Brief Report

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# Panax notoginseng root extract induces nuclear translocation of CRTC1 and *Bdnf* mRNA expression in cortical neurons

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**SUMMARY** Brain-derived neurotrophic factor (BDNF), a member of the neurotrophin family, is deeply involved in the development and higher function of the nervous system, including learning and memory. By contrast, a reduction in BDNF levels is associated with various neurological disorders such as dementia and depression. Therefore, the inducers of *Bdnf* expression might be valuable in ameliorating or protecting against a decline in brain functions. We previously reported that, through high-throughput screening to identify inducers of *Bdnf* expression in *Bdnf-luciferase* transgenic mice, several herbal extracts induced *Bdnf* expression in cortical neurons. In the present study, we found that Panax notoginseng root extract (PNRE) potently induced *Bdnf* expression in primary cultured cortical neurons primarily *via* the L-type voltage-dependent $Ca^{2+}$ channel (L-VDCC) and calcineurin. PNRE promoted nuclear translocation of cAMP-responsive element-binding protein-regulated transcription coactivator 1 (CRTC1). These findings suggest that PNRE activates the L-VDCC/calcineurin/CRTC1 axis, which is the primary signaling pathway involved in the neuronal activity-dependent expression of *Bdnf*. Moreover, we demonstrated that PNRE increased the dendritic complexity of cortical neurons *in vitro*. Thus, by upregulating *Bdnf* expression, PNRE is a potential candidate for improving cognitive impairment seen in several kinds of dementia.

***Keywords*** Panax notoginseng root extract, BDNF, CRTC1, calcineurin, L-VDCC

## 1. Introduction

Overcoming dementia, including Alzheimer disease, is an urgent global health issue, and many strategies for developing treatments have been proposed. Brain-derived neurotrophic factor (BDNF) is a member of the neurotrophin protein family and plays roles in memory and in the survival and differentiation of neurons (*1*). The level of BDNF mRNA is decreased in patients with Alzheimer disease (*2,3*). By contrast, antidepressant drugs may increase BDNF levels (*4,5*), and high expression of BDNF in the brain may delay the decline in cognitive function caused by aging (*6*). Therefore, upregulating *Bdnf* expression is a potential therapeutic target for the treatment of dementia.

Previously, we established a high-throughput screening method to identify inducers of *Bdnf* expression using primary cortical cell cultures derived from *Bdnf-luciferase* (alias *Bdnf-luc*) transgenic mice (*7*). We screened 120 herbal extracts and found that several extracts, including those from Panax notoginseng root (*8*), contained *Bdnf* inducers (*7*). The roots of *Panax notoginseng* (Burkill) F. H. Chen are traditionally used in the Chinese medicine and known for its various effects on the immune and cardiovascular systems and for its antitumor and antiatherosclerotic effects (*9*). A flavonol glycoside from the root of *P. notoginseng* decreases Aβ-mediated neurotoxicity (*10*). However, little is known about the effect of Panax notoginseng root extract (PNRE) on the nervous system, including its effect on regulating gene expression for neurotrophic factors, such as BDNF. In the present study, we found that PNRE induces *Bdnf* transcription *via* the L-type voltage-dependent calcium channel (L-VDCC) and calcineurin. PNRE is also a potent inducer of nuclear translocation of the cAMP-responsive element-binding protein (CREB)-

regulated transcription coactivator 1 (CRTC1), which is known as a substrate of calcineurin and a regulator of *Bdnf*. Furthermore, PNRE promoted dendritic complexity of cortical neurons.

Administration of the Panax notoginseng root saponin ginsenoside Rg1 to rats ameliorated $A\beta_{1-42}$-induced deficits in learning and memory by downregulating $A\beta_{1-42}$ production and promoting $A\beta_{1-42}$ degradation (*11*). In addition, *P. notoginseng* root saponins upregulated neurogenesis in the hippocampus, attenuated the reduction in BDNF protein levels caused by cerebral ischemia, activated the mTOR pathway, and ameliorated neurological deficits (*12*). Therefore, PNRE in this study might contribute to novel drug designs to improve cognitive impairment.

## 2. Materials and Methods

### 2.1. Animals

Pregnant female Sprague Dawley rats were purchased from Japan SLC (Hamamatsu, Shizuoka, Japan). All experiments were performed in accordance with the ARRIVE guidelines and the requirements of the Animal Care and Experimentation Committee of the University of Toyama, Sugitani Campus. The Committee approved the protocols with permit Nos. A2022PHA-6, A2022PHA-7, A2019PHA-7, A2019PHA-10, and A2016PHA-8. Every effort was made to minimize animal suffering and the number of animals used.

### 2.2. Cell culture

Primary cultured cortical cells were prepared and maintained as described previously (*7*). Plastic dishes (35 mm diameter, AGC Techno Glass, Shizuoka, Japan) were coated with poly-L-lysine (P9155, Sigma, St. Louis, MO, USA) and used for real-time quantitative PCR experiments. Cortical cells were seeded at $1.8 \times 10^6$ cells/dish. For immunostaining, coverslips (83-0233, Matsunami, Osaka, Japan) were coated with poly-D-lysine (P6407, Sigma) and placed into a well in 12-well plates. Cortical cells were seeded at $7.0 \times 10^5$ cells/well in 12-well plates. Half of the medium was exchanged for fresh medium every 3 days.

### 2.3. Reagents

PNRE was prepared by extracting Panax notoginseng root (purchased from Tochimoto Tenkaido (lot No. 029716001, Osaka, Japan) in autoclaved Milli-Q water (10× the volume of herbal medicine) at 100°C for 50 min, after which the infusion mixture was filtered. Then, the extract was freeze-dried to obtain a powder. The powdered extract was redissolved in autoclaved Milli-Q water before use. The PNRE was characterized using LC-IT-TOF ME ESJ (Shimadzu), and the results are shown in the Traditional Medical & Pharmaceutical Database of the University of Toyama at the following URL (*https://dentomed.toyama-wakan.net/en/information_on_experimental_crude_drug_extracts/Panax%20Notoginseng%20Root-T/EXC278004*). DL-APV (A5282), nicardipine (N7510) and FK506 (F4679) were obtained from Sigma. STO609 (570250, Calbiochem, La Jolla, CA, USA) and KN93 (AG-CR1-0065, AdipoGen Life Sciences, San Diego, USA) were used.

### 2.4. RNA isolation and quantitative (q)PCR

Total RNA isolation and the complementary DNA synthesis were performed as described previously (*7*). To detect *Bdnf* and *glyceraldehyde-3-phosphate dehydrogenase* gene (*Gapdh*) at the mRNA level, PCR was performed in 20 μL of 1× SYBR Select Master Mix (4472908, Thermo Fisher Scientific) containing 2 μL of cDNA solution and 0.2 μM of each primer. The primer sequences and PCR program is provided in the Supplementary material (*https://www.ddtjournal.com/action/getSupplementalData.php?ID=217*). The value indicating relative expression was obtained by the ratio of *Bdnf* mRNA level/*Gapdh* mRNA level, and was indicated as the fold change, where the vehicle control value was regarded as "1".

### 2.5. Plasmids and antibodies

Enhanced green fluorescent protein (pEGFP-C1) vector was purchased from Takara Bio USA (San Jose, CA, USA). The following primary goat antibodies were used: CF594-conjugated anti-mouse IgG (20110, Biotium, Fremont, CA, USA; 1:1,000) and CF488A-conjugated anti-rabbit IgG (20019, Biotium; 1:1,000). Polyclonal rabbit antibodies were used as follows: anti-GFP (598, Medical & Biological Laboratories, Tokyo, Japan; 1:1,000) and anti-transducer of CREB protein 1 (TORC1) (#A300-769A, Bethyl Lab, Montgomery, Texas, USA; 1:500) for detecting CRTC1. A mouse monoclonal antibody raised against microtubule-associated protein 2 (MAP2) (M4403, 1:1,000) was purchased from Sigma.

### 2.6. DNA transfection

Four-day primary cultured cortical cells were transfected using a previously described calcium phosphate precipitation method (*13*). The vector expressing EGFP (4 μg/well) was transfected.

### 2.7. Immunostaining

We used an existing method for immunostaining (*13*) with only minor modifications. 4′,6-diamidino-2-phenylindole (DAPI) was used for nuclear staining. MAP2-positive cells were regarded as neurons.

2.8. Localization of CRTC1

Immunofluorescence images of immunostained cells were acquired using an LSM 700 confocal microscope (Zeiss). Fluorescence intensities with nuclear or cytoplasmic localization were evaluated using ImageJ software (National Institutes of Health, *https://imagej.nih.gov/ij/*) by investigators blinded to the various treatments. The fluorescent area within the DAPI-positive area was regarded as the nuclear, and the area within the MAP2-positive area was regarded as cytoplasmic. At least 50 cells were examined in each of three independent experiments.

2.9. Morphological analysis

Sholl analysis was used to evaluate dendritic morphology (*13*). The immunofluorescence images were obtained using a fluorescence microscope (BX50-34LFA-1, Olympus). MAP2-positive cell processes were regarded as dendrites. The number of GFP and MAP2 double-positive dendrites crossing circles drawn at the center of the cell body (at 20, 40, and 60 μm radii) was counted and totaled to obtain the number of crossings.

2.10. Statistical analysis

Data sets were compared using a one-way ANOVA followed by a Scheffe *F* test, or by paired or Welch *t* tests. Differences were considered significant when $P < 0.05/x$ (where $x$ was the number of tests). Microsoft Excel 2013 [version 15.0.5127.1000] was used for all statistical analyses.

## 3. Results and Discussion

We had identified PNRE as an inducer of *Bdnf* expression using high-throughput screening (*7*). We initially examined whether PNRE induces endogenous *Bdnf* mRNA expression *via* activity-dependent $Ca^{2+}$ channels in primary cultured rat cortical cells. PNRE increased *Bdnf* mRNA expression, and an NMDA receptor antagonist, APV, inhibited this increase slightly (Figure 1A). By contrast, the PNRE-induced increase in *Bdnf* mRNA expression was strongly inhibited by nicardipine, an inhibitor of L-VDCC (Figure 1B). These findings suggest that PNRE might activate primarily *via* L-VDCC and subsequent $Ca^{2+}$ signaling in cortical neurons. Elevated $Ca^{2+}$ levels in neurons *via* the L-VDCC propagate multiple signals, thereby regulating

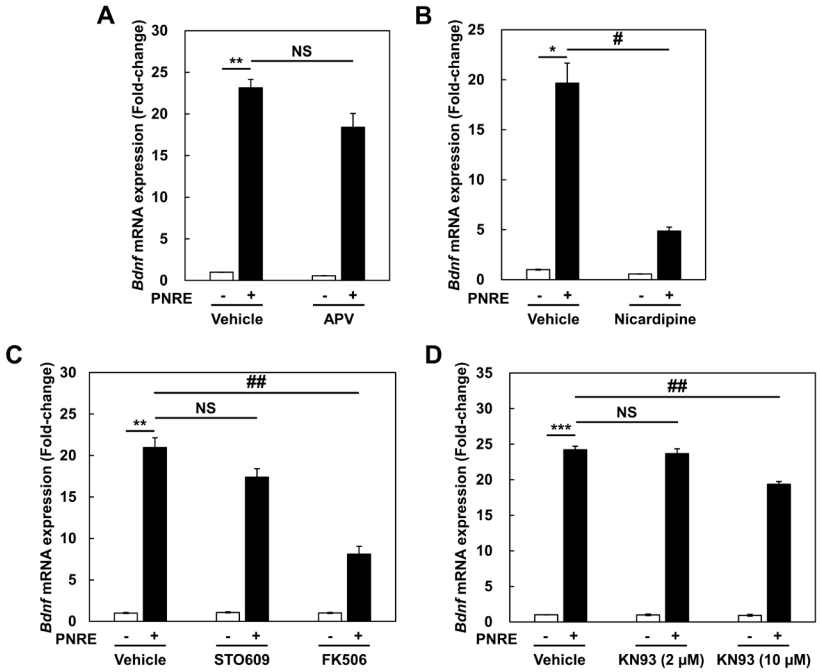


**Figure 1. PNRE induces *Bdnf* mRNA expression in primary cultured cortical cells and the effects of signaling inhibitors on this induction.** (**A-D**) Influence of signaling inhibitors on PNRE-induced *Bdnf* mRNA expression [days *in vitro* (DIV)13]. Inhibitors were added 10 min before administering PNRE (500 μg/mL). Three hours later, total RNA was extracted and subjected to qPCR. (**A**) APV (200 μM) partially inhibited *Bdnf* mRNA expression. The bars represent the means ± SEMs ($n$ = 3-6). Differences between treatment outcomes were analyzed using a Welch *t* test with post hoc Bonferroni correction ($^{**}P < 0.01/2$; NS, not significant). (**B**) Nicardipine (5 μM) significantly inhibited *Bdnf* mRNA expression. Bars represent the means ± SEMs ($n$ = 3). Differences between treatment outcomes were analyzed using a Welch *t* test with post hoc Bonferroni correction ($^{*}P < 0.05/2$; $^{\#}P < 0.05/2$). (**C**) FK506 (5 μM), but not STO609, significantly inhibited *Bdnf* mRNA expression. Bars represent the means ± SEMs ($n$ = 3-4). Differences between treatment outcomes were analyzed using one-way ANOVA with a post hoc Scheffe *F* test ($^{\#\#}P < 0.01$; NS, not significant) or a Welch *t* test with post hoc Bonferroni correction ($^{**}P < 0.01/3$). (**D**) KN93 (10 μM) partially inhibited *Bdnf* mRNA expression. The bars represent the means ± SEMs ($n$ = 4). Differences between treatment outcomes were analyzed using one-way ANOVA with a post hoc Scheffe *F* test ($^{\#\#}P < 0.01$; NS, not significant) or a Welch *t* test with a post hoc Bonferroni correction ($^{***}P < 0.001/3$).

neuronal activity-dependent gene expression (*14*). Therefore, we next determined the main $Ca^{2+}$ signaling pathway downstream of $Ca^{2+}$ channels. STO609, a $Ca^{2+}$/calmodulin-dependent protein kinase kinase (CaMKK) inhibitor, tended to inhibit the induction of *Bdnf*, but the effect was not significant (Figure 1C). A $Ca^{2+}$/calmodulin-dependent protein kinase (CaMK) inhibitor (KN93) appeared to inhibit the induction of *Bdnf* slightly at 10 μM, but not at 2 μM (Figure 1D), indicating that CaMK pathways, such as the CaMKIV–CREB axis, but not CaMKII, might also contribute to PNRE-induced *Bdnf* transcription, at least in part. Subsequently, we focused on calcineurin, a $Ca^{2+}$/calmodulin-dependent serine–threonine phosphatase. FK506, a calcineurin inhibitor, strongly inhibited PNRE-induced *Bdnf* mRNA expression (Figure 1C). These experiments suggested that L-VDCC/calcineurin signaling might be the primary pathway that mediates PNRE-induced *Bdnf*.

CREB and its cofactor, CRTC1, are transcription factors involved in *Bdnf* induction (*15*). CRTC1 is dephosphorylated by calcineurin, thereby translocating into the nucleus (*15*). As described above, our findings suggest that calcineurin is involved in PNRE-induced *Bdnf* (Figure 1). Therefore, we next investigated whether PNRE induces the nuclear translocation of CRTC1 in cortical neurons. Immunostaining of CRTC1 in cortical neurons with and without PNRE-treatment revealed that the extract strongly promoted nuclear translocation of CRTC1 (Figure 2A). PNRE increased the nuclear/cytoplasmic ratio of CRTC1, suggesting that the extract significantly induced the nuclear translocation of CRTC1 in cortical neurons (Figure 2B). Among the herbal and Kampo extracts we screened, ginseng extracts and the Kampo formula daikenchuto were identified as *Bdnf* inducers *via* L-VDCC and calcineurin activation (*7,16)*. The ginseng extract induces CREB phosphorylation and the translocation of CRTC1 into the nucleus in cortical neurons (*7*). Our screening method is considered appropriate for identifying *Bdnf* inducers mediated by the L-VDCC/calcineurin/CRTC1 axis, which is the primary

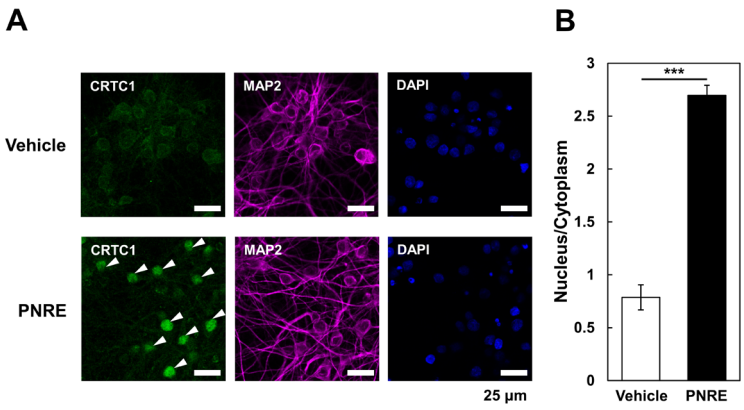


**Figure 2. PNRE promotes the translocation of CRTC1 into the nucleus in cortical neurons.** (**A**) Representative images showing the localization of CRTC1 (white arrowheads). Primary cultured rat cortical cells (DIV13) were stimulated with PNRE (500 μg/mL) for 15 min and immunostained. The cells were stained with an anti-CRTC1 antibody (left), an anti-MAP2 antibody (middle), and DAPI (right). Scale bars, 25 μm. (**B**) Bar graphs showing the nuclear/cytoplasmic ratio of CRTC1 under the experimental conditions shown in A. The bars represent the means ± SEMs ($n$ = 3). Intensities of the fluorescence immunostaining were quantified using ImageJ software (National Institutes of Health). Differences in treatment outcomes were analyzed using a paired $t$ test ($^{***}P < 0.001$).

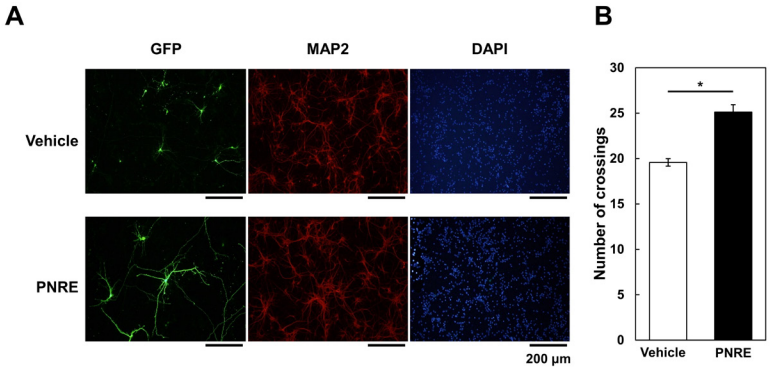


**Figure 3. Dendritic complexity is increased by PNRE.** (**A**) Primary cultured cortical cells (DIV4) were transfected with a green fluorescent protein (GFP) vector. Cells were stimulated with PNRE (500 μg/mL) 48 h posttransfection and incubated for 24 h. Then, the cells were immunostained with anti-GFP (left), anti-microtubule-associated protein 2 (MAP2) monoclonal antibodies (middle), and 4′,6-diamidino-2-phenylindole (DAPI) (right). Scale bars, 200 μm. (**B**) Bar graphs showing the total number of dendritic crossings of circles drawn around soma (at 20, 40, and 60 μm radii) as a proxy for dendritic complexity under the experimental conditions described for A. The bars represent the means ± SEMs ($n$ = 3). Differences between treatment outcomes were analyzed using a paired $t$ test ($^{*}P < 0.05$).

pathway involved in *BDNF* expression.

The activity-dependent expression of *Bdnf* may contribute to neurite outgrowth (*17*). As described above, we found that PNRE increased *Bdnf* induction in cortical neurons. These findings prompted us to investigate whether PNRE promotes the dendritic complexity of cortical neurons. Compared with vehicle control, PNRE induced greater dendritic complexity in GFP- and MAP2-double-positive neurons (Figure 3A). Sholl analysis to quantify dendritic morphology revealed that the total number of dendritic crossings across circles drawn around cell bodies increased significantly in response to treatment of cortical neurons with PNRE (Figure 3B), suggesting that PNRE had a neurotrophin-like effect on dendritic morphology. Daikenchuto, which includes the ginseng, induces *Bdnf* and promotes dendritic complexity (*16*). Additionally, we identified deltamethrin as an inducer of *Bdnf*, which mediates dendritic complexity in cortical neurons through endogenously expressed BDNF (*17*). Therefore, the dendritic complexity observed in the present study may be due to endogenous BDNF expression induced by PNRE.

Panax notoginseng root may be valuable for treating neurological disorders (*11,12,18*). The Panax notoginseng root saponin ginsenoside Rg1 may be a promising compound which induces *Bdnf* gene in our study because of identification of the Rg1 for ameliorating $A\beta_{1-42}$-induced deficits in learning and memory (*11*). The protection of Panax notoginseng root against traumatic brain injury is associated with inhibiting autophasic events *via* the mTOR pathway (*18*). Natural products, such as polygalasaponin XXXII (*19*), P7C3 (*20*), and Glehnia Root and Rhizome extract (*21*), reverse scopolamine-induced BDNF reduction and memory impairment. To our knowledge, the present study is the first to demonstrate that PNRE induces *Bdnf* expression at the mRNA level and to elucidate the mechanisms by which $Ca^{2+}$ signaling *via* the L-VDCC enhances the calcineurin/CRTC1 axis-mediated activation of *Bdnf* expression. This study on PNRE potentially contributes to the development of therapeutic strategies to upregulate *BDNF* expression for patients with neurological disorders associated with dysfunction of higher brain functions.

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*Conflict of Interest*: The authors have no conflicts of interest to disclose.

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