

Mechanism of the Dendritic Translation and Localization of Brain-derived Neurotrophic Factor

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ABSTRACT. Brain-derived neurotrophic factor (BDNF) is a neurotrophic factor critical for synaptic plasticity, neuronal development and neurite extension. BDNF mRNA is transported to dendrites and axons, where it is expressed locally. We previously reported that dendritic targeting elements in the BDNF 3' UTR are necessary for dendritic transport and interact with cytoplasmic polyadenylation element binding protein 1. Here, we demonstrated that the short 3' UTR directs local translation of BDNF and that locally synthesized BDNF exists in a novel compartment that does not co-localize with markers of endosomes, endoplasmic reticulum, Golgi or the trans-Golgi network. Further, locally synthesized BDNF vesicles co-localized with Bicaudal-D2 (BicD2), a member of dynein motor complex proteins. Silencing BicD2 significantly reduced BDNF local synthesis in dendrites. These new findings may underlie the mechanism of local neuronal response to environmental stimuli.

Key words: Brain-derived neurotrophic factor, Bicaudal-D2, RNA trafficking, local translation, hippocampal neuron

Introduction

Brain-derived neurotrophic factor (BDNF) is a neurotrophic factor imperative for rapid response to synaptic stimulation, long-lasting plasticity, and memory formation in the central nervous system (Minichiello, 2009). BDNF mRNA is not only translated in the cell body but is also transported to dendrites (Oe and Yoneda, 2010). The 3' untranslated region (UTR) is believed to be responsible for dendritic localization. We and other group previously reported that cytoplasmic polyadenylation elements (CPEs) within the short form of BDNF 3' UTR function as a dendritic targeting element (DTE) and that cytoplasmic polyadenylation elements binding protein 1 (CPEB1) binds directly to this sequence (Oe and Yoneda, 2010; Ma *et al.*, 2010). Local BDNF mRNA expression is triggered by environmental BDNF, which contributes to the rapid activity-dependent reaction in neurons (Tongiorgi *et al.*, 1997; Righi *et al.*, 2000). However, little is known about the post-translational trafficking of BDNF locally translated in distal dendrites.

It is inferred that Golgi outposts, the Golgi apparatus-like cisternae that exist in dendrites, contribute to local sorting of secretory proteins such as BDNF to proper membrane

compartments (Horton and Ehlers, 2003; Lu *et al.*, 2007). To date, several Golgi matrix proteins and enzymes, such as GM130, β -galactosyl transferase and mannosidase IIa1, have been reported to localize to Golgi outposts (Horton and Ehlers, 2003; Ye *et al.*, 2007), although the composition of Golgi outposts has not been completely elucidated. Bicaudal-D2 (BicD2) is a Golgi matrix protein that recruits Rab6 to the Golgi apparatus, functions as an adaptor protein in the dynein motor complex, and interacts with fragile X mental retardation protein (FMRP) (Short *et al.*, 2005; Kardon and Vale, 2009; Bianco *et al.*, 2010). In this study, we characterized the subcellular compartment in which locally translated BDNF resides and examined established organelle markers to elucidate post-translational trafficking in dendrites. Using various constructs that produce BDNF under the control of 3' UTR, we found that the short form of BDNF 3' UTR was sufficient for local dendritic translation. Additionally, locally synthesized BDNF resides in a novel compartment that does not co-localize with well-known markers of organelles, such as the endoplasmic reticulum (ER), the Golgi apparatus, endosomes or the trans-Golgi network (TGN). Moreover, we found that BicD2 interacts with locally translated BDNF and that BicD2 knockdown reduces the number of neurons with locally translated BDNF in distal dendrites to 40 to 60% of control siRNA transfected cells. These results suggest that BicD2 is required for local BDNF synthesis in response to BDNF stimulation.

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Materials and Methods

Generation of constructs

The vectors expressing GFP- or mCherry-fused BDNF with various 3' UTR sequences (Fig. 1B and Fig. 2A) were constructed as follows. The cDNA of BDNF (accession number NM_012513) was cloned with restriction sites Nhe I and Age I and inserted into expression vectors pAcGFP-C1 or pmCherry-C1 (Clontech, CA, USA), which produced RNA lacking 3' UTR localization sequences, referred to as BDNF(-) (Fig. 1B). The dendritic targeting elements, such as the CaMKII α 3' UTR and 3CPEs, were cloned as described previously (Oe and Yoneda, 2010) and inserted into BDNF-expressing constructs, referred to as BDNF-CaM and BDNF-3CPEs, respectively (Fig. 1B). An hnRNP A2 responsive element (A2RE) was generated by annealing the following oligonucleotides; 5'-TGAGCCAAGGAGCCAGAGAGCATGGTAC-3' and 5'-CATGCTCTCTGGCTCCTTGGCTCAAGCT-3', followed

by digesting with restriction enzymes and insertion into Sac I and Kpn I restriction sites of the BDNF in pAcGFP-C1 or pmCherry-C1 vectors, referred to as BDNF-A2RE (Fig. 1B). The BDNF 3' UTR sequences from 1 to 326 and from 326 to 2842 were cloned by PCR using rat brain cDNA and inserted into BDNF-GFP expressing constructs, referred to as BDNF(1-326) or BDNF(326-2842), respectively (Fig. 2A). All expression constructs used a SV40 polyadenylation signal from pAcGFP-C1 and pmCherry-C1 vectors.

Cell culture

To obtain hippocampal neurons from embryonic rat brain, we carried out primary cultures in accordance with the Animal Care and Use Committee of Jichi Medical University. Pregnant Wistar rats were anesthetized with vaporized 30% isoflurane, and embryos were extirpated and decapitated. To prepare hippocampal neuron cultures, embryonic day 18–21 rat brains were removed, and hip-

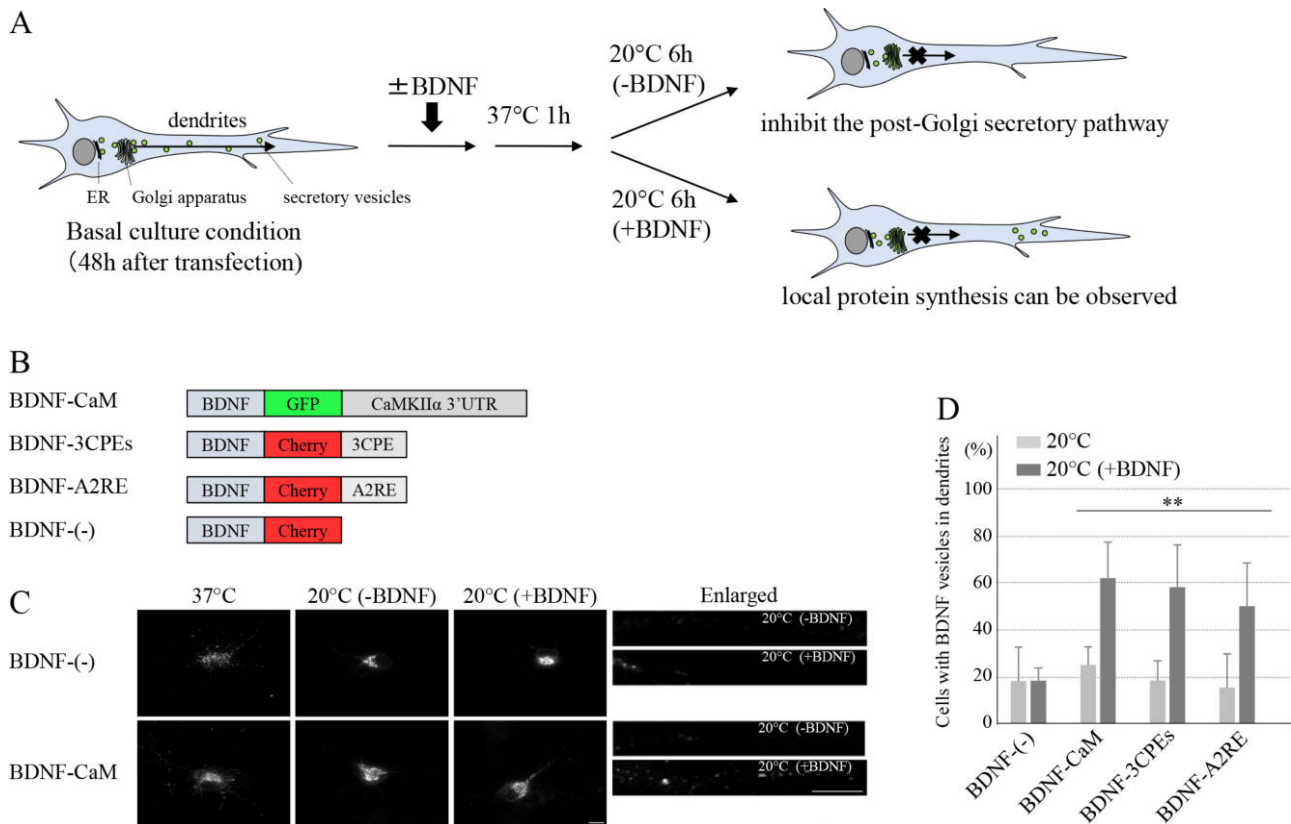


Fig. 1. Subcellular distribution of locally synthesized BDNF in distal dendrites. (A) Schematic representation of the experimental procedure. To discriminate locally synthesized proteins from proteins translated and transported from the cell body, after addition of BDNF to the media and incubation for one hour, neurons were cultured at 20°C for 6 h, which inhibits vesicle trafficking from the Golgi in the soma. Locally synthesized proteins were observed in distal dendrites. (B) Schematic diagram of expression constructs. The BDNF coding region was fused to fluorescent proteins at the C-terminal. DTEs, including CaMKII α 3' UTR, CPEs, or A2RE, were inserted into the 3' UTR. (C) Subcellular distribution of locally synthesized BDNF. Neurons were transfected and observed at 37°C, 20°C, and 20°C in the presence of recombinant BDNF, shown in the left, middle, and right column, respectively. Enlargement of dendrites from cells incubated for 6 h at 20°C after stimulated with BDNF (lower panels) or not (upper panels). (D) Quantitative analyses of cells with BDNF vesicles in dendrites transfected with different constructs with or without BDNF stimulation. (**) indicates $p < 0.01$. Scale bars, 10 μ m.

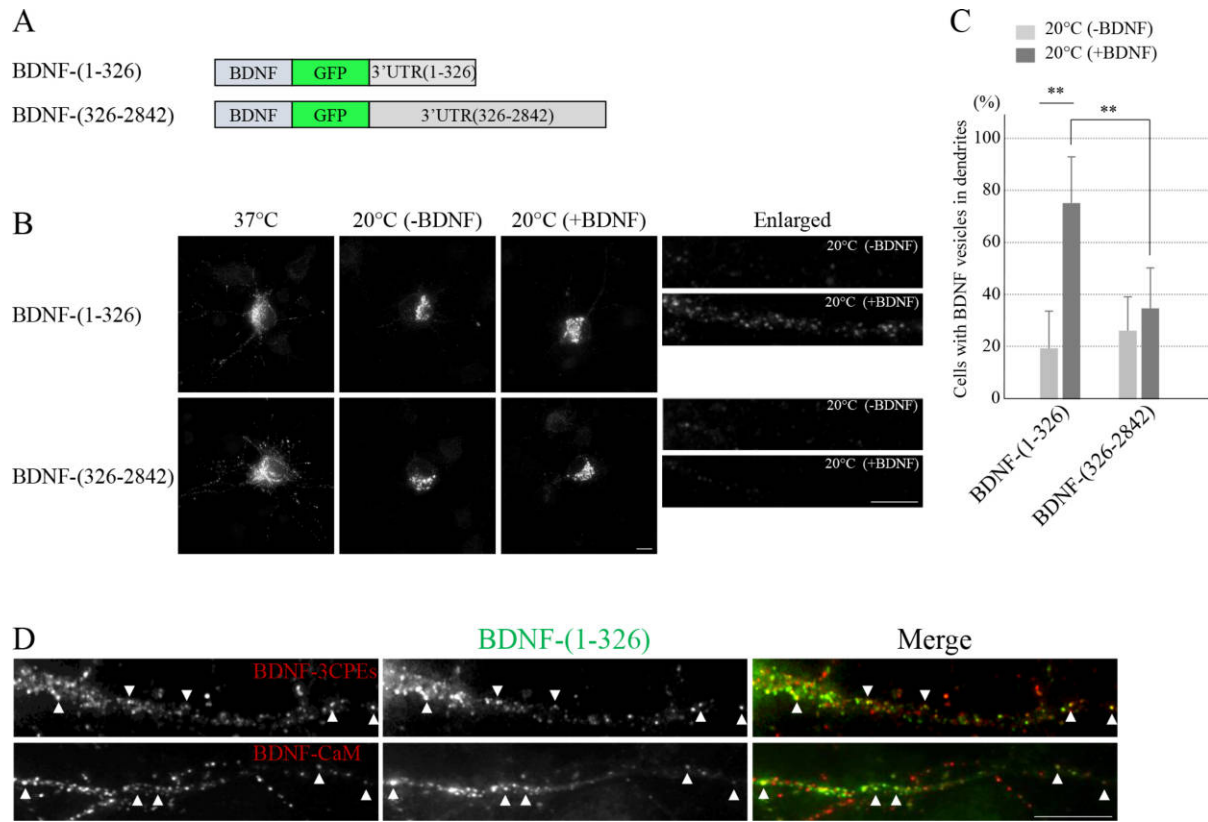


Fig. 2. Short isoform of endogenous BDNF mRNA 3' UTR is necessary for local synthesis. (A) Schematic diagram of constructs. The BDNF coding region was fused to that of a fluorescent protein to yield a C-terminal fusion protein, and two BDNF mRNA 3' UTR forms containing the sequence 1–326 and 326–2842, were inserted immediately downstream of the protein coding sequence. (B) Subcellular distributions of locally synthesized BDNF in neurons. DIV12 neurons were transfected and observed at 37°C or following incubation at 20°C without BDNF stimulation or 20°C after addition of recombinant BDNF, shown in the left, middle, and right column, respectively. Enlargement of dendrites from cells incubated for 6 h at 20°C with (lower column) or without (upper column) BDNF stimulation. (C) Quantitative analyses of cells with BDNF vesicles in the dendrites transfected with different constructs with the endogenous 3' UTR segment 1–326 or 326–2842 after incubation at 20°C in the absence or presence of BDNF. (**) indicates $p < 0.01$. (D) Subcellular distributions of BDNF derived from BDNF-(1–326) were compared with BDNF-3CPEs (upper panels) or BDNF-CaM (lower panels). Locally synthesized BDNF with the short BDNF 3' UTR co-localized with BDNF-3CPEs and BDNF-CaM. Scale bars, 10 μ m

pocampal regions were resected as described previously (Miki and Yoneda, 2004; Banker and Cowan, 1977). Briefly, hippocampal tissues were collected in ice cold Hanks balanced salt solution (HBSS) (Life Technologies, Carlsbad, CA, USA) containing 50 μ g/ml penicillin and 50 μ g/ml streptomycin and incubated in pre-warmed HBSS containing 0.25% trypsin (Life Technologies) and 0.1% DNase I (TaKaRa, Shiga, Japan) for 15 min at 37°C. Hippocampal tissues were then dissociated by pipetting, centrifuged, and suspended in Neurobasal medium (Life Technologies) containing 2% B27 supplement (Life Technologies/GIBCO) and 0.5% Gultamax™-I (Life Technologies). Cells were plated on polyethylenimine-coated 18 mm coverslips or 35 mm glass bottom dishes (IWAKI, Shizuoka, Japan) at a concentration of 10,000 cells/ml. On the seventh day *in vitro* (DIV), half of the culture medium volume was replaced with fresh media.

The rat pheochromocytoma PC12 cell line culture condition has been published previously (Greene and Tischler, 1976). Briefly, PC12 cells (ATCC) were maintained in RPMI Medium 1640 (Life

Technologies) supplemented with 10% horse serum and 5% fetal bovine serum.

Transient transfections

At DIV12, hippocampal neurons were transfected using the CalPhos™ Mammalian Transfection Kit (Clontech) following manufacturer's instructions, with some modifications as described previously (Jiang and Chen, 2006). Briefly, 4 μ g of plasmid DNA was mixed with 12.4 μ l of calcium chloride solution in a total of 100 μ l, and the mixture was added to 100 μ l of 2×HBS in one-eighths eight times with mild vortexing each time. The transfection solution was left for 20 min at room temperature and then added to neurons in 1 ml of fresh culture medium per 35 mm glass bottom dish. After 3 h of incubation at 37°C, calcium phosphate-containing medium was discarded, and cells were incubated at 37°C in 5% CO₂ for 25 min with 2 ml of Neurobasal medium, which was pre-warmed at 37°C in 10% CO₂ for 20 min. The cells

were then returned to 2 ml of culture medium. Expressed proteins were observed 48 hours after transfection.

siRNA transfection

Stealth RNAi™ siRNA (Life technologies) against BicD2 was designed based on the coding region (accession number, NM_001033674), and siRNA transfection was performed using the Lipofectamine™ RNAiMAX Transfection Reagent (Life technologies) according to manufacturer's instructions. Briefly, we mixed 150 pmol of siRNA duplex with 7.5 µl of transfection reagent in 500 µl of Opti-MEM I (Life Technologies) per glass bottom dish. The mixture was incubated at room temperature for 20 min and added to cells immediately thereafter. The cells were harvested 48 h after transfection, and knockdown efficiency was evaluated by Western blotting.

Western blotting

Cells were washed once in PBS and lysed with 150 µl of buffer containing 50 mM Tris HCl, pH 7.5, 0.1% SDS, and 1 mM DTT per 35 mm dish. Twenty microgram samples were run on a 10% SDS-PAGE gel, transferred to nitrocellulose membranes, blocked with 3% milk in PBS containing 0.1% Tween 20 (T-PBS), and incubated with primary antibodies overnight at 4°C. Membranes were washed and incubated with secondary antibody for 30 min at room temperature, washed, incubated with EMD Millipore Luminata Classico Western HRP substrate (Fisher Scientific, Waltham, Massachusetts, USA) for 5 min and exposed to Amersham Hyperfilm ECL (GE Healthcare Life Sciences, Little Chalfont, UK). Anti-BicD2 antibody (Abcam, Cambridge, Massachusetts, USA, 1:500) and anti-GAPDH antibody (Santa Cruz Biotechnology, CA, USA, 1:1000) were used as primary antibodies, and EnVision™ + Dual Link System-HRP (Dako, Glostrup, Denmark) was used as secondary antibody.

Live cell imaging

To image newly and locally synthesized proteins in dendrites of live neurons, neurons were transfected with the indicated reporter constructs. Forty-eight hours after transfection, neurons were stimulated with 50 ng/ml BDNF (Wako, Osaka, Japan) for one hour to initiate local translation. Subsequently, neurons were incubated at 20°C for 6 h to inhibit reporter proteins from being released from the Golgi apparatus in the soma and to eliminate pre-existing vesicles from dendrites (Matlin and Simons, 1983; Saraste and Kuismanen, 1984). The apical dendrites of live cells were observed using a DeltaVision Elite system (GE Healthcare Life Sciences). Dynamic imaging was performed by Leica AF7000.

RT-PCR analysis of PC12 cells

Total RNA was extracted from the cultured cells 96 hours after transfection of each siRNA using Sepasol-RNA I Super G (Nakarai Tesque, Kyoto, Japan). The concentration of purified

RNA was measured by spectrophotometer, followed by reverse transcription using SuperScript III First-Strand Synthesis System for RT-PCR (Life Technologies). Conventional PCR amplification was performed using the following primers: forward: 5'-CAT-CCGCAGCGAGATGGAGCA-3', reverse: 5'-CATTGACCAGG-GCTTCGGC-3' for detection of BicD2 (700 bp), and forward: 5'-CTACCCACGGCAAGTTCAAC-3', reverse: 5'-GAAGTCACAGGAGACAACCTG-3' for detection of GAPDH (727 bp).

Immunostaining of fixed cells

Neurons were fixed for 15 min in PBS containing 4% (w/v) paraformaldehyde, 4% (w/v) sucrose at RT, and permeabilized with 0.1% Triton X-100 for 5 min. Subsequently, neurons were incubated in 3% (w/v) BSA in PBS, and treated sequentially with primary and secondary antibodies. Microscopic observation was performed using LSM-510 meta confocal laser scanning microscope (Carl Zeiss). The antibodies used in this study are listed in Supplemental Table 1.

Statistical analysis

Quantitative analyses were performed to evaluate the number of cells locally synthesizing BDNF in dendrites. Data were collected from six independent experiments and are expressed as the mean ± SEM. In the control experiments, the number of cells analyzed were as follows: BDNF(-), $n=107$; BDNF-CaM, $n=104$; BDNF-3CPEs, $n=113$; BDNF-A2RE, $n=111$; BDNF-(1-326), $n=95$; BDNF-(326-2842), $n=109$. In the experiments with BDNF treatment, BDNF(-), $n=125$; BDNF-CaM, $n=126$; BDNF-3CPEs, $n=115$; BDNF-A2RE, $n=111$; BDNF-(1-326), $n=125$; and BDNF-(326-2842), $n=130$. Data were analyzed using two-way ANOVA and Tukey's *post hoc* multiple comparisons test using GraphPad 6 software. In the siRNA experiments, the number of cells analyzed were as follows: control siRNA, $n=103$; and BicD2 siRNA, $n=107$. Data were analyzed using Wilcoxon matched-pairs signed rank test using GraphPad 6 software. (*) and (**) indicate $p<0.05$ and $p<0.01$, respectively.

Results

Dendritic targeting elements enable BDNF local synthesis in dendrites

To observe the biosynthesis of BDNF in dendrites, neurons were incubated for one hour with BDNF to initiate local translation. Subsequently, neurons were incubated at 20°C for 6 h, which is expected to inhibit vesicles budding from the Golgi apparatus in the cell body and eliminates constitutively transported vesicles from dendrites while incubation at 37°C for 6 h did not inhibit transportation (Fig. 1A and Supplemental Fig. 1) (Matlin and Simons, 1983; Saraste and Kuismanen, 1984). Before the addition of BDNF at 37°C, the BDNF(-) construct, which lacks DTEs

(Fig. 1B), expressed BDNF-mCherry in puncta throughout the cytoplasm (Fig. 1C, left upper column)—which are similar to the endogenous expression of BDNF at 37°C without BDNF stimulation (Supplemental Fig. 2). However, after incubation for six hours without BDNF at 20°C, the expressed BDNF packed in the perinuclear Golgi apparatus where the release was blocked, and was not detected in dendrites (Fig. 1C, upper middle column). These data suggest that the 20°C treatment successfully blocked vesicle release of BDNF-mCherry protein from the Golgi. The number of BDNF(–)-transfected neurons exhibiting BDNF vesicles in dendrites decreased at 20°C (Fig. 1D). BDNF stimulation in advance did not significantly alter the ratio of cells with BDNF vesicles in dendrites: $17.96 \pm 14.39\%$ to $18.05 \pm 5.48\%$ (Fig. 1C, upper right column and 1D). These results suggest that without DTEs, no local translation occurred, as expected. Next, we used the GFP- or mCherry-tagged BDNF coding region fused to DTEs, such as CaMKII α 3' UTR, 3CPEs or hnRNP A2 response element (A2RE), referred to as BDNF-CaM, BDNF-3CPEs and BDNF-A2RE, respectively (Fig. 1B). In contrast to BDNF(–), dendritic BDNF vesicles appeared at 20°C in response to BDNF stimulation in neurons transfected with DTE-containing constructs (Fig. 1C, 1D and Supplemental Fig. 3). The percentage of cells containing dendritic BDNF increased more than two- to three-fold (Fig. 1D, $p < 0.01$). Effect of temperature block at 20°C was reversible since temperature shift from 20°C to 37°C released stacked BDNF at least partially in dendrites as well as in soma (Supplemental Figs. 4 and 5). These results collectively suggest that DTEs are necessary for local BDNF protein expression following BDNF stimulation.

Short BDNF 3' UTR is necessary for BDNF-stimulated local synthesis of BDNF in dendrites

To elucidate the involvement of intrinsic DTEs in the local synthesis of BDNF, we expressed the BDNF constructs BDNF-(1–326) and BDNF-(326–2842), which contain BDNF 3' UTR sequences from 1 to 326 and 326 to 2842, respectively (Fig. 2A). BDNF-induced dendritic synthesis of BDNF-GFP occurs preferentially through the translation of short BDNF-(1–326) transcripts but not of BDNF-(326–2842) (Fig. 2B). BDNF stimulation significantly increased the number of cells that contained dendritic BDNF when transfected with BDNF-(1–326) compared with those with BDNF-(326–2842) ($p < 0.01$, Fig. 2C). Further, BDNF-GFP expressed by BDNF-(1–326) co-localized with BDNF-mCherry from BDNF-3CPEs and BDNF-CaM (Fig. 2D). Given that these three DTEs contained common CPE-like sequences (Oe and Yoneda, 2010) and BDNF-(326–2842) did not, CPE-like sequences appear to be essential for endogenous local BDNF synthesis.

Bicaudal-D2 co-localizes with locally synthesized BDNF

To examine whether Golgi outposts are involved in the post-translational modification of BDNF, we observed the dendritic distribution of Golgi marker proteins. Interestingly, BicD2, a Golgi matrix protein, co-localized with BDNF expressed by BDNF-3CPEs, BDNF-CaM, and BDNF-(1–326) (Fig. 3A). Double transfection with Golgi markers, either β -galactosyl transferase (GalT) or Mannosidase IIa1 (ManIIa1), demonstrated that BDNF did not co-localize with these signals, although expression of the two

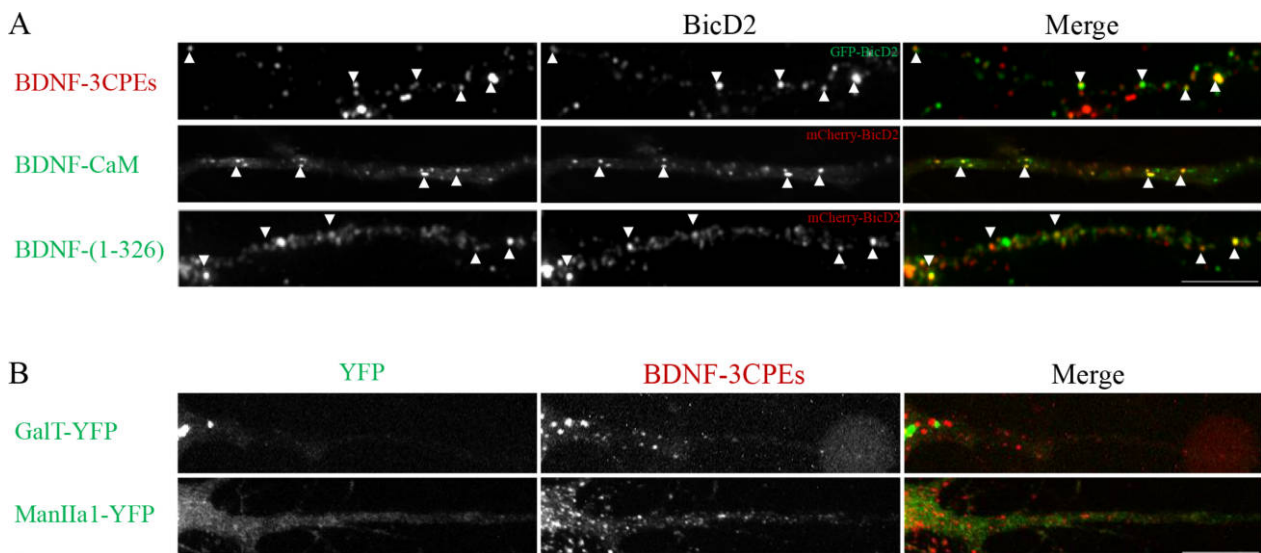


Fig. 3. Bicaudal-D2 co-localizes with locally synthesized proteins in dendrites. Neurons expressing BDNF were observed 48 h after transfection following BDNF stimulation and incubation at 20°C. Locally synthesized BDNF with three different DTEs co-localized with BicD2 in dendrites (A) but not with Golgi proteins, GalT or ManIIa1 (B). Scale bars, 10 μ m.

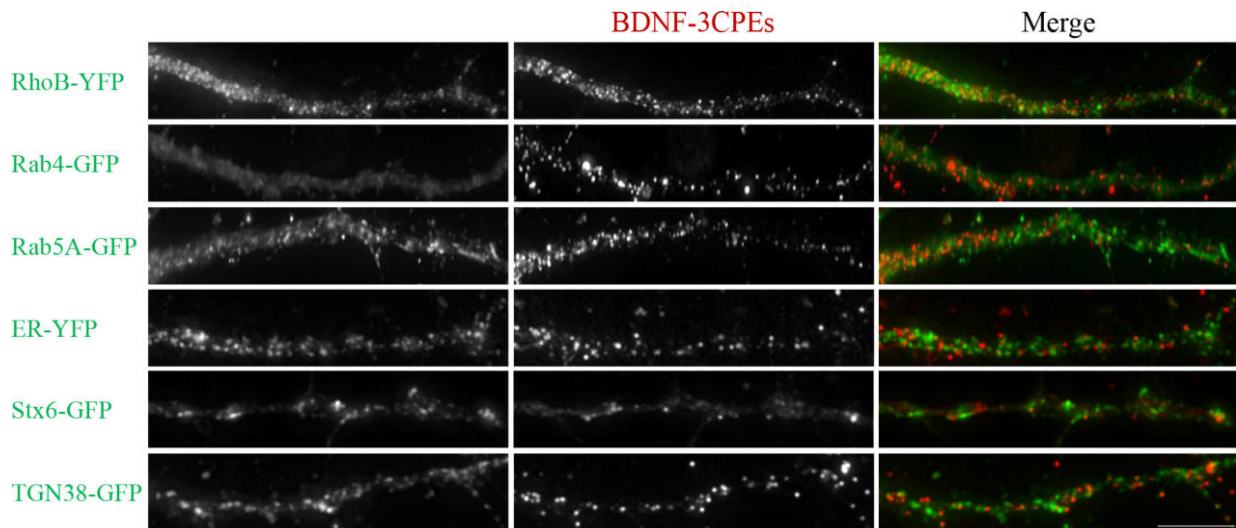


Fig. 4. Locally synthesized BDNF resides in a novel subcellular compartment. Neurons expressing BDNF and organelle marker proteins were observed following BDNF stimulation and incubation at 20°C. The intracellular organelle marker proteins were as follows; RhoB, Rab4A and Rab5A for endosomes, ER-YFP for endoplasmic reticulum, and syntaxin6 and TGN38 for the trans-Golgi network. Locally synthesized BDNF displayed localization distinct from the above intracellular organelle markers. Scale bar, 10 μ m.

Golgi enzymes displayed a vesicular distribution throughout dendrites (Fig. 3B). Immunocytochemistry revealed that GM130 was observed in the cell soma and proximal dendrites but rarely in distal dendrites, where most BDNF vesicles resided (data not shown).

Because BicD2 has been reported to interact with various cargoes, including Rab6, FMRP and the Golgi apparatus (Short *et al.*, 2005; Kardon and Vale, 2009; Bianco *et al.*, 2010), we investigated whether BDNF co-localizes with Rab6A in dendrites. However, we could not detect wild type Rab6A, the constitutively active form mimicking-mutant (Rab6A Q72L), or the constitutively inactive form mimicking-mutant (Rab6A T27N) in dendrites, although wild type and the constitutively active form localized to the Golgi in the cell body (data not shown). Thus, we could not evaluate whether Rab6A co-localizes with locally translated BDNF or Golgi outposts in dendrites.

Therefore, despite the co-localization of BicD2 with locally expressed BDNF vesicles, both proteins co-exist in a compartment that does not include previously known Golgi markers in dendrites.

Locally synthesized BDNF resides in a novel compartment

To identify the organelle in which locally synthesized BDNF resides, we expressed tagged forms of several organelle marker proteins, including RhoB, Rab4A and Rab5A for endosomes, ER-YFP for the endoplasmic reticulum, and syntaxin6 and TGN38 for the trans-Golgi network. These proteins did not co-localize with BDNF expressed by BDNF-3CPEs (Fig. 4), suggesting that locally

synthesized BDNF may reside in a novel subcellular compartment in distal dendrites.

Silencing of Bicaudal-D2 reduces local BDNF

To investigate the role of BicD2 in local BDNF, we knocked down BicD2 expression using siRNA. BicD2 siRNA effectively down-regulated mRNA and protein levels of BicD2 in PC12 cells compared with control siRNA (Fig. 5A and 5B). Treatment of PC12 cells with BicD2 siRNA#1 or #4 did not substantially change the GAPDH level. So, the BicD2 knockdown was estimated not to affect general protein synthesis. In dendrites of cultured neurons, BicD2 knockdown robustly decreased the number of vesicles expressed with BDNF-(1–326) (Fig. 5C). The percentage of cells that had BDNF vesicles decreased from $63.27 \pm 4.75\%$ to $38.56 \pm 6.23\%$ ($p < 0.05$, siRNA#1) or to $26.31 \pm 10.57\%$ ($p < 0.05$, siRNA#4, Fig. 5D). The results shown in Fig. 5 therefore suggest a particular role for BicD2 in local BDNF synthesis.

Discussion

The local translation and subsequent processing of secretory proteins in distal dendrites should consist of several events, including mRNA targeting, local translation and vesicle trafficking. In this study, we made three important observations concerning the local synthesis of BDNF in dendrites. First, the BDNF short 3' UTR, which contains CPEs, is essential for local BDNF synthesis. Second, Bicaudal-D2 (BicD2) may be involved in BDNF localiza-

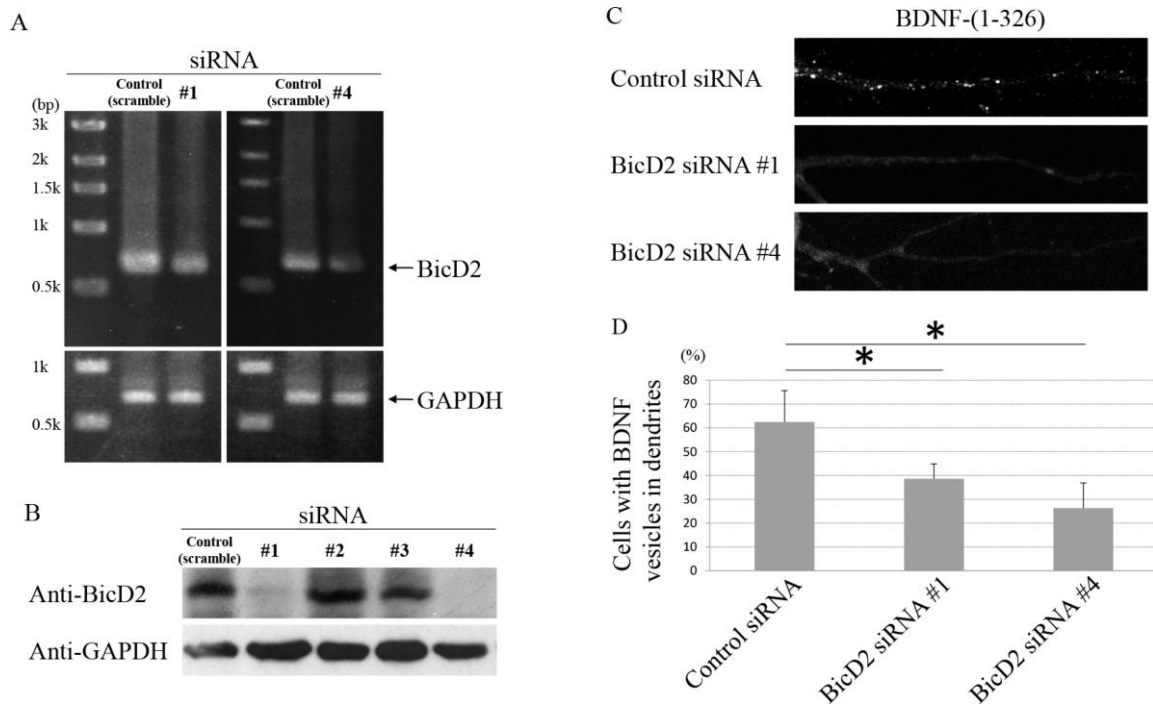


Fig. 5. Bicaudal-D2 is required for the expression of locally synthesized BDNF. (A, B) Results of semi-quantitative RT-PCR (A) and immunoblots (B) of PC12 cells transfected with each siRNA. siRNA #1 and #4 effectively inhibited mRNA (A) and protein (B) expression of BicD2. GAPDH was shown as an internal control. (C) Dendritic distributions of locally synthesized BDNF in hippocampal neurons transfected with BDNF-(1–326) and control siRNA, siRNA#1 or #4 directed against BicD2. (D) Quantitative analyses of cells with local synthesis of BDNF in dendrites. A significant difference in the number of cells with dendritic BDNF vesicles was observed between neurons transfected with BicD2 siRNAs and those with control siRNA. (*) indicates $p < 0.05$.

tion in dendrites. Third, local BDNF may localize to a novel subcellular compartment that contains BicD2.

First, the short BDNF 3' UTR is essential for local synthesis of BDNF. Because previous studies have demonstrated the importance of BDNF 3' UTR for local translation using EGFP derivatives alone, such as myristoylated EGFP and destabilized EGFP, the expression of these fluorescent proteins diffusely localized throughout the neurons (An *et al.*, 2008; Chiaruttini *et al.*, 2009). In this study, local expression of fluorescent protein-fused BDNF in dendrites was analyzed, and BDNF short 3' UTR was found to be an intrinsic DTE that contributes to mRNA trafficking and local post-translational processing of BDNF. After incubation at 20°C, BDNF-(1–326) expressed BDNF in dendrites in a vesicular pattern and partially co-localized with BDNF associated with other DTEs, whereas BDNF lacking any DTE was not observed in dendrites. Previous studies have established that vesicles are retained in the Golgi at 20°C (Matlin and Simons, 1983; Saraste and Kuismanen, 1984). Our results suggest that the endogenous BDNF short 3' UTR is necessary and sufficient for local synthesis of BDNF in distal dendrites.

Second, our results showed the first evidence that BicD2 may play a pivotal role in local BDNF. BDNF-(1–326)

expression was observed in 25 to 37% fewer neurons by silencing BicD2. Because BDNF protein co-localized with BicD2, BicD2 may be involved in the local synthesis or vesicle trafficking of BDNF protein in dendrites. However, as BicD/Egl complex has crucial roles in RNA transport involved in *Drosophila* oogenesis (Bullock and Ish-Horowicz, 2001), BicD2 may also be engaged in transporting BDNF mRNP complex.

The mRNAs of vasopressin and tissue plasminogen activator (tPA) or receptor proteins, such as tropomyosin related kinase B receptor (TrkB) and inositol 1,4,5-trisphosphate receptor (IP3R), display somatodendritic distribution in neurons (Prakash *et al.*, 1997; Shin *et al.*, 2004; Tongiorgi *et al.*, 1997; Fotuhi *et al.*, 1993). Although the dendritic targeting of the mRNA of these secretory proteins has been reported, little is known about local translation, subsequent vesicle trafficking and secretion. Analysis of colocalization of BDNF mRNP and BicD2 in dendrites would clarify precise role of BicD2 in local BDNF expression. Additional study is needed to further identify specific BicD2 functions in local BDNF.

Third, locally translated BDNF resides in a novel subcellular compartment. Previous studies on local translation in neurons did not observe the distribution of BDNF itself, as

discussed above (An *et al.*, 2008; Aakalu *et al.*, 2001; Lau *et al.*, 2010). To visualize locally synthesized BDNF more precisely, we combined three methods: 1) using the BDNF protein fused to a fluorescent protein and containing a DTE in the 3' UTR; 2) lowering the culture temperature to 20°C, which retains *de novo* synthesized BDNF in the Golgi apparatus (Matlin and Simons, 1983; Saraste and Kuismanen, 1984); 3) stimulating local translation by adding BDNF to the medium. The low-temperature incubation blocked vesicle budding from the Golgi in the cell body, as significantly fewer neurons had vesicles in dendrites. While proteins produced in the cell body are trapped in the Golgi, BDNF stimulation increased locally translated BDNF in dendrites. The construct lacking any DTE did not yield dendritic BDNF even after stimulated.

Given that constitutively translated BDNF undergoes post-translational modifications, such as glycosylation, cleavage and insertion into vesicles in the Golgi, we first speculated that Golgi outposts, the Golgi-like cisternae in dendrites, were involved in the post-translational modification and vesicle trafficking of locally synthesized BDNF in dendrites. Dendritic BDNF was associated with BicD2, a Golgi matrix protein that recruits Rab6 to the Golgi apparatus and functions as an adaptor protein in the dynein motor complex (Short *et al.*, 2005; Kardon and Vale, 2009; Bianco *et al.*, 2010). However, other Golgi markers did not co-localize with BDNF, suggesting the existence of heterogeneous Golgi-like compartments in dendrites. The dendritic distribution of BDNF differed from that of other well-known organelles, such as endosomes, endoplasmic reticulum, and the trans-Golgi network. There are two salient interpretations of these results. One explanation is that locally translated BDNF localizes to a *bona fide* novel subcellular compartment, and the other explanation is that a novel Golgi-like structure undetectable by the marker proteins used here exists and is involved in local BDNF synthesis in dendrites. Furthermore, some stacked granular BDNF signals in dendrites moved again and disappeared with increasing the temperature (Supplemental Figs. 4 and 5), while others retained. So, these other BDNF signals might be in non-physiological compartments or in waiting state for secretion. To characterize these new compartments needs further careful evaluation.

Taken together, this study reveals that locally translated BDNF localizes to a novel subcellular compartment and that the BDNF short 3' UTR is involved in local BDNF synthesis. Further investigation is now ongoing and expected to shed light on the mechanism of the local response in dendrites.

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References

- Aakalu, G., Smith, W.B., Nguyen, N., Jiang, C., and Schuman, E.M. 2001. Dynamic visualization of local protein synthesis in hippocampal neurons. *Neuron*, **30**: 489–502.
- An, J.J., Gharami, K., Liao, G.Y., Woo, N.H., Lau, A.G., Vanevski, F., Torre, E.R., Jones, K.R., Feng, Y., Lu, B., and Xu, B. 2008. Distinct role of long 3' UTR BDNF mRNA in spine morphology and synaptic plasticity in hippocampal neurons. *Cell*, **134**: 175–187.
- Banker, G.A. and Cowan, W.M. 1977. Rat hippocampal neurons in dispersed cell culture. *Brain Res.*, **126**: 397–442.
- Bianco, A., Dienstbier, M., Salter, H.K., Gatto, G., and Bullock, S.L. 2010. Bicaudal-D regulates fragile X mental retardation protein levels, motility, and function during neuronal morphogenesis. *Curr. Biol.*, **20**: 1487–1492.
- Bullock, S.L. and Ish-Horowicz, D. 2001. Conserved signals and machinery for RNA transport in *Drosophila* oogenesis and embryogenesis. *Nature*, **414**: 611–616.
- Chiaruttini, C., Vicario, A., Li, Z., Baj, G., Braiucă, P., Wu, Y., Lee, F.S., Gardossi, L., Baraban, J.M., and Tongiorgi, E. 2009. Dendritic trafficking of BDNF mRNA is mediated by translin and blocked by the G196A (Val66Met) mutation. *Proc. Natl. Acad. Sci. USA*, **106**: 16481–16486.
- Fotuhi, M., Sharp, A.H., Glatt, C.E., Hwang, P.M., von Krosigk, M., Snyder, S.H., and Dawson, T.M. 1993. Differential localization of phosphoinositide-linked metabotropic glutamate receptor (mGluR1) and the inositol 1,4,5-trisphosphate receptor in rat brain. *J. Neurosci.*, **13**: 2001–2012.
- Greene, L.A. and Tischler, A.S. 1976. Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. *Proc. Natl. Acad. Sci. USA*, **73**: 2424–2428.
- Horton, A.C. and Ehlers, M.D. 2003. Dual modes of endoplasmic reticulum-to-Golgi transport in dendrites revealed by live-cell imaging. *J. Neurosci.*, **23**: 6188–6199.
- Jiang, M. and Chen, G. 2006. High Ca²⁺-phosphate transfection efficiency in low-density neuronal cultures. *Nat. Protoc.*, **1**: 695–700.
- Kardon, J.R. and Vale, R.D. 2009. Regulators of the cytoplasmic dynein motor. *Nat. Rev. Mol. Cell Biol.*, **10**: 854–865.
- Lau, A.G., Irier, H.A., Gu, J., Tian, D., Ku, L., Liu, G., Xia, M., Fritsch, B., Zheng, J.Q., Dingleline, R., Xu, B., Lu, B., and Feng, Y. 2010. Distinct 3' UTRs differentially regulate activity-dependent translation of brain-derived neurotrophic factor (BDNF). *Proc. Natl. Acad. Sci. USA*, **107**: 15945–15950.
- Lu, Y., Christian, K., and Lu, B. 2007. BDNF: a key regulator for protein synthesis-dependent LTP and long-term memory? *Neurobiol. Learn. Mem.*, **89**: 312–323.
- Ma, B., Culver, B.P., Baj, G., Tongiorgi, E., Chao, M.V., and Tanese, N. 2010. Localization of BDNF mRNA with the Huntington's disease protein in rat brain. *Mol. Neurodegener.*, **5**: 22.
- Matlin, K.S. and Simons, K. 1983. Reduced temperature prevents transfer of a membrane glycoprotein to the cell surface but does not prevent terminal glycosylation. *Cell*, **34**: 233–243.
- Miki, T. and Yoneda, Y. 2004. Alternative splicing of Stauf2 creates the nuclear export signal for CRM1 (Exportin 1). *J. Biol. Chem.*, **279**: 47473–47479.
- Minichiello, L. 2009. TrkB signalling pathways in LTP and learning. *Nat. Rev. Neurosci.*, **10**: 850–860.
- Oe, S. and Yoneda, Y. 2010. Cytoplasmic polyadenylation element-like sequences are involved in dendritic targeting of BDNF mRNA in hippocampal neurons. *FEBS Lett.*, **584**: 3424–3430.
- Prakash, N., Fehr, S., Mohr, E., and Richter, D. 1997. Dendritic localization of rat vasopressin mRNA: ultrastructural analysis and mapping of targeting elements. *Eur. J. Neurosci.*, **9**: 523–532.
- Righi, M., Tongiorgi, E., and Cattaneo, A. 2000. Brain-derived neurotrophic factor (BDNF) induces dendritic targeting of BDNF and tyrosine

- kinase B mRNAs in hippocampal neurons through a phosphatidylinositol-3 kinase-dependent pathway. *J. Neurosci.*, **20**: 3165–3174.
- Saraste, J. and Kuismanen, E. 1984. Pre- and post-Golgi vacuoles operate in the transport of Semliki Forest virus membrane glycoproteins to the cell surface. *Cell*, **38**: 535–549.
- Shin, C.Y., Kundel, M., and Wells, D.G. 2004. Rapid, activity-induced increase in tissue plasminogen activator is mediated by metabotropic glutamate receptor-dependent mRNA translation. *J. Neurosci.*, **24**: 9425–9433.
- Short, B., Haas, A., and Barr, F.A. 2005. Golgins and GTPases, giving identity and structure to the Golgi apparatus. *Biochim. Biophys. Acta*, **1744**: 383–395.
- Tongiorgi, E., Righi, M., and Cattaneo, A. 1997. Activity-dependent dendritic targeting of BDNF and TrkB mRNAs in hippocampal neurons. *J. Neurosci.*, **17**: 9492–9505.
- Ye, B., Zhang, Y., Song, W., Younger, S.H., Jan, L.Y., and Jan, Y.N. 2007. Growing dendrites and axons differ in their reliance on the secretory pathway. *Cell*, **130**: 717–729.

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