Self-amplified BDNF transcription is a regulatory system for synaptic maturation in cultured cortical neurons

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Running title;

The role of self-amplifying of BDNF in neurons

Keywords;

BDNF, ERK, CREB, TrkB

1   **Abstract**

2   Neuronal cell survival and synaptic plasticity are controlled through expression of various
3   neurotrophic factors including brain-derived neurotrophic factor (BDNF). In the present study, we
4   examined the mechanism behind BDNF-induced *Bdnf* mRNA production and the physiological role
5   of its amplification system using cortical neurons. Exogenous BDNF was applied to the cultured
6   cortical neurons at days *in vitro* (DIV) 3 and DIV 7 with or without inhibitors for intracellular
7   signaling. Expression levels of total *Bdnf* and *Bdnf* variants (*exon I*, *exon IV*, and *exon VI*) were
8   biphasically increased after the BDNF application in different developing stage of neurons. Inhibitor
9   for extracellular signal-regulated kinase, calmodulin dependent protein kinase II, or protein kinase A
10   repressed the BDNF-induced *Bdnf* mRNA expression. Furthermore, we found that application of
11   TrkB-Fc, which scavenges produced endogenous BDNF, resulted in weakened BDNF/TrkB
12   signaling and decreased expression of postsynaptic proteins, suggesting that newly synthesized
13   BDNF induced by the self-amplification system contributes to the synaptic maturation and function.

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1. Introduction

Brain-derived neurotrophic factor (BDNF) is a pivotal regulator for neuronal development and function such as cell survival and synaptic plasticity (Park and Poo 2013). The expression of BDNF is regulated via neuronal activity at transcriptional levels. For example, stimulation with KCl and kainic acid increase mRNA levels of *Bdnf* and reduced neuronal activity with NMDA receptor antagonists downregulates *Bdnf* mRNA (Zefra et al. 1990; Katanuma et al. 2014). It has been reported that, among nine promoters existing in upstream of 5'-untranslated region (UTR) of *Bdnf*, promoters I and IV are highly responsive to the neuronal activity due to their multiple Ca^{2+} - and cyclic adenosine monophosphate (cAMP) responsive elements (Pruunsild et al. 2011).

Exogenous BDNF application increases *Bdnf* exon IV (Yasuda et al. 2007; Zheng and Wang. 2009) in cultured cortical neurons. BDNF binding to TrkB, a high-affinity receptor for BDNF, induces the activation of downstream signals including extracellular signal-regulated kinase (ERK), phosphatidylinositol-3 kinase (PI3K)/Akt, and phospholipase C-gamma (PLC γ) (Park and Poo 2013; Leal et al. 2014). Activation of L-type voltage-gated calcium channel and ERK signal by pulsed electromagnetic field upregulate protein levels of BDNF in neonatal dorsal root ganglion neurons (Li et al. 2014). Not only activity-dependent system, but also ERK contributes to the regulation of BDNF-induced *Bdnf* exon transcription in cortical neurons (Yasuda et al. 2007; Zheng and Wang 2009).

It has been suggested that activity-dependent synthesis and secretion of BDNF in both axon and dendrite is required for synaptic function including late-phase spine maturation (Kuczewski et al. 2010; Jakawich et al. 2010). Recent high-resolution visual analysis with stochastic optical reconstruction microscopy has revealed that high abundance of BDNF in glutamatergic presynapse of hippocampal neurons (Andreska et al. 2014). The activation of postsynaptic BDNF/TrkB systems also plays a role in the synaptic maturation and plasticity (Yoshii and Constantine-Paton 2010). Secreted BDNF acts as an autocrine regulator to enhance axon differentiation through the elevation of local cAMP levels and activation of protein kinase A (PKA) (Cheng et al. 2011). It has been demonstrated that the positive feedback loop of BDNF signaling contributes to hippocampal memory consolidation in rats in inhibitory avoidance test (Bambah-Mukku et al. 2014). Although these results suggest an importance of amplifying BDNF/TrkB signaling in the neuronal function, it is still unclear the contribution of accelerated self-induced BDNF production on BDNF-dependent synaptic regulation in cortical system.

Here, using cortical neurons at different developing stages, we explored a contribution of BDNF/TrkB signaling to BDNF-induced *Bdnf* mRNA expression and physiological role of produced BDNF in synaptic function.

2. Materials and Methods

2.1 Cell cultures

Cultured cortical neurons were prepared from cerebral cortex of postnatal Wistar rats (Japan SLC, Inc., Shizuoka, Japan) at the age of 1-2 days as described previously (Numakawa et al. 2009). Briefly, dissected cortical tissues were digested with 9 units/ml papain and 200 units/ml DNase I for 20 min at 37°C. After the removal of enzyme solution, dissociated cortical cells with pipetting was cultured with DMEM/F12 medium containing 5% fetal bovine serum, 5% horse serum, 18 mg/ml streptomycin and 18 units/ml penicillin. The cells were seeded on 3.5 cm dishes and 24-well plates at density of 5×10^5 cells/cm². After 1 day *in vitro* (DIV), the culture medium was replaced to DMEM/F12 medium containing 5% horse serum with AraC (cytarabine, 2 µM). BDNF (kindly gifted from Takeda Pharmaceutical company Ltd., and Sumitomo Co. Ltd.) or forskolin (Wako, Japan) was applied to cortical neurons at DIV 3-4 or 6-7 with/without 20 min pretreatment of U0126 (Cell signaling Technology Inc., MA, USA), LY294002 (Cell signaling), CREB (cAMP responsive element-binding protein) binding protein (CBP)-CREB interaction inhibitor (Calbiochem, MA, USA), KN-93 (Calbiochem), H-89 (Calbiochem), and K252a (Enzo Life Science, Inc., NY, USA). Recombinant Human TrkB-Fc Chimera (R&D systems Inc. Minneapolis, MN, USA) was added at 3 hours after the 10 ng/ml of BDNF application (DIV 6-7) considering of TrkB-Fc action to scavenge endogenous BDNF with/without washout of exogenous BDNF from the cultured medium. These experiments were performed several times using different series of cultures. These experiments were approved by the Ethics Review Committee for Animal Experimentation of the National Institute of Neuroscience, Japan (approval number; 2011019).

2.2 Real-time PCR

Total RNA was extracted using RNeasy mini kit (Qiagen, CA, USA) according to manufacturer's protocol. The reverse transcription of total RNA (100 ng) was performed with SuperScript® VIRO™ cDNA synthesis kit (Life Technologies Inc., CA, USA). Obtained cDNA with each specific Taqman® probe of *Bdnf* (Rn02531967_s1), *Bdnf exon I* (Rn01484924_m1), *Bdnf exon IV* (Rn01484927_m1), *Bdnf exon VI* (Rn01484928_m1), *Map2* (microtubule associated protein 2, Rn00565046_m1), *Ngf* (nerve growth factor, Rn01533872_m1), or *Gapdh* (4352338E) was applied to real-time PCR system (Life Technologies Inc.). The data were analyzed using SDS 2.2 real-time PCR data analysis software (Life Technologies Inc.). The expression value of target gene was normalized by expression levels of *Gapdh* in each sample, then relative expression against to that of control were calculated.

2.3 Western blotting

Total cell lysates were prepared with lysis buffer containing 1% SDS, 10 mM Tris-HCl, 10 mM

1 Na₄P₂O₇, 10 mM NaF, 5 mM EDTA, 2 mM Na₃VO₄ and 1 mM PMSF. The concentration of each
2 sample was determined by Pierce® BCA protein assay (Thermo scientific, IL, USA). The
3 equivalent amount of proteins was applied to 8% acrylamide gel, and then, separated total
4 proteins in the loaded gel were transferred to PVDF membrane (Millipore, MA, USA). The
5 primary antibody including anti-pTrk (Cell signaling, 1:500), anti-TrkB (BD biosciences, CA,
6 USA, 1:1000), anti-pERK (Cell signaling, 1:1000), anti-ERK (Cell signaling, 1:1000),
7 anti-pAkt (Cell signaling, 1:500), anti-Akt (Cell signaling, 1:1000), anti-NR2A (Sigma-Aldrich,
8 MO, USA, 1:500), anti-NR2B (Sigma-Aldrich, 1:500), anti-GluR1 (Sigma-Aldrich, 1:500),
9 anti-SNAP25 (Synaptic Systems, Germany, 1:1000), anti-synaptotagmin (BD Transduction
10 Laboratories, 1:1000), anti-syntaxin (Sigma-Aldrich, 1:3000), and anti-β-actin (Sigma-Aldrich,
11 1:5000) antibodies was applied overnight after blocking with 5% skim milk. The secondary
12 antibody of mouse (Jackson ImmunoResearch Europe Ltd., Suffolk, UK) or rabbit (Rockland
13 Immunochemicals, Inc., Gilbertsville, PA, USA) was applied for 1 hour. After the treatment of
14 ImmunoStar® reagent (Wako Pure Chemical Industries Ltd., Japan), immunoreactivity was
15 detected by ECL films (GE healthcare UK Ltd., Buckinghamshire, UK) or AE-9150 Ez-Capture
16 II (ATTO Co., Japan). The optical density of protein expression was measured by CS Analyzer
17 version 3.00.1011 (ATTO Co.).

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19 *2.4 Statistical analysis*

20 All data are expressed as means ± SEM. Data were analyzed by one- or two- way ANOVA with
21 Bonferroni's multiple comparison *post-hoc* test (IBM Japan, Japan). Statistically significance
22 was defined as probability values less than 5%.

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3. Results

3.1 Biphasic increase of *Bdnf* mRNA expression after exogenous BDNF addition in cortical neurons

When BDNF (100 ng/ml, for 1, 3, 6, 12, and 24 hours) was added to both developing stage of cortical neurons, endogenous levels of *Bdnf* mRNA was increased biphasically (see the initial peak at 3 hours after BDNF addition) compared with control (no treatment; 0 hour) (Fig. 1A). We confirmed dose-dependent influence of exogenous BDNF on the *Bdnf* mRNA expression at 3 and 24 hours after the BDNF application in DIV 7 (Fig. 1B). The significant effect of 10 ng/ml BDNF application for 24 hours was detected by student's *t*-test ($P > 0.01$) compared to control group although only higher dose of BDNF (100 and 500 ng/ml) significantly increased *Bdnf* mRNA in multiple comparison test. Further, mRNA levels of *Bdnf* variants (*exon I*, *exon IV*, and *exon VI*) were also increased by BDNF application similar to total *Bdnf* mRNA expression (Fig. 1C). Surprisingly, *Bdnf exon I* transcript was drastically increased after exogenous BDNF application, because *Bdnf exon I* transcript has been reported to be lower expressed compared to other transcripts (Katanuma et al., 2014). Activity-dependent regulation of *Ngf* mRNA expression has been investigated in addition to *Bdnf* (Zafra F et al., 1990), and MAP2 is well known as a neuronal marker (Andreska et al., 2014; Jakawich et al., 2010). To examine the specificity of exogenous BDNF application on its expression, we tested the effect of BDNF on mRNA levels of *Ngf* or *Map2*. Increase in *Ngf* or *Map2* mRNA by exogenous BDNF was lower than that in *Bdnf* mRNA expression (Fig. 1D). Because there was no significant difference between young (DIV 3) and mature (DIV7) neurons in BDNF-induced *Bdnf* mRNA production, cultured cortical neurons at DIV 7 were used for subsequent experiments unless otherwise noted.

3.2 Involvement of MAPK and PI3K/Akt signaling in the BDNF-induced *Bdnf* mRNA expression

As shown in Fig. 2A, BDNF treatment significantly increased activated ERK1/2 (phosphorylated ERK1/2, pERK1/2) and Akt (phosphorylated Akt, pAkt). At 3 hours after BDNF addition, blockade of both signal cascades by MEK inhibitor (U0126) and PI3K inhibitor (LY294002) caused significant decrease in the self-induced *Bdnf* mRNA production by BDNF (Fig. 2B). While, at 24 hours after BDNF application, only inhibition of ERK signal with U0126 completely blocked the self-induced *Bdnf* mRNA production by BDNF (Fig. 2C). However, U73122, an inhibitor for PLC γ pathway, did not show inhibitory effect on the BDNF-induced *Bdnf* mRNA expression at both time points (Figs. 2B and 2C).

At 3 hours after BDNF application, the expression levels of *Bdnf exon I* and *exon IV*, but not *exon VI*, were decreased by U0126 and LY294002 (Fig. 3A). Importantly, at 24 hours after BDNF addition, the *Bdnf* mRNA production from *exon I*, *exon IV*, and *exon VI* were abolished in the presence of U0126 similar to total *Bdnf* mRNA expression (Fig. 3B). Although LY294002 had no effect on total *Bdnf* mRNA expression at 24 hours after BDNF treatment (Fig. 2C), it reduced

1 BDNF-induced *Bdnf* expression from *exon I* and *exon IV* (Fig. 3B).

2 3 *3.3 CREB and Calmodulin dependent protein kinase (CaMK) II is involved in the self-induced Bdnf* 4 *mRNA production*

5 It has been shown that activity-dependent *Bdnf* mRNA expression is regulated by the activation of
6 CREB and CaMKII (Pruunsild et al. 2011). Forskolin which induces elevated cAMP levels also
7 increased *Bdnf* mRNA at 3 and 24 hours after the drug application and such an increment was
8 abolished by CBP-CREB interaction inhibitor (Fig. 4A, 4B). On the other hand, in the
9 BDNF-induced *Bdnf* mRNA expression, inhibitory effect of this CBP-CREB interaction inhibitor
10 was only observed at 24 hours after the BDNF treatment (Fig. 4A, 4B). Further, PKA inhibitor
11 (H-89) completely abolished the self-induced *Bdnf* transcription at 24 hours after BDNF addition,
12 but partial inhibitory effect by H-89 was observed at 3 hours (Fig. 4A, 4B). As shown in Fig. 4C, 4D,
13 the biphasic self-induced *Bdnf* mRNA production did not occur in the presence of CaMKII inhibitor
14 (KN-93) and NMDA-type glutamate receptors inhibitor (APV).

15 16 *3.4 Blocked BDNF/TrkB signaling failed to increase of BDNF transcript*

17 Next, to explore the mechanism underlying late phase increase in the levels of BDNF transcript, we
18 used TrkB inhibitors to block stimulation by endogenous BDNF produced after exogenous BDNF
19 application. Twenty minutes pretreatment of K252a (a nonspecific Trk receptors inhibitor)
20 significantly inhibited the BDNF-induced *Bdnf* mRNA expression (Fig. 5A). Although the removal
21 of remaining exogenous BDNF in the medium at 3 hours after BDNF addition weakened the *Bdnf*
22 mRNA production (12 and 24 hours, respectively) compared to no washing control, significant
23 increase of *Bdnf* mRNA was still observed (Fig. 5B). Furthermore, TrkB-Fc was added at 3 hours
24 after exogenous BDNF application to remove a contribution of BDNF including newly
25 synthesized-endogenous BDNF. Trapping of BDNF by TrkB-Fc from 3 hours after the BDNF
26 application blocked the increase in *Bdnf* mRNA levels (Fig. 5C). **TrkB-Fc still had an inhibitory**
27 **effect on BDNF-induced *Bdnf* mRNA expression after washout of exogenous BDNF (Fig. 5D). In**
28 **addition,** the activation of Trk and its downstream cascade including ERK- and Akt- signaling were
29 all attenuated by TrkB-Fc treatment (Fig. 5E).

30 31 *3.5 Self-induced BDNF production plays a role in synaptic protein maintenance*

32 In the presence of TrkB-Fc to scavenge **both exogenous BDNF and newly produced BDNF,**
33 increased postsynaptic protein glutamate receptors such as NR2A, NR2B, and GluR2/3 by BDNF
34 application was attenuated (Fig. 6B). In contrast, no blocking effect of TrkB-Fc on expression of
35 presynaptic proteins including SNAP25, synaptotagmin, and syntaxin was observed (Fig. 6C).

4. Discussion

In the present study, we found biphasic self-amplification of BDNF in different developing stages (DIV 3 and DIV 7) of cortical neurons. Signaling cascade via BDNF/TrkB including ERK regulated the self-induced *Bdnf* mRNA production. In our cortical neurons, sustainable activity of BDNF/TrkB signaling including self-induction contributed to its self-amplifying and upregulation of postsynaptic proteins.

It has been reported that the transcriptional activity of *Bdnf* exon IV is regulated by exogenous BDNF treatment and activity-dependent stimuli (Yasuda et al. 2007; Zhang and Wang 2009). ERK is a major signaling molecule to increase *Bdnf* IV transcript when exogenous BDNF is applied (Yasuda et al. 2007; Zheng and Wang 2009). Although PI3K signaling partially contributed to the BDNF-induced total *Bdnf* mRNA, our results also demonstrated that ERK is a main contributor to the self-production of *Bdnf* mRNA. Remarkably, upregulation of *Bdnf* exon I when exposing to activity-dependent stimuli (including BDNF treatment) is higher compared to exon IV and exon VI, although endogenous levels of exon I is much lower than exon IV and exon VI (Pruunsild et al. 2011; Katanuma et al., 2014). The inconsistency of *Bdnf* mRNA expression levels among experiments would reflect the difference in synaptic maturity and/or culture condition, which also supports the importance of neuronal activity for *Bdnf* transcription. On the other hand, U0126- or LY294002-reduced expression of exon I and exon IV, highly responsive regions to the neuronal activity, incompletely reflected to total *Bdnf* mRNA expression, supporting that BDNF-induced *Bdnf* expression is regulated via several systems but not only in activity-dependent manner.

CaMK I, II, and IV are also regulator for the *Bdnf* exon IV expression after treatment of exogenous BDNF and NMDA (Zheng and Wang, 2009; Zheng et al. 2011). Calcium-responsive transcription factor (CaRF) and CREB are recognized as main transcriptional factor for mRNA production of BDNF (Pruunsild et al. 2011). ERK, CaMKs (I, II, and IV), and PKA also stimulate transcriptional factors to increase *Bdnf* exon IV transcript (Zheng et al. 2011). Although PLC γ is involved in an increase in intracellular calcium concentration and activates CaMK II in various cell types (Kim et al. 2004), the influx of extracellular calcium also activates CaMK II (Nakanishi and Okazawa, 2006). The inhibition of NMDA receptors by APV reduced self-induction of BDNF in the present study, implying that CaMK II activation via Ca²⁺ influx is required for BDNF-induced *Bdnf* mRNA expression. Interestingly, we found possible contribution of CREB to the BDNF-induced total *Bdnf* mRNA in late phase (24 hours) (not in early phase; 3 hours), while CaMK, ERK, and PKA were all involved in the *Bdnf* mRNA production in early phase. The regulation of BDNF levels in each time course has been demonstrated (Yasuda et al. 2007; Zheng and Wang 2009). Yasuda et al. reported that the transcript of *Bdnf* exon IV is increased activity-dependently only in early phase (Yasuda et al. 2007). Notably, exon IV transcription by NMDA or BDNF are regulated differently (Zheng and Wang 2009), and we previously observed that reduced neuronal activity with blockade

1 of NMDA receptors rapidly decreased total and *exon IV* mRNA of BDNF through the inactivation of
2 CREB (Katanuma et al. 2014). It is possible that BDNF/TrkB system and neural activity
3 concurrently induce various intracellular signaling in early or late phase after BDNF binding to its
4 receptor, resulting in achievement of fine control in amplifying the self-induction of BDNF.

5 Signaling kinetics of BDNF/TrkB system is critical aspect in regulation of neuronal function.
6 Sustained TrkB activity by gradual application of BDNF or depolarization before the BDNF
7 stimulation enhances neurite branching and spine neck elongation (Ji et al. 2010; Guo et al. 2014). In
8 the present study, the scavenging of BDNF using TrkB-Fc from the first peak of BDNF transcription
9 levels (3 hours) suggested the possible contribution of self-amplifying BDNF on the sustainable
10 BDNF/TrkB signaling. Interestingly, self-amplifying BDNF acts as an autocrine factor to accelerate
11 axon development through local elevation of cAMP and PKA activity (Cheng et al. 2011). We found
12 that such a sustained BDNF/TrkB signaling is required for the further amplification of BDNF
13 production observed in late phase followed by upregulation of postsynaptic proteins. Because
14 postsynaptic BDNF/TrkB signaling is required for synaptic maturation and function (Yoshii and
15 Constantine-Paton, 2010), the acceleration of BDNF self-production may be important to regulate
16 synaptic plasticity. It is required that experiments such as specific downregulation of BDNF by
17 RNAi and/or stimulation with labeled BDNF to distinguish from endogenous BDNF in the future.
18 The present results have firstly demonstrated a physiological contribution of the elevated
19 endogenous BDNF levels via self-production system which prolongs BDNF/TrkB signaling and
20 increases postsynaptic proteins.

21 Decreased levels of *Bdnf* and *Trkb* mRNA expression in prefrontal cortex and hippocampus were
22 observed in Alzheimer's disease, schizophrenia, and mood disorders (Ray et al. 2014). The positive
23 effect of exercise on hippocampal *Bdnf* mRNA expression contributes to improve object recognition
24 and to increase cell proliferation (Garza et al. 2004; Bechara and Kelly 2013). Exercise and magnetic
25 stimulation also increases hippocampal TrkB transcript (Vaynman et al. 2003; Ma et al. 2013).
26 Recently, it has been demonstrated that positive BDNF-loop is important for the memory
27 consolidation mediated by the hippocampus (Bambah-Mukku et al. 2014). These studies including
28 our present results suggest that exploring external stimuli to trigger the self-amplifying BDNF
29 system has a beneficial effect to treat brain diseases.

31 **Acknowledgements**

32 This study was supported by grants from Takeda Science Foundation (T.N.), the Grant-in-Aid for
33 Challenging Exploratory Research (JSPS KAKENHI Grant Number 25640019) (T. N.), and the
34 Grant-in-Aid for Scientific Research (B) (JSPS KAKENHI 24300139) (T. N.) in the Ministry of
35 Education, Culture, Sports, Science, and Technology of Japan. This study also supported by the
36 Health and Labor Sciences Research Grants (H. K.), Intramural Research Grant for Neurological and

- 1 Psychiatric Disorders of NCNP (H.K.) and CREST, Japan Science and Technology Agency (JST)
- 2 (T.N., N.A. and H.K.).
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Figure legends

Figure 1 BDNF-induced *Bdnf* mRNA expression in cultured cortical neurons.

(A) The expression levels of total *Bdnf* mRNA induced by treatment with BDNF (100 ng/mL) at DIV 3 and DIV 7 (n=4). (B) The dose-dependent effect of BDNF treatment for 3 or 24 hours on cortical *Bdnf* mRNA expression at DIV 7 (n=4). (C) BDNF (100 ng/ml) increased *Bdnf* exon I, exon IV, and exon VI (n=4). (D) *Ngf* or *Map2* mRNA expression after BDNF (100 ng/mL) application (n=4). The values are mean $\pm$ SEM. * P < 0.05, ** P < 0.01, *** P < 0.001 vs control. # P < 0.05.

Figure 2 Involvement of ERK and Akt signaling in the *Bdnf* mRNA production induced by exogenous BDNF application

(A) Time course (1, 3, 6, 12, and 24 hours) of phosphorylated ERK (pERK) and Akt (pAkt) after the treatment of 100 ng/ml BDNF at DIV 7 (n=6). After stripping the antibody for pERK or pAkt, we performed re-blotting with antibody for ERK or Akt. The effect of U0126 (10 μ M), U73122 (10 μ M), and LY294002 (10 μ M) on BDNF-induced *Bdnf* mRNA expression at (B) 3 and (C) 24 hours after BDNF addition (n=5-6). The values are mean $\pm$ SEM. * P < 0.05, ** P < 0.01, *** P < 0.001 vs control, ††† P < 0.001 vs solo inhibitor application, ## P < 0.01, ### P < 0.001.

Figure 3 Changed levels of *Bdnf* exon mRNA by BDNF in the presence of inhibitor for ERK or PI3K signaling.

Production of each *Bdnf* exon (exon I, exon IV, and exon VI) in the absence or presence of U0126 or LY294002 at 3 (A) and 24 hours (B) after BDNF addition (n=4-6). The values are mean $\pm$ SEM. ** P < 0.01, *** P < 0.001 vs control, † P < 0.05, †† P < 0.01, ††† P < 0.001 vs solo inhibitor application, ## P < 0.01, ### P < 0.001.

Figure 4 CREB and CaMKII are involved in the self-induced *Bdnf* mRNA production

Forskolin (10 μ M) or BDNF (100 ng/mL)-induced *Bdnf* mRNA expression with or without CBP-CREB interaction inhibitor (30 μ M), H-89 (10 μ M), KN-93 (10 μ M), and APV (50 μ M) at 3 (A, C) and 24 hours (B, D) after BDNF stimulation (n=4-6). The values are mean $\pm$ SEM. * P < 0.05, ** P < 0.01, *** P < 0.001 vs control, ††† P < 0.001 vs solo inhibitor application, ## P < 0.01, ### P < 0.001.

Figure 5 Self-amplifying BDNF sustained BDNF/TrkB signaling

(A) The effect of K252a (an inhibitor for TrkB, 200 nM) on *Bdnf* mRNA expression induced by 100 ng/ml of BDNF (n=6). (B) *Bdnf* mRNA levels still increased after exogenous BDNF (100 ng/ml) washout (n=4-5) at DIV 3. (C) TrkB-Fc (1 μ g/ml) abolished the induction of *Bdnf* mRNA levels stimulated by 10 ng/ml of BDNF (n=6). (D) TrkB-Fc (1 μ g/ml) after washout of

1 exogenous BDNF (100 ng/ml, 3 hours) from the medium also blocked the increase of *Bdnf*
2 mRNA levels at DIV 3 (n=6). (E) BDNF-dependent activation of TrkB and downstream
3 signaling in the presence of TrkB-Fc. TrkB-Fc (1 µg/ml) was applied at 3 hours after exogenous
4 BDNF application (10 ng/ml) (n=5-6). The values are mean ± SEM. **P < 0.01, ***P < 0.001 vs
5 control, #*P* < 0.05, ##*P* < 0.01, ###*P* < 0.001 vs in the presence of TrkB-Fc. We stripped the antibody
6 for pERK or pAkt, then, re-blotted with antibody for ERK or Akt.

7
8 **Figure 6 Self-amplifying BDNF contributes to the upregulation of postsynaptic proteins**

9 (A) Representative images of expression of synaptic proteins after BDNF application. (B)
10 Postsynaptic glutamate receptors (NR2A, NR2B, and GluR2/3) and (C) presynaptic proteins
11 (SNAP25, synaptotagmin, and syntaxin) after BDNF (10 ng/ml, 24 hours) treatment with or without
12 TrkB-Fc (1 µg/ml) (n=8-9). The values are mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001 vs
13 control, #*P* < 0.05, ##*P* < 0.01, ###*P* < 0.001.

Figure 1**A** **B** **C** **D**

Figure 2**A** **B** 3 h **C** 24 h

Figure 3**A 3 h** **B 24 h**

Figure 4

Figure 5 **E**

Figure 6**A** **B** **C**