primary growth cones or axon shafts [11], and numerous actin-associated proteins that control actin organization are involved in neurite development [1,12,13].

The thymosin βs (Tβs) have G-actin sequestering activity and are key regulators of actin dynamics. They are small (5 kDa), highly conserved proteins [14], and at least three different isoforms (Tβ4, Tβ10, and Tβ15) have been identified in mammals [15,16]. Tβ4 and Tβ10 are abundant in neural tissue as well as in circulating cells such as platelets, leukocytes, and macrophages [15,17–19]. On the other hand, the expression of Tβ15 has not been thoroughly examined. Although the function of Tβs in the mammalian brain has yet to be elucidated, it is believed that they play a role in neurite development [15,19]. During zebrafish development, their expression is closely correlated with neuronal growth and differentiation, and inhibition of Tβ expression impairs axonal tract formation [15].

In this study, we examined the expression of Tβs in the developing rat brain, and assessed their function in

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neurite development by overexpressing and knocking down a number of Tβ isoforms in vitro. Our data indicate that Tβs, especially Tβ15, are involved in the regulation of axonal branches during brain development.

## Materials and methods

*Construction of expression vectors.* PCR-generated DNA fragments containing the full coding regions of various Tβs were ligated in-frame into the *Bam*HI and *Hin*dIII site of the N-terminally tagged expression vector pEYFP-C1 (Clonetech). Tβ15-T12P and -L18A were generated with a QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Western blotting confirmed that the YFP-tagged Tβs were properly expressed (data not shown). To construct the siTβ15 vector, pSilencer.U6.1.0 (Ambion) was digested with *Apa*I and *Eco*RI, and blunt-end ligated with insert fragments. The target sequence of siTβ15, derived from the NCBI database (Accession No.: U25684), was 5′-AGAATACTCTTCCTTCGAA-3′. A BLAST search revealed no significant sequence homology of this sequence to other genes. Sequences of 19–23 nt separated by a 9 nt hairpin loop from the reverse complementary repeat, with five thymidines as termination signal, were synthesized by Genotech (Korea) and inserted into pSilencer.U6.1.0.

*Cell culture and transfection.* Cultures were prepared from the cerebral cortex and hippocampus of gestation day 17 Sprague–Dawley rat embryos (E17) [20]. Briefly, cortical and hippocampal cells were isolated by trypsinization and cultured in serum-free conditions (Neurobasal media + B27 supplements) at a density of $5 \times 10^4$ cells/well in 24-well plates. Cultures were maintained in a 37 °C humidified atmosphere of 5% $CO_2$ for up to 2 days and then transfected with 1 μg of the various Tβ constructs using Lipofectamine 2000 (Invitrogen).

To examine the effect of Tβ15 on F-actin structure, COS-7 cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and antibiotics. One day before transfection, the cells were seeded in 24-well plates, and transfection was performed with a CalPhos Mammalian Transfection Kit (BD Bioscience).

*Immunocytochemistry.* Cells were fixed for 15 min with 4% paraformaldehyde in PBS. After blocking in 3% BSA and 0.2% Triton X-100 in PBS, primary antibodies (rabbit-anti-Tβ4, BIØDESIGN International; rabbit-anti-Tβ10, BIØDESIGN international; or chicken-anti-Tβ15 kindly provided by Dr. B.R. Zetter, Harvard University, 1:500 with mouse-anti-GAP43 (Sigma) 1:500, or mouse-anti-β-tubulin isotype III (Sigma) 1:1000) were applied to the slides. The slides were then rinsed with PBS and incubated in secondary antibody (goat-anti-rabbit (Vector Laboratories, Burlingame, CA) 1:500, or goat-anti-chicken (Amersham) 1:500 with goat anti-mouse (Vector Laboratories, Burlingame, CA) or TRITC labeled phalloidin (Sigma) 1:500) in 3% BSA and 0.2% Triton X-100 in PBS for 30 min at RT. Slides were rinsed with PBS and mounted.

*In situ hybridization.* In situ hybridization was performed as described previously [21,22]. Frozen sections (12 μm thick) were thaw-mounted, fixed in 4% paraformaldehyde, and incubated with 0.25% acetic anhydride in 0.1 M triethanolamine/0.9% NaCl (pH 8.0) for 10 min. They were then dehydrated/delipidated in ethanol and chloroform, and finally air-dried. Hybridization probes were prepared from pGEM-T Easy plasmids containing nt 1–499 of Tβ4 (AA819609), nt 32–445 of rat Tβ10 (M58405.1), and nt 1–437 of Tβ15 (AA997865) by transcription with appropriate RNA polymerases using a Riboprobe System (Promega, Madison, WI) and $^{35}$S-UTP (Amersham Pharmacia Biotech). Sections were hybridized with $^{35}$S-labeled probes ($1.2 \times 10^6$ cpm/slide) at 52 °C overnight, followed by four washes in 4× SSC at RT. The slides were then incubated with RNase buffer [RNase A (10 mg/ml in DW), 0.5 M NaCl, 10 mM Tris–HCl, and 1 mM EDTA, pH 8.0] at 37 °C for 30 min, washed with 2× SSC/10 mM DTT (twice for 5 min), 1× SSC/10 mM DTT for 10 min, 0.5× SSC/10 mM DTT for 10 min, and 0.1× SSC/10 mM DTT for 30 min at 62 °C. Finally, the sections were dehydrated, air-dried, and exposed to X-ray film (BioMAX-MR; Kodak) for 5 days. All experimental procedures using animals were in accordance with the NIH *Guide for the Care and Use of Laboratory Animals* and were approved by the Committee at Korea University College of Medicine.

*Quantitative analysis of neurites.* Stained and transfected cells were examined with a Zeiss Axioskop2 microscope equipped with a digital camera (CoolSNAP, Photometics) using metaview software, or a Zeiss Axiovert-equipped digital camera (CoolSNAP, Photometics) with IPlab3.0 software. The total number of neurites was determined for each cell. Neurons that were confounded by unresolvable processes were not included. All data were analyzed by one-way ANOVA and Student's $t$ test, with $P < 0.05$ as the accepted level of statistical significance.

## Results

### *Thymosin βs are strongly expressed in developing neuronal processes*

We examined the expression of three Tβs (Tβ4, 10, and 15) in the developing rat brain by in situ hybridization. At E18, expression of Tβ15 was predominantly observed in the central nervous system (CNS), whereas Tβ4 and Tβ10 were found throughout the embryo (Figs. 1A–C). There were differences in mRNA signal intensities (Tβ4 > Tβ10 > Tβ15), consistent with previous reports [23,24]. These differences in expression level were maintained on P3 (Figs. 1D–F). In the adult, Tβ4 expression was substantially reduced but a moderate level remained in most brain regions (Fig. 1G). Expression of Tβ10 was restricted to particular areas including olfactory bulb, deep layer of cerebral cortex, and hippocampal formation (Fig. 1H). At the same time a low level of Tβ15 expression was observed in the olfactory bulb (Fig. 1I).

Immunocytochemical analyses of cultured primary neurons demonstrated that the Tβs were enriched in developing neurite processes and growth cones (Figs. 1J–U). For instance, Tβ15 immunoreactivity (IR) colocalized with GAP43 (a marker for growth cones), phalloidin (a marker for F-actin), and Tuj1 (a marker for neurites). Similar cellular distributions of Tβ4 and Tβ10 were observed (data not shown).

### *Overexpression of the Tβs increases neurite branching in primary cultured neurons*

We tested whether overexpression of Tβs modified neurite development. Overexpression of YFP-tagged Tβ15 in DIV2 cerebral cortex neurons increased the number of neurite branches (Fig. 2A). Although overexpression of YFP-tagged Tβ4 and Tβ10 appeared to have similar branch-promoting effects, these effects did not reach statistical significance. To rule out a cell type-specific effect of Tβ15, we tested whether overexpression of Tβ15 had the same effect on cultured hippocampal

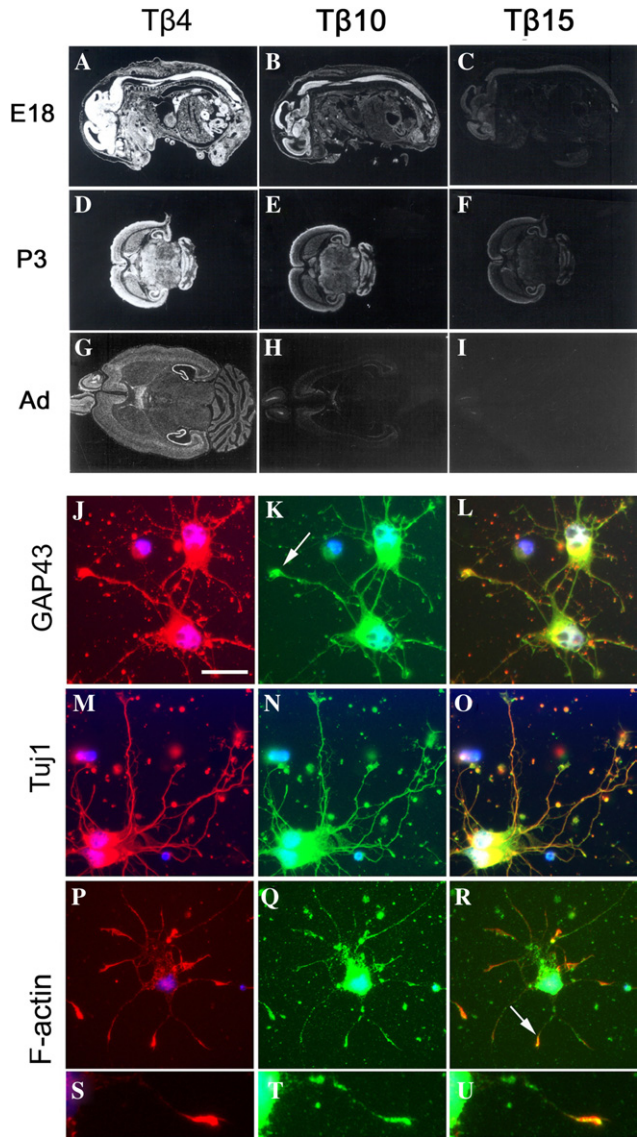


Fig. 1. Expression of Tβs during development and their distribution in primary cultured neurons. (A–I) Sagittal (E18, A–C) or horizontal (P3 and adult, D–I) sections were made, and the distribution of Tβ4 (A, D, and G), Tβ10 (B, E, and H), and Tβ15 (C, F, and I) mRNA was visualized by ISH. (J–U) DIV3 hippocampal neurons were double-labeled with Tβ15 (green) and GAP43 (J–L, red), Tuj1 (M–O, red), or F-actin (P–U, red). Scale bar, 10 μm.

neurons (Fig. 2). As shown in Figs. 2B and D, there was an approximately twofold increase in the number of branches in Tβ15-transfected hippocampal neurons compared to control YFP-transfected neurons. These results indicate that Tβ15 promotes branch formation in developing neurons.

### *The G-actin sequestering activity of Tβs is important for branch promotion*

We asked whether the branch-promoting effect of Tβ15 was related to its G-actin binding activity. Mutations of its actin-polymerization inhibitory domain (T12P) or G-actin binding domain (L18A) are known to disrupt G-actin binding by Tβ15 (Fig. 3A) [25], and we confirmed that transfection of these two Tβ15 mutants into COS-7 cells did not lead to disruption of F-actin structures, whereas transfection of wild type Tβ15 reduced F-actin intensity (Figs. 3B–N).

We then transfected these mutants into primary hippocampal neurons to see whether Tβ15 retained its branch-promoting activity in the absence of G-actin binding. In the event, the two Tβ15 mutants both failed to promote branching (Fig. 3O). We conclude that the ability of Tβ15 to bind G-actin is important for its branch-promoting effect.

### *Suppression of endogenous Tβ15 inhibits branching in primary neurons*

To block endogenous Tβ15 expression, we used an siRNA vector targeting Tβ15 RNA. Fig. 4B showed that expression of this siRNA substantially reduced Tβ15 immunoreactivity (IR) in growth cones. Tβ4-IR was not affected, showing that the siRNA effect was Tβ15-specific (data not shown). The number of branches 48 h after transfection of the Tβ15 siRNA vector into DIV2 hippocampal neurons was significantly lower than in cells receiving control scrambled siRNA (Figs. 4A and C).

## Discussion

We have presented several pieces of evidence suggesting that Tβ15 plays an important role in regulating neuronal branches during brain development: (1) Tβs were highly expressed in the CNS during neuritogenic periods, (2) they were enriched in the neurites and growth cones of early developing neurons, (3) overexpression of Tβ15 increased neurite branching, and (4) knockdown of endogenous Tβ15 using siRNA reduced branching.

We showed that Tβ15 is selectively expressed in the developing rat nervous system, and its expression declined after the major neuritogenic period. Reduced or marginal expression of Tβ15 in other peripheral tissues has also been reported by others [26]. We found that Tβ4 and Tβ10 mRNAs were present throughout the embryo, with relatively strong expression in the CNS, which is consistent with previous reports [23,27]. Although Tβ4 and Tβ10 mRNAs were abundant in the adult brain, their level of expression is also down-regulated after the major neuritogenic period [19,23,27]. The intracellular localization of Tβs also supported the idea that they are involved in neuritogenesis, because in cultured primary neurons all three Tβs were found to be enriched in developing neurite processes and growth cones where actin regulation is required.

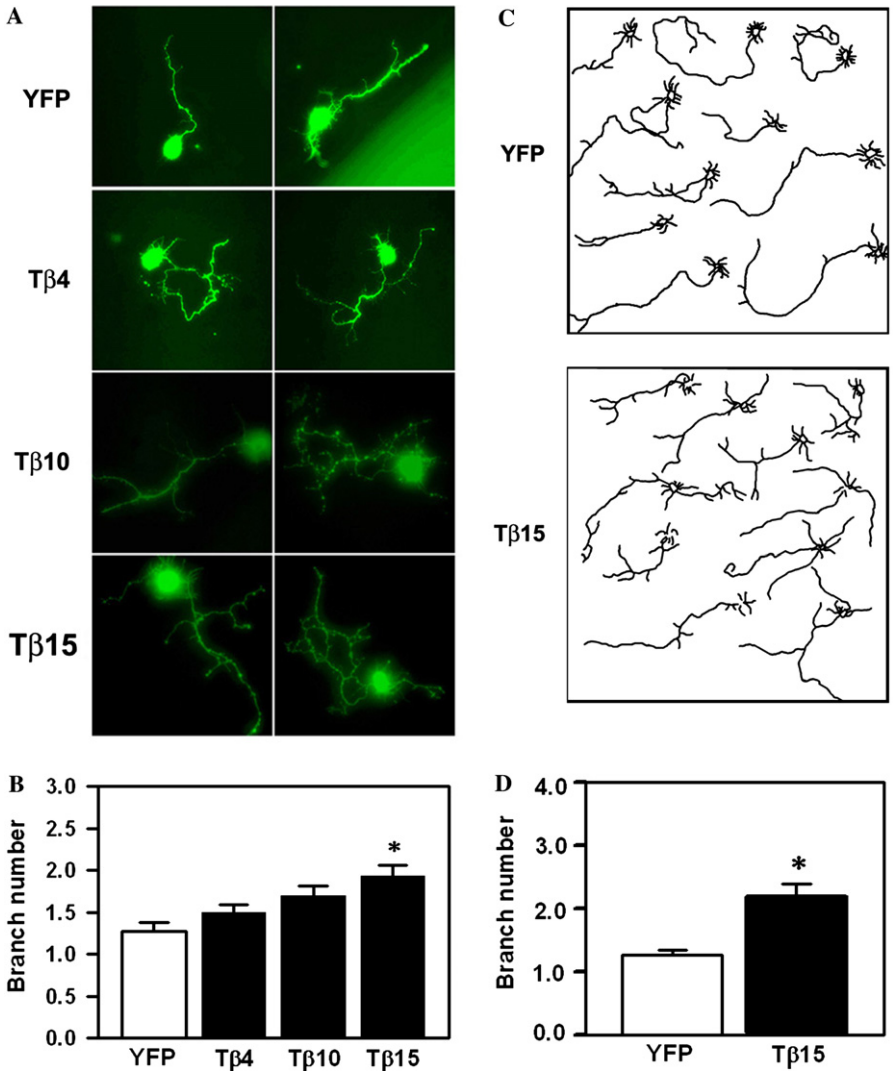


Fig. 2. Branch-promoting effect of Tβ15 in developing neurons. (A) Morphology of YFP-tagged Tβs in cortical neurons 24 h after transfection. (B) Quantitative analysis of the number of neuronal branches in the transfected cells. Data are means + SE. At least 50 neurons were measured in a minimum of three independent experiments. $*P < 0.05$ compared with control YFP-transfected cell. (C) Camera-lucida image of YFP-tagged Tβ15-transfected hippocampal neurons. (D) Quantitative analysis, as in the legend to Fig. 2(B). $*P < 0.05$.

We obtained more direct evidence for a role of Tβs in regulating neuritogenesis by overexpressing them in primary cultured neurons. Overexpression of Tβ15 resulted in the formation of more complex branching patterns. A well-known biochemical property of Tβs is that of depolymerizing F-actin by sequestering G-actin [14,17,28]. As a result, a short-term increase in the intracellular concentration of Tβs achieved by microinjection or transient transfection of many cell types results in the disassembly of actin stress fibers and the disappearance of membrane-associated actin bundles [29,30]. The ability of Tβ to depolymerize F-actin appears to be dependent on its concentration [14,31]. For instance, there is a report that the ability of Tβ4 to depolymerize F-actin decreased as its concentration was increased [32]. We were able to show that two point mutants of Tβ15 that lacked G-actin binding activity did not promote branching in primary cultured neurons, and we conclude from this that the branch-promoting effect of Tβ15 is related to its G-actin sequestering activity and its ability to influence actin dynamics. Interestingly, Tβ15 was the most effective of the three Tβs tested in promoting branch formation, followed by Tβ10, then Tβ4, and this apparent order is the same as the order of their actin binding affinities [25]. This is further evidence that their G-actin sequestering activity is related to branch formation. Most importantly, we found that reducing endogenous Tβ15 with siRNA reduced neurite branching.

In addition to observations on the role of Tβs, there are several reports that chemical and genetic effects on actin polymerization can enhance branch formation. For instance, it has been proposed that an actin binding protein, Ena, is involved in neurite elongation and branching [33,34]. Mammalian ena (Mena) is

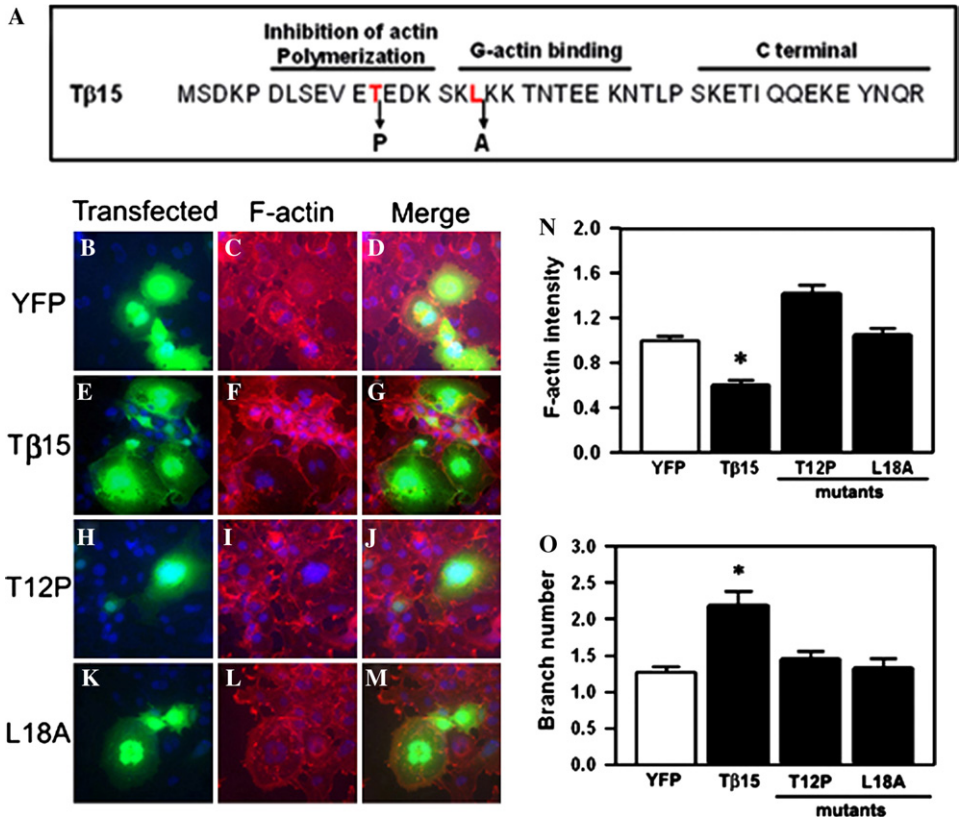


Fig. 3. Role of the G-actin-sequestering effect of Tβ15 in branch formation. (A) Sequence and domain structure of Tβ15 with sites of the point mutations used in this study. (B–M) YFP-tagged mutant forms were transfected into COS-7 cells and the F-actin fibers of the transfected cells were examined. (N) Quantitative analysis of F-actin intensity in the transfected cells. (O) Quantitative analysis of branch numbers per transfected hippocampal neuron. $*P < 0.05$ in comparison with control YFP vector. Error bars represent standard errors of the mean.

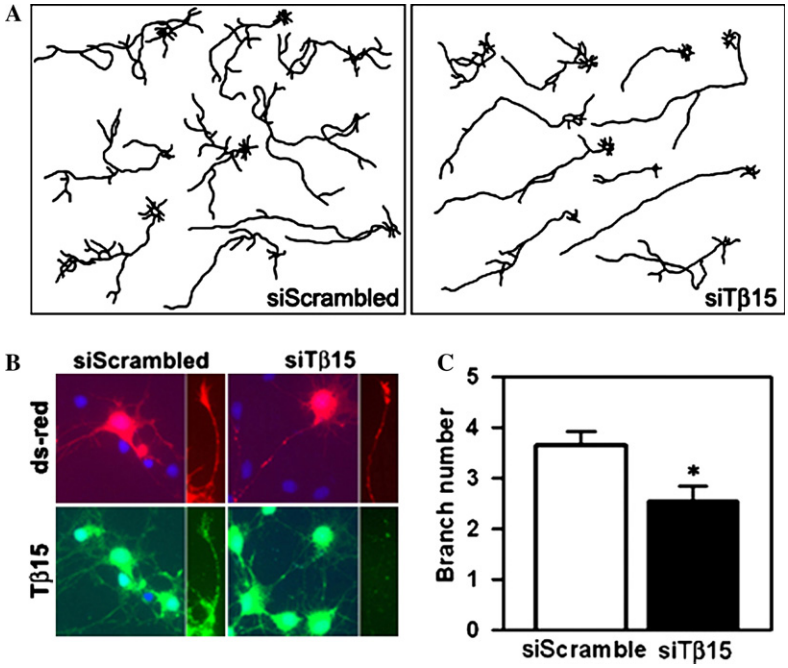


Fig. 4. Knock-down of endogenous Tβ15. (A) Camera-lucida images of control (siScrambled)- or Tβ15 antisense (siTβ15)-transfected neurons. (B) Either the siScrambled vector (control) or the siTβ15 vector was transfected together with the ds-red vector into DIV2 primary hippocampal neurons. Morphology of hippocampal neurons 48 h after transfection. (C) Quantitative analysis of branch number per transfected cell, as in the legend to Fig. 2(B). $*P < 0.05$.

concentrated at the distal tips of the filopodia of neuronal growth cones, and depletion of Mena from the growth cones resulted in increased neurite elongation and branching [35]. Furthermore, the actin polymerization protein, profilin, is reported to be a negative regulator of neurite sprouting and elongation in early neuronal differentiation, and it does so by increasing the F/G actin ratio [13]. Finally, exposure to a low concentration of the actin destabilizing agent, cytochalasin, caused cultured neurons to extend long neurites with a high degree of branching [13,36].

Although we have not addressed the question of whether Tβs are involved in the control of neuronal branching in vivo, Roth et al. [15] have reported that the introduction of Tβ antisense into developing zebrafish resulted in brain defects and impaired development of Tβ-associated axon tracts. Collectively, these results suggest that Tβ15 is a significant physiological regulator of branch formation in developing neurons.

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