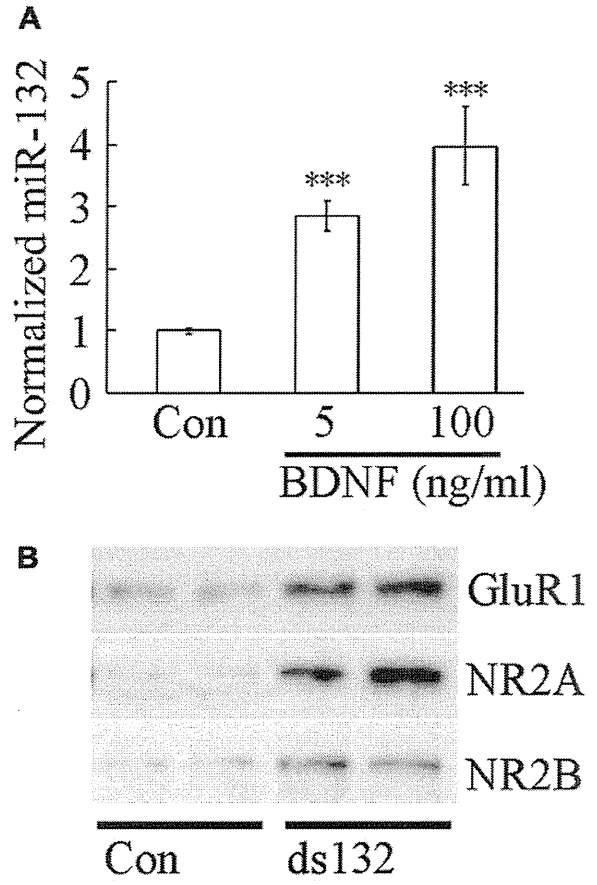

Fig. 4. BDNF induced increase in the levels of miR-132 in cultured cortical neurons. (A) BDNF increased the miR-132 levels in a dose-dependent manner. BDNF (5, or 100 ng/ml) was applied at 4 days *in vitro* (4DIV). Twenty-hours later, quantitative analysis of miR-132 was carried out by RT-PCR. Data represent mean ± S.D. ($n = 4$). ***$P < 0.001$. (B) Ds-miR-132 (double-stranded synthesized mature microRNA) transfection induced upregulation of glutamate receptors (GluR1, NR2A, and NR2B). The transfection was carried out at 9DIV. After an additional 2-day culture, the cortical neurons were harvested. (C) BDNF-induced upregulation of miR-132 is through the MAPK/ERK signaling cascade. U0126 (10 μM) is an inhibitor of the MAPK/ERK pathway. BDNF (100 ng/ml) was applied at 4DIV. Twenty-hours later, levels of miR-132 were determined by RT-PCR. Data represent mean ± S.D. ($n = 4$). ***$P < 0.001$.

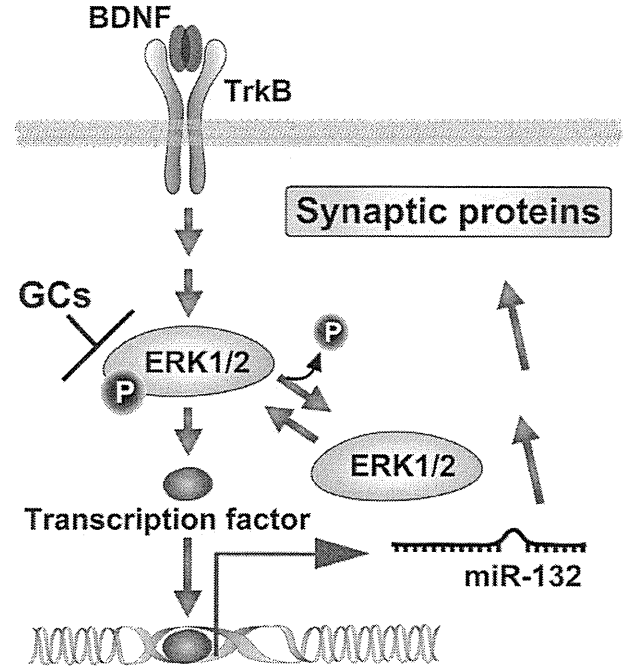

Fig. 5. BDNF increases levels of synaptic proteins via upregulation of miR-132. In cortical neurons, BDNF induces miR-132 expression, which is required for BDNF-increased postsynaptic proteins. The BDNF-induced miR-132 was inhibited by glucocorticoids (GCs). As GCs reduced the ERK1/2 activation, and both GCs and an inhibitor for the ERK1/2 pathway repressed the miR-132 expression, it is possible that BDNF upregulates synaptic proteins via stimulating the ERK/miR-132 system.

4.3. Regulation of BDNF levels by miRs

As shown above, some miRs are involved in BDNF functions including morphological change and regulation of neurotransmission. On the other hand, several recent reports indicate that BDNF expression is directly regulated by miRs. Mellios et al. showed that miR-30a-5p and miR-195 target the 3′UTR of BDNF (Mellios et al., 2008). In rat forebrain neurons, they observed that miR-30a-5p overexpression induced marked decreases in BDNF protein. In a postmortem study using the prefrontal cortex of schizophrenia and control subjects, the association among BDNF, GABAergic transcript [NPY(neuropeptide Y), SST (somatostatin), and PV (parvalbumin) mRNAs], and miRs (miR-195 and miR-30a-5p) was examined by the same group (Mellios et al., 2009). They showed deficits in NPY and PV mRNAs in the schizophrenic group, and levels of BDNF protein were positively correlated with NPY and SST levels. Importantly, BDNF levels showed a strong inverse association with miR-195 levels. NPY, SST, and PV are all well known GABAergic neuronal markers, and abnormalities in GABA-mediated neuronal function are putatively involved in the pathogenesis of schizophrenia (Hashimoto et al., 2008; Lewis et al., 2008). A plethora of evidence indicates that BDNF regulates the GABA system (Seil, 2003; Gottmann et al., 2009), therefore a possible association between miR-195 and schizophrenia is plausible. Though not in the CNS system, miR-15a-1 and miR-18a were reported to play a critical role in zebrafish inner ear development, and this is especially interesting given that BDNF mRNA is one of the miR-15a targets (Friedman et al., 2009). Furthermore, the possible involvement of miR-210 in BDNF regulation was also reported. HEK-293 cells transfected with plasmid encoding miR-210 showed downregulation of BDNF, while, in contrast, BDNF upregulation after transfection of anti-miR-210 oligonucleotide was observed (Fasanaro et al., 2009).

5. miRs and brain diseases

The cross relationship between BDNF and miRs in neurons suggests that miRs may be involved in brain disorders including mental illness. Interestingly, the prefrontal cortex of subjects with schizophrenia demonstrate downregulation of GABAergic genes (NPY and SST mRNAs). It is possible that miR-195 regulates BDNF levels, which may implicate miR-195 as being a key player in the disease-related deficits in the GABAergic genes (Mellios et al., 2009). Case-control studies (with panic disorder and control subjects) show that human miR-22, -138-2, -148a, and -488 are associated with panic disorder. Interestingly, reporter assays reveal that the BDNF gene is potentially repressed by miR-22 (Muiños-Gimeno et al., 2010). Remarkably, it was reported that methyl CpG binding protein 2, MeCP2, regulated striatal BDNF levels and cocaine addiction via homeostatic interaction with miR-212. In their system, a knockdown of striatal MeCP2 decreased the cocaine-seeking behavior of rats. Importantly, miR-212 increased after MeCP2 knockdown, while miR-212 inhibited MeCP2 expression. Considering that blockade of endogenous BDNF signaling in the dorsal striatum decreased cocaine intake, it is possible that the homeostatic interaction of MeCP2 with miR-212 is involved in cocaine addiction via regulation of striatal BDNF levels (Im et al., 2010).

Generally, it is recognized that the loss-of-function mutations in the MeCP2 gene contribute to Rett syndrome (RTT) (Amir et al., 1999). In MeCP2-null mice, reduced levels of BDNF and an increase in amplitude of spontaneous miniature and evoked EPSCs in nTS (nucleus tractus solitarius) neurons were observed. Such synaptic dysfunction in MeCP2-null mice was reversed by exogenous BDNF application (Kline et al., 2010). Recently, Wu et al. identified miRs whose level is changed in MeCP2-null mice before and after the onset of severe neurological symptoms (Wu et al., 2010). They showed that aberrantly increased miRs (including miR-30a/d, miR-381, miR-495) in the absence of MeCP2 may induce downregulation of BDNF in RTT brains. Furthermore, evidence suggests that miRs may influence levels of toxic molecules related to neurodegeneration (Eacker et al., 2009). In neurodegenerative diseases, including Alzheimer's disease (AD), a possible contribution of miRs have been suggested (Hébert and De Strooper, 2009). For example, a decrease in miR-29a/b-1 clusters in sporadic AD showed a correlation with upregulation of BACE1 (β-site APP-cleaving enzyme 1)/β-secretase, the AD related molecules (Hébert et al., 2008). Furthermore, Kim et al. reported miR-133b to be concentrated in the midbrain compared with measurements in the cerebellum and cerebral cortex, while also finding that the midbrain miR-133b levels were decreased in Parkinson's disease patient samples (Kim et al., 2007). Further studies are needed to elucidate the role of miRs expression as a biomarker for brain diseases.

6. Concluding remarks

Brain-specific miRs and BDNF are both involved in neuronal function. Dysfunction of either of these regulatory substances may result in the onset of brain diseases. Indeed, a close relationship between the downregulation of BDNF and the pathophysiology of brain diseases has been suggested (Numakawa et al., 2010b, 2011). Recently, new insight into miRs action has been shown. Some miRs are frequently dysfunctional in cancer, and are present in human plasma in a remarkably stable form. The detection method for measurement of tumor-derived miRs in serum or plasma has been well established (Mitchell et al., 2008). It was revealed that some miRs are released through exosome-dependent exocytosis. These secreted miRs are transferable and functional in the recipient cells (Kosaka et al., 2010), suggesting that specific miRs are promising biomarkers for disorders including cancer. Importantly, the miR-124 level is altered in the serum of patients following brain injury (Laterza et al., 2009). Therefore, in addition to measuring the amount of BDNF, evaluating levels of specific miRs in blood may be diagnostically beneficial in approaching brain diseases, though further study is necessary.

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Phencyclidine-Induced Decrease of Synaptic Connectivity via Inhibition of BDNF Secretion in Cultured Cortical Neurons

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Repeated administration of phencyclidine (PCP), a noncompetitive *N*-methyl-D-aspartate (NMDA) receptor blocker, produces schizophrenia-like behaviors in humans and rodents. Although impairment of synaptic function has been implicated in the effect of PCP, the molecular mechanisms have not yet been elucidated. Considering that brain-derived neurotrophic factor (BDNF) plays an important role in synaptic plasticity, we examined whether exposure to PCP leads to impaired BDNF function in cultured cortical neurons. We found that PCP caused a transient increase in the level of intracellular BDNF within 3 h. Despite the increased intracellular amount of BDNF, activation of Trk receptors and downstream signaling cascades, including MAPK/ERK1/2 and PI3K/Akt pathways, were decreased. The number of synaptic sites and expression of synaptic proteins were decreased 48 h after PCP application without any impact on cell viability. Both electrophysiological and biochemical analyses revealed that PCP diminished glutamatergic neurotransmission. Furthermore, we found that the secretion of BDNF from cortical neurons was suppressed by PCP. We also confirmed that PCP-caused down-regulation of Trk signalings and synaptic proteins were restored by exogenous BDNF application. It is possible that impaired secretion of BDNF and subsequent decreases in Trk signaling are responsible for the loss of synaptic connections caused by PCP.

Keywords: neurotrophin, NMDA receptors, schizophrenia, synaptic function, TrkB signaling pathways

Introduction

Phencyclidine (PCP), a noncompetitive and use-dependent blocker of the *N*-methyl-D-aspartate (NMDA)-type glutamate receptors, induces psychotic symptoms that are similar to schizophrenia in humans (Allen and Young 1978; Javitt and Zukin 1991). Unlike other psychotomimetic drugs, such as amphetamine, PCP induces negative symptoms (e.g., flattening of affect, alogia, anhedonia, and social withdrawal) and cognitive deficits in addition to positive symptoms (e.g., delusions, hallucinations, and formal thought disorder) of schizophrenia (Andreasen 1995; Olney and Farber 1995; Jentsch and Roth 1999). In rodents, PCP causes schizophrenia-related behaviors, such as disruption in prepulse inhibition (Mansbach and Geyer 1989), stereotyped behavior, and social isolation (Sams-Dodd 1996), increased immobility in forced swimming (Noda et al. 1995), and impaired learning and memory in various maze tasks (Kesner et al. 1983; Handelmann et al. 1987; Danysz et al. 1988; Wass et al. 2006). Extensive reduction in the number of spines in the rat prefrontal cortex has been demonstrated with subchronic PCP treatment (Hajszan et al. 2006), indicating a substantial contribution of abnormal synaptic function to the development of schizophrenia-related behaviors (Mirnics et al. 2001; Frankle et al. 2003; McCullumsmith et al. 2004). Moreover, recent findings suggest that altered expression of genes encoding synapse-associated proteins also play a critical role in the development of schizophrenia (Harrison and Weinberger 2005). Human post-mortem studies show reduced dendritic spine density of pyramidal cells in the prefrontal cortex of subjects with schizophrenia (Glantz and Lewis 1997, 2000; Knable et al. 2004). However, the molecular mechanisms underlying the effect of PCP on reduced synaptic connection have not yet been elucidated.

BDNF, a member of the neurotrophin family, plays an important role in synaptic plasticity (Stoop and Poo 1996; Lu 2003; Arancio and Chao 2007, Numakawa et al. 2011) through activation of its receptor Tropomyosin-related kinase B (TrkB) and consequent stimulation of downstream signaling pathways, including mitogen-activated protein/extracellular signal-regulated kinase (MAPK/ERK), phosphoinositide 3-kinase/Akt (PI3K/Akt), and phospholipase C-γ (PLC-γ). We have recently reported important regulatory roles of BDNF in synaptic functions of cortical neurons (Kumamaru et al. 2011). BDNF shows broad expression in the developing and adult mammalian brain (especially, the hippocampus, cerebral cortex, cerebellum, and amygdala) (Ernfors et al. 1990; Hofer et al. 1990; Yan et al. 1997; Conner et al. 1997). As expected, impairment of BDNF/TrkB function has been implicated in the pathogenesis of schizophrenia (Durany and Thome 2004; Angelucci et al. 2005; Lewis et al. 2005), as well as other neuropsychiatric diseases, such as depression (Altar 1999), drug addiction (Davis 2008), Huntington's disease (Zuccato et al. 2001; Gauthier et al. 2004), and Rett syndrome (Chen et al. 2003; Nelson et al. 2008). We found that disrupted-in-schizophrenia 1 (DISC1) and dysbindin, both of which confer susceptibility to schizophrenia, are involved in the regulation of ERK1/2 or Akt signaling (Numakawa et al. 2004; Hashimoto et al. 2006). We have also revealed that glucocorticoid, a stress hormone closely linked to depression (e.g., Kunugi et al. 2006), hampers the synaptic function of BDNF in cortical neurons (Numakawa et al. 2009).

Since expression and secretion of BDNF are facilitated by neuronal activity (Lessmann et al. 2003; Kuczewski et al. 2009),

it is likely that PCP may decrease the expression and/or secretion of BDNF via blockade of neuronal activity. Importantly, increased expression of BDNF was reported in rat hippocampal tissue after acute (Kalinichev et al. 2008) and chronic (Takahashi et al. 2006; Harte et al. 2007) treatment with PCP, although one study reported conflicting results (Semba et al. 2006). In the present study, we investigated changes in expression and secretion of BDNF, activity of downstream signaling cascades stimulated via Trk receptors and synaptic function after PCP exposure.

Materials and Methods

Cortical Cultures and PCP Treatment

Cortical cultures were prepared from postnatal day 1 or 2 old rats (Wistar, SLC, Shizuoka, Japan) as described previously (Numakawa et al. 2002). Dissociated cells were plated at a final density of $5 \times 10^5/cm^2$ on polyethyleneimine-coated culture dishes for immunoprecipitation, immunoblotting, and amino acid measurement. For Ca^{2+} imaging, cortical neurons were cultured on polyethylenimine-coated cover glasses (Matsunami, Osaka, Japan) with FlexiPERM (Greiner Bio-One GmbH, Germany). For immunostaining or electrophysiological recording, neurons were plated on glass-bottom dishes (Matsunami) with a glial feeder layer. The culture medium (5/5 DF) contained 5% fetal bovine serum, 5% heated-inactivated horse serum, 90% of a 1:1 mixture of Dulbecco's modified Eagle's medium, and Ham's F-12 medium. PCP (Sigma-Aldrich, MO) was added to the neurons by bath application at 10–11 days in vitro (DIVs), followed by incubation of the cultures for 3, 6, or 48 h in the presence of PCP before immunocytochemistry, immunoprecipitation, immunoblotting, amino acid measurement and electrophysiological recording. Pure astroglial cultures were prepared as described previously (Hatanaka et al. 1988). Astroglial cells were obtained from cerebral cortex of postnatal day 1 or 2 old rats. All animals were treated according to the institutional guidelines for the care and use of animals.

Immunoblotting

Cells were lysed in sodium dodecyl sulfate (SDS) lysis buffer containing 1% SDS, 20 mM Tris-HCl (pH 7.4), 5 mM ethylene-diamine-tetraacetic acid (EDTA) (pH 8.0), 10 mM NaF, 2 mM Na_3VO_4, 0.5 mM phenylarsine oxide, and 1 mM phenylmethylsulfonyl fluoride. The protein concentration was quantified using a BCA Protein Assay Kit (Pierce Biotechnology Inc., IL), and equivalent amounts of total protein were assayed for each immunoblotting. Primary antibodies were used at the following dilutions: anti-BDNF (1:500, Santa Cruz Biotechnology Inc., CA), anti-TrkB (1:1000, BD Biosciences, NJ), anti-Trk (1:1000, Santa Cruz Biotechnology Inc.), anti-pTyr (1:1000, Upstate, VA), anti-Akt (1:1000, Cell Signaling, MA), anti-pAkt (1:1000, Cell Signaling), anti-ERK (1:1000, Cell Signaling), anti-pERK (1:1000, Cell Signaling), anti-synaptotagmin (1:1000, Calbiochem, Darmstadt, Germany), anti-GluR1 (1:500, Sigma), anti-NR2B (1:500, Sigma), anti-SNAP25 (1:1000, Synaptic Systems, Goettingen, Germany), anti-Bcl-2 (1:1000, BD Biosciences), anti-Bad (1:1000, BD Biosciences), anti-TUJ1 (1:5000, Berkeley Antibody Company, CA), and βactin (1:5000, Sigma) antibodies. The immunoreactivity was quantified by using Lane and Spot Analyzer software (ATTO Corporation, Tokyo, Japan). At least 3 independent series of cultures were used for each set of experiments.

Immunoprecipitation

To detect the phosphorylation of Trk receptors, immunoprecipitation was carried out (Numakawa et al. 2002; Numakawa et al. 2009). After cells were lysed with 1% Triton-X buffer (20 mM Tris-HCl (pH 7.4), 5 mM EDTA, 150 mM NaCl, 1% Triton-X100), anti-Trk antibody (Santa Cruz Biotechnology Inc.) prebound Protein G-Sepharose beads (Amersham Pharmacia, NJ) were mixed with the lysates containing 300 μg of total protein and rotated for 3 h at 4 °C. After 3 washes with the lysis buffer, the proteins that bound the affinity beads were separated by SDS-polyacryl- amide gel electrophoresis (SDS-PAGE) and analyzed for immunoblotting with anti-pTyr (1:1000, Upstate) antibody.

Detection of Cell Surface TrkB

After a gentle wash with ice-cold phosphate-buffered saline (PBS), cells were incubated with 1.1 mg/mL of EZ-Link Sulfo-N-hydroxy- succinimidobiotin (NHS-biotin)-LC-Biotin (Pierce Biotechnology Inc.) in PBS at 4 °C for 20 min. Then, excessive NHS Biotin was quenched with 0.1 M of glycine in PBS and removed by extensive wash with ice-cold PBS. The biotinylated cells were lysed in 1% Triton-X buffer (20 mM of Tris-HCl (pH 7.4), 5 mM of EDTA, 150 mM of NaCl, 1% Triton-X100). Lysates containing equal amounts of protein were mixed with 50 μL of immobilized Neutravidin beads (UltraLink Immobilized NeutrAvidin protein, Pierce Biotechnology Inc.) and incubated at 4 °C for 1 h with gentle rotation. Centrifugated beads were washed 3 times with lysis buffer. The biotin-labeled cell surface proteins were separated by SDS-PAGE and immunoblotted with anti-TrkB antibody (1:1000, BD Biosciences).

Immunocytochemistry

For immunocytochemical staining, neurons were fixed with 4% paraformaldehyde (Sigma) and 4% sucrose in Dulbecco's PBS for 20 min at room temperature. The cells were incubated with PBS containing 0.2% Triton-X (Sigma) for 5 min and blocked by 10% horse serum in PBS for 1 h at 37 °C. Then, anti-MAP2 monoclonal antibody (isotype: IgG1, 1:250, Sigma), anti-BDNF polyclonal antibody (2 μg/μL, produced by Dr Ritsuko Katoh-Semba: Katoh-Semba et al. 1997), anti-GAD67 (glutamic acid decarboxylase 67 kD) monoclonal antibody (1:2000, Millipore, CA) and anti-synaptotagmin monoclonal antibody (isotype IgG2a, 1:100, Chemicon, CA) were applied overnight at 4 °C. BDNF and synaptotagmin were visualized by isotype-specific secondary antibody conjugated with Alexa 488 (1:200, Molecular Probes, CA). MAP2 was visualized by anti-mouse secondary antibody conjugated with Alexa 546 (1:2000, Molecular Probe). A fluorescent microscope (Axiovert 200, Carl Zeiss, Oberkochen, Germany) was used to obtain images. When quantification of BDNF immunoreactivity was conducted, we measured mean intensity of randomly selected areas of cell body or primary dendrites by using imaging software Slide Book TM 3.0 (Intelligent Imaging Innovations Inc., CO).

MTT Assay

To calculate cell viability, the metabolic activity of mitochondria was estimated by measuring the mitochondrial-dependent conversion of the tetrazolium salt, MTT (Sigma) (Numakawa et al. 2009). In brief, after treatment of PCP for 48 h, cultured cells were incubated with MTT solution. Two hours later, cultures were lysed and the metabolic activity of the mitochondrial reductase was estimated.

Detection of BDNF Secretion

Following washes with neurobasal medium and 0.1 mg/mL BSA, cultured neurons were incubated with or without PCP (1 μM) in neurobasal medium containing anti-BDNF antibody (2 μg/mL, Santa Cruz Biotechnology Inc.) for 6 h. Then, medium was carefully collected and the secreted BDNF captured by the antibody was immunoprecipitated. After 3 washes with the lysis buffer, BDNF in immunoprecipitates was detected by immunoblotting with the same anti-BDNF antibody (Santa Cruz Biotechnology Inc.) or ELISA assay (BDNF-ELISA E-max; Promega, WI) with another anti-BDNF antibody, a component of the ELISA kit. For detection of BDNF secretion from cortical acute slices, 200-μm-thick coronal sections were prepared using a microtome (VT1000S, Leica, Nussloch, Germany) from prefrontal cortex of postnatal 30–40 days old male rats in ice-cold HEPES-buffered solution (containing 120 mM NaCl, 4 mM KCl, 1.2 mM KH_2PO_4, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 30 mM glucose, and 20 mM HEPES, pH 7.4). Each slice, which was obtained from right and left cortical hemisphere, was assigned to control and PCP treatment. Freely floating slice sections were incubated with HEPES-buffered solution at 37 °C for 4 h before sampling. Then, after washing several times, secreted BDNF was determined in a similar way used in the culture experiments.

Detection of Amino Acid Neurotransmitters

The amount of amino acid released from cultured neurons was measured as described previously (Numakawa et al. 2002). Briefly, high-performance liquid chromatography (HPLC; Shimazu Co., Kyoto, Japan) was used to measure the amino acids released into the modified HEPES-buffered Krebs Ringer solution (KRH; containing 130 mM NaCl, 5 mM KCl, 1.2 mM NaH_2PO_4, 1.8 mM $CaCl_2$, 10 mM glucose, 1% bovine serum albumin, and 25 mM HEPES, pH 7.4). After the cultures were washed 3 times with KRH buffer, fresh KRH buffer was added to the cultures and collected without stimulation (1 min) as the basal release. Then, potassium (50 mM KCl for 1 min) was added to the cultures in order to induce depolarization.

Electrophysiology

Whole-cell voltage clamp recordings were performed on cultured cortical neurons using an AxoClamp 2B amplifier (Molecular Devices). Cells were continuously superfused with external solution containing 150 mM NaCl, 4 mM KCl, 2 mM CaCl, 1 mM $MgCl_2$, 10 mM HEPES, 10 mM D-glucose, 10 μM glycine (pH = 7.4, 310 mOsm). Miniature excitatory postsynaptic currents (mEPSCs) were isolated by adding picrotoxin (100 μM) and tetrodotoxin (0.5 μM) to the bath. Recordings were performed for 5 min, which enabled us to collect more than 1000 events. Patch electrodes (7–12 MΩ) contained 130 mM Cs-methane sulfonate, 10 mM CsCl, 10 mM $MgCl_2$, 10 mM HEPES, 5 mM MgATP, and 1 mM Na_2GTP (pH 7.3, 300 mOsm). All experiments were carried out at room temperature (27 °C). Cells with an input resistance >200 MΩ (range: 300–800 MΩ) were used. Each neuron was voltage-clamped at −70 mV. For each recording, series resistance and input resistance were continuously monitored, and if these values changed by >15%, the data were discarded. Signals were filtered at 3 kHz and digitized at 10 kHz (Digidata 1320A: Molecular Devices). Off-line analysis of mEPSCs was carried out using Clampfit v 9.2 (Molecular Devices). Miniature events were detected using Mini Analysis software (Synaptosoft) with an amplitude threshold of −5 pA. The events were further inspected visually to exclude inappropriate data, such as overlapped events or events with a noisy baseline.

Imaging of Intracellular Ca^{2+}

Ca^{2+} imaging was performed using fluo-3 dye (Molecular Probes) as previously reported (Numakawa et al. 2002). The changes in the fluo-3 intensity through the fluorescent microscope were analyzed and quantified using Slide Book TM 3.0 (Intelligent Imaging Innovations Inc.) from randomly selected cell bodies. Ca^{2+} imaging experiments were performed at least 3 times for each experimental condition.

Total RNA Extraction and Reverse Transcription

Total RNA was extracted from cultured cells using the RNeasy Plus Kit (QIAGEN, CA) according to the manufacturer's manual. Cells were homogenized by QIAshredder (QIAGEN). To prevent genomic DNA contamination, gDNA Eliminator column (QIAGEN) was used. Total RNA (1 μg) was mixed with SuperScript VILO enzyme and reaction mixes (Invitrogen, CA) in a total volume of 20 μL. After incubation at 25 °C for 10 min, the mixture was heated to 42 °C for 60 min and followed by an inactivation step (85 °C, 5 min). The cDNA solutions were stored at −80 °C until used.

Quantitative PCR

For real-time PCR analyses of mRNA, cDNA was prepared from cultured cortical neurons or pure astrocytes using a TaqMan Cells-to-Ct kit (Applied Biosystems, CA) according to the manufacturer's protocol. Each cDNA was amplified with specific TaqMan Gene Expression Assays (Rn02531967_s1 for rat BDNF; Rn00565046_m1 for rat MAP2; Rn00566603_m1 for rat GFAP: glial fibrillary acidic protein). ABI prism 7000 was used for amplifications. Ct of target mRNA was obtained using Sequence Detection System software (ABI). Serial dilution (1:1, 1:3.3, 1:10, 1:33, and 1:100) of pooled samples was used as a standard. Each gene amplification was normalized with rat GAPDH control (4352338E).

Statistical Analysis

Data are expressed as mean ± standard deviation, and statistical significance was calculated using a one-way ANOVA followed by Scheffe's post hoc test in SPSS ver.18 (SPSS Japan, Tokyo, Japan) if not otherwise specified. The probability values of less than 5% were considered significant.

Results

Transient Increase of Intracellular BDNF Levels by PCP

To determine the acute effects of PCP on neurons, cortical cultures (at DIV 10 and 11) were exposed to PCP, followed by examination of BDNF and its receptor expression. Interestingly, Western blot analyses revealed that 3-h PCP treatment significantly increased levels of intracellular BDNF (14 kD, mature BDNF) (e.g., PCP 1 μM: 152 ± 21.7% of control, $P < 0.01$) (Fig. 1*A,B*). BDNF expression was increased 6 h after 0.1 μM PCP application as well, though statistical significance was not reached (Fig. 1*A,B*). Expression of TrkB and p75 (low affinity receptor for BDNF) were not altered by 3- or 6-h PCP application (Fig. 1*A*, Supplementary Fig. 1*A*). Expression of β-actin and TUJ1 (both are controls) were also intact after PCP exposure (Fig. 1*A*). To check involvement of de novo synthesis in the up regulation of BDNF, we investigated BDNF mRNA after PCP stimulation. Unexpectedly, a significant reduction in BDNF mRNA expression was induced by PCP (1 μM, 3 h) (Supplementary Fig. 1*B*), suggesting that the PCP-induced increase in intracellular BDNF expression is not due to an increase of BDNF translation. Although the cell body size of neurons was not affected (Fig. 1*C*), immunostaining with anti-BDNF antibody showed that the intensity of granular signals in MAP2-positive neurons increased after PCP treatment (1 μM, 3 h) (cell body: 123 ± 10% of control, $P < 0.001$, dendrite: 115 ± 13% of control, $P < 0.001$, *t*-test) (Fig. 1*D,E*). Such granular signals of BDNF were observed in only non-GAD-positive neurons (Supplementary Fig. 1*C*), suggesting that the selected BDNF-positive neurons used to estimate immunoreactivity were glutamatergic neurons. In this study, we confirmed a very small proportion of glial cells in our cortical cultures (Supplementary Fig. 2*A*) and much higher expression of BDNF mRNA in the cortical cultures than that in pure astrocyte cultures. Furthermore, PCP decreased rather than increased the BDNF levels in the pure astrocytes (Supplementary Fig. 2*B*), indicating that the increased expression of BDNF was specific to neurons.

PCP Diminished Activity of BDNF/Trks Signaling

The effect of PCP on activation (phosphorylation) of Trk receptors and downstream signaling pathways was examined. In spite of the increased intracellular BDNF, phosphorylation of Trk receptors was suppressed by 3- and 6-h PCP treatment (PCP 1 μM, 3 h: 49.3 ± 16.3% of control; PCP 1 μM, 6 h: 56.8 ± 10.1% of control, $P < 0.001$ and $P < 0.05$, respectively) (Fig. 2*A*). Three-hour PCP treatment suppressed Trk phosphorylation in all doses tested. Six hours after PCP application, 1–25 μM doses achieved significant inhibition of Trk phosphorylation (Fig. 2*A*). When alterations in cell surface expression of the TrkB receptor were examined, we confirmed that PCP (10 μM) did not change the amount of surface TrkB, while the ligand BDNF indeed reduced surface TrkB levels (Ji et al. 2010) (Fig. 2*B*). As shown in Figure 2*C,D*, activation of Akt (a component of the PI3K pathway) and ERK1/2 (MAPK pathway) were also

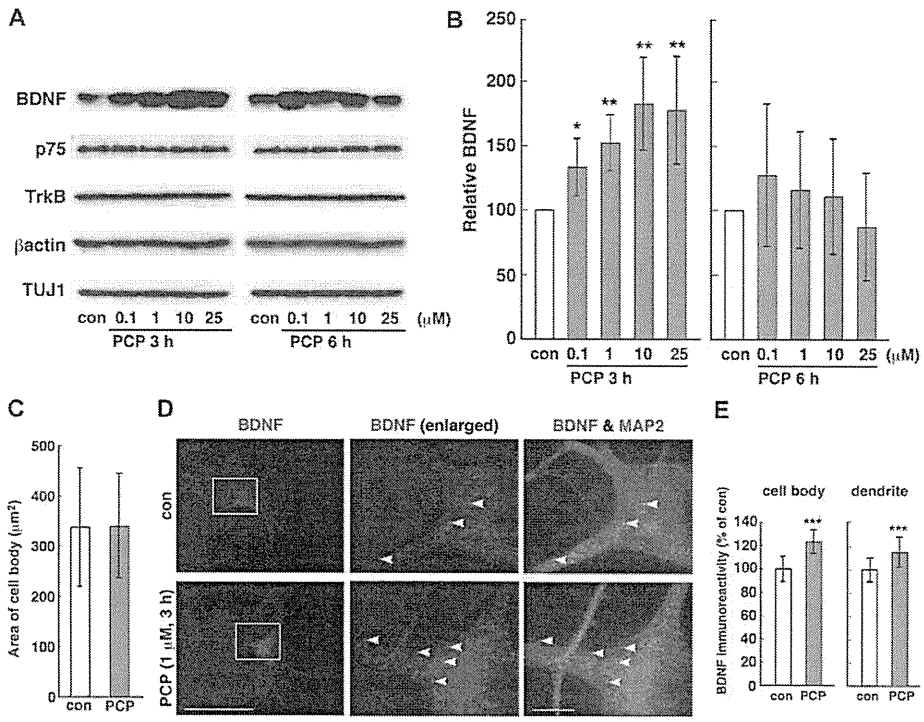

Figure 1. PCP increased levels of intracellular BDNF in cultured cortical neurons. (*A*) BDNF levels (14 kD, mature BDNF) in total lysate were rapidly increased after PCP treatment, whereas expression levels of TrkB and p75 were unchanged. Cultured cortical neurons at DIV 10 and 11 were treated with PCP at indicated doses. Samples were collected 3 or 6 h after PCP addition. TUJ1 and β-actin were represented as controls. (*B*) The mature BDNF expression was quantified. Data represent mean ± standard deviation ($n = 6$, obtained from 6 independent cultures. n indicates the number of experiments if not otherwise specified.). $^{**}P < 0.01$, $^{*}P < 0.05$ versus con (without PCP). Statistical significance was evaluated by one-way ANOVA followed by Scheffe's post hoc test. (*C*) Size of cell body area was not changed by PCP. After 48 h of PCP (10 μM) treatment, neuronal cell body sizes were measured (con: $n = 13$; PCP: $n = 12$, n indicates the number of neurons in each experimental condition). (*D*) Immunocytochemistry revealed a significant increase in BDNF immunoreactivity in cortical neurons in PCP-treated cultures. Cells were immunostained with anti-BDNF and anti-MAP2 antibodies. PCP (1 μM) was applied for 3 h. Left: images after staining with anti-BDNF antibody (green). Bar = 50 μm. Middle: magnified images of insets in Left panels. Right: double staining with anti-BDNF (green) and anti-MAP2 (red) antibodies. Typical BDNF-containing vesicles are indicated by arrowheads. Bar = 10 μm. (*E*) Quantification of BDNF immunoreactivity. Mean intensity of BDNF immunoreactivity in one or two areas of cell body per 1 cell (around 200 μm^2) and in 4–6 primary dendrites (around 50 μm^2) were measured (cell body: con, $n = 20$ and PCP, $n = 22$; primary dendrite: con, $n = 95$ and PCP, $n = 80$; n indicates the number of areas obtained from 14 randomly selected neurons in 4 different dishes of the same culture preparation). $^{***}P < 0.001$ (*t*-test).

decreased by PCP (e.g., PCP 1 μM, 3 h: phosphorylated Akt: 62.9 ± 11.7% of control, $P < 0.001$; phosphorylated ERK1: 61.4 ± 6.7% of control, $P < 0.01$, phosphorylated ERK2: 51.6 ± 10.3% of control, $P < 0.01$), whereas expression of total Akt and ERK1/2 were intact. The reduced activation of Akt and ERK1/2 were maintained until 6 h after PCP treatment (Fig. 2*C,D*). To elucidate which Trk receptor (i.e., TrkA, TrkB, or TrkC) is responsible for the activation of intracellular signaling, cortical neurons were treated with various neurotrophins (Fig. 2*E,F*). BDNF and neurotrophin 4/5 (NT-4/5), both of which are specific ligands for TrkB, strongly activated Trk receptors, Akt and ERK1/2, whereas nerve growth factor (NGF, a specific ligand for TrkA) did not. NT-3 (for TrkC, and weakly for TrkA and TrkB) also demonstrated low activation ability. These data suggest that TrkB signaling is responsible for the activation of Akt and ERK1/2 pathways in our cultures.

Impaired Secretion of BDNF Caused by PCP

In cortical neurons, intracellular BDNF was increased following PCP exposure, whereas activation of Trk and downstream signaling cascades were decreased. To examine whether BDNF secretion was affected by PCP, conditioned medium of cultures incubated in the presence of anti-BDNF antibody was collected. The immunoprecipitated BDNF using conditioned media showed the same molecular weight as that of recombinant mature BDNF in Western blot analysis, and we found reduced amounts of secreted BDNF after PCP exposure (1 μM, 6 h, Fig. 3*A*). A significant reduction in the secreted BDNF was observed (60.7 ± 9.9% of control, $P < 0.001$) in PCP-treated cultures (Fig. 3*B*). Such reduction in immunoprecipitated BDNF was further confirmed by enzyme-linked immunosorbent assay (ELISA) (Fig. 3*C*). Furthermore, we examined BDNF secretion in acute cortical slices and found significant suppression in the amount of secreted BDNF caused by PCP (1 μM, 3 h, Supplementary Fig. 3). It is well known that secretion of BDNF depends on neuronal activity (Hartmann et al. 2001; Lessmann et al. 2003). We tested the effect of glutamate (excitatory neurotransmitter) and tetrodotoxin (Na^+ channels blocker) on BDNF secretion in cultured neurons. As expected, increased BDNF by glutamate and decreased BDNF by TTX in conditioned media were observed (glutamate: 395 ± 114% of control, $P < 0.001$; TTX 49.1 ± 21.0% of control, $P < 0.001$) (Fig. 3*D,E*). These data suggest that PCP repressed the activity-dependent secretion of BDNF from cortical neurons.

Exposure to PCP for 48 h Did Not Affect Neuronal Viability

How does the suppression of BDNF/Trk signaling induced by PCP influence cortical neurons at the cellular and neuronal

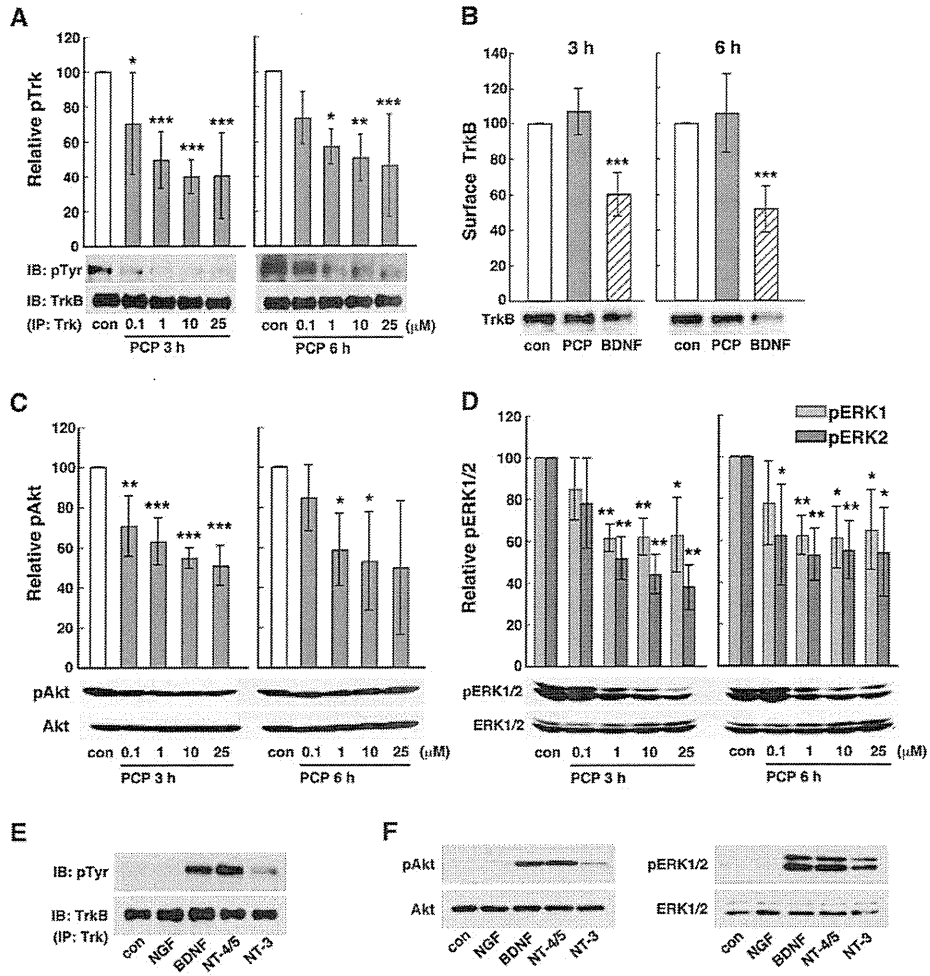

Figure 2. PCP decreased activation of Trk receptors, MAPK/ERK1/2 and PI3K/Akt pathways. (*A*) Phosphorylation of Trk receptors was suppressed after PCP treatment for 3 and 6 h. Cell lysates were immunoprecipitated with anti-Trk antibody. For immunoblotting, anti-phospho-Tyr (pTyr) or anti-TrkB antibodies were used. Phosphorylated Trk receptors (pTrk) were quantified (3 h: $n = 10$; 6 h: $n = 6$, obtained from 5 independent cultures). $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$ versus con (one-way ANOVA followed by Scheffe's post hoc test). (*B*) The amount of TrkB receptors on the cell surface after PCP (10 μM) treatment for 3 or 6 h was shown. Note that BDNF (100 ng/mL, positive control for TrkB internalization) exposure decreased surface expression of TrkB while PCP did not. $^{***}P < 0.001$ versus con. (*C,D*) Activation of Akt (a component of the PI3K pathway) and ERK1/2 (MAPK pathway) were reduced up to 6 h after PCP addition. Quantification of phosphorylated Akt (pAkt) (*C*) and ERK1/2 (pERK1/2) (*D*) was conducted ($n = 4$, respectively. Obtained from 4 independent cultures). $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$ versus con. (*E,F*) Trk signaling in cortical neurons stimulated by various neurotrophins. (*E*) Following immunoprecipitation with anti-Trk antibody, immunoblotting with anti-pTyr and TrkB antibodies was performed. Each neurotrophin was applied at 100 ng/mL. Note that BDNF and NT-4/5 significantly stimulated Trk receptors in cortical neurons. (*F*) PI3K/Akt (Left) and MAPK/ERK1/2 (Right) pathways.

network level? To approach this issue, the survival of neurons after exposure to PCP was examined. After 48 h of exposure to 1 μM PCP, cortical neurons were immunostained with anti-MAP2 antibody (Fig. 4*A*). Quantified data of MAP2-positive living cells revealed no difference in neuronal survival between control and PCP-treated cultures (Fig. 4*B*). MTT assay also indicated that PCP (0.1–25 μM, 48 h) treatment did not affect cell viability (Fig. 4*C*). As shown in Fig. 4*D*, expression of Bcl-2, an antiapoptotic protein, and Bad, a proapoptotic protein, were unchanged by PCP (1 μM, 48 h).

PCP Diminished the Number of Presynaptic Sites and Synaptic Transmission

We next investigated the effect of PCP (48 h) on synaptic function. Western blot analysis revealed decreased expression of presynaptic proteins (synaptotagmin and synaptosome-associated protein of 25 kD [SNAP25]) and postsynaptic glutamate receptors (NR2B and GluR1) (Fig. 5*A*). Immunostaining with anti-synaptotagmin antibody also demonstrated a decrease in the number of presynaptic puncta after PCP (1 μM, 48 h) exposure (13.8 ± 2.9 per 50 μm of dendrite in control and 8.3 ± 3.0 in PCP, $P < 0.001$) (Fig. 5*B,C*). Importantly, basal and depolarization-induced release of glutamate were both decreased after 48 h of PCP treatment (Fig. 5*D*), suggesting that PCP decreases the number of glutamatergic synapses. Electrophysiological recordings on cultured cortical neurons revealed that PCP (1 μM, 48 h) reduced the frequency of mEPSCs in both NMDA receptor and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor components (Fig. 6*A,B*). Cumulative plots also indicated a shift to longer intervals in both AMPA receptor- and NMDA receptor-mediated mEPSCs after PCP treatment (Fig. 6*C*), while the amplitude of mEPSCs was not changed (Fig. 6*D*).

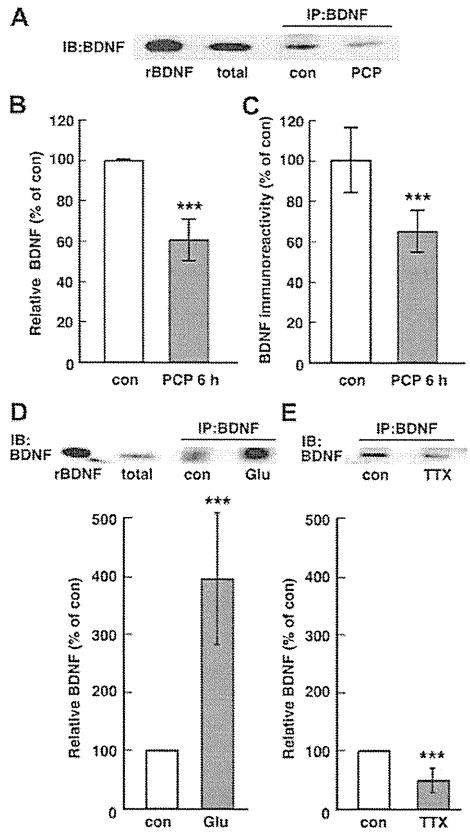

Figure 3. PCP decreased secretion of BDNF from cortical neurons. Secreted BDNF into culture medium was immunoprecipitated with anti-BDNF antibody. In the presence of anti-BDNF antibody (2 μg/mL), cortical neurons were incubated with or without PCP at 1 μM for 6 h, followed by immunoprecipitation. A representative Western blot (*A*) and quantified data (*B*) were shown ($n = 12$, from 5 independent cultures). $^{***}P < 0.001$ versus con (*t*-test). rBDNF: recombinant BDNF; total: total lysates (10% input). (*C*) ELISA analysis also showed a decreased amount of BDNF secretion by PCP (con: $n = 11$; PCP: $n = 12$, n indicates the number of dishes for each experimental condition). (*D*, *E*) Glutamate increased secretion of BDNF while TTX decreased it. Cortical neurons were treated with glutamate (1 μM, 15 min) (*D*) or TTX (3 μM, 6 h) (*E*). (Glutamate: $n = 6$, TTX: $n = 5$, from 5 independent cultures, respectively). $^{***}P < 0.001$ versus con (*t*-test).

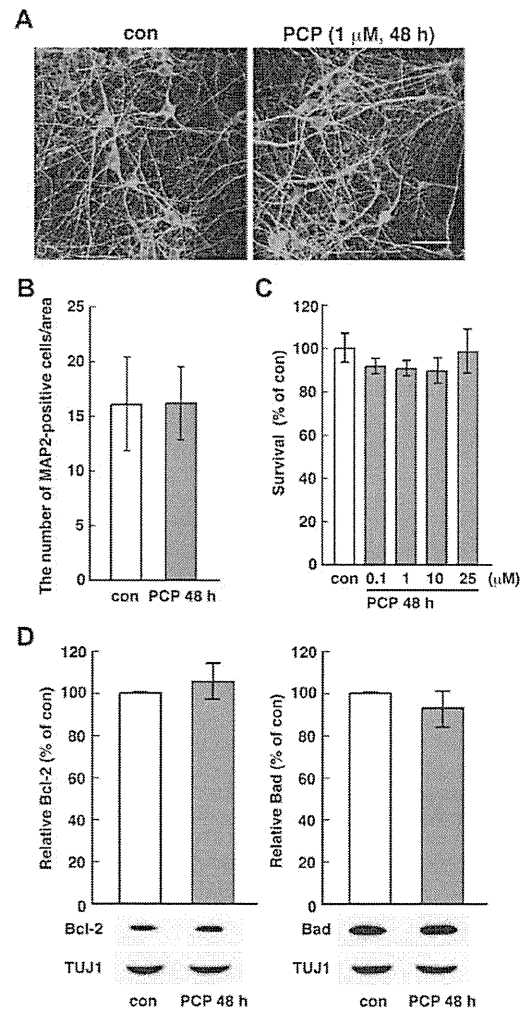

Figure 4. PCP did not affect neuronal viability. (*A*) Forty-eight hours after PCP (1 μM) treatment, cortical neurons were stained with anti-MAP2 antibody. Bar = 50 μm. (*B*) These MAP2-positive cells were counted ($n = 18$, n indicates the number of randomly selected 250 × 250 μm^2 fields from 5 dishes for each experimental condition). (*C*) PCP (0.1–25 μM, 48 h) treatment did not affect the cell viability, which was determined by MTT assay ($n = 4$). Normalization to control was carried out. (*D*) Both Bcl-2 (an antiapoptotic protein) and Bad (a proapoptotic protein) levels were not altered by PCP (1 μM, 48 h) ($n = 4$). TUJ1 is represented as control.

Exogenous BDNF Prevented PCP's Suppression of Intracellular Signaling and Synaptic Protein Expression

We investigated whether coapplication of BDNF with PCP could prevent the reduction in ERK1/2 and Akt activation at 3 h later as well as prevent the decrease in synaptic protein expression at 48 h later. As expected, exogenous BDNF (10 ng/mL) completely prevented the reduction of intracellular signaling (ERK 1/2 and Akt activation, Fig. 7*A*) and of synaptic proteins (synaptotagmin, SNAP25, NR2B, and GluR1, Fig. 7*B*). Delayed BDNF application at 3 h after PCP addition also prevented down regulation of synaptic proteins caused by PCP (Supplementary Fig. 4). These data suggest that the PCP-induced loss of synaptic connectivity is due to the impaired secretion of endogenous BDNF.

PCP Blocked Intracellular Ca^{2+} Mobilization and Other NMDA Receptor Antagonists Induced Intracellular BDNF Increase

Increase in the intracellular Ca^{2+} concentration is required for the activity-dependent secretion of BDNF (Hartmann et al. 2001; Lessmann et al. 2003). Thus, we assessed the possibility that the PCP-suppressed BDNF secretion is due to an inhibition of Ca^{2+} influx mediated by glutamate receptors. First, as shown in Fig. 8*A*, change in levels of glutamate receptors including NR2A, NR2B, and GluR1 during in vitro maturation were determined. We found that these proteins gradually increased during in vitro maturation, while very low expression levels at DIV 4 and 5 occurred (Fig. 8*A*). Synaptotagmin and SNAP25, presynaptic proteins, also increased during in vitro maturation in our cultures. Previously, we reported that cultured cortical neurons develop spontaneous Ca^{2+} oscillations during in vitro maturation, and the endogenous phenomenon is mediated via glutamatergic neurotransmission (Numakawa et al. 2002). Therefore, we determined whether PCP affected spontaneous Ca^{2+} oscillations at DIV 12 and found that PCP treatment (1 μM, 3 h) dramatically reduced the endogenous Ca^{2+} activity (Fig. 8*B,C*). In some cases, we observed a resting series of sister

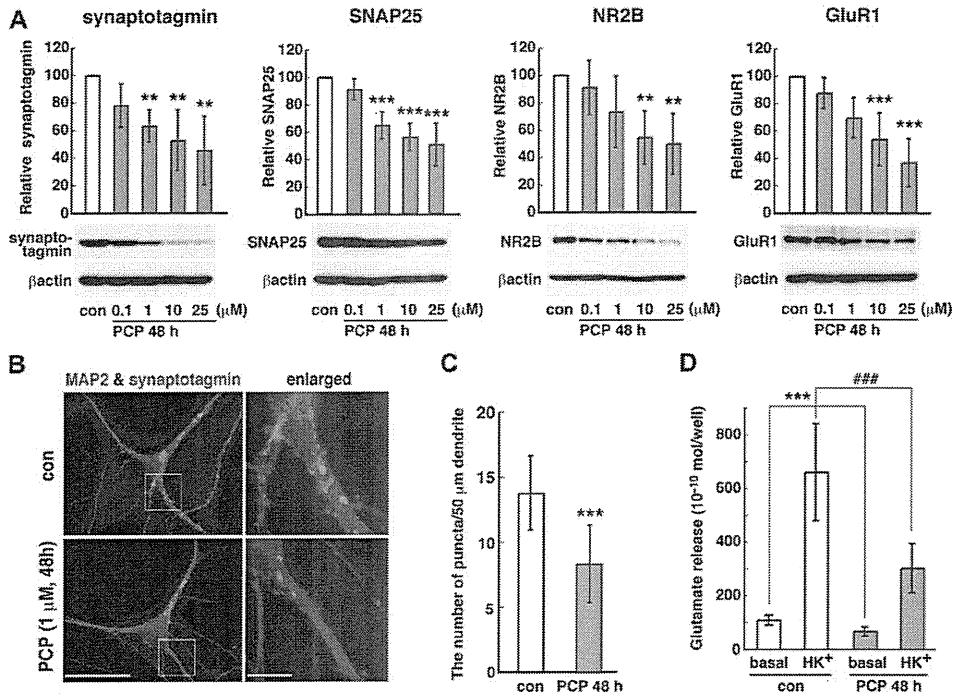

Figure 5. PCP reduced the number of synaptic sites, expression of synaptic proteins, and release of glutamate. (*A*) The amount of synaptic proteins, including synaptotagmin, SNAP25, NR2B, and GluR1, were reduced by PCP. Samples were collected and analyzed after 48 h PCP (0.1–25 μM) treatment. $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$ versus con (one-way ANOVA followed by Scheffe's post hoc test). ($n = 7$, from 5 independent cultures.) (*B*) The number of presynaptic sites was decreased by PCP (1 μM, 48 h). Representative images after synaptotagmin (red) and MAP2 (green) immunostaining are shown. Left: Bar = 50 μm, Right: inset in Left panels was magnified. Bar = 10 μm. (*C*) Synaptotagmin-positive presynaptic sites were counted. Thirty-eight randomly selected primary dendrites (per 50 μm) from 14 neurons in 4 randomly selected dishes for each experimental condition were selected. $^{***}P < 0.001$ versus con (*t*-test). (*D*) Glutamate release from cultured neurons was diminished by exposure to PCP. Glutamate from cortical cultures was collected for 1 min as nonstimulated basal and as high potassium-stimulated release. HK^{+}; high potassium (KCl, 50 mM) condition. $^{***}P < 0.001$, $^{\#\#\#}P < 0.001$ (*t*-test). $n = 6$. *n* indicates the number of dishes for each experimental condition.

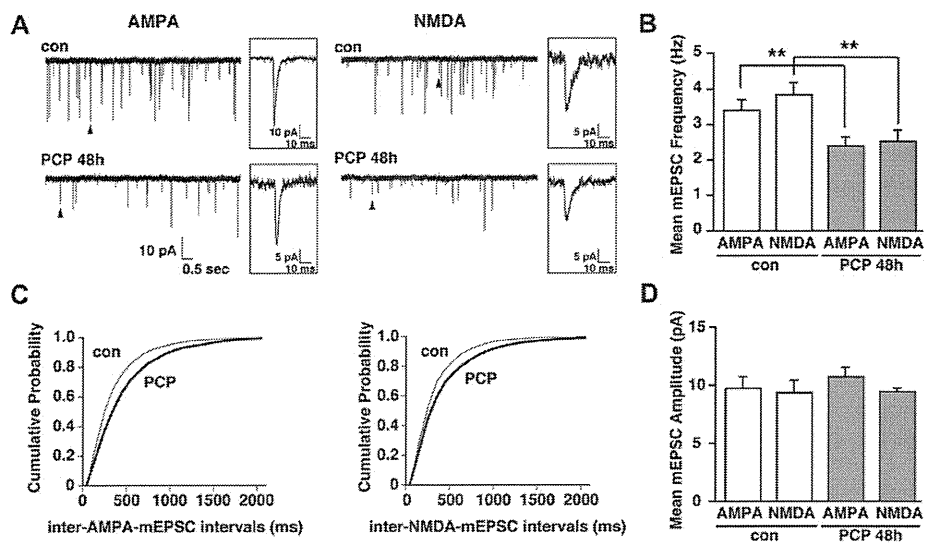

Figure 6. PCP decreased the frequency (but not amplitude) of mEPSCs. Cortical cultures were exposed to PCP (1 μM, 48 h), followed by recording of mEPSCs. (*A*) AMPA component of mEPSCs (AMPA-mEPSC) was recorded in control- and PCP-treated neurons in the presence of D-APV (25 μM) (Left). NMDA component of mEPSPs (NMDA-mEPSC) in both control and PCP conditions in the presence of CNQX (5 μM) with Mg^{2+} free was also measured (Right). Typical mEPSC for each condition (arrowhead) are shown in the inset. (*B*) Mean mEPSC frequency for control cells and PCP-treated cells. Error bars represent SEM. $^{**}P < 0.05$ (*t*-test). (*C*) Cumulative probability histogram for inter-AMPA-mEPSC (Left) and inter-NMDA-mEPSC intervals (Right) from control (thin) and PCP treatments (solid). Each data point represents the mean from 5 cells. (*D*) Mean amplitude of mEPSC in control and in PCP treatments. *n* indicates the number of cells obtained from 6 to 10 independent cultures.

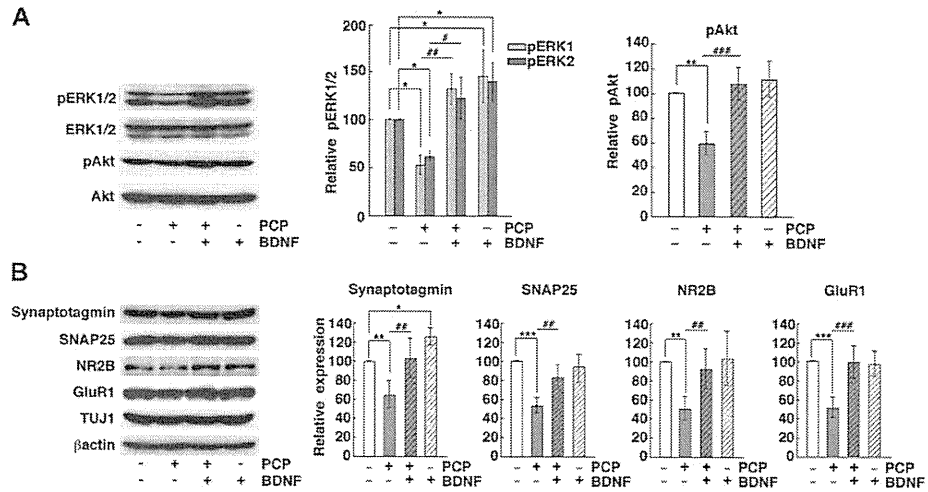

Figure 7. Exogenous BDNF prevented down regulation of intracellular signaling and synaptic protein expression. BDNF (10 ng/mL) was added immediately after PCP (10 μM) application. (*A*) Exogenous BDNF prevented the negative effect of PCP on ERK 1/2 and Akt activation. Samples were collected at 3 h after PCP application. ($n = 4$, from 2 independent cultures.) $^{**}P < 0.01$, $^{*}P < 0.05$ versus con; $^{\#\#\#}P < 0.001$, $^{\#\#}P < 0.01$, $^{\#}P < 0.05$ versus PCP (one-way ANOVA followed by Scheffe's post hoc test). (*B*) Decrease in levels of synaptic proteins, including synaptotagmin, SNAP25, NR2B, and GluR1 after PCP treatment (10 μM, 48 h) did not occur in the presence of BDNF (10 ng/mL). TUJ1 and β-actin are controls. ($n = 5$, from 3 independent cultures.) $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$ versus con; $^{\#\#\#}P < 0.001$, $^{\#\#}P < 0.01$ versus PCP (one-way ANOVA followed by Scheffe's post hoc test).

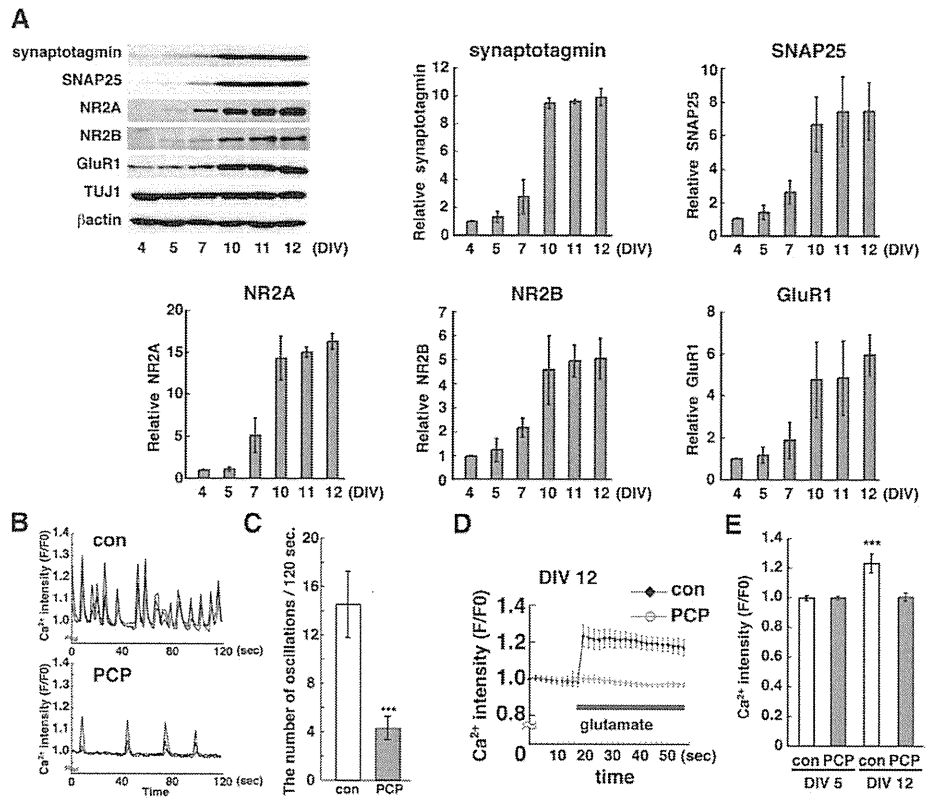

Figure 8. PCP inhibited intracellular Ca^{2+} elevation in mature cortical neurons. (*A*) Developmental change in the expression of glutamate receptors (NR2A, NR2B, and GluR1), in addition to synaptotagmin and SNAP25, were shown. Expression of these synaptic proteins was gradually increased during in vitro maturation and reached maximum levels around DIV 10. ($n = 3$, from 3 independent cultures). TUJ1 and β-actin are controls. (*B*) Spontaneous Ca^{2+} oscillations were decreased in cultured cortical neurons in the presence of PCP (1 μM, 3 h). Traces were obtained from 3 neurons at DIV 12 in the same dish for each condition. The fluorescence ratio (F/F_0, F_0; basal intensity) was calculated. (*C*) The number of the frequency in Ca^{2+} oscillations was counted. ($n = 6$, n indicates the number of dishes of a sister culture). $^{***}P < 0.001$ versus con. (*D*) Glutamate-triggered intracellular Ca^{2+} elevation was blocked by PCP. PCP (1 μM) was applied 3 h before glutamate (1 μM) stimulation. DIV 12. (*E*) Quantified data of the evoked Ca^{2+} in the presence or the absence of PCP at DIV 5 or at DIV 12. Summarized data from 50 randomly selected cells for each experimental condition. $^{***}P < 0.001$ versus con. (one-way ANOVA followed by Scheffe's post hoc test).

cultures, in which all neurons showed no Ca^{2+} oscillations as previously reported (Numakawa et al. 2002). Then, the glutamate-evoked increase in intracellular Ca^{2+} concentration was also checked using such resting neurons (Fig. 8*D,E* and Supplementary Fig. 5*A*). Importantly, PCP treatment for 3 h repressed the glutamate-stimulated Ca^{2+} (Fig. 8*D,E* and Supplementary Fig. 5*A*). Such suppression of Ca^{2+} elevation was still observed at 6 h after PCP addition (Supplementary Fig. 5*B*). In line with the low expression level of glutamate receptors, immature neurons at DIV 5 did not respond to glutamate with or without PCP (Fig. 8*E*). In the DIV 5 immature neurons, PCP had no effect on intracellular BDNF expression (Supplementary Table 1). Furthermore, we confirmed a positive effect of other noncompetitive and competitive antagonists for NMDA receptors (MK-801 and APV, respectively) on intracellular BDNF levels in DIV 12 neurons (but not in DIV 5 neurons) (Supplementary Table 2), indicating that the effect is not specific to PCP but it holds true for NMDA receptor antagonists in general. These results suggest that inhibition of Ca^{2+} influx via NMDA receptors was involved in the decrease in BDNF secretion by PCP.

Discussion

In the present study, we found that intracellular BDNF protein was transiently increased by 3-h PCP exposure. Despite such a BDNF increase, activation of Trks and downstream signaling pathways (ERK1/2 and Akt) were diminished. Importantly, the number of synaptic sites and expression of synaptic proteins were reduced 48 h after PCP application. Furthermore, both basal and depolarization-evoked glutamate release were decreased in PCP-treated neurons, and the electrophysiological studies also revealed a reduced frequency of mEPSCs by PCP. Interestingly, we discovered suppression in BDNF secretion after PCP treatment both in cortical cultures and acute slices. Application of exogenous BDNF prevented the PCP-induced reduction in expression of synaptic proteins in cortical cultures. It is possible that the PCP-induced impairment of BDNF secretion results in the transient accumulation of intracellular BDNF and leads to down regulation of BDNF/TrkB signaling, which is required for maintenance of synaptic protein expression. As PCP treatment significantly inhibited the spontaneous Ca^{2+} activity and evoked Ca^{2+} influx by glutamate, the inhibition of Ca^{2+} mobilization may contribute to the PCP-induced impairment of BDNF secretion.

In hippocampal and cortical neurons, BDNF showed a vesicular expression pattern in dendrites and axons and appeared to be sorted into a regulated pathway in which BDNF is secreted in response to neuronal activity (Goodman et al. 1996; Farhadi et al. 2000; Hartmann et al. 2001 Kojima et al. 2001; Kohara et al. 2001; Gartner and Staiger 2002; Lessmann et al. 2003; Wu et al. 2004; Adachi et al. 2005). The activity-dependent secretion of BDNF is triggered by an increase in intracellular Ca^{2+} concentration via ionotropic glutamate receptors, voltage-gated Ca^{2+} channels, and internal Ca^{2+} stores (Hartmann et al. 2001; Lessmann et al. 2003). In the present study, we examined the possibility that PCP attenuated an increase of intracellular Ca^{2+} concentration at basal and evoked conditions, contributing to the transient increase of intracellular BDNF and suppression of its secretion. In mature cortical neurons, we found that PCP induced an inhibition of Ca^{2+} mobilization, and that BDNF protein released into the culture medium was reduced after PCP application compared with control. This reduction in BDNF secretion was demonstrated by both immunoblotting and ELISA methods after immunoprecipitation with anti-BDNF antibody. Interestingly, when we examined the effect of PCP on de novo synthesis of BDNF, we found decreased levels of BDNF mRNA (Supplementary Fig. 1*B*), suggesting that PCP-dependent BDNF increase is not due to transcriptional activity. Resultant down regulation of BDNF protein levels might appear as a reduction in the amount of intracellular BDNF at 6 h or later. Importantly, other NMDA antagonists, MK-801 (noncompetitive) and APV (competitive), also elevated BDNF levels in neurons, suggesting that the PCP-increased BDNF would be due to the inhibitory effect of PCP on NMDA receptors. Furthermore, such an increase of BDNF by NMDA antagonists occurred only in mature cortical neurons (DIV 12) that express adequate glutamate receptors. PCP-dependent impairment of BDNF secretion and subsequent decrease in synaptic function may only occur in mature fully developed neurons that express adequate NMDA receptors.

The reduction of BDNF secretion is considered to be a neuronal response to PCP, as we confirmed that the majority of cultured cells in the experiment were indeed neurons. Furthermore, PCP did not cause an intracellular increase of BDNF in pure astroglial cultures. In our cortical cultures, vesicular expression of BDNF was observed only in GAD-negative neurons. If this vesicular pattern of BDNF expression reflects the activity-dependent population of BDNF secretion, it is possible that PCP specifically impacts the regulatory release of BDNF from glutamatergic (not GAD-positive) neurons. Importantly, some reports suggest that preferential binding of PCP to NMDA receptors on GABAergic interneurons results in the activation of glutamatergic pyramidal neurons in vivo (Homayoun and Moghaddam 2007; Kargieman et al. 2007). Homayoun and Moghaddam reported that firing rates in ~69% of GABAergic neurons and 86% of pyramidal neurons were decreased after PCP injection (Homayoun and Moghaddam 2007). Interestingly, Kargieman et al. showed that PCP increases and decreases the activity of 45% and 33% of the pyramidal neurons, respectively (Kargieman et al. 2007). Our results indicated a decreased synaptic activity in cultured cortical neurons after 48 h PCP exposure. Furthermore, PCP-dependent decrease in the secretion of BDNF from acute cortical slices, in which local neuronal circuits remain intact, was confirmed. It is possible that differences in experimental conditions including dose of PCP and neuronal maturity may influence such a different neuronal response to PCP, although future studies will be needed using in vivo and in vitro systems.

Secretion of BDNF to the extracellular space is required to generate its biological effects via activation of TrkB. Indeed, activation of Trk receptors and downstream signaling cascades (ERK1/2 and Akt) were reduced by PCP. In our system, BDNF and NT-4/5 functioned as major contributors for the activation of Trk receptors, suggesting that TrkB signaling is predominant. A subset of the BDNF/TrkB downstream signaling molecules, especially ERK1/2 activity, is known to be regulated by NMDA receptor-mediated Ca^{2+} influx (Xia et al. 1996; Sutton and Chandler 2002). Therefore, it is possible that decreased activity of ERK1/2 may be attributable, at least in part, to the blockade of the NMDA receptor by PCP directly. However, simultaneous application of exogenous BDNF blocked the PCP-dependent suppression of synaptic protein levels as well as ERK1/2 and Akt signaling pathways even when NMDA receptors were

blocked in the presence of PCP. Recently, we reported that ERK1/2 activity is involved in the maintenance of synaptic protein expression (Kumamaru et al. 2011). Furthermore, delayed application of exogenous BDNF reversed the suppression of some synaptic proteins inhibited by PCP. All things considered, impaired BDNF secretion substantially contributed to the down regulation of ERK1/2 activity and synaptic protein expression.

The number of synaptic sites was decreased when chronic PCP exposure was administered. There are 2 lines of evidence: 1) the reduced expression of pre- and postsynaptic proteins assessed with Western blotting and 2) the decreased number of presynaptic sites estimated with immunostaining. We also obtained evidence for functional changes, showing a marked reduction in glutamate release as well as a decreased frequency of mEPSCs mediated by NMDA and AMPA receptors. Taken together, excitatory neurotransmission is suppressed by 48 h of PCP treatment.

It is well known that BDNF/TrkB signaling plays an important role in synaptic plasticity. BDNF stabilizes and increases dendritic synapse density in the optic tectum (Hu et al. 2005; Sanchez et al. 2006). BDNF increases spine density in hippocampal neurons through ERK1/2 activation (Alonso et al. 2004). We also reported that BDNF increases the expression of pre- and postsynaptic proteins via ERK1/2 signaling in cultured cortical neurons (Kumamaru et al. 2011). Overexpression of TrkB or activation of PI3K/Akt signaling enhances motility of dendritic filopodia and synaptic density (Luikart et al. 2008). These findings, including our current results, suggest that decreased BDNF secretion caused by PCP is one of the major factors for loss of synaptic connections and/or overall neuronal function.

In the present study, 48 h of PCP treatment did not change both the number of MAP2-positive cells and mitochondrial activity in cortical cultures. Mitochondrial activity was not influenced even when a critically high concentration of PCP (25 μM) was applied. Furthermore, expression levels of both an antiapoptotic protein Bcl-2 and a proapoptotic protein Bad were unchanged after PCP application. These data suggest that PCP has no major influence on survival of cultured cortical neurons, although it does inhibit synaptic connectivity and function. Interestingly, Lei et al. (2008) reported that PCP causes apoptosis in cultured cortical neurons through suppression of Akt activity and activation of GSK3β and caspase-3. This discrepancy between Lei et al. (2008) and our study may be attributable to differences in culture conditions, as their neurobasal medium contained B27 while our 5/5 DF medium contained serums.

PCP induces schizophrenia-like behaviors in humans (Allen and Young 1978; Javitt and Zukin 1991) and rodents (Noda et al. 1995; Furuta and Kunugi 2008). In vivo administration of PCP causes extensive reduction in the number of spines in the rat prefrontal cortex (Hajszan et al. 2006) and suppression of glutamate release in the prefrontal cortex of mice (Nabeshima et al. 2006; Murai et al. 2007). Postmortem brain studies from schizophrenia patients demonstrate that the number of neurons in the prefrontal cortex is not decreased (Pakkenberg 1993; Akbarian et al. 1995), although synaptophysin immunoreactivity and dendritic spine density of pyramidal cells are reduced (Glantz and Lewis 1997; Glantz and Lewis 2000; Knable et al. 2004). These findings are consistent with our observation of synaptic loss and decreased glutamatergic transmission without any change in cell viability in PCP-treated cortical neurons. Therefore, impairment in BDNF secretion and downstream signaling may be involved in the pathogenesis of schizophrenia-like behaviors. In fact, altered expression of BDNF and TrkB has been reported in postmortem brains of schizophrenia patients in several studies (e.g., Takahashi et al. 2000; Durany et al. 2001; Weickert et al. 2003; Weickert et al. 2005; Hashimoto et al. 2005; Altar et al. 2009). Recently, biological functions of proneurotrophins through the p75 receptor were revealed (Lee et al. 2001; Dechant and Barde 2002; Pagadala et al. 2006). Considering this, it may be valuable to study not only TrkB-stimulated signaling but also p75-stimulated signaling, during PCP exposure.

In conclusion, our results suggest that impaired secretion of BDNF and the resultant decrease in activation of Trk receptor signaling pathways are responsible, at least in part, for the PCP-dependent reduction in synaptic connectivity and function, which may be involved in PCP's ability to elicit schizophrenia-like behaviors. Our experimental system might be a "cell model" suitable for studies to clarify the molecular mechanisms of schizophrenia.

Supplementary Material

Supplementary material can be found at: http://www.cercor.oxfordjournals.org/

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Growth factors stimulate expression of neuronal and glial miR-132

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ABSTRACT

Brain-specific microRNAs (miRs) and brain-derived neurotrophic factor (BDNF) are both involved in synaptic function. We previously reported that upregulation of miR-132 is involved in BDNF-increased synaptic proteins, including glutamate receptors (NR2A, NR2B, and GluR1) in mature cortical neurons [7]. However, the potential role of other growth factors in miR-132 induction has not been clarified. Here, we examined the effect of growth factors including basic fibroblast growth factor (bFGF), insulin-like growth factor-1 (IGF-1), glial cell line-derived neurotrophic factor (GDNF), and epidermal growth factor (EGF), on expression of miR-132 and glutamate receptors in immature cortical neurons. We found that BDNF and bFGF upregulated levels of miR-132 in cortical cultures, though bFGF failed to increase glutamate receptors such as NR2A, NR2B, and GluR1. IGF-1, GDNF, and EGF did not have a positive influence on miR-132 and glutamate receptors in neuronal cultures. Furthermore, bFGF significantly upregulated miR-132 in cultured astroglial cells, while other growth factors failed to elicit such a response. It is possible that the growth factor-stimulated neuronal and glial action of miR-132 plays a critical role in brain function.

1. Introduction

Multiple cellular mechanisms including differentiation, death, and metabolism are influenced by miRs via regulating expression of target genes [1,2,19]. It is possible that brain-specific miRs play an important role in synaptic plasticity. Deletion of the Dicer1 gene in mouse forebrain caused diminished levels of brain-specific miRs including miR-124, -132, -137, -138, -29a, and -29c, resulting in increased hippocampal synaptic activity and more robust translation of BDNF [8]. Importantly, Schratt et al. showed that miR-134 helps to regulate hippocampal spine size [21]. They reported that miR-134 negatively regulates spine size via inhibiting the translation of Limk1 mRNA (LIM kinase 1), and that BDNF relieves suppression of Limk1 caused by miR-134 [21].

It is well known that BDNF, which is an important neurotrophin, contributes to regulation of synaptic plasticity in addition to neuronal survival and differentiation via stimulating the mitogen-activated protein kinase/extracellular signal-regulated protein kinase (MAPK/ERK), phospholipase Cγ (PLCγ), and phosphatidylinositol 3-kinase (PI3K) intracellular signaling pathways [5,14,15]. Recent studies revealed an interaction between brain-specific miRs and BDNF. Interestingly, Vo et al. showed that miR-132, one of the brain-specific miRs, is involved in the biological effect of BDNF. They found that marked upregulation of miR-132 was induced by BDNF in cultured cortical neurons. Overexpression of miR-132 increased the outgrowth of primary neurites, while an application of antisense for miR-132 decreased neurite outgrowth [23]. We also reported that BDNF stimulates miR-132 expression, contributing to greater BDNF-mediated upregulation of glutamate receptors including NR2A, NR2B, and GluR1 in cultured cortical neurons [7]. Importantly, we confirmed that the MAPK/ERK pathway is essential for BDNF-increased miR-132 levels and maintenance of glutamate receptor expression [7,9,22]. Taken together, it is possible that miR-132 plays a role in BDNF functions. However, the possible contribution of other growth factors in the regulation of miRs is not well known in the central nervous system (CNS).

Here, we examined the effect of growth factors including BDNF, bFGF, IGF-1, GDNF, and EGF on the expression of miR-132 in both neuronal and astroglial cultures. We found that BDNF and bFGF upregulated miR-132 in cortical cultures, though IGF-1, GDNF, and EGF did not. Furthermore, we found a significant miR-132 increase stimulated by bFGF, but not by other factors, in glial cultures.

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2. Materials and methods

2.1. Cortical neurons and astroglial cultures

Cortical neuronal cultures were prepared as previously reported [16]. Dissociated cortical neurons obtained from postnatal 1- to 2-day-old rats were plated at a final density of $5 \times 10^5/cm^2$ on polyethyleneimine-coated plates or dishes (BD Falcon, CA, USA). Neural cultures from the cerebral cortex of rats were maintained with a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium containing 5% fetal bovine serum (FBS) and 5% heated-inactivated horse serum. Astroglial cultures were also prepared from rat cerebral cortex [4]. Dissociated glial cells were plated on non-coating dishes. The pure astroglial cells, judging from immunostaining with anti-glial fibrillary acidic protein (GFAP) antibody (1:1000, CHEMICON, CA, USA), were grown on dishes in medium containing 10% FBS. Cells were used for assay after reaching 70–80% confluence.

2.2. Growth factor treatment

To stimulate cortical neurons, growth factors including BDNF, bFGF, IGF-1, GDNF, and EGF, were applied at 4 days in vitro (4 DIV). Twenty-four hours later, neuronal cells were harvested for sample collection for PCR and immunoblotting. When the glial cell contribution in cortical neuronal cultures was checked, arabinosylcytosine (1.0 μM, SIGMA, MO, USA) was applied at 24 h after cell plating to block proliferation of glial cells. We found no change in BDNF-stimulated intracellular signaling with or without pretreatment with arabinosylcytosine as previously reported [12]. Growth factors (BDNF, bFGF, IGF-1, GDNF, and EGF) were also added to pure astroglial cultures. Treatment with growth factors was maintained for 24 h before sample collection for PCR and immunoblotting. U0126 (10 μM) was applied 20 min before growth factor addition. Regeneron Pharmaceutical Co., Takeda Chemical Industries Ltd., and Sumitomo Co. Ltd. donated the BDNF and bFGF. IGF-1, GDNF, and EGF were purchased from R&D Systems (MN, USA), Wako Pure Chemical Industries Ltd. (Osaka, Japan) and from Invitrogen (CA, USA), respectively.

2.3. RNA isolation and quantification of mature miR-132

Total RNA, including miRs, was extracted from cultured cells using the mirVana miRNA isolation kit (Ambion, Austin, TX, USA) at room temperature according to the manufacturer's instructions. The total RNA was eluted with 50 μl of elution buffer provided by the manufacturer. 100 ng of total RNA was reverse transcribed using the TaqMan miRNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA) and miR-specific stem-loop primers (part of TaqMan miRNA assay kit; Applied Biosystems). The expression levels of miR-132 were quantified by real-time PCR using specific primers with the 7900HT Fast Real-Time PCR System (Applied Biosystems) according to the manufacturer's instructions.

Expression of miR-9 and -124a were similarly determined using specific primers. There is no current consensus on the use of an internal control for real-time PCR analysis of miR. Therefore, we used a fixed amount of starting total RNA (100 ng) and inserted this into the RT reaction (5 μl) in each assay for technical consistency. All values were normalized to the same amount of miR-16 as an endogenous control. Data analysis was performed by SDS 2.2 real-time PCR data analysis software (Applied Biosystems).

2.4. Immunoblotting

Lysate collection and western blotting were prepared as described [13]. Briefly, neuronal or glial cultures were lysed in SDS lysis buffer (1% SDS, 20 mM Tris–HCl (pH 7.4), 5 mM EDTA (pH 8.0), 10 mM NaF, 2 mM Na_3VO_4, 0.5 mM phenylarsine oxide, and 1 mM phenylmethylsulfonyl fluoride). To load an equivalent amount of protein for each immunoblotting, protein concentration in each sample was quantified using a BCA Protein Assay Kit (PIERCE, IL, USA). Primary antibodies were used at the following dilutions: anti-Akt (1:1000, Cell Signaling, MA, USA), anti-pAkt (1:1000, Cell Signaling), anti-ERK (1:1000, Cell Signaling), anti-pERK (1:1000, Cell Signaling), anti-NR2A (1:500, SIGMA), anti-NR2B (1:500, SIGMA), anti-GluR1 (1:1000, CHEMICON, CA, USA), and anti-βactin (1:5000, SIGMA) antibodies. The immunoreactivity was quantified by using Lane & Spot Analyzer software (ATTO Corporation, Tokyo, Japan).

2.5. Statistical analysis

Data shown are presented as mean ± standard deviation (SD). Statistical significance was evaluated using a one-way ANOVA followed by Scheffe's post hoc test in SPSS ver11 (SPSS Japan, Tokyo, Japan). Probability values less than 5% were considered statistically significant.

3. Results

We used cultured cortical neurons to investigate the effect of several growth factors (including BDNF, bFGF, IGF-1, GDNF, and EGF) on induction of miR-132 expression. BDNF application was performed at 4 DIV and a significant increase in expression of miR-132 was observed 24 h later (Fig. 1A). bFGF also increased miR-132 expression, though the induced levels were lower compared with the levels achieved by BDNF. On the other hand, other factors including IGF-1, GDNF, and EGF all failed to upregulate miR-132 in neuronal cultures. In the induction of miR-132 expression, the dose-dependency of BDNF's effect was determined (Fig. 1B). A dose-dependent effect of bFGF was also observed (Supplementary Fig. S1). As shown in Fig. 1C, we investigated the effect of growth factors on miR-9 and miR-124a as the control, and no change in these miRs expression by growth factors were observed. In the present study, the mature form of miRs was quantified.

Previously, we reported that BDNF upregulated miR-132 and that the upregulation of miR-132 was important for a BDNF-mediated increase in postsynaptic protein levels. Therefore, in the present study, a change of glutamate receptor expression after exposure to growth factors was investigated. As expected, 24 h-BDNF treatment induced upregulation of NR2A, NR2B, and GluR1, which are glutamate receptor subunits, in cortical cultures (Fig. 2A and B). Interestingly, bFGF did not increase the levels of these glutamate receptors. Though IGF-1 demonstrated an increased trend in the expression of these postsynaptic proteins (especially on GluR1), the IGF-1 effect did not reach statistical significance (Fig. 2A and B). Both GDNF and EGF had no influence on levels of NR2A, NR2B, and GluR1 in cultured cortical neurons (Fig. 2A and B).

Next, we examined the effect of growth factors on miR-132 expression in pure astroglial cells. In astroglial cultures, BDNF had no effect (Fig. 3A). On the other hand, bFGF positively influenced astroglial miR-132 expression. All other growth factors such as IGF-1, GDNF, and EGF did not have any effect on glial miR-132 levels (Fig. 3A). The dose-dependent effect of bFGF on glial miR-132 expression was determined (Fig. 3B). In astroglial cultures, no influence on miR-9 and -124a levels by all growth factors was observed (Fig. 3C).

In the present study, we compared endogenous miR-132 levels between cortical neurons and pure astrocytes. Neuronal expression of miR-132 was approximately three times higher than that of astrocytes (Supplementary Fig. S2A). As expected, mRNA

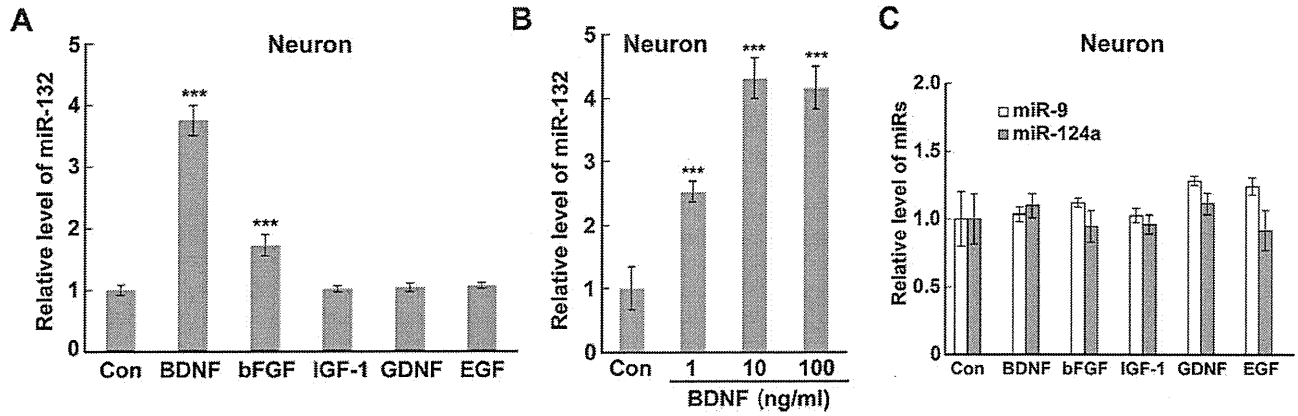

Fig. 1. Effect of growth factors (including BDNF, bFGF, IGF-1, GDNF, and EGF) on induction of miR-132 in cultured cortical neurons. (A) Following growth factor application for 24 h, levels of miR-132 were determined by real-time PCR. All values were normalized to miR-16 levels. Growth factors were applied at the final concentration of 100 ng/ml, respectively. Data represent mean ± SD ($n = 4$, n indicates the number of dishes for each experimental condition). ***$P < 0.001$. Reproducibility was confirmed with separated cortical cultures. (B) Dose-dependency of BDNF effect on miR-132 upregulation ($n = 4$). ***$P < 0.001$. (C) Levels of miR-9 and -124a after growth factor stimulation (100 ng/ml, 24 h) were shown ($n = 3$).

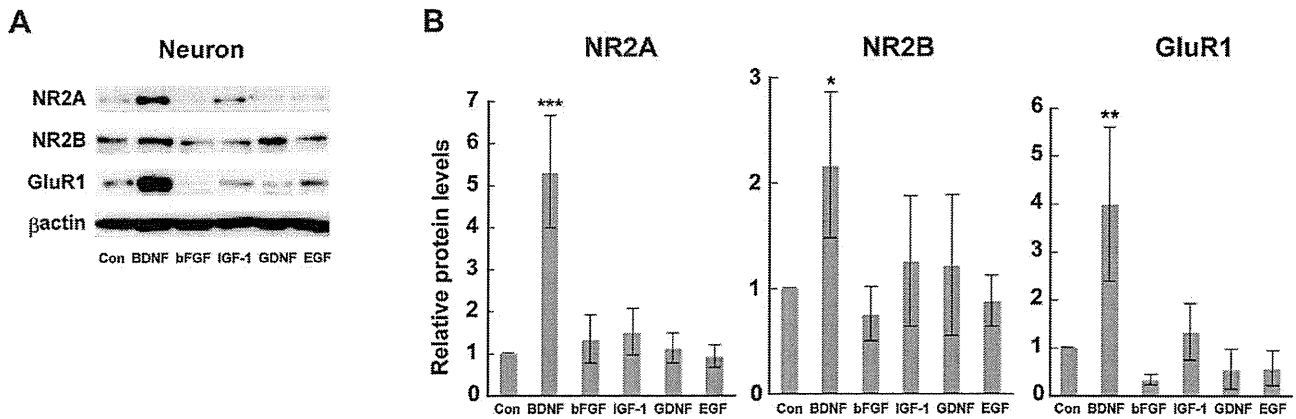

Fig. 2. Effect of growth factors (BDNF, bFGF, IGF-1, GDNF, and EGF) on expression of glutamate receptor subunits in cultured cortical neurons. (A) Levels of NR2A, NR2B, and GluR1 were determined by immunoblotting after growth factor application for 24 h. Each growth factor was applied at 100 ng/ml. βactin is a control. (B) Quantification was carried out. Normalization to a level in control (Con, without growth factors) was performed. Data represent mean ± SD (NR2A, $n = 7$, NR2B, $n = 6$, GluR1, $n = 5$). ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$.

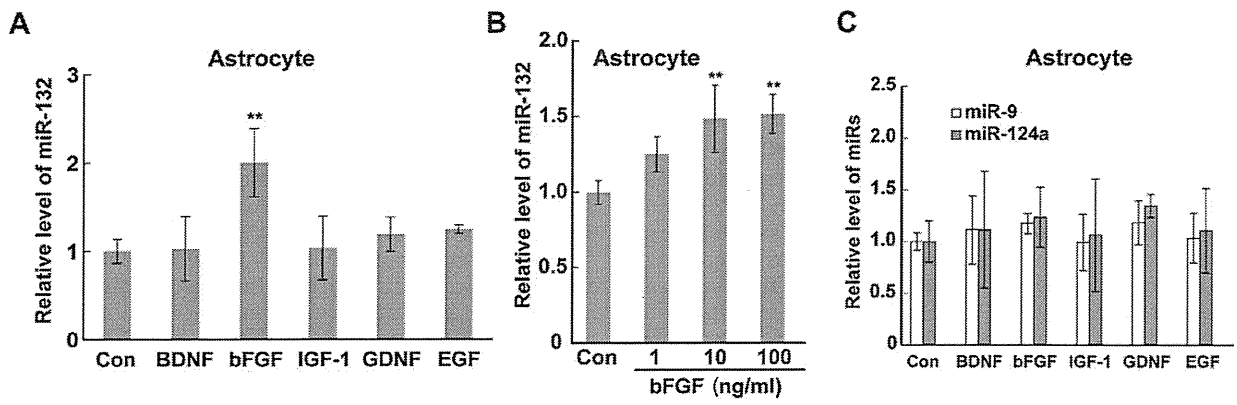

Fig. 3. Effect of growth factors on miR-132 expression in cultured astroglial cells. (A) Astroglial expression of miR-132 was determined by real-time PCR. Growth factors, including BDNF, bFGF, IGF-1, GDNF, and EGF, were applied at 100 ng/ml for 24 h, respectively. Data represent mean ± SD ($n = 5$, n indicates the number of dishes for each experimental condition). **$P < 0.01$. (B) Dose-dependency of bFGF effect on glial miR-132 ($n = 4$). **$P < 0.01$. (C) Growth factors did not change levels of glial miR-9 and -124a. Growth factors were applied at 100 ng/ml for 24 h, respectively ($n = 3$).

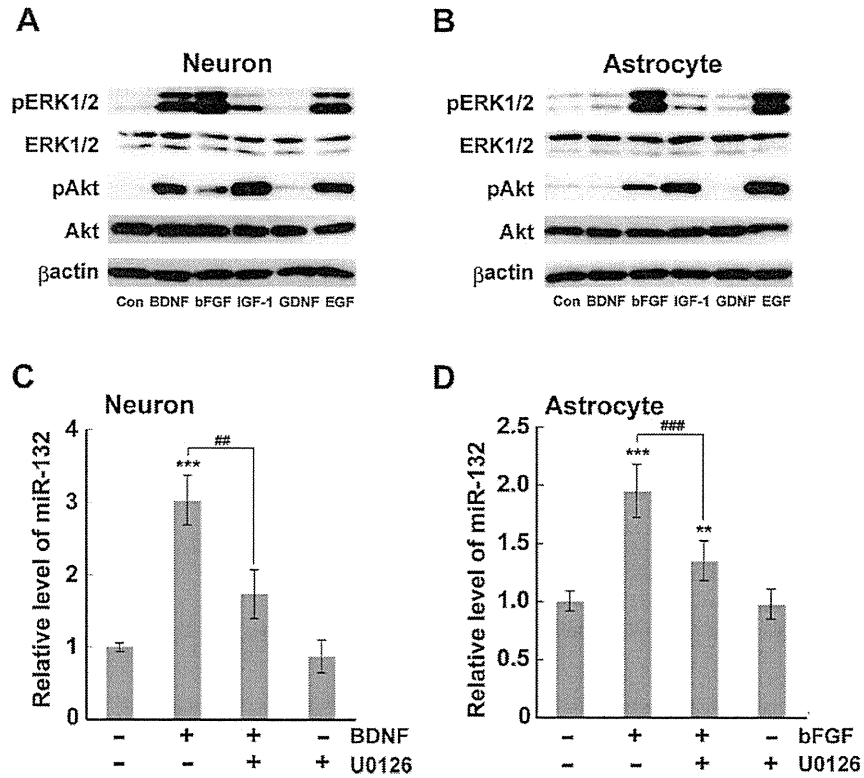

Fig. 4. MAPK/ERK signaling is involved in upregulation of miR-132. Activation of intracellular signaling including MAPK/ERK and PI3K/Akt pathways after growth factor (BDNF, bFGF, IGF-1, GDNF, and EGF) stimulation was examined in neuronal or glial cultures. (A) Activated ERK1/2 (phosphorylated ERK1/2, pERK1/2), activated Akt (phosphorylated Akt, pAkt), total ERK1/2, and total Akt in cortical neurons. Growth factors were applied at 100 ng/ml for 20 min. (B) Levels of pERK1/2, pAkt, total ERK1/2, and total Akt in astroglial cultures. Treatment with growth factors was carried out at 100 ng/ml for 20 min. (C) U0126, an inhibitor for ERK1/2 signaling, inhibited BDNF-induced miR-132 expression in neurons. BDNF (100 ng/ml) was applied for 24 h ($n=3$). $^{***}P<0.001$, $^{\#\#}P<0.01$. (D) U0126 inhibited bFGF-induced miR-132 expression in astrocytes. bFGF (100 ng/ml) was applied for 24 h ($n=5$). $^{***}P<0.001$, $^{**}P<0.01$, $^{\#\#\#}P<0.001$.

expression of microtubule-associated protein 2 (MAP2, neuronal marker) in our cortical cultures was robust, while very low levels of GFAP mRNA were confirmed (Supplementary Fig. S2B and C). Astroglial cultures expressed high levels of GFAP mRNA (Supplementary Fig. S2C).

As shown in Fig. 4A, we examined whether BDNF, bFGF, IGF-1, GDNF, and EGF elicited intracellular signaling cascades, including MAPK/ERK and PI3K/Akt pathways, in neuronal cultures, as BDNF-stimulated increases in miR-132 expression in mature cortical neurons is via activation of MAPK/ERK [7]. BDNF, bFGF, IGF-1, and EGF stimulated both MAPK/ERK and PI3K/Akt pathways, while GDNF did not (Fig. 4A). In pure glial cultures, BDNF could not stimulate these intracellular signaling cascades (Fig. 4B). Importantly, bFGF, IGF-1, and EGF activated MAPK/ERK and PI3K/Akt pathways in glial cultures, whereas GDNF did not have any effect on these signaling cascades (Fig. 4B). As shown in Fig. 4C and D, BDNF and bFGF induced miR-132 expression in neuronal and glial cultures, respectively, while both of these responses were suppressed by U0126, an inhibitor for ERK signaling. bFGF-induced miR-132 expression in neuronal cultures was also blocked by U0126 (Supplementary Fig. S3). Recently, it was reported that the expression of pri/pre-miR-132 is altered to a larger extent than mature miR-132 after growth factor stimulation [20]. Pre-miRs are processed and approximately 22nt mature miRs are generated following cleavage by Dicer in the cytoplasm [1]. Thus, we examined possible alterations in pre-miR-132 expression with Northern blotting, but failed to detect pre-miR-132, although expression of mature miR-132 was significant (Supplementary Fig. S4).

4. Discussion

In the present study, BDNF induced upregulation of miR-132 and increased expression of glutamate receptors in cultured cortical neurons, though astroglia miR-132 expression was not influenced. Others including IGF-1, GDNF and EGF did not change neuronal miR-132 and glutamate receptor levels. Interestingly, bFGF increased levels of neuronal and glial miR-132, but did not impact the expression of glutamate receptors in neuronal cultures.

Upregulation of miR-132 is involved in neuronal BDNF functioning in the CNS. Vo et al. showed that BDNF induces upregulation of miR-132 and that miR plays a critical role in the outgrowth of primary neurites in cultured cortical neurons [23]. We previously reported that 24 h-treatment with BDNF was able to induce miR-132 expression in 10–11 DIV matured cortical neurons. Furthermore, BDNF-mediated miR-132 expression enhanced the overall expression of glutamate receptors including NR2A, NR2B, and GluR1 in mature neurons [7]. In the present study, we found that 4 DIV of immature cortical neurons also responded to BDNF in a significant manner. We confirmed that BDNF upregulated these glutamate receptors in immature neurons, suggesting that BDNF plays a role in synaptic development, in addition to regulation of synaptic plasticity in a matured cortical system. Interestingly, induction of miR-132 appeared when bFGF was used to stimulate cortical neurons, while IGF-1, GDNF, and EGF had no effect. Many studies reported that IGF-1, GDNF, and EGF exert a biological effect on the neuronal population [6,18,24]. Though experimental conditions such as neuronal maturity, brain region, or neuron