Brief Report

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The BDNF-ERK/MAPK axis reduces *phosphatase and actin regulator1*, *2* and *3* (*PHACTR1*, *2* and *3*) mRNA expressions in cortical neurons

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SUMMARY Actin rearrangement and phosphorylation-dephosphorylation in the nervous system contribute to plastic alteration of neuronal structure and function. Phosphatase and actin regulator (PHACTR) family members are actin- and protein phosphatase 1 (PP1)-binding proteins. Because some family members act as regulators of neuronal morphology, studying the regulatory mechanisms of *PHACTR* is valuable for understanding the basis of neuronal circuit formation. Although expression patterns of PHACTR family molecules (PHACTR1–4) vary across distinct brain areas, little is known about the extracellular ligands that influence their mRNA levels. In this study, we focused on an important neurotrophin, brain-derived neurotrophic factor (BDNF), and examined its effect on mRNA expression of *PHACTR* family member in cortical neurons. *PHACTR1-3*, but not *PHACTR4*, were affected by stimulation of primary cultured cortical neurons with BDNF; namely, sustained downregulation of their mRNA levels was observed. The observed downregulation was blocked by an inhibitor of the extracellular signal-regulated protein kinase/mitogen-activated protein kinase (ERK/MAPK) pathway, U0126, suggesting that ERK/MAPK plays an inhibitory role for gene induction of *PHACTR1-3*. These findings aid the elucidation of how BDNF regulates actin- and PP1-related neuronal functions.

Keywords BDNF, PHACTR, megakaryoblastic leukemia, myocardin-related transcription factor, extracellular signal-regulated protein kinase, serum response factor

1. Introduction

Actin dynamics and phosphorylation events in the nervous system play essential roles in brain circuit formation and functions (*1-3*). A series of proteins containing the amino acid sequence RPXXXEL (RPEL motif) have been identified as important regulators of cell motility, cell shape, and gene expression (*4-7*). One of these RPEL-containing proteins, megakaryoblastic leukemia/myocardin-related transcription factor, is a serum response factor transcriptional coactivator that regulates immediate early and cytoskeletal genes, as well as neuronal morphology (*8,9*). The phosphatase and actin regulator (PHACTR) family is an RPEL-containing protein family that binds to actin and protein phosphatase 1 (PP1) (*10,11*). There are four PHACTRs (PHACTR1–4) that are spatiotemporally expressed in the brain (*10,12*). Overexpression of PHACTR3 in neurons results in decreased axonal elongation *via* RPEL motifs (*13*) and increased dendritic complexity *via* the PP1-binding domain (*14*), suggesting that PHACTR proteins may contribute to the construction of brain structure. Although such regulation of neuronal morphology is thought to require modulation of PHACTRs mediated by extracellular stimuli at the protein level, only a few studies have identified ligands that control PHACTR functions [*e.g.*, serum-induced nuclear translocation of PHACTR1 (*15*) and Slack channel stimulation-induced dissociation of PHACTR1 from the channel (*16*)]. Moreover, with the exception of vascular endothelial growth factor (*17*), it is largely unknown what ligands control *PHACTRs* at the mRNA level. Here, we investigate whether brain-derived neurotrophic factor (BDNF), a representative neurotrophin involved in neuronal plasticity and development (*18*), influences *PHACTR* mRNA expression in cortical neurons. In this study, we demonstrate differential expression profiles of *PHACTR1–4* after BDNF stimulation and identify the signaling route required for BDNF-mediated alteration of mRNA levels.

2. Materials and Methods

2.1. Animals

For primary cultures of rat cortical neurons, we used rat embryos from pregnant female Sprague-Dawley rats (Japan SLC, Hamamatsu, Shizuoka, Japan) in compliance with guidelines of the Animal Care and Experimentation Committee of University of Toyama, Sugitani Campus (Approval Nos. A2022PHA-6, A2019PHA-7, A2016PHA-8, A2013PHA-4, A2012PHA-1, and A2011PHA-5) and the ARRIVE guidelines.

2.2. Reagents

Human recombinant BDNF protein was a gift from Sumitomo Pharma Co., Ltd. (Osaka, Japan). We used U0126 from Calbiochem (La Jolla, CA, USA, 662005) as a MEK inhibitor. According to previously established concentrations of each inhibitor, U0126 (*19*), LY294002 [L9908, Sigma-Aldrich (St. Louis, MO, USA)] (*20*), and U73122 (U6756, Sigma-Aldrich) (*21*) were added to the medium before BDNF stimulation.

2.3. Cell culture

Rat cortical neurons were prepared from rats aged embryonic day 17, seeded at a density of 8×10^5 cells/well in poly-D-lysine (P6407, Sigma)-coated 12-well plates, and maintained in Neurobasal medium (21103-049, Invitrogen, Carlsbad, CA, USA) including 1× B27 supplement (17504-044, Invitrogen), 2 μg/mL gentamicin (15750-060, Invitrogen), and 0.5 mM glutamine (25030-081, Invitrogen), as previously described (*22*). Medium exchange was carried out by replacing half of the conditioned medium with fresh medium every 3 days.

2.4. RNA preparation and quantitative (q)PCR

RNA preparation followed by reverse-transcription (RT)-qPCR was executed by previously reported methods (*22*). TRIsure (BIO-38032; Bioline, London, UK) was used for RNA extraction and, subsequently, the isolated total RNA was reverse-transcribed for complementary DNA (cDNA) synthesis using SuperScript II (18064-014; Invitrogen). To detect *PHACTR1–4* and *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*), SYBR Select Master Mix (4472908; Thermo Fisher Scientific, Waltham, MA, USA) was used for qPCR according to the manufacturer's experimental conditions. The PCR program was as follows: initial preheating to 50°C for 2 min; subsequent denaturing at 95°C for 2 min; and 40 cycles of denaturing at 95°C for 15 s, annealing at 57°C for 15 s, and extending at 72°C for 1 min. qPCR primer sequences were as follows:

GAPDH-sense: 5′-ATCGTGGAAGGGCTCATGAC-3′,
GAPDH-antisense: 5′-TAGCCCAGGATGCCCTTTAGT-3′,
PHACTR1-sense: 5′-GAGCTCTCCCTGGCATCCTACAC-3′,
PHACTR1-antisense: 5′-CTGCATGGTCATAGCAAGTGTC-3′,
PHACTR2-sense: 5′-TGTCCCCCAACACAGTCACTTC-3′,
PHACTR2-antisense: 5′-GTCCATCACTGACTAGGACCATG-3′,
PHACTR3-sense: 5′-CACCCAAGGCCAGCTCTA-3′,
PHACTR3-antisense: 5′-TTGAAAACTGTCCTGACGGTGC-3′,
PHACTR4-sense: 5′-GCTGAACTGTCCCAAGCAATG-3′,
PHACTR4-antisense: 5′-TTGTCAGCGGTGGTTCCAAAC-3′.

PCR reactions were performed using standard vectors carrying DNA fragments of interest and cDNA samples simultaneously to acquire the standard curve and levels of mRNA expression. Standard vectors containing *PHACTR1*, *PHACTR2*, and *PHACTR4* were made by PCR and subsequent ligation of partial cDNA into the vector. The HA-rat *PHACTR3* vector, which was generated and reported previously (*14*), was also used as a standard vector. The internal control in this experiment was *GAPDH* mRNA expression.

2.5. Statistical analysis

All data are expressed as the mean ± S.E. ($n = 4$, n indicates the number of animals). Statistics were executed using Microsoft Excel 2013 (version 15.0.5127.1000). Data were analyzed by paired *t*-tests with Bonferroni's correction were applied; $p < 0.05/x$ (where x was the number of tests) was regarded as significant (see figure legends).

3. Results and Discussion

Initially, we investigated whether BDNF influenced *PHACTR1–4* mRNA levels in cortical neurons. We found that mRNA expression of *PHACTR1–3* was downregulated by BDNF stimulation (Figure 1). Specifically, downregulation was observed at 1 h for *PHACTR1* and *PHACTR3*, at 3 h for *PHACTR1–3*, at 6 h for *PHACTR3*, and at 12 h for *PHACTR2* and *PHACTR3*. *PHACTR2* displayed the lowest mRNA level among all *PHACTRs*. *PHACTR4* mRNA expression was unchanged at the indicated times (Figure 1D), suggesting that the responsiveness of *PHACTR4* gene to BDNF is less or too minute to be detected. Previously, Allen et al. studied the expression patterns of *PHACTRs* in the brain using *in situ* hybridization (*10*) and Kim et al. reported differential mRNA expression patterns of *PHACTR* family members in the developing and injured brain following traumatic brain injury (*12*). Our findings demonstrating high expression of *PHACTR1* and *PHACTR3* are consistent with these previous studies (*10,12*).

There are three intracellular signaling routes activated by BDNF (*18*). Therefore, we subsequently investigated which signaling pathway is involved in the reduction of *PHACTR1–3* mRNA by using three inhibitors to block

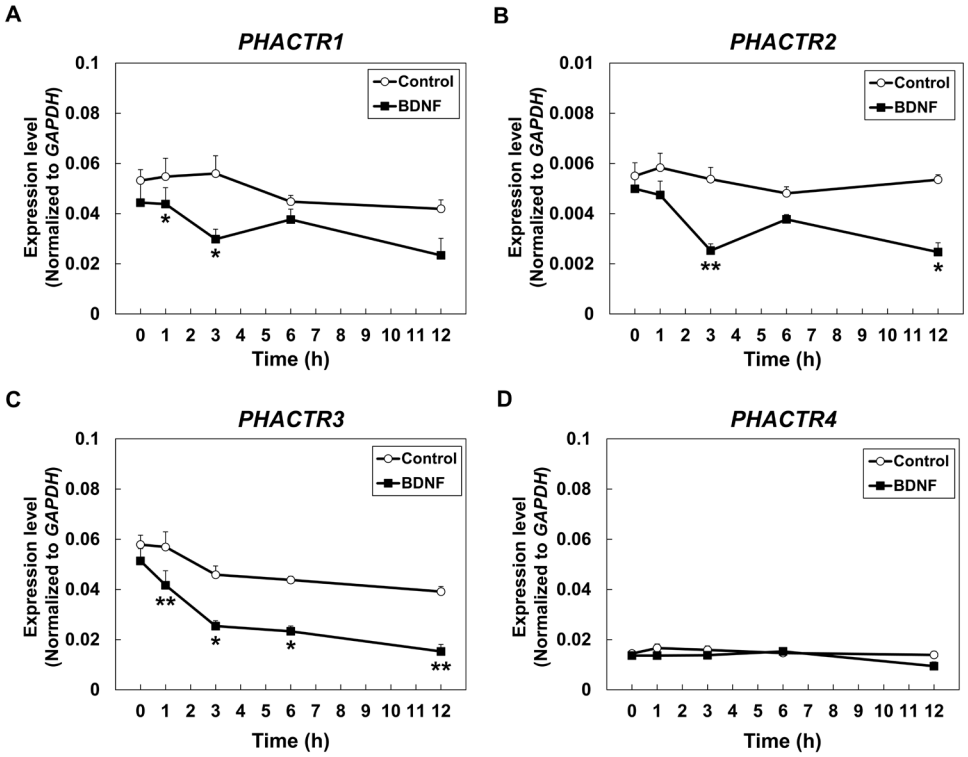

Figure 1. Downregulation of *PHACTRs1*, *2* and *3*, but not *PHACTR4* genes by BDNF stimulation. Primary cultured cortical neurons (9 days *in vitro*) were stimulated with vehicle or 100 ng/mL BDNF. At the time indicated, RNA was isolated and subjected to RT-qPCR for measuring mRNA levels. Time course of *PHACTR1* **(A)**, *PHACTR2* **(B)**, *PHACTR3* **(C)**, and *PHACTR4* **(D)** $^{*}p < 0.05/5$ and $^{**}p < 0.01/5$ (*vs*. vehicle control at the same time point).

the main signals propagated by BDNF. Administration of U0126, a Ras–Raf–mitogen-activated protein kinase (MEK) inhibitor for extracellular signal-regulated protein kinase (ERK)/mitogen-activated protein kinase (MAPK), significantly blocked the decreases of *PHACTR1–3* mRNA levels induced by BDNF (Figures 2A–2C, closed bars). In contrast, the phosphatidylinositol-3 kinase (PI3K) inhibitor LY294002 did not block BDNF-induced reductions of *PHACTR1–3* mRNA levels (Figures 2D–2F, closed bars). Similarly, the phospholipase C inhibitor U73122 did not inhibit the observed reductions (Figures 2G–2I, closed bars). These findings suggest that the MEK–ERK/MAPK cascade is deeply involved in BDNF-mediated attenuation of *PHACTR1–3* mRNA levels. Because transcription of a few genes is reportedly downregulated by ERK/MAPK-mediated C-terminal binding protein corepressor activation (*23*), such a similar mechanism might occur in this study.

The treatment with U0126 alone in the absence of BDNF altered the mRNA level of *PHACTR3* (Figure 2C). Unlike *PHACTR1* (Figure 2A, open bars) and *PHACTR2* (Figure 2B, open bars), *PHACTR3* mRNA level was upregulated by U0126 alone (the third bar from the left) compared with the untreated control sample (the first bar from the left) (Figure 2C). This finding suggests that the MEK–ERK/MAPK suppresses *PHACTR3* gene induction at the resting state of cortical neurons.

Although PHACTRs are expected to play versatile roles in health and disease of the brain, primarily because of their abundant expression (*10*), only a few reports describe the possible involvement of PHACTRs in function or dysfunction of the nervous system, such as roles in neuronal morphology (*13,14*) and epilepsy (*24*). In terms of neuronal morphology, the regulatory roles of PHACTR3 have been examined by its overexpression in primary cultured neurons, whereby it disrupts axonal elongation (*13*), increases dendritic complexity, upregulates percentages of matured spines, and decreases spine densities (*14*). As it remains largely unknown how endogenously expressed PHACTRs influence the nervous system, future studies applying knockout or knockdown technologies to *PHACTR1–4* expression are needed. In this study, we show downregulation of *PHACTR1–3* gene expression caused by BDNF. As described above, the regulatory ligands that regulate *PHACTR1–4* gene expression and role of endogenous PHACTRs remain to be fully elucidated. This study provides valuable insights into the contribution of endogenous PHACTRs and upstream signaling regulating their expression to brain structure and function.

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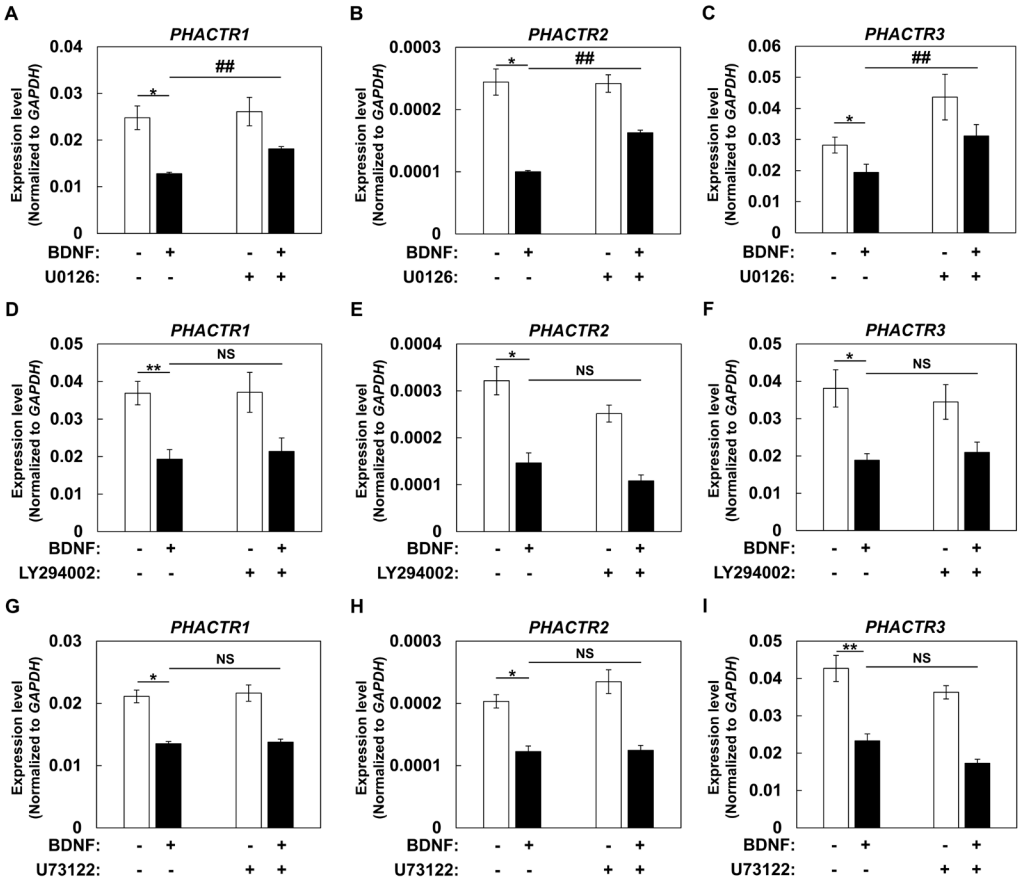

Figure 2. Contribution of ERK/MAPK pathway to BDNF-induced decrease of *PHACTRs1*, *2* and *3* mRNA. The mRNA levels in terms of *PHACTR1* **(A, D, G)**, *PHACTR2* **(B, E, H)** and *PHACTR3* **(C, F, I)**. Three inhibitors, U0126 (20 μM), LY294002 (10 μM) or U73122 (10 μM), were administered into cortical neurons (9 days *in vitro*) 30 min before BDNF stimulation (100 ng/mL). Three hours later, RNA was isolated and subjected to RT-qPCR for measuring mRNA levels. $^{*}p < 0.05/2$ and $^{**}p < 0.01/2$ (*vs*. vehicle control), $^{\#\#}p < 0.01/2$ and NS, not significant (*vs*. BDNF alone).

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