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GLUCOCORTICOID ATTENUATES BRAIN-DERIVED NEUROTROPHIC FACTOR-DEPENDENT UPREGULATION OF GLUTAMATE RECEPTORS VIA THE SUPPRESSION OF MICRORNA-132 EXPRESSION

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Abstract—Brain-specific microRNAs (miRs) may be involved in synaptic plasticity through the control of target mRNA translation. Brain-derived neurotrophic factor (BDNF) also contributes to the regulation of synaptic function. However, the possible involvement of miRs in BDNF-regulated synaptic function is poorly understood. Importantly, an increase in glucocorticoid levels and the downregulation of BDNF are supposed to be involved in the pathophysiology of depressive disorders. Previously, we reported that glucocorticoid exposure inhibited BDNF-regulated synaptic function via weakening mitogen-activated protein kinase/extracellular signal-regulated kinase1/2 (MAPK/ERK) and/or phospholipase C-γ (PLC-γ) intracellular signaling in cultured neurons [Kumamaru et al (2008) Mol Endocrinol 22:546–558; Numakawa et al (2009) Proc Natl Acad Sci U S A 106:647–652]. Therefore, in this study, we investigate the possible influence of glucocorticoid on BDNF/miRs-stimulated biological responses in cultured cortical neurons. Significant upregulation of miR-132 was caused by BDNF, although miR-9, -124, -128a, -128b, -134, -138, and -16 were intact. Transfection of exogenous ds-miR-132 induced marked upregulation of glutamate receptors (NR2A, NR2B, and GluR1), suggesting that miR-132 has a positive effect on the increase in postsynaptic proteins levels. Consistently, transfection of antisense RNA to inhibit miR-132 function decreased the BDNF-dependent increase in the expression of postsynaptic proteins. U0126, an inhibitor of the MAPK/ERK pathway, suppressed the BDNF-increased miR-132, suggesting that BDNF upregulates miR-132 via the MAPK/ERK1/2 pathway. Interestingly, pretreatment with glucocorticoid (dexamethasone, DEX) reduced BDNF-increased ERK1/2 activation, miR-132 expression, and postsynaptic proteins. We demonstrate that the exposure of neurons to an excess glucocorticoid results in a decrease in the BDNF-dependent neuronal function via suppressing miR-132 expression.

Key words: BDNF, MAPK, ERK, synaptic function, neurotrophin.

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Abbreviations: BDNF, brain-derived neurotrophic factor; DEX, dexamethasone; miRs, microRNAs; DIV, days *in vitro*; GR, glucocorticoid receptor; MAPK/ERK1/2, mitogen-activated protein kinase/extracellular signal-regulated kinase1/2; PI3K, phosphatidylinositol 3-kinase; PLC-γ, phospholipase C-γ; NMDA, N-methyl-D-aspartate.

MicroRNAs (miRs) are endogenous ~22 nt RNAs that regulate various gene expression profiles via targeting mRNAs for cleavage or translational repression (Bartel, 2004). A growing number of reports suggest that miRs are important for various cellular processes, including differentiation, apoptosis, and metabolism in both animals and plants (Schratt et al., 2006; Chapman and Carrington, 2007; Pillai et al., 2007). Recently, the neuronal roles of miRs have been proposed. MiR-132 is increased by (brain-derived neurotrophic factor) BDNF and is involved in the promotion of neuronal outgrowth (Vo et al., 2005). Conversely, miR-134 negatively regulates the size of postsynaptic sites through inhibiting the translation of an mRNA encoding a protein kinase, Limk1 (Schratt et al., 2006).

BDNF, a neurotrophin, is essential for a variety of neuronal aspects, including proliferation, differentiation, and survival in the CNS. In addition, BDNF promotes synaptic maturation and modulates synaptic plasticity, including long-term potentiation (LTP) (Bibel and Barde, 2000). Importantly, BDNF may be a key molecule related to the pathophysiology of mental disorders. For example, expression of BDNF is low in the brains of suicide victims with depressive disorder (Karege et al., 2005). Reduction in the level of BDNF was also observed in chronically stressed rats (Smith et al., 1995; Hansson et al., 2003). Interestingly, a failure in the control of glucocorticoid (a stress hormone) homeostasis is thought to be involved in the symptoms of depressive disorder (Holsboer, 2000; Kunugi et al., 2006). We have recently reported that glucocorticoid suppressed the BDNF-induced synaptic maturation and excitatory neurotransmitter glutamate release (Kumamaru et al., 2008; Numakawa et al., 2009), while antidepressants reinforced the BDNF-triggered glutamate release (Yagasaki et al., 2006). Collectively, these results suggest that the prevention of BDNF action by increased glucocorticoid is closely related to depressive disorders.

It has been suggested that miR-132 is involved in BDNF function; however, the mechanism underlying the induction of miR-132 and the change in its function after exposure to glucocorticoid has not been fully elucidated. Here, we investigated the effect of BDNF on expression of miR-132 and its function with or without glucocorticoid pretreatment.

EXPERIMENTAL PROCEDURES

Chemicals

Dexamethasone (DEX) (Biomol International LP, PA, USA), a synthetic glucocorticoid receptor (GR)-selective agonist, was dis-

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solved in dimethyl sulfoxide (DMSO, Wako Pure Chemical Industries, Ltd., Osaka, Japan). DMSO alone had no effect compared with no treatment (data not shown). U0126 (an inhibitor for MEK, an upstream molecule of MAPK/ERK1/2) was purchased from Promega (WI, USA), and used at a final concentration of 10 μM. PD98059 (an inhibitor for MEK, Calbiochem-Novabiochem GmbH, CA, USA) was used at 50 μM. LY294002 (a PI3 kinase inhibitor, Calbiochem-Novabiochem GmbH) and U73122 (a phospholipase C-γ (PLC-γ) inhibitor, Wako) were applied at 1.0 μM, respectively. D-(−)-2-amino-5-phosphonopentanoic acid (APV), and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) were purchased from Tocris Bioscience (Bristol, UK). Other reagents were obtained from Sigma (MO, USA). Regeneron Pharmaceutical Co., (NY, USA), Takeda Chemical Industries, Ltd. (Osaka, Japan) and Dainippon Sumitomo Pharma Co. Ltd. (Osaka, Japan) donated the BDNF.

Cell cultures

Primary cultures were prepared from postnatal 2 day-old rat (SLC, Shizuoka, Japan) cerebral cortex, as reported previously (Numakawa et al., 2002a,b). Dissociated cortical cells were plated at a final density of $5\times10^5/cm^2$ on polyethyleneimine-coated culture dishes (Corning, NY, USA). The culture medium (5/5 DF) consisted of 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 containing 15 mM HEPES buffer, pH 7.4, 30 nM Na_2SeO_3 and 1.9 mg/ml $NaHCO_3$. All animals were treated according to the institutional guidelines for the care and use of animals. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Drug application

Cortical cultures were maintained for 7 days (7 days *in vitro*, DIV7) before DEX (final 1.0 μM) application. Seventy two hours later (at DIV10), BDNF was applied, and an additional 24 h of maintenance was carried out before sample collection for Northern blotting, RT-PCR, and immunoblotting. U0126, PD98059, LY294002, or U73122 was applied 20 min before BDNF addition.

Transfection of miR-132 antisense or ds-miR-132

To examine the function of endogenous miR-132, 2′-o-met-miR-132 antisense oligonucleotide (5′-CGACCAUGGCUGUAGACUGUUA-3′, Hokkaido system science, Hokkaido, Japan) transfection was carried out at DIV9 for 24 h prior to BDNF addition at DIV10. As a control, 2′-o-met-negative control oligonucleotide (5′-AGACUAGCGGUAUCUUAAACC-3′) (Tsuchiya et al., 2006) was used. Ds-miR-132 (double-stranded synthesized mature microRNA "miCENTURY OX miNatural," B-Bridge, Tokyo, Japan, sense strand 5′-CAACCGUGGCUUUCGAUUGUUACU-3′, antisense strand 5′-UAACAGUCUACAGCCAUGGUCGCC-3′), or negative control siRNA (5′-ATCCGCGCGATAGTACGTA-3′, B-Bridge) was also transferred. The ds-miR-132 transfection was conducted at DIV9. Forty eight hours later, the cortical cultures were harvested for immunoblotting. When an influence of U0126 was examined, U0126 (10 μM) was applied just after the ds-miR-132 transfection. RNA transfection (final 100 ng/ml, respectively) was performed using Lipofectamine 2000 reagent (Invitrogen, CA, USA). After estimating the efficiency of transfection using Lipofectamine in our cortical neurons (with GFP plasmid, pAcGFP1-N1, Clontech, CA, USA), we confirmed at least 39.1±4.6% (n=9) of cells were transfected.

Northern blotting

Small RNAs (<200 nt) were isolated using a *mir*Vana™ miRNA Isolation Kit (Ambion, TX, USA). The RNAs were separated on 15% acrylamide TBE-urea mini-gel and then electroblotted onto a Hybond N+ nylon filter membrane (Amersham, Buckinghamshire, UK). Antisense oligonucleotides for miR-9 (5′-TCATACAGCTAGATAACCAAAGA-3′), miR-124a (5′-TGGCATTCACCGCGTGCCTTAA-3′), miR-128a (5′-AAAAGAGACCGGTTCACTGTGA-3′), miR-128b (5′-GAAAGAGACCGGTTCACTGTGA-3′), miR-132 (5′-CGACCATGGCTGTAGACTGTTA-3′), miR-134 (5′-CCCTCTGGTCAACCAGTCACA-3′), miR-138 (5′-GATTCACAACACCAGCT-3′), and miR-16 (5′-CGCCAATATTTACGTGCTGCTA-3′) were labeled with [γ-32P] ATP using T4polynucleotide kinase and hybridized to the filter in ULTRAhyb®-Ultrasensitive Hybridization Buffer (Ambion) according to the manufacturer's instructions. To confirm equal loading, the blots were reprobed to detect U6 snRNA. Quantitation was performed using a Bioimageanalyser (BAS2500, Fuji film, Tokyo, Japan) system. In the present study, we quantified the mature miR-132 (around 20 nt) because the expression of pre-miR-132 (around 60 nt) could not be detected (Supplemental figure).

Polymerase chain reaction (PCR)

Total RNAs were isolated using the *mir*Vana miRNA Isolation Kit (Ambion). Quantitative analysis of miR-132 was carried out on RNA samples using the specific stem-loop primers for reverse transcription, followed by real-time TaqMan reagents (Applied Biosystems, CA, USA). All values were normalized to achieve endogenous control of miR-16. All amplicons were analyzed using Prism 7900HT sequence detection system 2.2 software (Applied Biosystems).

Immunoblotting

Cultured cells were lysed in a sodium dodecyl sulfate (SDS) lysis buffer containing 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. After boiling for 5 min, lysates were centrifuged at 15,000 rpm for 60 min, and the supernatants were collected. For equal loading, the protein concentration of the supernatants was determined with a BCA Protein Assay Kit (Pierce, IL, USA). As primary antibodies, anti-NR2A (1:500, Sigma, MO, USA), anti-NR2B (1:500, Sigma, MO, USA), anti-GluR1 (1:1000, Chemicon, CA, USA), anti-synapsin I (1:2000, Chemicon, CA, USA), anti-syntaxin (1:10,000; Sigma, MO, USA), anti-SNAP25 (1:1000, Synaptic Systems, Göttingen, Germany), anti-TUJ1 (1:5000, Berkeley antibody company, CA, USA), anti-phospho-ERK (1:1000, Cell Signaling, MA, USA), anti-ERK (1:1000, Cell Signaling), anti-TrkB (1:1000, BD Biosciences, CA, USA), and anti-phospho-Trk antibodies (Stephens et al., 1994, 1:1000, Cell Signaling, MA, USA) were used. The n indicates the number of experiments performed with separate cultures. The intensity of the immunoreactivity was quantified by using Lane and Spot Analyzer software (ATTO Corporation, Tokyo, Japan).

Immunocytochemistry

The cortical neurons were fixed with methanol at −20 °C for 10 min. The cells were permeabilized, and the non-specific binding of antibodies was blocked with 10% goat serum, 0.2% Triton X-100 in PBS for 30 min at room temperature. As the primary antibody, anti-MAP 2 (1:1000, Sigma) was applied overnight at 4 °C. Alexa Fluor 488-conjugated anti-mouse IgG (1:1000, Invitrogen) was used as a secondary antibody. For the Hoechst staining, nuclei of cells were stained with 2 mg/ml Hoechst 33342 (Molecular Probes, Eugene, OR, USA) at 37 °C for 1 h before the number of condensed and/or fragmentation nuclei were counted (Hetman et al., 1999).

MTT assay

To measure the cell viability, the metabolic activity of mitochondria was estimated by measuring the mitochondrial-dependent conversion of the tetrazolium salt, MTT (Sigma) as described previously (Numakawa et al., 2007). Seventy two hours DEX incubation was started at DIV7 before BDNF addition at DIV10. Twenty four hours later, cultured neurons were incubated with MTT solution. Two hours later, cultures were lysed and the metabolic activity of the mitochondrial reductase was determined. To investigate the effect of intracellular signaling inhibitors, 24 h U0126, LY293002, or U73122 incubation was started at DIV10. Twenty four hours later, neurons were incubated with MTT solution for estimating the cell viability.

Statistical analysis

Data shown in this study are expressed as mean±standard deviation (SD). Statistical significance was evaluated using Student's *t*-test, or one-way ANOVA followed by Bonferroni's multiple comparison test performed by GraphPad Prism ver.5 (GraphPad Software Inc., CA, USA). The probability values less than 5% were considered significant.

RESULTS

BDNF increased the expression of miR-132 in cultured cortical neurons

We first investigated the changes of expression of various miRs after BDNF application in cultured cortical neurons. Mir-9, -124, -128a, -128b, -132, -134, and -138 are brain-specific miRs (Lagos-Quintana et al., 2002); on the other hand, miR-16 is known to be ubiquitously expressed (Hayes et al., 2008). As shown in Fig. 1A, northern blot analysis indicates that an increase in the expression of miR-132 was induced by BDNF application for 24 h in cortical neurons. In contrast, the levels of the other miRs, including miR-9, -124, -128a, -128b, -134, -138, and -16 were unchanged. Quantitative analysis was performed (Fig. 1B). Using the RT-PCR method, we checked the upregulation of miR-132 after BDNF stimulation for various durations. A significant increase of miR-132 was observed after BDNF application for 6–24 h, although acute BDNF stimulation (10 min, or 1 h) failed to increase the miR-132 (Fig. 1C). In the following experiment, incubation with BDNF was performed for 24 h. In our cultures, we examined the changes in the expression of miR-132 during maturation *in vitro* (at DIV 3, DIV7, and DIV 10), however, significant increase in the endogenous miR-132 during *in vitro* maturation was not observed (DIV7, 116±2.2; DIV10, 111±3.6, % (per DIV3), $n=3$).

Many BDNF actions are dependent on the activation of a specific receptor, TrkB, which is broadly expressed in the brain. In contrast, the expression of TrkA, a receptor for NGF (nerve growth factor), is restricted and limited neuronal populations (for instance, cholinergic neurons in the basal forebrain) exhibit biological responses to NGF (Hatanaka et al., 1988; Fagan et al., 1997). BDNF increased the expression of miR-132 in a dose-dependent manner (Fig. 1D). Importantly, NGF had no effect on the miR-132 expression, suggesting that BDNF-stimulated miR-132 upregulation is via the activation of TrkB. In cortical neurons, BDNF induces rapid and transient release of glutamate

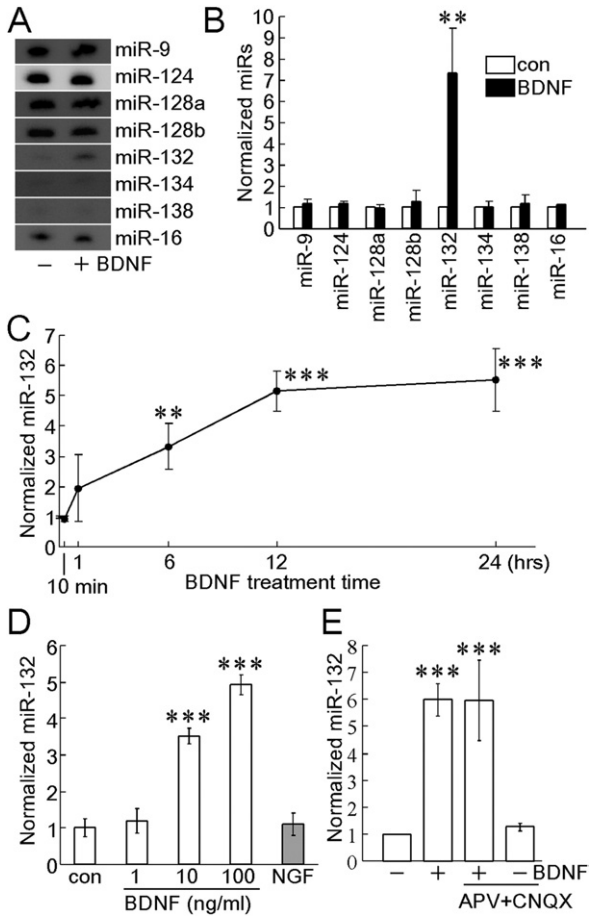

Fig. 1. Expression of miR-132 was increased by brain-derived neurotrophic factor (BDNF) in cultured cortical neurons. (A) Levels of microRNAs (miRs) including miR-9, -124, -128a, -128b, -132, -134, -138, and -16 with or without BDNF stimulation in cultured cortical neurons. The miR-132 was upregulated by BDNF. The remaining miRs (miR-9, -124, -128a, -128b, -134, -138, and -16) were unchanged. Representative data obtained from sister cultures are shown. Samples for Northern blotting were collected from days *in vitro* (DIV)11 neurons with or without BDNF (100 ng/ml for 24 h). (B) Quantitative analysis of (A) Normalization to a non-treated control was performed. Data represent mean±SD ($n=3$). ** $P<0.01$ (*t*-test). (C) Upregulation of miR-132 after various durations of BDNF application. RT-PCR was conducted to examine the increase of miR-132 after BDNF addition. Data represent mean±SD ($n=4$). Normalization to a level in control (0 time) was performed. One-way ANOVA followed by Bonferroni's multiple comparison test was performed. *** $P<0.001$, ** $P<0.01$. (D) Dose-dependent effect of BDNF on the upregulation of miR-132. BDNF (24 h) was applied at the indicated concentration. As a negative control, NGF (100 ng/ml) was also applied. RT-PCR analysis was performed. Data represent mean±SD ($n=4$). One-way ANOVA followed by Bonferroni's multiple comparison test was performed. *** $P<0.001$. (E) BDNF still increased miR-132 in the presence of APV (a NMDA receptor inhibitor, 10 μM) and CNQX (an AMPA receptor inhibitor, 10 μM). Northern blotting was performed. Data represent mean±SD ($n=4$). One-way ANOVA followed by Bonferroni's multiple comparison test was performed. *** $P<0.001$.

(Numakawa et al., 2009), implying that activation of glutamate receptors may be involved in the miR-132 upregulation. Therefore, we confirmed that BDNF still increased miR-132 in the presence of APV (an N-methyl-D-aspartate (NMDA) receptor inhibitor) and CNQX (an AMPA receptor

inhibitor), indicating that BDNF has a direct effect on miR-132 upregulation, not via activation of glutamate receptors (Fig. 1E).

DEX pretreatment suppressed BDNF-increased miR-132 expression

Next, we investigated whether chronic DEX (a synthetic GR-selective agonist) exposure influences BDNF-increased miR-132 in cultured cortical neurons. We monitored the effect of various durations of DEX pretreatment on BDNF-increased miR-132 using the RT-PCR method. As shown in Fig. 2A, the upregulation of miR-132 by BDNF was decreased by the DEX pretreatment for 48 or 72 h. We found a trend for decreasing levels of miR-132 after 24 h DEX pretreatment, although a significant decrease was not detected. By using northern blot analysis, we confirmed the suppression of the BDNF-increased miR-132 after 72 h exposure to DEX (a, b in Fig. 2B). U6 is displayed as a control.

To check the influence of DEX exposure on neuronal survival, immunocytochemistry with anti-microtubule-associated protein 2 (MAP2, a neuronal cell marker) antibody was performed, and no change in the number of MAP2-positive cells was observed (a in Fig. 2C). Consistently, none of the treatments increased apoptosis, as measured by Hoechst staining [BDNF, 99±12; DEX, 99±9.7; DEX+

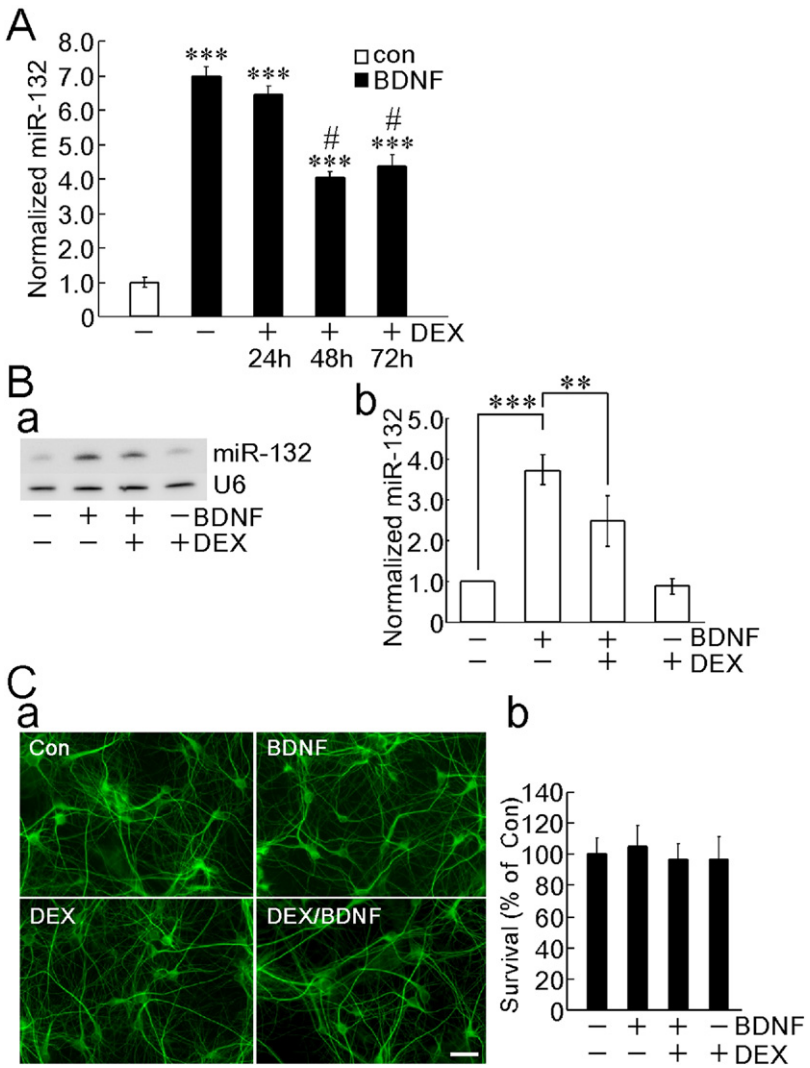

Fig. 2. Pretreatment with dexamethasone (DEX) reduced BDNF-stimulated miR-132 increase. (A) Inhibitory effect of various durations of DEX (1.0 μM) pretreatment on the BDNF-increased miR-132. DEX incubation was performed for the indicated times before BDNF addition (at DIV10, 24 h). Normalization to control (without DEX and BDNF) was carried out. Data was obtained through RT-PCR and represent mean±SD (n=4). *** P<0.001 vs. none. # P<0.05 vs. BDNF-increased in without DEX. One-way ANOVA followed by Bonferroni's multiple comparison test. (B) (a) Northern blotting indicates an inhibitory effect of DEX on BDNF-increased miR-132. Seventy two hours DEX incubation was started at DIV7 before BDNF addition at DIV10. Twenty four hours later, samples were collected for Northern blotting. As a control, the blots were reprobed to detect U6 snRNA. (b) Quantitative analysis is shown. Data represent mean±SD (n=4). *** P<0.001, ** P<0.01. One-way ANOVA followed by Bonferroni's multiple comparison test was performed. (C) DEX did not affect neuronal survival. (a) The number of MAP2-positive cells was not altered under any conditions described in (B). (b) Cell viability was determined with MTT assay. No changes in survival were observed under any conditions. Data represent mean±SD (n=8). The n indicates the number of wells for each experimental condition on a plate. Bar=50 μM.

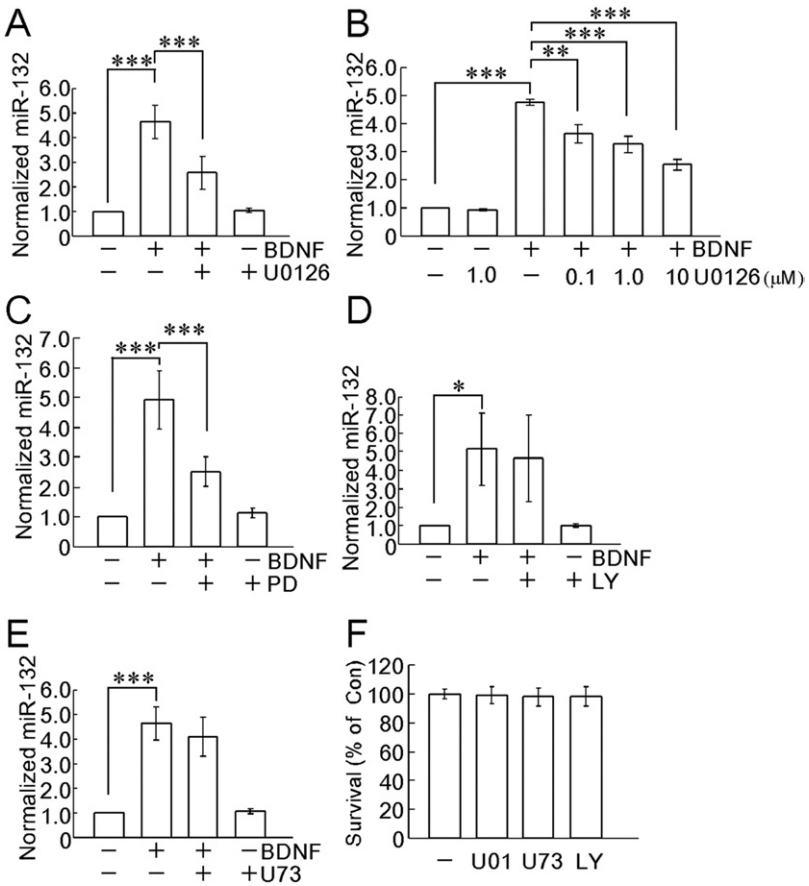

Fig. 3. Activation of the MAPK/ERK1/2 pathway was required for the BDNF-increased miR-132. (A) Effect of the MAPK/ERK1/2 pathway inhibitor, U0126, on the BDNF-dependent miR-132 increase. U0126 (final 10 μM) was applied 20 min before BDNF addition. BDNF was added at DIV10 for 24 h. Quantitative analysis was performed after Northern blotting. Normalization to a level in non-treated control was performed. Data represent mean±SD (n=4). *** P<0.001. One-way ANOVA followed by Bonferroni's multiple comparison test. (B) U0126 reduced the miR-132 increase in a dose-dependent manner. Data represent mean±SD (n=4). *** P<0.001, ** P<0.01. One-way ANOVA followed by Bonferroni's multiple comparison test. (C) PD98059 (50 μM, a MAPK/ERK1/2 pathway inhibitor) decreased the BDNF-dependent miR-132 increase. Data represent mean±SD (n=4). *** P<0.001. One-way ANOVA followed by Bonferroni's multiple comparison test. PD: PD98059. (D) LY294002, a phosphatidylinositol 3-kinase (PI3K) inhibitor, did not block the BDNF-increased miR-132 level. LY294002 (1.0 μM) was applied 20 min before BDNF addition. Data represent mean±SD (n=4). * P<0.05. One-way ANOVA followed by Bonferroni's multiple comparison test. LY: LY294002. (E) U73122 (1.0 μM), a phospholipase C-γ (PLC-γ) pathway inhibitor, had no effect on the BDNF-increased miR-132. U73122 was applied 20 min before BDNF addition. Data represent mean±SD (n=4). *** P<0.001. One-way ANOVA followed by Bonferroni's multiple comparison test. U73: U73122. (F) Cell viability after 24 h incubation with the U0126 (final 10 μM), U73122 (1.0 μM), or LY294002 (1.0 μM) was not changed. MTT assay. Data represent mean±SD (n=6). The n indicates the number of wells for each experimental condition on a plate. U01: U0126.

BDNF, 98±12, % (per control), scoring nuclear condensation and/or fragmentation per field, n=5]. Furthermore, an MTT assay to estimate cell survival was conducted and no change in the cell viability was detected after DEX and/or BDNF application in our cultures (b in Fig. 2C). These results suggest that the inhibitory effect of DEX on the BDNF-increased miR-132 is not due to a decline in the cell viability.

MAPK/ERK1/2 pathway was important for BDNF-increased miR-132

BDNF exerts its biological effects through activating intracellular signaling, including MAPK/ERK1/2, phosphatidylinositol 3-kinase (PI3K), and PLC-γ pathways, after the activation of TrkB. To identify essential signaling for the BDNF-dependent miR-132 increase, the effect of each pathway inhibitor was examined. We found that the BDNF-dependent miR-132 increase was reduced by the ERK1/2 pathway inhibitor, U0126, by northern blot analysis (Fig. 3A). The dose-dependency of U0126 on the decrease in the miR-132 expression was confirmed (Fig. 3B). PD98059, a distinct inhibitor of the ERK1/2 pathway, also decreased the BDNF-dependent miR-132 increase (Fig. 3C). The contribution of the other pathways activated by TrkB, i.e., the PI3K and PLC-γ pathways, were also examined. As illustrated, BDNF still increased the miR-132 level in the presence of LY294002, a PI3K inhibitor (Fig. 3D). U73122, a PLC-γ pathway inhibitor, did not inhibit BDNF-increased miR-132 expression (Fig. 3E). As shown in Fig. 3F, the viability of the cultured cells after a 24 h incubation with the U0126, U73122, or LY294002 was not changed. These results suggest that activation of the ERK1/2 is necessary,

and functions as the upstream regulator of the BDNF-potentiated miR-132 expression.

The miR-132 was involved in the BDNF-dependent increase in expression of postsynaptic proteins

Previously, we reported that BDNF increases synaptic proteins via the MAPK/ERK1/2 pathway (Matsumoto et al., 2006; Kumamaru et al., 2008). Therefore, to verify the possible involvement of miR-132 in the regulation of pre- and postsynaptic protein expression, the effect of an antisense oligonucleotide to inhibit the miR-132 function was examined. Consistent with our previous studies, BDNF significantly increased postsynaptic proteins (ionotropic glutamate receptors) NR2A, NR2B, and GluR1. Interestingly, these increases were weakened by the antisense transfection (Fig. 4A). As shown, miR-132 antisense partly reduced the BDNF-induced upregulation of glutamate receptor subunits. There may be an alternative mechanism (including regulation by other unknown miRs) that also controls the BDNF-increased glutamate receptors. The level of TUJ1 (class III β-tubulin, a neuronal marker) is shown as a control (Fig. 4A). Levels of presynaptic proteins, including synapsin I, SNAP25, and syntaxin were also checked. In contrast to postsynaptic proteins, no change in the levels of these presynaptic proteins was caused by miR-132 antisense with or without BDNF (Fig. 4B). Quantification of these pre- and postsynaptic proteins was performed (Table 1). Furthermore, the effect of exogenous ds-miR-132 (double-stranded synthesized precursor of mature microRNA) transfection was examined. As shown in Fig. 4C, marked upregulation of NR2A, NR2B, and GluR1 was induced by the transfection of ds-miR-132. These results suggest that miR-132 is involved in the BDNF-mediated upregulation of postsynaptic proteins.

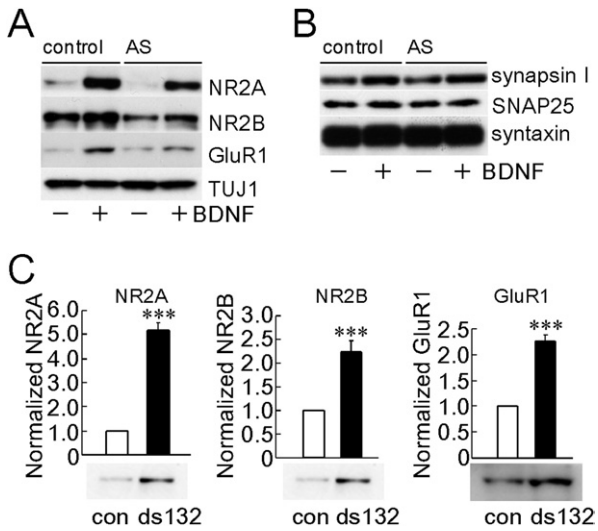

Fig. 4. Antisense RNA transfection to obstruct the function of endogenous miR-132 inhibited the BDNF-increased postsynaptic proteins, and the transfection of ds-miR-132 mimicked the BDNF action. (A) BDNF-increased NR2A, NR2B, and GluR1 were decreased by the miR-132 antisense transfection. TUJ1 (a neuronal marker) is shown as a control. Samples for immunoblotting were collected after BDNF incubation for 24 h. Antisense transfection was carried out 24 h prior to BDNF addition. Quantitative data are shown in Table 1. AS: antisense (B) Presynaptic synapsin I, SNAP25, and syntaxin were also checked. No change in the expression of these presynaptic proteins was caused by miR-132 antisense transfection. Quantitative data are shown in Table 1. (C) Effect of exogenous ds-miR-132 on the postsynaptic proteins. Marked upregulation of NR2A, NR2B, and GluR1 was induced by the transfection of ds-miR-132. ds132: ds-miR-132. After immunoblotting was performed, quantification was carried out. Data represent mean±SD (NR2A; n=5, NR2B; n=4, GluR1; n=5). *** $P<0.001$ (t-test).

Table 1. Antisense for miR-132 decreased the BDNF-enhanced expression of postsynaptic proteins, but not presynaptic proteins

	con	BDNF	Antisense	antisense+BDNF
NR2A	0.16±0.13	1.00***	0.11±0.02	0.61±0.15***
NR2B	0.43±0.25	1.00**	0.22±0.13	0.69±0.13
GluR1	0.32±0.09	1.00***	0.38±0.12	0.49±0.12***
Synapsin I	0.84±0.38	1.00	0.54±0.23	1.18±0.24
SNAP25	0.96±0.14	1.00	0.87±0.28	0.93±0.08

Quantification was performed by densitometry after Western blotting. Antisense and BDNF were applied as indicated in the legend for Fig. 4. To quantify Western blot, normalization using response to sole BDNF application was performed. Increase in NR2A and GluR1 expressions by BDNF was significantly attenuated by the antisense for miR-132. The decreasing tendency in the NR2B levels by the antisense was also observed although the statistical significance was not confirmed. In contrast, the levels of presynaptic proteins, including synapsin I and SNAP25 were not changed. Data represent the mean±SD (n=4), ** $P<0.01$ (con vs. BDNF), *** $P<0.001$ (BDNF vs. antisense+BDNF). One-way ANOVA followed by Bonferroni's multiple comparison test.

Previously, we showed that DEX inhibits BDNF-increased synaptic proteins via inhibiting ERK1/2 activation in immature hippocampal neurons (Kumamaru et al., 2008). Thus, in the present system, we examined whether there was a decline in the BDNF-stimulated ERK1/2 activation after DEX pretreatment. As expected, DEX suppressed the BDNF-dependent phosphorylation of ERK1/2 (activated ERK1/2 form, pERK1/2) in cultured cortical neurons (a in Fig. 5A). Total ERK1/2 expression was not affected (a in Fig. 5A). Quantification of pERK1/2 levels was carried out (b in Fig. 5A).

As shown in Fig. 3, it is possible that miR-132 expression was regulated via the ERK1/2 pathway. Thus, to further clarify whether ERK1/2 activation is upstream of the BDNF-potentiated miR-132 expression, the effect of miR-132 antisense transfection on the ERK1/2 activation was examined. Surprisingly, ERK1/2 activation by BDNF was diminished by the miR-132 antisense (a, b in Fig. 5B), implying that miR-132 also affects ERK1/2 activation. We examined an effect of miR-132 antisense transfection on the level of TrkB protein because the TrkB receptor is upstream of ERK1/2 activation. As shown, TrkB expression was not influenced by the antisense transfection (a in Fig. 5C). Furthermore, we examined whether BDNF-induced Trk activation is altered by miR-132 antisense. Western blotting using phospho-Trk antibody (Stephens et al., 1994) revealed that an inhibitory effect of miR-132 antisense on Trk activation was not induced (b in Fig. 5C). Lastly, the effect of U0126, an inhibitor of the ERK1/2 pathway, on the upregulation of NR2A, NR2B, and GluR1

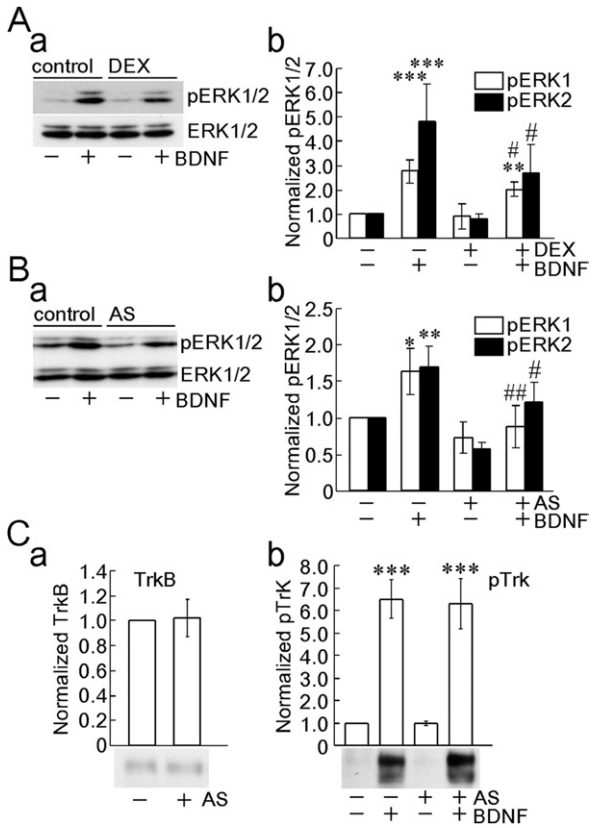

Fig. 5. DEX and miR-132 antisense RNA attenuated the MAPK/ERK1/2 activation stimulated by BDNF. (A) (a) Pretreatment with DEX suppressed the phosphorylation (activation) of ERK1/2 (pERK1/2) stimulated by BDNF. Total ERK1/2 expression was intact. DEX (1.0 μM) was applied at DIV7. Seventy-two hours later, BDNF was added. An additional 24 h of maintenance was conducted before sample collection. (b) Quantification of levels of pERK1/2 was performed. Data represent mean±SD (n=4). *** P<0.001, ** P<0.01 vs. non-treated, $^{\#}$ P <0.05 vs. BDNF-treated samples without DEX. One-way ANOVA followed by Bonferroni's multiple comparison test. (B) (a) BDNF-stimulated ERK1/2 activation was diminished by miR-132 antisense. (b) Quantification of levels of pERK1/2. Data represent mean±SD (n=4). ** P<0.01, * P<0.05 vs. non-treated, $^{\#\#}$ P <0.01, $^{\#}$ P <0.05 vs. BDNF-treated samples without antisense. One-way ANOVA followed by Bonferroni's multiple comparison test. (C) (a) Effect of miR-132 antisense transfection on the level of TrkB protein. TrkB expression was not influenced. Data represent mean±SD (n=6). (b) BDNF-induced Trk activation is not altered by miR-132 antisense. Data represent mean±SD (n=8). *** P<0.001 vs. non-treated. One-way ANOVA followed by Bonferroni's multiple comparison test.

caused by ds-miR-132 transfection was examined. U0126 partly but significantly decreased the upregulation of these glutamate receptors, implying that unknown regulation, including involvement of other unknown miRs downstream of ERK1/2 activation, may also contribute to the BDNF-stimulated increase in glutamate receptors (Fig. 6).

DISCUSSION

In the present study, we found that BDNF induced the upregulation of miR-132 through the MAPK/ERK1/2 pathway in cultured cortical neurons. Transfection of antisense RNA to inhibit the miR-132 function diminished the BDNF-dependent increase in the expression of postsynaptic proteins. Transfection of ds-miR-132 upregulated these synaptic proteins in the absence of BDNF. Remarkably, pretreatment with DEX, a synthetic glucocorticoid, decreased BDNF-increased ERK1/2 activation, miR-132 expression, and postsynaptic protein levels.

MiR-132 is critical for BDNF-stimulated dendritic outgrowth (Vo et al., 2005). Morphological change in dendrites might be associated with synaptic connections. Thus, a possible involvement of miR-132 in the maintenance of pre- and postsynaptic protein expressions is an interesting issue. We previously reported that BDNF upregulates synaptic proteins in cultured hippocampal and cortical neurons (Matsumoto et al., 2006; Kumamaru et al., 2008). Thus, in the present study, a possible role of miR-132 in the BDNF-regulated expression of synaptic proteins was examined. BDNF dramatically increased various synaptic proteins, including NR2A, NR2B, GluR1, and synapsin I in cultured cortical neurons. Importantly, the inhibitory effect of miR-132 antisense on the BDNF-increased postsynaptic proteins (especially, NR2A and GluR1) was considerable. On the other hand, presynaptic synapsin I was not influenced by the miR-132 antisense. These results suggest that miR-132 plays a role in postsynaptic protein expression regulated by BDNF, although roles of miR-132 in presynaptic regulation may be further elucidated if neuronal maturity or cell type is altered.

In the present study, upregulation of post-synaptic proteins following the ds-miR-132 transfection occurred. Le et al. reported that endogenous levels of miR-125b are relatively low, and that consequent transfection of the miR-125b duplex brings miR-125b levels up by ~2^7 fold (Le et al., 2009). In their study, ectopic expression of miR-125b downregulates the target protein p53 by ~40%. Notably, the suppression of p53 achieved by the miR-125b duplex was not stronger compared to the p53 siRNA, implying that a higher concentration of ectopic miR is required. In our case, though endogenous miR-132 was also relatively low, the miR-132 level was significantly upregulated by BDNF application. In our preliminary experiment (using a SH-SY5Y neuroblastoma cell line), transfection of ds-miR-132 caused the upregulation of miR-132 expression by ~2^{10} fold. As a result, the strong expression of miR-132 may influence the expression of post-synaptic proteins via suppression of target gene expression. Recently, Wayman reported that the miR132-p250GAP pathway plays a key role in activity-dependent dendrite growth in cultured hippocampal neurons (Wayman et al., 2008). In their system, bicuculline-mediated inhibition of $GABA_A$ inhibitory tone (to increase spontaneous synaptic activity) and transfection of miR-132 increased total dendritic length. Interestingly, in their data, inhibition of miR-132 by 2′-O-methyl RNA antagonists blocked the bicuculline and exogenous miR-132 actions. However, marked decrease of dendritic length was not achieved by solo 2′-O-methyl RNA antagonist application compared with control. In our present study, the significant downregulation of post-synaptic proteins was not induced by the miR-132 antagonist alone. It is possible that the amount of the miR132 above threshold

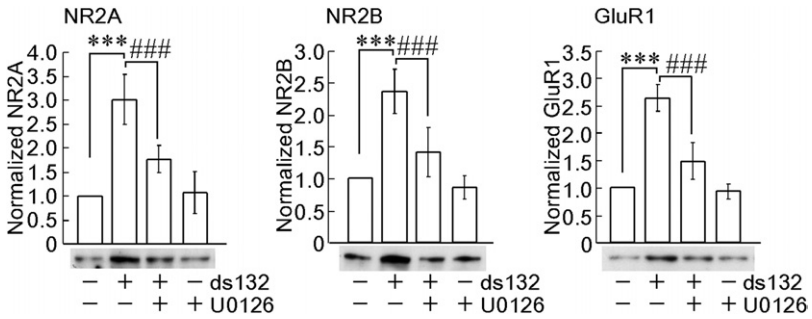

Fig. 6. Effect of U0126 on the upregulation of NR2A, NR2B, and GluR1 caused by ds-miR-132. U0126, an inhibitor of the ERK1/2 pathway, partly but significantly decreased the upregulation of ionotropic glutamate receptors caused by ds-miR-132. Data represent mean±SD (NR2A; $n=8$, NR2B; $n=7$, GluR1; $n=10$). *** $P<0.001$ vs. non-treated. ### $P<0.001$ vs. solo ds-miR-132. One-way ANOVA followed by Bonferroni's multiple comparison test.

may be required for enhanced action of miR-132 in dendritic outgrowth and upregulation of post-synaptic proteins. Moreover, there is a possibility that the miR-132 is important for enhancement of expression of these proteins, but not for basal level maintenance of these proteins.

Chronic stress and glucocorticoid administration causes dendritic atrophy in hippocampal neurons (Woolley et al., 1990; Watanabe et al., 1992; Magariños et al., 1996; Liu et al., 2006). The MAPK/ERK1/2 pathway is important for dendritic formation (Miller and Kaplan, 2003) and we had also confirmed the importance of the activation of ERK1/2 in the neurite outgrowth in developing cortical neurons (Numakawa et al., 2004). Furthermore, we recently found that DEX exhibits an inhibitory effect on BDNF-increased synaptic proteins through preventing ERK1/2 activation in immature hippocampal neurons (Kumamaru et al., 2008). In the present study, we found that BDNF-increased miR-132 was decreased by DEX in mature cortical neurons, and that the BDNF-dependent increase in the miR-132 level was repressed by MAPK/ERK1/2 pathway inhibitors (U0126 and PD98059). In addition to the MAPK/ERK1/2 pathway, the TrkB receptor activates the PI3K pathways, which is critical for cell survival (Patapoutian and Reichardt, 2001; Rodgers and Theibert, 2002; Zheng and Quirion, 2004). In our system, LY294002 (a PI3K inhibitor) had no effect on BDNF-stimulated miR-132 increase. Consistently, neuronal survival was not changed after DEX exposure. Inhibition of the PLC-γ pathway, another downstream signaling of TrkB, had no effect on the miR-132 increase. Therefore, the DEX-dependent prevention of the increase in postsynaptic proteins may be due to diminishing MAPK/ERK1/2 signaling, especially. In our recent study, neurotransmitter release and the activation of PLC-γ stimulated by acute BDNF application after long-term glucocorticoid exposure (24–48 h) were decreased in developing cortical neurons (Numakawa et al., 2009). Collectively, it is possible that glucocorticoids play a variety of functions depending on exposure time to glucocorticoids, neuronal cell types, or their maturity.

The MAPK/ERK1/2 pathway is an important intracellular signaling for the upregulation of miR-132 during BDNF stimulation. With regard to a blocking experiment, the BDNF-dependent miR-132 increase was prevented by U0126 in a dose-dependent manner, suggesting that ERK1/2 activation is upstream of miR-132 upregulation. Unexpectedly, the decrease in the ERK1/2 activation was observed after transfection of the miR-132 antisense, implying that miR-132 has a positive influence on the ERK1/2 activation. Actually, following the miRNA specific target detection algorithm (TargetScan, available at: http://www.targetscan.org/) was used to identify the predicted mRNA targets, we found two putative binding sites of miR-132 within 3′ UTR of ERK1 mRNA (unpublished data). As the first binding site was conserved among vertebrates, we focused on the first binding site for reporter analysis and found that miR-132 decreased reporter activity (unpublished data). However, as shown above, total protein level of ERK1/2 was not affected by DEX and miR-132 antisense in our cultured neurons. In general, it is reasonable that miR-132 antisense blocks the endogenous miR-132 action and increases the levels of both pERK1 and total ERK1, if ERK1 is a target for miR-132. Furthermore, we confirmed that the level of TrkB after miR-132 antisense application was not altered. In addition, BDNF-stimulated Trk activation was not altered by miR-132 antisense. Thus, other molecules including phosphatase targeted by miR-132, that prevents the activation of ERK1/2 or up-stream molecules of ERK1/2, might be a potential target of miR-132 (Fig. 7). Taken together, it is possible that a positive feedback system functions between ERK1/2 signaling and miR-132 for BDNF-dependent upregulation in expression of postsynaptic proteins.

Interestingly, miR-21 regulates the MAPK/ERK signaling pathway in cardiac fibroblasts (Thum et al., 2008). The miR-21 is increased selectively in fibroblasts of the failing heart, augmenting MAPK/ERK activity through inhibition of sprouty homologue 1 (Spry1). Indeed, after the miRNA specific target detection algorithm (TargetScan, available at: http://www.targetscan.org/) was used, a putative binding site of miR-132 within 3′ UTR of Spry1 mRNA was found (data not shown). As Spry1 is a potent inhibitor of the MAPK/ERK pathway (Hanafusa et al., 2002), it is possible that Spry1 may be involved in miR-132-regulated MAPK/ERK signaling, although additional investigation is necessary.

In our cortical cultures, miR-132 expression could be detected, although its level was very low compared with that of miR-9, -124, -128a, or -128b. Interestingly, miR-132

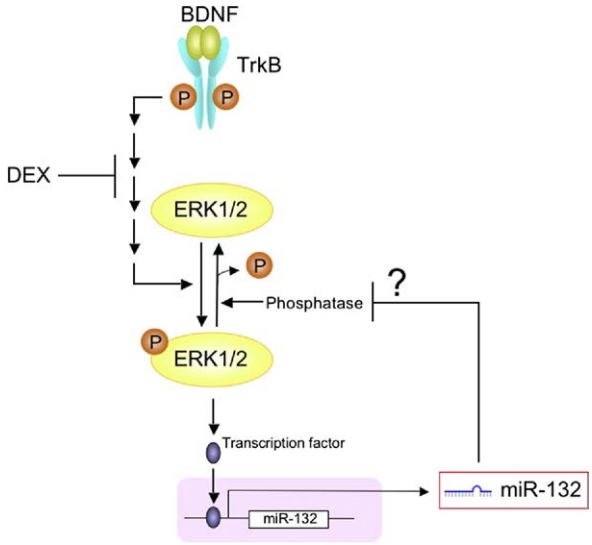

Fig. 7. A positive feedback system functions between ERK1/2 signaling and miR-132. In our system, the MAPK/ERK1/2 pathway is important for the upregulation of miR-132 during BDNF stimulation. Pretreatment with DEX declined BDNF-increased ERK1/2 activation and miR-132 expression. The BDNF-dependent miR-132 increase was attenuated by U0126 and PD98059 (both are ERK1/2 pathway inhibitors), suggesting that activation of ERK1/2 is upstream of miR-132 upregulation. In contrast, miR-132 antisense transfection resulted in decrease in the ERK1/2 activation, implying that miR-132 also has a positive influence on the ERK1/2 pathway. In our cultures, total level of ERK1/2, and expression and activation of Trk were not changed by DEX and miR-132 antisense application. Therefore, unknown phosphatase, that prevents the activations of ERK1/2 or up-stream molecules of ERK1/2, might be targets of miR-132. It is possible that a positive feedback system functions between ERK1/2 signaling and miR-132.

expression was significantly upregulated after BDNF addition. In contrast, the levels of miR-9, -124, -128a and -128b were not affected by BDNF. Thus, it is possible that the function of miR-132 in the cortical system is strictly controlled by BDNF. Computer analysis predicted many candidate genes as targets of miR-132; however, we were unable to identify a direct target involved in the upregulation of ERK1/2 activation or postsynaptic protein expression. In primary human preadipocytes and *in vitro* differentiated adipocytes, miR-132 plays a role in activation of nuclear factor-kappaB (Strum et al., 2009). Very recently, Tai et al. reported that serum- and glucocorticoid-inducible kinase 1, SGK1, directly phosphorylates IKKα at Thr-23 and indirectly activates IKKα at Ser-180, and that the SGK1 phosphorylation of IKKα results in the phosphorylation and activation of nuclear factor-kappaB that consequently upregulates NR2A and NR2B expression (Tai et al., 2009). Klein et al. showed that methyl CpG–binding protein 2 (MeCP2) translation is regulated by miR132 (Klein et al., 2007). Importantly, MeCP2 controls excitatory synaptic strength by regulating the glutamatergic synapse number in hippocampal neurons (Chao et al., 2007). In our cortical cultures, BDNF significantly induced the increase in miR-132 levels and it is possible that miR-132 is important for the BDNF-induced postsynaptic protein expression. The miR-132 is also increased in an activity-dependent manner (Wayman et al., 2008). They showed that neuronal activity inhibited translation of p250GAP (Rho family GTPase-activating protein), a miR-132 target, and siRNA-mediated knockdown of p250GAP mimicked miR-132-induced dendrite growth in hippocampal neurons (Wayman et al., 2008). Taken together, it may be valuable to study whether these miR-132-regulated molecules including nuclear factor-kappaB, MeCP2, and p250GAP are involved in the BDNF-induced postsynaptic protein expression.

Here, we found that DEX exposure negatively regulated BDNF/miR-132 system-mediated glutamate receptor expression in cortical cultures. BDNF is produced and secreted in an activity-dependent manner (Schinder and Poo, 2000; Hartmann et al., 2001; Balkowiec and Katz, 2002). Thus, continuous weak synaptic activity due to the downregulation of glutamate receptors, which is induced by exposure with increased glucocorticoid, may result in the reduction of BDNF protein levels, as observed in depressive disorder (Gervasoni et al., 2005; Karege et al., 2005). Thus, our system, where the upregulation of synaptic proteins occurs through the BDNF/miR-132-dependent signaling, could be used as an *in vitro* model for evaluating novel analogs as antidepressant candidates.

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APPENDIX

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.neuroscience.2009.11.057.

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