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Negative regulation of microRNA-132 in expression of synaptic proteins in neuronal differentiation of embryonic neural stem cells

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ABSTRACT

MicroRNAs (miRs) play important roles in neuronal differentiation, maturation, and synaptic function in the central nervous system. They have also been suggested to be implicated in the pathogenesis of neurodegenerative and psychiatric diseases. Although miR-132 is one of the well-studied brain-specific miRs, which regulates synaptic structure and function in the postnatal brain, its function in the prenatal brain is still unclear. Here, we investigated miR-132 function during differentiation of rat embryonic neural stem cells (eNSCs). We found that miR-132 expression significantly increased during the fetal rat brain development and neural differentiation of eNSCs *in vitro*. Furthermore, miR-132 expression was increased during differentiation through MAPK/ERK1/2 pathway. Inhibition of ERK1/2 activation resulted in increased levels of synaptic proteins including PSD-95, GluR1 and synapsin I. Silencing of miR-132 also increased PSD-95 and GluR1. Considering that miR-132 increases synaptic proteins in differentiated cortical neurons, our result shows a novel function of miR-132 as a negative regulator for synaptic maturation in the neuronal differentiation of eNSCs.

1. Introduction

Neural stem cells (NSCs) have the ability of self-renewing and differentiating into neurons and glial cells (astrocytes and oligodendrocytes) in the developing and adult brain. MicroRNAs (miRs) are small (~22 nt) non-coding RNAs that act as post-translational regulators through target mRNA degradation and/or translational repression by binding to the 3' untranslated region of mRNAs (Bartel, 2004). miRs are essential for developmental and physiological processes including cell apoptosis, differentiation and metabolism in animals and plants (Carrington and Ambros, 2003). The miRs studies in the developing brain and differentiating cultured embryonic NSCs (eNSCs) have demonstrated that brain-enriched miRs are important regulators for neuronal differentiation (Krichevsky et al., 2003; Sempere et al., 2004; Miska et al., 2004; Smith et al., 2010; Kawahara et al., 2012; Meza-Sosa et al., 2014). Moreover miRs offer novel insights into brain-related disease research. Recently, the crucial roles of miRs are increasingly reported in the pathogenesis of neurodegenerative and psychiatric diseases (Im and Kenny, 2012; Rao et al., 2013).

As a single microRNA can repress several targeting mRNAs, multiple functions of a miR has been shown, depending not only on cell types but also on spatial and temporal factors. For example, our group recently reported that glial miR-134 enhanced by bFGF positively regulated astroglial cell maturation (Numakawa et al., 2015). On the other hand, it was demonstrated that miR-134 targets Chordin-like 1 (Chrdl-1) and Doublecortin (Dcx) in the developing brain, and it has important roles in the stage-specific modulation of eNSC proliferation, neuron migration and neuronal differentiation via interacting with Chrdl-1 and Dcx (Gaughwin et al., 2011).

In this study, to investigate further functions of miRs specifically expressed during fetal neurodevelopment, we investigated miRs

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expression during differentiation of rat eNSCs *in vitro*. Among examined miRs, miR-132 showed the most dramatic increase during the differentiation into neuron from eNSCs. miR-132 is a well-studied miR in the central nervous system (CNS). It functions in neurotrophin signaling pathway including brain-derived neurotrophic factor (BDNF) and enhances neurite outgrowth (Wanet et al., 2012). Interestingly, miR-132 has been implicated in depression disorder (Li et al., 2013) in addition to neurodegenerative disorders such as Alzheimer's disease (AD) (Wong et al., 2013) and Parkinson's disease (PD) (Lungu et al., 2013). Methyl-CpG-binding protein 2 (MeCP2), a transcriptional repressor, was reported as a target of miR-132 (Klein et al., 2007). Mutation in MeCP2 causes progressive neurological disorder Rett syndrome (RTT) in female as a result of deficits in synaptic maturation. Whereas functions of miR-132 associated with postnatal CNS have been well studied, its role in the prenatal brain still uncertain. Here we examined the function and the induction mechanism of miR-132 during neuronal differentiation of eNSCs *in vitro*. Our results suggested that miR-132 negatively regulated the expression of synaptic proteins in the neuronal maturation of eNSCs.

2. Materials and methods

2.1. Animals

All animals used in this study were Wistar rats, and were treated according to the guidelines of the Animal Ethics Committee for the care and use of animals in the National Institute of Neuroscience, National Center of Neurology and Psychiatry, Japan. Brain samples for the quantitative PCR were harvested from embryonic stage day 14.5 and 19.5 (E14.5 and E19.5) rats (CLEA JAPAN, Tokyo, Japan). The tissues were frozen in liquid nitrogen quickly and stored at −125 °C until RNA extraction.

2.2. Neural stem cell culture, differentiation, and transfection

eNSCs were obtained from telencephalon tissues of E14.5 rats (Japan SLC, Shizuoka, Japan). Isolated eNSCs were maintained as neurospheres in proliferation medium, KBM neural stem cell (KOHJIN BIO, Saitama, Japan) with 0.2% KBM supplement containing EGF and bFGF (KOHJIN BIO), at 37 °C in 5% CO_2. After 4 days, these neurospheres were dissociated by trypsin (Sigma–Aldrich, MO, USA), and cells were subsequently seeded onto tissue culture dishes pre-coated with 0.2% polyethyleneimine (PEI) (Wako, Osaka, Japan), with differentiation medium, KBM containing 2% B-27 serum-free supplement containing vitamin A (Life technologies, CA, USA). Dissociated cells were plated at the density of 6×10^5 cells/35 mm culture dish (Becton Dickinson, NJ, USA). For immunocytochemistry, 9.375×10^4 cells/0.2 mL were re-seeded on glass region of pre-coated glass-bottom dish (Matsunami, Osaka, Japan). The culture medium was changed at 3 days *in vitro* (DIV3). The differentiated eNSCs were treated with 10 μM U0126 (Millipore, MA, USA) at DIV3 to DIV7 to inhibit MAPK/ERK signaling pathway. To activate TrkB signaling pathways, 100 ng/mL BDNF (kindly gifted from Takeda Chemical Industries, Ltd., and Dainippon Sumitomo Pharma Co. Ltd.) was applied to the cells at DIV4 and 6, and 1 μg/mL TrkB-Fc chimeric protein (R&D Systems, MN, USA) was added daily during DIV3-7 to block endogenous BDNF/TrkB-mediated signalings. The eNSCs differentiation was observed using an inverted microscope (IX71, OLYMPUS, Tokyo, Japan) with a microscope digital camera (DP21, OLYMPUS).

miRNA mimics (50 nM) (ID: MC10166, Life technologies), or miRNA inhibitors (25 nM) (ID: MH10166, Life technologies) for miR-132 or each negative controls (NC) (Life technologies) were transfected into differentiating eNSCs at DIV3 using Lipofectamine 2000 or Lipofectamine RNAiMAX (Life technologies). After 6 h, the transfection medium was replaced with fresh differentiation medium to reduce cellular toxicity of the reagent, and the transfected cells were collected at DIV7.

2.3. Rat primary cortical culture

Primary cortical neurons were prepared from the cerebral cortex of postnatal 1–2 old (P1-2) rats (Japan SLC) as described previously (Numakawa et al., 2009). Primary neurons were maintained in DMEM/F12 (Life technologies) containing 5% fetal bovine serum, 5% horse serum, and penicillin-streptomycin at 37 °C in 5% CO_2. U0126 (10 μM) treatment in the neuronal cell cultures was performed at DIV3 to DIV7.

2.4. Immunocytochemistry

eNSCs undergoing differentiation were fixed with 4% paraformaldehyde in PBS for 20 min at room temperature. The fixed cells were incubated with primary antibodies in blocking solution containing 0.2% Triton X-100 (Sigma–Aldrich) and 10% FBS in PBS, overnight at 4 °C. Triple staining was performed to estimate the degree of differentiation to neuron. Anti-nestin monoclonal antibody (1:1000, Millipore) was used as neural stem cell marker. The differentiation to neurons or glial cells was confirmed by neuronal marker, anti-MAP2 monoclonal antibody (1:500, Sigma–Aldrich), or astrocyte marker, anti-GFAP polyclonal antibody (1:1000, Abcam, Cambridge, UK). After three times wash by PBS, the cells were incubated with following secondary antibodies for 1 h at room temperature: Alexa Fluor 647 mouse IgG_{2A} (1:200, Life technologies), Alexa Fluor 546 mouse IgG_1 (1:2000, Life technologies) and Alexa Fluor 488 rabbit IgG (1:200, Life technologies). Furthermore, to confirm the developmental maturation of the synapse during the cell differentiation, the cells at DIV7 were incubated with anti-PSD-95 monoclonal (1:500, Thermo Fisher Scientific, CA, USA), anti-synapsin I monoclonal (1:500, Synaptic Systems, Goettingen, Germany) and anti-βIII-tubulin (TUJ1) polyclonal (1:1000, Sigma–Aldrich) antibodies followed by the incubation with secondary antibodies, Alexa Fluor 647 rabbit IgG (1:200, Life technologies), Alexa Fluor 546 mouse IgG_1 (1:2000, Life technologies) and Alexa Fluor 488 mouse IgG_{2a} (1:200, Life technologies). DAPI was also used to stain cell nuclei. The images were obtained using a fluorescence microscopy (Axiovert 200, Carl Zeiss, Oberkochen, Germany).

2.5. Immunoblotting

For total protein extraction, cultured cells were lysed using sodium dodecyl sulfate (SDS) lysis buffer (1% SDS, 10 mM Tris–HCl pH 7.5, 5 mM EDTA, 10 mM Sodium Pyrophosphate, 10 mM NaF, 1 mM PMSF and 2 mM Na_3VO_4). The cell lysates were heated at 100 °C for 5 min and sonicated, then, the lysates were centrifuged and the supernatants were collected. Protein concentration was measured using Pierce BCA Protein Assay Kit (Life technologies).

Total proteins were separated in 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred to Immobilon-P transfer membranes (Millipore). After blocking in 5% skim milk, membranes were incubated with primary mouse monoclonal antibodies for syntaxin (1:1000, Sigma–Aldrich), TrkB (1:500, BD), TUJ1 (1:1000, Berkekey Antibody Company, CA, USA), and β-actin (1:5000, Sigma–Aldrich) or primary rabbit polyclonal antibodies for DCX (1:2000, Cell Signaling Technology, MA, USA), GFAP (1:1000, Millipore), GluR1 (1:500, Millipore), PSD-95 (1:1000, Cell Signaling Technology), SOX1 (1:1000, Cell Signaling Technology), SOX2 (1:1000, Abcam),

synapsin I (1:1000, Millipore), anti-phospho-MAPK/ERK (1:1000, Cell Signaling Technology), anti-MAPK/ERK (1:1000, Cell Signaling Technology) and GAPDH (1:1000, Cell Signaling Technology). Membranes were subsequently incubated with peroxidase-conjugated anti-mouse IgG (1:1000, Jackson Immuno Research, PA, USA) or rabbit IgG secondary antibody (1:1000, Rockland, PA, USA) for 1 h at room temperature. The relative intensities of bands were quantified using Lane and Spot Analyzer soft ware (ATTO Corporation, Tokyo, Japan).

2.6. RNA isolation and real-time quantitative PCR (qPCR)

Total RNAs including mRNAs and small RNAs from brain tissues and cultured cells were extracted by *mir*Vana™ miRNA Isolation Kit (Ambion, TX, USA). Complementary DNA (cDNA) for miRNAs was generated using TaqMan microRNA Reverse Transcription (RT) kit (Applied Biosystems, MA, USA) and SuperScript VILO cDNA synthesis kit (Life technologies) for mRNAs. The cDNA was subjected to StepOnePlus Real Time PCR System (Applied Biosystems) with TaqMan® MicroRNA Assays or TaqMan® Gene Expression Assays; miR-9 (ID: 000583, Life technologies), miR-124a (ID: 001182), miR-132 (ID: 000457), miR-134 (ID: 001186), miR-206 (ID: 000510), and BDNF mRNA (ID: Rn02531976). The relative expression levels of RNAs were calculated by the $\Delta\Delta C_t$ method. MicroRNAs and BDNF mRNA levels were normalized to miR-16 (ID: 000391) and GAPDH (ID: 4352338E) levels, respectively.

2.7. Statistical analysis

The statistical differences were compared by the Student's *t*-test or one-way ANOVA with Bonferroni's *post-hoc* test in SPSS (SPSS Japan, Tokyo, Japan). A *P*-value of <0.05 was considered significant.

3. Results

3.1. The expression of miRs in the developing fetal brain

To investigate changes in miRs expression levels, we analyzed five miRs (miR-9, -124a, -132, -134 and -206) in the developing fetal brains by real-time qPCR. These miRs are suggested to be involved in psychiatric and/or neurological disorders (Mouillet-Richard et al., 2012; Lee et al., 2012; Rao et al., 2013). Total RNAs were isolated from the rat telencephalon (TE) at E14.5, cerebral cortex (CTX) and hippocampus (HIP) at E19.5. BDNF mRNA expression levels was increased, especially in HIP, with the growth of the fetal brain ($P < 0.01$) (Sup. Fig. 1A). miR-132 levels in CTX and HIP at E19.5 showed dramatic increase (nearly twentyfold) compared with TE at E14.5 ($P < 0.05$) (Fig. 1).

3.2. eNSC in vitro and differentiation to neurons

To investigate the mechanisms underlying the miRs expression changes observed in the fetal brain, we established an *in vitro* model for eNSCs differentiation. eNSCs obtained from E14.5 rat TE formed neurospheres in the presence of EGF and bFGF. Dissociated neurospheres were re-seeded and differentiated in EGF- and bFGF-free medium. After 7-days differentiation, the eNSCs developed neuronal morphology with long and branched neurites (Fig. 2A). The differentiated eNSCs showed decreased expression of nestin, a neural stem cell marker, and most cells differentiated into neuron (MAP2 only, 77.23%) while little population became astrocyte (GFAP only, 1.98%) (Fig. 2B and D). Synapses were observed by immunostaining with post- (PSD-95) and presynaptic (synapsin I) markers at DIV7 (Fig. 2C). Western blot analysis confirmed decreasing expression of neural stem cell markers SOX1 and SOX2, and upregulation of neuron specific proteins DCX and TUJ1, presynaptic proteins (synapsin I and syntaxin), and postsynaptic proteins (GluR1 and PSD-95) during *in vitro* differentiation (Fig. 2E).

3.3. Increased miR-132 expression required activation of MAPK/ERK pathway

Using *in vitro* differentiation model for eNSCs, we examined the expression of five miRs (miR-9, -124a, -132, -134 and -206) by real-time qPCR. While miR-9 (data not shown) and miR-134 levels showed no significant changes during the differentiation, ~30-fold elevated expression level of miR-132 was confirmed ($P < 0.001$) (Fig. 2F). Both miR-124 and miR-206, showed a tendency to increase the expression at DIV4 and DIV3 to DIV7, respectively (data not shown).

We previously reported that exogenous BDNF stimulated miR-132 expression via MAPK/ERK1/2 pathway and subsequently upregulated synaptic proteins in cultured cortical neurons (Kawashima et al., 2010; Numakawa et al., 2011). To determine whether BDNF and MAPK/ERK pathway were involved in the upregulation of miR-132 and synaptic proteins in differentiating eNSCs, the relationships among BDNF, ERK1/2, and miR-132 were investigated. Interestingly, BDNF mRNA showed a similar expression pattern to miR-132 (Sup. Fig. 1B). BDNF receptor TrkB, leading to the activation of MAPK/ERK signaling, was increased from DIV4 to 7 (Sup. Fig. 1C). ERK1/2 activity was transiently reduced at DIV3 and increased again after DIV4 (Fig. 3A). When spontaneous ERK1/2 activity was suppressed by 10 μM U0126 (an inhibitor of MAPK/ERK kinase) during DIV3-7 (Fig. 3B), miR-132 expression was dramatically reduced at DIV7 (Fig. 3C) and synaptic proteins including PSD-95 ($P < 0.05$), GluR1 ($P < 0.05$) and synapsin I ($P < 0.01$) were significantly increased (Fig. 3D). Immunostaining analysis with synaptic markers (PSD-95 and synapsin I), however, did not show a significant difference in the number of synaptic sites after U0126 treatment because of the variation in the number of synaptic puncta among neurites even in the same neuron (Fig. 3E). On the other hand, primary cortical neurons (already differentiated) did not show any miR-132 increase during neuronal maturation without exogenous BDNF (Sup. Fig. 2A), despite upregulation in ERK1/2 activation and synaptic proteins (PSD-95, synapsin I, and syntaxin) after DIV5 (Sup. Fig. 2B and C). In contrast to eNSCs, U0126 slightly reduced synaptic proteins such as PSD-95 ($P < 0.001$) and synapsin I ($P < 0.05$) in cultured cortical neuron (Sup. Fig. 2D, E and F). To examine whether the elevation of miR-132 during the eNSCs differentiation was regulated through MAPK/ERK1/2 activation by "endogenous" BDNF, a BDNF scavenger TrkB-Fc chimeric protein was used. Although TrkB-Fc treatment from DIV3 to 7 reduced ERK1/2 activation (Sup. Fig. 3A), it had no effect on the expression of miR-132 (Sup. Fig. 3B). Exogenous BDNF application also did not have a significant effect on the miR-132 expression although it significantly induced ERK1/2 activation (Sup. Fig. 3C and D). These results suggested different regulatory systems for miR-132 expression in differentiating and differentiated neurons, and indicated that miR-132 in eNSCs negatively regulated synaptic proteins expression.

3.4. Downregulation of synaptic proteins by miR-132 during differentiation of eNSCs

We next determined miR-132 function in synaptic maturation of eNSCs. Transfection of miR-132 mimic at DIV3 decreased expression levels of synaptic proteins compared with negative controls, by 13–20% in presynaptic proteins, synapsin I ($P < 0.05$) and syntaxin ($P < 0.01$), and 22–28% in postsynaptic proteins, PSD-95 ($P < 0.05$) and GluR1 ($P < 0.01$) (Fig. 4A). Cytoskeleton-associated proteins

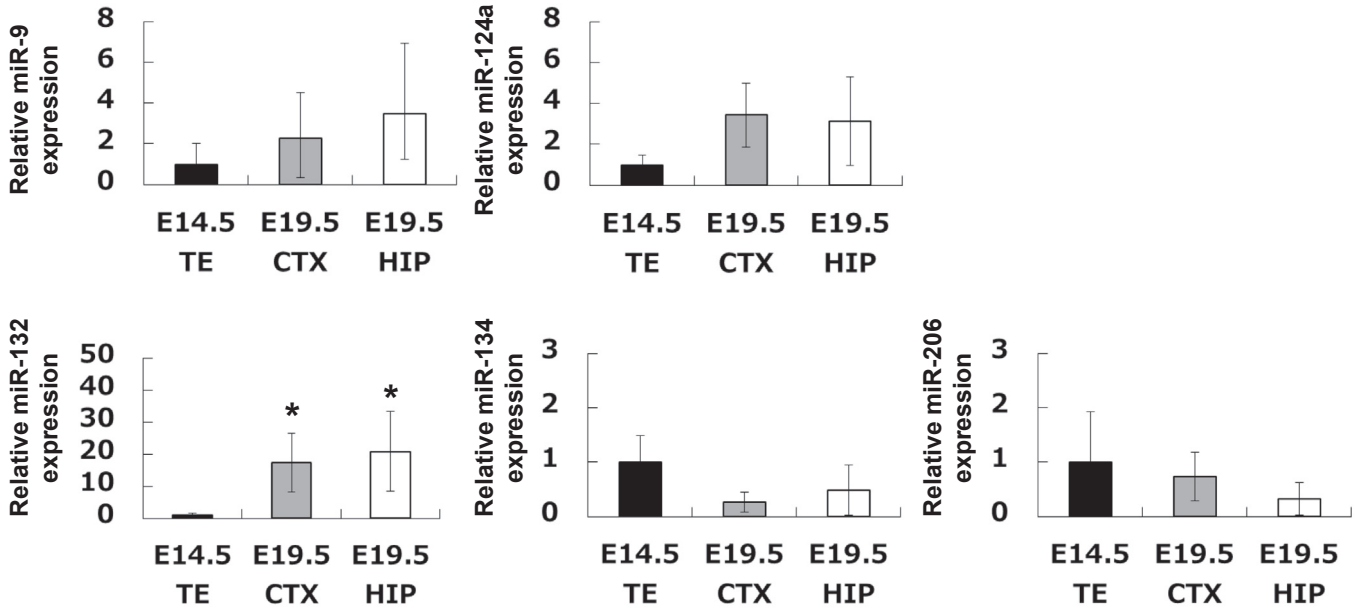

Fig. 1. Quantitative PCR analysis of miRs including miR-9, -124a, -132, -134 and -206 during rat prenatal brain development. The RNA samples were harvested from the telencephalon (TE) at embryonic stage day 14.5 (E14.5), cerebral cortex (CTX) and hippocampus (HIP) at E19.5 (n = 3). Relative expression of each miR is normalized to miR-16, and error bars present SD. *$P < 0.05$ versus TE at E14.5 (*t*-test).

(TUJ1 and β-actin) also showed a decreased expression while GAPDH, a house keeping gene, was unchanged. Conversely, miR-132 inhibitor upregulated postsynaptic proteins PSD-95 and GluR1 ($P < 0.05$) while it did not change levels of presynaptic proteins including synapsin I, syntaxin, SNAP25, and synaptotagmin (Fig. 4B and C, and data not shown). These results supported that miR-132 played a suppressive role in synaptic maturation of eNSCs.

4. Discussion

Previous studies have reported positive effects of miR-132 on neuronal functions including synaptic plasticity, dendrite outgrowth, and cell survival (Edbauer et al., 2010; Magill et al., 2010; Luikart et al., 2011; Wanet et al., 2012; Remenyi et al., 2013; Hwang et al., 2014). Down-regulation of miR-132 has also been implicated in several neurological disorders such as AD (Wong et al., 2013), PD (Lungu et al., 2013), schizophrenia (Miller et al., 2012) and depression (Li et al., 2013). Most of the studies, however, focused on the miR-132 functions in differentiated neurons. Here we found a novel function of miR-132 during neuronal differentiation of eNSCs to negatively regulate expression of synaptic proteins. We also showed that miR-132 significantly increased during both prenatal brain development and differentiation of eNSCs into neurons while it showed little change during maturation of cultured cortical neurons. Furthermore, ERK1/2 activation during DIV3-7 suppressed the upregulation of synaptic proteins in eNSCs via elevating miR-132 expression.

Because miR-132 expression was increased through MAPK/ERK1/2 signaling pathway activated by exogenous BDNF in differentiated cortical neurons (Kawashima et al., 2010; Numakawa et al., 2011) and BDNF mRNA and TrkB were elevated in parallel with the miR-132 increase during the neuronal differentiation observed in this study, we had expected that miR-132 was also upregulated in eNSCs by endogenous BDNF via ERK1/2 activation. Although miR-132 expression was almost completely dependent on ERK1/2 activity during DIV4-7, both activating and inhibiting of endogenous BDNF/TrkB signaling by exogenous application of BDNF and TrkB-Fc, respectively, did not cause a significant effect on miR-132 expression. Considering that miR-132 was reported to be induced by neuronal activity (Wayman et al., 2008) and ERK1/2 activity in eNSCs was also partially suppressed by TrkB-Fc, the ERK signaling-dependent miR-132 expression in eNSCs would be regulated by other activity-dependent factors than BDNF/TrkB signaling, or the other signaling pathways which repress the miR-132 expression might be stimulated simultaneously after TrkB activation.

MAPK/ERK1/2 pathway is one of the key regulators of neuronal differentiation and neurite outgrowth in the developing CNS (Li et al., 2006). In the *in vitro* neuronal differentiation model used here, an intriguing activation pattern of ERK1/2 was observed. Strong ERK1/2 activation immediate after plating the dissociated neurospheres was transiently reduced at DIV3 and the activity was recovered during DIV4-7. It was reported that miR-34, a repressor for neuronal differentiation of eNSC, was increased after several hours of differentiation induction, and decreased at DIV1, then, remained low expression levels throughout neuronal differentiation and maturation until DIV12 (Morgado et al., 2014). Furthermore, blockade of ERK1/2 signaling immediate after the induction of differentiation lead to reduce the number of NeuN-positive cells and expression of presynaptic protein synaptophysin (Li et al., 2006). Based upon these results, the first ERK1/2 activation might be required for the initiation of the eNSCs differentiation and the second activation (after DIV4) would be responsible for further enhancement of neuronal differentiation and/or maturation.

Reduced ERK1/2 activation with U0126 during DIV3-7 promoted the expression of synaptic proteins (PSD-95, GluR1 and synapsin I) in eNSCs. Similar results were obtained in the silencing of miR-132, in which postsynaptic proteins (PSD-95 and GluR1) were increased, suggesting a negative regulation of ERK1/2 on these synaptic protein expression via inducing miR-132 expression. Interestingly, miR-132 inhibitor treatment increased only postsynaptic proteins without affecting presynaptic proteins. Our previous study reported that antisense for miR-132 changed the levels of postsynaptic proteins (NR2A, NR2B and GluR1) but not presynaptic

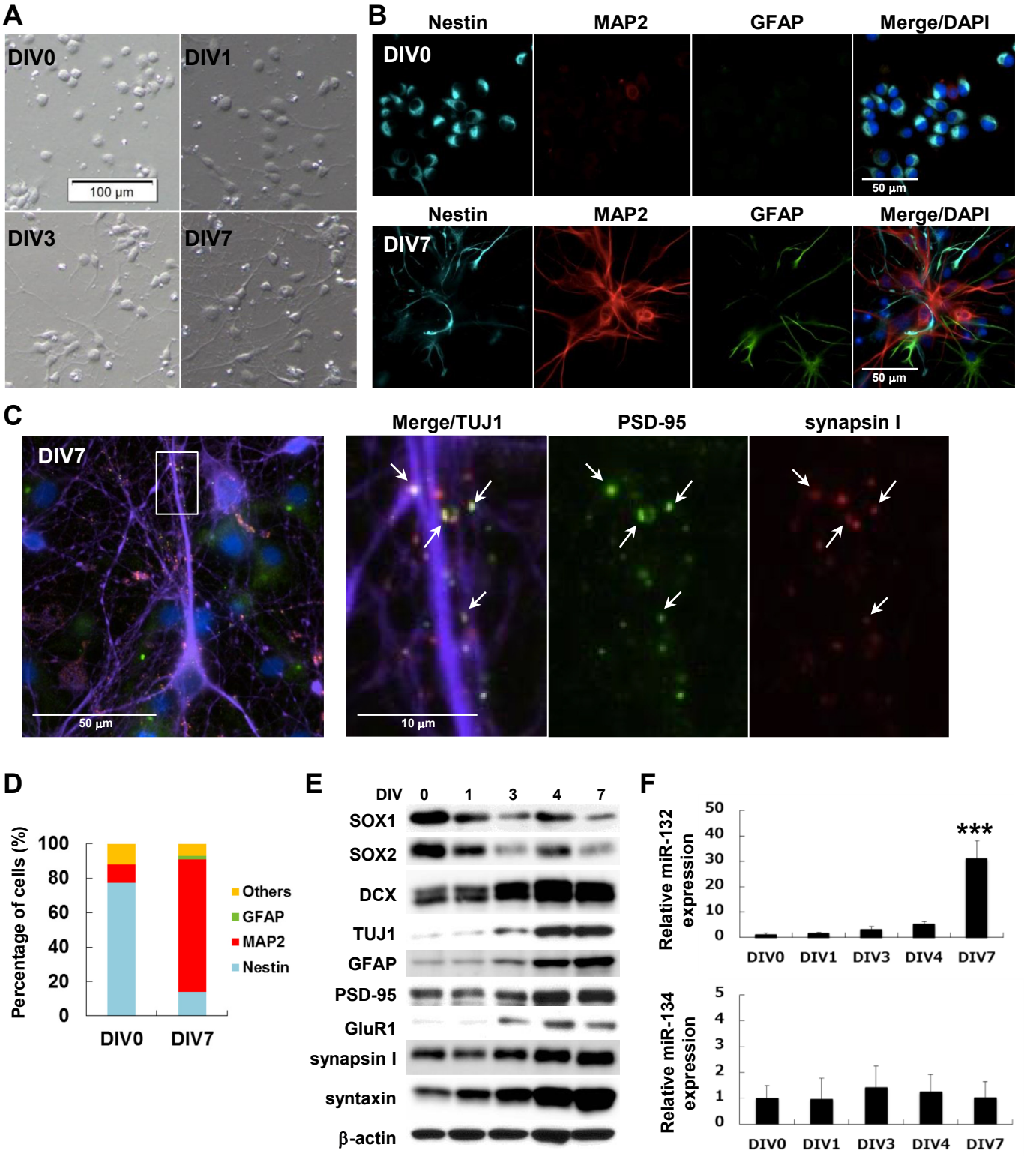

Fig. 2. Neuronal differentiation of eNSCs obtained from the rat embryonic telencephalon (E14.5) *in vitro*. eNSCs were initially cultured in the proliferation medium for 4 days, and then the cells were cultured with the differentiation medium for 7 days. (A) Microscopic images were taken 7 days after the differentiation initiation. Scale bar = 100 μm. (B) At 0 day *in vitro* (DIV0) and DIV7, the cells were immunostained with antibodies against nestin (cyan), MAP2 (red), GFAP (green) and DAPI (blue). Scale bars = 50 μm. (C) Immunostaining with antibodies against TUJ1 (purple), PSD-95 (green) and synapsin I (red) at DIV7 revealed developmental synapse formation in differentiated neurons. Nuclei were visualized by DAPI (blue). Magnified images of the white square region are shown in the right panels. Arrows indicate co-localization of presynaptic terminals and postsynaptic sites. Scale bars = 50 μm and 10 μm. (D) Quantification of the percentage of MAP2-positive differentiated neuronal cells, GFAP-positive astrocytes, or nestin-positive undifferentiated cells. The others include nestin/MAP2-positive or nestin/GFAP-positive cells (n = 89 at DIV0, n = 101 at DIV7). (E) Protein expression profiles during differentiation of eNSCs. Markers for neurons (DCX and TUJ1), astrocytes (GFAP), neural stem cells (SOX1 and SOX2), presynaptic proteins (synapsin I and syntaxin), and postsynaptic proteins (GluR1 and PSD-95) were examined. (F) Quantitative PCR analysis of miR-132 and -134 expression during neuronal differentiation of eNSCs (n = 3–4). Relative expression of miRs were normalized to miR-16, and error bars present SD. $^{***}P < 0.001$ versus DIV0 (one-way ANOVA with Bonferroni's *post-hoc* test).

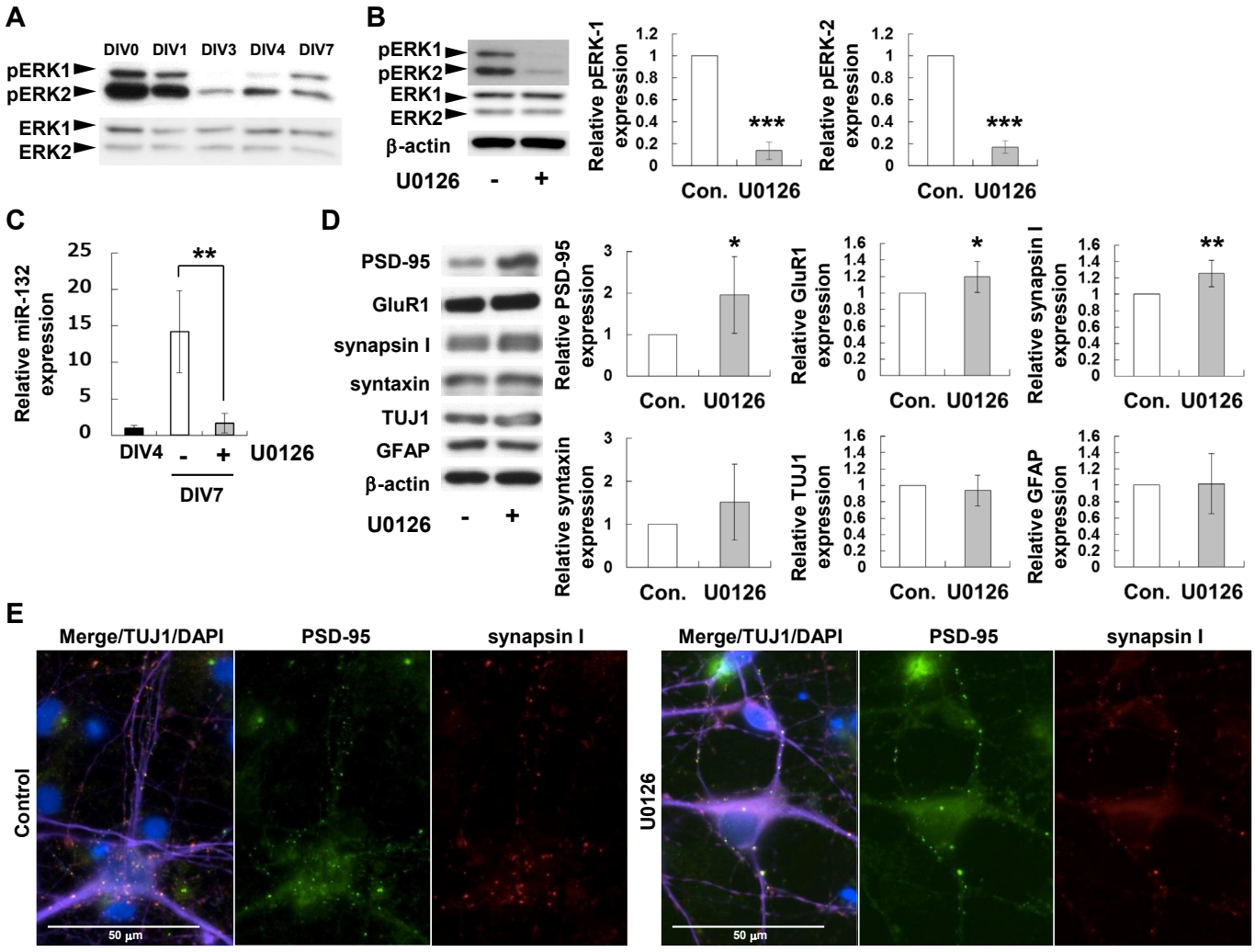

Fig. 3. Activity of MAPK/ERK signaling pathway induced miR-132 and suppressed synaptic proteins expression. U0126 (10 μM), a MAPK/ERK kinase inhibitor, was administered during neuronal differentiation of eNSCs. (A) Western blotting of phosphorylation levels of ERK1/2 (pERK1/2) in differentiating eNSCs. (B) U0126 almost completely diminished phospho-ERK1/2 levels in eNSCs (n = 5). (C) Quantitative PCR measurement of miR-132 in differentiating eNSCs in the presence or absence of U0126 (n = 4). Relative expression of miR-132 is normalized to miR-16. (D) Increased levels of PSD-95, GluR1 and synapsin I after the U0126 treatment were observed. Data are obtained from two independent cultures (n = 5). Error bars present SD. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ versus control (*t*-test). (E) The cells after U0126 treatment were immunostained with antibodies against PSD-95 (green), synapsin I (red) and TUJ1 (purple). Nuclei were stained with DAPI (blue). Scale bars = 50 μm.

proteins (SNAP25 and synapsin I) in differentiated cortical neurons (Kawashima et al., 2010). These results suggested that miR-132 tends to play a regulatory role in postsynaptic proteins in neural cells. It is possible that miR-132 regulates indirectly expression levels of these synaptic proteins because they have not been listed as the targets of miR-132 in miRs' target databases. There is a report about a miR-132-dependent negative regulation for differentiation of dopaminergic neurons developed from embryonic stem cells, where miR-132 was endogenously increased after DIV6 of differentiation (Yang et al., 2012). As mentioned above, our previous study demonstrated a miR-132-dependent positive regulation in the expression of synaptic proteins in differentiated cortical neurons (Kawashima et al., 2010). Although the exact reason why miR-132 regulations for synaptic proteins in eNSCs and differentiated neurons were different has remained unclear, the main target of miR-132 might be changed, depending on the developmental stages of neural cells.

The miR-132 overexpression in this study caused a reduced level of β-actin as well as synaptic proteins. β-actin, a cytoskeletal protein, is essential for dendritic spine structure and its synthesis in dendrites is stimulated by local synaptic activity (Buxbaum et al., 2014). The inhibitor for miR-132 had little influence on the expression of β-actin compared to its reduction induced by miR-132 overexpression, suggesting that an excess amount of β-actin would be expressed in eNSCs and β-actin levels was reduced only when miR-132 was overexpressed. PSD-95, which was also sensitive to miR-132, is a member of postsynaptic scaffolding proteins and associates with glutamate receptors (AMPA and NMDA receptors) and cytoskeletal proteins, supporting postsynaptic structure (Chen et al., 2011). Moreover, PSD-95 plays a role to regulate for surface expression of glutamate receptors at postsynaptic sites (Ehrlich and Malinow, 2004). Using immature neurons, it was reported that excess expression of PSD-95 caused aberrant spine formation (Mizui et al., 2005). These results indicate that miR-132 might have a modulating role in appropriate postsynaptic formation during neuronal differentiation of eNSCs.

Recent studies have revealed the important relationship between miRs and brain-related diseases. Zucchi and colleagues reported that prenatal stress modulated an epigenetic regulation of expression of miRs associated with psychiatric/neurological

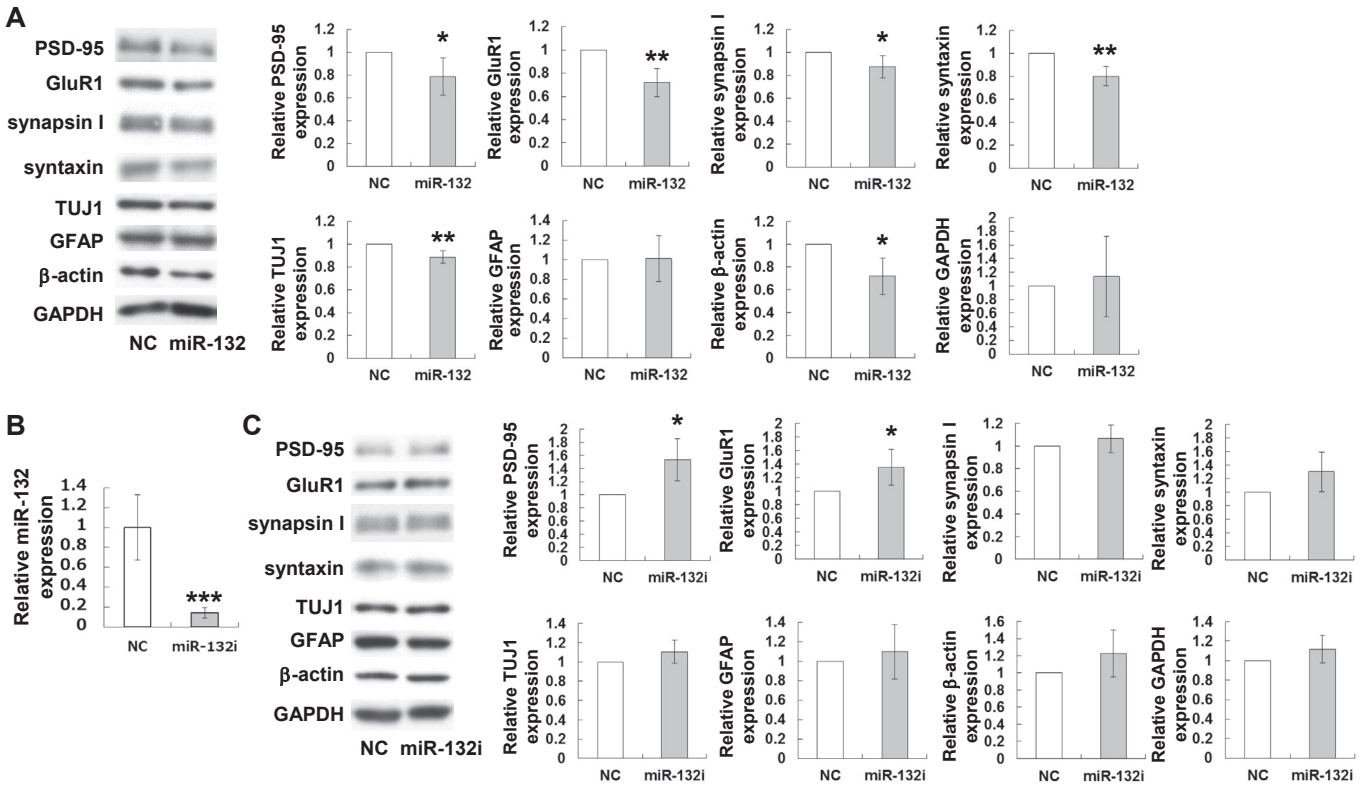

Fig. 4. Effect of increased/decreased miR-132 levels on expression of synaptic proteins. (A) The miR-132 mimic was transfected at DIV3. Decreased postsynaptic proteins (PSD-95 and GluR1), presynaptic proteins (synapsin I and syntaxin) and cytoskeleton-associated proteins (TUJ1 and β-actin) at DIV7 (n = 4). GAPDH was used as a loading control. (B) Quantitative PCR measurement of miR-132 in differentiating eNSCs in the presence or absence of miR-132 inhibitor (n = 5). Relative expression of miR-132 is normalized to miR-16. (C) An inhibitor for miR-132 was treated during DIV3-7. Increased PSD-95 and GluR1 levels were observed (n = 4). Samples for miR-132 mimic and inhibitor data were collected one time from a sister culture. Error bars present SD. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ versus control (*t*-test).

disorders in the offspring (Zucchi et al., 2013). Moreover, miRs levels in the prenatal brain would also be affected by maternally-mediated exposure to drug and alcohol (Ignacio et al., 2014). Our previous study showed a stress-related hormone (glucocorticoid)-induced reduction in BDNF-stimulated miR-132 expression in cortical neurons (Kawashima et al., 2010). These findings indicate the importance of miR functions for normal synapse formation in the developing brain and of the future research focusing on miR functions in stress-related brain disorders.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.neuint.2016.04.013.

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